Ammonia-oxidizing archaea and ammonia-oxidizing bacteria in six full-scale wastewater treatment bioreactors

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Abstract In this study, dideoxy sequencing and 454 high-throughput sequencing were used to analyze diversities of the ammonia monooxygenase (*amo*A) genes and the 16S rRNA genes of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in six municipal wastewater treatment plants. The results showed that AOB *amo*A genes were quite diverse in different wastewater treatment plants while the 16S rRNA genes were relatively conserved. Based on the observed complexity of *amo*A and 16S rRNA genes, most of the AOB can be assigned to the *Nitrosomonas* genus, with *Nitrosomonas ureae*, *Nitrosomonas oligotropha*, *Nitrosomonas marina*, and *Nitrosomonas aestuarii* being the four most dominant species. From the sequences of the AOA *amo*A genes, most AOA observed in this study belong to the CGI.1b group, i.e., the soil lineage. The AOB *amo*A and 16S rRNA genes were quantified by quantitative PCR and 454 high-throughput pyrosequencing, respectively. Although the results from the two approaches show some disconcordance, they both indicated that the abundance of AOB in activated sludge was very low.

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Introduction

Nitrification, the biological oxidation of ammonium to nitrite and nitrate, is an essential process in nitrogen cycling and wastewater treatment bioreactors. Several groups of microorganisms are involved in the two-step process: ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB). In the first step, AOA and/or AOB oxidize NH_3 to NO_2^-, and in the second step NOB oxidize NO_2^- to NO_3^-. The first step is rate limiting and has been relatively well studied (Limpiyakorn et al. 2006; Park and Noguera 2004; Tokutomi et al. 2010). Although some heterotrophic bacteria (Robertson and Kuenen 1990) and anaerobic ammonia-oxidizing (anammox) bacteria (Strous et al. 1999) can also oxidize ammonia to nitrite, AOA and AOB are thought to be the main contributors for environmental ammonia oxidation and in bioreactors (Nicol and Schleper 2006). According to the previous reports, *Nitrosomonas* and *Nitrosospira* are the most important genera of AOB in activated sludge (Park and Noguera 2004; Purkhold et al. 2000). Of the two genera, *Nitrosomonas* has been shown to be the dominant AOB in many bioreactors (Limpiyakorn et al. 2006; Park and Noguera 2004; Wells et al. 2009). By contrast, *Nitrosospira* sp. were rarely found in activated sludge (Schramm et al. 1999), probably due to their relative low growth rate (Siripong and Rittmann 2007) resulting in an underrepresentation in bioreactors. The limited studies of AOA species in activated sludge showed a composition different from those

existing in other environments such as soil, water column, and sediment (Park et al. 2006). Moreover, their abundances seemed to be much lower (four orders or more) than AOB based on analysis of *amo*A gene copy number (Jin et al. 2010; Limpiyakorn et al. 2010; Wells et al. 2009).

In this study, the AOA and AOB diversity in six bioreactors from the wastewater treatment plants (WWTPs) in four countries was investigated using *amo*A and 16S rRNA genes as the biomarkers. High-throughput pyrosequencing of the 16S rRNA genes was used to tabulate the AOB diversity and their relative abundance in the total bacterial community. Quantitative polymerase chain reaction (qPCR) was also used to quantify AOB *amo*A genes in the same activated sludge samples. Several SYBR green-based qPCR systems have been tried to quantify AOA *amo*A gene. However, non-specific amplification and/or the formation of primer dimer hindered the accurate quantification of AOA *amo*A using this approach.

Materials and methods

Activated sludge sampling and DNA extraction

In this study, activated sludge samples were taken from aeration tanks of eight full-scale WWTPs treating municipal wastewater in China, Singapore, Canada, and the USA. Relevant parameters about these WWTPs are shown in Supplementary Table S1. Sludge samples from the aeration tank were fixed with 50% ethanol (v/v) on site before transporting to the laboratory for DNA extraction. Ten milliliters of fixed activated sludge samples was centrifuged at 4,000 rpm for 10 min at 4 °C. Approximately 200 mg of sample pellet was recovered for DNA extraction in duplicate with a FastDNA® SPIN Kit for Soil (Qiagen, CA, USA), which was found to be the most suitable DNA extraction method for the samples in this study, as being compared with other commercial reagents.

PCR and quantitative PCR

Primer set amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe et al. 1997) was used to amplify bacterial *amo*A gene in a 30-μl mixture containing 0.2 μl of TaKaRa Ex Taq^TM, 3 μl of 10× Ex Taq Buffer (TaKaRa), 3 μl of 10 mM dNTP mixture (TaKaRa), 0.2 μM of each primer, and 20–50 ng of genomic DNA. Thermal cycling parameters followed the protocol of Rotthauwe and colleagues (1997). AOB *amo*A gene copy numbers were quantified by using an iCycler IQ System (Bio-Rad, Hercules, CA, USA) in triplicate with primer set amoA-1F/amoA-2R. Quantitative real-time PCR amplification was performed in a total

volume of 30 μl containing 15 μl of iQ^TM SYBR® Green Supermix (Bio-Rad), 5 μl of DNA template with the concentration of about 1 ng/μl, and 0.3 μM of each primer using the same cycling conditions. The archaeal *amo*A gene was amplified by PCR using the primer set Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis et al. 2005). PCR amplification was performed in a 50-μl volume comprising 25 μl 2× MightyAmp Buffer (TaKaRa), 1 μl (1.25 U) MightyAmp DNA Polymerase (TaKaRa), 0.3 μM of each primer, and 20–50 ng of genomic DNA. PCR was first incubated at 98 °C for 1 min and was followed by 35 cycles at 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 60 s. The PCR products were visualized by agarose (1%) gel electrophoresis in the presence of suitable size markers.

