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Research Article

RESEARCH ARTICLE

An RNA-based quantitative and compositional study of ammonium-oxidizing bacteria and archaea in Lake Taihu, a eutrophic freshwater lake

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One sentence summary: The quantification study of amoA gene based on the RNA level was performed on the surface sediment of Lake Taihu, and suggests that active ammonium-oxidizing bacteria are the dominant ammonia oxidizer.

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ABSTRACT

Ammonium-oxidizing archaea (AOA) and bacteria (AOB) play crucial roles in ammonium oxidation in freshwater lake sediment. However, previous reports on the predominance of AOA and AOB in the surface sediment of Lake Taihu have been based on DNA levels, detecting the total abundance of microbiota (including inactive cells), and have resulted in numerous contradictory conclusions. Existing RNA-level studies detecting active transcription are very limited. The current study, using RNA-based real-time quantification and clone library analysis, demonstrated that the *amoA* gene abundance of active AOB was higher than that of active AOA, despite conflicting results at the DNA level. Further exploration revealed a significant positive correlation between the potential nitrification rate (PNR) and the abundance of AOA and AOB at the RNA level, with irregular or contradictory correlation found at the DNA level. Ultimately, using quantitative analysis of RNA levels, we show AOB to be the active dominant contributor to ammonium oxidation. Our investigations also indicated that AOB were more diverse in high-ammonium lake regions, with *Nitrosomonas* being the active and dominating cluster, but that AOA had an advantage in the low-ammonium lake regions.

Keywords: ammonium-oxidizing archaea; ammonium-oxidizing bacteria; *amoA* gene; DNA and RNA levels; spatiotemporal changes; surface sediment

INTRODUCTION

Ammonium oxidation (aerobically oxidizing NH_4^+ to NO_2^-), the first and rate-limiting step of the nitrification process, is basically mediated by ammonium-oxidizing bacteria (AOB) and archaea (AOA). Unlike AOB, which were discovered more than 100 years ago, AOA were discovered relatively recently (Konneke *et al.* 2005). However, current findings show that the nitrite-oxidizing bacteria (NOB) genus *Nitrospira* (Daims *et al.* 2015; van Kessel *et al.* 2015; Kits *et al.* 2017), the members of which are capable of complete ammonia oxidation (comammox) to nitrate, does not adhere to the traditional concept that nitrification is a two-step process. To analyze the diversity and abundance of ammonium oxidizers in natural environments, the *amoA* gene, encoding the *alpha* subunit of ammonium monooxygenase, has broadly served as a functional gene marker (Stahl and de la Torre 2012; Yang *et al.* 2013), for environments such as soil (Boyle-Yarwood, Bottomley and Myrold 2008), oceans (Bouskill *et al.* 2012; Dang *et al.* 2010), estuaries (Puthiya Veettil *et al.* 2015), salt

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lakes (Jiang *et al.* 2009) and freshwater lakes (Bollmann, Bullerjahn and McKay 2014).

Lake Taihu is a large (2338 km^2), shallow (mean depth 1.9 m) subtropical, freshwater lake located in the Yangtze River Delta in China (Zhang *et al.* 2007; Cai, Gong and Qin 2012). Anthropogenic influence, such as industrial development, has gradually led to the constant discharge of pollutants into different regions of Lake Taihu. As a result, eutrophication and algal blooms have become very severe due to nutrient increases, such as excessive nitrogen pollution. Hence, the removal of excess nitrogen by ammonium-oxidizing prokaryotes in Lake Taihu has been extensively reported (Schloss *et al.* 2009; Wu *et al.* 2010; Hou *et al.* 2013; Zhao *et al.* 2013, 2014; Huang *et al.* 2016).

However, in these studies, there were conflicting conclusions about AOA and AOB, which perform a leading role in the process of aerobic ammoxidation. For example, a higher total abundance for AOA compared with AOB in the surface sediment of Lake Taihu was reported, as illustrated in early observations (Schloss *et al.* 2009; Wu *et al.* 2010; Hou *et al.* 2013; Zhao *et al.* 2013, 2014); however, another study reached the opposite conclusion, that the total abundance of AOB surpassed that of AOA (Huang *et al.* 2016). To our knowledge, identification of *amoA* genomic DNA abundance (total abundance) is primarily based on the DNA-level analyses, which cannot distinguish between microbial cells that are active or inactive (including dead and dormant cells) (Ostle *et al.* 2003; Keer and Birch 2003). Considering that mRNA-based technology can indicate the specific activity of living cells or complex microbial communities (Aoi *et al.* 2002), demonstrating that the use of *amoA* transcripts to assess functional ammonium-oxidizing communities is valid and reliable (Wu *et al.* 2017; Pelissari *et al.* 2018), we chose RNA-based detection for this study in parallel with DNA methods to reveal differences.

Lake Taihu provides an ideal freshwater ecosystem model for studying the effects of microorganisms on the environment due to the gradient of trophic levels in different lake regions (Yang, Zhang and Chen 2008). Thus, we selected three different lake regions in Lake Taihu with gradient nutrient levels (hypertrophic or eutrophic (ZS and ML) and mesotrophic (QD) lake regions) to conduct a comparable study of ammonium-oxidizing microorganisms (AOA and AOB) at DNA and RNA levels to reveal the following: (i) spatiotemporal variations of 'total' and 'active' *amoA* abundance; (ii) diversity changes between different lake regions; and (iii) active contributors of AOA and AOB. Comparing the total genomic abundance and active transcripts of the *amoA* gene will contribute to a deeper understanding of ammonia-oxidizing microorganisms.

METHODS

Site description

In Lake Taihu (30°55′40″-31°32′58″N, 119°52′32″-120°36′10″E), China's third largest freshwater lake, with high spatial variability the water quality decreases from southeast to northwest due to the anthropogenic emissions of pollutants (Zhang *et al.* 2007; Cai, Gong and Qin 2012). Based on these studies, samples were collected in three regions within Lake Taihu: Meiliang Bay (ML) and Zhushan (ZS) Bay (the hypertrophic or eutrophic states) and Qidu (QD) (East Lake Taihu, the mesotrophic state) (Fig. 1).

