

Ammonia-oxidizing bacteria and archaea in wastewater treatment plant sludge and nearby coastal sediment in an industrial area in China

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Abstract Under the increasing pressure of human activities, Hangzhou Bay has become one of the most seriously polluted waters along China's coast. Considering the excessive inorganic nitrogen detected in the bay, in this study, the impact of an effluent from a coastal industrial park on ammonia-oxidizing microorganisms (AOMs) of the receiving area was interpreted for the first time by molecular technologies. Revealed by real-time PCR, the ratio of archaeal *amoA*/bacterial *amoA* ranged from 5.68×10^{-6} to 4.79×10^{-5} in the activated sludge from two wastewater treatment plants (WWTPs) and 0.54–3.44 in the sediments from the effluent receiving coastal area. Analyzed by clone and pyrosequencing libraries, genus *Nitrosomonas* was the predominant ammonia-oxidizing bacteria (AOB), but no ammonia-oxidizing archaea (AOA) was abundant enough for sequencing in the activated sludge from the WWTPs; genus *Nitrosomonas* and *Nitrosopumilus* were the dominant AOB and AOA, respectively, in the coastal sediments. The different abundance of AOA but similar

structure of AOB between the WWTPs and nearby coastal area probably indicated an anthropogenic impact on the microbial ecology in Hangzhou Bay.

Keywords Ammonia-oxidizing archaea (AOA) · Ammonia-oxidizing bacteria (AOB) · Wastewater treatment plant (WWTP) · Industrial effluent · Hangzhou Bay

Introduction

Nitrogen, a prevalent pollution in aquatic environments, can be eliminated through denitrification following nitrification, in which ammonia oxidation is the first and rate-limiting step. For a long time, ammonia-oxidizing bacteria (AOB) were considered as the only group responsible for the biological oxidation of ammonia in both natural and artificial environments. Since a marine *Thaumarchaeota*, isolated from the rocky substratum of a tropical marine aquarium tank, was found to possess the ability of oxidizing ammonia into nitrite (Konneke et al. 2005), the ammonia oxidizers had extended to archaea.

By detecting the α subunit gene of ammonia monooxygenase (*amoA*), the widespread distribution of ammonia-oxidizing archaea (AOA) has been confirmed in various natural environments, i.e., soils (Bates et al. 2011), oceans (Bouskill et al. 2012), hot springs (Hatzenpichler et al. 2008), lakes (Hu et al. 2010), and rivers (Liu et al. 2013). Different from natural aquatic environments, AOA was discovered at low frequency in wastewater treatment plants (WWTPs) (Mussmann et al. 2011; Park et al. 2006; Wu et al. 2013; Zhang et al. 2009; Zhang et al. 2011). For instance, AOA *amoA* gene was only detected in 14 out of 52 WWTPs

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by real-time PCR in Mussmann et al.'s research. In Park et al.'s research, AOA was found to occur in five out of nine WWTPs by PCR and clone library. In four pilot-scale wastewater treatment reactors (Gomez-Silvan et al. 2010), AOA *amoA* gene was not detected in any of the 23 tested activated sludge samples by PCR. Focusing on the relative abundance, AOA overwhelmed AOB in some natural aquatic ecosystems (Beman et al. 2010; Mincer et al. 2007; Newell et al. 2011; Santoro et al. 2010), while AOB overtook AOA in some others (Christman et al. 2011; Santoro et al. 2008). In the majority of AOA reported WWTPs (Jin et al. 2010; Limpiyakorn et al. 2011; Wells et al. 2009; Yapsakli 2010), the abundance of AOB *amoA* gene was 2–4 orders of magnitude higher than that of AOA. Only in several municipal WWTPs reported by Kayee (Kayee et al. 2011) and Bai (Bai et al. 2012b), AOA outnumbered AOB in the activated sludge.

Based on the published *amoA* gene sequences, Pester et al. (2012) provided evidence for the diversification of AOA into five major clusters, *Nitrosopumilis* cluster, *Nitrososphaera* cluster, *Nitrosocaldus* cluster, *Nitrosotalea* cluster, and *Nitrososphaera* sister cluster. Later, Cao et al. (2013) also summarized the global ecological pattern of AOA based on *amoA* phylogeny; however, the non-natural environments like WWTPs were not included (Cao et al. 2013). Previous investigation indicated that AOA were mainly *Nitrososphaera* in WWTPs (Gao et al. 2013; Kayee et al. 2011; Limpiyakorn et al. 2011; Sonthiphand and Limpiyakorn 2011) and *Nitrosopumilis* cluster in marine environments (Jin et al. 2011; Li et al. 2013; Santoro et al. 2008; Zheng et al. 2013).

The discovered AOB are classified into two groups in the taxonomy, *Betaproteobacteria* and *Gammaproteobacteria*. In marine sediment, *Gammaproteobacteria* are considered as the minority of ammonia-oxidizing communities (Nold et al. 2000). *Nitrosospira* and *Nitrosomonas*, two genera in *Betaproteobacteria*, compose the majority of AOB in natural aquatic environments. In WWTPs, the high pollution aquatic environments, *Nitrosomonas* was found to be the ascendant AOB in most previous studies (Cao et al. 2011; Gao et al. 2013; Kayee et al. 2011; Limpiyakorn et al. 2011; Wang et al. 2012; Zhang et al. 2011).

The ammonia-oxidizing microorganisms (AOMs) between artificial WWTPs and natural aquatic environments exhibit putative differences in the abundance and predominant genera of AOA and AOB. In a natural environment being contaminated by WWTPs' effluent, some pollutants might homologize the AOM community structure of the environment with that of the WWTPs, because pollution could alter the environmental factors and further the ecological niche of AOM. From previous research studies, salinity (Caffrey et al. 2007), dissolved oxygen (Bouskill et al. 2012; Molina et al. 2010), and ammonia (Ando et al. 2009; Wuchter et al. 2006) have been preliminarily suspected to affect the composition of AOM in aquatic environments, though the influences are still

not clarified. Ratio of AOA *amoA* gene copies to AOB *amoA* gene copies and *Nitrosomonas* genus were put forward by Sims et al. (2012) and Cao et al. (2012), respectively, as a potential biological indicator for the contamination of natural aquatic environments.

