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ENVIRONMENTAL BIOTECHNOLOGY

The influence of salinity on the abundance, transcriptional activity, and diversity of AOA and AOB in an estuarine sediment: a microcosm study

Yan Zhang[1,4] · **Lujun Chen**[1,2] · **Tianjiao Dai**[3] · **Jinping Tian**[1] · **Donghui Wen**[3]

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Abstract Estuarine sediment-seawater microcosms were established to evaluate the influence of salinity on the population, transcriptional activity, and diversity of ammonia-oxidizing archaea (AOA) and bacteria (AOB). AOA was found to show the most abundant and the highest transcriptional activity under moderate salinity; on the other hand, AOB abundance was not sensitive to salinity variation but showed the highest transcriptional activity in the low-salinity microcosms. AOA exhibited more advantages than AOB on growth and ammonia-oxidizing activity under moderate- and high-salinity environments. The highest richness and diversity of active AOA were found under salinity of 15 psu. All the active AOA detected under the salinities studied were clustered into *Nitrosopumilus maritimus* linage, with the composition shifted from *N. maritimus* C12 cluster, *N. maritimus* like 1.1 cluster, *N. maritimus* SCM1 cluster, and *N. maritimus* like 1.2 cluster to *N. maritimus* C12 and *N. maritimus* A10 clusters when salinity was increased from 5 to 30 psu.

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✉ Donghui Wen
dhwen@pku.edu.cn

[1] School of Environment, Tsinghua University, Beijing 100084, China

[2] Zhejiang Provincial Key Laboratory of Water Science and Technology, Department of Environmental Technology and Ecology, Yangtze Delta Region Institute of Tsinghua University, Zhejiang Jiaxing 314050, China

[3] College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

[4] Present address: Department of Civil and Environmental Engineering, University of Alberta, Edmonton T6G 2 W2, Canada

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Introduction

Ammonia oxidation is the first and rate-limited step of nitrification, which is the only oxidative process that links the reduced and oxidized pools of inorganic nitrogen (Martens-Habbena et al. 2009) and thus plays a critical role to sustain the global nitrogen cycle. Two narrow bacterial clades of *betaproteobacteria* and *gammaproteobacteria* (Kowalchuk and Stephen 2001; Purkhold et al. 2000) and the new archaeal phylum *Thaumarchaeota* (Brochier-Armanet et al. 2008; Spang et al. 2010) respectively contains the ammonia-oxidizing bacteria (AOB) and archaea (AOA) who possess the gene encoding ammonia monooxygenase (AMO) and own the ability of transform ammonia to nitrite via hydroxylamine. After the discovery of AOA in 2005 (Francis et al. 2005; Konneke et al. 2005; Venter et al. 2004), AOA, as well as AOB, has been found in diverse environments including soil (He et al. 2007; Le Roux et al. 2008), freshwater (Liu et al. 2013; Wu et al. 2010), ocean (Mosier and Francis 2008; Pitcher et al. 2011), and salt lake (Jiang et al. 2009).

The abundance and activity of AOA and AOB vary in different environments with the variation of environmental factors. As the substrate of ammonia oxidation, ammonia has been considered as a primary element to manipulate the abundance of AOA and AOB in environments owing to the lower half-saturation constant and substratethreshold of AOA (Martens-Habbena et al. 2009). Microcosm studies has proved the preference of AOA and AOB to ammonia-limited and ammonia-rich niches, respectively, in both soil (Di et al. 2009, 2010; Pratscher et al. 2011; Verhamme et al. 2011)

and aquatic environments (Zhang et al. 2015). In addition to ammonia, in soil environments, pH is another important driver segregating ammonia-oxidizing microorganism (AOM). Shen et al. (2008) revealed the significant correlation of AOB abundance and soil pH in an alkaline sandy loam (pH 8.3–8.7); Gubry-Rangin et al. (2010) found the significant growth of AOA but not AOB in microcosms of two acidic soils (pH 4.5 and 6) without ammonia supplement; higher abundance of AOA than that of AOB was detected in the highly acidic soils (pH<3.5) in the investigation of 713 soil samples of Scotland (Yao et al. 2013). Yao et al. (2011) and Hu et al. (2013) respectively revealed that the ratios of AOA to AOB *amoA* gene copy numbers significantly decreased with the increasing pH in acidic tea orchard soils (pH 3.58–6.29) and in 65 soil samples across North to South China (pH 3–9), suggesting a competitive advantage of AOA over AOB in acidic soils. As ammonia rather than ammonium (NH_4^+) is the substrate for AOB, the sensitivity of AOB at low pH is due to the absence of NH_3 because NH_3 concentration decreases exponentially with the decrease of pH ($NH_3+H^+ \rightarrow NH_4^+$; $pK_a=9.25$) (Frijlink et al. 1992; Wang and Gu 2014).