Sample collection

Sediment samples were collected in triplicate at each site over four months (December (2016), March (2017), Jun (2017) and September (2017)) from the surface to a depth of 0–5 cm using a sterile tube (diameter, 28 mm) for transport in a mini-icebox. Pretreatment of the sediment samples in the laboratory involved removal of excess water (same low-speed centrifugation (3000 rpm) to ensure consistency of sampling quantity). Subsamples used for examination of physicochemical characteristics within 48 h were placed at 4°C; samples used for nucleic acid extraction were stored at −80°C (RNA was extracted immediately from the central layer of the sediment after arrival at the laboratory).

Environmental factor analysis

We determined NH^+_4-N, NO_2^--N, and NO_3^--N concentrations according to the protocol described in HJ 634–2012 (ISO/TS14256-1, http://english.mee.gov.cn/Resources/standards): determination of total ammonium, nitrite and nitrate by extraction with a potassium chloride solution (i.e. spectrophotometric methods). Briefly, total ammonium, nitrate and nitrite were extracted with 1 M KCl. Total ammonium was determined spectrophotometrically at 630 nm using phenol. Nitrite was determined spectrophotometrically at 543 nm using N-(1-Naphthyl) ethylenediamine dihydrochloride. Nitrate was first reduced to nitrite, and then the total amount of nitrate and nitrite was measured spectrophotometrically at 543 nm using N-(1-Naphthyl) ethylenediamine dihydrochloride. The difference between the total amount of nitrate nitrogen and nitrite was considered the nitrate concentration.

Potential ammonium oxidation rate analysis

The potential ammonium-oxidation rate (PNR) was measured using the chlorate inhibition method (Kurola *et al.* 2005). The potential ammonium-oxidation rate was calculated based on the change in nitrite concentration and used as an indicator index for the capacity of active *amoA* amounts in the sediment.

Nucleic acid extraction

A Power Soil DNA/RNA Isolation Kit (MO BIO Laboratories, San Diego, CA, USA) was used to extract DNA/RNA from the sediment samples, and we used PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) to synthesize complementary DNA. All DNA, RNA and cDNA samples were stored at −80°C. The quantity and integrity of the extracted DNA and RNA were checked. The absence of contaminating DNA in RNA was checked by performing a general bacteria 16S rRNA gene-based PCR with RNA extract without reverse transcriptase as the negative control and DNA extract as the positive control.

Quantitive analysis of archaeal and bacterial *amoA* gene

SYBR Green I chemistry was used in our quantification, run on a Master cycler gradient thermocycler (Realplex 4 s, Eppendorf, Germany). The abundance of AOA and AOB were quantified by real-time PCR, with the primers Arch-*amo*AF/R (Francis *et al.* 2005) and *amo*A-1F/2R (Rotthauwe, Witzel and Liesack 1997), respectively. The thermal cycling protocol was performed based on the previous studies (Hou *et al.* 2013: 2285-96). The amplification efficiency for archaeal and bacterial *amoA* was 85% ($R^2 = 0.998$) and 94% ($R^2 = 0.999$), respectively.

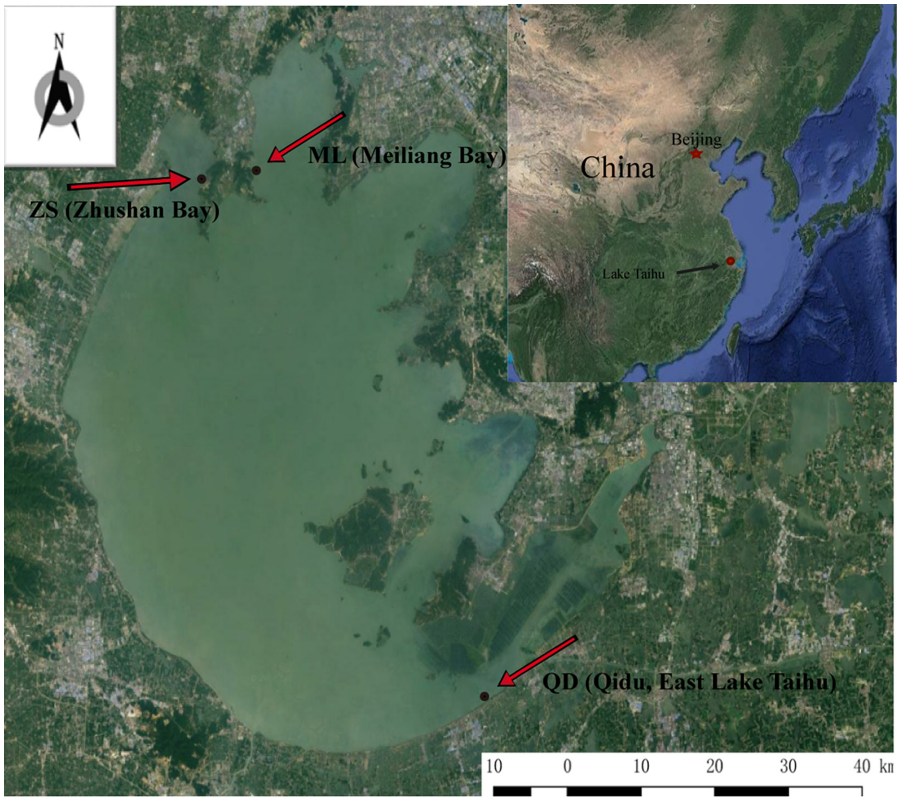


Figure 1. Sampling sites in our study of Lake Taihu. QD: Qidu, East Lake Taihu. ML: Meiliang Bay. ZS: Zhushan Bay.

Clone libraries, sequence and phylogenetic analysis

cDNA extracts (December 2016) were used to construct the clone libraries of AOA and AOB, with the primers as described above. Details can be seen in Sun *et al.* (2014). The inserts were checked for correctness, and the positive PCR products were digested with the restriction endonucleases *Taq*I (AOA-*amo*A) and *Hha*I (AOB-*amo*A) in 20 μl reactions according to the manufacturer's protocol and were analyzed by electrophoresis. All restriction enzymes were purchased from TaKaRa (TaKaRa, Dalian, China). One or two representative clones of each restricted fragment length polymorphism (RFLP) pattern were chosen for sequencing with an ABI DNA analyzer (ABI, Applied Biosystems).

