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Key Points:
- Significant shifts in ammonia-oxidizing community structure were found along the salinity gradient
- Both of AOA *amoA* gene abundance and potential nitrification rates were significantly and negatively correlated with salinity
- Potential nitrification rates were significantly related to AOA *amoA* gene abundance, but not with AOB

Supporting Information:
- Supporting Information S1

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Shifts in the Community Dynamics and Activity of Ammonia-Oxidizing Prokaryotes Along the Yangtze Estuarine Salinity Gradient

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Abstract Ammonia oxidation, the first and rate-limiting step in nitrification, plays a critical role in the nitrogen cycle. However, the links between the dynamics of ammonia-oxidizing communities and ecosystem processes along the estuarine salinity gradient remain uncertain. In this study, we examined the diversity, abundance, and community structure of ammonia-oxidizing prokaryotes, and the potential nitrification rates along the Yangtze estuarine salinity gradient. Phylogenetic analysis showed that the predominant ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) fell within the *Nitrosospira* and *Nitrosopumilus* clusters, respectively. The AOB *amoA* gene abundance (4.67×10^5 to 3.90×10^7 copies per gram of dry sediment) outnumbered AOA (5.14×10^4 to 8.88×10^6 copies per gram of dry sediment). The potential nitrification rates varied between 0.13 and 0.63 μg $N \cdot g^{-1} \cdot day^{-1}$ and related only to AOA *amoA* gene abundance. Salinity had significant effects on AOA *amoA* gene abundance, nitrification rates, and the community structure of ammonia-oxidizing prokaryotes. Principal coordinate analysis showed that the AOB *amoA* gene clones derived from the middle- and high-salinity regions behaved as a cohesive group, while all the low-salinity clone libraries were grouped together. Moreover, the distribution of AOA communities showed a distinct salinity differentiation. Overall, this study improves the understanding of the dynamic shifts in ammonia-oxidizing microorganisms in the Yangtze Estuary.

1. Introduction

Nitrogen overload is recognized as a severe environmental problem in this century (Galloway et al., 2014). Anthropogenic reactive nitrogen has increased by 120% over the past few decades, mainly because of excessive application of nitrogen fertilizers and vast combustion of fossil fuels (Galloway et al., 2008; Gruber & Galloway, 2008; Kim et al., 2008). Most of the anthropogenic nitrogen is delivered to estuarine and coastal zones through groundwater, river, and atmosphere (Diaz & Rosenberg, 2008; Hou, Yin, et al., 2015; Seitzinger, 2008). The estuarine ecosystem generally harbors steep environmental gradients due to the mixing of both freshwater and seawater, which has significant influence on nitrogen transformations (Crump et al., 2004; Hou, Zheng, et al., 2015; Moore, 1999). Of all the nitrogen transformation processes, nitrification, oxidizing ammonium (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-), plays a critical role in the nitrogen cycle of estuarine ecosystems (Dang et al., 2010; Jin et al., 2011; Santoro et al., 2008; Zheng et al., 2017). The process is often the first step in nitrogen removal from estuaries, as it links the mineralization of organic matter-derived nitrogen to the ultimate nitrogen loss via denitrification and anaerobic ammonium oxidation (Lam et al., 2007; Santoro et al., 2008). Additionally, nitrification is an important detoxification process by removing excess ammonia from eutrophic environments (Camargo & Alonso, 2006) and controls the distribution of reduced and oxidized forms of nitrogen (Bouskill et al., 2012; Gruber & Galloway, 2008).

Ammonia oxidation is the first and rate-limiting step of nitrification, which has been widely concerned due to its important ecological role (Kowalchuk & Stephen, 2001; Y. F. Wang & Gu, 2014). In particular, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) communities are identified to be the dominant contributors of ammonia oxidation (Caffrey et al., 2007; Francis et al., 2003). Ammonia monooxygenase (*amoA*) gene is frequently used as a biomarker for AOA and AOB in various environments due to its wide distribution and conserved phylogeny (He et al., 2007; Jin et al., 2011; Rotthauwe et al., 1997). Numerous studies have explored the diversity and abundance of AOB and AOA in mangrove sediments (M. Li et al., 2011), lakes (Mukherjee et al., 2016), and estuarine ecosystems (Mosier & Francis,

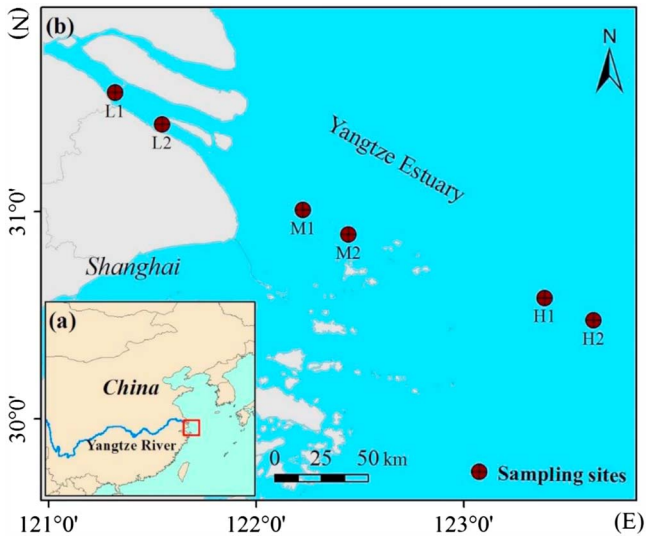

Figure 1. Map showing the location of the Yangtze Estuary (a) and sampling stations along a salinity gradient (b).

2008) and the response of ammonia-oxidizing prokaryotes to fertilization (Y. L. Chen et al., 2014). However, how physicochemical factors modulate the activity and community structure of ammonia-oxidizing prokaryotes remains poorly understood in both terrestrial and aquatic ecosystems.

Salinity has been suggested to be a crucial factor modulating the community structure of AOB and AOA (M. Li et al., 2011; Zheng et al., 2014) and nitrification rates (Bernhard et al., 2007) in estuarine systems where salinity as well as other environmental factors is highly dynamic (Campbell & Kirchman, 2013). The Yangtze River is the largest river in the Euro-Asian continent. Its estuary is characterized by a wide range of salinity gradient (Zheng et al., 2016). However, to our knowledge, few reports have demonstrated the underlying interactions among the dynamics of ammonia-oxidizing microorganisms and nitrification rates along the salinity gradient of the Yangtze Estuary. In the present study, we elucidated (i) the diversity, abundance, and community composition of AOB and AOA in the Yangtze estuarine and coastal zone based on *amoA* gene; (ii) the activity of ammonia-oxidizing microorganisms; and (iii) the correlations among AOB and AOA community structure, activity, and salinity in the study area. This work may improve our understanding of the microbial nitrogen cycles in the transition zone of both land and sea systems.

