ISEIS-Environmental Informatics

Characteristics of Ammonia-oxidizing and Denitrifying Bacteria at the River-sediment Interface

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Abstract

The hyporheic zone is essential in retaining nutrients and regulating downstream nutrient fluxes, but little is known about microbial communities in this area. The community structure of ammonia-oxidizing and denitrifying bacteria were studied at the river-sediment interface along a transect across an urban river. Using denaturing gradient gel electrophoresis (DGGE) analysis, the study showed that the ammonia-oxidizing and denitrifying bacteria presented the highest diversity at the oxic/fluctuating position, due to the flexible variations of water level and oxygen content. At this position, phylogenetic analysis revealed that amoA sequences mainly grouped with Nitrosospira and Nitrosomonas, which were belonged to β-Proteobacteria. Meanwhile, for denitrifying bacteria, they were mainly belong to α-, β-, γ-Proteobacteria, distributed across four broad clusters. Most of nosZ genes were closely related to uncultured environmental clones, indicating a high level of species richness in the denitrifying bacterial population. Thus, the ammonia-oxidizing and denitrifying bacteria could coexist at the river-sediment interface, which might play an important role in nitrogen removal of eutrophic rivers.

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Keywords: River-sediment interface; Nitrifier; Denitrifier; Eutrophication.

1. Introduction

Increasing population, agricultural advancement and urbanization have resulted in higher nitrogen (N) loading to rivers through aqueous wastes from several key industries (e.g. fertilizer, fish canning, refinery and tannery), agricultural runoff and domestic wastes [1, 2]. The additional nitrogen may contribute to the eutrophication of both freshwater and marine environments, harm the ecosystem, weaken the water body vitality, and deprive water use applications [3]. Recent studies have emphasized the importance of the hyporheic zone in the reduction of nitrogen in N-rich streams [4, 5]. Advections of oxygen and nutrients between the surface and subsurface water result in the
oxidized/anoxic area at the river-sediment interface of the hyporheic zone. And antagonistic processes, i.e., nitrification and denitrification, can simultaneously occur at the interfaces where oxygen content fluctuates frequently. Accordingly, the interface is an active site of biogeochemical transformations in the river that regulated the flux of nutrients between ecosystems [6]. Thus, this interface can enhance nitrogen cycling and is regarded as a hot spot of aquatic systems [7]. In nitrogen cycling processes, nitrification followed by denitrification are regarded as the main biological loss terms for fixed nitrogen. Studies have shown that the composition of microbial communities is important in nutrient cycling and organic matter transformation [8]. Thus, it is reasonable to assume that nitrogen cycling processes in the hyporheic zone are associated with different community structures.

However, most studies conducted to evaluate nitrogen cycling processes in the hyporheic zone have focused on the importance of physicochemical conditions, including water exchanges, nutrients flux, and the hydraulic conductivity of hyporheic sediments [4]. Indeed, little attention has been given to the bacterial diversity in nitrogen removal processes [9]. Accordingly, there is an urgent need for an improved understanding of the diversity of ammonia-oxidizing bacteria (AOB) and denitrifying bacteria in this area. To address this knowledge gap, we evaluated the bacterial diversity at the water-sediment interface along a transect across an urban river. Previous studies have investigated the entire bacterial community using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments [10]. However, the 16S rRNA gene sequence similarities among different AOB are so high that the ammonia monooxygenase (amoA) primer set is highly specific for AOB and is suitable for assessing community shifts [11]. For denitrifying bacteria, targeting 16S rRNA genes also cannot reveal their presence and physiological role in the study area as they are spread among diverse phylogenetic groups [12]. Despite this, analysis of the functional genes (e.g., nitrous oxide reductase (nosZ)) involved in denitrification represents a suitable alternative.

In this study, a portion of the Fuhe River, which is mainly composed of urban sewage and treated wastewater from Baoding city in northern China [13], was evaluated. We investigated the bacterial diversity at the river-sediment interface using polymerase chain reaction (PCR) amplification and DGGE targeting the amoA and nosZ genes. The objectives of this study were the following: (1) to investigate the spatial variations of AOB and denitrifying bacteria at the river-sediment interface; (2) to determine how the environmental factors influenced the distribution of AOB and denitrifying bacteria in this aquatic system.