The sequences obtained were checked for chimeras using the RDP pipeline and then searched using blast in NCBI to obtain the best matching sequences. The sequences were aligned using MEGA6.0 (Kumar, Stecher and Tamura 2016). Mothur v. 1.38.1 was employed to determine operational taxonomic units (OTUs) sharing 97% similarity by utilizing a method (furthest neighbor) modified from a previous report (Schloss *et al.* 2009). The maximum likelihood method (the Kimura 2-parameter model) was applied for constructing phylogenetic trees in MEGA6.0, with bootstrap support (1000 replications). The sequences obtained in our study were deposited in the GenBank database under accession numbers MH846128-MH846149 for AOA and MH846200-MH846222 for AOB.

Statistical analysis

The libraries' coverage was analyzed with the algorithm of $C = [1–(n/N)] \times 100\%$, where 'n' represents unique OTUs and 'N' represents clones. The Shannon and Chao index were computed by Mothur v. 1.38.1. The rarefaction (Fig. S1) was analyzed by EstimateS version 9 (Colwell and Elsensohn 2014). One-way and two-way analysis of variance (ANOVA) and Spearman correlation analyses were obtained by SPSS version 20. 0 software (IBM, USA). Redundancy analysis (RDA) was performed by R software version 3.5.1.

RESULTS

Physicochemical properties and PNR

Fluctuations in environmental factors were found in the surface sediment samples (Table 1). The concentrations of total ammonium and nitrate generally showed significant spatiotemporal variation ($P < 0.05$, one-way ANOVA), but the concentration of nitrite was not significantly changed ($P > 0.05$, one-way ANOVA). Two-way ANOVA analysis indicated significant spatial and seasonal changes for the concentration of nitrate and total ammonium ($P < 0.05$ and $P < 0.01$). Our results showed that the N salt (total ammonium, nitrate and nitrite) concentration of ZS was generally higher than that of ML, with QD having the lowest concentration. Therefore, combining our results with data provided by other studies researching the nutrient levels of Lake Taihu's different regions (Zhang *et al.* 2007; Cai, Gong and Qin 2012), we defined the three lake regions as high nutrient (HN), medium nutrient (MN) and low nutrient (LN).

Significant changes in the PNR, used as an indicator index for the capacity of active amounts of *amo*A, were observed within and between groups (sites and seasons) ($P < 0.01$), which indicated an active metabolism of ammonium oxidizers over seasons in each lake region.

Table 1. The physicochemical parameters and PNR of the surface sediment collected from Lake Taihu.

Site	Season	NO_3^--N (mg/kg) N = 3	NH_4^+-N (mg/kg) N = 3	NO_2^--N (mg/kg) N = 3	PNR ($\mu g \cdot kg^{-1} \cdot h^{-1}$) N = 3
	Dec.	0.62 ± 0.14^{c}	3.76 ± 2.29^{b}	0.02 ± 0.01^{a}	0.44 ± 0.02^{a}
QD (LN)	Mar.	1.31 ± 0.08^{b}	82.1 ± 23.69^{a}	0.03 ± 0.01^{a}	0.62 ± 0.01^{b}
	Jun.	1.79 ± 0.22^{a}	45.38 ± 18.21^{ab}	0.03 ± 0^{a}	1.14 ± 0.02^{c}
	Sep.	0.81 ± 0.04^{c}	77.3 ± 7.87^{a}	0.03 ± 0.01^{a}	1.32 ± 0^{d}
	Dec.	0.74 ± 0.21^{bc}	47.67 ± 9.02^{a}	0.02 ± 0.02^{a}	0.64 ± 0.02^{a}
ML (MN)	Mar.	1.42 ± 0.26^{a}	38.04 ± 8.73^{ab}	0.02 ± 0.01^{a}	0.82 ± 0.01^{b}
	Jun.	1.37 ± 0.24^{ab}	9.61 ± 1.02^{c}	0.05 ± 0.02^{a}	1.49 ± 0.06^{c}
	Sep.	0.55 ± 0.05^{c}	26.59 ± 3.41 bc	0.03 ± 0.01^{a}	2.13 ± 0.08^{d}
	Dec.	0.71 ± 0.1^{c}	39.29 ± 2.56^{a}	0.02 ± 0.01^{a}	0.72 ± 0.01^{a}
ZS (HN)	Mar.	1.57 ± 0.3^{ab}	36.76 ± 8.11^{a}	0.02 ± 0.01^{a}	0.9 ± 0.01^{b}
	Jun.	2.04 ± 0.28^{a}	43.38 ± 11.27^{a}	0.04 ± 0.01^{a}	1.59 ± 0.05^{c}
	Sep.	1.07 ± 0.13^{bc}	61.63 ± 6.99^{a}	0.05 ± 0.02^{a}	2.82 ± 0.06^{d}
Two-way ANOVA					
Site	*	*	ns	**	
Season	**	**	*	**	
Site * Season	**	ns	ns	**	

Means in the same column (Dec. to Sep.) with different superscripts were significantly different at $P < 0.05$. All the parameters were tested by Dunnett's T3. QD = Qidu (East Lake Taihu); ML = Meiliang Bay; ZS = Zhushan Bay. LN = low nutrition level; MN = medium nutrition level; HN = high nutrition level. *$P < 0.05$, **$P < 0.01$, ns = not significant.

Spatiotemporal variations of AOA and AOB abundance

Further exploration revealed that the *amoA* gene abundance of AOA and AOB had significant spatial variations (two-way ANOVA, $P < 0.01$, Fig. 2a and b), not only in terms of total abundance but also in active transcription. Nonetheless, AOA and AOB exhibited different distribution patterns. A higher abundance (both total abundance and active transcription) in MN and HN than in LN was found for AOB, with AOA showing the opposite, with a generally higher abundance in LN. The total abundance of AOA and AOB ranged from 7.22×10^4 to 6.79×10^5 and 1.66×10^5 to 3.64×10^8 copies per gram of sediment, respectively. The active transcription of AOA and AOB ranged from 7.80×10^3 to 2.61×10^5 and 4.06×10^5 to 3.63×10^8 copies per gram of sediment, respectively.

