

ABUNDANCE AND DIVERSITY OF AMMONIA-OXIDIZING BACTERIA IN RELATION TO AMMONIUM IN A CHINESE SHALLOW EUTROPHIC URBAN LAKE

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Submitted: February 11, 2009; Returned to authors for corrections: May 01, 2009; Approved: July 24, 2009.

ABSTRACT

The measures of most-probable-number and restriction fragment length polymorphism analysis were used to analyze the abundance and diversity of ammonia-oxidizing bacteria in sediment of a Chinese shallow eutrophic urban lake (Lake Yuehu). Among the 5 sampling sites, ammonia concentration in interstitial water was positively proportional not only to the content of organic matter, but also to ammonia-oxidizing bacteria numbers (at a magnitude of 10^5 cells g^{-1} dry weight) in sediment significantly. Furthermore, the diversity of ammonia-oxidizing bacteria were determined by means of PCR primers targeting the *amoA* gene with five gene libraries created and restriction pattern analysis. The 13 restriction patterns were recorded with 4 ones being common among all sampling sites. The 8 restriction patterns including 4 unique ones were found at the site with the highest NH_4^+ concentrations in interstitial water, while, there were only common patterns without unique ones at the site with the lowest NH_4^+ concentrations in interstitial water. Phylogenetic analysis showed that the *amoA* fragments retrieved belong to *Nitrosomonas oligotropha* & *ureae* lineage, *N. europaea* lineage, *N. communis* lineage and *Nitrosospira* lineage, most of which were affiliated with the genus *Nitrosomonas*. The *N. oligotropha* & *ureae*-like bacteria were the dominant species. Thus, the abundance and diversity of sediment AOB is closely linked to ammonium status in eutrophic lakes.

Key words: Ammonia-oxidizing bacteria, Abundance, *amoA*, Diversity.

INTRODUCTION

Nitrification, defined as the oxidation of ammonia to nitrate via nitrite, is a key process of the nitrogen cycling in freshwater lakes. The former is the rate-limiting step (31), which is mediated by the ammonia-oxidizing bacteria (AOB). Therefore AOB are of great ecological significance in freshwater lakes.

Ammonia is the essential energy source for the AOB that can adapt to a broad range of ammonia concentrations in the diverse environments, as reflected by different affinity

constants for ammonia. On the other hand, ammonia is a toxic compound for AOB. Consequently, their succession and colonization in different systems requires tolerance of increasing ammonia concentrations (20). So, ammonia is one of the most important factors, influencing the distribution patterns of AOB (19, 37, 38, 39), and its accumulation may occur in eutrophic lakes with the concentration being up to 4 mg l^{-1} in Onondaga Lake (11). The ammonia concentrations in 33 Chinese lakes ranged from 0.358-1.295 mg l^{-1} on average (49). However, its linkage to AOB was inadequately studied.

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Traditionally, the abundance and population structure of AOB were investigated by cultivation-dependant methods. To quantify AOB, the most probable number (MPN) technique was often used. However, different media may produce different results (1), since some media were unfit for the growth of some AOB under special conditions in nature due to unsuitable ammonia concentration, salinity, or pH value (20). Therefore, the optimum medium (33) was chosen for AOB counting in lake sediments.

Molecular techniques have been allowed to obtain reliable information on the bacterial diversity in different environments (22, 42). The *amoA* gene coding for the catalytic subunit of ammonia monooxygenase reported by McTavish *et al.* (24) has emerged as a useful target for population studies on AOB in nature (5, 25, 34, 43, 50). In freshwater environments, molecular analysis of 16S rDNA and *amoA* genes demonstrated that the majority of AOB belong to β subclass of the *Proteobacteria* including members of the genera *Nitrosomonas* (as well as *Nitrosococcus mobilis*) and *Nitrospira* (as well as *Nitrosolobus* and *Nitrosovibrio*) (15, 41, 44). Furthermore, the natural diversity of AOB in various environments has extensively been studied by comparative sequence analysis of environmental *amoA* clones. Consistent with the 16S rDNA-based AOB diversity surveys, most *amoA* clones obtained are affiliated with 7 lineages defined by cultured AOB. These lineages are described as *Nitrospira* lineage, *Nitrosomonas marina* lineage, *Nitrosomonas oligotropha* lineage, *Nitrosomonas europaea/Nitrosococcus mobilis* lineage, *Nitrosomonas communis* lineage, *Nitrosomonas* sp. Nm143 lineage and *Nitrosomonas cryotolerans* lineage (20). However, few data were available about the AOB composition in lake sediments with different ammonia status.