In aquatic environments, salinity is another putative factor that shapes the structure and abundance of ammonia-oxidizing microbial community. An ecophysiology study revealed the preference for low-salinity habitat of an enriched AOA, Candidatus *Nitrosoarchaeum limnia* strain SFB1. *N. limnia* (Mosier et al. 2012). The strain was capable of growing at 75 % of seawater salinity; nevertheless, there was a longer lag time, incomplete oxidation of ammonia to nitrite, and slower overall growth rate (Mosier et al. 2012). Consistently, in San Francisco Bay estuary (Mosier and Francis 2008) and a subterranean estuary at Huntington Beach (Santoro et al. 2008), AOA was found to be more prosperous than AOB in the low-salinity region of the estuary, and AOB *amoA* copy numbers were greater than AOA *amoA* in the higher salinity regions. In Plum Island Sound estuary, however, AOA abundance exceeded that of AOB with the AOA/AOB ratio of 3.0–107.1 along the full salinity gradient (Bernhard et al. 2010). In contrast, despite of the salinity variation of 1.5–26.8 psu in a whole year, the abundance of AOB *amoA* gene was found to be always greater than that of AOA *amoA* gene in the sediments of Douro River estuary (Magalhaes et al. 2009). In a study at Cochin estuary, although salinity was below 3 psu at almost all sampling sites, AOB overnumbered AOA was revealed (Puthiya Veettil et al. 2015). So far, the abundance of bacteria and archaea in ammonia oxidation by salinity remains ambiguous.

The change of AOM is the comprehensive outcome under many environmental factors, including salinity. It is better to understand the influence of salinity by minimizing the impacts of other environmental factors. Therefore, in order to distinguish the influence of salinity on the abundance, activity, and diversity of AOA and AOB, a series of estuarine sediment-water microcosms were constructed and cultivated under different salinity in this study. The abundance and transcriptional activity of AOA and AOB were estimated by real-time PCR of *amoA* genes. 454 pyrosequencing was adopted to investigate the transcriptional diversity of AOA and AOB *amoA* genes. This is the first microcosm study that focuses on salinity influence on AOM community in an estuarine environment.

Materials and methods

Samples' source and properties

The estuarine water and sediment used for microcosms' construction were sampled from the Hangzhou Bay at N 30° 12.780′, E 120° 51.144′, the same with a previous study (Zhang et al. 2015). The properties of the water and sediment samples were described in the previous study. Briefly, the water properties were DO 5.82 mg L^{-1}, pH 7.80, salinity 5.36 psu, nitrite nitrogen (NO_2^--N) 0.03 mg L^{-1}, nitrate nitrogen (NO_3^--N) 2.89 mg L^{-1}, ammonia nitrogen (NH_4^+-N) 0.17 mg L^{-1}, chemical oxygen demand (COD) 3.07 mg L^{-1}; and the sediment properties were pH 8.53, water content 31.6 %, total organic carbon (TOC) 361.4 mg g^{-1} dried sediment, NO_2^--N 0.04 μg g^{-1} dried sediment, NO_3^--N 8.97 μg g^{-1} dried sediment, NH_4^+-N 22.69 μg g^{-1} dried sediment, TP 0.53 mg g^{-1} dried sediment.

Microcosm incubation

Before the construction of microcosms, two 200 ml of salty water samples were respectively prepared to salinity of 15 and 30 psu by adding NaCl. Each microcosm, containing 10 g of sediment sample and 50 ml of raw water or one of the salty water sample, was constructed in a 120-ml flask. Three groups of microcosms with three replicated microcosms in each group were constructed. The groups with salinity of 5, 15, and 30 psu were labeled as S-5, S-15, and S-30, respectively. Each flask was covered with a sterile sealing film and cultivated under 25 °C in the dark. For simulating the ammonia concentration of the sampling site and sustaining the activity of AOM, 5 μg of NH_4^+-N (equal to 0.1 mg L^{-1} in the microcosms) was supplemented to each microcosm every day by adding $(NH_4)_2SO_4$ solution (50 mg L^{-1} NH_4^+-N), followed by a moderate shaking for air exchange. The incubation lasted for 56 days.

