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ENVIRONMENTAL BIOTECHNOLOGY

Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR

Tao Jin · Tong Zhang · Qingmei Yan

Abstract Using ammonia monooxygenase α-subunit (*amo*A) gene and 16S rRNA gene, the community structure and abundance of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in a nitrogen-removing reactor, which was operated for five phases, were characterized and quantified by cloning, terminal restriction fragment length polymorphism (T-RFLP), and quantitative polymerase chain reaction (qPCR). The results suggested that the dominant AOB in the reactor fell to the genus *Nitrosomonas*, while the dominant AOA belonged to Crenarchaeotal Group I.1a in phylum Crenarchaeota. Real-time PCR results demonstrated that the levels of AOB *amo*A varied from 2.9×10^3 to 2.3×10^5 copies per nanogram DNA, greatly (about 60 times) higher than those of AOA, which ranged from 1.7×10^2 to 3.8×10^3 copies per nanogram DNA. This indicated the possible leading role of AOB in the nitrification process in this study. T-RFLP results showed that the AOB community structure significantly shifted in different phases while AOA only showed one major peak for all the phases. The analyses also suggested that the AOB community was more sensitive than that of AOA to operational conditions, such as ammonia loading and dissolved oxygen.

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Introduction

Nitrification, the key and often rate-limiting step in nitrogen removal, includes two steps, i.e., oxidation of ammonia to nitrite catalyzed by ammonia-oxidizing bacteria (AOB), and nitrite to nitrate by nitrite-oxidizing bacteria. AOB are thought to be largely responsible for the oxidation of ammonia to nitrite in wastewater treatment plants (WWTPs) and natural environments. In particular, members of the betaproteobacterial genera *Nitrosomonas* and *Nitrosospira* are considered as the most important AOB in activated sludge (Purkhold et al. 2000).

Recently, however, it was discovered that autotrophic oxidation of ammonia is not restricted to the *Bacteria* domain (Treusch et al. 2005; Könneke et al. 2005). The first cultivated representative of ammonia-oxidizing archaea (AOA), named *Nitrosopumilus maritimus*, which, like AOB, grows chemolithoautotrophically by oxidizing ammonia to nitrite, was isolated from a marine aquarium tank (Könneke et al. 2005). Both AOA and AOB have ammonia monooxygenase (AMO), one of the key enzymes responsible for the conversion of ammonia to nitrite. AMO is composed of three subunits encoded by genes of *amo*A, *amo*B, and *amo*C (Klotz et al. 1997). Among these three genes, the *amo*A gene has been much more extensively used for the study of ammonia oxidizers and has a well-established database, compared with *amo*B and *amo*C (Francis et al. 2005; Okano et al. 2004).

By using culture-independent DNA-based techniques, AOA has been found quite diverse and abundant in various

natural environments, such as marine sediment (Francis et al. 2005; Park et al. 2008), soil (He et al. 2007), estuary (Beman and Francis 2006), and seawater (Coolen et al. 2007). So far, most studies on AOA have been focused on their diversity and quantity in various natural environments, revealing its high diversity and widespread distribution. However, only very limited works on the presence of AOA in wastewater treatment reactors/plants have been done (Park et al. 2006; Zhang et al. 2009). Most studies of nitrifying activated sludge only focused on the presence and abundance of AOB (Dionisi et al. 2002; Geets et al. 2007). So far, there was only one new report to indicate that AOB was predominant rather than AOA in targeted full-scale bioreactors (Wells et al. 2009). Lacking more data, it remains unknown whether it will be the same or not in other bioreactors.

AOB are highly sensitive to several environmental factors, such as temperature, salinity, pH, dissolved oxygen (DO), ammonium concentration, and hydraulic retention time (HRT; Limpiyakorn et al. 2007; Lydmark et al. 2007). Any improper condition may result in nitrification failure in wastewater treatment. Although the study on the factors affecting AOA is quite limited, it is believed that AOA responds to different environmental conditions (Erguder et al. 2009). Therefore, knowledge on the diversity and dynamics of AOA and AOB in wastewater treatment will be helpful to improve the reactor performance.

In this study, a laboratory-scale nitrogen-removing reactor was carefully controlled to gradually change the running conditions (DO, ammonium concentration, and HRT) in five phases in order to investigate the response of AOA and AOB. Using culture-independent DNA-based approaches, the goals of this study are (1) to investigate the diversity of AOA and AOB, as well as the total archaeal and bacterial community based on clone libraries of 16S rDNA and amoA, (2) to monitor changes of composition of AOA/AOB populations under different running conditions

using terminal restriction fragment length polymorphism (T-RFLP), and (3) to quantify the AOA/AOB amoA gene copies using quantitative polymerase chain reaction (qPCR).

