Research Article

Response of Spatial Patterns of Denitrifying Bacteria Communities to Water Properties in the Stream Inlets at Dianchi Lake, China

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Streams are an important sink for anthropogenic N owing to their hydrological connections with terrestrial systems, but main factors influencing the community structure and abundance of denitrifiers in stream water remain unclear. To elucidate the potential impact of varying water properties of different streams on denitrifiers, the abundance and community of three denitrifying genes coding for nitrite (nirK, nirS) and nitrous oxide (nosZ) reductase were investigated in 11 streams inlets at the north part of Dianchi Lake. The DGGE results showed the significant pairwise differences in community structure of nirK, nirS, and nosZ genes among different streams. The results of redundancy analysis (RDA) confirmed that nitrogen and phosphorus concentrations, pH, and temperature in waters were the main environmental factors leading to a significant alteration in the community structure of denitrifiers among different streams. The denitrifying community size was assessed by quantitative PCR (qPCR) of the nirS, nirK, and nosZ genes. The abundance of nirK, nirS, and nosZ was positively associated with concentrations of total N (TN) and PO₄³⁻ (p < 0.001). The difference in spatial patterns between nirK and nirS community diversity, in combination with the spatial distribution of the nirS/nirK ratio, indicated the occurrence of habitat selection for these two types of denitrifiers in the different streams. The results indicated that the varying of N species and PO₄³⁻ together with pH and temperature would be the main factors shaping the community structure of denitrifiers. Meanwhile, the levels of N in water, together with PO₄³⁻, tend to affect the abundance of denitrifiers.

1. Introduction

It has been commonly observed that a large but variable proportion of aquatic N removal occurred in freshwater ecosystems, including groundwater, streams, lakes, and wetlands [1, 2]. The variation in microbial abundance and community structure among different aquatic ecosystems has been recognized as one of the most important factors which contributed to the changing of N biogeochemical cycling process in different aquatic ecosystems [3, 4]. Therefore, studying the spatial patterns of functional microbial guilds can help us to understand the relationships between microbial community ecology and the related ecosystem functions. Canonical denitrification has been generally considered as the main mechanism for permanent removal of N from aquatic ecosystem through returning of N in water to the atmosphere in the form of N₂O and N₂, although some alternative pathway, such as anaerobic ammonium oxidation (Anammox), has been discovered [5]. Denitrification in aquatic ecosystems has been widely found removing large proportion of the total N inputs to watersheds and thus providing a valuable ecosystem service by alleviating the impact of increased human N inputs [1]. Several studies have reported that the function of denitrifying bacteria communities was correlated with their abundance and community structure [6–8]. Hence, studying the variation in
abundance and structure of denitrifying community will help to understand the variable denitrification potential as well as variable proportion of aquatic N removal that occurred among different aquatic ecosystems.

Streams are an important sink for anthropogenic N owing to their hydrological connections with terrestrial systems. The microbial communities in streams adapted to changes in the concentration and makeup of organic matter [9] and nutrients [10, 11]. Some recent studies have been concentrated on the link between the freshwater bacterioplankton dynamics and the environmental changes [12]. Numerous studies have reported that denitrifying bacteria can be affected by physical and chemical parameters such as pH, temperature (T), dissolved oxygen (DO), and N forms [12, 13] in series of laboratory incubation experiments. However, it is hard to identify the factors driving the variation in abundance and community structure of denitrifying bacteria in complicated aquatic ecosystems. Nowadays, functional markers include nitrite reductase (nirK and nirS) and nitrous oxide reductase (nosZ) genes have been frequently used to analyze the diversity and abundance of denitrifying bacteria community in the processes of denitrification and their response to the changing of environmental factors [8, 14–18]. Based on the analysis of these functional markers, recent research has demonstrated that the variation in the assemblage of nirS, nirK, and nosZ populations in soil was closely related to temperature, pH, and DO [19–21]. Furthermore, the abundance of these denitrifies varied in response to different nitrogen concentrations in soil, and a differential response of denitrifies communities structure to environmental gradients has also been reported [22]. These mean that functional genes of nirS, nirK, and nosZ could be sensitive indicators when studying the response of denitrifier community to variation of environmental gradients in complicated ecosystems. So far, limited studies incorporated the phosphorus concentration into the analysis of environmental gradient resulting in denitrifies community change in freshwater ecosystems [17], despite phosphorus being a vital element influencing microbial spatial patterns [23]. Therefore, in this study, we will focus on the environmental factors that have been reported to be closely related with denitrification and the variation of denitrifier community (e.g., nitrogen forms, nitrogen concentration, pH, water temperature, and DO) and the less addressed factor such as phosphorus concentration.