For high-throughput 454 pyrosequencing, the bacterial DNA was amplified with a set of primers targeting the hypervariable V4 region of the 16S rRNA gene. The forward primer is 5′-AYTGGGYDTAAAGNG-3′ and the reverse primers are the mixture of four equally mixed primers: 5′-TACCRGGGTHTCTAATCC-3′, 5′-TACCA-GAGTATCTAATTC-3′, 5′-CTACDSRGGTMTCTAATCC-3′, and 5′-TACNVGGGTATCTAATCC-3′ (RDP's Pyrosequencing Pipeline, http://pyro.cme.msu.edu/pyro/help.jsp). Barcodes that allowed sample multiplexing during pyrosequencing were incorporated between the 454 adaptor A and the forward primer.

Dideoxy DNA sequencing

PCR products were purified by using PCRquick-spin^TM PCR Product Purification Kit (iNtRON Biotechnology, Korea). The purified PCR products were ligated to *pMD®18-T* Vector (TaKaRa). Recombinant plasmid was transformed into *E. coli* and white colonies that grew on LB plates containing ampicillin (60 μg/ml), X-Gal, and IPTG were picked to conduct colony PCR amplification with the primer set M13F and M13R. The PCR products were purified and sequenced by ABI 3730xl capillary sequencers (Applied Biosystems, Foster City, CA, USA) with the primer M13F.

OTU definition and phylogenetic analysis

An AOB *amo*A gene clone library was constructed for each of the eight activated sludge samples (sample IDs—CN-NJ-SJ, CN-HR-UN, SG-SG-UP, CN-QD-TD, CN-SH-TS, CN-BJ-JX, US-CO-CO, and CA-GP-GP). Individual AOA *amo*A gene clone libraries were successfully constructed for samples CN-NJ-SJ, CN-HR-UN, and SG-SG-UP, while the other samples failed to generate library products. Approximately 20 clones in each clone library were selected randomly and sequenced. The resulting AOB *amo*A gene sequences were

aligned and the Jukes–Cantor distances between subsequent pairs of sequences were calculated with DNADIST from the PHYLIP package (http://www.phylip.com/), and were grouped into 43 OTUs with a distance cut-off of 3%. In order to construct the phylogenetic tree, one sequence in each OTU was selected, merged, and aligned with the reference sequences from NCBI Entrez Database. The neighbor-joining phylogenetic tree of AOB *amo*A gene sequences was created by MEGA software (Kumar et al. 2008). The translated protein sequences were assigned to different OTUs with a 3% distance cut-off. The phylogenetic tree of AOB AmoA protein sequences was also created by MEGA software. AOA *amo*A gene and AOA AmoA protein sequences were analyzed in the same manner as that of AOB. The AOB 16S rRNA gene sequences were also classified into different OTUs with a 3% distance cut-off. In addition, Good's estimator of coverage (Good 1953) was calculated for each AOA and AOB *amo*A gene clone library under 3% distance cut-off (Table S2).

High-throughput pyrosequencing

PCR amplicon libraries were prepared using a minimal number of amplification cycles (25 cycles) to minimize the accumulation of PCR artifacts. Amplicons were purified using PCRquick-spin™ columns (iNtRON Biotechnology). Equal amounts of amplicon products bearing individual sequence barcode for each sludge samples were combined for multiplex pyrosequencing on the Roche 454 FLX Titanium platform (Roche, Nutley, NJ, USA).

Sequence analysis and phylogenetic assignment

Following pyrosequencing, Python scripts were written to (1) remove sequences containing more than one ambiguous base ('N'), (2) check the sequence integrity of the barcodes and partitioned the multiplex reads to the individual samples, and (3) remove sequence reads shorter than 150 bases. The resulting filtered reads were then compared with the Greengenes 16S rRNA gene database (DeSantis et al. 2006) using NCBI's BLASTN tool (Altschul et al. 1990) with default parameters set to a maximum hit number of 100 (Claesson et al. 2009). Sequences were then assigned to NCBI taxonomies with MEGAN (Huson et al. 2007) using the Lowest Common Ancestor (LCA) algorithm and default parameters (absolute cut-off—BLAST bitscore 35; relative cut-off—10% of the top hits).

Accession numbers

The sequences obtained from clone library in this study were deposited in GenBank under accession numbers JF271927–JF271985. The pyrosequencing results are deposited into the NCBI short reads archive database (accession number SRA026842.2).

Results

Diversity of AOB *amo*A gene and 16S rRNA gene in different activated sludge samples

Table S2 suggested that the coverage of the AOB *amo*A gene clone libraries was over 70% except sample CN-SH-TS with a relatively low coverage (65%). As shown in Fig. 1, a total of 43 OTUs were generated based on 163 AOB *amo*A gene sequences in eight clone libraries with a 3% distance cut-off. Figure 1 reveals an interesting phenomenon that relatively few *amo*A OTUs were shared among the activated sludge samples, indicating that the *amo*A genes were quite diverse and not widely disseminated.

