RESEARCH ARTICLE

Abundance of ammonia-oxidizing bacteria and archaea in industrial and domestic wastewater treatment systems

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Received 5 October 2011; revised 24 December 2011; accepted 28 December 2011. Final version published online 3 February 2012.

DOI: 10.1111/j.1574-6941.2012.01296.x

Editor: Gary King

Keywords
ammonia-oxidizing archaea; ammonia-oxidizing bacteria; wastewater treatment; nitrification.

Abstract

Nitrification plays a significant role in the global nitrogen cycle. Ammonia oxidation, the first step of nitrification, is performed in wastewater treatment by both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Most previous studies focused on their distribution in natural environments. In this study we qualified and quantified AOB, AOA, total bacteria, and total archaea in six different wastewater treatment systems (WTSs) using clone library and real-time PCR techniques. The results revealed that wastewater quality was an essential factor for the distribution of AOB and AOA in aerobic reactors. Although both AOB and AOA were present in all samples and contributed to nitrification simultaneously, AOB were the dominant nitrifiers in the three industrial WTSs, whereas AOA were dominant in the three domestic WTSs. This indicates AOA may be more sensitive to some toxic compounds than AOB. In addition, the dominant groups of AOB in the industrial WTSs were *Nitrosomonas* and *Nitrosospira*; the composition of AOA in the domestic WTSs was very similar, possibly due to the same source of raw sewage.

Introduction

Ammonium is a prevalent pollutant in both natural and artificial environments. Chemolithotrophic nitrification plays a significant role in the transformation of ammonium, and nitrogen loss is typically due to the processes of nitrification followed by denitrification (Kowalchuk & Stephen, 2001). A failure of NH_3-N transformation and N removal can occur easily in the activated sludge process for wastewater treatment because the microbial communities capable of nitrification grow slowly and are sensitive to external factors (e.g. temperature, pH, dissolved oxygen, and chemical toxins; Whang *et al.*, 2009). Therefore, in recent years the study of the ecology and physiology of microbial community for nitrification has become a hotspot subject with the wide application of culture-independent techniques.

Ammonia oxidation, the first and rate-limited step in nitrification, was considered to be executed only by the chemolithoautotrophic ammonia-oxidizing bacteria (AOB) until the recent discovery of ammonia-oxidizing archaea (AOA; Francis *et al.*, 2005; Könneke *et al.*, 2005; Zhang *et al.*, 2010). Recent studies demonstrated the presence of AOA in oceans (Francis *et al.*, 2005), estuaries (Dang *et al.*, 2008), gulfs (Beman *et al.*, 2008, 2010), lakes (Schubert *et al.*, 2006) and bioreactors in wastewater treatment plants (Park *et al.*, 2006). The abundance of AOA was even higher than that of AOB in natural environments (Francis *et al.*, 2005; Herrmann *et al.*, 2008). Although AOA is important in ammonia oxidation, the relative contributions to nitrification by AOA and AOB in different environments are still unknown (Park *et al.*, 2006; Wells *et al.*, 2009). In addition, most studies about AOA have been conducted in marine, soil or other natural environments. AOA in wastewater treatment systems (WTSs) have rarely been investigated and should be studied extensively to quantify the abundance of AOA and determine the factors

determining the ratio of AOA to AOB in artificial environments.

In this study, we investigated the diversity and composition of bacteria, archaea, and the ammonia-oxidizing prokaryotes in six typical WTSs. By analyzing the data and information from clone libraries and qPCR, we aimed to detect any connections between the microbial communities for nitrification and the specific wastewater environments.

Materials and methods

Wastewater treatment systems

Six typical WTSs were adopted for the investigation of ammonia-oxidizing prokaryotes and their diversity. The WTSs treated industrial (A–C) and domestic (D–F) wastewater, in which nitrification was active in the aerobic biological units. The details of the WTSs and the influent wastewater characteristics are listed in Table 1.

DNA extraction

Samples of activated sludge or biofilm were collected and centrifuged from each aerated bioreactor in the WTSs. The total DNA extraction was conducted in duplicate for each sample (about 0.5 g) by a Power soil DNA isolation kit (Mo Bio, Carlsbad, CA) according to the manual's recommendation. The duplicate total DNA of the same sample was merged as one total DNA sample of the corresponding WTS. All total DNA samples were stored at −20 °C in a freezer for further application.