2. Materials and Methods

2.1. Study area

As the only inflow river with perennial flows, the Fuhe River discharges a great deal of nitrogen pollutants into the Baiyangdian Lake, which is the largest natural freshwater body in the North China Plain. Sediments were sampled on 31 March 2010 from the NS site in the Fuhe River (Latitude: 38°50′18.5″N; Longitude: 115°35′30.7″E). The sampling locations were positioned at the water-sediment interface along a transect across the river (water depths of 0, 0.25, 0.5, 0.75, and 1 m; Fig. 1). Surface sediment samples (< 10 cm depth) were collected and homogenized, after which they were stored in sterile centrifuge tubes, transported to the laboratory in the dark in refrigerated ice chests and then stored in a refrigerator at 4°C. The sediment sample characteristics are shown in Table 1.

The soil pH was measured using a soil: water (1:1) extract. The total organic carbon (TOC) and total organic nitrogen (TON) contents of the sediment samples were measured using an Elemental Analyzer (EA, Vario EL, Elementar, Germany). To extract mineral nitrogen from the sediment, 5 g aliquots of moist sediment were taken from each bag and shaken in 25 ml of 1 M KC1 for 1 h before filtration through 0.45 μm Whatman GF/C glass fiber filters. The NH4+-N and NO3--N concentrations in the filtered extracts were then determined by standard colorimetric methods using an autoanalyzer. The oxidation reduction potential (ORP) was measured on site (Thermo Orion, America).
2.2. Potential activity assays

The potential NO$_3^-$ plus nitrite (NO$_2^-$) accumulation rates were determined as an indication of nitrification activity in the samples [14]. Approximately 5 g of fresh sediment were placed in 250-ml flasks in triplicate and then diluted in 100 ml of sterile isotonic solution with (NH$_4$)$_2$SO$_4$ (140 mg l$^{-1}$ NH$_4^+$-N, final concentration). The flasks were then incubated at 30°C at 150 rpm for 24 h. Meanwhile, the potential NO$_3^-$ plus nitrite (NO$_2^-$) reduction rates were used to indicate the denitrification activity in the samples [14]. Approximately 5 g of fresh sediment were placed in 250-ml flasks in triplicate and then diluted in 100 ml of sterile isotonic solution. The atmosphere in the flasks was exchanged after five evacuation and filling cycles with N$_2$. After agitation for 30 min, the samples were supplemented with KNO$_3$ (140 mg l$^{-1}$ NO$_3^-$-N, final concentration) and sodium citrate (5 g l$^{-1}$). The flasks were then incubated at 30°C for about 24 h. Controls with sterilized sediments were also incubated. Samples for the analysis of NO$_3^-$-N and NO$_2^-$-N concentrations (2 ml) were collected directly from the slurries every 4 h, centrifuged for 2 min at 12,000 g and then filtered.

2.3. Number of AOB and denitrifying bacteria

The most probable number (MPN) assay was used to enumerate the AOB and denitrifying bacteria [15]. Briefly, fresh sediment (10 g) was blended in 100 ml of sterile phosphate-buffered saline (PBS), after which the sediment suspensions were diluted 10-fold to $10^{-7}$. Sterilized Hungate tubes containing the nitrification medium were inoculated with 1 ml of each dilution from $10^{-3}$ to $10^{-7}$ in triplicate. The composition of the nitrification medium used in this experiment was as follows (g l$^{-1}$): (NH$_4$)$_2$SO$_4$ 2.0 g; K$_2$HPO$_4$ 0.75 g; MnSO$_4$$\cdot$$4$H$_2$O 0.01 g; NaH$_2$PO$_4$ 0.3 g; CaCO$_3$ 5.0 g; MgSO$_4$$\cdot$$7$H$_2$O 0.03 g (pH 7.0). The tubes were incubated at 30°C for about 2 weeks, and scored for those in which NO$_3^-$-N and NO$_2^-$-N accumulated. And Sterilized Hungate tubes containing the denitrification
medium and inverted glass Durham tubes were then inoculated with 1 ml of each dilution from $10^{-3}$ to $10^{-7}$ in triplicate. The composition of the denitrification medium used in this experiment was as follows (g l$^{-1}$): sodium citrate 5.0; KNO$_3$ 2.0; KH$_2$PO$_4$ 1.0; K$_2$HPO$_4·3$H$_2$O 1.0; MgSO$_4·7$H$_2$O 0.2 (pH 7.0) [16]. The tubes were incubated at 30°C for about 2 weeks, after which the NO$_3^-$ removal was tested and those with growth and gas production were scored. Tubes that met the following three criteria (growth, nitrate removal, gas production) were considered positive for denitrification. NO$_3^-$-N and NO$_2^-$-N in the medium was confirmed by observing the color formation.

