Variation in the nitrous oxide reductase gene (nosZ)-denitrifying bacterial community in different primary succession stages in the Hailuogou Glacier retreat area, China

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Background: The Hailuogou Glacier in the Gongga Mountain region (SW China), on the southeastern edge of the Tibetan Plateau, is well known for its low-elevation modern glaciers. Since the end of the Little Ice Age (LIA), the Hailuogou Glacier has retreated continuously due to global warming, primary vegetation succession and soil chronosequence have developed in this retreat area. The retreated area of Hailuogou Glacier has not been strongly disturbed by human activities, thus it is an ideal models for exploring the biological colonization of nitrogen in the primary successional stages of ecosystem. The nosZ gene encodes the catalytic center of nitrous oxide reductase and is an ideal molecular marker in studying the variation in the denitrifying bacterial community.

Methods: Soil properties as well as abundance and composition of the denitrifying bacterial community were determined via chemical analysis, quantitative polymerase chain reaction (qPCR), and terminal restriction fragment length polymorphism (T-RFLP), respectively. The relationships between the nosZ denitrifying bacterial community and soil properties were determined using redundancy analysis (RDA). Soil properties, potential denitrify activity (PDA), and the nitrous oxide reductase gene (nosZ)-denitrifying bacterial communities significantly differed among successional stages.

Results: Soil properties, potential denitrify activity (PDA), and the nitrous oxide reductase gene (nosZ)-denitrifying bacterial communities significantly differed among successional stages. Soil pH in the topsoil decreased from 8.42 to 7.19 in the course of primary succession, while soil organic carbon (SOC) and total nitrogen (TN) gradually increased with primary succession. Available phosphorus (AP) and available potassium (AK), as well as potential denitrify activity (PDA), increased gradually and peaked at the 40-year-old site. The abundance of the nosZ denitrifying bacterial community followed a similar trend. The variation in the denitrifying community composition was complex; Mesorhizobium dominated the soil in the early successional stages (0-20 years) and in the mature phase (60 years), with a relative abundance greater than 55%. Brachybacterium was increased in the 40-year-old site, with a relative abundance of 62.74%, while Azospirillum dominated the early successional stages (0-20 years). Redundancy analysis (RDA) showed that the nosZ denitrifying bacterial community correlated with soil available phosphorus and available potassium levels (P < 0.01).
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Abstract:

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Keywords: Hailuogou glacier retreat area; primary succession; nosZ denitrifying bacterial community; T-RFLP; qPCR
Primary successional ecosystems, such as glacier forelands and volcanoes, are ideal models for exploring the biological colonization of various substrates. Since the ice covers of many glaciers have receded over the past century, glacier forelands have released substrates for soil formation and development (Rangwala et al., 2012; Anesio et al., 2012). In this process, new bare lands have emerged because of glacial retreats, starting ecological succession towards a zonal ecosystem. Autotrophic microbes play a critical role in the initial stages of primary community assembly (Cavicchioli et al., 2002). However, heterotrophic colonizers, decomposing organic material, are also important in the initial establishment of functional communities (Hodkinson et al., 2003). Previous studies in this field have mainly focused on the diversity and composition of the soil microbial community in the primary succession of receding glaciers (Ambrosini et al., 2017; Fernández-Martínez et al., 2017). Only a few studies have employed molecular tools to understand the diversity and composition of the functional microbial community along the forelands of receding glaciers (Kandeler et al., 2006; Töwe et al., 2010). For example, in a study about the activity and composition of the denitrifying bacterial community at the Rotmoosferner Glacier, Austria, the successional age shaped the N cycling processes as well as the microbial community composition (Nicol et al., 2010).

The Gongga Mountain is a typical mountain glacier in southwest China and extremely sensitive to climate change. The Hailuogou Glacier, located on the Gongga Mountain, has been retreating continuously since the end of the Little Ice Age (LIA) (Li et al., 2018) with the development of a soil and primary vegetation succession. Previous studies in this area have investigated specific processes such as pedogenesis (He et al., 2008; Zhou et al., 2013), plant succession (Yang et al., 2014), and microbial community changes (Sun et al., 2016). However, to date, there is no information on the density of the functional community involved in the denitrifying process in the primary succession of the Hailuogou Glacier.