In this study, MPN technique was used to enumerate AOB and restriction fragment length polymorphism analysis (RFLP) based on *amoA* gene was used to identify its population structure at five sampling sites in sediments of a Chinese shallow eutrophic urban lake (Lake Yuehu). Additionally, the ammonium concentrations in interstitial water and the contents

of organic matter (OM) in sediments were also detected. The aims of this study are (1) to determine the abundance and diversity of AOB in the eutrophic shallow lakes, and (2) to extend our understanding of AOB species occurred in sediments of freshwater lake with different ammonium status.

MATERIALS AND METHODS

Study lakes and sampling sites

Lake Yuehu (114°14'~114°15'E, 30°33'N, 0.66 km², 1.2 m mean depth), is a shallow eutrophic lake located in Wuhan City, Hubei Province. Due to the discharge of vast untreated domestic sewage in the past decades, the contents of organic matter (84.56-158.48 g kg⁻¹), TP (2.23-3.61g kg⁻¹) and TN (3.72-5.84g kg⁻¹) were high in surface sediment (7). Here, surface sediments (0-3 cm) were sampled at five sites in March, 2007 (Fig. 1).

Measurement of chemical parameters in interstitial water and sediment

Interstitial water was obtained from sediment by centrifugation at 4000 rpm for 20 min, and then filtered through 0.45 μ m polycarbonate filter membranes. Ammonium concentration was determined by the indophenol-blue method with spectrophotometry (46). Sediments at each sampling site were oven-dried at 80 °C for 2 days and ignited in a muffle furnace at 550 °C for 3 h to determine the values of loss on ignition (LOI) (16). Sediment pH values were determined using PHS-3C numerical pH meters using a sediment-to-water ratio of 1:5 after shaking for 1 h.

MPN enumeration

MPN enumeration was performed according to Matulewich *et al.* (23). Serial 10-fold dilutions of the suspension were prepared in 1mM phosphate buffer solution (pH 7.2), and 1-ml portions were transferred to 5 replicate tubes per dilution with the designated MSF medium (23). Samples were incubated at 28 \pm 1 °C in the dark. After 6 weeks incubation, the AOB medium was examined by removing a

few drops to a spot plate depression with the Griess reagent. If the medium gave a strong reaction (dark red) compared with the uninoculated control, the tube was scored positive for

AOB. Counting the positive tubes and subsequent quantification could be made using a table (26).