Clone library and phylogenetic analysis

PCR amplification of 16S rRNA gene or *amoA* gene of bacteria and archaea from the total DNA sample was performed with the primer pairs used frequently in previous studies. The primer sequences and thermal programs for the PCR are listed in Table 2. Four PCR products were obtained for constructing clone libraries of the four genes. Each PCR product was separated by agarose (0.8%) gel electrophoresis, and the target DNA fragment was cut with a blade and purified with a QIAquick® gel extraction kit (Qiagen, Hilden, Germany). Four purified DNA fragments were cloned into pGEM-T Easy vectors (Promega, Madison, WI) separately according to the manufacturer's instructions. The recombinant plasmids were transformed into competent *Escherichia coli* TOP 10 (TianGen, China). Positive clones were randomly selected and sequenced with the vector-specific primers M13F and M13R (*amoA* gene), 8F (bacterial 16S rRNA gene), and 21F (archaeal 16S rRNA gene) by an ABI 3730*xl* DNA Analyzer (Applied Biosystems, Carlsbad, CA). Chimeric 16S rRNA gene sequences were detected using the BELLEROPHON (Huber *et al.*, 2004) and MALLARD (Ashelford *et al.*, 2006) programs, and were excluded from the subsequent analysis. Rarefaction and diversity statistics, including library coverage (Good, 1953) and Shannon index, were calculated using the MOTHUR software (Schloss *et al.*, 2009). Operational taxonomic units (OTUs) were defined as groups in which the sequence similarities were greater than 98% (for *amoA*) or 97% (for 16S rRNA gene). Representative OTUs sequences were blasted against the National

Table 1. The WTSs and the influent wastewater characteristics*

Code	Treatment process†	Wastewater treated‡	pH	COD (mg L^{-1})	TN (mg L^{-1})	NH_3-N (mg L^{-1})	NO_3^--N (mg L^{-1})	NO_2^--N (mg L^{-1})
A	Full scale A/O	100% Coking	8.52	2730	399.8	262.0	135.4	1.12
B	Lab scale A^2/O MBR	100% Coking	7.22	524	179.3	67.4	13.7	0.50
C	Full scale SBR	80% Dyeing + 20% Domestic	7.35	750	45.3	35.2	3.58	0.89
D	Lab scale A/O MBR	100% Domestic	7.23	580	79.3	76.6	0.97	–§
E	Lab scale BAF	100% Domestic	7.30	104	11.9	9.80	–§	–§
F	Full scale MBR	100% Domestic	7.31	115	18.7	16.3	0.77	–§

*The wastewater was the influent of aerobic unit of each WTS. The data represent the mean values during the operation period in the recent 3 months.

†MBR, membrane biological reactor; A/O, anoxic-oxic process; A/O MBR, anoxic-oxic process with the oxic tank being an MBR; A^2/O MBR, anaerobic-anoxic-oxic process with the oxic tank being an MBR.

‡The sources of wastewater are as follows: A and B, coking wastewater of the Capital Iron and Steel Corporation, Beijing; C, 80% of dyeing wastewater and 20% of domestic wastewater in Chongfu, Zhejiang Province; D, E, and F, domestic wastewater from the Zijin Students Community in Tsinghua University, Beijing.

§Below the detectable level.