Hangzhou Bay, surrounded by many industrial aggregations, is located in the estuary of Qiantang River and the south adjacent sea area of the Yangtze River. Large amount of industrial effluents are discharged into the bay; consequently, it has become one of the most severely polluted waters along China's coast. As indicated by 2011 Marine Environment Quality Bulletin of Zhejiang Province, the bay was placed in the seawater quality level of inferior to class IV, the worst level for seawater quality according to the National Seawater Quality Standard (GB3097-1997). Inorganic nitrogen (up to 4 mg L^{-1}) was a primary pollutant in Hangzhou Bay, which might influence the AOM community but lacks evidence up to now.

In this study, the Shangyu Industrial Area (SYIA), a fine chemical industrial park located on the south bank of Hangzhou Bay, was selected as our research object. The SYIA consists of over 100 enterprises, mainly producing pharmaceuticals, dyes, and amine compound. Industrial wastewaters were treated preliminarily by enterprise-owned WWTPs and followed by a SYIA-owned WWTP. The final effluent is discharged to Hangzhou Bay.

The effect of wastewater disposal on the bacterial and archaeal community of the coastal sediment of Hangzhou Bay was not distinct, though a correlation between effluent connection and bacterial communities was found (Zhang et al. 2014). AOM, the key role in nitrogen cycle, may be a more evident bioindicator for the contamination of inorganic nitrogen from wastewater disposal.

For the purpose of exploring the impact of wastewater disposal on the AOM community of the effluent receiving area, we investigated the occurrence, abundance, and compositions of bacterial and archaeal *amoA* genes in the samples of WWTPs' activated sludge and coastal sediments by several molecular technologies. The results of our study would improve the knowledge of the AOM communities in industrial WWTPs and nearby coastal environment.

Materials and methods

Activated sludge and sediment sampling

About 50 g of activated sludge were sampled from the aerobic biological units of the WWTP (named as GB) in an antibiotic production factory and the WWTP (named as SY) for the whole SYIA. About 500-g surface sediment was collected by a surface sediment sampler (Van Veen, HYDRO-BIOS, Germany) at the effluent discharged site (named as HW) of $30^{\circ} 12.890' \text{ N}$, $120^{\circ} 51.550' \text{ E}$ in Hangzhou Bay.

Table 1 The PCR primer pairs and thermal programs

Target gene	Sequence of primers (5'-3')	Technology applied	Thermal program	Reference
Bacterial <i>amoA</i>	<i>amoA</i> -1F: GGT TTC TAC TGG TGG T	Clone library	5 min at 94 °C, 35 cycles of 60 s at 94 °C, 60 s at 60 °C, and 90 s at 72 °C, followed by 10 min at 72 °C	Rothauwe et al. (1997)
	<i>amoA</i> -2R: CCC CTC KGS AAA GCC TTC TTC			
Archaeal <i>amoA</i>	Arch- <i>amoA</i> F: STA ATG GTC TGG CTT AGA CG	Real-time PCR	3 min at 94 °C, 40 cycles of 30 s at 94 °C, 55 s at 60 °C, and 45 s at 72 °C	Chen et al. (2008)
	Arch- <i>amoA</i> R: GCG GCC ATC CAT CTG TAT GT			
	Arch- <i>amoA</i> F: STA ATG GTC TGG CTT AGA CG	Clone library	5 min at 95 °C, 35 cycles of 45 s at 94 °C, 60 s at 53 °C, and 60 s at 72 °C, followed by 10 min at 72 °C	Francis et al. (2005)
	Arch- <i>amoA</i> R: GCG GCC ATC CAT CTG TAT GT			
Bacterial 16S rRNA	341F: CCT ACG GGA GGC AGC AG	Real-time PCR	3 min at 94 °C, 40 cycles of 30 s at 94 °C, 60 s at 53 °C, and 60 s at 72 °C	Chen et al. (2008)
	518R: ATT ACC GCG GCT GCT GG			
Archaeal 16S rRNA	711F: ACG GTG AGG GAT GAA AGCT	Real-time PCR	3 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 40 s at 72 °C	He et al. (2007)
	934R: GTG CTC CCC CGC CAA TTC CT			

The location of the SYIA and the two WWTPs, the characteristics of the wastewaters, and the seawater qualities were described in our previous article (Zhang et al. 2014). The samplings were conducted in winter (February 24) and summer (August 24) in 2012. Each sample was labeled as “sampling site-sampling month,” e.g., SY-August. The activated sludge and sediment samples were transported from field to our lab under 4 °C within 24 h, and stored at −70 °C until use.

DNA extraction and clone library

Total DNA was extracted from 0.5 g each sample using a PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA) according to the manual and stored at −20 °C for further application.

AOA and AOB *amoA* genes of the February samples were amplified to construct clone libraries. The amplifications were operated with primer pairs and thermal programs as listed in Table 1. The clone libraries were constructed as described previously (Bai et al. 2012b). Briefly, the target DNA fragments in the PCR products was firstly separated by agarose (0.8 %) gel electrophoresis and subsequently purified with a QIAquick® gel extraction kit (Qiagen, Hilden, Germany). The purified gene fragments were cloned into pGEM-T Easy vectors (Promega, Madison, WI) and then transformed into competent *Escherichia coli* JM109 (Promega, Madison, WI). By blue-white screening, positive clones were randomly selected and sequenced by an ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA). The rarefaction and diversity statistics were calculated by Mothur (Schloss et al. 2009). The sequences were clustered into different operational taxonomic units (OTUs) with 95 and 97 % of sequence identity threshold, respectively, for archeal (Beman et al. 2008; Mosier and Francis 2008) and bacterial (Gao et al. 2013; Wang et al. 2012) *amoA* genes. The representative OTU sequences were blasted against the National Center for Biotechnology Information (NCBI) database to obtain the closest published sequences. The sequences were assembled to construct phylogenetic trees applying the neighbor-joining method with the MEGA 5.2 software (Kumar et al. 2008). The representative sequences constructed on the tree were deposited in the GenBank under accession numbers of KF704381, KF704384 to KF704386, and KF704388 to KF704390.