2. Materials and Methods

2.1. Study Area and Sampling

The Yangtze Estuary, located in the central eastern coast of China, experiences a great salinity gradient (approximately 0–33‰) due to the interaction between freshwater and seawater. Field surveys were conducted in July 2015 and March 2016. According to the salinity gradient, six sampling sites were chosen in this work (Figure 1). These sites belong to three salinity groups: sites L1 and L2, representing low salinity (0.1‰ to 0.4‰); sites M1 and M2, representing middle salinity (6.5‰ to 16.9‰); and sites H1 and H2, representing high salinity (27.6‰ to 33.7‰). Triplicate surface sediment samples (0–5 cm) were collected from each site with plexiglass tubes and stored at low temperature (4–5 °C). The bottom water samples (0.5 m over the surface sediment) were also obtained. After sampling, sediment in each tube was homogenized under helium to form a mixed sample. One part of the mixed sample was incubated for measurement of nitrification rates, and the other part was used for analyses of sediment characteristics and microbial dynamics.

2.2. Environmental Variables

The overlying water salinity was measured in situ using a YSI Model 30 salinity meter. Sediment pH was determined with a pH meter (Mettler-Toledo), after mixing the sediments with deionized water free of CO_2 at a sediment/water volume ratio of 1:2.5 (Lin et al., 2016). Concentrations of sediment ammonium (NH_4^+) and nitrate plus nitrite (NO_x^-) were determined on a continuous flow nutrient analyzer (SAN plus, *Skalar Analytical* B.V., the Netherlands), after extracted with 2-M KCl from fresh sediments (Zheng et al., 2014). Content of sediment sulfide was analyzed using a H_2S microsensor (Unisense, Aarhus, Denmark), with a detection limit of 0.03 μM. Sediment mean size was analyzed with a LS 13 320 Laser grain sizer. Total organic carbon (TOC) of sediment was measured with a thermal combustion furnace analyzer (VVarioELIII, Elementary, Germany) after removing carbonate by leaching with 0.1-M HCl (Hou et al., 2013).

2.3. DNA Extraction and *amoA* Gene Library Analyses

Nucleic acids were extracted from ~0.2-g wet sediment using PowerSoil DNA isolation kits (MoBio, USA). AOB *amoA* gene fragments (~491 bp) were amplified with primers amoA1F/2R (Rotthauwe et al., 1997). AOA *amoA* gene amplicons (~635 bp) were amplified from the extracted DNA using primers Arch-amoAF/R (Francis et al., 2005). Related information on the primers and PCR protocols is given in Table S1 in the supporting information. The *amoA* gene fragments were visualized by electrophoresis on 1.0% agarose gels and purified with the Gel Advance gel extraction system (Viogene, China). Then 100 clones were randomly screened and sequenced with an ABI 3370XL Prism genetic analyzer (Applied Biosystems, Canada) by Sangon (Shanghai,

China). The DNAstar software package (DNASTAR, USA) was applied to assemble, remove low-quality and short sequences, and then edit and put in order the remaining reads. All sequences were detected initially with the BLASTn tool (https://www.ncbi.nlm.nih.gov/genbank/) in GenBank (e-value cutoff of 1×10^{-10} and the query between 95% and 100%; Thompson et al., 1997). The *amoA* sequences having >97% similarity were grouped into one operational taxonomic unit (OTU) using the Mothur program (version 1.31.2; http://www.mothur.org/; Y. L. Chen et al., 2014; Schloss et al., 2009). Phylogenetic trees were performed using Mega 5 software using the neighbor-joining method (Kumar et al., 2004). The related confidence of the tree topologies was performed by 1,000 bootstrap replicates (Tamura et al., 2007). In addition, the maximum likelihood trees were also built for comparison (Figures S12 and S13). The unique AOB and AOA *amoA* gene sequences obtained in this study have been submitted in GenBank, with accession numbers MF355822 to MF356206 and MF355421 to MF355821, respectively.

2.4. Quantification of the *amoA* Genes

Primers amoA1F/2R and Arch-amoAF/R were applied to quantify the AOB and AOA *amoA* genes (Francis et al., 2005; Rotthauwe et al., 1997). Related information of the primers and amplification conditions is given in Table S1. Plasmids containing *amoA* genes were extracted from *Escherichia coli* hosts by using Qiagen Miniprep Spin Kit (QIAGEN, Hilden, Germany). The quantitative polymerase chain reaction (Q-PCR) standard curves were formed as described by Gao et al. (2016). Each of the triplicate DNA samples was quantified with the ABI 7500 Sequence detection system (Applied Biosystems, Canada). The melting curves were shown by the ABI 7500 system, and the threshold cycle values were estimated by comparison with standard curves.

2.5. Nitrification Rates

Potential nitrification rates were determined in triplicate with the chlorate inhibition method (Kurola et al., 2005). Approximately 5.0 g of sediment and 25 ml of phosphate buffer solution (pH 7.4; g/L: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 0.2; NaH_2PO_4, 0.2) with 1-mM $(NH_4)_2SO_4$ were added to the 50-ml centrifuge tubes. Potassium chlorate (final concentration 10 mM) was added to inhibit nitrite oxidation. The suspension was then incubated in dark at in situ temperature for 24 hr. After incubation, nitrite was extracted with 2-M KCl and measured on a continuous flow nutrient analyzer (SAN plus, *Skalar Analytical* B.V., the Netherlands). The rates of nitrification were quantified on the basis of the changes in nitrite concentrations within the incubations.

2.6. Statistical Analysis

The rarefaction curves, Chao1 species richness, and Shannon-Wiener diversity index of clone libraries were calculated by the Mothur program with 97% identity (sequences >97% similarity were grouped into one OTU; Schloss et al., 2009), and the command and parameters used for Mothur were shown in the supporting information. Correlations of AOB and AOA assemblages with environmental parameters were explored using the redundancy analysis (RDA) and canonical correspondence analysis (CCA) by Canoco 4.5 software (ter Braak & Šmilauer, 2002). Community classification for ammonia-oxidizing communities was explored with principal coordinate analysis (PCoA) using the Mothur program and phylogeny inference package (version 3.695) based on the distance matrix (Felenstein, 1989). To assess the temporal and spatial variance of AOA and AOB assemblages, one-way analysis of variance (ANOVA) was applied using Statistical Package of Social Sciences (SPSS, version 16.0). The Pearson and partial correlation analyses were also performed by the SPSS software.