Figure 2 illustrated that most of these bacterial *amo*A OTUs were affiliated to *Nitrosomonas* genus with *Nitrosomonas ureae*, *Nitrosomonas oligotropha*, *Nitrosomonas marina*, and *Nitrosomonas aestuarii* being the four most dominant species. However, only two OTUs (OTU-17 and OTU-32) were in the *Nitrosospira* lineage and these two OTUs represented only eight sequences in the total 163 sequences, indicating the low abundance of *Nitrosospira* in various types of activated sludge.

Figure S1 shows the relative abundance and distribution of AOB AmoA protein OTUs in different activated sludge samples. Compared with AOB *amo*A gene OTUs (shown in Fig. 1), the diversity of AmoA protein (a total of 26 OTUs) was less than that of *amo*A gene (43 OTUs) due to codon wobble positions. Twelve OTUs of AOB AmoA protein were shared by at least two activated sludge samples. Our findings indicate that a number of different *amo*A genotypes encode essentially the identical or very similar proteins possibly reflecting the functional constraints in efficient ammonia oxidation. As shown in the phylogenetic tree of AOB AmoA protein in Fig. S2, most OTUs were affiliated with *N. ureae*, *N. oligotropha*, *N. marina*, and *N. aestuarii* lineages, exactly the same as the *amo*A gene phylogenetic tree. There were only a few OTUs in *Nitrosospira* lineage and *N. europaea* lineage, which was also identical with the above *amo*A gene phylogenetic analysis.

In addition to the *amo*A gene, the 16S rRNA gene was also used to investigate the AOB community in six samples (i.e., CN-NJ-SJ, CN-QD-TD, CN-HR-UN, US-CO-CO, SG-SG-UP, and CA-GP-GP) by high-throughput pyrosequencing. As shown in Table 1, more than 20,000 16S rRNA gene fragment sequences were obtained for each sample site. These sequences were assigned to NCBI
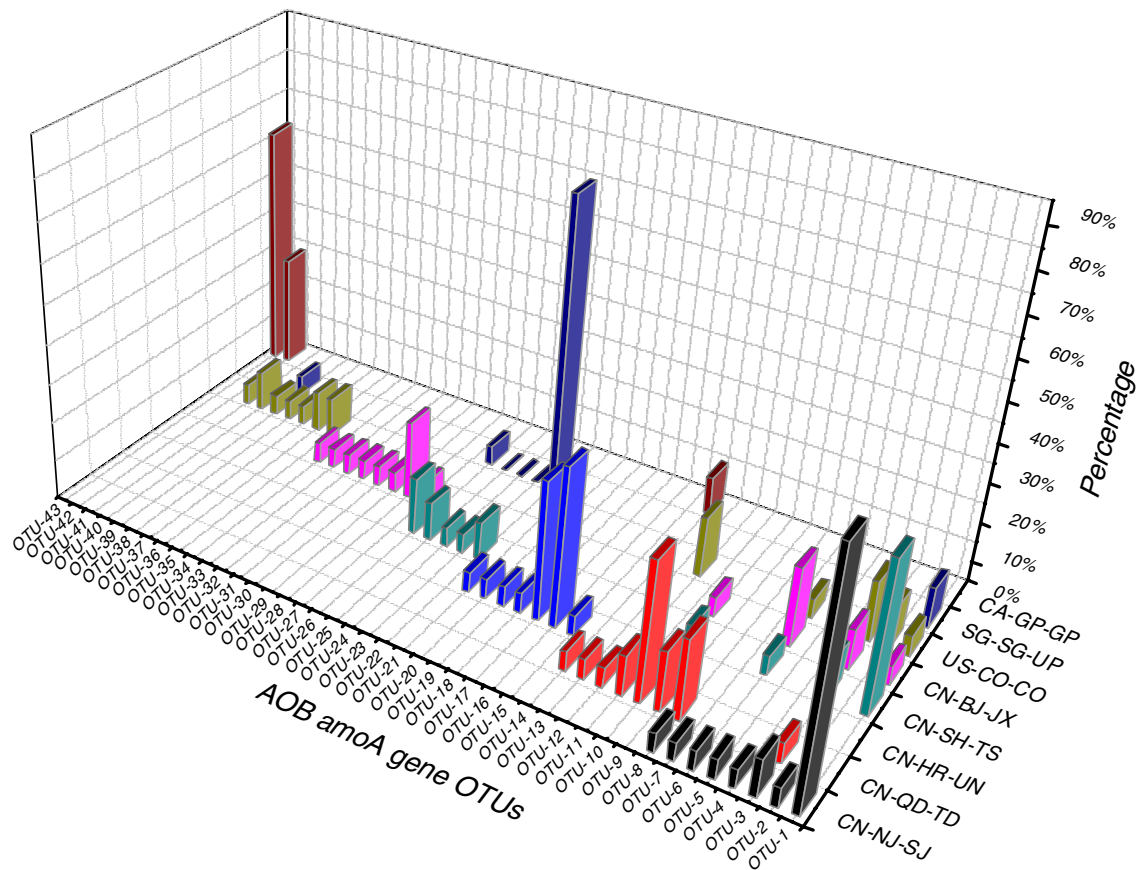
Fig. 1 Relative abundances and distribution of AOB *amo*A gene OTUs in different activated sludge samples

taxonomies using BLAST and MEGAN software. A tabulation of AOB-like sequences is shown in Table 1. The filtered reads were merged, aligned, and classified into 18 OTUs with a Jukes–Cantor distance cut-off of 3%. Figure 3 shows that most (about 61%) AOB 16S rRNA OTUs were shared by more than two sample sites, and two major OTUs, i.e., OTU-14 and OTU-15, accounted for 49% of total sequences. These results implied the AOB 16S rRNA genes are much more conservative than *amo*A gene and AmoA protein.