Pyrosequencing

The *amoA* genes of August samples were amplified using barcoded primers to construct libraries through high-throughput pyrosequencing. The primers and thermal programs are listed in Table 1. Pyrosequencing was performed as described previously (Bai et al. 2012a). Briefly, PCR products were purified with an AxyPrep DNA Gel Extraction Kit (Axygen, USA). The amplicon libraries were generated by

emulsion PCR according to the recommendation of 454 Life Sciences. The sequencing was performed on a Roche Genome Sequencer GS-FLX using the 454/Roche B sequencing primer kit according to the protocol. After the pyrosequencing flowgrams were converted to sequence reads without assembling by Mothur software, the sequence reads were initially trimmed to remove the barcodes, then filtered and denoised to eliminate ambiguous and low-quality reads, i.e., the reads with average sequence quality <25, ambiguous base >0, or sequence lengths <440 bp. The putative chimeras were detected and excluded with UCHIME. The remained sequences were clustered into OTUs using Mothur software with 95 and 97 % of sequence identity threshold for archeal (Beman et al. 2008; Mosier and Francis 2008) and bacterial (Cao et al. 2013; Wang et al. 2012) *amoA* genes, respectively. Rarefaction and diversity statistics were calculated for each sample after OTUs were clustered. To avoid the bias of libraries' alpha diversity comparison caused by the great disparity in sequence numbers between clone libraries and pyrosequencing libraries, a hundred sequences in each pyrosequencing library were subsampled randomly by Mothur and calculated alpha diversity indexes. All the trimmed 454 sequences from this study are archived at the NCBI Sequence Read Archive (SRA) under accession SRP030141.

Real-time PCR

Real-time PCR was performed on a quantitative thermocycler (IQ5, Bio-Rad, CA) based on SYBR Green I method. The *amoA* genes and 16S ribosomal RNA (rRNA) genes were amplified using primers and thermal programs as listed in Table 1. The 20- μ L reaction mixture consisted of 0.4 μ L of primers, 10 μ L of SYBRs Premix Ex Taq™ (Takara, Dalian, China), and 1.0 μ L of template DNA. Standard curves were constructed as described previously (Bai et al. 2012b). The amplified efficiencies of real-time PCR ranged from 99.4 to 102.2 %, and the correlation coefficients (R^2) were all >0.99. After real-time PCR detection, gene copy in the 1 μ L of template DNA were determined, while the total gene copy extracted from the sample were obtained by multiplying the gene copy in template DNA and the total DNA volume acquired by extraction. The gene abundance was calculated by dividing total gene copies extracted from the sample by the weight of the sample used for DNA extraction.

Results

Gene abundance

The abundance of archaeal and bacterial *amoA* genes as well as 16S rRNA genes measured by qPCR are shown in Fig. 1.

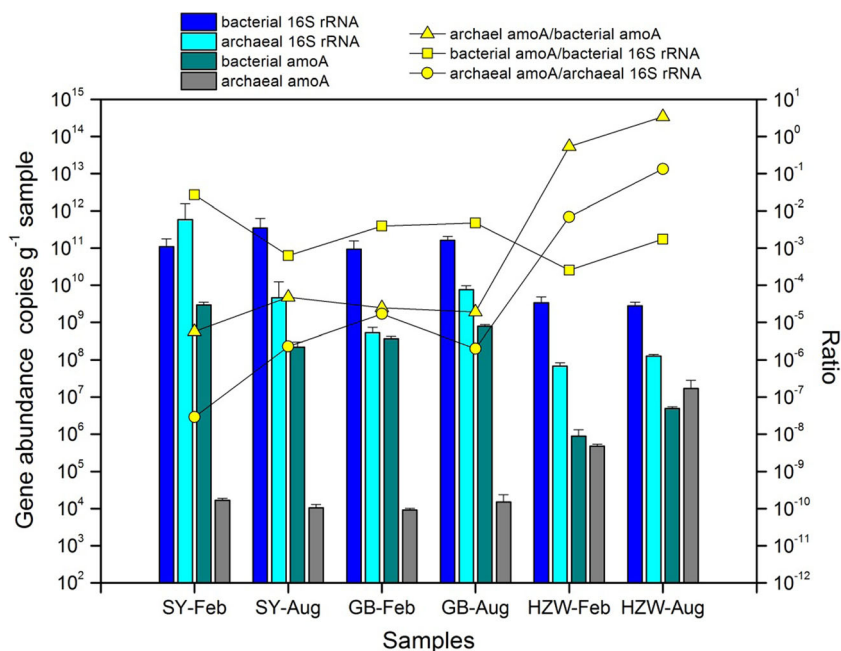
Based on ANOVA analysis by SPSS 17.0, significant changes were found between the two sampling seasons for the abundance of AOA and AOB *amoA* gene in SY, the abundance of archaeal 16S rRNA and AOB *amoA* gene in GB, and the abundance of archaeal 16S rRNA and AOB *amoA* gene in HZW. The abundance of archaeal and bacterial 16S rRNA genes in sludge samples were much higher than that in sediments.

The WWTPs, SY and GB, possessed abundant AOB *amoA* ranged in 2.19×10^8 – 2.96×10^9 gene copies per gram of activated sludge but much less AOA *amoA* ranged in 9.15×10^3 – 1.68×10^4 gene copies per gram of activated sludge. The ratio of AOA *amoA*/AOB *amoA* ranged from 5.68×10^{-6} to 4.79×10^{-5} , revealing that AOB was the predominant AOM in the activated sludge. The ratio of AOB *amoA*/bacterial 16S rRNA was 2–6 orders of magnitude higher than the ratio of AOA *amoA*/archaeal 16S rRNA, indicating larger proportion of bacteria, not archaea, in the sludge might participate in ammonia oxidation. In the sediment of HZW, the abundance of AOB *amoA* was 8.73×10^5 gene copies per gram in winter and 4.92×10^6 gene copies per gram in summer, and the abundance of AOA *amoA* gene shifted from 4.71×10^5 gene copies per gram in February to 1.69×10^7 gene copies per gram in August. The ratio of AOB *amoA*/bacterial 16S rRNA of the sediment samples (2.6×10^{-4} in HZW-February and 1.8×10^{-3} in HZW-August) were close to that in the sludge samples (6.3×10^{-4} – 2.7×10^{-2}), while the ratio of AOA *amoA*/archaeal 16S rRNA of the sediment samples (6.9×10^{-3} in HZW-February and 1.3×10^{-1} in HZW-August) were much higher than that in the sludge samples (2.9×10^{-8} – 1.7×10^{-5}). The ratio of AOA *amoA*/AOB *amoA* at HZW was 0.54 in winter and 3.44 in summer, revealing the close abundance of AOA and AOB in the sediments in the effluent receiving area in Hangzhou Bay.