Studying the dynamics of nitrogen cycling in the glacier retreat area is critical to evaluate the responses and feedbacks of biogeochemistry cycles to global climate change. Inorganic nitrogen compounds are primarily transformed through biochemical reactions mediated by microbial communities, and their activities regulate the input or output of nitrogen from ecosystems (Hallin et al., 2018). Nitrous dioxide (N$_2$O) is a potent greenhouse gas and generated via the microbially mediated processes nitrification and denitrification. Its impact on global warming is about 300 times higher than that of CO$_2$, and it’s half-life in the atmosphere is about 120 years; most atmospheric N$_2$O is the result of soil processes (Solomon et al., 2007; Wuebbles, 2009). Increasing denitrification is the main pathway to reduce N$_2$O production, and this process can be mediated by bacteria, archaea, or fungi. More than 50 genera of bacteria have been identified as denitrifying bacteria; hence, given the high phylogenetic diversity among denitrifying bacteria, detection methods based on gene assessment, such as 16S rRNA, are not adequate and require the use of functional genes which encode for enzymes directly involved in the denitrification process (Gu et al., 2017).
The enzyme nitrous oxide reductase catalyzes the final step of the denitrification process, namely the conversion of nitrous oxide (N\textsubscript{2}O) to nitrogen (N\textsubscript{2}), and the nosZ encodes the catalytic center of nitrous oxide reductase; therefore, it is generally used as a key functional gene of molecular markers to detect whether denitrification is complete (Philippot, 2002). Wang et al. (2012) have shown that in the case of nosZ gene abundance, N\textsubscript{2}O reduction both in the surface soils of the land area and in the soil core of the transition site was the dominant process. According to a previous study (Brankatschk et al., 2011), nosZ gene copy numbers were lowest in the early successional stages of a glacier retreat and significantly correlated with the potential enzyme activities, but not with the abundances of nirK and nirS. In another study (Kandeler et al., 2006), the nosZ gene was an ideal molecular marker in studying the variation in the denitrifying bacterial community throughout primary succession in a glacier retreat. We therefore infer that nosZ gene abundance and the dynamics of the denitrifying bacterial community of glacier retreat areas are important factors to understand the ecological role of bacteria involved in denitrification processes and, subsequently, in soil development (Brankatschk et al., 2011; Kandeler et al., 2006).

In this study, in combination with the measurement of soil properties, potential denitrification activities as well as the abundance and structural diversity of nosZ denitrifying bacteria in four successional stages (time of exposure after ice melting of 0, 20, 40, and 60 years, respectively) were assessed by chemical analysis, quantitative polymerase chain reaction (qPCR), and terminal restriction fragment length polymorphism (T-RFLP), respectively. The objectives of this study were (i) to investigate the variation in the nosZ denitrifying bacterial community in the four different successional stages found in the Hailuogou Glacier retreat and (ii) to understand the relationships between the nosZ denitrifying bacterial community and soil properties. We hypothesized that the variation in the activity and composition of the nosZ denitrifying bacterial community is strongly correlated with the successional stages.

**Material and methods**

**Study area**

The Gongga Mountain is located on the southeastern edge of the Tibetan Plateau, with a summit elevation of 7,556 m above sea level (ASL) (Liu et al., 2010). The Hailuogou Glacier (29° 34’ 07.83″ N, 101° 59’ 40.74″ E, Sichuan Province, China) is the single most developed glacier on the eastern slope of the Gongga Mountain. Against the background of a warming climate, the Hailuogou Glacier is shrinking continuously and is melting more rapidly now. This glacier retreat area currently covers an elevation range of 2,855 to 2,982 m above sea level. Average annual temperature is 4.2°C, with an average annual precipitation of 1,947 mm. The retreat area of the Hailuogou Glacier provides an excellent opportunity to study the relationship between vegetation succession and soil development, as its relatively mild and humid climate allows for rapid moraine colonization by plants and promotes fast ecosystem development. Along the approximately 2-km-long belt, there is a complete primary succession series from bare land to climax vegetation communities (Mapelli et al., 2018; Jiang et al., 2018). In the area with time of exposure after ice melting of about 60 years, a community resembling a mature phase
Primary succession was marked by the following stages: (i) bare land for 8-15 years, (ii) *Willow-Hippophae rhamnoides-Populus purdomii*, with a duration of about 35 years, (iii) *Populus purdomii-Betulautilis-Abies fabri*, about 60 years after glacier retreat, (iv) *Populus purdomii-Betulautilis-Abies fabri* community was gradually replaced by the *Abies fabri* community, with high biomass productivity of the plant community, (v) *Picea brachytyla, Abies fabri*, mature phase species. The soil parent material is mainly composed of biotite schist, granodiorite, and quartzite, with small amounts of phyllite, slate, and chlorite schist (Zhou et al., 2013). Table 1 provides information about the different sampling points.

### Soil sampling

Soil samples were collected from four sites, representing four successional stages (0, 20, 40, and 60 years) (Fig. 1). Site T0 is a bare land and contain with gravel, without any vegetation; successional time is below 5 years. Site T1 represents a bare-dwarf vegetation stage with the pioneer species *Astragalus Membranaceus, Hippophae rhamnoides* and willow; successional time is about 20 years, and the horizontal distance from the end of the glacier is about 300 m. Site T2 represents the stage of the *Willow-Hippophae rhamnoides-Populus purdomii* community, and the pioneer species are *Willow, Hippophae rhamnoides* and *Populus purdomii*; successional time is about 40 years, and the horizontal distance from the end of the glacier is about 600 m. Site T3 represents the stage of the *Populus purdomii-Betulautilis-Abies fabri* community, where the dominant plant species are *Populus purdomii* and *Betulautilis*, and some pine species (*Abies fabri*) begin to appear. Succession age is about 60 years, and the horizontal distance from the end of the glacier is about 1,200 m (Table 1). To avoid erosion and deposition effects, all sampling plots were located in the middle of gentle slopes, far from the small streams inside the primary succession areas. Moreover, all plots (except those in the T0 site) were located under the canopies of the dominant plant species. For each successional stage, five sampling plots of at least 20 × 20 m², with a minimum distance of 100 m between plots, were selected (Fig. 1). From each plot, we collected four soil samples, using a 6.5-cm diameter steel auger, from the surface layer (0-20 cm, roots and residue were removed and the samples were mixed to obtain one composite sample for each plot. Subsequently, the samples were stored in plastic bags on ice and divided into two portions: one was air-dried and sieved through a 2-mm mesh screen for physicochemical analyses, while the other one was stored at -80°C for soil total DNA extraction.