Abundance of AOB in activated sludge

Along with direct tabulation of AOB 16S rRNA genes by pyrosequencing, we also quantify AOB abundances in the activated sludge samples by qPCR, normalizing the AOB *amo*A gene copy number against per nanogram of the genomic DNA extracted from activated sludge. Table 1 shows the abundances of AOB *amo*A genes in different activated sludge samples quantified by qPCR. Sample CN-SH-TS has the most abundant AOB *amo*A gene copy numbers among the eight activated sludge samples tested. Table 1 lists the

total sequences obtained from pyrosequencing and the AOB-like sequences in each of the six samples. AOB sequences were successfully obtained in five out of the six samples, and the percentages range from 0.29% to 0.64%. For US-CO-CO, a sample from Columbia, USA, no AOB sequences were obtained, probably due to the low abundance of AOB in this wastewater treatment plant, in agreement with *amo*A gene quantification results.

Diversity of AOA *amo*A gene in different activated sludge samples

In the present study, multiple experiments were attempted to amplify *amo*A gene from the eight samples using four PCR systems (including TaKaRa Ex Taq, TaKaRa Mighty Amp DNA Polymerase, Sigma *Taq* DNA Polymerase, and Bio-Rad SYBR® Green Supermix) at different thermal conditions (annealing temperature from 50 to 60 °C). Amplification was successful from only five samples as shown in Fig. S3 and only three samples were eventually successfully cloned and sequenced. Additionally, the presence of primer dimers was very serious in all samples

Fig. 2 Neighbor-joining phylo-genetic tree based on bacterial *amo*A gene sequences. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. Bootstrap values are indicated on branch nodes. Sequences obtained in this study are shown with "*OTU-*" in the names. Other sequences were obtained from GenBank. The tree was out-grouped with *Nitrosococcus halophilus* (gammaproteobacterial AOB) *amo*A sequence
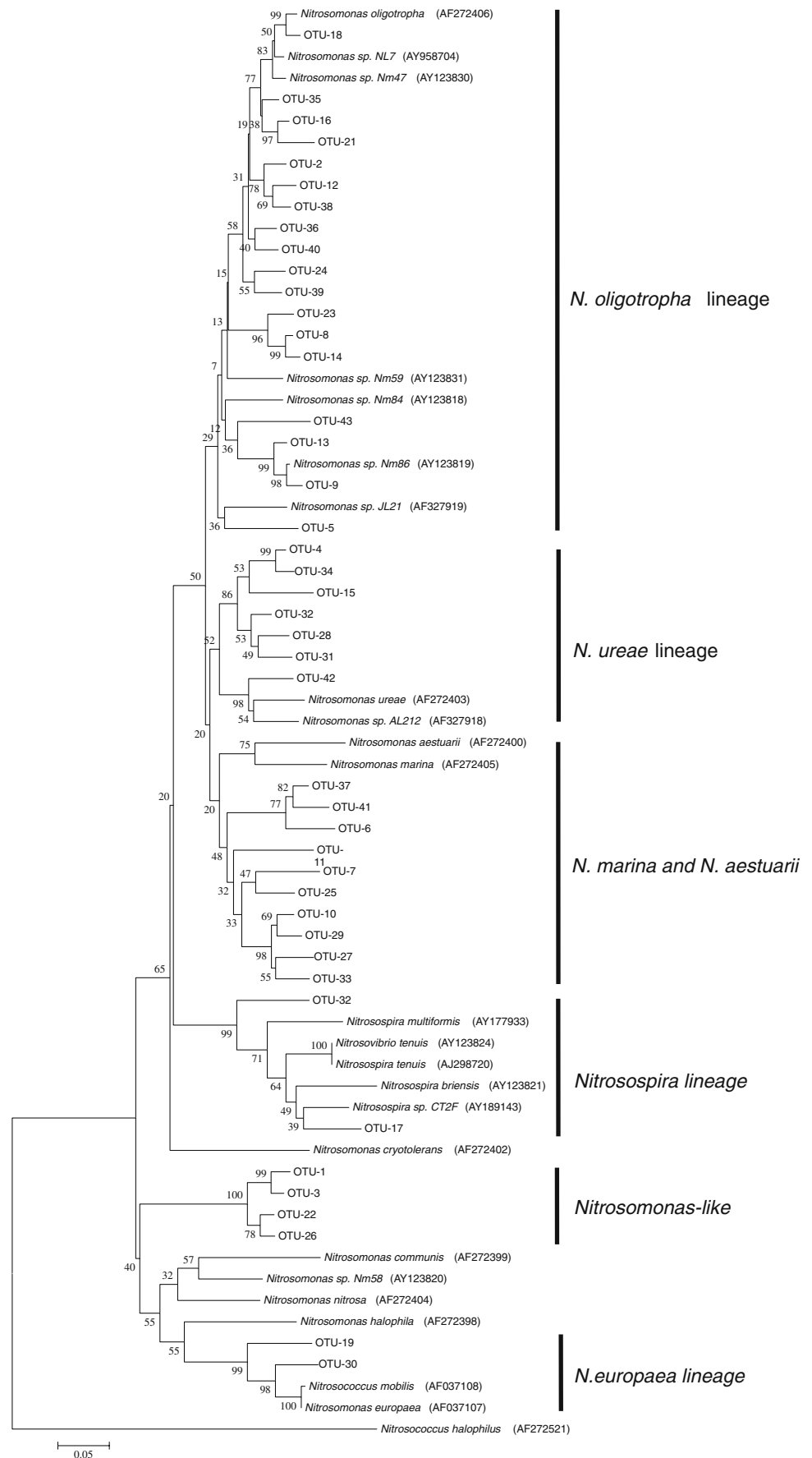
Table 1 Summary of the diversity and abundance of AOA and AOB in eight WWTPs