Materials and methods

Reactor operation

Nitrifying activated sludge was collected from Hong Kong Shatin sewage treatment plant, which treats 150,000 m^3 saline sewage containing an average of 6,000 mg Cl$^-$/L, 300 mg COD/L, and 20 mg NH$_4^+$–N/L, and used as the sludge seed for an automatically controlled continuous stirred-tank reactor (CSTR) of 2.6 L (working volume) followed by a cylindered sedimentation separation unit of 300 ml (length 15 cm and diameter 9 cm). The influent of the reactor was synthesized by deionized water (67%) and seawater (33%) to simulate the salinity of sewage in Hong Kong. NH$_4$Cl was added at different concentrations in the five phases. For each gram of NH$_4^+$-N in the synthetic wastewater, 7 g of NaHCO$_3$ and 0.07 g of phosphorus were added. Table 1 shows the detailed operational conditions and the wastewater compositions in five different phases. The pH was kept at 7.0 by adding NaHCO$_3$ solution. DO was controlled by the combination of aeration and stirring. Temperature was 23±2°C throughout the whole operation. Mixed liquor suspended solid (MLSS), nitrate, nitrite, and ammonium were measured accordingly to the standard method (APHA 2005).

DNA extraction and PCR

Genomic DNA was extracted from 1.5 ml of sludge sample using a FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC, Illkirch, France) following the instruction of the producer. The amount of DNA was determined by NanoDrop®

Table 1 Operational conditions for nitrogen-removing CSTR

	phase I	phase II	phase III	phase IV	phase V
Time range (days)	1–45	46–86	87–142	143–204	205–286
Feed flow rate (L day^{-1})	5	5	5	5	7.5
HRT (h)	12.5	12.5	12.5	12.5	8.3
Feed nitrogen concentration (mg NH$_4^+$–N L^{-1})	100	100	100	200	200
Feed COD concentration (mg COD L^{-1})	100	100	0	0	0
DO (mg L^{-1})	1.0	0.5	0.5	0.5	0.5
Nitrogen loading rate (mg NH$_4^+$–N L^{-1} day^{-1})	192	192	192	384	576
Ammonium-oxidizing rate (g N g SS^{-1} day^{-1})	No data	No data	0.13	0.21	0.26
Nitrogen removal rate (g N g SS^{-1} day^{-1})	No data	No data	0.09	0.17	0.20
MLSS concentration (g L^{-1})	1.1	1.7	1.5	1.8	2.2

Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The primers used were listed in Table 2. The PCR was performed in a total volume of 25 µl containing 20 ng of DNA template, 1 U of AmpliTaq® DNA polymerase (Applied Biosystem, Branchburg, USA), 0.1 µM of each primer, and 12.5 µl FailSafe™ PCR Premix F (EPICENTRE Biotechnologies, Madison, USA). Thermal cycling was carried out by an initial denaturation step at 94°C for 4 min. The major cycling program for each primer set was listed in Table 2. The presence and sizes of the PCR amplification products were determined by agarose (1%) gel electrophoresis.

Cloning and sequencing

The PCR products were purified by using PCRquick-spin™ PCR Products Purification Kit (iNtRON Biotechnology, Seongnam-Si, Korea). Then the PCR products were cloned using InsT/Aclone™ PCR Cloning Kit (Fermentas, Vilnius, Lithuania). White colonies were picked for insert screening using PCR with M13F and M13R primers, and then grouped by RFLP with restriction enzymes *Rsa*I and/or *Msp*I. For each RFLP group, representatives were selected and sequenced. The sequencing was performed using M13F primer on ABI 3730xl capillary sequencers (PE Applied Biosystems, Foster City, USA).

Phylogenetic analyses

The nucleotide sequences were compared with those from the GenBank using BLASTn in the National Center for the Biotechnology Information server (http://www.ncbi.nlm. nih.gov). The sequences in this study and the reference sequences retrieved from the GenBank were aligned by ARB (http://www.arb-home.de; Ludwig et al. 2004) to construct phylogenetic trees using the neighbor-joining method (based on Jukes–Cantor corrected distance). Bootstrap value was calculated based on 1,000 replications.

Quantitative PCR

qPCR was performed using an iCycler IQ System (Bio-Rad, Hercules, USA), two replicates for each sample. For AOA, the PCR was performed in a total volume of 30 µl containing 15 µl of FailSafe™ PCR Premix F, 5 µl of DNA template, 0.1 µM of each primer, 1.5 U of AmpliTaq® DNA polymerase, 15 mM MgCl$_2$, and 0.5× SYBR® Green I (Invitrogen, Eugene, USA). For AOB, the PCR was performed in a total volume of 30 µl containing 15 µl of iQ™ SYBR® Green Super Mix (Bio-Rad, Hercules, USA), 5 µl of DNA template, and 0.1 µM of each primer. The real-time PCR thermocycling steps were set as follows: 95°C for 4 min and 45 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s. Cycling was completed by a final elongation step at 72°C for 10 min. The negative control containing no DNA was subjected to the same procedure to exclude or detect any possible contamination. After real-time PCR assay, the specificity of amplification was verified by generation of melting curves and agarose gel electrophoresis.