2.4. DNA extraction and PCR amplification

DNA was extracted from 1 g to 1.5 g (wet weight) of homogenized sediment using an Ultra Clean Soil DNA Isolation Kit (Qbiogene, CA, USA). Fragments of nosZ genes were amplified using the following primer set: nosZ-F (5'-CGC TGT TCA TCG ACA GCC AG-3') or nosZ-F-GC before DGGE, and nosZ-R (5'-CAT GTG CAG AGC ATG GCA GAA-3') which amplify ~700 bp of the nosZ gene [17]. The primer pair amoA-F (5'-GGGG GTT TCT GCT GGT GGT-3') and amoA-R (5'--CCCCTC (GT)G(GC) AAA GCC TTC TTC-3') was used for the amplification of the ammonia monooxygenase gene amoA [18]. PCR amplification was conducted in a total volume of 25 μl containing 2.5 μl of 10×PCR buffer (containing 1.5 mM MgCl$_2$), 0.2 mM of each deoxynucleotide triphosphate, 2.0 U of Taq polymerase (Sangon, Shanghai, China), 25 pM of each primer and 10–100 ng DNA. For amplification of the environmental samples, 400 ng μl$^{-1}$ BSA was added to the reaction mixtures. PCR for denitrifying bacteria was conducted by subjecting the samples to the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. And for AOB, PCRs were performed with initial denaturation at 94°C for 10 min, followed by 25 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s with a final extension at 72°C for 15 min.

2.5. DGGE analysis of the amoA and nosZ genes

DGGE was conducted using a D-code system (Bio-Rad, Hercules, California, USA) in accordance with the manufacturer’s instructions. The PCR amplification products were loaded onto 8% polyacrylamide gels in 1.0 × TAE (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA; pH 8.4). The polyacrylamide gels contained denaturant gradients of 35–60% for the amoA-F-GC plus amoA-R PCR products and 30–60% for separation of the nosZ-F-GC plus nosZ-R PCR products. Gels were run for 10 h at 120 V and then stained for 30 min with SYBR Green I (1:10,000 dilution) for visualization under UV excitation. The DGGE images were analyzed using the Quantity One software (Bio-Rad). Analysis of the band patterns was conducted using the Dice coefficient with 4% position tolerances and 0.5% optimization for the band migration distance. Clustering of the patterns was performed using Ward’s dendrogram.

Individual DGGE bands were excised, reamplified by PCR, then rerun on denaturing gradient gels to verify the purity of the PCR reamplification products. Automated sequencing of the purified PCR products was performed on an ABI sequencer using the ABI PRISM dye terminator cycle sequencing reaction kit (Perkin-Elmer, Foster City, Calif.). To estimate the level of similarity with the published sequences, all sequences generated in this study were compared with the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database sequences using a BLAST search. Alignment of the DNA sequences was conducted with the Clustal X program [19], and phylogenograms were edited using MEGA version 4.1 [20]. Phylogenograms were constructed by the neighbor-joining method with 1,000 replicate trees [21]. The method described by Kimura was employed to correct for multiple substitutions [19].

3. Results

3.1. Physical-chemical characteristics at the river-sediment interface

As shown in Table 1, depth-related gradients of physical parameters were observed at the water-sediment interface along the transect. The ORP decreased from -15 mV to -334 mV from site 1 to site 5, indicating that the study area had a relatively reductive environment. The moisture and TOC increased with water depth. Additionally,
the NO$_3^-$-N concentration increased from site 2 to site 5, whereas the NH$_4^+$-N concentration decreased. However, the NH$_4^+$-N and NO$_3^-$-N concentrations showed sudden changes at site 1 when compared with other sampling sites. The water fluctuated frequently at site 1, which resulted in the sediment at this position being semi-saturated and oxic/fluctuating. These conditions may have lead to the unique characteristics observed at this site.