Sample	WWTP	City and country	Diversity					Abundance of AOB	
			AOA		AOB				
			amoA sequence	No. of OTUs	amoA sequence	No. of OTUs	16S rRNA gene sequence	16S rRNA gene percentage in total sequences	amoA gene (copies/ng DNA)
CN-NJ-SJ	Suojincun	Nanjing, China	20	6	21	8	87	0.42% (20,955)	34.5
CN-QD-TD	Tuandao	Qingdao, China	ND	ND	21	8	156	0.64% (24,437)	0.538
CN-HR-UN	Harbin	Harbin, China	16	5	20	7	105	0.46% (22,593)	2.81
CN-BJ-JX	Jiuxianqiao	Beijing, China	ND	ND	21	9	ND	ND	142
CN-HR-UN	Tianshan	Shanghai, China	ND	ND	20	12	ND	ND	5.55
US-CO-CO	Columbia Regional	Columbia, USA	ND	ND	20	12	0	0% (25,499)	1.29
SG-SG-UP	Ulu Pandan	Singapore	20	10	20	5	81	0.34% (23,928)	74.3
CA-GP-GP	Guelph	Guelph, Canada	ND	ND	21	3	66	0.29% (22,593)	1.72

ND: No Data

and it was not possible to quantify $amoA$ using SYBR® Green qPCR approach applied in this study (Supporting information Fig. S3).

However, it is still of interest to have a preliminary assessment of the diversity of AOA $amoA$ gene in the activated sludge samples by exploring the sequences obtained from the clone libraries of the samples CN-NJ-SJ, CN-HR-UN, and SG-SG-UP, all of which had Good's estimator of coverage over 80% (Table S2). The 56 sequences from the three samples were classified into 15 OTUs using a distance cut-off of 3% (Fig. 4). Different from AOB $amoA$ OTUs (Fig. 1), some AOA $amoA$ OTUs were shared among multiple samples. Judging from the results in this study, it was found that the AOA $amoA$ genes are not as diverse as AOB $amoA$ gene in different activated sludge samples. While it should be mentioned that the sample amount in this study was kind of limited, thus this issue needs more studies to be confirmed in the future. As shown in the phylogenetic tree of AOA $amoA$ gene sequences in Fig. 6, most of the sequences obtained in this study were distinctly different from the previously reported sequences, especially the sequences recovered from marine and sediment. Only two OTUs (OTU-10 and OTU-11) were grouped into the marine and sediment lineages.

Discussion

Diversity of AOB $amoA$ gene and 16S rRNA gene in different activated sludge samples

A previous study reported that the $amoA$ gene similarities among different AOB species ranged from 65% to 100% (Purkhold et al. 2000). The similarities of the OTUs obtained in this study were 62% to 100%. The high diversity of the $amoA$ gene found in this study was likely due to the different geographic locations of these sludge samples and the different operation/sewage conditions in these WWTPs.

The results in this study were consistent with previous reports that showed *Nitrosomonas* instead of *Nitrosospira* being the dominant AOB in nitrification bioreactors in WWTP (Park and Noguera 2004; Wells et al. 2009). Notably, seven OTU-17-associated sequences were all from the sample CN-HR-UN taken from Harbin, a city in northern China. The latitude of this city is the highest among those sampled in this study. Our observation is consistent with the previous report that *Nitrosospira* spp. prefer low temperatures of 4–10 °C (Avrahami et al. 2003; Siripong and Rittmann 2007). We also observe a small number of OTUs belonging to *Nitrosomonas europaea* lineage and several OTUs (OTU-1, OTU-3, OTU-22, and OTU-26) grouped far away from the known AOB species.