T-RFLP analysis

PCR was performed by using fluorescently labeled primers amoAR-Hex and amoA-2R-Hex for AOA and AOB, respectively. PCR conditions were the same as those for clone library construction. *Rsa*I and *Hha*I were selected to digest PCR products of AOA and AOB *amo*A gene purified with PCRquick-spin™ PCR Products Purification Kit, respectively, as these two enzymes may classify the different operational taxonomic units (OTUs) of AOA or AOB into unique terminal restriction fragments (T-RFs) as shown in the Table 3.

The restriction–digestion mixture, containing 17 µl of purified PCR product (about 200 ng DNA), 2 µl of buffer, and 1 µl of restriction enzyme (10 U/µl), was incubated at 37°C for 5 h. The digested DNA was directly precipitated

Table 2 Primers used for PCR amplification

Target gene		Primer	Sequence (5′–3′)	PCR program	Reference
16S rRNA	Bacterial	EBU8F	AGAGTTTGATCMTGGCTCAG	(94°C, 60 s; 55°C, 45 s; 72°C, 60 s)×30	Heuer et al. 1997
		UNIV1392R	ACGGGCGGTGTGTRC		Ferris et al. 1996
	Archaeal	ARC23F	TGCAGAYCTGGTYGATYCTGCC	(94°C, 45 s; 56°C, 45 s; 72°C, 60 s)×35	Burggraf et al. 1991
		ARC934R	GTGCTCCCCGCCAATTCCT		Giovannoni et al. 1998
*amo*A	AOA	cren amo_F	ATGGTCTGGCTAAGACGMTGTA	(94°C, 45 s; 55°C, 45 s; 72°C, 45 s)×35	Hallam et al. 2006
		amoAR[a]	GCGGCCATCCATCTGTATGT		Francis et al. 2005
	AOB	amoA-1F	GGGGTTTCTACTGGTGGT	(94°C, 60 s; 55°C, 45 s; 72°C, 60 s)×40	Rotthauwe et al. 1997
		amoA-2R[a]	CCCCTCKGSAAAGCCTTCTTC		Rotthauwe et al. 1997

[a] Primers used for T-RFLP analysis were labeled by Hex

Table 3 *amo*A OTUs and clones of AOA and AOB and their corresponding T-RFs

	T-RF	OTU	Clones
AOA	247	AOA-OTU-1, 4	D30-C-1, D30-C-8, D30-C-33
	56	AOA-OTU-3	D30-C-3
AOB	214	AOB-OTU-1	F-AOB-5, F-AOB-19, F-AOB-40
	371	AOB-OTU-3	AS-1
	421	AOB-OTU-2	F-AOB-1, F-AOB-25
	486	AOB-OTU-4	AS-60

with 50 µl 100% ethanol by centrifugation at 14,000×*g* for 15 min at 4°C. The DNA pellets were washed with 100 µl 70% (*v/v*) ethanol and then air dried. The precipitate (about 70–100 ng) was dissolved in 15 µl of distilled water. The fluorescently labeled T-RFs were run through an ABI 3730xl capillary sequencer in the GeneScan mode. T-RFLP data was analyzed using GeneMarker V1.6 (SoftGenetics LLC, Pennsylvania, USA). Because of the detection range of internal marker GS500, T-RFs smaller than 50 bp and larger than 500 bp were excluded from further analysis. The peaks were first selected by the default parameters setting of the software GeneMarker with the threshold cutoff of peak intensity of 100. The relative abundance of each T-RF was determined by calculating the ratio between the area of each peak and the total area of all peaks in one sample. The peaks with relative abundance <1% were neglected in this study.

Accession number

The sequences reported in this study were deposited in the GenBank under accession number FJ917560–FJ917595.

Results

Reactor performance

As shown in Table 1, the ammonium-oxidizing rate was increased from 0.13 to 0.21 and 0.26 mg N g SS^{-1} day^{-1} from phase III to phase V. Correspondingly, the nitrogen removal rates were 0.09, 0.17, and 0.20 mg N g SS^{-1} day^{-1} and the removal percentages were 64%, 70%, and 71%, respectively.

Bacterial community

Eighteen OTUs were recovered from 75 clones of a library based on bacterial 16S rRNA gene using 3% nucleotide cutoff. Most of these sequences were closely related to unidentified clones from environmental samples. Bacteria in the sludge of this study belonged to four divisions: Proteobacteria (11 OTUs), Chloroflexi (two OTUs), Bacteroidetes (three OTUs), and Planctomycetales (two OTUs; Fig. 1). In the subdivision of betaproteobacteria, the OTUs FN-4, 5, 7, and 72 were affiliated with bacteria *Nitrosomonas* spp. at similarities of 97% to 99%.