In particular, active transcription of the *amoA* gene displayed greater seasonal changes than did total abundance (Fig. 2c). Notably, the maximal cDNA-targeted *amoA* gene abundance for both AOA and AOB occurred in June and September; the minimal values were observed in December and March. However, opposite results were observed for the total abundance of AOA and AOB, especially for the latter (Fig. 2a and c). Additionally, active transcription of the *amoA* gene, but not total abundance, exhibited a significant difference in pairwise comparison of all months (two-way ANOVA, $P < 0.001$, Fig. 2c). These results indicated obvious spatiotemporal variations in AOA and AOB abundance in the surface sediment, and the cDNA-targeted *amoA* gene abundance of AOA and AOB had a marked correlation with temperature. Changes in AOB were particularly prominent.

Changes in the AOB/AOA ratio

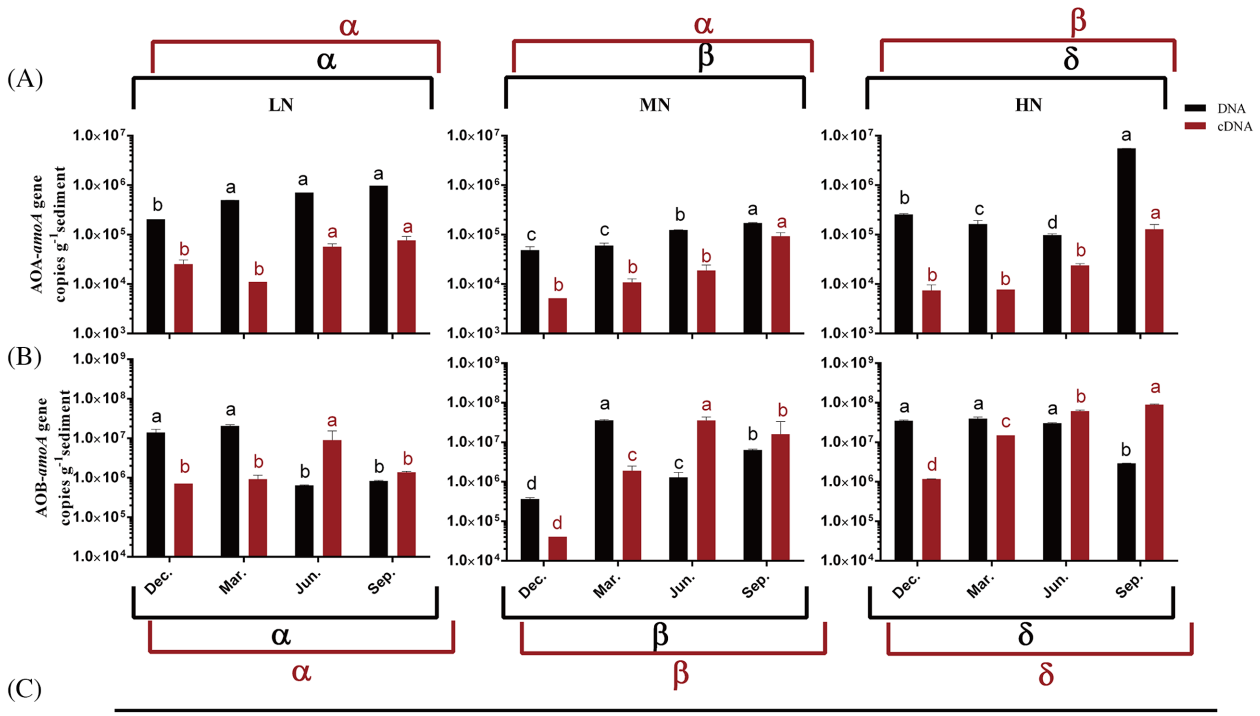
To identify the dominant aerobic ammonium oxidizer in the surface sediment of Lake Taihu, further investigation was performed, revealing that the active transcription of AOB surpassed that of AOA, even though the total abundance of AOA was sometimes higher than that of AOB: the AOB/AOA ratios at the DNA and RNA levels ranged from 0.76- to 1398.69- and from 7.83- to 40 380.05-fold, respectively (Fig. 3).

Overall, the observed changes in AOB/AOA ratios were associated with seasonal changes, but the opposite was found between DNA and RNA levels (Fig. 3). The maximal value of the AOB/AOA ratio was observed in December and the minimal value in June and September at the DNA level. At the RNA level, the maximum value was observed mostly in June, and except for LN, the minimum value was observed in December. These observations emphasize a clear difference in quantitative methods based on DNA and RNA levels.

Correlation analysis with physicochemical properties and PNR

Different correlations were found between *amoA* gene abundance and fluctuating physicochemical properties (Fig. 4). No significant correlation was found between the cDNA-targeted *amoA* gene abundance of AOA and physicochemical properties ($P > 0.05$). The total abundance of AOA only showed a significant correlation with the concentration of total ammonium. Regarding AOB, contradictory results were found between total abundance and active transcription: a significant positive correlation was found between the *amoA* gene abundance of AOB and physicochemical parameters (nitrite and nitrate) at the RNA level ($P < 0.01$, $r > 0$), but this was negative (but not significant) at the DNA level ($P > 0.05$, $r < 0$).

Interestingly, the PNR had a positive correlation with active transcription of the *amoA* gene but correlated inconsistently with the total abundance of the *amoA* gene (Fig. 5). The PNR also exhibited a significantly positive correlation with the cDNA-targeted *amoA* gene abundance of AOA ($P < 0.01$, $r = 0.650$), but a positive, though not significant, correlation with the total *amoA* gene abundance of AOB ($P > 0.05$, $r = 0.194$). Our observations also showed that the PNR had a different correlation with the abundance of AOB, which was negative at the DNA level ($P < 0.01$, $r = -0.469$) and positive at the RNA level ($P < 0.01$, $r = 0.855$).



(C)

	Dec. – Mar.	Dec. – Jun.	Dec. – Sep.	Mar. – Jun.	Mar. – Sep.	Jun. – Sep.
AOA-DNA	**0.193**	<0.001	0.023	**0.707**	**0.719**	**0.114**
AOA-cDNA	0.002	<0.001	<0.001	0.012	<0.001	<0.001
AOB-DNA	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
AOB-cDNA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Figure 2. Spatiotemporal variation in the *amoA* gene. (**A**) Spatiotemporal variation in the *amoA* gene of AOA. (**B**) Spatiotemporal variation in the *amoA* gene of AOB. Significant differences within a site in different seasons are indicated by letters above the season (one-way ANOVA, $P < 0.05$). Significant differences between sample sites are indicated by Greek symbols (two-way ANOVA, $P < 0.05$). Values are presented as means of triplicate values and error bars represent standard errors. (**C**) *P*-values from an analysis of molecular variance of the amoA gene abundance within different seasons. *P*-values are analyzed by SPSS (Tukey), $\alpha = 0.05$. The data in the table in bold indicates the *P*-values were not significant. LN = low nutrition level (lake regions of QD); MN = medium nutrition level (lake regions of ML); HN = high nutrition level (lake regions of ZS).