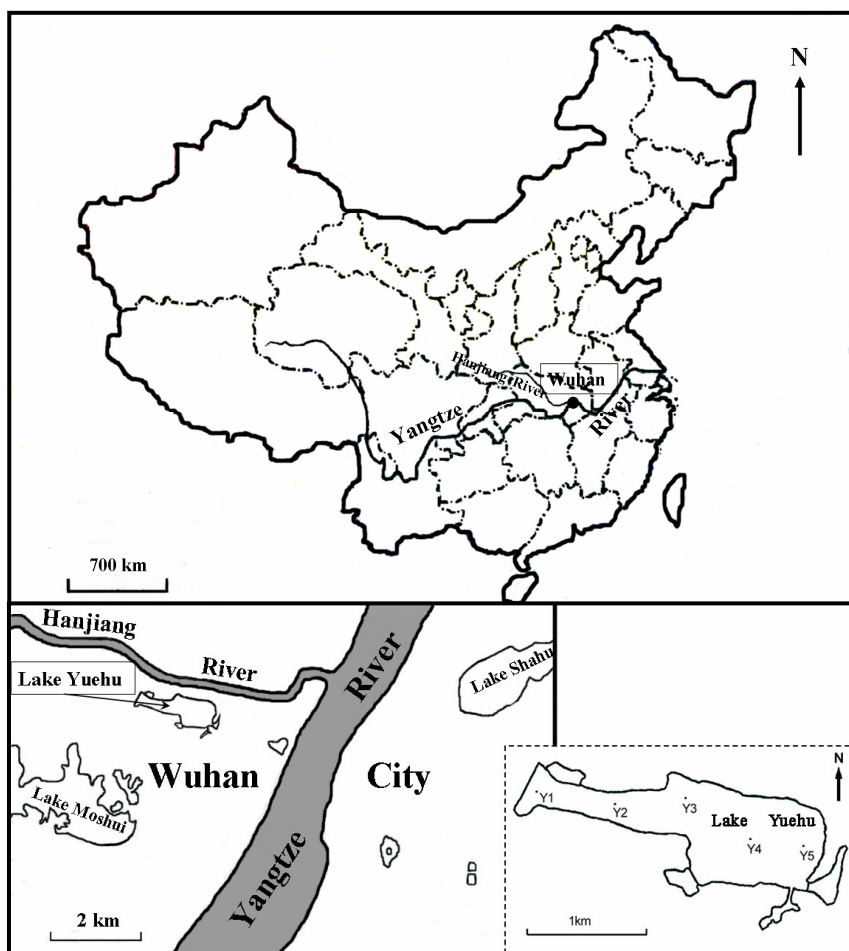


Figure 1. A location map of Wuhan City, China and that of Lake Yuehu. Y1-Y5 represent the five sampling sites.

DNA extraction from sediments and PCR of *amoA* fragment

The protocol of Zhou *et al.* (51) was used for DNA extraction from sediments at the five sites. PCR amplification of a 491-bp fragment of the *amoA* gene was carried out as described by Rotthauwe *et al.* (34) by using the *amoA*-1F and *amoA*-2R primer with a DNA thermocycler (model PTC-200, Bio-Rad, USA).

RFLP analysis of *amoA* gene and sequencing

Five clone libraries were constructed for the five sites. The amplified *amoA* PCR fragments were excised from the agarose

gel, and purified with an agarose gel extraction kit (Doupson, China). The 491-bp *amoA* DNA fragments from the five sites were cloned into the pMD18-T vector (TaKaRa, Japan) according to the manufacturer's instructions, respectively. 100 clones from each library were randomly selected for further analysis. The cloned inserts were reamplified with *amoA* primers and then digested with the *MspI* enzyme (TaKaRa). Restriction patterns were analyzed after gel electrophoresis on 2% agarose gels. Clones representative of each restriction pattern were chosen for sequence analysis on a DNA Sequencer (model Prism 377, ABI, USA).

Phylogenetic analysis

The sequences were aligned using ClustalX program. Phylogenetic analysis of representative sequences including three outgroups was performed to reconstruct bifurcating trees using neighbor-joining (NJ) and maximum-parsimony (MP) approaches using PAUP* 4.0b10 (40). For MP analysis, heuristic searches were performed using tree bisection-reconnection (TBR) branch-swapping and 10 random sequence addition replicates. All sites were equally weighted and gaps were treated as missing characters. Support for recovered clades was measured using a non-parametric bootstrap analysis (13) with 1000 replicates. For NJ analysis, Modeltest 3.7 (30) was used to find the model of nucleotide evolution with AIC criterion. To assess statistical support for hypothesized clades, bootstrap analysis was performed with 1000 replicates for NJ analysis (under the GTR+I+G model of evolution, $-\ln L = 8265.6465$).

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database under accession numbers: FJ498909-FJ498961.

