Diversity and abundance of ammonia-oxidizing bacteria in eutrophic and oligotrophic basins of a shallow Chinese lake (Lake Donghu)

Guo-Yuan Chen \textsuperscript{a,b}, Shan-Lian Qiu \textsuperscript{a,b}, Yi-Yong Zhou \textsuperscript{a,*}

\textsuperscript{a} Laboratory of Water Environmental Biochemistry, Institute of Hydrobiology, The Chinese Academy of Sciences, 7 Donghu Nan Road, Wuhan, Hubei 430072, China
\textsuperscript{b} Graduate School of the Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

Received 30 August 2008; accepted 21 January 2009
Available online 4 February 2009

Abstract

Classical cultivation and molecular methods based on the ammonia monooxygenase gene (\textit{amoA}) were used to study the abundance and diversity of beta-proteobacterial ammonia-oxidizing bacteria (AOB) in lake sediments. The eutrophic and oligotrophic basins of a Chinese shallow lake (Lake Donghu), in terms of ammonium (NH$_4$\textsuperscript{+}) concentrations, were sampled. The AOB number was significantly lower in the oligotrophic basin, but significantly higher in the eutrophic basin. In addition, using restriction fragment length polymorphism targeting the \textit{amoA}, ten restriction patterns including six unique ones were found in the eutrophic basin, while five patterns were observed in the oligotrophic basin with only one unique restriction group. Phylogenetic analysis for AOB revealed that \textit{Nitrosomonas oligotropha}- and \textit{Nitrosomonas ureae}-related AOB and \textit{Nitrosospira}-affiliated AOB were ubiquitous; the former dominated in the eutrophic basin (87.2%), while the latter dominated in the oligotrophic basin (65.5%). Furthermore, \textit{Nitrosomonas commutans}-related AOB was only detected in the eutrophic basin, at a small proportion (3.2%). These results indicate significant selection and adaptation of sediment AOB in lakes with differing trophic status.

Keywords: Ammonia-oxidizing bacteria; Ammonia monooxygenase gene; Restriction fragment length polymorphism; Shallow lakes

1. Introduction

Nitrification is a major pathway in the overall nitrogen cycle [6]. It consists of oxidation of ammonia (NH$_3$) to nitrite (NO$_2$) and then to nitrate (NO$_3$). The first step is carried out by chemolithotrophic ammonia-oxidizing bacteria (AOB), which form a tight cluster within the beta subclass of \textit{Proteobacteria} and include members of the genera \textit{Nitrosomonas} (as well as \textit{‘Nitrosococcus mobilis’} and \textit{Nitrospira} (as well as \textit{Nitrosolobus} and \textit{‘Nitrosovibrio’}) [27]. At present, AOB within \textit{Nitrosomonas} are divided into six lineages: \textit{Nitrosomonas europaea} ‘N. mobilis’, \textit{Nitrosomonas communis}, \textit{Nitrosomonas oligotropha} and \textit{Nitrosomonas ureae}, \textit{Nitrosomonas marina}, \textit{Nitrosomonas cryotolerans} and \textit{Nitrosomonas} sp. Nm143 [17].

It is noteworthy that there are differences in the generation time, specific oxidation rates and growth yield between the ammonia-oxidizing genera [3]. For example, \textit{Nitrosospira} have lower growth rate and specific activity than \textit{Nitrosomonas} [3]. \textit{Nitrosomonas} traditionally have been considered the most important bacteria for NH$_3$ oxidation [23]. Furthermore, \textit{Nitrosospira} and \textit{Nitrosomonas} have both been recently found in activated sludge [1], freshwater lakes [30] and estuaries [5]. There is culture-based evidence that \textit{Nitrosospira} strains may be more common in soils [2]. Therefore, it is necessary to quantify and identify AOB in sediment of freshwater lakes; however, relevant information is lacking [8,30]. Moreover, environmental conditions such as oxygen (O$_2$) concentration and substrate availability can affect the species distribution of AOB [5]. Thus, it is hypothesized that trophic status will shape the AOB composition in lake sediments.

At present, molecular methods based on 16S rRNA and the \textit{amoA} gene which encodes the active site subunit of the enzyme...
ammonia monooxygenase [16] have been widely used to study the population structure of AOB (e.g. [27,5,25,8,22,30]). Amplification of functional genes such as amoA could confirm the presence of species that cannot be detected by primers targeting the 16S rRNA gene [22,8,19]. As a functional gene target, amoA is advantageous over the 16S rRNA approach for studying AOB community structure [19] because the latter has reduced specificity, and co-amplification of 16S rRNA sequences from non-nitrifying bacteria occurs [25].