### Soil properties and denitrifying enzymatic activity analysis

Soil pH was measured potentiometrically. Soil total nitrogen was measured using the semi-micro Kjeldahl method, while soil organic carbon was determined via the potassium dichromate oxidation-external heating method. Soil available phosphorus (AP) and available potassium (AK) were assessed via the sodium bicarbonate extraction-Mo-Sb colorimetry methods and ammonium acetate extraction-flame photometry, respectively (Bao, 2007). Soil potential denitrifying activity (PDA) was determined using the C$_2$H$_2$ inhibition method (Dambreville et al., 2006).
Total soil DNA extraction

Total soil DNA was extracted from 0.5 g of fresh soil using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer’s instructions. The concentration and quality of extracted DNA were measured with a Nano-200 spectrophotometer (Aosheng, Hangzhou, China); DNA samples were stored at -20°C for further analysis.

Quantitative PCR

Quantitative PCR (qPCR) of the \textit{nosZ} gene was carried out using the ABI7500 sequence detection system (Bio-Rad) in 25 μL of the reaction mixture containing 12.5 μL of ABI Power SybrGreen qPCR Master Mix (Applied Biosystems, USA), 1 μM of each primer, 1.25 μL of template DNA, adjusted to a concentration of 20 ng μL\(^{-1}\); sterile distilled water was used to bring the final volume up to 25 μL. The \textit{nosZ} gene was amplified with primer pairs used for qPCR of \textit{nosZ} gene were \textit{nosZ}2F (5'-CGYTGTTCMTCGACAGCCAG-3') and \textit{nosZ}2R (5'-CGSACCTTSTTGCCSTYGCG-3') (\textit{Henry et al., 2006}). For \textit{nosZ}, the qPCR program consisted of an initial denaturation step at 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s. The \textit{nosZ} gene was quantified via qPCR with six technical replicates per sample.

Standard curves to assess \textit{nosZ} gene abundance consisted of a 10-fold serial dilution of a plasmid containing the \textit{nosZ} gene fragments.

The PCR amplification and T-RFLP of the \textit{nosZ} gene

Terminal restriction fragment length polymorphism (T-RFLP) analysis of the \textit{nosZ} gene was carried out using the primer pair \textit{nosZ}1126F (5'-GIGICTBGGICCRYTGCAYAC-3') and \textit{nosZ}1884R (5'-CATYTCSAKR-TGCAKGGCRTG-3'); the forward primer was labeled with 6-carboxyfluoroscein (FAM) (\textit{Chen et al., 2012}). The PCR reaction mixture volume was 50 μL, which consisted of 60 ng of template DNA, 0.4 μM of each primer, 25 μL of PCR master mix (Tiangen, China), and sterile distilled water to obtain a final volume of 50 μL. The PCR was performed with the following thermal profile: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR products were checked via 1.0% agarose gels electrophoresis and visualized with a UV Transilluminator Model M-26 (UVP, USA) after ethidium bromide staining. The amplified \textit{nosZ} gene fragments were digested with Bstul and Hhal endonucleases (NEB, Ipswich, Massachusetts, USA) at 37°C for 6 h, followed by further purification with a Tiangen DNA purification kit (Tiangen, China). The T-RFLP profiles were then generated by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer at Sangong Corporation (Shanghai, China).

Statistical analysis

The means and standard deviations of the soil physicochemical parameters and \textit{nosZ} gene diversities were calculated and tested for normality and homogeneity of variances. One-way analysis of variance (ANOVA) was applied to test for differences between sites, followed by Tukey’s multiple comparison tests. In the two-way tests, the significance level was \( p \leq 0.05 \). Pearson’s correlation analysis was conducted to examine the relationships between soil properties and \textit{nosZ} gene diversity, using the software package SPSS 21.0. Analysis of the T-RFLP profiles was performed using the software package Peak Scanner version 1.0 (Applied
Biosystems, Inc.). The peak heights of terminal restriction fragments (T-RFs) with size differences ≤ 2 bp in an individual profile were combined and considered to be one fragment. The T-RFs with a relative abundance < 1% were excluded from further analysis (Zhang et al., 2017). Principal components analysis (PCA) was performed to compare the dissimilarities among the denitrifying communities across different successional stages, based on Bray-Curtis dissimilarity. Correlation best-of-fit model for nosZ gene abundance and soil properties was carried out by curve estimation in SPSS. Redundancy analysis (RDA) was performed to assess the relationships between the nosZ denitrifying bacterial community composition and the soil properties, using the software package CANOCO 5.0.