3. Results

3.1. Site Physicochemical Characteristics

The environmental variables of sampling sites are given in Table S2. The sampling sites covered a salinity gradient from 0.1‰ to 33.7‰. The median grain size of sediment was in the range of 64.2–98.8, 6.5–20.0, and 70.7–96.6 μm at the low-, middle-, and high-salinity sites, respectively. The concentration of TOC in sediments showed a negative correlation with salinity ($R = -0.927$, $P = 0.001$). The value of pH varied from 7.87 to 8.66 throughout the estuary. The concentration of sediment NO_x^- ranged from 0.01 to 0.35 μmol/g, with the highest value at high-salinity site H1 and the lowest value at middle-salinity site M1. Additionally, the content of sediment NH_4^+ in summer (0.32 to 0.61 μmol/g) was higher than in winter (0.06 to 0.09 μmol/g). Sulfide

Table 1
Diversity Estimators of AOB and AOA amoA Gene Clone Libraries in the Sediments Along the Salinity Gradient of the Yangtze Estuary

amoA gene and sample	Summer/winter value				
	No. of clones	OTUs[a]	Chao1[b]	Shannon[c]	Coverage (%)[d]
AOB					
L1	70/69	11/3	11.8/3.1	1.65/0.49	93.6/98.4
L2	73/76	6/12	6.5/12.5	0.65/1.56	92.3/96.0
M1	73/72	12/2	13.5/2.1	2.11/0.07	88.9/97.6
M2	73/73	10/8	12.0/8.1	1.86/1.78	83.3/99.4
H1	70/77	7/6	7.3/6.3	1.67/0.54	96.6/94.7
H2	71/71	12/4	15.0/4.0	1.97/0.71	80.0/95.2
AOA					
L1	65/71	8/7	8.4/7.3	1.87/1.54	95.8/96.6
L2	63/72	9/3	9.3/3.3	1.91/0.20	97.3/92.3
M1	64/67	4/5	5.0/5.5	0.73/0.93	80.0/90.9
M2	63/65	5/8	5.3/8.3	1.08/1.47	95.2/96.9
H1	65/74	2/22	2.1/24.6	0.69/2.69	97.6/89.3
H2	63/62	11/11	12.5/11.3	1.75/1.90	88.0/97.8

Note. AOA = ammonia-oxidizing archaea; AOB = ammonia-oxidizing bacteria; OTU = operational taxonomic unit.
[a]OTUs are defined based on 3% nucleotide acid divergence. [b]Nonparametric statistical predictions of total richness of OTUs based on distribution of singletons and doubletons. [c]Shannon-Weiner index. Higher number represents more diversity. [d]Percentage of observed number of OTUs divided by Chao1 estimate.

contents in sediments varied from 0.40 to 4.90 μmol/g, with higher sulfide concentration in summer (0.84 to 4.90 μmol/g) than in winter (0.40 to 1.51 μmol/g).

3.2. Diversity of AOA and AOB

In this study, 69 to 76 AOB *amoA* gene clones and 62 to 74 AOA *amoA* gene clones were successfully sequenced at each site (Table 1). To characterize the diversity of *amoA* genes, 3% divergence at the nucleotide level was applied to define OTU. In clone libraries of each site, 2 to 12 AOB or 2 to 22 AOA OTUs were recovered. The high clone library coverage (80.0% to 99.4% for AOB and 80.0% to 97.8% for AOA) indicates that the majority of ammonia-oxidizing prokaryotes were selected, which was further verified by the smooth rarefaction curves (Figure S1).

The Shannon-Wiener index of AOB and AOA ranged from 0.07 to 2.11 and 0.20 to 2.69, respectively (Table 1). There was significant seasonal shift in the richness of AOB (one-way ANOVA, $P = 0.045$). The maximal AOB diversity was found at the middle-salinity sites in summer, while the lowest occurred at the high-salinity sites in winter. For AOA diversity, the highest appeared at the high-salinity sites, while the lowest occurred at the low-salinity sites. However, no distinctive seasonal difference was detected in the diversity of AOA *amoA* gene in the Yangtze Estuary (one-way ANOVA, $P > 0.05$).

3.3. Phylogenetic Analysis of *amoA* Gene Sequences

AOB *amoA* gene sequences were divided into five distinctive clusters based on the evolutionary distance (Figure 2). Three of these clusters were affiliated with *Nitrosospira* lineage, whereas the other two clusters belonged to *Nitrosomonas* lineage. Additionally, AOB *amoA* gene sequences in this study were also affiliated with sequences retrieved from various sediment habitats, including the East China Sea, North Sea Oyster Ground, and aquaculture pond and mangrove (Lipsewers et al., 2014; Lu et al., 2016; Yu et al., 2016). The *Nitrosospira* cluster, occupying 87% of the total sequences, was the dominant group in all the samples (Figure 4). In contrast, *Nitrosomonas* lineage only occupied a few clones (13% of the total sequences). The sequences retrieved from the low-salinity sites were dominant in *Nitrosomonas* lineage (accounting for 82% of all the *Nitrosomonas* lineage sequences); however, sequences retrieved from the middle- and high-salinity sites only contributed 11% and 7%, respectively.

The AOA sequences were phylogenetically grouped into six distinct clusters (Figure 3). These OTUs were mainly affiliated to *Nitrosopumilus* (approximately 57% of sequences), *Nitrososphaera* (approximately 10% of sequences), and *Nitrosotenuis* (approximately 10% of sequences) lineage. The other two clusters fell into uncultured cluster comprised of marine/estuary sediment clones from the Yangtze Estuary, East China Sea, and intertidal mudflat (Y. Y. Li et al., 2016; Ma et al., 2015; Pester et al., 2012; Yu et al., 2016; Zheng et al., 2014). *Nitrosopumilus*-like sequences were amplified in all samples (Figure 4). In contrast, most of the *Nitrososphaera*-like clones (88%) or *Nitrosotenuis*-associated clones (97%) were obtained from the low-salinity sites. Additionally, the sequences acquired from the middle-salinity sites accounted for 66% of the uncultured cluster clones.