Diversity of AOA

Owing to the extremely low abundance of AOA *amoA* gene, the amplifications of the gene from all sludge samples failed. Two libraries of AOA *amoA* gene from sediment samples were constructed. The coverage, diversity, and richness indexes of AOA *amoA* gene libraries are listed in Table 2. The Good's coverage indicates that both libraries could well reflect the diversity of archaeal *amoA* gene. Six operational taxonomic units (OTUs) were detected in the winter sediment by clone library analysis, and 23 OTUs were determined in the summer sediment by 454 high-throughput pyrosequencing. The Chao1 and Shannon indexes revealed a lower richness and diversity of AOA in HZW-August than in HZW-February. The constitutions of OTUs in the two AOA libraries are shown in Fig. 2. In each library, there appeared one superdominant OTU, i.e., OTU-C3 accounted for 69.32 % of the total sequences in the

Fig. 1 Quantitative analysis of archaeal and bacterial 16S rRNA and *amoA* genes



winter sample and OTU-1 accounted for 96.83 % of the total sequences in the summer sample.

A phylogenetic tree was built based on the OTUs of AOA *amoA* gene, as shown in Fig. 3a. The OTUs were classified into two clusters, *Nitrosopumilus* with 10 OTUs and *Nitrososphaera* with 1 OTU. The *Nitrosopumilus* cluster was further divided into three subclusters. The relative abundance of the clusters in the two seasons was analyzed in Fig. 4. *Nitrosopumilus* cluster predominated in both winter and summer sediments, accounting for 95.45 and 99.18 % in the total OTUs, respectively, and *Nitrososphaera* cluster only appeared in the February sediment with a small abundance of 4.55 %.

Diversity of AOB

A total of six libraries of AOB *amoA* gene were constructed from all the six samples, including three clone libraries and three pyrosequencing libraries. The coverage, diversity, and richness indexes of all AOB *amoA* gene libraries are listed in Table 3. All libraries covered the majority of bacterial *amoA* gene diversity, as Good’s coverage ranged from 98.1 to 100 %. In winter samples, 1, 6, and 6 OTUs were detected at SY, GB, and HZW, respectively. When applying 454 high-

throughput pyrosequencing to the summer samples, 26, 27, and 13 OTUs were detected at SY, GB, and HZW, respectively. Through comparison of the diversity index of clone libraries and the 100 subsamples from pyrosequencing libraries, SY-August was considered to possess higher diversity than SY-February; the diversity of GB-August was slightly lower than that of GB-February; and HZW-August exhibited similar diversity with HZW-February.

A total of 10 OTUs were drawn from the three AOB clone libraries (Fig. 5a), and 35 OTUs were obtained from the three pyrosequencing libraries. The main OTUs from pyrosequencing libraries are shown in Fig. 5b. In the clone libraries, the only OTU in the sample SY-February, OTU-C1, distributed in all three winter samples and accounted for 52.83 and 75.45 % of total sequences in GB-February and HZW-February, respectively. In the pyrosequencing libraries, 12 OTUs were shared by all three libraries, the all-shared OTUs accounted for 46.15, 44.44, and 92.31 % of total OTUs, and 97.20, 98.69, and 99.96 % of total sequences in SY-August, GB-August, and HZW-August, respectively. One common OTU in particular, OTU-1, accounted for the largest proportion of total sequences in all samples, as 87.34 % in SY-August, 76.49 % in GB-August, and 74.64 % in HZW-August.

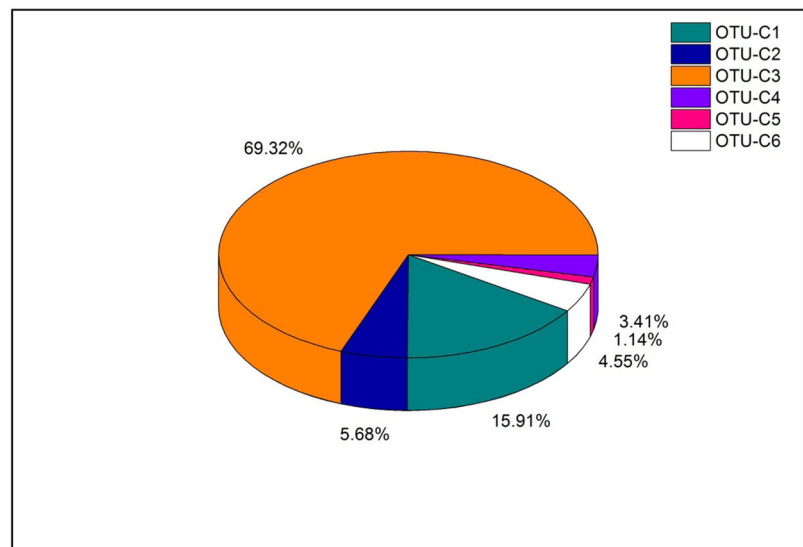
Table 2 Coverage, diversity, and richness indexes of AOA *amoA* gene libraries

Samples	No. of filtered sequences	No. of OTUs	Coverage (%)	Chao1 values	Shannon index
HZW-February	88	6	98.9	6.0	1.02
HZW-August	5268 (100) ^a	23 (3.5)	99.8 (98.0)	28.6 (5.0)	0.21 (0.17)