Dianchi Lake is the sixth largest freshwater lake in China. There are 35 streams radially flowing into the Dianchi Lake, which is a shallow plateau freshwater lake in the south-west of Kunming city, Yunnan province of China. Streams around the lake serve as ecohydrological channels that impose anthropogenic stress on the lake ecosystem and eventually cause water quality deterioration [24]. Due to the sedimentation, land reclamation, and excessive pollution, the water quality of 35 streams continued degrading from the level of drinking water quality in 1975 to the level of landscape-use only water in 2009 [24, 25]. The water quality in the north part of Dianchi Lake is the worst grade of national water quality standard. There were relatively higher NH_4^+ and NO_3^- concentration even up to 12–20 mg/L in some rivers. The pH values of all sites were alkalescent [24, 26]. More than 6 sewage treatment plants (STPs) had been in operation near 11 streams in the north lake side in recent years. The effluents from the STPs are a major cause of degraded water quality in the down streams within the basin. Generally, effluents are characterized by high concentrations of nitrogen and organic matter [27]. Along with high concentration of nitrogen, many microorganisms especially denitrifying bacterial community entrained in effluents were domesticated [28].

In the present study, in order to address the response of spatial patterns of denitrifying communities to variation in environmental factors among different streams, we investigated the water properties and the abundance and diversity of denitrifying bacterial community in 11 stream inlets with different pollution sources, some of which were receiving effluents from different sewage treatment plants in the north part of Dianchi Lake, an eutrophic lake located in Southwest China (Table 1). We hypothesized that the variation in pollution sources and effluents types, such as the main eutrophication elements of N and P, may modulate

| Streams | NO_3^- (mg L^-1) | TN (mg L^-1) | PO_4^- (mg L^-1) | TP (mg L^-1) | ORP (mg L^-1) | DO (mg L^-1) | pH | T (°C) |
|---------|-----------------|--------------|-----------------|-------------|--------------|--------------|-----|--------|
| XBX     | 4.79 ± 0.39     | 7.44 ± 0.19  | 0.33 ± 0.01    | 0.39 ± 0.01 | 18.45 ± 1.63 | 2.15 ± 0.15  | 7.83 ± 0.06 | 19.60 ± 0.00 |
| H       | 0.16 ± 0.02     | 23.69 ± 0.79 | 2.07 ± 0.07    | 2.10 ± 0.07 | -156.80 ± 29.56 | 0.20 ± 0.10  | 7.90 ± 0.01 | 18.80 ± 0.00 |
| XB      | 0.56 ± 0.14     | 6.35 ± 0.21  | 0.39 ± 0.01    | 0.45 ± 0.03 | -46.55 ± 9.83 | 0.55 ± 0.25  | 7.78 ± 0.06 | 17.95 ± 0.05 |
| YA      | 8.99 ± 0.00     | 15.08 ± 0.59 | 0.22 ± 0.01    | 0.28 ± 0.01 | -15.95 ± 4.03 | 1.05 ± 0.35  | 7.85 ± 0.07 | 17.60 ± 0.10 |
| JJ      | 0.43 ± 0.02     | 8.14 ± 0.54  | 0.36 ± 0.01    | 0.41 ± 0.01 | 59.55 ± 2.19  | 1.30 ± 0.10  | 7.90 ± 0.01 | 19.50 ± 0.00 |
| GPG     | 0.08 ± 0.02     | 23.66 ± 0.16 | 1.78 ± 0.11    | 1.86 ± 0.04 | -225.75 ± 12.80 | 0.20 ± 0.10  | 7.83 ± 0.00 | 17.90 ± 0.00 |
| PLJ     | 5.86 ± 0.01     | 8.08 ± 0.19  | 0.16 ± 0.01    | 0.28 ± 0.00 | 48.05 ± 0.78  | 2.80 ± 0.00  | 8.03 ± 0.00 | 19.70 ± 0.00 |
| XBH     | 5.92 ± 0.25     | 8.60 ± 0.19  | 0.13 ± 0.01    | 0.19 ± 0.01 | 37.53 ± 1.34  | 0.60 ± 0.00  | 7.77 ± 0.00 | 21.60 ± 0.00 |
| CF      | 5.09 ± 0.03     | 8.63 ± 0.10  | 0.10 ± 0.01    | 0.13 ± 0.01 | 49.50 ± 3.25  | 3.80 ± 0.10  | 7.90 ± 0.06 | 21.75 ± 0.05 |
| DG      | 12.00 ± 0.67    | 12.91 ± 0.62 | 0.06 ± 0.00    | 0.12 ± 0.02 | 43.90 ± 0.28  | 2.70 ± 0.13  | 7.56 ± 0.04 | 22.15 ± 0.05 |
| XYL     | 0.08 ± 0.02     | 21.40 ± 2.78 | 0.98 ± 0.09    | 2.52 ± 0.16 | -246.75 ± 17.18 | 0.45 ± 0.05  | 7.78 ± 0.02 | 21.55 ± 0.05 |

Stream names: XBX = Xinbaoxiang, H = Haixue, XB = Xiaba, YA = Yaoan, JJ = Jinjiang, GPG = Guangpugou, PLJ = Panlongjiang, XBH = Xibahe, CF = Chuangfang, DG = Duguang, and XYL = Xinyunliang.