The NO$_3^+$ plus NO$_2^-$ accumulation rate increased from site 5 to site 1 (Fig. 2), indicating that the nitrifying activity of AOB increased with the rise of ORP. As oxygen content is the major impact factor of nitrification, the oxic/fluctuating position (e.g. site 1) favours the activity and the growth of nitrifying bacteria. Meanwhile, The NO$_3^+$ plus NO$_2^-$ reduction rate increased from site 2 to site 5 due to the increase in TOC and NO$_3^-$-N concentration (Fig. 2). However, it was higher at site 1 when compared with the sediments from other sampling sites. Additionally, the population of denitrifying bacteria varied in accordance with the potential denitrifying rates, indicating that the activity and number of denitrifying bacteria were highest at site 1.

Table 1. Characteristics of sediment samples [N=3, mean (stderr)]

<table>
<thead>
<tr>
<th>Site</th>
<th>Depth (cm)</th>
<th>pH</th>
<th>Moisture content (%)</th>
<th>ORP (mV)</th>
<th>TOC (g kg$^{-1}$ DW)</th>
<th>TON (g kg$^{-1}$ DW)</th>
<th>NH$_4^+$-N (mg kg$^{-1}$ DW)</th>
<th>NO$_3^-$-N (mg kg$^{-1}$ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>6.6</td>
<td>40.5</td>
<td>-15 (5)</td>
<td>21.68 (1.91)</td>
<td>1.15 (0.34)</td>
<td>44.87 (2.32)</td>
<td>4.04 (0.21)</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>6.5</td>
<td>63.6</td>
<td>-125 (11)</td>
<td>29.78 (0.73)</td>
<td>2.02 (0.78)</td>
<td>145.22 (4.57)</td>
<td>2.87 (0.32)</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>6.5</td>
<td>64.8</td>
<td>-257 (7)</td>
<td>43.55 (2.34)</td>
<td>3.69 (0.69)</td>
<td>144.73 (6.71)</td>
<td>2.81 (0.21)</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>6.4</td>
<td>61.1</td>
<td>-348 (8)</td>
<td>40.28 (4.32)</td>
<td>2.75 (0.45)</td>
<td>101.06 (6.72)</td>
<td>4.20 (0.23)</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>6.5</td>
<td>66.6</td>
<td>-334 (17)</td>
<td>47.18 (2.31)</td>
<td>3.41 (0.22)</td>
<td>121.30 (7.83)</td>
<td>5.07 (0.44)</td>
</tr>
</tbody>
</table>

DW: dry weight

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(a) AOB bacteria population (h$^{10^4}$ g$^{-1}$ DW)

(b) NO$_3^+$ + NO$_2^-$ accumulation rate (mg N kg$^{-1}$ h$^{-1}$ DW)
Fig. 2. The AOB / denitrifying bacteria population (a) and NO$_3^-$ + NO$_2^-$ accumulation / reduction rate (b) along the transect across the river

3.2. Distribution of amoA and genotypes

In recent years, amoA genes are used as the popular gene target to investigate the phylogenetic relationships of AOB communities. As shown in Fig. 3a, the DGGE bands were grouped to three main clusters of AOB. The dominant bands (bands A, B and C) were classified into the *Nitrosomonas eutrophaea* (Fig. 4). Band D was affiliated to *Nitrosospira* sp. And the other three bands were grouped with *Nitrosolobus multiformis*. The results showed that the AOB detected at river-sediment interface were mainly belonged to *Nitrosospira* and *Nitrosomonas eutrophaea*, which were belonged to β-Proteobacteria. Since the digital signal intensity of bands is an indicator of bacteria population, we conclude that *Nitrosomonas eutrophaea* was more abundant in the study area.

Even though AOB did exist at the river-sediment interface (see Fig. 2a), the amoA genes were not successfully amplified from site 2 to site 5. Since the amoA primers used in this study were mainly targeting the β-Proteobacteria, the inability of detecting sequences at these positions might be due to inadequacy of the primer set, as well as low population levels, or biases associated with the DNA extraction procedure.

Fig. 3. DGGE fingerprints of amoA of AOB (a) and nosZ genes of denitrifying bacteria (b) at different sampling sites (site 1 to site 5). From the negative image of DGGE gels, profiles of the highlighted bands were compared. The corresponding dendogram calculated on the basis of the Dice’s similarity coefficient, was constructed with the Ward’s clustering algorithm. Distances are measured in arbitrary units.