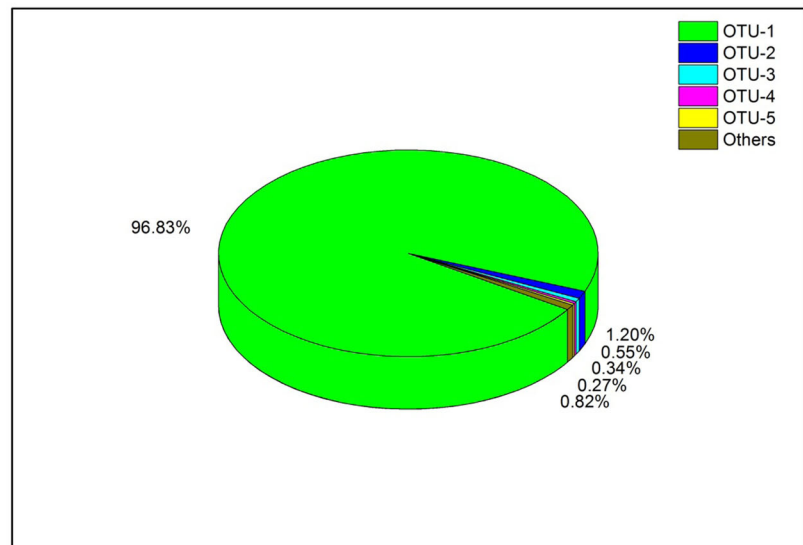
The library of February sample was constructed by clone, and the library of August sample was constructed by pyrosequencing

^a The numbers in the parentheses are the subsampled sequence number or the indexes calculated from the subsamples

Fig. 2 Distribution and relative abundance of AOA *amoA* gene OTUs: **a** HZW-February and **b** HZW-August. In the pyrosequencing library of HZW-August, the OTUs with relative abundance of smaller than 0.5 % were classified as others. OTU-* and OTU-C* were the OTUs from pyrosequencing and clone libraries, respectively



(a) HZW-Feb

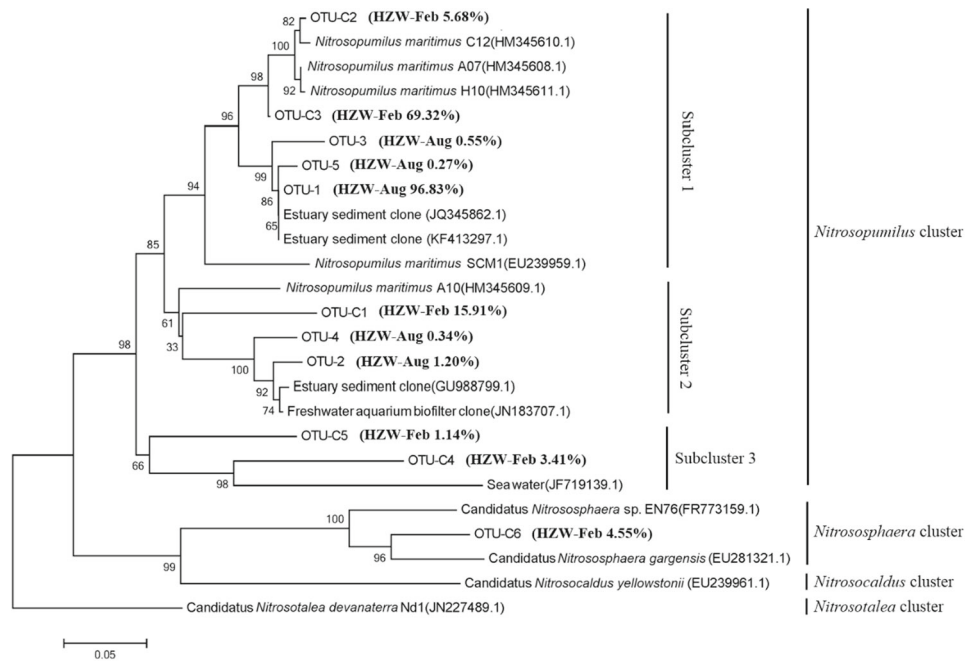


(b) HZW-Aug

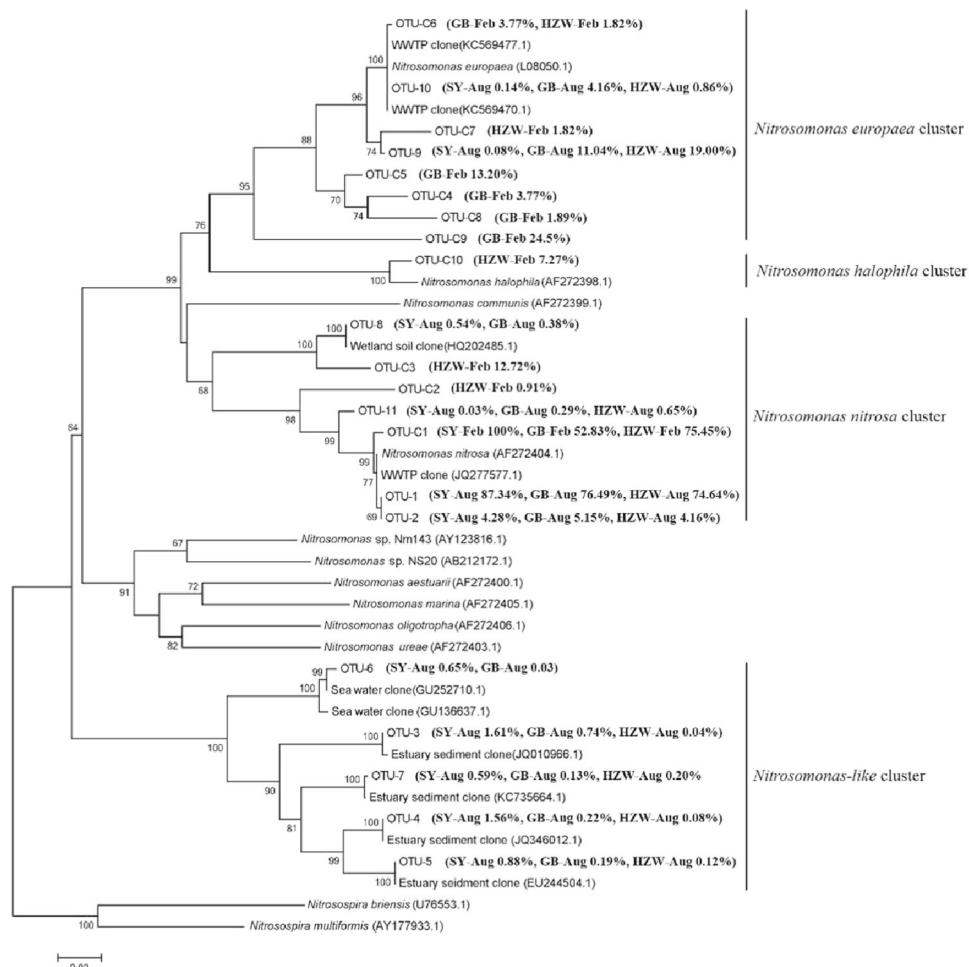
A phylogenetic tree was built based on the OTUs of AOB *amoA* gene (Fig. 3b). As a majority of the OTUs in the pyrosequencing libraries accounted for very small proportion in the total sequences, only the OTUs with relative abundance of more than 0.5 % were analyzed. All OTUs were classified into four clusters: *Nitrosomonas europaea*, *Nitrosomonas halophilla*, *Nitrosomonas nitrosa*, and *Nitrosomonas*-like, possessing 8, 1, 7, and 5 OTUs, respectively. The distribution of phylogenetic AOB clusters was further analyzed, as shown in Fig. 6. *N. nitrosa* cluster appeared in all six samples and was the most predominant AOB group in all samples. *N. europaea* cluster occurred in five samples and was the second abundant group in the samples of GB-February, GB-August, and HZW-August. *Nitrosomonas*-like cluster was only detected in the three August samples. Regarding the different sampling sites,