In this study, molecular methods based on the amoA gene were used to identify the population composition of AOB, and the most probable number (MPN) and MPN-PCR techniques were used to enumerate AOB in eutrophic and oligotrophic basins of a Chinese shallow lake (Lake Donghu). The phylogenetic makeup of the population from the two basins was determined by sequence analysis of the amoA gene. The beta-proteobacterial AOB that mostly occur in freshwater, rather than entire ammonia oxidizers, were targeted. The aims of this study were to quantify AOB populations and identify their diversity in sediments, as affected by trophic status of lake basins, and consequently obtain a better understanding of the mechanisms used by AOB to adapt to eutrophic conditions.

2. Materials and methods
2.1. Site description
Lake Donghu is on the northeastern outskirts of Wuchang, Wuhan City, China. It is a component of a large drainage system with a catchment area of 187 km². The lake itself is composed of several basins separated by artificial dikes, with a total surface area of 32 km². The muddy lake bottom is flat and sparsely covered by hydrophytes. The lake is generally 3–4 m in depth with a maximum depth of 4.5 m [33].

Lake Donghu is contaminated by domestic wastewater (80%–90%), non-point sources and industrial wastewater (10%–20%), which contains large amounts of total nitrogen and phosphorus [31]. The pollutants are discharged into Yujia Basin, leading to higher nutrient loading in it. In contrast, Tuanhu Basin was not polluted by point sources. As a larger area (Fig. 1) and the littoral zone are covered by aquatic plants, Tuanhu Basin shows a lower trophic level.

The study was conducted in June, 2007 at sites Y1, Y2 and Y3 in the Yujia Basin, and sites T1, T2 and T3 in the Tuanhu Basin (Fig. 1).

2.2. Sample collection and physicochemical analysis
Surface sediments (0–3 cm) were collected and transported to the laboratory on ice in the dark within 2–4 h for routine analysis. Interstitial water was obtained by centrifugation at 4000 rpm for 20 min. Ammonium (NH₄⁺) concentrations were measured with spectrophotometry by the indophenol-blue method using phenol [29], in interstitial water filtered through pre-washed 0.45-μm polycarbonate filter membranes. The centrifuged sediments were extracted by 2 M KCl, and the aqueous phase was used for NH₄⁺ assay in sediments using the same method. Sediment pH values were determined by PHS-3C numerical pH meters using a sediment to water ratio of 1:5 after shaking for 1 h. Total nitrogen (TN) content in sediments was determined using the semi-micro Kjeldahl method [14].

2.3. MPN enumeration of AOB
The protocol used for MPN enumeration was based on that of Matulewich et al. [11]. 10 g of sediment was mixed with 95 ml of deionized water in the flask with several glass beads and shaken vigorously for 20 min. Serial 10-fold dilutions of the suspension were prepared in 1 mM phosphate buffer solution (pH 7.2), and 1 ml portions were transferred to 5 replicate tubes per dilution with the designated MSF medium [11], which gave the highest MPN count among the three media [18]. Samples were incubated at 28 ℃ in the dark. After 6 weeks of incubation, the AOB medium in each tube containing 6 ml liquid was examined by removing a few drops to a spot plate depression with Griess reagent. If the AOB medium gave a strong reaction (dark red) compared with the uninoculated control, the tube was scored positive for AOB. Counting of positive tubes and subsequent quantification were carried out using the Taylor table [12].

2.4. Extraction and PCR amplification of DNA from sediment samples
The protocol of Zhou et al. [32] was used for DNA extraction from sediments at the six sites. PCR amplification of a 491 bp fragment of the amoA gene was carried out by using the amoA-1F and amoA-2R primer set specific for AOB belonging to the beta subclass of Proteobacteria [19]. Amplification was performed in a total volume of 50 μl in 0.2-ml Eppendorf tubes using a DNA thermocycler (model PTC-200, Bio Rad, USA). The reaction mix was prepared using the following: 1 × PCR buffer (20 mM Tris–HCl, 25 mM KCl, 1.5 mM MgCl₂, 0.5% Tween 20, 100 μg
of bovine serum albumin per ml), 20 nmol of each deoxy-nucleoside triphosphate, 30 pmol of each primer, 1 µl of template DNA, and 2.5 U of Taq DNA polymerase (Fermentas, California, USA). The enzyme was added after the first denaturation step. The standard thermal profile used for amplification of the amoA target sequence was as follows: 5 min at 94 °C; pause at 80 °C to add polymerase; then 48 cycles (environmental samples) consisting of 60 s at 94 °C (denaturation), 90 s at 60 °C (annealing), and 90 s at 72 °C (elongation); and a final cycle consisting of 90 s at 60 °C and 10 min at 72 °C. Aliquots (10 µl) of the PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures.