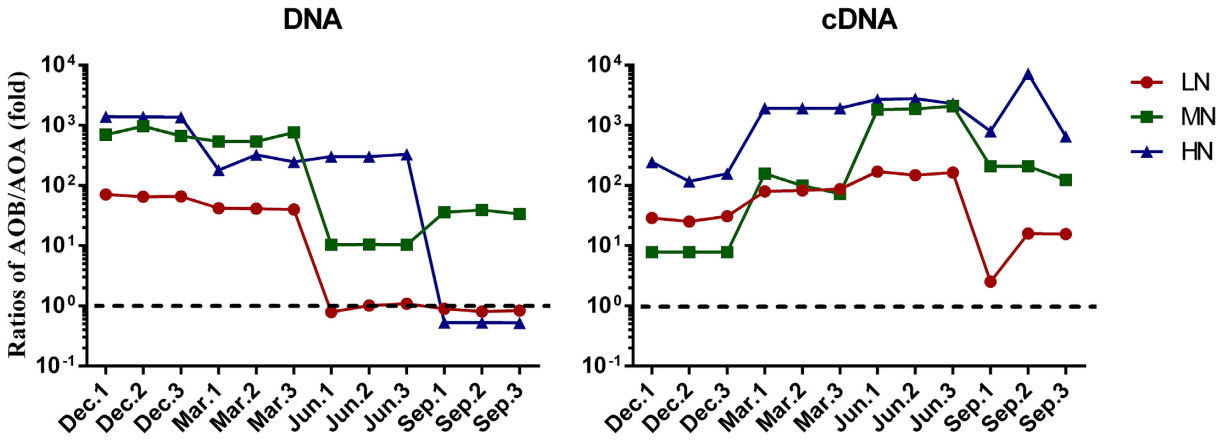


Figure 3. AOB/AOA ratios at the DNA and RNA levels. The dashed line starts at the ratio of 1 (10^0). LN = low nutrition level (lake regions of QD); MN = medium nutrition level (lake regions of ML); HN = high nutrition level (lake regions of ZS).

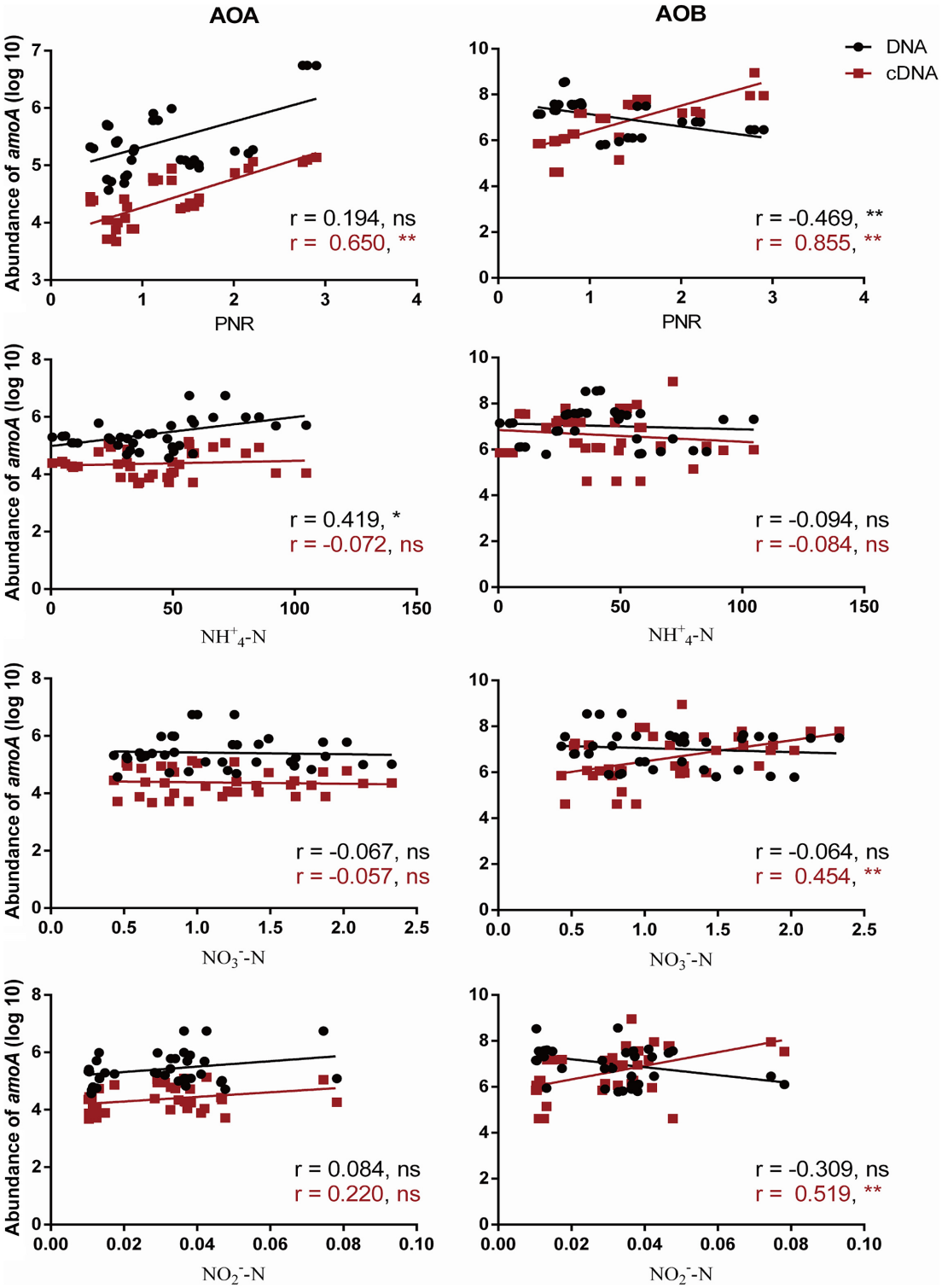


Figure 4. Correlation analysis between the physicochemical parameters, potential ammonia oxidation rate (PNR) and the abundance of *amoA* gene (log 10). 'r' and *P*-values were produced by Spearman correlation coefficient analysis; **correlation is significant at the 0.01 level (two-tailed); *correlation is significant at the 0.05 level (two-tailed); ns: not significant, n = 36.