AOB community diversity based on *amo*A gene

As shown in Fig. 2 and Table 3, two AOB *amo*A OTUs (based on the 3% nucleotide cutoff), i.e., AOB-OTU-1 and AOB-OTU-2, obtained from the sludge formed two clusters. The sequences of all these OTUs were closely related to those of *Nitrosomonas* spp., instead of *Nitrosospira* spp., in agreement with results of bacterial 16S rRNA gene clone library. Most of the sequences in cluster II were from activated sludges of different WWTPs. Especially, *Nitrosomonas* sp. ML1 (AY958703) was isolated from a bioreactor operated under low DO (0.12–0.24 mg L^{-1}; Park and Noguera 2007), which was even lower than DO levels of 0.5–1.0 mg L^{-1} in this study.

Archaeal and AOA community

The results of the archaeal 16S rRNA gene clone library showed very low diversity of archaeal community. RFLP analysis of 16 clones showed only one OTU. Three representatives were sequenced, as shown in Fig. 3a. It was affiliated with an archaeon clone ZES-168 (EF367605) from a tropical estuary, belonging to Crenarchaeotal Group I.1a (CGI.1a) in phylum Crenarchaeota. As reported before (Zhang et al. 2009), there were four AOA *amo*A OTUs (on the basis of the 5% nucleotide cutoff) recovered by using two primer sets in this sludge. As shown in Fig. 3b, they were dominantly distributed in the CGI.1a cluster and only one OTU fell in the CGI.1b cluster, according to the classification of Park et al. (2008), in agreement with the results of the archaeal 16S rRNA gene clone library.

AOA and AOB abundance

Figure 4 shows the change of AOA and AOB population abundance in the whole microbial community of the CSTR in terms of the *amo*A gene copy number per nanogram DNA in the extract. AOA *amo*A gene copy number ranged from 1.7×10^2 to 3.8×10^3 copies per nanogram DNA in the
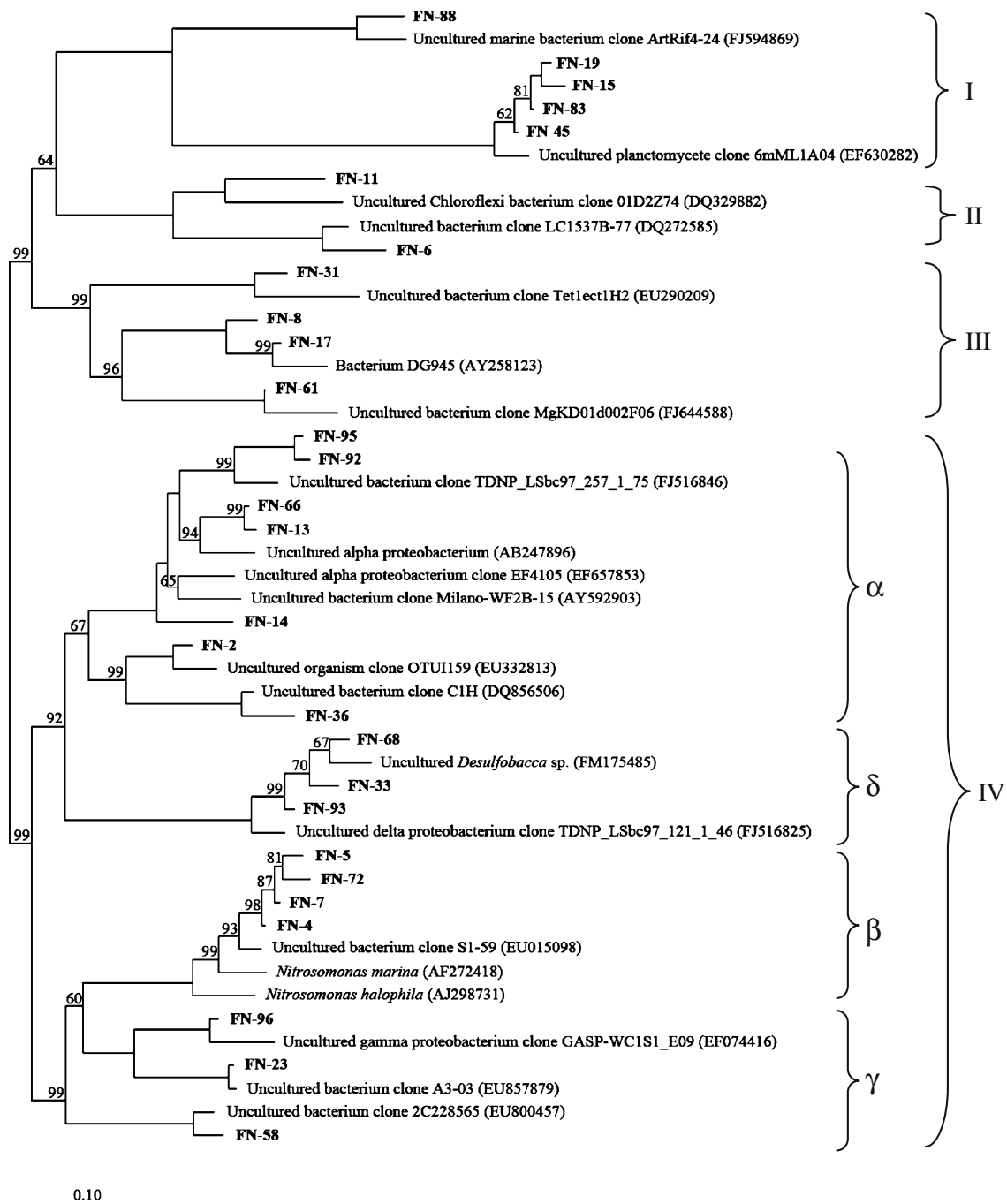
Fig. 1 Neighbor-joining tree based on Jukes–Cantor corrected DNA distances showing the phylogenetic affiliation of bacterial 16S rRNA gene sequences from activated sludge of CSTR and reference sequences from other environmental samples or pure stains. The sequences obtained in this study are printed in *bold*. All the sequences were grouped as *I* Planctomycetales, *II* Chloroflexi, *III* Bacteroidetes, and *IV* Proteobacteria