2.5. Construction and analysis of amoA gene fragment libraries

Two clone libraries were constructed for the two basins in Lake Donghu. The amplified amoA PCR fragments were excised from agarose gel and purified with an agarose gel extraction kit. The 491-bp amoA DNA fragments from two basins were cloned using the TA cloning kit in a pMD18-T vector (TACloning kit; TaKaRa) according to the manufacturer’s recommendations. 144 clones from the Yujia Basin library and 100 clones from the Tuanhu Basin library were randomly selected for further analysis. The cloned inserts were amplified with amoA primers and then digested with theMspI enzyme. Restriction patterns were analyzed after gel electrophoresis on 2% agarose gels. Clones representative of each restriction pattern were chosen for sequence analysis. Selected plasmids were then subjected to sequencing by the manufacturer on a 377 DNA sequencer (PRISM, ABI, USA).

2.6. Sequence alignment and phylogenetic analysis

All sequences have been deposited in GenBank database under accession numbers: EU744309–EU744332. Multiple alignment of sequences was performed using Clustal X [28]. Phylogenetic analysis of all sequences including three out-group sequences retrieved from continuous culture enrichments [4] and 10 groups from water of the Lower Seine River and Estuary [5]. The analyzed Yujia Basin clones clustered into 10 different patterns, while those for Tuanhu Basin were clustered in 5 different patterns (Fig. 3), indicating greater diversity of the amoA gene in sediments of Yujia Basin. Furthermore, the major groups were basin-specific, with restriction group A dominating in the Yujia Basin and restriction group D dominating in the Tuanhu Basin. In addition, 6 groups (restriction groups C, E, F, H, I and J) were unique to the Yujia Basin, but only one group (restriction group K) was unique to the Tuanhu Basin (Table 2), indicating a shift in the community of AOB to take advantage of different substrate concentrations.

Phylogenetic analysis showed that N. oligotropha- and N. ureae-related AOB and Nitrosospira-affiliated AOB were readily detected in both basins; N. communis-related AOB was

Table 1
Basic parameters and nitrogen concentrations in interstitial water and sediments in the study basins.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>T (°C)</th>
<th>pH</th>
<th>NH$_4^+$ in interstitial water (mg/l)</th>
<th>NH$_4^+$ in sediments (mg/kg of dry sediment)</th>
<th>TN in sediments (g/kg of dry sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>26.7</td>
<td>7.3 ± 0.02</td>
<td>3.96 ± 0.134</td>
<td>108.06 ± 1.631</td>
<td>4.31 ± 0.162</td>
</tr>
<tr>
<td>Y2</td>
<td>26.9</td>
<td>7.3 ± 0.04</td>
<td>5.02 ± 0.112</td>
<td>144.82 ± 5.726</td>
<td>4.79 ± 0.169</td>
</tr>
<tr>
<td>Y3</td>
<td>27.0</td>
<td>7.1 ± 0.02</td>
<td>4.88 ± 0.076</td>
<td>139.25 ± 6.410</td>
<td>3.56 ± 0.147</td>
</tr>
<tr>
<td>T1</td>
<td>26.8</td>
<td>6.8 ± 0.01</td>
<td>1.13 ± 0.038</td>
<td>40.30 ± 0.352</td>
<td>2.93 ± 0.108</td>
</tr>
<tr>
<td>T2</td>
<td>26.9</td>
<td>6.9 ± 0.03</td>
<td>0.58 ± 0.048</td>
<td>17.79 ± 2.993</td>
<td>2.58 ± 0.096</td>
</tr>
<tr>
<td>T3</td>
<td>26.8</td>
<td>6.8 ± 0.02</td>
<td>0.98 ± 0.075</td>
<td>29.14 ± 1.693</td>
<td>2.58 ± 0.127</td>
</tr>
</tbody>
</table>
only detected in the eutrophic Yujia Basin (Fig. 4). This spatial heterogeneity can be explained mainly by nitrogen status in the study basins. Both species belonging to *N. oligotropha*—*N. ureae* and *Nitrosospira* lineages would appear to represent K strategists, as defined by their high affinity with resources required for their growth and demand for a selective environment [5]. *N. oligotropha*- and *N. ureae*-related AOB can easily adapt to low NH$_4^+$ concentrations and grow in various environments [4]. Most of the AOB belonging to the *N. oligotropha*-N. ureae lineage were obtained from oligotrophic freshwater environments (rivers and lakes), and some originate from neutral and often moderately acid (pH around 6) soils [10]. Moreover, with high substrate affinity, *Nitrosospira*-affiliated AOB would appear to be more competitive in environments with low NH$_4^+$ concentrations. Thus, their ubiquity in freshwater and terrestrial environments was well documented [8,25]. In addition, urea was an alternative NH$_4^+$ source for many but not all species of AOB [9]. Many species belonging to *N. oligotropha*- *N. ureae* and *Nitrosospira* lineages have been shown to be urease-positive [10,24], which may also help them to inhabit substrate-poor environments.