3.4. Distribution of Ammonia-Oxidizing Community

The dissimilarity matrix of the AOB and AOA community from the sampling sites was determined using PCoA (Figures 5 and S2). The first two ordination axes explained 52.3% (Figure 5a) and 46.8% (Figure 5b) of the variability for AOB and AOA community compositions, respectively. All the low-salinity AOB clone libraries were grouped together (Group 1). However, the AOB communities retrieved from the middle- and high-salinity sites were not different from each other (Figure 5a). The PCoA analysis indicated that the distribution of AOA communities showed a distinct salinity shift (Figure 5b). AOA clone libraries obtained from the lower-salinity sites (L1 and L2) were clustered together, which shared the freshwater AOA community characteristics. The middle-salinity clone libraries (M1 and M2) were grouped together,

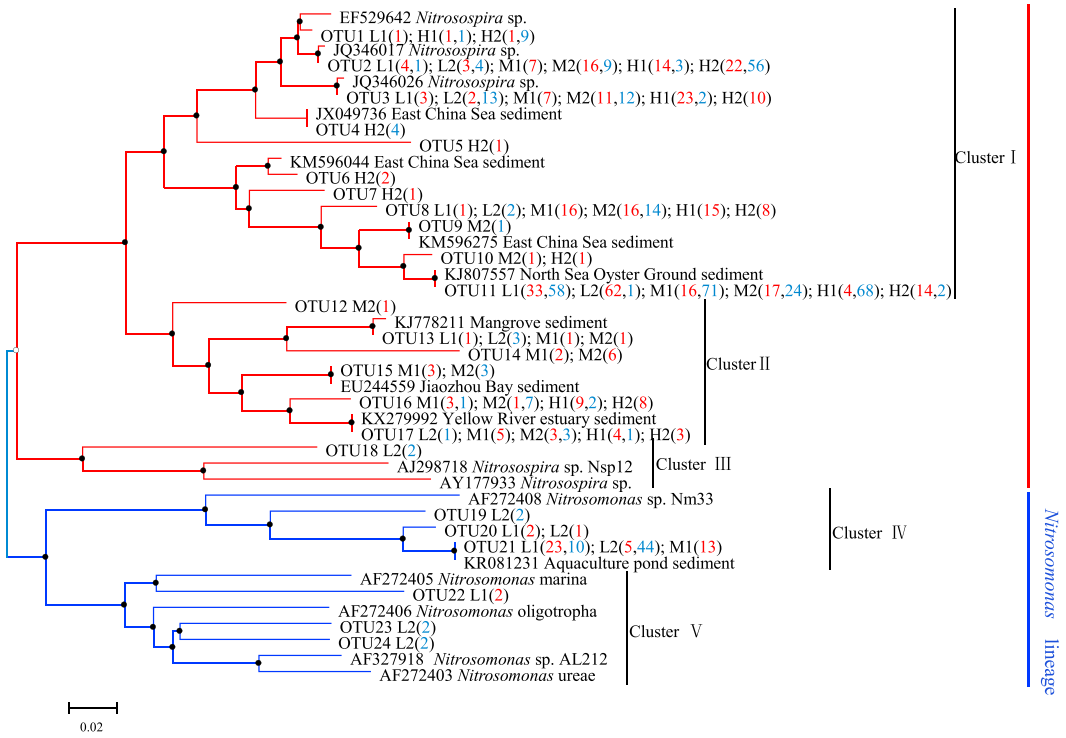

Figure 2. Dendrogram of ammonia-oxidizing bacteria *amoA* gene sequences obtained from the Yangtze Estuary sediments, showing the affiliations between ammonia-oxidizing bacteria *amoA* gene fragments derived from the Yangtze Estuary and reference sequences in the databases. Clone names include the sample name and the number of sequences recovered from each sampling site in summer (red) and winter (blue). Bootstrap values greater than 50% (n = 1,000) are shown with solid circle, and those less than 50% are shown with open circle on the corresponding nodes.
OTU = operational taxonomic unit.

belonged to the estuarine group. The remaining high-salinity clone libraries (H1 and H2) fell into the marine group. Neither AOB nor AOA community structure showed a significant seasonal shift (Figure S2).

3.5. Abundance of the *amoA* Gene

The *amoA* gene copies in the Yangtze Estuary sediments were quantified using Q-PCR. The AOB *amoA* gene abundance (4.67×10^5 to 3.90×10^7 copies per gram of dry sediment) was slightly greater than AOA *amoA* gene abundance (5.14×10^4 to 8.88×10^6 copies per gram of dry sediment; Figure 6 and Table S3). However, no significant difference occurred between the two *amoA* gene abundances (one-way ANOVA, $P > 0.05$). In the present study, the abundance of AOB and AOA *amoA* genes showed a significant seasonal shift (one-way ANOVA, P = 0.028 and 0.026, respectively; Figures S3 and S4).

In addition, the abundance of *amoA* genes displayed a significant spatial heterogeneity. The lowest AOB and AOA *amoA* gene abundance was detected at the high-salinity site H2 and the low-salinity site L2, respectively, whereas the highest *amoA* gene abundance for both AOB and AOA was detected at the middle-salinity sites. AOB and AOA *amoA* gene copies in the middle-salinity sediments were significantly greater than those of the low- or high-salinity sediments (one-way ANOVA, P = 0.001, 0.001, 0.048, and 0.001, respectively).

3.6. Nitrification Rates

Chlorate inhibition method was performed to evaluate the nitrification rates in the Yangtze estuarine sediments. Results showed that the potential nitrification rates varied from 0.13 to 0.63 μg $N \cdot g^{-1} \cdot day^{-1}$ (Figure 7 and Table S3). The nitrification rates were significantly higher in summer than in winter (one-way ANOVA, P = 0.030), with respective average rates of 0.43 and 0.24 μg $N \cdot g^{-1} \cdot day^{-1}$ (Figure S5). A significant shift in nitrification rates was detected along the Yangtze Estuary. The maximum potential nitrification rates occurred at the low-salinity sites, followed by the middle-salinity sites and high-salinity sites, with respective