whole process. It was firstly down from 8.9×10^2 to 1.7×10^2 copies per nanogram DNA in the initial 20 days (phase I), recovered slightly in phase II, subsequently increased up to reach the highest 3.8×10^3 copies per nanogram DNA in phase III, and finally decreased slightly in last two phases. The average abundance for AOA was 1.9×10^3 copies per nanogram DNA for five phases.

The levels of AOB *amo*A varied from 2.9×10^3 to 2.3×10^5 copies per nanogram DNA during the five phases, fluctuating more significantly than that of AOA. In phases I, II, and V, AOB abundance was over 1,000-fold higher than that of AOA. In phases III and IV, the abundances of AOA and AOB were in the same magnitude. Overall, the average abundance for AOB was 1.2×10^5

Fig. 2 Neighbor-joining tree based on Jukes–Cantor corrected DNA distances showing the phylogenetic affiliation of AOB *amo*A gene sequences from activated sludge of CSTR and other environmental samples or pure strains. The sequences obtained in this study are printed in *bold*
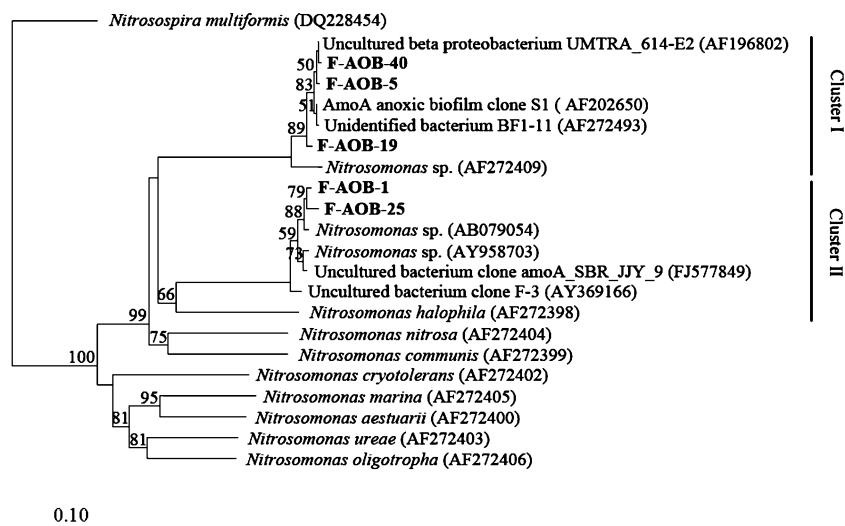
copies per nanogram DNA for five phases. Thus, AOB were about 60 times more abundant than AOA in terms of *amo*A copy number. Assuming 2.5 copies of *amo*A gene per AOB cell (Norton et al. 2002) and one copy per AOA cell (Hallam et al. 2006), AOB cell numbers were about 24 times higher than that of AOA, indicating their dominance in the nitrifying community and possible leading role in the nitrification process of the CSTR in this study.

T-RFLP analysis of AOA and AOB

The AOA T-RFLP profiles showed that there was only one major T-RF of 247 bp, corresponding to the dominant AOA-OTU-1 and another AOA-OTU-4 (Table 3), which were also derived from this bioreactor (Zhang et al. 2009). This result indicated that AOA-OTU-1 was the most dominant AOA in activated sludge for all phases, and the AOA community was stable and had relatively low diversity in this CSTR (Fig. 5).