Table 2. The PCR primer pairs and thermal programs

Target prokaryote	Target gene	Sequence (5′-3′) of primer pairs	Thermal program	Reference
Bacteria (Clone library)	16S rRNA gene	8F: AGAGTTTGATCCTGGCTCAG 806R: GGACTACCAGGGTATCTAAT	5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C, followed by 8 min at 72 °C	Bik *et al.* (2006)
Bacteria (qPCR)	16S rRNA gene	341F: CCTACGGGAGGCAGCAG 518R: ATTACCGCGGCTGCTGG	5 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)
Archaea (Clone library)	16S rRNA gene	21F: TTCCGGTTGATCCYGCCGGA 958R: YCCGGCGTTGAMTCCAATT	5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, followed by 10 min at 72 °C	Bano *et al.* (2004)
Archaea (qPCR)	16S rRNA gene	349F: GYGCASCAGKCGMGAAW 806R: GGACTACVSGGGTATCTAAT	5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 40 s at 72 °C	Swan *et al.* (2010)
AOA (Clone library)	Arch-*amoA*	*amoA*F: STAATGGTCTGGCTTAGACG *amoA*R: GCGGCCATCCATCTGTATGT	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 15 min at 72 °C	Francis *et al.* (2005)
AOA (qPCR)	Arch-*amoA*		3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 53 °C, and 1 min at 72 °C	Chen *et al.* (2008)
AOB (Clone library)	*amoA*	*amoA*F: GGGGTTTCTACTGGTGGT *amoA*R: CCCCTCKGSAAAGCCTTCTTC	5 min at 94 °C, 35 cycles of 60 s at 94 °C, 90 s at 60 °C, and 90 s at 72 °C, and a final cycle consisting of 90 s at 60 °C and 10 min at 72 °C	Rotthauwe *et al.* (1997)
AOB (qPCR)	*amoA*		3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C	Chen *et al.* (2008)

Center for Biotechnology Information (NCBI) database to obtain the closest published sequences. The selected sequences and the detected OTUs sequences were assembled and the phylogenetic trees were constructed using the neighbor joining method with the MEGA 4.0 software (Tamura *et al.*, 2007).

Real-time PCR

Bacterial and archaeal 16S rRNA and *amoA* genes were quantified by an ABI Prism 7900HT Thermocycler based on SYBR Green I method. The primer pairs and thermal programs of the PCR amplification are also listed in Table 2. The 20-μL reaction mixture consisted of 10 μL of SYBRs Premix Ex TaqTM (Takara, Dalian, China), 0.5 μL of primers, and 1.0 μL of template DNA. The specificity of the PCR for each target gene was checked using melting curve analysis and gel electrophoresis.

Standard curves for real-time PCR assays were performed as follows: the bacterial and archaeal 16S rRNA and *amoA* genes were amplified from the extracted total DNA with the same primers to the qPCR, respectively. Each PCR product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy Vector (Promega). The resulting ligation mix was transformed into *E. coli* JM109 competent cells (Promega). Plasmids used as standards for quantitative analysis of the four genes were extracted from the positive clones and sent for sequencing. The plasmid DNA concentration was determined by an Eppendorf BioPhotometer Plus (Hamburg, Germany). The copy number of each target gene was calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to a real-time PCR assay in triplicate to generate an external standard curve and to check the amplification efficiency. The qPCR assay efficiencies ranged from 102% to 107%, and the correlation coefficients (R^2) were all > 0.98. The parameter of threshold cycle (C_t) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

Table 3. The PCR amplification for constructing clone libraries of the aerobic biological units in different WTSs

WTS code	16S rRNA gene	Arch-16S rRNA gene	*amoA*	Arch-*amoA*
A	+	–	+	–
B	+	–	+	–
C	+	–	+	–
D	+	+	–	+
E	+	–	–	+
F	+	+	–	+

+, PCR amplification; –, no PCR amplification.

Results and discussion

PCR amplification of 16S rRNA gene and *amoA* for clone libraries

The results of PCR amplification are shown in Table 3. Due to the low concentrations of some target genes in the total DNA samples, the PCR program was improved to 42 cycles when there was no obvious PCR product band in the gel. Nevertheless, some target genes still had no amplification.

Bacterial and archaeal diversities

Six clone libraries were constructed to investigate the total bacterial composition in the different WTSs. After chimeras check, 55.3–77.6% of the total sequences in the six clone libraries were kept for further analysis. Estimations of species coverage and diversity were calculated for 16S rRNA gene clone libraries (Table 4). Good's coverage ranged from 23.1% to 90.0%. The Shannon indices showed that the diversities of bacterial 16S rRNA gene sequences in the oxic tanks for coking wastewater treatment (A and B) were lower than those in the other WTSs. Figure 1a shows the taxonomic classification of the six clone libraries and the relative abundance of each group. *Proteobacteria* was a dominant phylum in each clone library (32–100%) and the proportions in the coking WTSs were much higher (100% for A and 97% for B). Among the different classes of *Proteobacteria*, the *Betaproteobacteria* was presented in all libraries and its proportion was always higher than the other classes of *Proteobacteria* except F library. *Bacteroidetes* was another dominant phylum in the clone libraries of C–F (25–45%), but was subordinate in A (0%) and B (3%) libraries. These two phyla are frequently retrieved from the activated sludge or biofilm in sewage treatment systems (Wagner & Loy, 2002).