Nucleic acid extraction and reverse transcription

DNA was extracted from 0.5 g of sediment sample with the DNA PowerSoil® Total DNA Isolation Kit (Mo Bio, Carlsbad, CA). RNA was extracted from 3 g of sediment with

the RNA PowerSoil® Total RNA Isolation Kit (Mo Bio, Carlsbad). After RNA extraction, a RTS™ DNase Kit (Mo Bio, Carlsbad, CA) was adopted to eliminate the residual DNA from the extracted RNA. Subsequently, the purified RNA was converted to complementary DNA (cDNA) by reverse transcription using a PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the product instruction. The DNA and cDNA samples were frozen at −70 °C for further analysis.

Real-time PCR

Real-time PCR was conducted on an iCycler IQ5 thermocycler (Bio-Rad, CA, USA) using SYBR Green I method as previously described (Zhang et al. 2015). Each reaction consisted of 10 μL of SYBRs Premix Ex TaqTM (Takara, Dalian, China), 1.0 μL of template DNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 8.2 μL of ddH_2O. The primers and thermal programs for AOA and AOB *amoA* genes were listed in Table 1. The standard curves were performed using standard plasmids obtained from the positive clones of the target genes amplified from the sediment sample. The amplification efficiencies ranged from 94.8 to 104.7 %, and the correlation coefficients (R^2) of the standard curves were all >0.99. Every sample was tested in triplicate.

Pyrosequencing and phylogenetic analysis

The AOA and AOB *amoA* genes in the cDNA samples were analyzed by pyrosequencing to investigate the transcriptional diversity. The pyrosequencing was performed as previously described (Zhang et al. 2015). Briefly, the target genes were amplified in triplicate on an ABI9700 thermocycler (ABI, Foster City, USA) using barcoded primers and TransStart Fastpfu DNA polymerase (TransGen, Beijing, China). The adopted primers were the same with that used in the real-time PCR, while the used thermal programs were also listed in Table 1. The triplicate PCR products were mixed and tested with 2 % agarose gel electrophoresis, followed by purification with AxyPrep DNA gel extraction kit (Axygen, Union City, CA). The amplicon libraries were generated by emulsion PCR with the purified PCR products and sequenced on the Roche GS-FLX Titanium Sequencer (Roche Diagnostics Corporation, Branford, CT).

After sequencing, QIIME (Caporaso et al. 2010) was adopted to convert the flowgrams to sequences for further analysis. Firstly, the reads with ambiguous base>0, length< 440 bp, or average sequence quality<25 were eliminated from the sequences set. Subsequently, putative chimeras were identified and removed with Chimera-uchime. Finally, Mother (Schloss et al. 2009) was applied to cluster the filtered sequences into OTUs with sequence identity threshold of 97 and 95 % for AOB *amoA* and AOA *amoA*, respectively, and to calculate the rarefaction and diversity indexes. The representative sequences in the main OTUs as well as the closest sequences in the National Center for Biotechnology Information (NCBI) database were used to construct phylogenetic tree using neighbor-joining method with MEGA 5.2 software (Kumar et al. 2008). All original nucleotide sequence reads were archived at the NCBI Sequence Read Archive (SRA) under accession SRP039379.

Results

Gene abundance

After 56-day incubation, nitrate accumulated, whereas nitrite kept in low concentrations in the aqueous phase of all the microcosms (Fig. 1), which indicated that the nitrification was well conducted in the microcosms. The accumulated nitrate in S-5 was significantly higher than that in S-15 and S-30, illustrating that the ammonia-oxidizing ability of the sediment of S-5 might be higher than that in the other two groups of microcosms.

The *amoA* gene abundance before and after the incubation was shown in Fig. 2. Before cultivation, AOA and AOB *amoA* genes were 8.94×10^6 and 1.12×10^6 copies g^{-1} sediment, respectively, revealing the dominance of AOA in the sediment. After 56-day cultivation, slight decreases of AOB

Table 1 The PCR primer pairs and thermal programs

Target gene	Sequence of primers (5′-3′)	Thermal program	Technology applied
AOB *amoA*	amoA-1 F: GGT TTC TAC TGG TGG T amoA-2 R: CCC CTC KGS AAA GCC TTC TTC	3 min at 94 °C, 40 cycles of 30 s at 94 °C, 55 s at 60 °C, and 45 s at 72 °C (plate read)	Real-time PCR
		2 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 5 min at 72 °C	Pyrosequencing
AOA *amoA*	Arch-amoA F: STA ATG GTC TGG CTT AGA CG Arch-amoA R: GCG GCC ATC CAT CTG TAT GT	3 min at 94 °C, 40 cycles of 30 s at 94 °C, 60 s at 53 °C, and 60 s at 72 °C (plate read)	Real-time PCR
		2 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 5 min at 72 °C	Pyrosequencing