In contrast, the five major T-RFs in AOB T-RFLP profiles varied significantly in the five phases in terms of the percentage of each T-RF, indicating the dynamic shift of the AOB community subject to change of operation conditions. As shown in Table 3, two of five T-RFs (214 and 421 bp) may correspond to AOB-OTU-1 and AOB-OTU-2, respectively, which were recovered from the activated sludge in this CSTR. The other two T-RFs (371 and 486 bp) may correspond to AOB-OTU-3 and AOB-OTU-4, respectively, which were recovered from the seed sludge of the CSTR. For T-RF of 117 bp, there was no OTU related to it. From 20 to 180 days, T-RF of 214 bp, i.e., AOB-OTU-1, was the most dominant AOB, accounting for 32–85% of the total AOB, but showed low abundance (4–25%) in the following 100 days (Fig. 5). Oppositely, AOB-OTU-2 (i.e., T-RF of 421 bp) was the most dominant (48–70%) AOB after 200 days although its relative abundance was lower than 10% in

previous phases. The other three T-RFs (117, 371, and 486 bp) also showed fluctuation in different phases, but not as significantly as AOB-OTU-1 and AOB-OTU-2, and not the dominant AOB in the sludge.
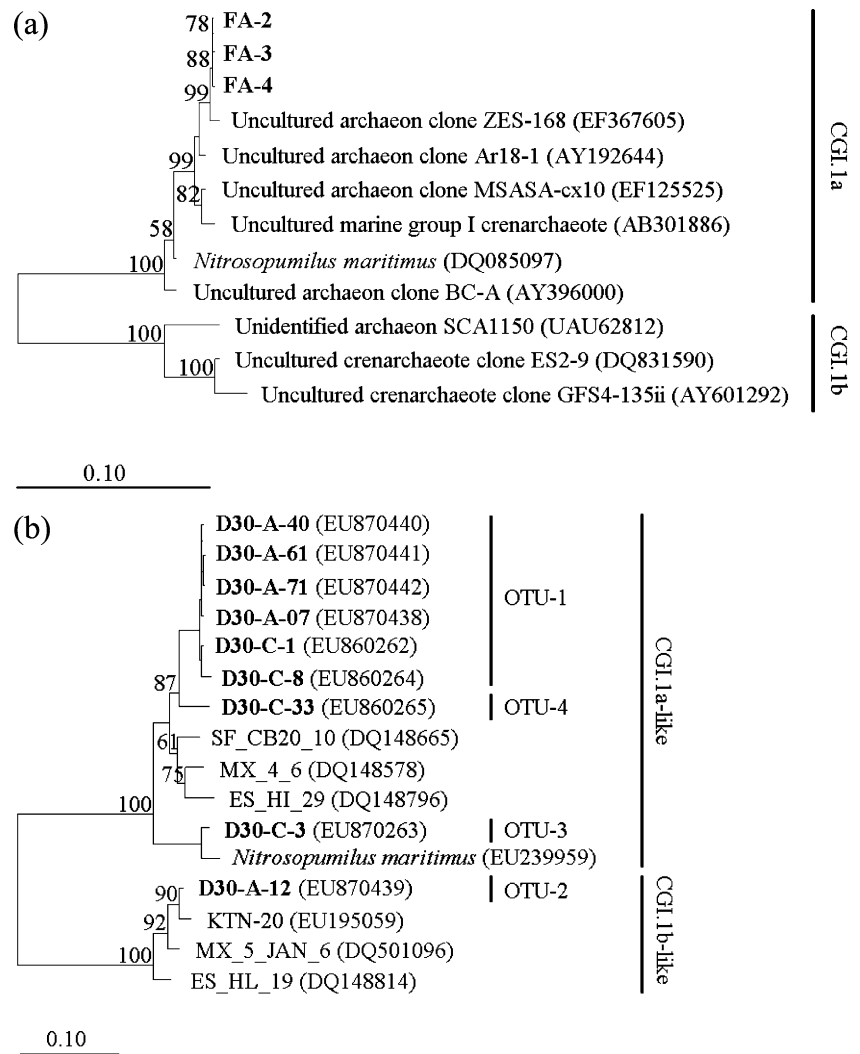
Discussion

Phylogenic analysis of microbial community

The reactor in this study was operated to conduct nitrification of saline (about 1% salinity) wastewater. The bacterial 16S rRNA gene clone library revealed that sequences of many clones, including OTUs FN-8, 11, 13, 15, 23, 58, 61, 66 and 88, were closely related to bacteria from seawater and marine sediment, indicating that salinity was a key factor shaping the bacterial community in this study. Additionally, both 16S rRNA gene clone library and AOB *amo*A gene clone library confirmed the presence of the nitrifying genus *Nitrosomonas*, which had been identified as the major AOB in many nitrifying bioreactors (Egli et al. 2003; Hallin et al. 2005; LaPara and Ghosh 2006; Limpiyakorn et al. 2007; Park and Noguera 2004; Persson et al. 2002; Wells et al. 2009). However, the sludge in this study did not contain another AOB group, the genus *Nitrosospira*, which was rarely found in nitrogen-removing bioreactors (Schramm et al. 1998; Rowan et al. 2003). *Nitrosospira* spp. preferred low temperatures of 4–10°C and grew slowly (Avrahami et al. 2003; Siripong and Rittmann 2007) and may not be able to dominate in the reactor operated at 23°C in this study.