[Diagram of the dendrogram and DGGE fingerprints]

**Band E**

**Band F**

**Band G**

*Nitrosolobus multiformis* (X90822)

**Nitrosovibrio tenus* (L76552)

**Nitrosospira* sp. (AF042170)

**Band D**

**Band B**

**Band C**

*Nitrosomonas europaea* (AF058692)

*Nitrosomonas europaea* (L08050)

**Band A**

Distances measured in arbitrary units.
3.3. Distribution of nosZ and genotypes

As shown in other studies of different types of aquatic habitats, DGGE can reveal differences in bacterial assemblages under different environmental conditions [22]. The DGGE banding pattern of nosZ showed spatial changes in the presence of bands according to depth (Fig. 3b). Ward’s dendrogram analysis of the nosZ profiles revealed that all samples were clustered together except for those collected from site 1, indicating that the DGGE pattern of the nosZ genotypes from the site 1 sample was different from the other sediment samples. All bands were observed in samples collected from site 1, while only bands C, F and G were present in all DGGE profiles. Additionally, the number and intensity of the bands increased from site 2 to site 5, indicating a depth-related change in sample types.

Comparison with the NCBI database using a BLAST search, phylogenetic analysis of sequences of nosZ revealed that they fell into four distinct clusters (Fig. 5). nosZ genes of cluster 1 detected in this study were 81–95% similar to environmental sequences retrieved from paddy soils (unpublished) and aquatic environments [23]. Cluster 2 sequences were most similar to Rubrivivax gelatinosus, belonged to β-Proteobacteria. Cluster 3 sequences were 82–92% similar to Pseudomonas mandelii and Pseudomonas sp., which belonged to γ-Proteobacteria and retrieved from samples of cultivated cropping soils [24]. nosZ genes of cluster 4 were most similar to nosZ from bacterial isolates affiliated with α-Proteobacteria.
4. Discussion

Zhang et al. (2007) [25] observed the coexistence of AOB and anammox bacteria at the water-sediment interface of the bank in the Xinyi River. In the present study, AOB and denitrifying bacteria were found to coexist at the river-sediment interface. Additionally, the microbial abundance and diversity were subjected to gradients and fluctuations of oxygen and nutrient concentrations at different water depths, except for site 1. It is generally accepted that denitrification requires anoxic conditions, although there have been some reports of aerobic denitrification [26, 27]. Thus, the DGGE pattern of nosZ genotypes was found to be richest at site 1 due to the coexistence of aerobic and anoxic denitrifiers. Meanwhile, compared with the anoxic positions of the river, the abundance and activity of AOB showed relatively higher at site 1. Thus, microorganisms were abundant and presented high diversities at site 1, the oxic/fluxuating position, indicating the especially important role that this area played in the nitrogen cycling of the river.

Compared to a large river, denitrification in small, shallow rivers is especially important since more contact and exchange of river water with the hyporheic zone were found [28]. The water depth of the Fuhe River ranges from 0.8 m to 2.7 m. In this shallow urban river, the increased organic loading accelerates the oxygen consumption and results in the formation of the anaerobic or anoxic areas in the hyporheic zone, which stimulates the process of denitrification compared with the nitrification process. Using nosZ genes as functional markers of the denitrifying community, the PCR-based approach has revealed the presence of diverse denitrifier communities in different natural ecosystems, including continental shelf and estuarine sediments [29], stream sediments [23], meadow and forest soils [17, 30]. In this study, most of nosZ genes were similar to the uncultured environmental clones retrieved from aquatic ecosystem or soils, indicating a high level of species richness in the denitrifying bacterial population at the water-sediment interface. The presence of so many denitrifying bacteria indicated that conventional denitrification has played an important role in nitrogen removal.

Therefore, in the study, nitrifying and denitrifying bacteria could coexist at the water-sediment interface of the hyporheic zone. Nitrogen removal could be enhanced by taking advantage of the hyporheic zone as “hot spots” for biological processes. However, the contribution of different groups to nitrogen reduction process in this area is hard to distinguish and thus an essential issue for future research.

Acknowledgements

This research was jointly supported by National Natural Science Foundation of China (50979007) and National Water Environment Management Projects of China Foundation (2008ZX07209-007).

References