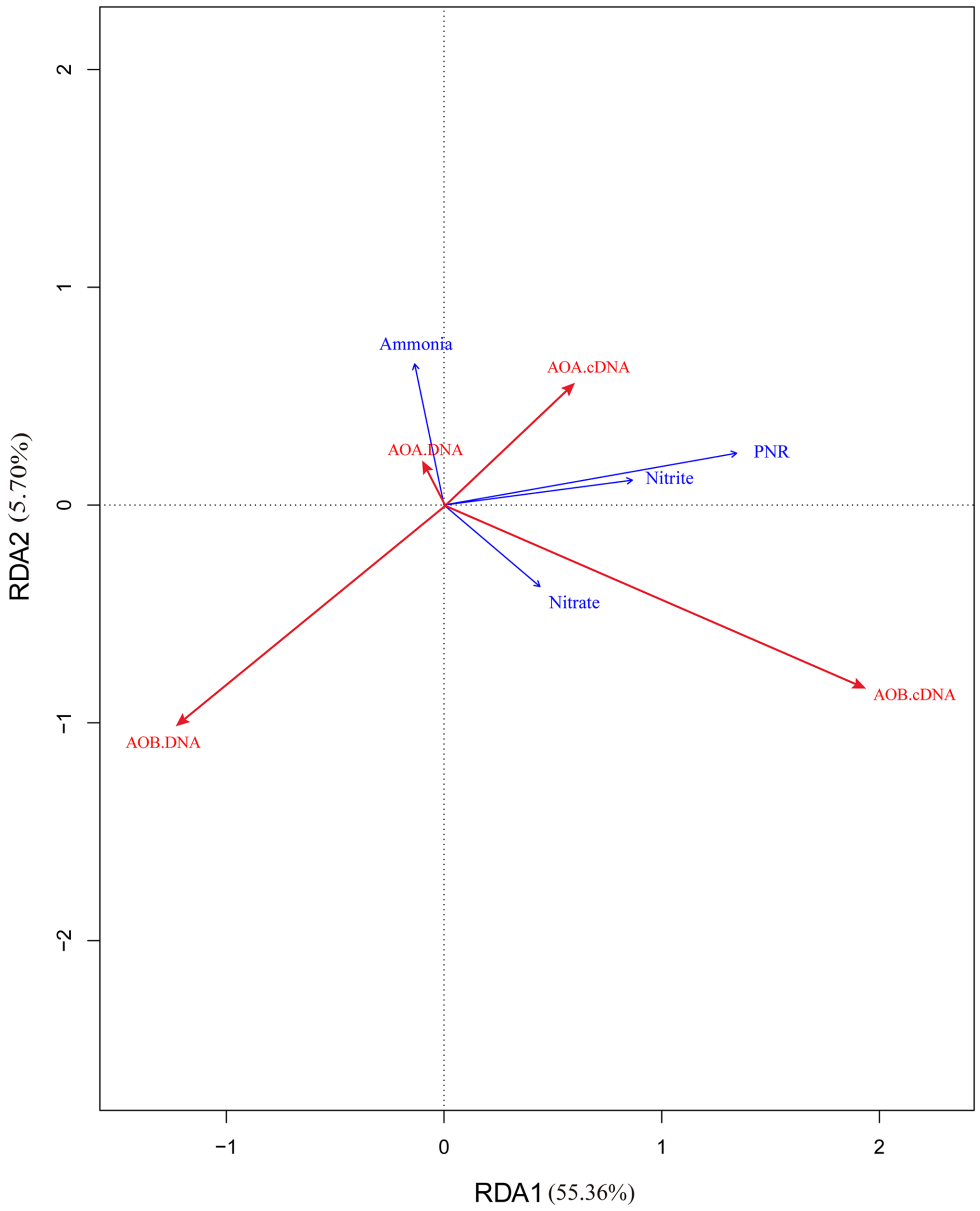


Figure 5. Redundancy analysis biplot of log gene copy numbers of *amoA* gene and environmental parameters obtained from Lake Taihu. AOA-DNA: the total *amoA* gene abundance of AOA; AOA-cDNA: the cDNA-targeted *amoA* gene abundance of AOA; AOB-DNA: the total *amoA* gene abundance of AOB; AOB-cDNA: the cDNA-targeted *amoA* gene abundance of AOB.

According to RDA, the PNR had a strong effect on the abundance of AOA and AOB, especially the latter.

Community diversity analysis

Based on our observations, the diversity of AOA and AOB exhibited an opposite distribution in the surface sediment: the diversity (the OTUs, Shannon and Chao index) of AOA was in the order LN > MN > HN and that for AOB was in the order HN > MN > LN (Table 2). Interestingly, the biodiversity and richness of AOA in LN were larger than those for AOB; however, in MN and HN, AOB showed higher biodiversity and richness than AOA did. Overall, these results indicate that the diversity of AOA and AOB is strongly linked to a lake's trophic status.

Table 2. Biodiversity and predicted richness of AOA and AOB *amoA* genes recovered from the different sediment sampling sites in Lake Taihu.

	Site	No. of clones	No. of OTUs	Coverage (100%)	Shannon	Chao
AOA	LN	81	11	0.93	2.00	20
	MN	34	5	1.00	1.61	15
	HN	29	6	1.00	1.60	6
AOB	LN	23	5	0.87	1.61	15
	MN	30	6	0.93	1.79	21
	HN	48	12	0.94	2.48	34

Community structure analysis

The significant variations in abundance and diversity of AOA and AOB prompted us to further investigate their composition in the surface sediment of different nutrient-level lake regions. The phylogenetic tree demonstrated that all OTUs of AOA belong to Thaumarchaeota, comprising Group I.1a (average 25.2%), Group I.1b (average 7.0%), Group I.1a-associated (average 18.6%), *Candidatus Nitrososcosmicus exaquare* (average 43.9%) and *Nitrososphaera* (average 5.3%) clusters. However, different compositions were found between the lake regions (Fig. 6a and c). For example, the *Candidatus Nitrososcosmicus exaquare* cluster, the most abundant AOA group, showed the highest proportion in ZS (69.0%), followed by MN (44.1%) and LN (18.5%). Group I.1b and the *Nitrososphaera* cluster only appeared in LN. In general, LN, compared with HN and MN, contained more AOA groups (Group I.1a, Group I.1b, *Candidatus Nitrososcosmicus* and *Nitrososphaera* cluster).