Previous studies have suggested that most of AOA belonged to clusters of CGI.1a and CGI.1b in the phylum Crenarchaeota (Hatzenpichler et al. 2008). The clone library of archaeal 16S rRNA gene in this study recovered only one dominant OTU in CGI.1a, indicating the low

Fig. 3 Neighbor-joining tree based on Jukes–Cantor corrected DNA distances showing the phylogenetic affiliation of sequences from activated sludge of CSTR and reference sequences from other environmental samples or pure stains: **a** archaeal 16S rRNA gene; **b** AOA *amo*A gene. The sequences obtained in this study are printed in *bold*

(a)

```
        78 ┌ FA-2
        88 ├ FA-3
        99 ├ FA-4
           ├ Uncultured archaeon clone ZES-168 (EF367605)
        99 ├ Uncultured archaeon clone Ar18-1 (AY192644)        CGI.1a
        82 ├ Uncultured archaeon clone MSASA-cx10 (EF125525)
           └ Uncultured marine group I crenarchaeote (AB301886)
     58
    100 ┌ Nitrosopumilus maritimus (DQ085097)
        └ Uncultured archaeon clone BC-A (AY396000)
    100 ┌ Unidentified archaeon SCA1150 (UAU62812)
     100┌ Uncultured crenarchaeote clone ES2-9 (DQ831590)       CGI.1b
        └ Uncultured crenarchaeote clone GFS4-135ii (AY601292)
```

0.10

(b)