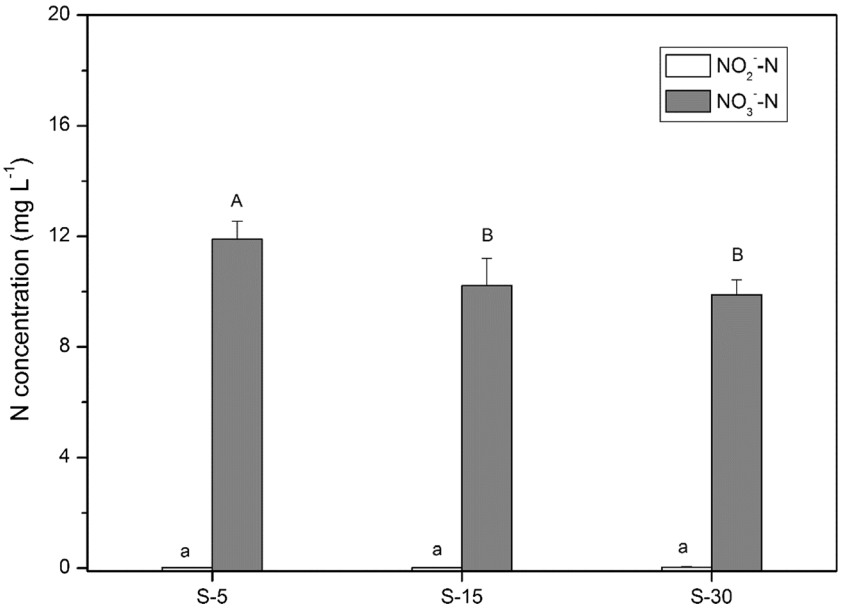

Fig. 1 Nitrite- and nitrate-N in the aqueous phase of the 56-day cultivated microcosms. "S-x" represents the microcosm with salinity of "x" psu. *Error bars* represent the standard deviation of the triplicate samples. *Different letters above the bars* indicate a significant difference ($P<0.05$) by ANOVA analysis using Student-Newman-Keulstest method; *lowercase letters* are for nitrite-N, and *capital letters* are for nitrate-N

amoA was found in all of the three groups of microcosms; however, the decreases were not statistically significant, indicating the relative stability of AOB in the sediments under diverse salinities. The AOA *amoA* in the sediment of S-5 was close to that before incubation, but AOA *amoA* in S-15 and S-30, especially in S-15 (statistically significant), were much higher than that before incubation and in S-5, revealing the preference for higher salinity of AOA. The AOA *amoA*/AOB *amoA* in S-5, S-15, and S-30 were 9.5, 207.3, and 60.1, respectively. No strong correlation between salinity and abundance of AOA or AOB was found by Pearson correlation analysis using SPSS 17.0.

The transcribed AOA and AOB *amoA* genes' abundance were measured by RT-qPCR (shown in Fig. 3). As more transcribed AOA *amoA* gene was detected under each salinity, higher abundance of active AOA than active AOB was revealed in the sediments. The AOB *amoA* transcripts in S-15 and S-30 were significantly lower than that in S-5, which indicated that AOB might perform higher transcriptional activity in lower salinity environments. On the contrary, AOA *amoA* transcripts in both S-15 and S-30 were more abundant than that in S-5 and exhibited the highest in S-15, which indicated that AOA might be more transcriptionally active under moderate-salinity environments.

Transcriptional diversity

The transcribed *amoA* genes' diversities were analyzed by 454 pyrosequencing of the cDNA samples. As the AOB *amoA* gene abundance in the cDNA samples were relatively low,