RESULTS

Distributions of chemical parameters in interstitial water and sediment

As shown in Table 1, among all the sampling sites, the NH_4^+ concentration in interstitial water and the sediment LOI were the lowest at Site Y3 and the highest at Site Y5. These two variables were significantly related with each other ($P < 0.01$; $r = 0.989$). Moreover, pH values in sediments were similar.

AOB abundance in sediment

At all sampling sites, the MPN numbers of AOB were at a magnitude of 10^5 cells g^{-1} dry weight (Table 1) and positively related to NH_4^+ concentrations ($P < 0.05$; $r = 0.955$).

Table 1. Some characteristics of sampling sites in Lake Yuehu in March 2007^a

characteristics	Y1	Y2	Y3	Y4	Y5
pH of sediment	7.65±0.03	7.82±0.02	7.80±0.01	7.66±0.04	7.64±0.07
NH_4^+ in porewater (mg l^{-1})	8.169±0.146	8.501±0.325	5.819±0.107	9.321±0.304	10.740±0.615
LOI ^b	0.082±0.000	0.083±0.001	0.063±0.001	0.086±0.001	0.095±0.000
AOB ^c numbers in sediment ($\times 10^5$ cells g^{-1} dry weight)	1.14±0.50	1.20±0.33	1.07±0.25	1.22±0.35	1.24±0.35

^aValues are mean \pm SD of triple determinations; ^bLOI = loss on ignition; ^cAOB = ammonia-oxidizing bacteria.

AOB diversity in sediment

The RFLP analysis for the sediment at all sampling sites gave 13 different restriction patterns (A-M) for total 500 *amoA* clones. The former 4 patterns (A-D) were shared by the five sites (Fig. 2). The 8 patterns including 4 unique ones were found at Site Y5, the 6 patterns including 2 unique ones were found at Site Y1, Site Y2 and Site Y4 respectively, while only common patterns without unique ones were found at Site Y3 (Table 2). Thus, the diversity of *amoA* gene was greatest at Site

Y5. Restriction group A was the major component for all sites, accounting for 81% - 89% of 100 clones in each library (Table 2).

As shown in Fig. 3, the *amoA* fragments retrieved from the sediments at the five sampling sites belonged to four lineages: *N. oligotropha* & *ureae* lineage, *N. europaealNc. mobilis* lineage, *N. communis* lineage and *Nitrosospira* lineage. Most restriction patterns including A, B, E, G, H, I, J, K, L, M groups belonged to *Nitrosomonas* group, and the remaining C,

D, F groups belonged to *Nitrosospira* lineage. According to phylogenetic tree and Table 2, from Site Y1 to Site Y5, the clones of *Nitrosomonas* group made up 92%-87% of the total analyzed clones, and the clones of *Nitrosospira* lineage represented 8%-13% (Fig. 4).

The restriction group A was the predominant member in libraries for all sites, and most of this group was affiliated with the *N. oligotropha* & *ureae* lineage. Thus *N. oligotropha* & *ureae*-like bacteria were the dominant AOB species in the sediment of Lake Yuehu.

Table 2. Numbers of restriction patterns in each site and percentages of analyzed clones in each restriction pattern in each library

Sites	Numbers of restriction patterns	Percentages of analyzed clones in each restriction pattern in 100 <i>amoA</i> clones of each library (%)												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Y1	6	89	2	5	2		1	1						
Y2	6	88	1	5	4					1	1			
Y3	4	89	2	7	2									
Y4	6	87	1	3	7		1		1					
Y5	8	81	1	6	7	2						1	1	1