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              ┌ D30-A-40 (EU870440)
              ├ D30-A-61 (EU870441)
              ├ D30-A-71 (EU870442)              OTU-1
              ├ D30-A-07 (EU870438)
              ├ D30-C-1 (EU860262)
         87   └ D30-C-8 (EU860264)
              ┌ D30-C-33 (EU860265)              OTU-4       CGI.1a-like
         61 ┌ SF_CB20_10 (DQ148665)
         75 ├ MX_4_6 (DQ148578)
            └ ES_HI_29 (DQ148796)
        100 ┌ D30-C-3 (EU870263)                 OTU-3
            └ Nitrosopumilus maritimus (EU239959)
         90 ┌ D30-A-12 (EU870439)                OTU-2
         92 └ KTN-20 (EU195059)                               CGI.1b-like
        100 ┌ MX_5_JAN_6 (DQ501096)
            └ ES_HL_19 (DQ148814)
```

0.10

archaeal diversity. Although four OTUs were recovered from clone libraries of the AOA *amo*A gene, the most dominant AOA-OTU-1 also belonged to the CGI.1a cluster, showing that the dominant OTU of 16S rRNA gene and *amo*A gene might represent the same archaeal species. T-RFLP of AOA *amo*A also showed only one major peak, confirming the results of the two clone libraries. Possibly due to the low abundances, the counterparts of the other three OTUs of AOA *amo*A were not detected in the T-RFLP profile and the clone library of 16S rRNA gene.

AOA and AOB abundances

It was reported that AOA abundance was remarkably 1–3 orders of magnitude higher than AOB in soil and marine environments (Leininger et al. 2006; Wuchter et al. 2006). Oppositely, AOB was dominant than AOA in estuarine sediment (Mosier and Francis 2008; Santoro et al. 2008). However, little is known about AOA in biological nitrogen removal reactors.

So far, only limited AOA-containing activated sludge were reported for a few WWTPs operated with aerated-anoxic processes in which extremely low DO concentrations were maintained, enabling simultaneous nitrification and denitrification (Park et al. 2006; Zhang et al. 2009). Additionally, AOA-positive samples were collected from WWTPs operating with sludge retention times longer than 15 days and HRT longer than 24 h. It seems that these features (low DO levels and long retention times) might facilitate the growth of AOA (Park et al. 2006).

This study determined the AOA/AOB abundance and ratio of AOA/AOB in activated sludge. The range of AOA *amo*A gene copy number was from 10^2 to 10^3 copies per nanogram DNA at the high end of the levels for marine sediments which was 10^{-1} to 10^3 copies per nanogram DNA (Mosier and Francis 2008). The fluctuation of AOA abundance in five phases was <10-fold. However, the AOB *amo*A copy number fluctuated from 10^3 to 10^5 copies per nanogram DNA, much larger than that of AOA. Overall, the abundance of AOA was relative stable, indicating that
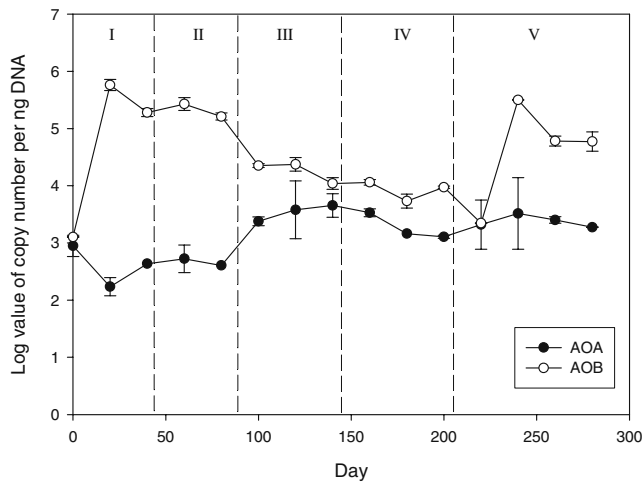
Fig. 4 AOB (*open circles*) and AOA (*solid circles*) *amo*A gene abundance over five phases, as measured by qPCR. Standard deviations (*n*=2) are indicated by *error bars*. The five phases were separated by *vertical dashed lines*

the change of reactor conditions did not significantly affect AOA, in good agreement with the conclusion by Wells et al. (2009). In contrast, the great change of AOB indicated that AOB might be more sensitive to bioreactor conditions than AOA. It was suggested that AOA are adapted to a broad range of growth conditions and, therefore, have a more versatile metabolism than AOB (Leininger et al. 2006).

The results of phases I to II indicated that lower DO possibly decreased the AOB abundance, while the results of phases IV to V showed that higher ammonia concentration and feeding rate were beneficial for AOB. Similarly, a previous study also suggested that AOB abundance was higher at an ammonia load of 250 mg N–NH$_4$ day^{-1} than that of 130 mg N–NH$_4$ day^{-1} (Cydzik-Kwiatkowska and Wojnowska-Baryła 2008).

The average abundance of AOB in five phases was much higher (averagely 24 times) than AOA, indicating that AOB played more important roles than AOA in this study.
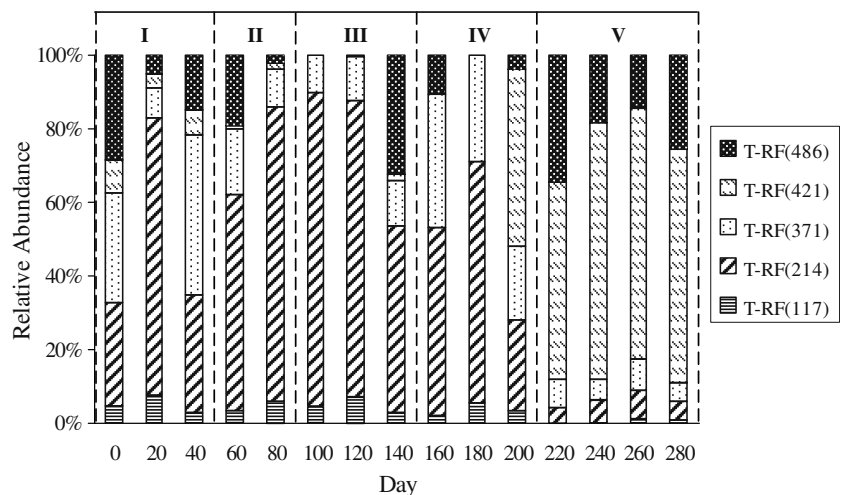
However, in phases III and IV, AOA and AOB were almost equally important, judging from their *amo*A abundance. Compared to the initial activated sludge (day 0), AOB was enriched more significantly than AOA, suggesting that the overall conditions in this study were more favorable for AOB than AOA.

AOA and AOB community shift

Generally, the AOA community was relatively less diversified and stable than AOB in this reactor. The T-RFLP profile showed that only one AOA *amo*A OTU remained dominant in activated sludge during the five phases. Another OTU, AOA-OTU-3, only appeared in the clone library with relatively low abundance (Zhang et al. 2009).

In contrast, the AOB community showed obvious shift through the five phases, indicating that AOB were more sensitive to bioreactor conditions, i.e., ammonium concentration and loading rate, than AOA. Previous studies suggested that bioreactors with low or high ammonia loadings enriched different species of *Nitrosomonas* (Limpiyakorn et al. 2007). The same phenomenon was observed in this study although the specific species identities of these two *Nitrosomonas* spp., corresponding to T-RFs of 214 and 421 bp, respectively, were still unknown. The AOB-OTU-1 (T-RF of 214 bp) might prefer the lower ammonium loading, while the higher ammonium loading was favorable to AOB-OTU-2 (T-RF of 421 bp).

The DO level may have also significantly affected the AOB community (Wen et al. 2008). However, in this study, when DO was decreased in the first two phases, the AOB community did not show obvious shift although the total abundance of AOB decreased. Wells et al. (2009) reported that the *Nitrosospira* lineage showed strong negative correlations to DO while the *Nitrosomonas*-like phylotype showed no significant correlation to DO. In this study, the dominant AOB belonged to genus *Nitrosomonas*. Thus, the

Fig. 5 Temporal dynamics of five AOB OTUs detected from the bioreactor in the five phases (total 280 days). Relative abundance (in percent) of each AOB OTU was determined by the corresponding normalized T-RFLP peak area

results were consistent with their finding, indicating that various AOB might have different responses to DO change.

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