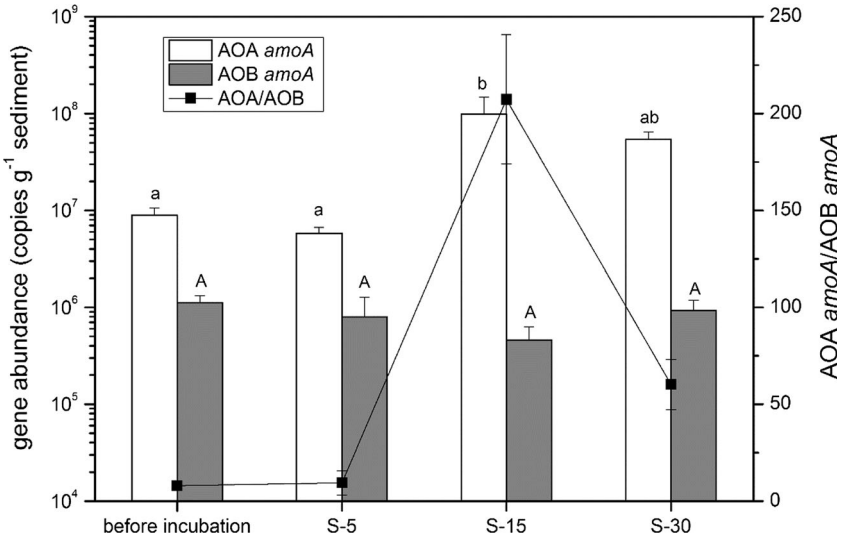

Fig. 2 Abundance of *amoA* genes in the sediments before and after cultivation. *Error bars* represent standard errors of triplicate samples. *Different letters above the bars* indicate a significant difference ($P<0.05$). *Lowercase letters* are for AOA *amoA*, and *capital letters* are for AOB *amoA*

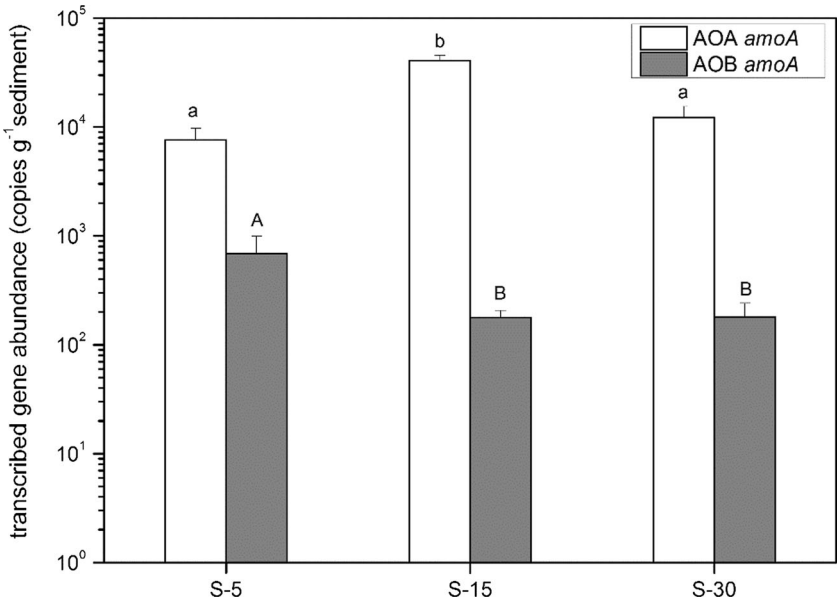

Fig. 3 Abundance of transcribed *amoA* genes in the 56-day cultivated sediments. *Error bars* represent standard errors of triplicate samples. *Different letters above the bars* indicate a significant difference ($P<0.05$). *Lowercase letters* are for AOA *amoA*, and *capital letters* are for AOB *amoA*

the amount was not enough for pyrosequencing, and the pyrosequencing of AOB *amoA* gene failed. Therefore, only three AOA *amoA* gene libraries were constructed. The coverage, diversity, and richness indexes of AOA *amoA* gene libraries are listed in Table 2. The rarefaction curves are shown in Fig. S1. The coverage exceeded 98 % in all the three libraries, which indicated that the majority of AOA *amoA* gene diversity in the cDNA samples were well covered in the libraries. A total of 111 OTUs were obtained from 4463 sequences. The order of detected OTUs abundance was S-15>S-5>S-30, which was consistent with the diversity sequence indicated by Shannon index and the richness sequence indicated by Chao 1. The distribution of the main OTUs (relative abundance>0.5 %) in each library is shown in Fig. S2. Evident shift of AOA *amoA* transcriptional diversity under different salinity was revealed by the OTUs' distribution analysis: (1) In S-5, OTU21 was the dominant OTU in the library; (2) in S-15, OTU16, OTU19, OTU21, OTU28, and OTU30 became the relative abundant OTUs; and (3) in S-30, the AOA *amoA* transcriptional diversity reduced, a number of main OTUs in S-5 and S-15 were not detected in S-30, and the dominant OTUs shifted to OTU20 and OTU23.