(1) TSN = total soluble nitrogen.
the communities of denitrifiers, which may further lead to changes in the biogeochemical cycling of N in the streams and lake. The results would also provide valuable information on how the abundance and diversities of denitrifiers in various streams responded to the variation of water properties such as nitrogen forms, nitrogen concentration, phosphorus concentration, pH, water temperature, and DO. It was also expected to shed some lights on understanding the variable denitrification potential as well as variable proportion of aquatic N removal that occurred among different streams.

2. Materials and Methods

2.1. Site Description, Sampling, and Water Properties. There are 11 streams around Dianchi Lake located from 24°9’ to 25°0’ latitude and 102°6’ to 102°7’ longitude that were investigated (Figure 1).

Dissolved oxygen (DO), oxidation reduction potential (ORP), pH, and water temperature were measured in situ using portable meter (YSI ProPlus, USA) at all sampling sites. Three replicates of surface water (0–0.5 m) samples were randomly collected at three sampling locations from each sampling site of eleven streams using a cylinder sampler on 25 September 2012. Basically, the three sampling locations at each sampling site were from the upper, middle, and lower sections of a stream. One-liter water samples were reserved at −4°C with addition of HgCl2/acid solution for chemical analysis, and two-liter water samples were filtered immediately for further molecular DNA extraction after being transported to laboratory. The concentrations of total nitrogen (TN), total phosphorus (TP), nitrate (NO₃⁻), and phosphates (PO₄³⁻) in the water samples were analyzed using a SEAL AutoAnalyzer 3 (SEAL Analytical Co., Hampshire, UK).

2.2. DNA Extraction. All water samples were kept in an ice box, transferred to the laboratory, and filtered through a 5 µm pore size sterilized filter to remove the impurities. The resultant filtrate of each sample (500 mL) was filtered through 0.22 µm Millipore membrane filters using a vacuum air pump and the membranes were stored at −80°C for DNA extraction. The membranes were cut into pieces with sterilized scissors and used immediately for DNA extraction. DNA extractions were performed using an E.Z.N.A. Water DNA Kit (OMEGA Bio-Tek Inc., Doraville, GA, USA) by following the manufacturer’s instructions. The DNA samples were stored in a −20°C freezer until use.

2.3. Real-Time Polymerase Chain Reaction Assay. The plasmids containing nirK, nirS, and nosZ fragments from environmental samples were used to create standard curve. The PCR amplified products were cloned into vector pMD19-T using the pMD19-T vector system I kit according to the manufacturer’s instructions (Takara, Dalian, China). The recombinant plasmids were inoculated into LB broth with ampicillin and incubated at 37°C overnight. Plasmid DNA was then extracted from the correct insert clones of each
target gene using the E.Z.N.A. Plasmid Mini Kit II (OMEGA Bio-Tek Inc., Doraville, GA, USA) according to the manufacturer’s instructions. The plasmids DNA concentration was determined by NanoVue spectrophotometer (GE Healthcare Europe, Munich, Germany), and then the copy numbers of target genes were calculated. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate to generate an external standard curve.

The real-time polymerase chain reaction (qPCR) was performed on ABI 7500 real-time system (Life technologies, USA). The plasmids DNA concentration was determined by NanoVuespectrophotometer (GE Healthcare) according to the manufacturer’s instructions. The plasmids DNA concentration was determined by NanoVue spectrophotometer (GE Healthcare Europe, Munich, Germany) and then the copy numbers of target genes were calculated. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate to generate an external standard curve.

The real-time polymerase chain reaction (qPCR) was performed on ABI 7500 real-time system (Life technologies, USA) to assess gene abundance. Amplification was performed in a 20-µL reaction mixture using SYBR Premix Ex Taq as PCR Kit provided by the suppliers (Takara bio, Dalian, China). The DNA diluted template corresponding to 1–10 ng of total DNA extracts was used in each reaction mixture. The primers and procedures used to amplify each target gene when performing real-time PCR were listed in Table 2. Data was analyzed using the 7500 software (version 2.0.6, Life technologies, USA). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. Standard curves for real-time PCR assays were created according to the method described by Henry et al. [34].

The amplified products were pooled and resolved on DGGE gels using a Dcode system (Bio-Rad Laboratories Inc. Hercules, USA). PCR samples (50 µL) containing approximately equal amounts of PCR amplicons were loaded onto the 1 mm thick 8% (w/v) polyacrylamide (37.5:1, acrylamide:bisacrylamide) gels in 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA) with denaturing gradients of 50–75% for 15 h (nirS), 50–70% for 12 h (nirK), and 50–70% (nosZ) (100% denaturant contains 7 mol/L urea and 40% (v/v) formamide) for 15 h at 100 V and 60 °C, respectively. After being stained with silver nitrate according to the protocol [35], polaroid pictures of the DGGE gels were scanned using an EPSON (Perfection V700 Photo) scanner and stored as TIFF files and digitized and then analyzed with the Quantity One software (version 4.5, Bio-Rad, USA).