Even ubiquitous, *N. oligotropha*- and *N. ureae*-related AOB dominated in the sediments of Yujia Basin, accounting for 87.2%, while, *Nitrosospira*-affiliated AOB dominated in the sediments of Tuanhu Basin accounting for 65.5% (Fig. 4). In Yujia Basin, the NH$_4^+$ concentration was significantly higher, which favored growth of *N. oligotropha*- and *N. ureae*-related AOB with higher Km values ranging from 30 to 75 μM NH$_4^+$ [24]. In addition, the decomposition of organic matter may immediately deplete the O$_2$, and *Nitrosomonas* strains constituted about one-fourth of the microbial biomass in an anoxic-tricking filter biofilm [20]. On the other hand, Tuanhu Basin gave a lower NH$_4^+$ concentration, to which *Nitrosospira*-affiliated AOB were more adaptive because of their lower Km values (40 μM NH$_4^+$) [21].

AOB belonging to *N. communis* lineage exhibit strong heterogeneity, according to the ecophysiological traits of their members [10]. They were infrequently detected in freshwater environments, but often obtained from neutral agricultural soils [10] and sewage from wastewater treatment plants [16]. Interestingly, a small proportion of this species (3.2%) was recorded in Yujia Basin (Fig. 4), and this was assumed to derive from wastewater discharge. At the lower NH$_4^+$ concentration in interstitial water in Yujia Basin, these exotic species could grow but not accumulate.

In conclusion, in the two basins studied, higher nitrogen loading in terms of significantly higher NH$_4^+$ concentrations in both sediments and interstitial water was coupled with more abundant sediment AOB, whose *amoA* gene is more diverse as well. Among the AOB species often found in freshwater, *N. oligotropha*- *N. ureae* and *Nitrosospira* strains were commonly detected in the lake under study, but they dominated in the two different basins, respectively, while *N. communis*-related AOB was found only in the eutrophic basin. This characteristic of AOB population structures can be explained by sharp differences in nitrogen status between the two basins and heterogeneity in substrate affinity of the ammonia-oxidizing system among the dominant AOB species. Therefore, the increasing diversity of *amoA* genes and changing dominant

<table>
<thead>
<tr>
<th>Restriction patterns</th>
<th>% of analyzed clones</th>
<th>Representative clones for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yujia Basin</td>
<td>Tuanhu Basin</td>
<td>Yujia Basin</td>
</tr>
<tr>
<td>A</td>
<td>80.9</td>
<td>30.1</td>
</tr>
<tr>
<td>B</td>
<td>5.6</td>
<td>3.3</td>
</tr>
<tr>
<td>C</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>3.2</td>
<td>62.2</td>
</tr>
<tr>
<td>E</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>H</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>K</td>
<td>0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 2: Proportions of analyzed clones in each restriction pattern for the two libraries and representative clones for sequencing.
species are effective strategies for adapting AOB to a eutrophic environment, which is of great ecological significance.

Acknowledgements

This work was supported by the National Basic Research Program of China (2008CB418005) and grants (KZCX2-YW-426-01 and KZCX1-YW-14-1) from the Chinese Academy of Sciences. The guidance offered by Dr. Z. Li is gratefully acknowledged. We thank Miss Y. Li and Mr. H. Shang for competent technical assistance. Mr. L. Peng, Y. Du, L. Chen, W. Xu and Y. Zeng are thanked for their assistance throughout the course of this study.

References

K.H. Schleifer (Eds.), The Prokaryotes (pp. 2625–2637). New York: Springer Verlag.