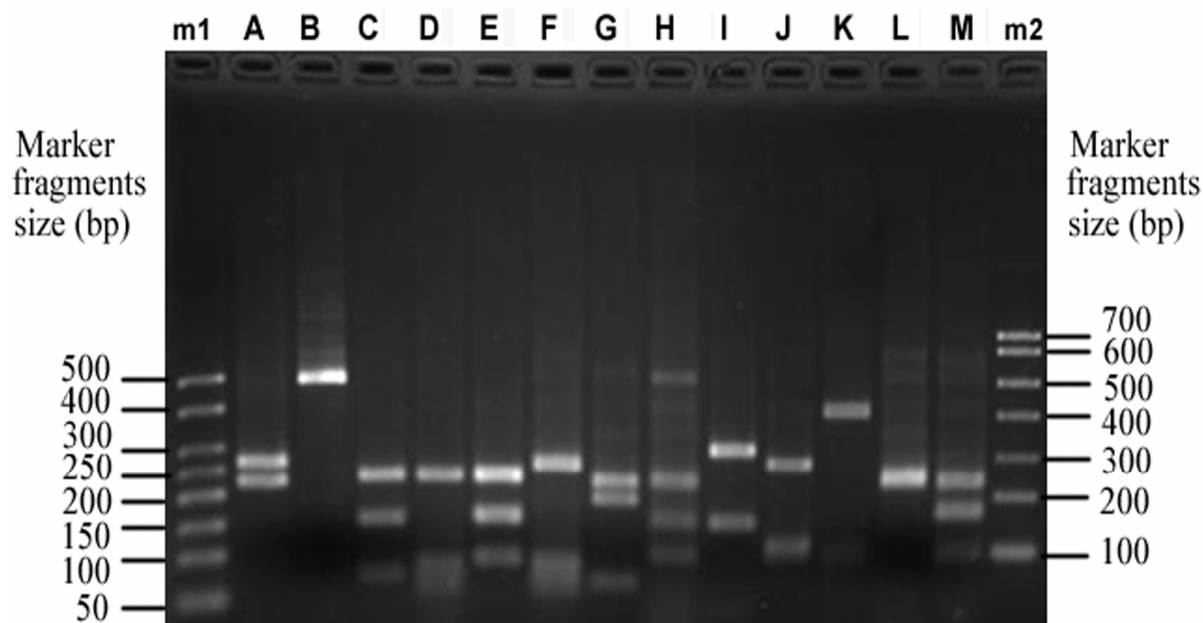


Figure 2. Gel with different *MspI* restriction patterns of analysed *amoA* clones from five sites in March 2007. Lanes A-M represent different restriction patterns of *amoA* gene; Lane m1 is 50bp ladder DNA (Transgen, China); Lane m2 is Marker 1 (Transgen, China).