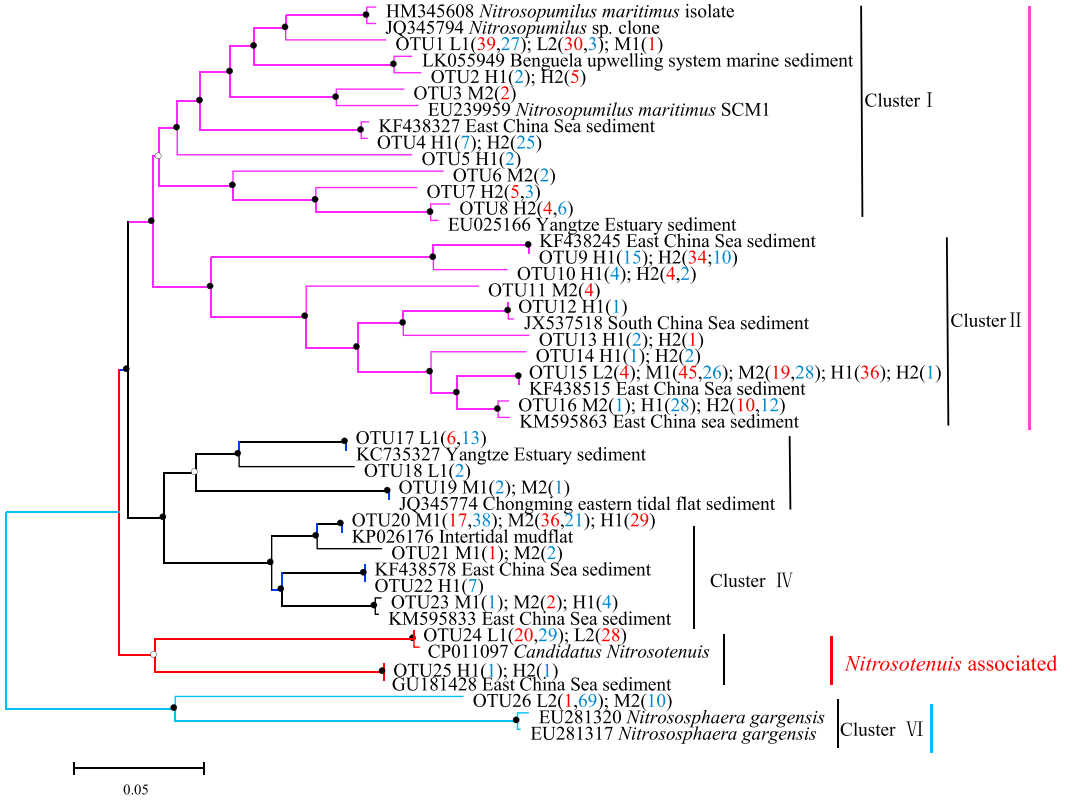

Figure 3. Dendrogram of ammonia-oxidizing archaea *amoA* gene sequences obtained from the Yangtze Estuary sediments, showing the affiliations between ammonia-oxidizing archaea *amoA* gene fragments derived from the Yangtze Estuary sediments and reference sequences in the databases. Clone names include the sample name and the number of sequences recovered from each sampling site in summer (red) and winter (blue). Bootstrap values greater than 50% (n = 1,000) are shown with solid circle, and those less than 50% are shown with open circle on the corresponding nodes. OTU = operational taxonomic unit.

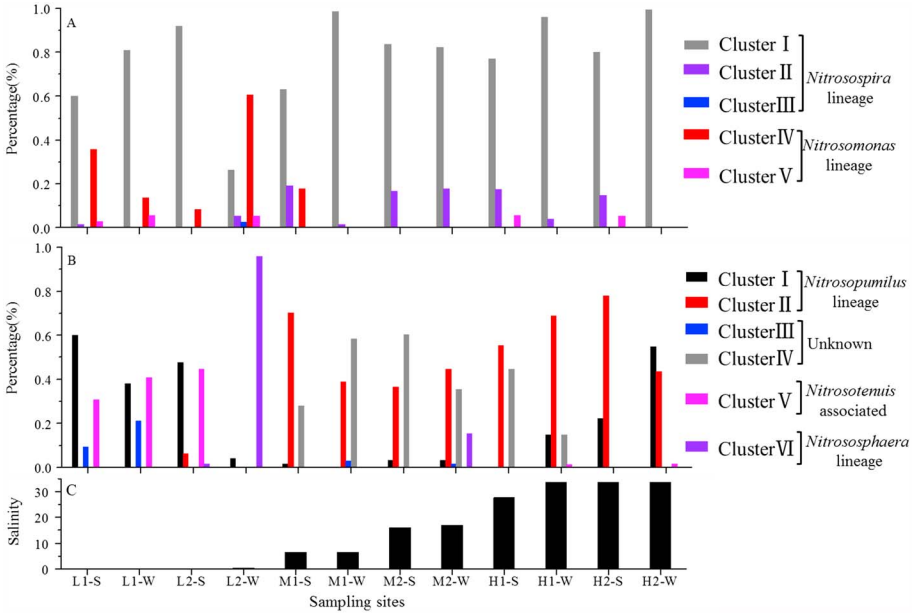

Figure 4. The composition of (a) ammonia-oxidizing bacteria and (b) ammonia-oxidizing archaea communities along the (c) salinity gradient of the Yangtze Estuary. L, M, and H represent low, middle, and high salinity, respectively. S and W represent summer and winter samples, respectively.