The main AOA *amoA* OTUs from the microcosms with different salinity were assorted by phylogenetic tree (Fig. 4). The main AOA *amoA* OTUs were placed into five clusters, i.e., *Nitrosopumilus maritimus* C12 cluster, *N. maritimus* like cluster 1.1, *N. maritimus* like cluster 1.2, *N. maritimus* SCM1 cluster, and *N. maritimus* A10 cluster, which contained 15, 9, 1, 4, and 3 OTUs, respectively.

The distribution and relative abundance of the phylogenetic AOA groups are shown in Fig. 5. *N. maritimus* C12 cluster was the main transcriptional active AOA group in all the three microcosms, accounted for 47.9, 62.8, and 56.4 % in S-5, S-15, and S-30, respectively. The other active AOA in S-5 consisted of *N. maritimus* like cluster 1.1 (24.6 %), *N. maritimus* SCM1 cluster (18.2 %), and *N. maritimus* like cluster 1.2 (2.3 %). In S-15, *N. maritimus* SCM1 cluster was almost not detected, *N. maritimus* like cluster 1.1 decreased to 5.4 %, and *N. maritimus* like cluster 1.2 increased to 19.0 %. Whereas in S-30, *N. maritimus* A10 cluster became the second abundant active AOA group with an abundance of 35.7 %.

Discussion

The estuarine sediment-water microcosms under three different salinities were incubated in this study. The accumulated aqueous nitrate (Fig. 1) indicated the achievement of nitrification, which was confirmed by the detection of the transcription of both AOA and AOB *amoA* genes (Fig. 3). The abundance

Table 2 Coverage, diversity, and richness indexes of AOA *amoA* gene libraries

Samples	No. of filtered sequences	No. of OTUs	Coverage (%)	Chao 1 values	Shannon index
S-5	1588	68	98.74	87 (74, 124)	2.55
S-15	1195	75	98.24	98 (83, 143)	2.89
S-30	1680	48	99.05	65 (53, 104)	1.96