Table 2: Primers and thermal profiles used for the qPCR and DGGE.

| Target gene | primers                  | Thermal profile                                           |
|-------------|--------------------------|----------------------------------------------------------|
| qPCR        | nosZ-F [29]              | qPCR: 94°C/2 min; 6 cycles of 94°C/30 s, 57°C/30 s (–1°C/cycle), and 72°C/45 s; 30 cycles of 94°C/30 s, 52°C/30 s, and 72°C/45 s. |
|             | nosZ1622R [29]           | DGGE: 94°C/2 min; 10 cycles of 94°C/30 s, 58°C/30 s (–0.5°C/cycle), and 72°C/60 s; 30 cycles of 94°C/30 s, 53°C/30 s, and 72°C/60 s; 72°C/10 min. |
| DGGE        | nosZ-F [29]              | q-PCR: 94°C/2 min; 6 cycles of 94°C/30 s, 57°C/30 s (–1°C/cycle), and 72°C/45 s; 30 cycles of 94°C/30 s, 52°C/30 s, and 72°C/45 s. |
|             | nosZ1622-GC* [30]        | DGGE: 94°C/2 min; 10 cycles of 94°C/30 s, 57°C/30 s (–0.5°C/cycle), and 72°C/45 s; 30 cycles of 94°C/30 s, 53°C/30 s, and 72°C/45 s; 72°C/10 min. |
| qPCR        | Cd3aF [31]               | q-PCR: 95°C/3 min; 6 cycles of 95°C/30 s, 63°C/30 s (–1°C/cycle), and 72°C/30 s; 32 cycles of 95°C/30 s, 58°C/30 s, and 72°C/45 s. |
|             | R3cd [31]                | DGGE: 95°C/3 min; 32 cycles of 95°C/30 s, 58°C/30 s, and 72°C/45 s. |
| DGGE        | Cd3aF [31]               | DGGE: 95°C/3 min; 32 cycles of 95°C/30 s, 58°C/30 s, and 72°C/45 s; 72°C/10 min. |
|             | R3cd-GC* [32]            |                                                                 |

* (GGGCGCCGCGCGCGCGGCCCGCCTCGCCC) was attached to the 5’ end of the primers.

2.5. Data Analysis. Three replicates were used in all parameter analysis. Data were presented as the mean values of triplicates and the maximum difference (mean ± SD) among triplicate results was 5%. One way analysis of variance (ANOVA) was performed to test whether there were any significant differences among the means at the 95% confidence level. Potential relationships between all denitrifying bacteria abundance and environmental data sets were tested by Pearson correlation analysis. All data were analyse using SPSS software.

DGGE banding profiles for nirS, nirK, and nosZ communities were digitized after average background subtraction for entire gels. Band position and intensity date for each sample were exported to an excel spreadsheet prior to further statistical analyses. The relative intensity of a specific band was transformed according to the sum of intensities of all bands in a pattern [36]. Redundancy analysis (RDA) for community ordination was conducted using CANOCO (version 4.5, Centre for Biometry, Wageningen, The Netherlands) for Windows using relative band intensity data obtained from the Quantity One analysis [37, 38]. Among all environmental variables, eight parameters, including water temperature, pH, DO, oxidation reduction potential nitrate, total nitrogen, total phosphorus (TP), and phosphates (PO₄³⁻), were selected to perform RDA by Monte Carlo reduced model tests with 499 unrestricted permutations to statistically evaluate the significance of the first canonical axis and of all canonical axes together. Statistical significance was kept at p < 0.05 for all analyses (Table 4).
Table 3: Shannon index (H) and richness (S) values of nirK, nirS, and nosZ genes.