Because concentrations of the amplified archaeal 16S rRNA gene were lower, only two archaeal clone libraries (D and F) were obtained (Table 4). Good's coverage was 98.9% for the F and 97.7% for the D library, indicating that the clone libraries represented the majority of the archaeal community in both membrane bioreactors (MBRs). The Shannon indices showed that the diversities of archaeal 16S rRNA gene sequences were much lower than the corresponding bacterial diversities of both MBRs. *Crenarchaeota* was dominant in both clone libraries and Euryarchaeota was another dominant phylum only in D library (Fig. 1b). In addition, a large number of OTUs in both clone libraries had never been

Table 4. The sequence diversity and library coverage estimations of the clone libraries

WTS code (target gene)	No. of sequences	No. of clear sequences	No. of OTUs*	Library coverage (%)	Shannon index
A (16S rRNA gene)	88	50	9	90.0	1.52
B (16S rRNA gene)	88	61	16	77.0	1.28
C (16S rRNA gene)	87	55	39	47.3	3.52
D (16S rRNA gene)	85	47	38	31.9	3.55
E (16S rRNA gene)	85	66	40	54.5	3.42
F (16S rRNA gene)	88	52	39	23.1	3.52
D (Arch-16S rRNA gene)	87	86	7	97.7	1.16
F (Arch-16S rRNA gene)	88	88	2	98.9	0.06
A (*amoA*)	46	46	5	95.7	0.98
B (*amoA*)	46	46	7	91.3	1.22
C (*amoA*)	56	56	1	100	0
D (Arch-*amoA*)	77	77	6	97.4	1.32
E (Arch-*amoA*)	77	77	7	94.8	0.67
F (Arch-*amoA*)	78	78	8	97.4	1.43

*OTUs were defined as groups in which the sequence similarities were > 97% (for 16S rRNA gene) and 98% (for *amoA*).

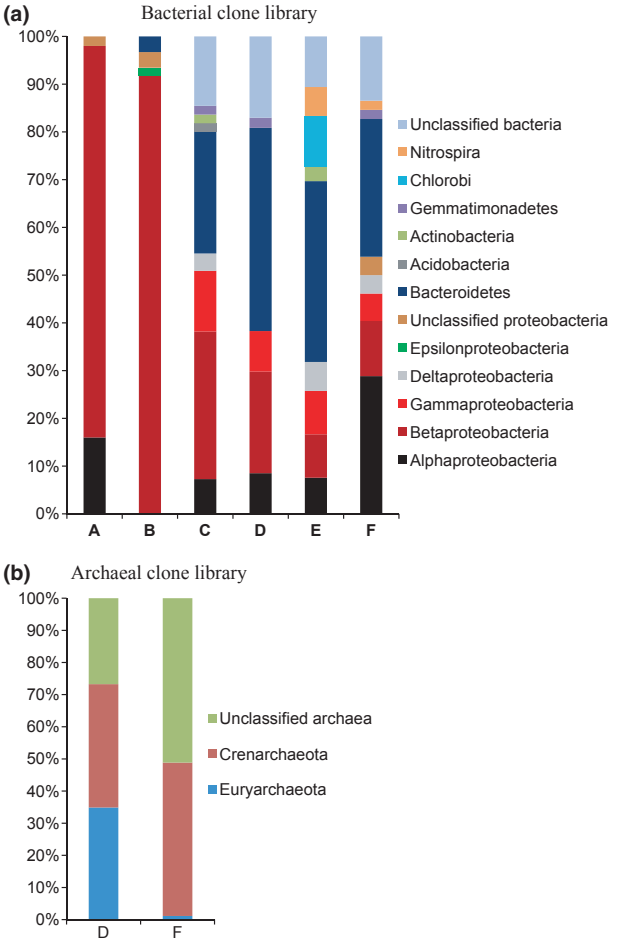

Fig. 1. Taxonomic classification of the 16S rRNA gene clone libraries using online Ribosome Database Project classifier. (a) Bacterial clone library. (b) Archaeal clone library.

described previously. Therefore, the treatment of domestic wastewater in MBRs could be regarded as a special artificial environment, in which the archaeal community remains largely unexplored (Yu *et al.*, 2011).