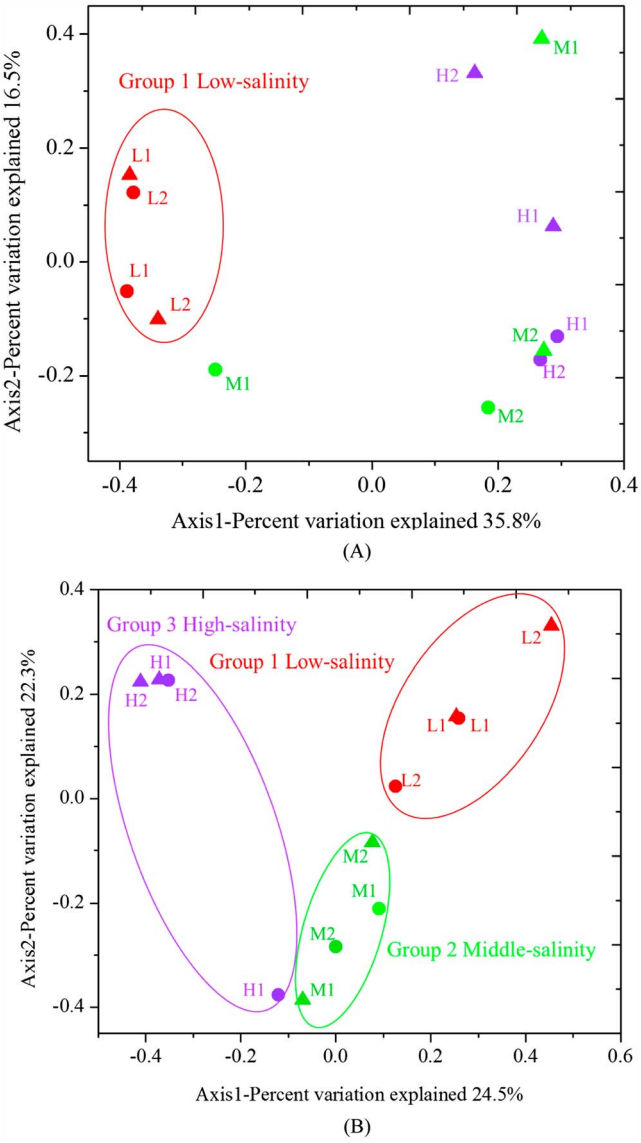

Figure 5. Principal coordinate analysis of (a) ammonia-oxidizing bacteria and (b) ammonia-oxidizing archaea communities along the salinity gradient of the Yangtze Estuary. Triangle and circle represent winter and summer samples, respectively. Red, green, and purple sign represent samples from low-, middle-, and high-salinity sites, respectively.

average values of 0.47, 0.33, and 0.21 μg $N \cdot g^{-1} \cdot day^{-1}$ (Figure S6). The nitrification rates were significantly related to the AOA *amoA* gene abundance (R = 0.586, P = 0.001; Figure S8). However, no significant relationship was observed between AOB *amoA* gene abundance and nitrification rates ($P > 0.05$; Figure S7).

3.7. Relationships of Community Structure, Abundance, and Nitrification Rates With Environmental Factors

RDA and CCA analyses were used to explore the influence of the environmental variables on the structure of AOB and AOA communities, respectively (Figure 8). The first two dimensions provided 73.5% of the accumulative variance of the AOB community-environment correlation, while they explained 55.5% in the AOA CCA ordination plot. Results indicated that the AOB community structure was significantly related to salinity and TOC (P = 0.027 and 0.026, respectively; 499 Monte Carlo permutations), which in total accounted for 47% of the total RDA expositive power. Similar to AOB, AOA community structure was also significantly correlated with salinity and TOC (P = 0.026 and 0.025, respectively; 499 Monte Carlo permutations). These two environmental variables explained 49% of the entire variance.

The influence of environmental parameters on gene abundance and nitrification rates was also explored using SPSS software. The statistical analysis showed that there were negative correlations of AOA *amoA* gene copy numbers (R = −0.387, P = 0.020) and nitrification rates (R = −0.711, P = 0.001) with salinity (Figures S10 and S11). No similar relationship was observed between AOB *amoA* gene abundance and salinity ($P > 0.05$; Figure S9), but a negative correlation was found between AOB *amoA* gene abundance and sediment mean size (R = −0.684, P = 0.021). AOA *amoA* gene abundance was also significantly related to the concentration of NH_4^+ (R = 0.615, P = 0.020). Additionally, the nitrification rates were negatively correlated to the concentration of TOC (R = 0.686, P = 0.015).

4. Discussion

Although salinity is known to be an important factor affecting the diversity and abundance of ammonia-oxidizing microorganisms, corresponding changes in their composition, distribution, and activity in estuarine systems are less described. This study explored the community diversity, distribution, and abundance of ammonia-oxidizing prokaryotes and potential nitrification rates along the salinity gradient of the Yangtze Estuary. Diversity estimations showed that there was a significant seasonal shift in AOB communities (one-way ANOVA, P = 0.045), indicating that high temperature might favor the coexistence of diverse AOB communities (L. M. Wang et al., 2013). And the diversity of AOA communities was slightly higher than AOB along the Yangtze estuarine salinity gradient (0.20–2.69 and 0.07–2.11, respectively, predicted by Shannon-Wiener index; Table 1). This might be because archaea communities have competitive advantage in severe environments (e.g., high salinity; Martens-Habbena et al., 2009; Yao et al., 2011), which do not support the development of bacteria and eukaryotes (Valentine, 2007). Actually, the OTU number of AOA communities (based on 3% nucleotide acid divergence) in the high-salinity libraries (a total of 46 OTUs) was twice as in the low- or middle-salinity libraries (27 and 22 OTUs, respectively). These results indicated that salinity might be an important factor causing the shift of the ammonia-oxidizing communities in estuarine ecosystems.

The predominant AOB and AOA microorganisms recovered in this research fell within *Nitrosospira* (87% of the total AOB sequences) and *Nitrosopumilus* clusters (57% of the total AOA sequences), respectively (Figures 2 and 3), which are also known as marine/estuarine sediment lineages (Cao et al., 2011; Pester et al., 2012).

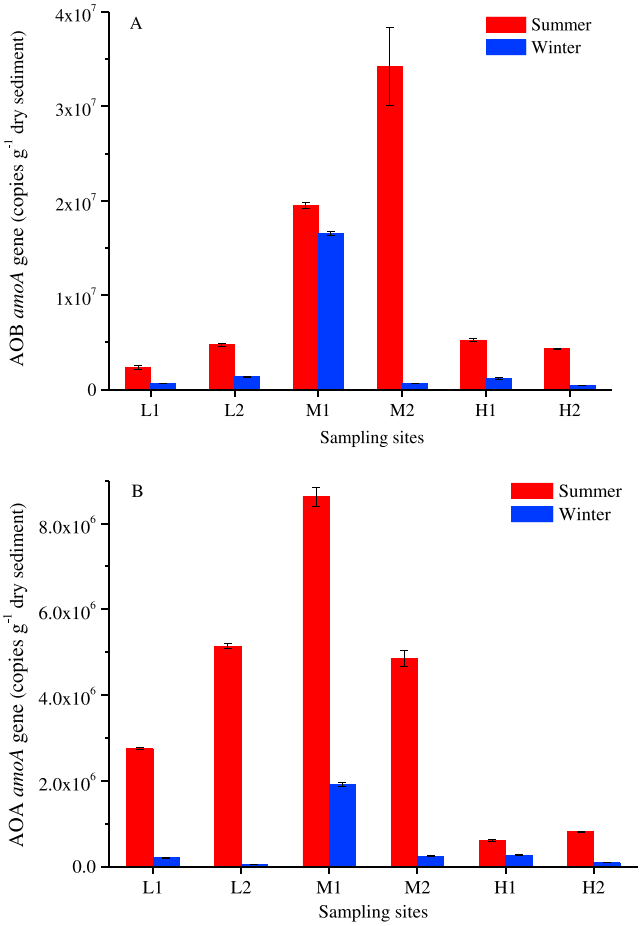

Figure 6. The spatiotemporal variations of (a) ammonia-oxidizing bacteria (AOB) *amoA* gene and (b) ammonia-oxidizing archaea (AOA) *amoA* gene abundance along the salinity gradient of the Yangtze Estuary. Vertical bars indicate standard error (n = 3).