| Streams | S  | H    | S  | H    | S  | H    |
|---------|----|------|----|------|----|------|
| XB      | 10.00 ± 1.00cd | 2.09 ± 0.12b | 17.00 ± 1.40f | 5.00 ± 0.80b | 6.00 ± 1.40e | 1.00 ± 1.90f |
| H       | 13.33 ± 1.53bc | 2.33 ± 0.27ab | 14.33 ± 1.58b | 2.47 ± 0.29ab | 11.00 ± 1.00def | 1.95 ± 0.19a |
| XB      | 11.33 ± 0.58cd | 2.20 ± 0.15ab | 18.00 ± 1.00a | 2.50 ± 0.20a | 12.67 ± 0.58cde | 2.34 ± 0.22a |
| YA      | 11.00 ± 0.00cd | 2.10 ± 0.06b | 14.33 ± 0.58b | 2.42 ± 0.25ab | 15.33 ± 1.15ab | 2.55 ± 0.21a |
| JJ      | 17.33 ± 1.58a | 2.64 ± 0.14a | 10.00 ± 1.00cd | 1.92 ± 0.19b | 10.33 ± 0.58ef | 2.09 ± 0.25a |
| GPG     | 15.00 ± 0.00ab | 2.47 ± 0.17ab | 15.33 ± 0.58b | 2.43 ± 0.31ab | 17.33 ± 0.58a | 2.60 ± 0.18a |
| PLJ     | 13.00 ± 1.00bc | 2.22 ± 0.11ab | 8.33 ± 1.53cd | 1.92 ± 0.07ab | 9.67 ± 0.58f | 1.94 ± 0.29a |
| XBH     | 15.00 ± 1.00ab | 2.36 ± 0.09ab | 15.33 ± 1.15b | 2.50 ± 0.40ab | 14.67 ± 0.58bc | 2.56 ± 0.15a |
| CF      | 11.33 ± 1.15cd | 2.34 ± 0.19ab | 10.33 ± 1.15cd | 2.26 ± 0.28ab | 13.33 ± 1.15bcd | 2.39 ± 0.25a |
| DG      | 11.00 ± 1.00cd | 2.32 ± 0.33ab | 13.67 ± 0.58bc | 2.37 ± 0.16ab | 12.00 ± 1.00cde | 2.43 ± 0.32a |
| XYL     | 9.67 ± 1.52d | 2.08 ± 0.06b | 8.00 ± 0.00cd | 1.91 ± 0.07ab | 8.67 ± 0.58f | 1.89 ± 0.19a |

Stream names: XB = Xinbaoxiang, H = Haihe, XB = Xiaba, YA = Yaoan, JJ = Jinjia, GPG = Guangpugou, PLJ = Panlongjiang, XBH = Xibahe, CF = Chuangfang, DG = Daguang, and XYL = Xinyunliang. The different letters indicate significant differences (p < 0.05).

Table 4: Eigen values, F values, and p values obtained from the partial RDAs testing the influence of the significant parameters on the denitrifying bacterial community composition.

| Samples | Environmental variables | Eigen value | % variation explains solely | F value | p value |
|---------|-------------------------|-------------|-----------------------------|--------|--------|
| nirK    | PO4<sup>3−</sup>        | 0.21        | 21                          | 2.37   | 0.002  |
|         | TN                      | 0.19        | 19                          | 1.32   | 0.190  |
|         | pH                      | 0.10        | 10                          | 1.12   | 0.398  |
|         | All the above together  | 0.80        |                             |        |        |
| nirS    | pH                      | 0.10        | 10                          | 1.20   | 0.298  |
|         | Temperature             | 0.11        | 11                          | 1.20   | 0.360  |
|         | DO                      | 0.08        | 8                           | 1.10   | 0.388  |
|         | All the above together  | 0.77        |                             |        |        |
| nosZ    | Temperature             | 0.17        | 17                          | 1.83   | 0.048  |
|         | NO3<sup>−</sup>         | 0.13        | 13                          | 1.17   | 0.060  |
|         | TP                      | 0.08        | 8                           | 1.31   | 0.278  |
|         | All the above together  | 0.83        |                             |        |        |

* Only keeping the first three significant parameters in models of RDAs based on Monte Carlo permutation (n = 499). Sum of all Eigen values for both partial RDAs was 1.000.

3. Results

3.1. Spatial Patterns of Structure and Size of Denitrifier Communities. All digitized data of the three replicates were used in statistics analysis. The Shannon index (H) of nirK, nirS, and nosZ calculated from DGGE gels ranged from 2.08 to 2.64, 1.73 to 2.80, and 1.89 to 2.60, respectively. The significant differences among them were observed statistically (p < 0.05). There were relatively lower richness and diversity of nirK, nirS, and nosZ in Xinyunliang stream (Table 3), while the highest of the Shannon values and richness of nirK, nirS, and nosZ occurred in Guangpugou stream.

The results of qPCR showed that the abundance of nirS, nirK, and nosZ gene copies per mL water ranged from 2.79 × 10<sup>3</sup> to 1.20 × 10<sup>5</sup>, 3.23 × 10<sup>3</sup> to 1.76 × 10<sup>5</sup>, and 8.66 × 10<sup>2</sup> to 1.90 × 10<sup>5</sup>, respectively. The abundance of nirK, nirS, and nosZ denitrifiers in Xinbaoxiang, Daguan, Chuangfang, and Panlongjiang streams was relatively stable and low. The results of nirK, nirS, and nosZ genes abundance in group Xiaba and Yaoan streams were consistent. The absolute abundance of denitrification genes in the other five streams varied widely (Figure 2). These results indicated that different pollution source would influence the abundance of denitrifiers in streams. The denitrifiers (nirK, nirS, and nosZ) abundance of the water samples in Haihe stream and Guangpugou stream was significantly higher than that in other stream samples (p < 0.05). The abundance of nirK- and nosZ-type denitrifiers had similar trends in the streams, with higher number in Haihe stream and Guangpugou stream and intermediate levels in Xinbaoxiang, Xiaba, Yaoan, Jinjia, Panlongjiang, and Xiaba streams but lower levels in Chuangfang stream, Daguang stream, and Xinyunliang stream. However, the nirS-type abundance in all sites did not totally follow this trend and no significant differences were detected in qPCR data among Haihe, Guangpugou, and Daguang streams.