the phylogenetic distribution of AOB was similar between winter and summer at GB, e.g., *N. nitrosa* and *N. europaea* accounted for 52.83 and 47.17 % in winter, while 82.31 and 15.20 % in summer. At SY, more AOB clusters were detected in summer. At HZW, the dominant *N. nitrosa* cluster maintained a large proportion of 89.10 % in winter and 79.45 % in summer, *N. halophilla* only occurred in winter at considerable abundance (7.27 %), and *N. europaea* cluster presented

Fig. 3 Phylogenetic tree constructed for **a** partial AOA *amoA* OTUs' sequences and **b** partial AOB *amoA* OTU 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 resamplings. Numbers in the brackets are the GenBank accession numbers of the strains in the NCBI. OTU-* and OTU-C* were the OTUs from pyrosequencing and clone libraries, respectively

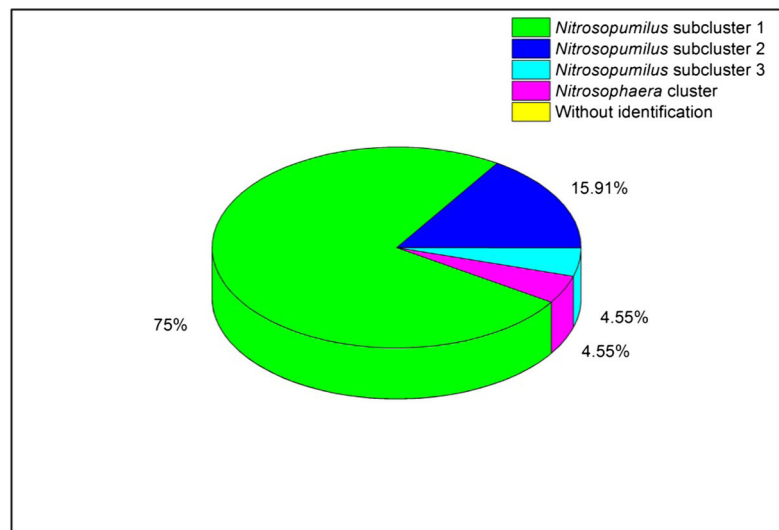


(a) AOA *amoA* OTUs

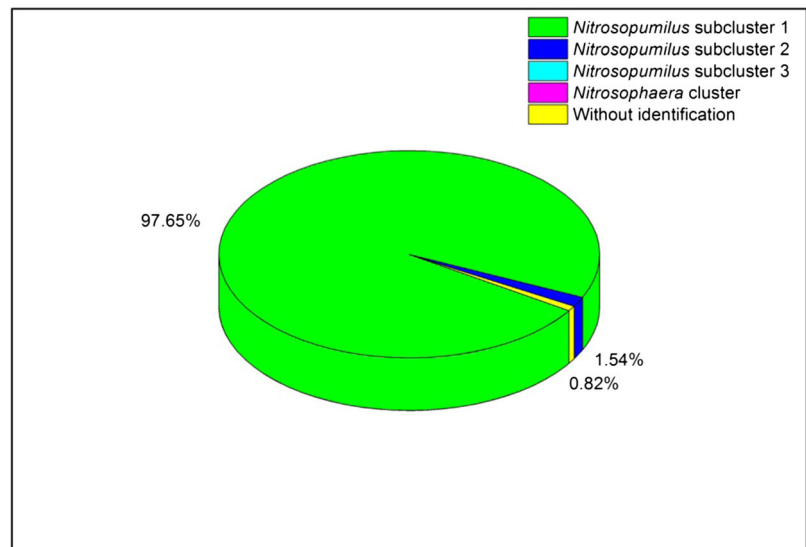


(b) AOB *amoA* OTUs

Fig. 4 Distribution and relative abundance of phylogenetic AOA groups: **a** HZW-February and **b** HZW-August. The group of “without identification” consisted of the OTUs that accounted for less than 0.5 % in a pyrosequencing library



(a) HZW-Feb



(b) HZW-Aug

3.64 % in winter and 19.85 % in summer. Focusing on the three libraries of summer samples, the all-shared 12 OTUs were identified into three clusters (shown in Table 4), in which *N. nitrosa* was the most abundant cluster, accounting for 91.65, 81.93, and 79.45 % of total sequences in SY-August, GB-August, and HZW-August, respectively.