Regarding AOB, all OTUs belong to the *Nitrosomonas* and *Nitrosospira* cluster, accounting for an average of 94.4% and 5.6%, respectively (Fig. 6b and d). Furthermore, the *Nitrosomonas* cluster contained four branches, which exhibited different proportions between the lake regions. The *Nitrosomonas* sp. Is79A3 (average 46.7%) was the dominant group of AOB, especially in LN. The *Nitrosomonas nitrosa* (average 19.6%) was only found in high-total ammonium lake regions (MN and HN). The other two groups were the *Nitrosomonas* sp. strain Nm173 (average 12.2%) and *Nitrosomonas oligtropha* (average 15.9%). In general, the clusters of AOB and relative abundance (OTUs) were highest in the surface sediment in HN, followed by MN and LN (Fig. 6d).

DISCUSSION

Active AOB dominated the ammonium oxidation

In our study, the total abundance of AOB was generally higher than that of AOA (Fig. 3), which is contrary to previous studies on Lake Taihu reporting that the total abundance of AOA, not AOB, dominated the surface sediment (Schloss *et al.* 2009; Wu *et al.* 2010; Hou *et al.* 2013; Zhao *et al.* 2013, 2014). These contradictory conclusions may be related to experimental methods or actual biological differences, such as different sampling times or the regions of Lake Taihu sampled. However, comparative analysis showed that quantitative analyses in previous studies were essentially based on DNA-level techniques. However, DNA-based methods cannot distinguish between the abundance of total (including inactive and active cells) and active cells (Keer and Birch 2003; Ostle *et al.* 2003), which may also be an important reason for the contradictory conclusions to date. Thus, comparative analysis based on the DNA level cannot accurately identify dominant ammoxidation agents. Indeed, transcriptional level-based quantitative analysis is needed to identify active and dominating ammonium oxidizers of AOA and AOB.

Consequently, we quantified the *amoA* gene in situ at the transcription level, and based on consistent spatiotemporal results, we found that the cDNA-targeted *amoA* gene abundance of AOB was much higher than that of AOA, even though contradictory results were found at the DNA level (such as for September in HN and LN) (Fig. 3). We compared our observations with previously obtained results indicating that AOB strains isolated and cultured from freshwater habitats with the addition of ammonium nitrogen ($>$ 10 μM) exhibited a strongly higher growth rate than that of AOA (French *et al.* 2012). In addition, the K_m of AOB for NH_3/NH_4^+ is approximately 1000 times higher than the K_m of AOA (Park *et al.* 2010; Jung *et al.* 2011), and the $K_{m(app)}$ for NH_3 of *Nitrospira inopinata* (a pure culture of a comammox bacterium) is 4- to 2500-fold below the values reported for AOB (Kits *et al.* 2017). Considering all these data, we propose that the high NH_4^+ concentration ($>$10 μM; the lowest NH_4^+ concentration in LN was approximately 3.76 mg/kg, approximately 210 μM; Table 1) promoted expression of the *amoA* gene in AOB. Further exploration revealed that RNA-based quantification had a precise conclusion: the PNR showed a significant positive correlation with the abundance of AOA and AOB at the RNA level ($P < 0.01$, $r > 0$), but irregular or contradictory correlations were found at the DNA level (Figs 4 and 5). Taken together, we believe that quantitative methods based on RNA levels, rather than on DNA levels, more accurately demonstrate that active AOB are the predominant ammonium oxidizers in the surface sediment of Lake Taihu.

Spatiotemporal changes of AOA and AOB

Regarding temporal variations, compared with active transcription, which was significantly correlated with seasonal change, the total abundance of AOA and AOB was irregularly related to seasonal changes (Fig. 2). As mentioned above, DNA-based quantification includes inactive cells (Ostle *et al.* 2003), which are rarely affected by environmental changes or external influences. Nevertheless, the high number of *amoA* gene transcripts in the surface sediment during high-temperature months may be attributable to high metabolic activity due to the mixing of the surface sediment with water and the subsequent increases in nutrient availability. Considering the positive effects of elevated temperature on microbial metabolic efficiency and transcriptional activity (Tourna *et al.* 2008; Allison, Wallenstein and Bradford 2010), we speculate that elevated temperatures lead to increased activity of AOB and AOA, resulting in higher transcriptional activity.

In contrast to seasonal changes, our results showed different distribution patterns under gradient nutrients for the *amoA* gene abundance of AOA and AOB, especially for AOB (Fig. 2). We suggest that the different nutrient levels significantly impacted the spatial distribution of the *amoA* gene. In fact, different nutrient levels strongly affect the growth of AOA and AOB in their habitats (Verhamme, Prosser and Nicol 2011; Lehtovirta-Morley 2018). Moreover, AOA has a higher affinity for substrates such as ammonium nitrogen than do AOB (Verhamme, Prosser and Nicol 2011; Lehtovirta-Morley 2018); the former was therefore more abundant in LN with low nutrient levels, whereas AOB showed higher abundance in MN and HN (Fig. 2). In addition, the high transcription levels of AOB in the surface sediment of the high-nutrient lake regions may be traceable to suitable substrate environments, such as high ammonium, affecting the metabolic level of active transcription.