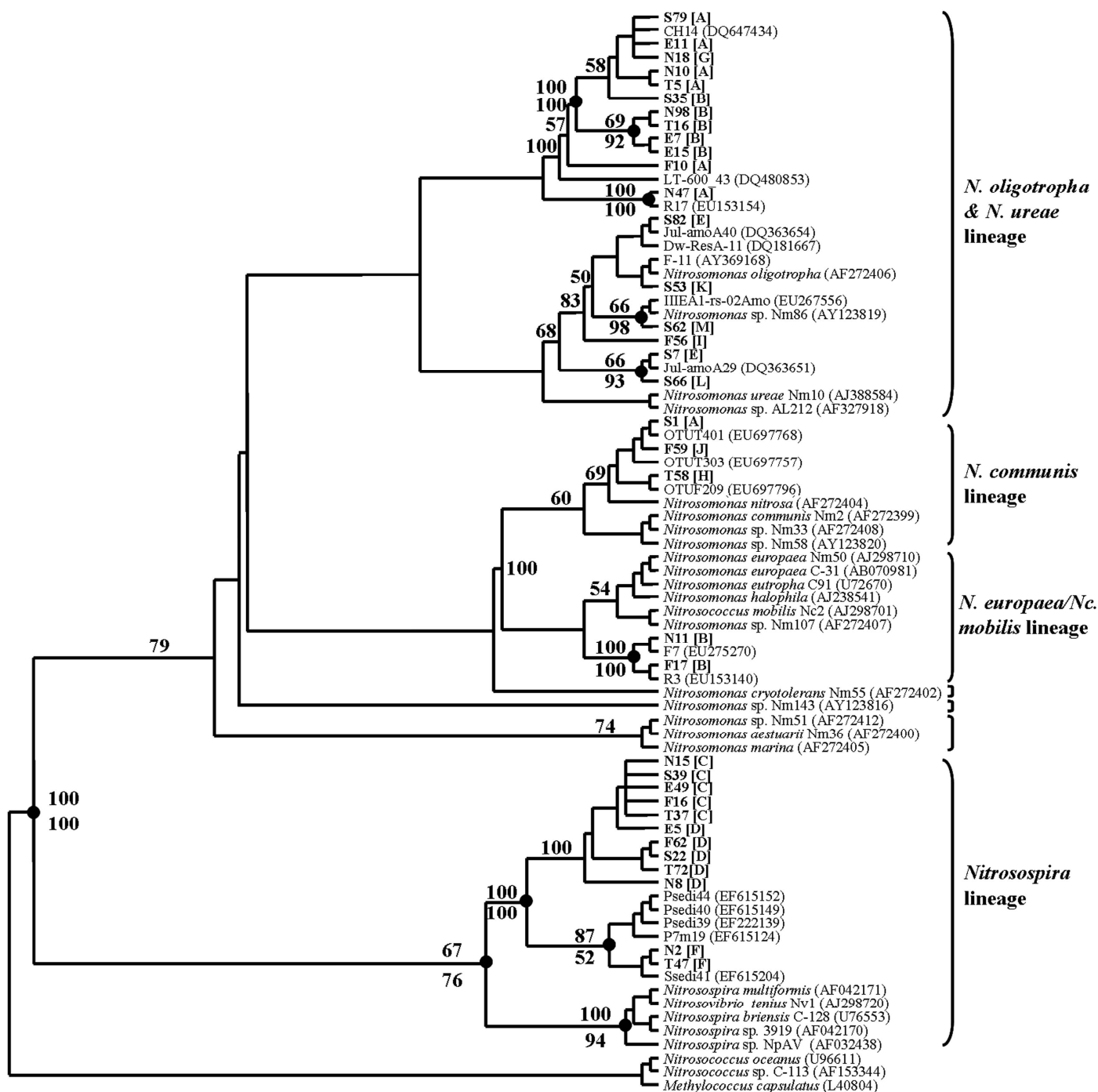


Figure 3. *amoA*-based MP tree of the betaproteobacterial AOB. The clone sequences reported in this study are depicted in bold. The tree was rooted with the *pmoA* sequence of *Methylococcus capsulatus* and the two *amoA* sequences of the gammaproteobacteria. The branch nodes supported by two phylogenetic analysis (MP and NJ) are indicated as solid circles. Numbers at branch nodes are bootstrap values obtained from MP analysis (above the branch) and NJ analysis (below the branch); only values greater than 50% and important to define the phylogenetically major lineages are indicated. Prefixes N, F, E, T and S of clone names represent Y1, Y2, Y3, Y4 and Y5, respectively.

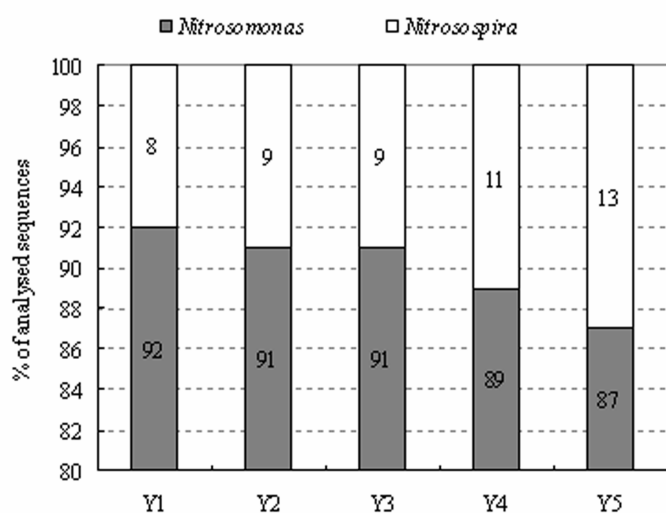


Figure 4. Proportions of analyzed clones belonging to *Nitrosomonas* and *Nitrospira* in sediment AOB at the five sampling sites (Y1-Y5) in Lake Yuehu, respectively.