The qPCR results (Table 5) showed that the abundance of bacterial 16S rRNA gene in the different WTSs was similar, ranging from 7.8×10^{10} to 1.3×10^{11} gene copies per gram of dry weight of activated sludge or biofilm, but the abundance of archaeal 16S rRNA gene differed, varying from 5.4×10^{5} to 3.0×10^{8} gene copies per gram of dry weight. The copy numbers of the bacterial 16S rRNA genes were much higher than those of the archaeal 16S rRNA genes, with ratios ranging from 3.4×10^{2} to 2.2×10^{5}. This indicates that bacteria dominated quantitatively over archaea and played the most significant role in the aerobic biological treatment of both domestic and industrial wastewaters.

AOA and AOB diversities

Six clone libraries, including three AOB and three AOA libraries, were constructed to explore the diversities of AOB and archaea according to the PCR amplification of *amoA* gene from the total DNA samples. Although the primer pairs used in this study were designed based on currently available target sequences, the complete bacterial information could not be obtained due to the specificity of PCR primers. As shown in Table 4, Good's coverage ranged from 91.3% to 100% for *amoA* gene, showing that the clone libraries covered the greatest diversity of the *amoA* gene community. For the 100% coverage community in the sequencing batch reactor (SBR) of the dyeing WTS (C library), only a single type of *amoA* gene was amplified, and the diversity index was 0.

Figure 2a shows the phylogenetic tree based on the *amoA* gene for AOB in the three industrial WTSs. All 11 *amoA* gene sequences originated were deposited in the NCBI database under the accession numbers JN813545 to JN813555. The single genus of AOB in C library belonged to *Nitrosomonas* [99% identity with *Nitrosomonas nitrosa* (AF272404)]. All AOBs in A library also belonged to the genus of *Nitrosomonas*. *Nitrosospira* was another important genus with a great proportion in B library. Figure 2b

Table 5. Quantitative analysis* for 16S rRNA and *amoA* genes

	Target gene copy number/dry weight of activated sludge or biofilm (g^{-1})				Ratio			
WTS code	Bacterial 16S rRNA gene	Archaeal 16S rRNA gene	*amoA*	Arch-*amoA*	Bacterial 16S rRNA gene/Archaeal 16S rRNA gene	*amoA*/Bacterial 16S rRNA gene	Arch-*amoA*/Archaeal 16S rRNA gene	*amoA*/Arch-*amoA*
A	1.2×10^{11}	5.4×10^{5}	2.6×10^{7}	9.6×10^{3}	2.2×10^{5}	2.2×10^{-4}	1.8×10^{-2}	2.7×10^{3}
B	9.0×10^{10}	2.8×10^{7}	5.4×10^{8}	9.9×10^{3}	3.2×10^{3}	6.0×10^{-3}	3.5×10^{-4}	5.5×10^{4}
C	1.3×10^{11}	6.6×10^{6}	3.6×10^{9}	5.7×10^{3}	2.0×10^{4}	2.8×10^{-2}	8.6×10^{-4}	6.3×10^{5}
D	1.3×10^{11}	3.0×10^{8}	1.7×10^{5}	4.5×10^{6}	4.3×10^{2}	1.3×10^{-6}	1.5×10^{-2}	3.8×10^{-2}
E	1.1×10^{11}	1.8×10^{7}	3.6×10^{4}	6.0×10^{5}	6.1×10^{3}	3.3×10^{-7}	3.3×10^{-2}	6.0×10^{-2}
F	7.8×10^{10}	2.3×10^{8}	7.2×10^{3}	6.3×10^{5}	3.4×10^{2}	9.2×10^{-8}	2.7×10^{-3}	1.1×10^{-2}

*Data represent the mean of three independent measurements.