Results

Soil properties and denitrifying activities in different successional stages

Based on our results, the soil nutrient status changed substantially with primary succession (Table 2). Soil pH gradually decreased from the T0 (8.42) to the T3 site (7.19). Soil organic carbon (SOC) was significantly higher in the T3 site (30.77 g·kg⁻¹) than in the other sites. Soil total nitrogen (TN) ranged from 0.29 to 1.11 g kg⁻¹. The site T3 had the highest concentrations of soil total nitrogen (TN), while the lowest level was found in the site T0. Soil available phosphorus (AP) varied from 0.98 to 5.54 mg kg⁻¹, with the highest value observed in the T2 site and the lowest in the T0 site. Soil available potassium (AK) ranged from 13.60 to 120.80 mg kg⁻¹; the highest concentration was observed in the T2 site and the lowest in the T0 site. Potential denitrifying activities ranged from 0.08 to 0.31 μg N₂O-N g⁻¹ dry soil h⁻¹.

Principal components analysis (PCA) was based on the analyzed soil properties of the four different successional stages. The PCA ordinated samples along PCA1 (89.51% of variance) and PCA2 (7.74% of variance), based on the successional stages. The soil micro-environment of the sites T0 and T1 was similar, with significant differences compared to the other stages (Fig. 1).

The nosZ denitrifying bacterial community abundance and diversity in different successional stages

Soil nosZ gene copy numbers ranged from 4.47 log₁₀ per gram dry soil in T0 to 5.86 log₁₀ per gram dry soil in T2, showing that the nosZ denitrifying bacterial community abundance changed significantly throughout primary succession (Fig. 2). The nosZ gene abundance was negatively correlated with soil pH and positively correlated with available soil potassium (P < 0.05) (Fig. 3).

The diversity of the nosZ denitrifying bacterial community varied significantly among the four different successional stages (P < 0.05) (Table 3). Shannon-Weiner index (H), richness (S), and evenness (Eh) ranged from 1.82 to 2.54, 8.99 to 18.41, and 1.03 to 1.30, respectively. The T2 site had the highest Shannon-Weiner index (H) and richness (S) values, while the lowest values were found in T1. Evenness (Eh) increased gradually throughout primary succession, with the highest values observed in the T3 site.

Pearson’s correlation analysis revealed that Shannon-Weiner index (H) was significantly linked to soil AP (P < 0.01) and AK (P < 0.05), while richness (S) was positively correlated with AP, AK, and PDA (P < 0.05) and evenness (Eh) was negatively correlated with soil pH (P < 0.01).
In the nosZ gene T-RFLP analysis, the dominant T-RFs were not significantly affected by the composition of the nosZ denitrifying bacterial community; however, some minor T-RFs were impacted (Fig. 3). In the T0 stage, 20- and 25-bp T-RFs were dominant nosZ T-RFs, with relative abundances of 62.6 and 10.5%, respectively. At T1, 15-, 20-, and 25-bp T-RFs dominated, with relative abundances of 22.8, 43.4, and 10.6%, respectively. In the site T2, 10-, 15-, and 20-bp T-RFs were most abundant, with relative abundances of 10.7, 41.7, and 24.7%, respectively. In the site T3, the dominant nosZ T-RFs had 15 and 20 bps, with relative abundances of 55.2 and 12.3%, respectively. Across all successional stages, the 40-bp T-RFs was present, with a relative abundance of 2.2-2.6%. All sites also showed specific minor nosZ T-RFs. For example, the 50-bp T-RFs were found in the sites T0, T1, and T2, and their relative abundances gradually decreased over time. The 45- and 70-bp T-RFs were found at the site T1, while 55- and 95-bp T-RFs were present in the successional stages T2 and T3. At the sites T0 and T1, the 90-bp T-RFs were dominant. Redundancy analysis (RDA) showed that denitrifying bacterial communities in the different successional stages formed separate clusters, expect for the sites T0 and T1 (Fig. 4).

In the in-silico T-RFLP analysis of the nosZ sequences obtained in this study and from the NCBI database, the nosZ-denitrifying bacterial community in the clone library was generally similar to those detected in the actual T-RFLP. The dominant 15-bp T-RF was consistent with the T-RFs of Brachybacterium, the 20-bp T-RF was consistent with Mesorhizobium, and the T-RFs with 25, 40, 50, and 55 bp were consistent with Azospirillum, Sulfuritalea, Pseudomonas, and Nitrospirillum, respectively (Fig. 3).