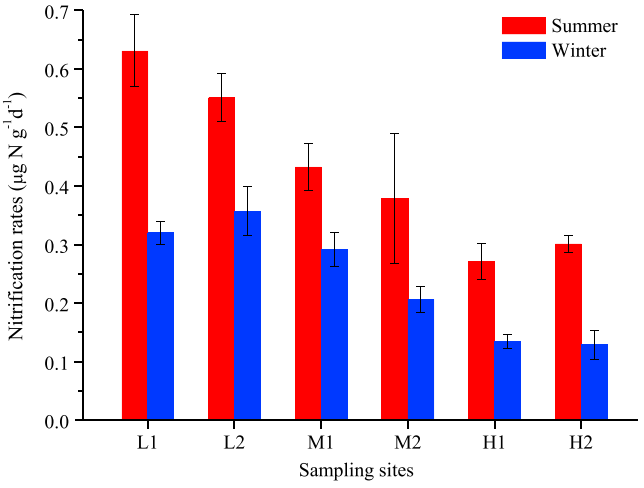

Figure 7. The spatiotemporal variations of nitrification rates along the salinity gradient of the Yangtze Estuary. Vertical bars indicate standard error (n = 3).

These results indicated that most ammonia-oxidizing prokaryotes along the Yangtze estuarine salinity gradient were halotolerant microorganisms (Bollmann & Laanbroek, 2002). A part of the AOB and AOA sequences was also affiliated with *Nitrosomonas* (13% of the total AOB sequences) and *Nitrososphaera* lineages (10% of the total AOB sequences), respectively, and their reference sequences originally retrieved from terrestrial soil (Cao et al., 2011; Francis et al., 2003). Both marine and terrestrial ammonia-oxidizing species were detected in the Yangtze Estuary, probably due to the land-sea interaction (Hou et al., 2013; Zhang et al., 2014). The Yangtze River could carry massive terrestrial sediment to the estuarine zone via its runoff (Z. Y. Chen et al., 2004). Additionally, it is interesting to note that the terrestrial-lineage sequences in the present study mainly retrieved from low-salinity sites (accounting for 82% in AOB and 88% in AOA terrestrial lineage). Similar distribution patterns were also found in other estuarine/marine ecosystems, such as the southern North Sea, San Francisco Bay estuary, and Chesapeake Bay (Francis et al., 2003; Lipsewers et al., 2014; Mosier & Francis, 2008), suggesting that there was a distinctive spatial heterogeneity in the ammonia-oxidizing community composition along unique estuarine gradients created by the interaction of both freshwater and seawater.

The distribution of AOA microorganisms showed a distinct shift along the salinity gradient according to the PCoA analysis (Figure 5). The AOA sequences recovered from the low-, middle-, and high-salinity regions were clustered independently (Figure 5b), indicating the niche specificity along the salinity gradient in the present study. Significant shifts of AOB and AOA communities along the salinity gradient have been reported in other estuarine systems, such as Plum Island Sound estuary, Schelde estuary, and Pearl River estuary (Bernhard et al., 2005; Cao et al., 2011; de Bie et al., 2001). Bollmann and Laanbroek (2002), with continuous culture experiments, demonstrated that salinity was the main factor controlling the shift of AOB communities. In this study, only AOB communities from the low-salinity habitats showed site specificity, while AOB communities obtained from the middle- and high-salinity samples behaved as a cohesive group (Figure 5a). It is predicted that the ammonia-oxidizing microorganisms, especially AOA assembles, contain selectable species to adapt sharp salinity gradients in the Yangtze Estuary. This is further confirmed by the RDA and CCA analyses, indicating that both AOB and AOA community structures were significantly related to salinity (P = 0.027 and 0.026, respectively; Figure 8). However, neither of the ammonia-oxidizing prokaryotes distribution showed significant seasonal difference (Figure S2).

In the present study, AOB *amoA* gene abundance outnumbered AOA except for site L1 in summer, with 4.67×10^5 to 3.90×10^7 and 5.14×10^4 to 8.88×10^6 copies per gram of dry sediment, respectively (Figure 6). Although previous studies demonstrated that the abundance of AOA *amoA* gene was greater than AOB in some marine or estuarine systems (Beman et al., 2008; Mincer et al., 2007; Park et al., 2008; Zhang et al., 2015), opposite results were also reported in high-salinity zones of San Francisco Bay and the Elkhorn Slough estuary (Mosier & Francis, 2008; Wankel et al., 2010). In any case, the contributions of both AOB and AOA to nitrification remain a debate topic (Yao et al., 2011). The difference of AOB and AOA *amoA* gene abundance may be due to the variance of physicochemical properties in marine and estuarine systems (Zhang et al., 2015). Both of the AOB and AOA *amoA* gene abundances in the middle-