Community analysis of AOA and AOB

Further exploration of the community distribution patterns of AOA revealed that the OTUs of the Group I.1a cluster were the dominant cluster in LN (average 44%), which may be associated with its high affinity for ammonium. Studies have demonstrated that AOA are mainly distributed in regions with low levels of ammonium (French *et al.* 2012; Hou *et al.* 2013; Lehtovirta-Morley 2018). Interestingly, we found the highest proportion (average 43.9%) of the *Candidatus Nitrososcosmicus exaquare* cluster in the surface sediment, especially in HN (Fig. 6c), which might be due

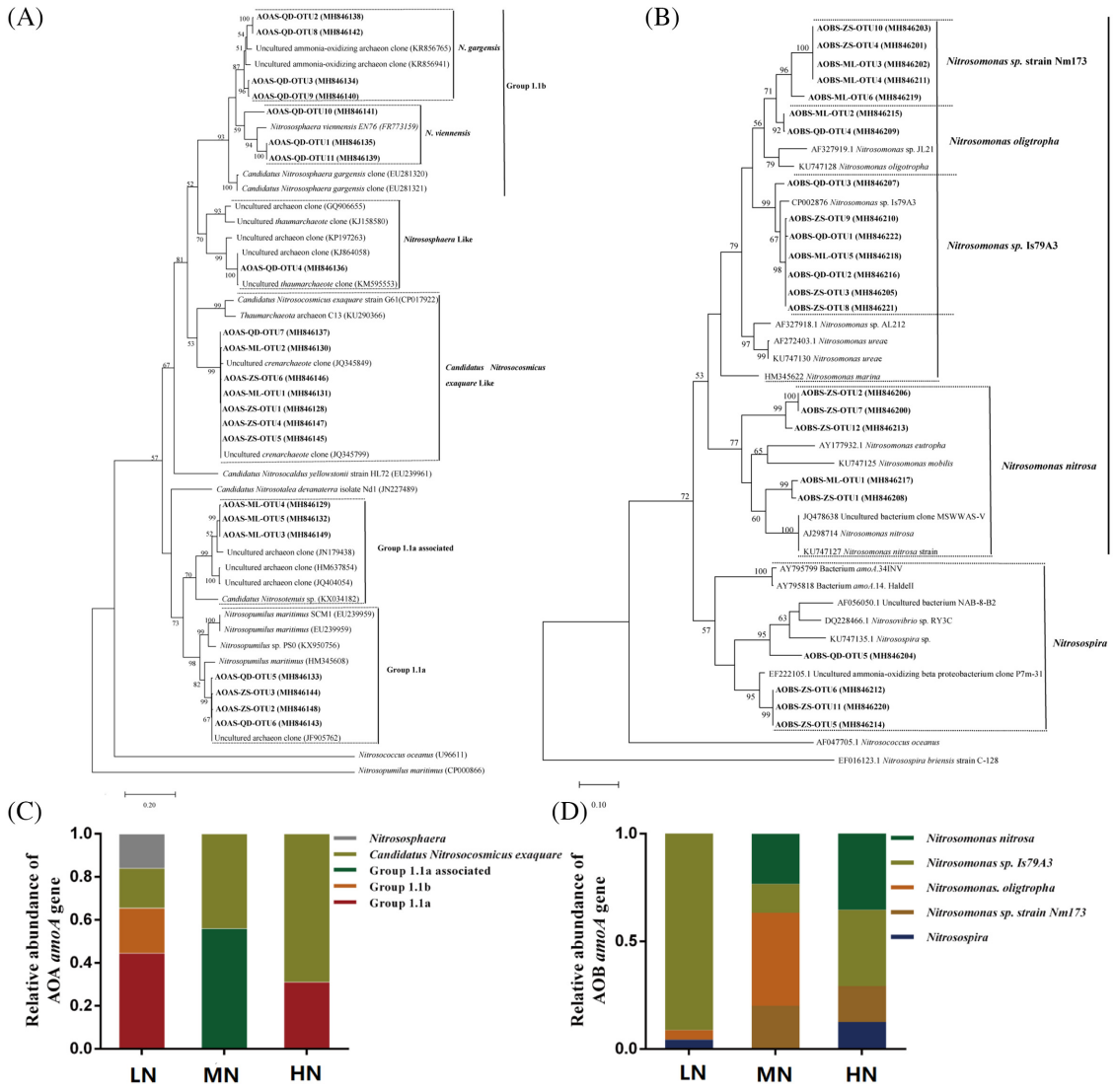


Figure 6. Community structure and composition of AOA and AOB *amoA* gene sequences in the sediment of Lake Taihu. Phylogenetic tree of archaeal *amoA* gene sequences (**A**) and bacterial *amoA* gene sequences (**B**); relative abundance of archaeal *amoA* gene sequences (**C**) and bacterial *amoA* gene sequences (**D**). The phylogenetic tree is constructed by the Maximum Likelihood method. All reference sequences are gained from GenBank. Two capital letters and six numbers in brackets represent the sequences accession number. The OTUs in bold are designated by sample name. For example, AOAS-QD-OTU1 indicates the OTU of AOA detected from the sediment of QD. The numbers close to the nodes represent the bootstrap values of ≥50% (n = 1000 replicates). The scale bar represents 0.20/0.10 nucleic acid substitutions per nucleotide position.

to its adaption to high-ammonium environments (Lehtovirta-Morley 2018). Therefore, these observations illustrate that specific AOA groups adapt to heterogeneous nutritional statuses.

The clusters of AOB were also affected by nutrient levels, especially with regard to ammonium nitrogen. We found that the *Nitrosomonas* cluster (average 94.4%) was predominant in the surface sediment (Fig. 6d), demonstrating that *Nitrosomonas* was the active and dominating cluster of AOB in the high-NH_4^+-N concentration environment. The *Nitrosomonas* cluster exhibited higher tolerance to ammonium nitrogen and cell-specific activity than did the *Nitrosospira* cluster, and the *Nitrosomonas* cluster is reported to be a dominant group of AOB in high-concentration complex nutrient environments (Bollmann *et al.* 2005; Ke and Lu 2012).

As previous reports on the predominance of AOA and AOB on the surface sediment of Lake Taihu have been based on DNA levels and have resulted in numerous contradictory conclusions, RNA level-based studies are urgently needed. In our study, by comparing the total abundance and active transcription of the *amoA* gene, we found RNA-based experimental results to be more convincing for uncovering dominant ammonia oxidizers and may more accurately illustrate the spatiotemporal dynamics of the *amoA* gene.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

Authors' contributions

Tongtong Liu and Hong Yang designed the experiments. Tongtong Liu performed the experiments. Tongtong Liu and Hong

Yang analyzed the data. Tongtong Liu and Hong Yang wrote the manuscript.

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