Redundancy analysis of nosZ denitrifying bacterial community composition and soil properties

The relationships between soil properties and nosZ denitrifying bacterial community were analyzed via redundancy analysis (RDA). Based on the RDA pattern, the nosZ denitrifying bacterial communities from the soils of the four different successional stages could be partitioned into four separate groups (Fig. 4), indicating that the nosZ denitrifying bacterial communities from these four different soils related to primary succession were significantly different (P < 0.05). The RDA results further confirmed that the nosZ denitrifying bacterial community composition in the young soils (0-20 years) differed from that in the older sites (40-60 years). Among the measured soil properties, soil AP and AK appeared to be the major factors in determining the nosZ denitrifying bacterial community composition during primary succession of the Hailuogou Glacier (P < 0.01). Soil nosZ denitrifying bacterial community variance was also significantly linked to soil pH and PDA (P < 0.05).
Discussion

Soil properties at different successional stages

Soil properties reflect the primary succession of the glacier retreat areas, with significant consequences for soil formation, soil microbial community development, and plant growth (Rangwala et al., 2012; Ambrosini et al., 2017). In the course of primary succession, soil is generally considered as a nutrient pool for plant and microbial growth (Roubíčková et al., 2009). In this sense, soil development and soil microbial community dynamics affect the species replacement during primary succession, with feedback mechanisms for plants (Frouz et al., 2016). On the other hand, plants directly affect soil properties and have an indirect effect via modifying the soil microbial community, which in turn may affect soil formation (Frouz et al., 2008). Liter decomposition redistributes organic matter, increases soil porosity, and enhances soil aggregate formation, which greatly alters soil sorption capacity, water holding capacity, and other soil properties (Ponge et al., 2003; Six et al., 2004). Roots and associated soil organisms also affect the formation and development of soil, which in turn affects subsequent generations of plants and other soil organisms, a phenomenon referred to as “plant-soil feedback” (Six et al., 2004). In this study, SOC, TN, AP, and AK increased in the course of primary succession. During primary succession, pioneer plants such as herbs initially colonize the bare land, followed by shrubs; such processes facilitate colonization by soil microorganisms and fauna, ultimately resulting in altered soil properties (Frouz et al., 2008). Vegetation growth leads to an increase in litter residue and root exudate secretion, contributing to soil nutrient cycling (Cotrufo et al., 2013; Schmidt et al., 2011) and increased soil nutrient levels. However, later-successional species require higher amounts of nutrients (Püschel et al., 2007), leading to decreased soil nutrient levels. In addition, loss through runoff may also lead to a decrease in soil nutrients. Soil pH is an important indicator reflecting soil quality, and its value indicates the degree of acidity and alkalinity of the soil and its effect on the survival of plants and animals (Li et al., 2017). In this study, soil pH decreased in the course of succession, which was not in line with some previous studies that found no correlation between soil pH and the distance from the glacier (Zeng et al., 2016; Strauss et al., 2009).

Potential denitrifying activity in different successional stages

Potential denitrifying activity is significantly affected by a variety of environmental factors such as plant species richness, soil moisture, soil carbon, and soil nitrogen (Leloup et al., 2018). Zeng et al. have indicated that soil carbon is the key factor affecting soil potential denitrifying activity. Besides, soil pH, available phosphorus, and available potassium also significantly influenced soil activity such as urease, deaminase, and protease activities; these soil enzyme activities were grouped together for newly exposed soils on young moraines (0-5 years), while older soils (17-44 years) were well-separated from each other (Zeng et al., 2015). In another study, soil potential denitrifying activity gradually increased with the succession of salt marshland and reached the peak in the middle of the succession period. Soil potential denitrifying activity was negatively correlated with soil pH and positively correlated with soil
organic carbon, nitrate, and soil moisture (Salles et al., 2017). Similarly, in our study, soil potential denitrifying activity gradually increased and peaked in T2. The variation in soil parameters such as plant litter, soil microbial activity, and plant species could lead to an increase in PDA (Zeng et al., 2015). Plant litter decomposition increases soil organic carbon levels, thereby facilitating soil microbial growth and survival and increasing soil metabolism and enzyme activity (Ponge et al., 2003). In addition, a rich vegetation cover also positively affects the activity of denitrifying enzymes (Means et al., 2017).

The nosZ denitrifying bacterial community abundance response to different successional stages

Denitrifying bacteria are widely distributed in different ecosystems and strongly shaped by the ecological conditions; soil depth and seasonal changes will significantly affect the composition and abundance of denitrifying bacterial communities (Henry et al., 2006; Wu et al., 2017; Tang et al., 2016). On the contrary, the abundance of functional genes (nirS/K, nosZ) could also indirectly reflect the denitrification activity of the soil (Wu et al., 2017). Therefore, quantitative analysis of denitrifying functional genes is often used to study denitrification changes in a given ecosystem. In previous studies, nosZ gene abundance gradually increased and peaked at the early successional stages, with a subsequent decreased in the mature stage (Kandeler et al., 2006; Henry et al., 2006). Similarly, in our study, nosZ gene abundance reached the peak at T2 and subsequently decreased. Soil formation and the accumulation of soil nutrients, processes which occur in primary succession, provide a stable micro-habitat for soil microorganisms. Plant species, richness, and litter also lead to variations in soil microbial community dynamics and abundance (Zhang et al., 2017).