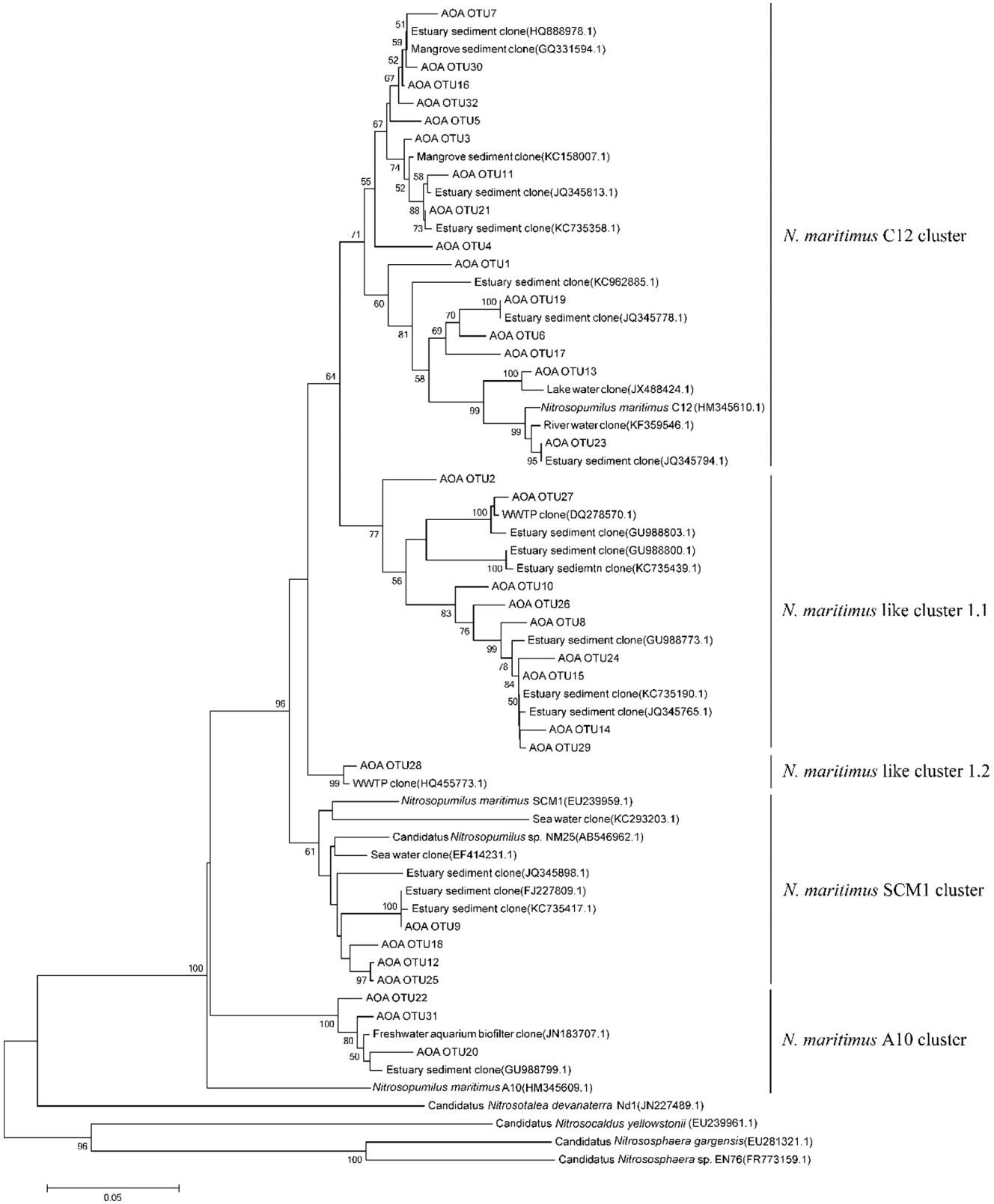

Fig. 4 Phylogenetic tree of the main AOA *amoA* based on OTUs' sequences. The *numbers* (only those >50 % are shown) *on the branch nodes* indicate the percentages of bootstrap support for the clades based on 1000 bootstrap resampling. *Numbers in the brackets* are the GenBank accession numbers of the sequences in the NCBI

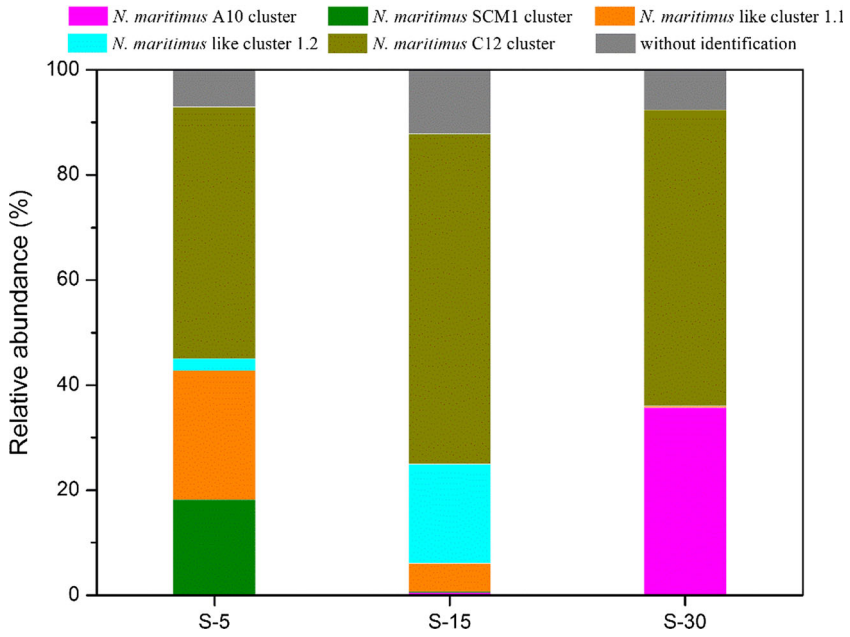

Fig. 5 Distribution and relative abundance of the phylogenetic AOA and AOB groups. The group "*without identification*" consists of the low-abundant OTUs that were not analyzed by phylogenetic tree

of AOA and AOB (Fig. 2) as well as the transcriptional abundance of AOA and AOB *amoA* genes (Fig. 3) in the sediments exhibited different changes under different salinity. AOA overnumbered AOB in the sediments with 5.83×10^{6}–9.92×10^{7} and 4.61×10^{5}–9.28×10^{5} *amoA* gene copies g^{-1} sediment, respectively. Previous studies revealed that archaeal *amoA* gene and bacterial *amoA* gene, respectively, ranged in 10^{4}–10^{9} (Moin et al. 2009; Mosier and Francis 2008; Santoro et al. 2008) and 10^{4}–10^{8} (Bernhard et al. 2007; Moin et al. 2009; Mosier and Francis 2008; Santoro et al. 2008) copies g^{-1} sediment in estuaries. The magnitude of range reflects the significant variation of AOA and AOB abundance at various sites, which may be due to the differences of physicochemical properties of the environment, as the methodology differences were minimized by using the same method and detection system (Moin et al. 2009). In this study, the AOA and AOB *amoA* gene abundance fell into the ranges reported by the previous studies, which demonstrated that AOA and AOB abundance could be stable under salinity variation in an estuarine region. The higher AOA abundance and AOA *amoA* transcripts demonstrated that AOA was the dominant one in ammonia-oxidizing community and may be the major contributor of ammonia oxidation in the sediment under a wide range of salinity.