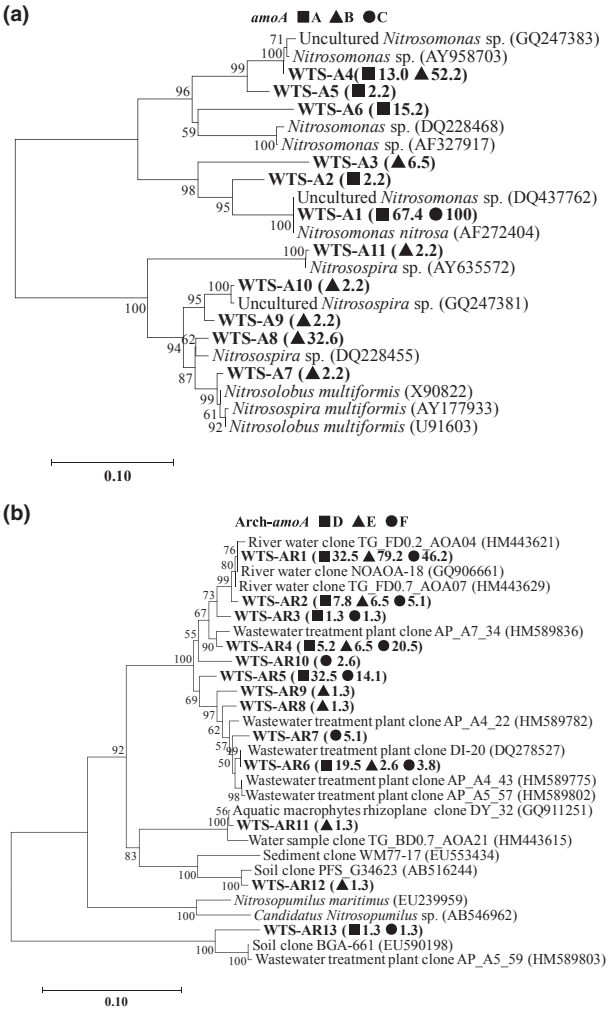

Fig. 2. Phylogenetic trees constructed for partial *amoA* OTU sequences retrieved from the total DNA samples from different WTSs. The numbers (only those > 50% are shown) on the branch nodes indicate the percentages of bootstrap support for the clades based on 1000 bootstrap resamplings. Numbers in the brackets are the GenBank accession numbers of the strains in the NCBI. The relative abundances of OTUs in the libraries are shown in parentheses. (a) *amoA*: ■, A; ▲, B; ●, C. (b) Arch-*amoA*: ■, D; ▲, E; ●, F .

describes the AOA distribution in the three domestic WTSs. All 13 Arch-*amoA* gene sequences were deposited in the NCBI database under the accession numbers JN813556 to JN813568. Phylogenetic analysis revealed that all of the archaeal *amoA* sequences retrieved from activated sludge (MBR of D and F WTSs) or biofilm [biological aerated filter (BAF) of E WTS) were closely related to the sequences previously obtained from river water, sediment, and wastewater treatment plants. Four OTUs (WTS-AR1, WTS-AR2, WTS-AR4, and WTS-AR6) were present in all three samples and their relative abundance ranged from 65.0% to 94.8%. The clone of WTS-AR1 made up the greatest proportion (32.5–79.2%) in each archaeal *amoA* sample. These results indicate that the composition and distribution of AOA were very similar in the three aerobic biological units in different WTSs, where the influents originated from a same source.