Previous studies have revealed that the abundance of functional genes differed among different ecosystems. For example, the high soil pH of salt marshes is the most important factor limiting microbial growth, and nosZ gene abundance was more or less constant throughout the succession of such ecosystems (Salles et al., 2017). In forest ecosystems, plant species and plant litter are abundant, facilitating the survival and growth of soil microorganisms, and the functional genes gradually increased throughout succession (Meng et al., 2017). Generally, the abundance of denitrifying bacterial communities is affected by soil properties and vegetation factors such as soil organic carbon, total nitrogen, and the appearance of plant patches (Brankatschk et al., 2011; Frouz et al., 2016).

Variation in nosZ denitrifying bacterial communities in response to primary succession

We hypothesized that the variation in the composition of the nosZ denitrifying bacterial community would strongly correlate with the successional stage. Although we did find that the soils from the different sites, representing different successional stages, hosted distinct denitrifying bacterial communities, this hypothesis was not clearly supported. Based on the terminal restriction fragments (T-RFs), the microbial communities of the sites T0 and T1 stages were similar and differed from those of the sites T2 and T3. In addition, more minor T-RFs differed between the four successional stages, with each stage having its specific T-RFs. This
leads us to infer that the communities were relatively stable in the early stages of primary
succession, and only minor T-RFs contributed to the structural differences. Similar results have
been found in previous studies, suggesting that successional patterns of the compositions and
abundances of different denitrifying bacteria provide evidence that different denitrifying
bacterial communities develop in the course of primary succession. The relative abundance of
the denitrifying bacterial community increased with the development of the newly formed soils,
but significant differences were recorded only for older sites ([Sigler et al., 2002; Kandeler et al.,
2006]). The minor T-RFs contributed to the community structural differences that were tested in
different ecological systems, and [Baldrian et al. (2013) and Gu et al. (2017)] indicated that the
core denitrifying bacteria may not be sensitive to wetland degradation, whereas some minor
denitrifying bacteria were sensitive to primary succession (environmental changes).

Ollivier et al. (2011) have indicated that N cycling processes start from diazotrophs at the
initial succession sites of the glacier retreat areas, which can directly transform N₂ into
ammonium which is sufficient to activate the nitrification process and further influence
denitrification. Our findings show that denitrification processes were initiated in the T0 site,
representing the starting point of primary succession, as evidenced by the predominance of
Mesorhizobium in the early stages (0 and 20 years); this genus has a strong nitrogen fixation
ability and promotes plant growth ([Velez et al., 2017]). At the T2 site, Brachybacterium was the
dominant genus, with a high nitrogen fixation and denitrification capacity; it mostly grows in
acidic-neutral soil ([Saeki et al., 2017]). Pseudomonas was found in the three sites T0, T1, and T2;
it has the ability to degrade cellulose, produce amylase, and dissolve calcium phosphate, and
based on its antibacterial properties, it has a certain certain inhibitory effect on soil diseases
([Meyer et al., 2017; Ramette et al., 2006; Almario et al., 2014]). Sulfuritalea was stable
throughout succession; it is a facultative anaerobic bacterial genus that is autotrophic under
denitrification conditions and still abundant in low-oxygen nitrate-free environments ([Watanabe
et al., 2017]). In our study, those common nosZ denitrifying bacterial taxa were highly adapted to
the environmental conditions. The minor T-RFs differed among the four stages, and 45- and 70-
bp T-RFs were mainly found in the T1 site. Nitrospirillum and 95-bp T-RFs were found in T2
and T3, while the 90-bp T-RF was specific for the T0 and T1 sites. Those specific T-RFs were
sensitive to any changes in the course of primary succession, and the denitrification mechanism
of those specific T-RFs in the primary succession of glacier retreat areas deserves further
investigation.

Relationships between nosZ denitrifying bacterial communities and soil
properties

Environmental factors can significantly affect both the denitrification process and the
functional genes involved in this microbially mediated pathway, and factors such as carbon
substrate availability, moisture, pH, and other edaphic nutrients play crucial roles ([Wallenstein et
al., 2006]). A previous study has shown that organic carbon is the limiting factor in microbial
processes and activities in the early successional stages without vegetation cover, and carbon
accumulation affects the availability of soil nitrogen ([Wardle et al., 2004]), in this sense, soil
organic carbon and nitrogen levels in retreating glaciers can become the main driving factor in shaping the composition of nitrogen-related microbial communities, and soil pH is the most important factor impacting denitrification processes (Zeng et al., 2016; Čuhel et al., 2011; Kim et al., 2015). Jha et al. (2017) have pointed out that the nosZ gene community structure had the strongest correlation with soil moisture content and available phosphorus. In a similar study, Tang et al. (2016) showed that phosphorus had a strong influence on the abundance of nitrogen-cycling microorganisms in forest plantations, with a strong positive correlation between soil available phosphorus and nitrogen cycling-related microbial functional gene abundance. Our study also revealed that soil pH, AP, AK, and PDA significantly affect the composition of the nosZ denitrifying bacterial communities. Soil pH was significantly negatively correlated with denitrifying bacterial evenness (Eh), while soil AP and AK were positively correlated with the Shannon diversity index (H). Soil AP, AK, and PDA were positively linked to richness (S). Consistent with previous studies, AP and AK were the most important factors affecting the composition of nosZ denitrifying bacterial communities (Tang et al., 2016). According to a previous study, soil AP limits plant productivity and indirectly affects soil microbial communities (Rangwala et al., 2012). Variations in the soil mineral elements stimulate the microbial metabolic rates and activities, consequently changing the microbial community composition. Soil microorganisms also significantly impact nutrient levels via nutrient cycling (Gaimster et al., 2017). However, in forest ecosystems, carbon and nitrogen are generally abundant, while phosphorus is the main factor affecting the abundance of microbial functional genes. The different environmental factors had different influences on the microbial communities and abundances. Nitrogen and carbon accumulation and nutrient availability shape the soil microenvironment, although the geographic location of glaciers significantly affects microbial succession (Zeng et al., 2015). However, microbial activities also influence and shape the soil microenvironment through feedback mechanisms, regulating the stability and diversity of ecosystems.