Ratios of nosZ abundance to the abundance of nirK + nirS for all samples varied widely. The highest nosZ/(nirK + nirS) ratio occurred in Haihe stream (0.64), the lowest
nosZ/(nirK + nirS) ratio occurred in Daguang stream (0.08), and the nosZ/(nirK + nirS) ratios in the other streams were similar and ranged from 0.13 to 0.35. Ratios of nirK abundance nirK/nirS to nirS abundance in different streams were also different. The highest (nirK/nirS) ratio occurred in Daguang stream (18.79), the lowest (nirK/nirS) ratios occurred in Xinyunliang stream (0.48), and the (nirK/nirS) ratios in the other streams were between 0.90 and 2.15. These results implied that the abundance of nirS was not always greater than that of nirK in stream inlet water column.

3.2. Water Parameters Controlling Denitrifier Communities. In order to determine to what extent the eight environmental properties affected the three types of denitrifying genes on their community compositions, DGGE fingerprints were analyzed by redundancy analysis. The results showed that PO₄³⁻, pH, and water temperature were the relatively important environmental parameters for denitrifiers (Table 5). For nirK-type denitrifier, PO₄³⁻, TN, and pH explained 50% variations of microbial communities. Variation partitioning analysis showed that PO₄³⁻, TN, and pH separately explained 21% ($p = 0.002$), 19% ($p = 0.190$), and 10% ($p = 0.398$) of the variation, respectively. The analysis did not reveal significant relationship between nirS-type denitrifier communities and any environmental parameters. For nosZ-type denitrifier, temperature (17%, $p = 0.048$), NO₃⁻ (13%, $p = 0.06$), and total P (8%, $p = 0.278$) explained 38% variations of microbial communities.

nirS abundance was significantly and positively correlated with nirK and nosZ abundance. nirK abundance was significantly correlated in a positive direction with nosZ abundance. These results suggested that all of three denitrifiers can interact with each other. The abundance diversification of nirK, nirS, and nosZ was strongly and positively associated with TN and PO₄³⁻ ($p < 0.001$). The analysis did not reveal significant relationship between pH and any denitrifying bacteria gene abundance. All relationships between nirS, nirK, and nosZ genes abundance and chemical variables were positively correlated except for DO, ORP, T, and NO₃⁻, which were negatively correlated with the copy numbers of nirS, nirK, and nosZ genes. The NO₃⁻ concentration was a key parameter influencing the ratios of nirK abundance of nirS.
A significant correlated correlation existed between ratios of nosZ abundance to the abundance of nirK + nirS and the concentration total nitrogen.

4. Discussion

4.1. The Variation of Community Pattern of Denitrifying Bacteria according to Pollution Sources and Effluent Types of Different Streams. Nitrogen cycle in aquatic ecosystems is predominantly controlled by bacteria, and their activities determine the fate of nitrogen compounds. Meanwhile, environmental conditions that regulate the activity of bacteria determine where each nitrogen transformation process occurs and the degree of exchange among various nitrogen pools. Thus, chemical information of different nitrogen species alone is not sufficient to predict rates of nitrogen transformation processes in the environment, and information concerning characteristics of nitrogen cycling bacterial community under various environmental conditions is essential for understanding the related nitrogen cycle process. With regard to the denitrification process, previous studies have shown that it was regulated by various environmental factors such as oxygen and nitrogen concentration, quality, temperature, and pH [5, 39]. However, how the denitrifying bacteria communities were correlated with the environmental factors in streams, receiving massive amount of nutrients and pollutants, remains unclear.