With increased salinity, AOB kept relative stability of abundance but transcribed less AOB *amoA* gene; however, the abundance of AOA and transcribed AOA *amoA* gene were increased in S-15 and S-30. These indicated that AOA has more advantages than AOB on growth and ammonia-oxidizing activity under moderate- and high-salinity environments, especially under moderate-salinity environment, as the AOA *amoA*/AOB *amoA* in S-15 (207.3) was much higher than that in S-5 (9.5) and S-30 (60.1). Our results was consistent with that revealed in Plum Island Sound estuary, where the abundance of AOA was found to be always greater than that of AOB along the salinity gradient, and AOA abundance was the highest at intermediate salinity (Bernhard et al. 2010) but different to that in San Francisco Bay (Mosier and Francis 2008) and in a subterranean estuary at Huntington Beach (Santoro et al. 2008), where AOA was more prosperous than AOB in the low-salinity region and reversed in the higher salinity regions and different to that in Weeks Bay (Caffrey et al. 2007), Douro River (Magalhaes et al. 2009), and Cochin estuary (Puthiya Veettil et al. 2015), where AOB dominated in the ammonia-oxidizing community. In addition, unlike the studies on coastal area of California (Mosier and Francis 2008; Santoro et al. 2008), where a correlation between salinity and AOB abundance was set up, this study did not find strong correlation between salinity and abundance of AOA or AOB. The variable populations of AOA and AOB in the estuaries may be a result of the complex influences of various environmental factors. In a previous study, increasing C/N ratio was found to be significantly correlated with an increase of the number of AOA and a decrease of the number of AOB (Mosier and Francis 2008). Moreover, strong negative correlations have been reported between AOA abundance and pore water sulfide (Caffrey et al. 2007), pH (Moin et al. 2009), and lead concentration and clay percentage (Mosier and Francis 2008). By minimizing the impacts of other environmental factors, this microcosm study provided

a strong evidence that AOA get the advantage over AOB in the water environments with moderate and high salinity.

Regarding the active AOM species that conducted the transcription in the microcosms, the detection for the diversity of active AOB was failed; all the detected active AOA were clustered to *N. maritimus* linage. This revealed the well adaptability of *N. maritimus* linage to a broad salinity range, though the first discovered AOA species, *N. maritimus* SCM1, was isolated from a marine tropical fish tank with a high salinity (Konneke et al. 2005). Similarly, the low-salinity habitats of an enriched AOA, Candidatus *N. limnia* strain SFB1, was capable of growing at 75 % of seawater salinity (Mosier et al. 2012). The phylogenetic study by Mosier and Francis (2008) observed that a clade of AOA *amoA* sequences was dominated by sequences from low-salinity environments; however, Moin et al. (2009) found that some new AOA *amoA* sequences from a salty marsh were also clustered into this clade. These findings indicate the wide salinity adaptability of AOA species. The active AOA shift in *N. maritimus* linage with salinity increase was revealed in this study, indicating the different activities of AOA under various salinity. The AOA in *N. maritimus* A10 cluster may have higher activity under high salinity, while the AOA in *N. maritimus* like cluster 1.1 and *N. maritimus* SCM1 cluster may have higher activity under low salinity.

In conclusion, based on estuarine sediment-water microcosm study, more advantages of AOA than AOB on growth and ammonia-oxidizing activity in moderate- and high-salinity environments was revealed. The abundance of AOB was not sensitive to salinity variation but exhibited less transcriptional activity with salinity increase. AOA was the most abundant and exhibited the highest transcriptional activity under moderate salinity. The highest and lowest transcriptional diversities of AOA *amoA* gene were found under salinity of 15 and 30 psu, respectively. All the active AOA detected under various salinities were clustered into *N. maritimus* linage, with the composition shifted from *N. maritimus* C12 cluster, *N. maritimus* like 1.1 cluster, *N. maritimus* SCM1 cluster, and *N. maritimus* like 1.2 cluster to *N. maritimus* C12 and *N. maritimus* A10 clusters when salinity was increased from 5 to 30 psu.

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Conflicts of interest All contributing authors declare no conflicts of interest.

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