Conclusions

We could show that the variation in soil properties, potential denitrifying activity, abundance, and composition of the nosZ denitrifying bacterial community differed among different successional stages. Soil pH decreased gradually in the course of succession, while SOC and TN significantly increased with primary succession, and AP and AK increased gradually and peaked at T2. The potential denitrifying activity and the nosZ gene copy numbers followed the same trend as AP and AK, while the composition of the nosZ denitrifying bacterial community differed among successional stages. Overall, 13 nosZ T-RFs were detected in the primary succession stages; Mesorhizobium was the dominant genus across all stages, followed by Brachybacterium and Azospirillum. The minor T-RFs were sensitive to the environmental variation and contributed to the structural differences in the microbial community. Among the measured soil properties, AP and AK were the key factors affecting the composition of the denitrifying bacterial community (P < 0.01). The results of this study could contribute to support
a theoretical foundation for predicting the soil denitrifying bacterial community structure variations in the primary succession of Hailuogou Glacier retreat areas.

Acknowledgments

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References


Wu X, et al., 2017. Effects of land-use change and fertilization on N_{2}O and NO fluxes, the abundance of nitrifying and denitrifying microbial communities in a hilly red soil region of southern China. *Applied Soil Ecology* **120:**111-120 DOI: 10.1016/j.apsoil.2017.08.004.


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Fig.1 Soil samples were collected in sites representing a chronosequence of primary succession in the Hailuogou Glacier area. Triangles correspond to Site 1 - 0 years, Site 2 - 20 years, Site 3 - 40 years, and Site 4 - 60 years.

Fig.2 Principal components analysis of the soil properties in different soil successional stages. T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years.

Fig.3 The nosZ gene copy numbers of different soil successional stages. T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years. Error bars indicate ± SD.

Fig.4 The nosZ-denitrifying bacterial community composition of different soil successional stages. T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years.

Fig.5 Redundancy analysis ordination diagram of the nosZ-denitrifying bacterial community composition associated with environmental variables. T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years. SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium; PDA: Potential denitrifying activity.
**Table 1** (on next page)

Details of the sampling sites within the Hailuogou Glacier retreat area.

**Note:** Horizontal distance refers to the distance from the glacier.
Table 1 Details of the sampling sites within the Hailuogou Glacier retreat area.

<table>
<thead>
<tr>
<th>Site code</th>
<th>Successional time (years)</th>
<th>Longitude</th>
<th>Latitude</th>
<th>Horizontal distance (m)</th>
<th>Elevation (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>≤5</td>
<td>101° 59′ 32.8″</td>
<td>29°34′ 03.6″</td>
<td>100</td>
<td>2,956</td>
</tr>
<tr>
<td>T1</td>
<td>≤20</td>
<td>101° 59′ 40.2″</td>
<td>29° 34′ 04.1″</td>
<td>300</td>
<td>2,948</td>
</tr>
<tr>
<td>T2</td>
<td>≤40</td>
<td>101° 59′ 43.7″</td>
<td>29° 34′ 05.7″</td>
<td>600</td>
<td>2,940</td>
</tr>
<tr>
<td>T3</td>
<td>≤60</td>
<td>101°59′ 48.9″</td>
<td>29° 34′ 07.9″</td>
<td>1,200</td>
<td>2,926</td>
</tr>
</tbody>
</table>

**Note.**

Horizontal distance refers to the distance from the glacier.
**Table 2** (on next page)

Soil properties of the different sampling sites

**Note:** Values represent mean ± standard error (n = 5). Values within the same column followed by the same letter do not differ at P < 0.05. SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium. PDA: Potential denitrifying activity.
<table>
<thead>
<tr>
<th>Sampling code</th>
<th>pH</th>
<th>SOC (g/kg)</th>
<th>TN (g/kg)</th>
<th>AP (mg/kg)</th>
<th>AK (mg/kg)</th>
<th>PDA (µg/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>8.41 ± 0.08a</td>
<td>4.87 ± 0.14d</td>
<td>0.29 ± 0.01d</td>
<td>0.98 ± 0.07c</td>
<td>13.60 ± 0.45d</td>
<td>0.08 ± 0.00d</td>
</tr>
<tr>
<td>T1</td>
<td>7.85 ± 0.03b</td>
<td>9.36 ± 0.35c</td>
<td>0.49 ± 0.02c</td>
<td>1.24 ± 0.04c</td>
<td>40.45 ± 1.78c</td>
<td>0.10 ± 0.01c</td>
</tr>
<tr>
<td>T2</td>
<td>7.31 ± 0.05c</td>
<td>16.19 ± 0.63b</td>
<td>0.76 ± 0.04a</td>
<td>5.54 ± 0.34a</td>
<td>120.80 ± 3.36a</td>
<td>0.31 ± 0.01a</td>
</tr>
<tr>
<td>T3</td>
<td>7.19 ± 0.15c</td>
<td>30.77 ± 1.93a</td>
<td>1.11 ± 0.09b</td>
<td>3.70 ± 0.15b</td>
<td>73.59 ± 2.23b</td>
<td>0.13 ± 0.00b</td>
</tr>
</tbody>
</table>