DISCUSSION

The spatial heterogeneity in NH_4^+ concentrations was found in interstitial water of Lake Yuehu, which was causatively linked to the sediment OM as evidenced by the significantly positive relation between the two variables (Table 1). In fact, potential N mineralization was positively correlated with the OM content (45). What is more, the most important impact of high concentration of OM is its contribution of additional oxygen demand (8) leading to the anaerobic condition in sediment, under which the rate of NH_4^+ releasing in the interface between water and sediment was 2-8 times higher than that at aerobic condition (12).

The AOB numbers were significantly proportional to the NH_4^+ concentrations in interstitial water ($P < 0.05$; $r = 0.955$), which was in agreement with other studies (28, 48). Interestingly, the numbers of restriction pattern were also positively correlated with NH_4^+ concentrations in interstitial water ($P < 0.01$; $r = 0.966$) as well. In details, the clones were clustered in 8 different restriction patterns at Site Y5 with the highest ammonium concentration, but, only 4 different patterns were found at site Y3 with the lowest ammonium concentration. All the remaining sampling sites gave 6 patterns.

It means that the different NH_4^+ concentrations could shape the population structure of AOB in nature, which held true in various environments (6, 14, 21, 47).

In freshwater environments, the *N. oligotropha* & *ureae*-like bacteria are generally the predominant AOB group, as evidenced by repeated isolation of members of this lineage from high MPN dilutions (18, 19, 37) and molecular data including 16S rDNA and *amoA* (5, 21, 32, 36, 50). Our result strengthened this conclusion. This lineage that well adapts to low ammonium concentrations can grow in various environments (2, 3). Moreover, isolates of this lineage (e.g., *N. oligotropha*, *N. ureae*, *Nitrosomonas* sp. AL212, etc.) are inhibited at high ammonium concentrations (exceeding 10 mM) (4, 35, 37, 39). Therefore, the ammonium concentrations in Lake Yuehu, ranging from 0.32 mM to 0.60 mM, appeared to favor their growth.

The *N. europaealNc. mobilis* lineage is always dominant in waster-water treatment plants (WWTP) (9, 17, 21), since its growth requires high ammonium concentrations. While, the ammonium concentration in Lake Yuehu was not high enough to support its growth, so the small numbers (2 *amoA* sequences) of total 500 clones were affiliated with this lineage, which is assumed to be derived from effluent discharge.

The *N. communis* lineage comprises the *N. communis* sublineage and *N. nitrosa* sublineage. The former prefers agricultural soils, while the latter is commonly distributed in more or less eutrophicated freshwater (20). From the phylogenetic tree, 3 clones (S1, F59, T58) in Lake Yuehu belonged to the *N. nitrosa* subgroup. Noticeably, they were retrieved from diverse environments like WWTP (10), activated sludge (29), freshwater estuary (5), soil (27), showing the ecological versatility.

The clones of the *Nitrospira* lineage represented only 8%-13% of the total analyzed clones, probably due to the conditions in Lake Yuehu unsuitable for growth of these species that prefer terrestrial habitats (20).

Conclusively, this study revealed the abundance and diversity of sediment AOB and their significant relationships with ammonium status in a Chinese shallow eutrophic lake.

ACKNOWLEDGEMENTS

This work was supported by the the National Basic Research Program of China (2008CB418005) the grant (KZCX2-YW-426) from the Chinese Academy of Sciences and the funding (40730528) from the National Science Foundation of China.

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