Fig. 3 Relative abundances and distribution of AOB 16S rRNA gene OTUs in different activated sludge samples
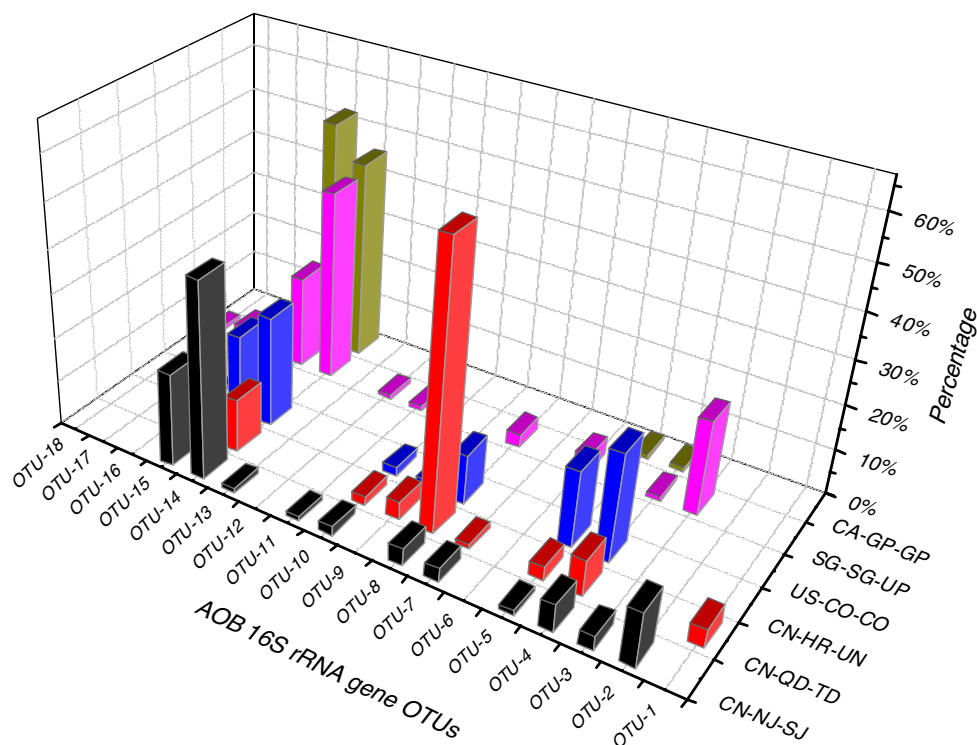

It was reported that the AOB 16S rRNA gene similarities generally ranged from 90% to 100%, much higher than those of *amo*A gene (about 65–100%) and AmoA protein (about 72.5–100%) (Purkhold et al. 2000). The phylogenetic tree in Fig. 5 shows that all of these sequences obtained in this study were grouped in *Nitrosomonas* genus. There were 12 OTUs in *Nitrosomonas oligotropha* group, four OTUs in the group of *Nitrosomonas ureae*, and one OTU