From the qPCR results (Table 5), the *amoA* gene abundance, reflecting the AOB community size, was in the range of 7.2×10^3 to 3.6×10^9 gene copies per gram of dry weight of activated sludge or biofilm. The numbers of *amoA* gene copies in the three industrial activated sludges were higher than those in the three domestic activated sludges or biofilms. The numbers of Arch-*amoA* gene copies corresponding to the AOA community were in the range of 5.7×10^3 to 4.5×10^6 copies per gram of dry weight, with higher numbers in the domestic activated sludge and biofilm. The ratios of *amoA*/bacterial 16S rRNA gene varied from 9.2×10^{-8} to 2.8×10^{-2}, with higher ratios in the three industrial samples. However, the ratios of Arch-*amoA*/Archaeal 16S rRNA gene reveal less variation, ranging from 3.5×10^{-4} to 3.3×10^{-2}. The ratios of *amoA*/Arch-*amoA* indicated that AOB might play a crucial role in the ammonia oxidation in the industrial WTSs, and AOA might dominate the ammonia oxidation in the domestic WTSs.

Relationship between the wastewater characteristics and ammonia oxidation

Qualification and quantification of bacteria and archaea capable of nitrification are important for an understanding of nitrifying activity and NH_3-N removal in a WTS (Baek *et al.*, 2010). The ratios of bacteria to archaea and AOB to AOA among the samples may reflect not only the different sources of wastewater but also the differences in nitrification process. In some extracted total DNA samples, we were unable to detect archaea, AOA or AOB by cloning-sequencing. This did not prove the absence of them in the samples, because errors may have been introduced from incomplete extraction, PCR or other factors, but it strongly suggests that they were of little significance in the pollutants (including ammonia) removal. This speculation is further confirmed by the qPCR results. Coking wastewater is a typical refractory industrial wastewater containing a great number of persistent and toxic substances (Bai *et al.*, 2011). The COD, TN, NH_3-N, and NO_3^--N of the influent of the A coking WTS were much higher than in the other types of wastewaters (Table 1). Many types of bacteria cannot endure the toxicity of coking wastewater, therefore the bacterial diversity was lower and most bacteria belonged to *Betaproteobacteria*. We found that the most abundant OTU in AOA communities (Fig. 2b) in the three domestic WTSs

was the same. This might be attributed to the similar wastewater characteristics.

PCR amplification and qPCR results demonstrated that AOB dominated the ammonia oxidation in the three industrial WTSs, that is AOB was the main contributor to the process of nitrification. Conversely, AOA dominated the nitrification in the three domestic WTSs. Previous studies proved that the growth rate, cell production, and nitrification activity of AOA were similar to AOB, being even higher than AOB in some cases (Könneke *et al.*, 2005; Ingalls *et al.*, 2006; Wuchter *et al.*, 2006; You *et al.*, 2009). Our investigation of the domestic WTSs supported this conclusion but our investigation of the industrial WTSs showed the opposite results. On the basis of the findings, we presume that wastewater quality was a key factor in the abundance and distribution of AOA, in addition to DO and wastewater temperature (Park *et al.*, 2006). AOA seemed to be more fragile than AOB in toxic industrial wastewaters. But in the moderate domestic wastewater, AOA could grow with higher abundance in the artificial aerobic environment. This phenomenon disagrees with the study from Wells *et al.* (2009), which proposed that AOA might have a minor role in highly aerated activated sludge. AOA and AOB were widely present in the aerobic biological treatment systems, and their proportions were determined by the influent water quality rather than the treatment process.

Conclusions

Our experimental results revealed a significant effect of wastewater quality on the abundance of AOB, AOA, bacteria, and archaea in different WTSs. Bacterial diversity of coking activated sludge was lower due mainly to the many toxic compounds present in the coking wastewater. Compared with archaea, bacteria were dominant in all samples and played the most significant role in the removal of pollutants. AOB were the dominant nitrifiers in the industrial wastewater treatment, a common dominant group of AOB being *Nitrosomonas*. In the domestic wastewater treatment, AOA were dominant, and the dominant groups of AOA were similar, possibly due to the same source of raw sewage. Further study is needed to clarify the relationship between nitrification rate and the distribution of AOB and AOA.

Acknowledgements

This study was supported by two General Projects (No. 51008003 and 51178002) granted by the National Natural Science Foundation of China and a special financial grant (201104016) from the Postdoctoral Science Foundation of China. The authors thank Xiaobiao Zhu, Xiaolan Peng, and Yan Zhang from Tsinghua University as well as Montcho Leon Monthero and Man Ding from Peking University for providing the activated sludge or biofilm samples and the data on wastewater quality.

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