In this study, we evaluated differences in the genetic makeup of the communities by comparing DGGE profiles for denitrification genes encoding nitrite and nitrous oxide reductase (nirK, nirS, and nosZ). It has been suggested that DGGE was a powerful tool for identifying and comparing the dominant of these communities [40, 41]. The DGGE results in this study revealed the significant pairwise differences in the community structure of denitrifying bacteria containing the nirK, nirS, or nosZ genes among the water samples collected from different streams. When comparing the diversities of denitrifier communities from all sites, similar trends emerged with low richness and diversity in the Xinyunliang stream. According to previous studies, Xinyunliang streams run through a historical area of old Kunming city, where there are many industries (such as Yunnan smelter), high population density, and poor sewage networks [42]. This serious industrial pollution could reduce bacterial diversity and damage microbial ecological system [43, 44], although the concentration of NO$_3^-$, the substrates for denitrification, in Xinyunliang stream was higher than most of other streams with exception of Guangpugou and Haihe streams. Our studied streams differed substantially in the amount of inorganic nutrients which were potentially available to denitrifiers and other microbial populations during the development of microbial community in the water column. The three large streams around Dianchi Lake, Panlongjiang, Daguan, and Chuanfang streams [42], were the important sites receiving effluents from the STPs, with the characteristics of high nitrate concentrations. Nevertheless, the fast-flowing water and irregular discharge of effluents prevented the stream from developing a stable environment for microbial colonization and propagation. Therefore, these streams also represented relatively low diversities of denitrifying bacteria. It has been reported that the high stream flow and nitrate concentration of streams were the major factors controlling the development of planktonic denitrifier populations [45]. On the contrary, the streams of Haihe and Guangpugou were of narrow and slow-flowing, which leading to the long residence time for nitrogen-containing pollutants and well-established hypoxic ($\sim$0.2 mg L$^{-1}$ in DO) environment. Therefore, the abundance of denitrifiers was much higher in Haihe and Guangpugou streams than the other streams, which may enhance denitrification in Haihe and Guangpugou streams [46]. This result was consistent with the previous reports that the most transformation of inorganic nitrogen occurred in narrow streams [47]. Simultaneously, an ecological engineering project using Eichhornia crassipes for nutrient removal has been conducted in the 11 streams around Dianchi Lake since June 2011. The roots of E. crassipes [48, 49] in streams provided a large specific area for denitrifiers to attach, which would benefit the formation

|   | nirK | nirS | nosZ | nirK/nirS | nosZ/(nirK + nirS) |
|---|------|------|------|-----------|-------------------|
| nirK | 1    |      |      |           |                   |
| NirS | 0.933** | 1    |      |           |                   |
| nosZ | 0.886** | 0.770** | 1    |           |                   |
| nirK/nirS | -0.011 | -0.225 | -0.1743 | 1           |                   |
| nosZ/(nirK + nirS) | 0.349* | 0.264 | 0.632*** | -0.396 | 1               |
| DO   | -0.513*** | -0.411* | -0.420* | -0.175 | -0.333 |
| pH   | 0.111 | 0.201 | 0.244 | 0.019 | 0.021 |
| Temp (°C) | -0.507*** | -0.543** | -0.459** | 0.357* | -0.378* |
| ORP  | -0.582*** | -0.649*** | -0.525** | 0.272 | 0.337* |
| NO$_3^-$ (mg L$^{-1}$) | -0.394* | -0.519** | -0.449*** | 0.665*** | -0.047 |
| TN (mg L$^{-1}$) | 0.709*** | 0.698*** | 0.676*** | 0.008 | 0.426** |
| PO$_4^{3-}$ (mg L$^{-1}$) | 0.868*** | 0.872*** | 0.875*** | 0.007 | -0.334* |
| TP (mg L$^{-1}$) | 0.560*** | 0.601*** | 0.601*** | 0.283 | 0.377* |

* is significant at the 0.05 level (two-tailed); ** is significant at the 0.01 level (two-tailed); *** is significant at the 0.001 level (two-tailed).
of biofilms and therefore may further change or modify the 
diversity and abundance of denitrifiers in water [50]. Pre-
vious studies suggested that microbial biofilms were highly 
efficient and successful ecological communities that might 
also contribute to the influence of the headwater streams 
on streams, estuaries, and even oceans [51]. Therefore, the 
slow-moving flows such as Haihe and Guangpugou streams 
could be considered as living zones of transient storage, 
where roots and other biofilms bring hydrodynamic retention 
and biochemical processing into close spatial proximity and 
influence biogeochemical processes and patterns in streams. 
All of these results coincided with our hypothesis that the 
pollution sources and effluent types of different streams 
would modulate the community composition of denitrifying 
bacteria to a great extent, although a complex picture of 
denitrifier community similarity emerged depending on 
which functional denitrification gene was evaluated. 