in the *N. marina* and *N. aestuarii* lineages, indicating the dominance of these species in the activated sludge

Fig. 4 Relative abundances and distribution of AOA *amo*A gene OTUs in different activated sludge samples
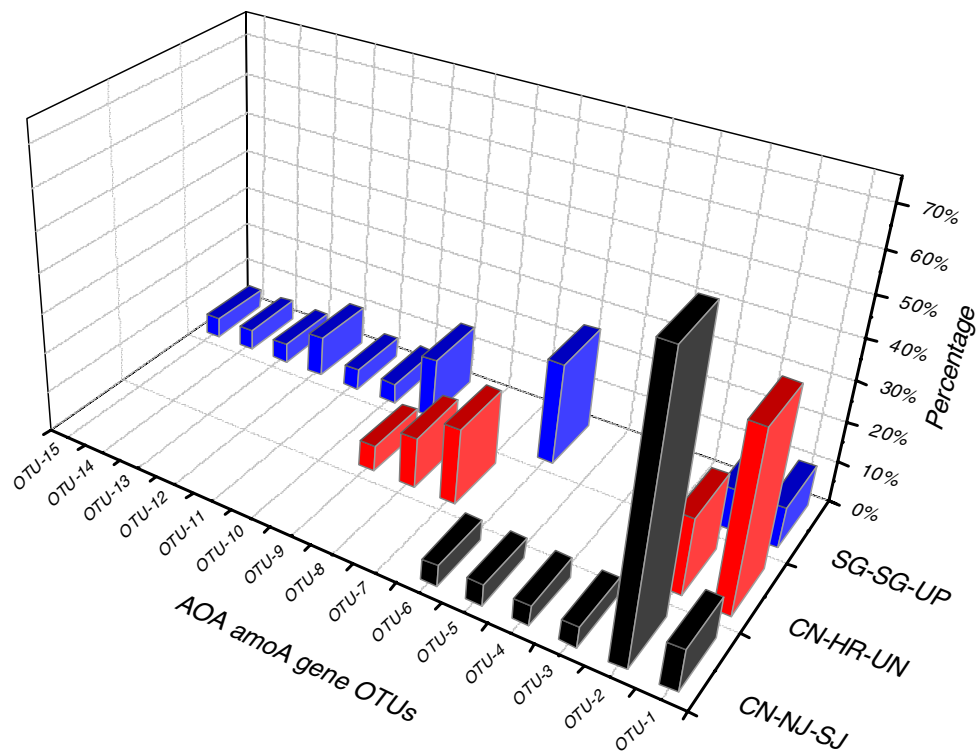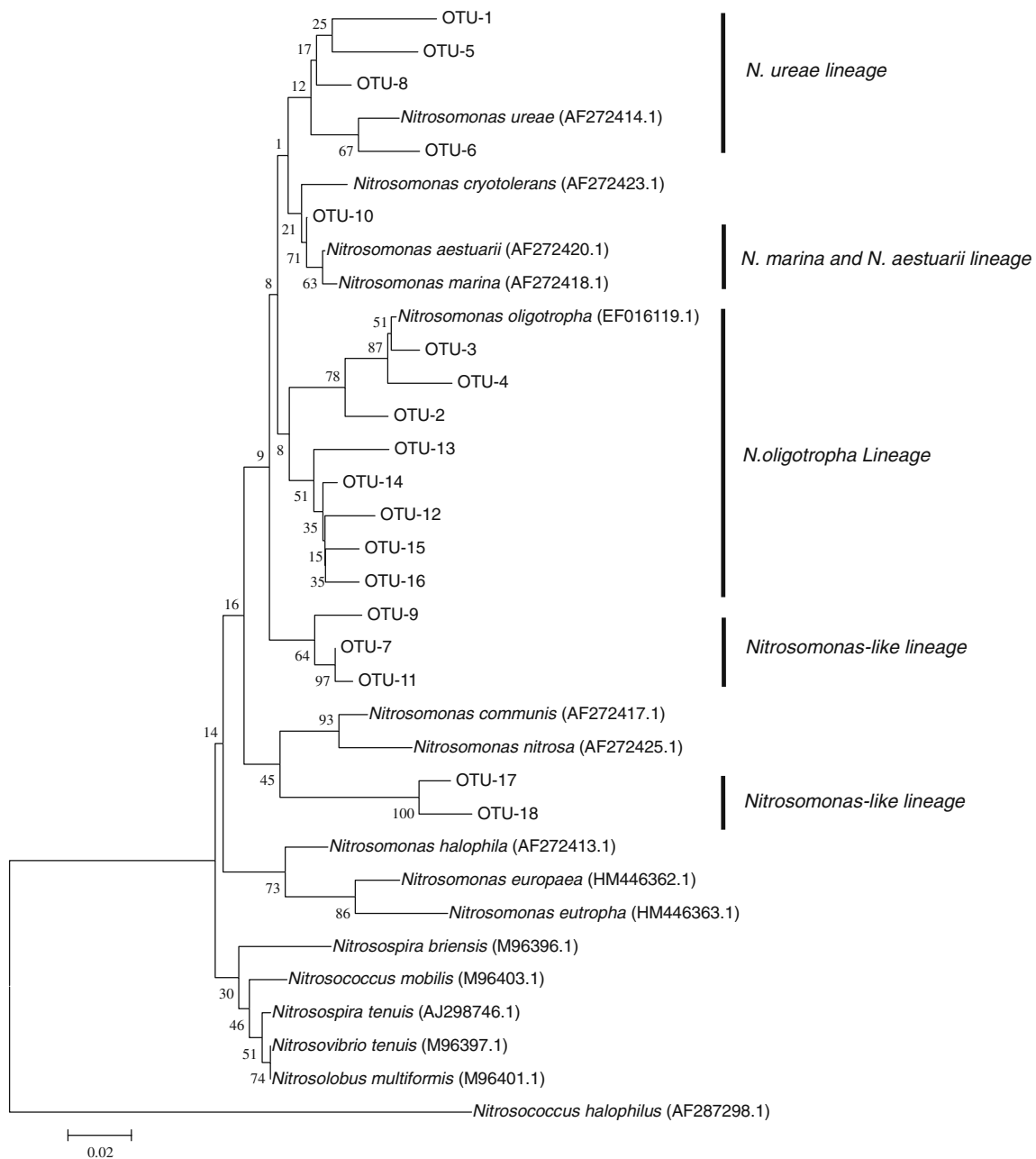
Fig. 5 Neighbor-joining phylogenetic tree based on AOB 16S rRNA gene sequences. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. Bootstrap values are indicated on branch nodes. Sequences obtained in this study are shown with "*OTU-*" in the names. Other sequences were obtained from GenBank