Discussion

Gene abundance

The wastewater quality varied between the two WWTPs and fluctuated between two samplings in the same WWTP. The Spearman correlation between the wastewater

characteristics and *amoA* gene abundance of AOA and AOB was analyzed using SPSS 17.0, but no significant correlation was discovered (data not shown). However, lower abundance of AOA detected in the WWTPs (Fig. 1) in this study was consistent with some previous studies with $\text{NH}_4^+\text{-N}$ of the wastewaters ranged from 14.0 to 422.3 mg/L (Limpiyakorn et al. 2011; Mussmann et al. 2011; Ozdemir et al. 2011; Park et al. 2006; Wells et al. 2009; Zhang et al. 2011). The influent $\text{NH}_4^+\text{-N}$ of both WWTPs in this study ranged from 4.7 to 158.1 mg/L (Zhang et al. 2014). Relatively high abundance of AOA in sludge samples was also reported (Kayee et al. 2011; Limpiyakorn et al. 2011; Mussmann et al. 2011), where the WWTPs' influent $\text{NH}_4^+\text{-N}$ was in the range of 5.4–38.6 mg/L. Both pure culture-based and environmental studies indicated that at least some AOA have a high substrate affinity for ammonia

Table 3 Coverage, diversity, and richness indexes of AOB *amoA* gene libraries

Samples	No. of filtered sequences	No. of OTUs	Coverage (%)	Chao1 values	Shannon index
SY-February	82	1	100	1.0	0
GB-February	53	6	98.1	6.0	1.27
HZW-February	110	6	99.1	6.0	0.854
SY-August	3532 (100) ^a	26 (7.8)	99.9 (95.8)	27.5 (12.7)	0.68 (0.59)
GB-August	3126 (100)	27 (6.9)	99.8 (97.4)	29.5 (9.2)	0.93 (0.86)
HZW-August	2453 (100)	13 (4.8)	99.9 (98.5)	13.8 (5.6)	0.79 (0.75)

The library of February sample was constructed by clone, and the library of August sample was constructed by pyrosequencing

^a The numbers in the parentheses are the subsampled sequence number or the indexes calculated from subsamples

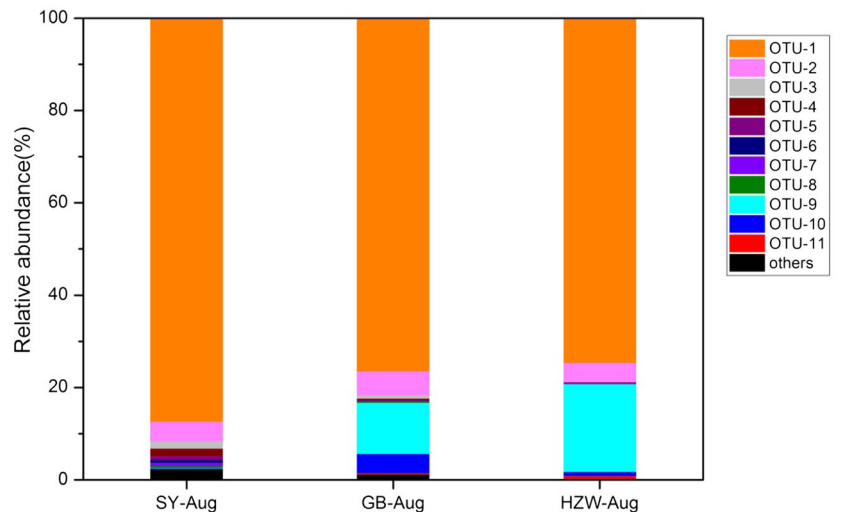
and are able to grow under extremely oligotrophic conditions (Schleper and Nicol 2010). The kinetic study of the first isolated AOA strain, *N. maritimus* SCM1, obtained the lowest half-saturation constant ($K_m=133$ nM) and the

minimum ammonia concentration (less than 20 nM), which was over 100-fold lower than that required by AOB (Martens-Habbena et al. 2009). Therefore, AOA may prefer to live in environments with low ammonia concentration. The

Fig. 5 Distribution and relative abundance of AOB *amoA* gene OTUs: **a** February and **b** August. The OTUs with relative abundance of smaller than 0.5 % were classified as others. OTU-* and OTU-C* were the OTUs from pyrosequencing and clone libraries, respectively

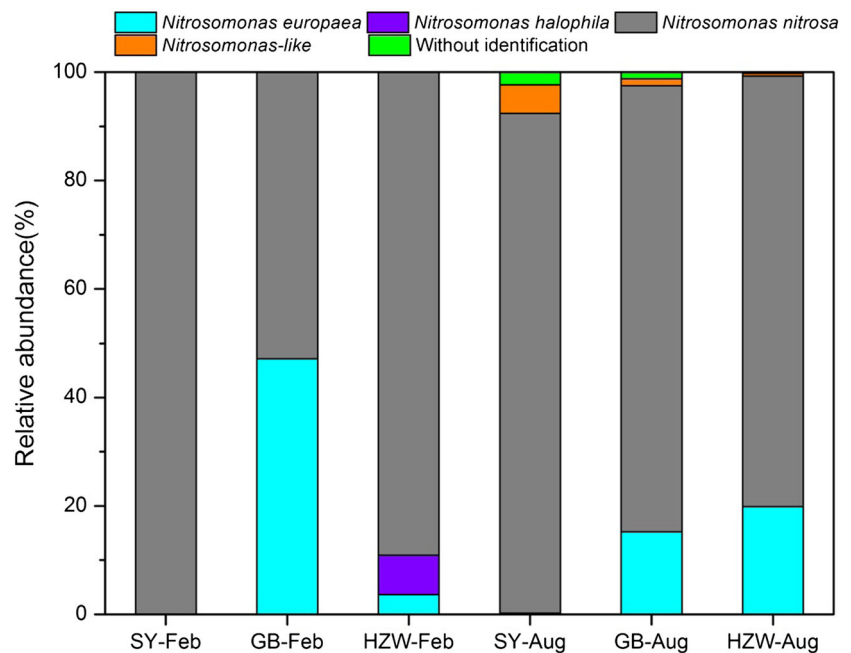


(a) Feb.



(b) Aug.