**Note.**
Values represent mean ± standard error (n = 5). Values within the same column followed by the same letter do not differ at $P < 0.05$. SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium. PDA: Potential denitrifying activity.
Table 3 (on next page)

The *nosZ* gene diversity along successional stages

**Note:** Values represent mean ± standard error (*n* = 5). Values within the same column followed by the same letter do not differ at *P* < 0.05.
<table>
<thead>
<tr>
<th>Sample codes</th>
<th>Shannon-Wiener index (H)</th>
<th>Richness (S)</th>
<th>Evenness (E_h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>1.82 ± 0.03c</td>
<td>8.99 ± 0.30c</td>
<td>1.03 ± 0.01c</td>
</tr>
<tr>
<td>T1</td>
<td>1.87 ± 0.03c</td>
<td>10.04 ± 0.38c</td>
<td>1.13 ± 0.04b</td>
</tr>
<tr>
<td>T2</td>
<td>2.54 ± 0.05a</td>
<td>18.41 ± 0.36a</td>
<td>1.28 ± 0.01a</td>
</tr>
<tr>
<td>T3</td>
<td>2.32 ± 0.07b</td>
<td>11.57 ± 0.47b</td>
<td>1.30 ± 0.02a</td>
</tr>
</tbody>
</table>

**Note.**

Values represent mean ± standard error (n = 5). Values within the same column followed by the same letter do not differ at P < 0.05.
**Table 4** (on next page)

Pearson’s correlations between soil properties, potential denitrifying activity, and *nosZ* gene diversity

**Note:** SOC: Soil organic carbon; TN: Total nitrogen; AP: Available phosphorus; AK: Available potassium; PDA: Potential denitrifying activity. **P < 0.01, *P < 0.05.**
Table 4 Pearson's correlations between soil properties, potential denitrifying activity, and nosZ gene diversity

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>SOC</th>
<th>TN</th>
<th>AP</th>
<th>AK</th>
<th>PDA</th>
<th>nosZ gene abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shannon-Weiner index ($H$)</td>
<td>-0.879</td>
<td>0.684</td>
<td>0.776</td>
<td>0.995**</td>
<td>0.968*</td>
<td>0.871</td>
<td>0.901</td>
</tr>
<tr>
<td>Richness ($S$)</td>
<td>-0.658</td>
<td>0.292</td>
<td>0.428</td>
<td>0.932</td>
<td>0.953*</td>
<td>0.999**</td>
<td>0.995**</td>
</tr>
<tr>
<td>Evenness ($Eh$)</td>
<td>-0.995**</td>
<td>0.879</td>
<td>0.944</td>
<td>0.872</td>
<td>0.872</td>
<td>0.647</td>
<td>0.728</td>
</tr>
<tr>
<td>nosZ gene abundance</td>
<td>-0.709</td>
<td>0.335</td>
<td>0.473</td>
<td>0.935</td>
<td>0.970*</td>
<td>0.990**</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Note.
SOC: Soil organic carbon; TN: Total nitrogen; AP: Available phosphorus; AK: Available potassium; PDA: Potential denitrifying activity. **$P < 0.01$, *$P < 0.05$. 
Soil samples were collected in sites representing a chronosequence of primary succession in the Hailuogou Glacier area.

Triangles correspond to Site 1 - 0 years, Site 2 - 20 years, Site 3 - 40 years, and Site 4 - 60 years.
Principal components analysis of the soil properties in different soil successional stages.

T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years.
Figure 3 (on next page)

The nosZ gene copy numbers of different soil successional stages.

T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years. Error bars indicate ± SD.
The bar chart shows the log_{10} number of nosZ gene copies g^{-1} dry soil across different successional stages. The stages are labeled as T0, T1, T2, and T3. The chart indicates a trend where the log_{10} number of nosZ gene copies increases from T0 to T3.
The nosZ-denitrifying bacterial community composition of different soil successional stages.

T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years.
**Figure 5 (on next page)**

Redundancy analysis ordination diagram of the *nosZ*-denitrifying bacterial community composition associated with environmental variables.

T0: Soil successional age ≤ 5 years; T1: Soil successional age ≤ 20 years; T2: Soil successional age ≤ 40 years; T3: Soil successional age ≤ 60 years. SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium; PDA: Potential denitrifying activity.