samples. This almost perfectly matches the above results of this study using *amo*A gene as the biomarker. A minor exception is that *Nitrospira* 16S rRNA gene was not found by pyrosequencing, probably due to the low abundance of these AOB species in activated sludge and that the sequencing depth (>20,000 reads per sample) of the pyrosequencing applied in this study was not of sufficient depth to represent the complexity of the rare AOB sequences. Although we identified 495 sequence reads to

the betaproteobacterial AOB, no reads can be assigned to the gammaproteobacterial AOB 16S rRNA genes, indicating the dominance of betaproteobacterial AOB in samples of this study.

Abundance of AOB in activated sludge

The abundances of AOB in activated sludge were quantified using qPCR and pyrosequencing. However, the two data sets

show a certain degree of discordance that might be due to the following reasons: (1) primer bias in qPCR amplification may introduce many uncertainties, especially when the target gene copy numbers are at low levels (Herrmann et al. 2008; Smith et al. 2006); (2) the 454 pyrosequencing conducted in this study may not be at a sufficient read depth to reflect the AOB complexity since AOB sequences was not detected in one of these samples; (3) the qPCR normalization step made use of total activated sludge DNA, which contains nucleic acids from eukaryotes, bacteria, archaea, and viruses to which any variations in the relative distribution of these components between samples would distort the qPCR normalization process; and (4) 16S rRNA gene copy numbers in prokaryotic microorganisms (Klappenbach et al.
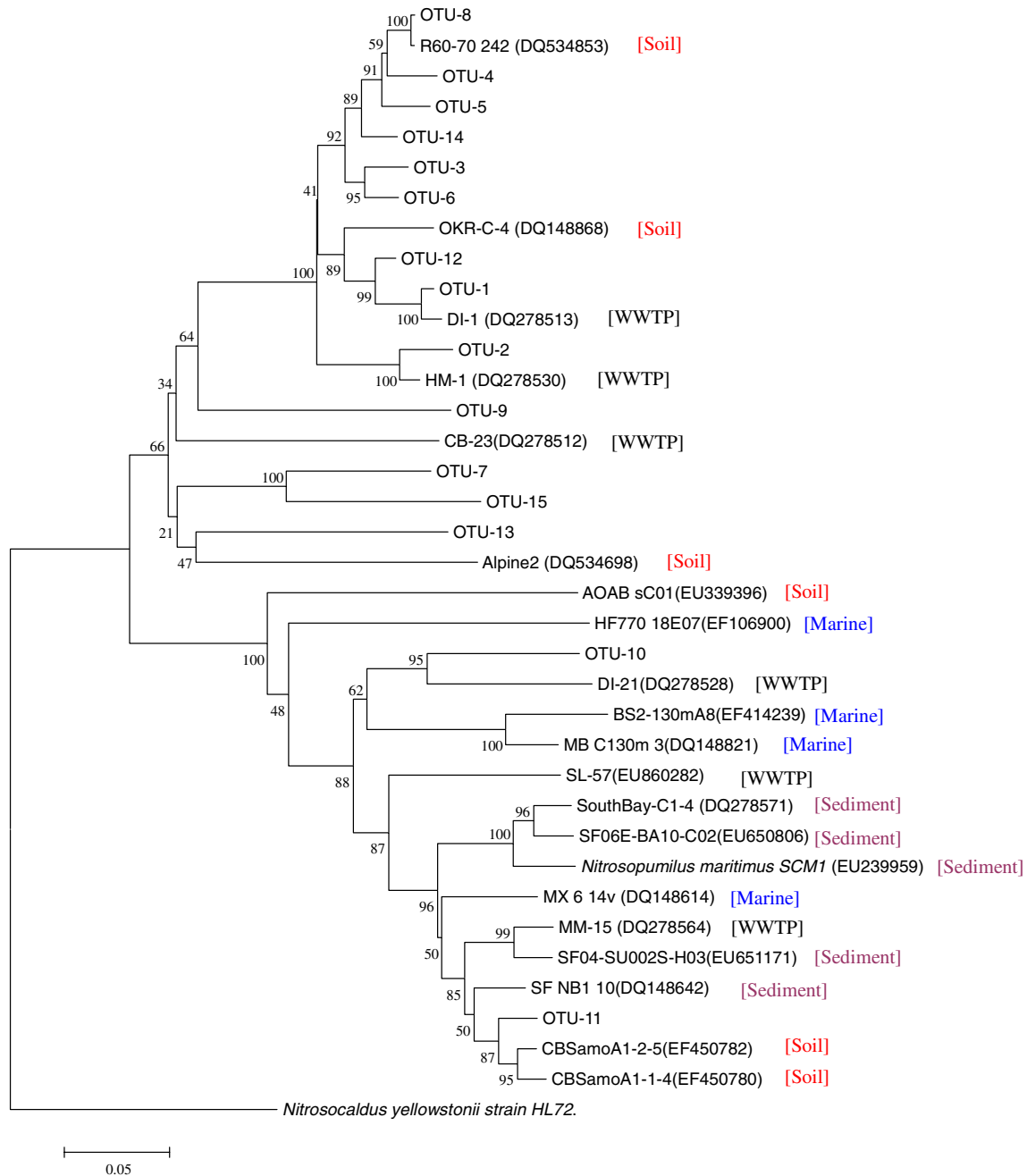
Fig. 6 Neighbor-joining phylogenetic tree based on AOA *amoA* gene sequences. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. Bootstrap values are indicated on branch nodes. Sequences recovered in this study are shown with "*OTU*-" in the names. Other sequences were obtained from GenBank. The words in *square brackets* indicate the source the sequences originated from

2001) are different and the *amo*A gene copy numbers in different AOB are also different (Norton et al. 2002).

Diversity of AOA *amo*A gene in different activated sludge samples

Although many reports described the diversity and abundance of AOAs in the natural environments, only limited information is available describing AOA diversity in the activated sludge (Limpiyakorn et al. 2010; Park et al. 2006; Wells et al. 2009; Zhang et al. 2009). One possible reason for this situation could be due to the difficulty in PCR amplification of AOA *amo*A from DNA of sludge samples. In this study, only five out of eight samples were successfully amplified, and only three samples were eventually successfully cloned and sequenced.

Previous studies revealed that most of AOA should be assigned to the clusters of CGI.1a (the marine and sediment lineage) and CGI.1b (the soil lineage) in the phylum *Crenarchaeota* (Hatzenpichler et al. 2008). In this study, it was found that most of the AOA in the activated sludge samples have a closer distance to those AOA found in soil and thus belonged to the soil lineage. This result was in agreement with the previous studies on AOA communities in activated sludge (Limpiyakorn et al. 2010; Park et al. 2006), except for a study on the activated sludge in bioreactors treating saline sewage (Jin, et al. 2010) which had dominant AOA communities belonging to the marine and sediment lineage. AOA AmoA protein sequences were classified into nine OTUs using 3% distance cut-off. Figure S4 shows the relative abundance and distribution of AOA AmoA protein OTUs in different activated sludge samples, indicating lower diversity compared with AOA *amo*A gene and that most of the AOA AmoA protein OTUs were shared among the three samples. The neighbor-joining phylogenetic tree based on AOA AmoA protein sequences in Fig. S5 demonstrates the same pattern as shown by the AOA *amo*A gene tree (Fig. 6), that is, most of the OTUs in this study were grouped together and had a far distance from those sequences of marine and sediment. Interestingly, there are two OTUs (OTU-6 and OTU-7 in Fig. S5) closely related to the marine and sediment AOA lineage. The two OTUs were only detected in the sludge SG-SG-UP, a sample from Singapore, implying that the occurrence of these OTUs might be due to the geographic location of Singapore, which is near the ocean. The other two samples were taken from cities far from the ocean and thus their AOA populations mainly come from soil.

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