In this study, we found that the distribution of nir-
denitrification genes was much patchier, which was 
consistent with earlier observations in other streams [52, 53]. 
Studies of changes in composition and diversity of nirK 
and nirS genes communities support the hypothesis of niche 
differentiation among denitrifying bacteria [54–56]. 
Meanwhile, this study found the abundance of nirS was not 
consistently greater than that of nirK in stream inlet water 
column, which was different from some of the previously 
published results [53, 57]. However, in a similar way, some of 
previous studies also suggested that the spatial distribution 
of nirS and nirK genes abundance differs in other types of 
urban streams, reflecting different habitat preferences [52, 
53]. The results of the nirK/nirS ratios suggested that the 
nirK-type denitrifiers might be more abundant than nirS-
type denitrifiers in Daguang stream (nirK/nirS ratio, 18.79), in 
contrast to Panlongjiang stream (nirK/nirS ratio, 0.90). How-
ever, the concentrations of DO in the two streams were both 
relatively higher (2.8 mg L−1) than others. This contradicted 
with previous studies that nirK often prevailed in condi-
tionally O2−-exposed environments [53, 58]. This discrepancy 
probably was due to other environmental parameters such 
as nitrogen and phosphorus concentrations [21, 53] which 
varied significantly in the two streams. Even though the nirK 
and nirS are functionally equivalent, denitrifying bacteria 
harboring either nitrite reductase was likely not under the 
same community assembly rules [59]. Philippot et al. [60] 
suggested that the existence of the two types of nitrite 
reductase (nir-gene) was due to differential niche preferences. 
The different community patterns, together with the spatial 
distribution of the nirS/nirK abundance ratio, can suggest 
habitat selection for the nirS- and nirK-type denitrifiers [17]. 
In the present study, the different spatial patterns of nirK and 
nirS community diversities, in combination with the spatial 
distribution of the nirS/nirK ratio, indicated habitat selection 
for the two types of denitrifiers. Denitrifying organism 
includes either nirS or nirK, but not both of the two-type 
nitrite reductase genes [61, 62], and experiments have shown 
the two nitrite reductases to be functionally redundant, as 
one nir-type gene in denitrifying bacteria can be eliminated 
and replaced by the other type [63]. This, however, did not 
necessarily indicate that nirK-type denitrifiers contributed 
more or less in denitrification than nirK-type ones. Hence, 
gene expression analysis is necessary to further investigate 
which is more important in denitrification in the stream inlet 
water column around Dianchi Lake.

4.2. Relationship between Water Properties and Spatial Pat-
terns of Denitrifying Bacteria. In the present study, the 
RDA charts of nirK, nirS, and nosZ genes indicated that 
diversity of denitrifying populations had varying response to 
environmental factors, and the concentrations of P (PO4−3– or 
TP) and N (NO3− or TN) were the most important 
environmental factors causing a significant alteration in the 
denitrifier community structure among different streams by 
serving as essential nutrients for microorganisms growth 
in streams. Meanwhile, abundance of all denitrifiers in this 
study was, by and large, controlled by the water parame-
ters, especially nutrient (phosphorous and nitrogen) con-
centration (Table 3). Certainly, microbes need phosphorus 
for their growth and function. Finlay et al. found increasing 
phosphorous inputs associated with eutrophication could 
indirectly promote N losses via enhancing denitrification 
[64]. Additions of P have been demonstrated to increase N 
removal in whole-ecosystem experiments in both lakes and 
streams [65], which provides further support for the 
role of P as an important control over N cycling and fate 
in freshwater ecosystems. In addition, different responses 
of community diversities of nirS- and nirK-denitrifiers to 
the changes of phosphorous concentrations agreed with a 
study suggesting that nirK-denitrifiers were most sensitive 
to alteration of phosphorous concentration [21]. Contrary 
to previous studies [17, 46], our results implied that variation 
of phosphorous content in water was positively linked to 
the abundance of nirS and nirK genes and resulted in 
shift of community structure of nir-containing denitrifiers 
populations. This may further clarify the important function 
of phosphorous in shaping microorganisms structure in 
environments [23, 46]. However, the mechanisms concerning 
how phosphorus would affect growth of denitrifying bacteria 
in water are still not well understood [21]. Further studies 
are needed to explain underlying mechanism related to the 
role of P in regulating the denitrifiers’ community, although 
our results have built some supporting evidence for the related 
phenomenon. In addition, the water temperature and pH 
were the main factors driving the changes in the denitrifying 
bacterial community composition among different streams. 
The genes nirS, nirK, and nosZ abundance was shown by 
Pearson correlation coefficient (r) to be mightily influenced 
by water temperature and oxidation reduction potential 
(ORP). The quality of inlet water in 11 streams differed with 
water origin and pollution sources [24, 25, 42]. It has been 
shown that the changes in denitrifying community structures 
responded to their habitat conditions like temperature and 
DO gradient and N forms [12, 13, 66, 67]. The pH was 
known to generally affect denitrifier community diversity and 
richness [15]. Generally, the effect of temperature on driving 
biogeochemical processes is either to alter the functioning 
bacteria without changing the microbial communities or 
Restructuring communities, thus modifying the fundamental
physiologies [68]. Previous studies suggested that temperature could directly or indirectly affect the communities' diversity and abundance of denitrifying organisms [16, 20, 69–71].

5. Conclusions

The results showed that abundance and diversities of denitrifying genes (nirK, nirS, and nosZ) were variable in the streams of Dianchi Lake. Nutrient concentrations (nitrogen and phosphorous), water temperature, and pH were important environmental factors to alter abundance and community structure of the denitrifiers significantly. The different community patterns, together with the spatial distribution of the nirS/nirK abundance ratio, suggest habitat selection for the nirS- and nirK-type denitrifiers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Neng Yi and Yan Gao contributed equally to this paper.

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