Fig. 6 Distribution and relative abundance of phylogenetic AOB groups. The group of “without identification” consisted of the OTUs that accounted for less than 0.5 % in a pyrosequencing library



high ammonia concentration may be the reason for the low abundance of AOA in the wastewater environment.

The much higher ratio of AOA *amoA*/archaeal 16S rRNA of the sediment samples than that in the sludge samples (Fig. 1) revealed larger proportion of archaea in the estuarine sediment than in the activated sludge could take part in ammonia oxidation. Focusing on AOA *amoA*/AOB *amoA*, the ratio in HZW (0.54 in winter and 3.44 in summer) was almost 5 orders of magnitude higher than that in WWTPs. In a subterranean estuary, Huntington Beach, log ratio of AOB to AOA was -1.0 – 1.5 (Santoro et al. 2008); in the low salinity area of San Francisco Bay, log ratio of AOA to AOB was 0.5 – 2.0 (Mosier and Francis 2008); in the Pearl River Estuary, AOA *amoA* gene copy numbers (9.6×10^6 – 5.1×10^7 copies per gram of sediment) overwhelmed AOB *amoA* gene copy numbers (9.5×10^4 – 6.2×10^5 copies per gram of sediment) (Jin et al. 2011); in Douro estuary, the sediments possessed AOB *amoA*/AOA *amoA* of 1–39, and the AOB abundance slightly

exceeded the AOA's (Magalhaes et al. 2009). In this study, the ratio of AOA/AOB in the effluent receiving area was similar with that in other estuaries, but much different from WWTPs, though the seawater had been contaminated.

Diversity of AOM

As shown in Fig. 3a, the OTUs classified in *Nitrosopumilus* cluster were similar to the AOA sequences from ocean (Francis et al. 2005), estuaries (Jin et al. 2011; Li et al. 2013; Santoro et al. 2008; Zheng et al. 2013), and a biofilter treating artificial seawater (Brown et al. 2013), while the only OTU classified in *Nitrososphaera* cluster has been detected with high abundance in WWTPs in previous studies (Gao et al. 2013; Kayee et al. 2011; Limpiyakorn et al. 2011; Sonthiphand and Limpiyakorn 2011). In a study on eight WWTPs (Gao et al. 2013), for example, 18 out of 19 AOA *amoA* OTUs were affiliated to *Nitrososphaera* cluster, only

Table 4 Shared AOB groups among the summer samples at SY, GB, and HZW

Groups	Shared OTUs	Shared sequences		
		SY-August	GB-August	HZW-August
<i>N. nitrosa</i> cluster	3	3237 (91.65 %) ^a	2561 (81.93 %)	1949 (79.45 %)
<i>N. europaea</i> cluster	2	8 (0.23 %)	475 (15.20 %)	487 (19.85 %)
<i>Nitrosomonas-like</i> cluster	4	164 (4.64 %)	40 (1.28 %)	11 (0.45 %)
Without identification ^b	3	24 (0.68 %)	9 (0.29 %)	5 (0.20 %)
Total	12	3433 (97.20 %)	3085 (98.69 %)	2452 (99.96 %)

^a The numbers in parentheses were the percentage that the corresponding cluster accounted for

^b The group of “without identification” consisted of the OTUs that accounted for less than 0.5 % and were not analyzed in the phylogenetic tree

one OTU (two sequences) was in *Nitrosopumilus* cluster. It seems that *Nitrososphaera* cluster is more likely to predominate in wastewater systems, while *Nitrosopumilus* cluster prefers to prevail in marine and estuary environments.

In this study, the AOB groups in the WWTPs and the effluent receiving area in Hangzhou Bay were all classified as *Nitrosomonas*, but another AOB genus *Nitrospira* was not detected. The same phenomena were confirmed in many WWTPs. Gao et al. (2013) found all AOB classified in *Nitrosomonas* lineage in eight full-scale WWTPs, and *Nitrospira* lineage was not detected. In another group of eight WWTPs treating municipal wastewater (Kayee et al. 2011), *N. oligotropha* cluster dominated in seven WWTPs, *N. communis* cluster dominated in the last WWTP, and AOB of *Nitrospira* cluster was not detected. In a study on six full-scaled wastewater treatment bioreactors (Zhang et al. 2011), though a lower abundance of *Nitrospira* was detected, *Nitrosomonas* genus was predominant in all bioreactors. The similar results were also reported from a municipal WWTP in Palo Alto (Newell et al. 2011), seven full-scale WWTPs in Thailand (Limpiyakorn et al. 2011), and a pilot-scale WWTP in China (Wang et al. 2012). It seems that *Nitrosomonas* genus is more likely to flourish in the aerobic artificial environment for wastewater treatment.

However, the AOB composition in the sediments in Hangzhou Bay was different from that in other sea areas. The AOB *amoA* sequences in the intertidal sediments of the Yangtze River estuary (Zheng et al. 2013) as well as in the sediments of the Pearl River estuary (Jin et al. 2011) were grouped into two genera, *Nitrospira* and *Nitrosomonas*. Both genera distributed in the San Francisco Bay estuary (Mosier and Francis 2008) and the offshore Rovinj, Croatia (Bayer et al. 2008). The *amoA* gene sequence lineages of *Nitrospira* clade contained the sequences mainly from estuarine, coastal, and deep sea, while *Nitrosomonas* genus could be a potential bioindicator for pollution or land-sourced effluents (freshwater or wastewater) flowing into coastal environments (Cao et al. 2012). As for Hangzhou Bay, *Nitrospira* cluster was determined as the dominant AOB group in the Qiantang River (Liu et al. 2013); thus, the low salinity of the industrial effluent receiving area might be a minor reason for the dominance of *Nitrosomonas*. Moreover, we deduced that the pollution, mainly caused by industrial effluent discharge, should be the key factor that determined the dominance of *Nitrosomonas* and absence of *Nitrospira* in the coastal sediments of Hangzhou Bay.

In conclusion, although the ratio of AOA to AOB in the coastal sediment was 5–6 orders of magnitude different from that in the activated sludge, the homologous AOB between the effluent receiving coastal area and the WWTPs probably indicated an impact on the microbial ecology in Hangzhou Bay by the effluent disposal.

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