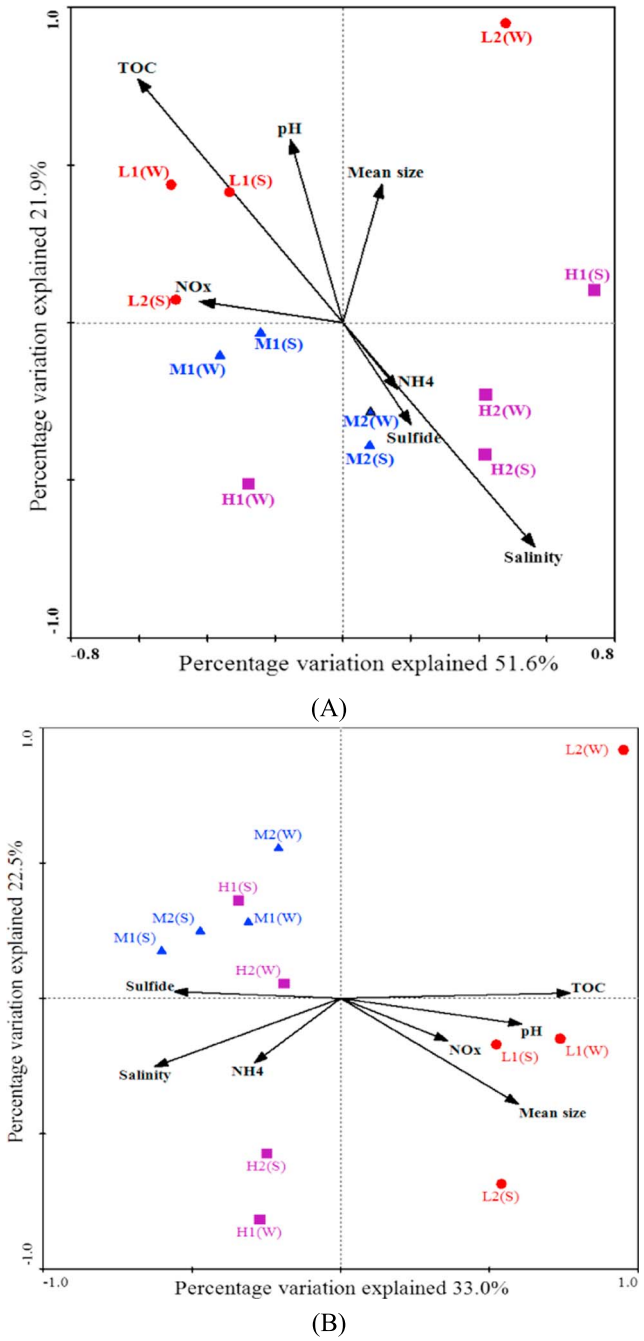

Figure 8. Redundancy analysis and canonical correspondence analysis ordination plot for the effect of environmental variables on the structure of AOB (a) and AOA (b) communities. NO*x* represents the concentration of nitrate plus nitrite in sediment. Details of other environmental variables are the same as shown in Table S2. AOA = ammonia-oxidizing archaea; AOB = ammonia-oxidizing bacteria; TOC = total organic carbon.

salinity sediments were significantly higher than those derived from the low- or high-salinity sediments (one-way ANOVA, $P = 0.001$, 0.001, 0.048, and 0.001, respectively), which was similar to those detected in a New England estuary (Bernhard et al., 2007) and the Plum Island Sound estuary (Bernhard et al., 2010). It is possible that cations of the middle-salinity substrate could exchange more NH_4^+ than low salinity, while excessive salinity might inhibit the activity of ammonia-oxidizing prokaryotes (Y. F. Wang & Gu, 2014), which is further supported by the negative relationship between potential nitrification rates and salinity ($R = -0.711$, $P = 0.001$) and the positive correlation between AOA *amoA* gene abundance and the concentration of NH_4^+ ($R = 0.615$, $P = 0.020$).

In this study, a negative correlation was observed between AOB *amoA* gene abundance and sediment mean size ($R = -0.684$, $P = 0.021$). This relationship shows that fine-grained sediments could provide more nutritional sources than coarse-grained sediments (Cammen, 1982; Dale, 1974). In addition, significant correlations were detected between TOC and nitrification rates ($R = 0.686$, $P = 0.015$), as well as the community structure of AOB and AOA ($P = 0.026$ and 0.025, respectively; Figure 8). Interestingly, the concentration of TOC showed significant negative correlation with salinity ($P = 0.001$). Consequently, we speculated that there might be a primary factor contributing to the shift of ammonia-oxidizing community structure in the Yangtze Estuary. Lozupone and Knight (2007), analyzing 21,752 sequences retrieved from various systems in 111 previous studies, found that the crucial environmental determination of microorganism structure was salinity rather than other physicochemical parameters. Recent studies also demonstrated that salinity was a major environmental parameter affecting AOB and AOA community structure (Bernhard et al., 2010; Shen et al., 2008; Wankel et al., 2010). In this study, when we further used partial correlation analysis in which the effect of salinity was controlled to explore the influence of TOC on nitrification rates, it was shown that the relationship was not significant ($P > 0.05$). These results confirmed that salinity was a crucial factor affecting the structure of ammonia-oxidizing microorganisms.

The nitrification rates showed a significant seasonal shift (one-way ANOVA, $P = 0.030$), with average rates of 0.43 and 0.24 μg $N \cdot g^{-1} \cdot day^{-1}$ in summer and winter, respectively (Figure S5), which might be attributed to the higher *amoA* gene numbers in summer (Zheng et al., 2014). Previous studies also demonstrated that nitrification rates may be reflected by *amoA* gene abundance (Bernhard et al., 2010; Di et al., 2009; Moin et al., 2009), so we predicted that the two parameters were correlated with each other. In the present study, the nitrification rates were only significantly related to the AOA *amoA* gene abundance ($R = 0.586$, $P = 0.001$; Figures S7 and S8), although AOB *amoA* gene abundance outnumbered AOA. This might be because, although the niche occupancy of AOB was larger than AOA, the activity of AOB might be inhibited by the salinity shift. Previous studies also revealed that with the increase of salinity, AOB *amoA* gene abundance was stable, while the gene transcription decreased; in contrast, the transcription of AOA *amoA* gene increased (Smith et al., 2014; Zhang et al., 2015). These results implied that AOA microorganisms were probably more responsible for nitrification along the salinity gradient of the Yangtze Estuary. In the present study, the decreased nitrification rates along the Yangtze Estuary with increasing salinity (Figures 7 and S11) was in agreement with previous reports (Caffrey et al., 2007; Rysgaard et al., 1999). Moreover, both of the AOA *amoA* gene abundance and nitrification rates were negatively correlated with salinity ($R = -0.387$,

$P = 0.020$; $R = -0.711$, $P = 0.001$, respectively; Figures S10 and S11), suggesting that salinity might be a critical environmental factor modulating nitrification process along the Yangtze Estuary. However, further efforts are required to explore the underlying mechanisms.

5. Conclusions

In this study, we explored the variation of ammonia oxidizers along the salinity gradient of the Yangtze Estuary. Distinct shifts in ammonia-oxidizing community composition were found along the salinity gradient. The predominant of AOB and AOA microorganisms fell within *Nitrosospira* and *Nitrosopumilus* clusters, respectively. Nitrification rates were only significantly correlated with the AOA *amoA* gene abundance, indicating that AOA communities were more responsible for nitrification in the estuarine system. Salinity showed significant effects on the community composition and distribution of ammonia oxidizers, the abundance of AOA, and the potential nitrification rates. These results suggested that salinity might be an important factor regulating nitrification process in the Yangtze Estuary. Overall, this research improves the understanding of the dynamics and control of ammonia-oxidizing prokaryotes in the estuarine and coastal environment.

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