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Changes of abundance and diversity of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in three nitrifying bioreactors with different ammonia concentrations

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ABSTRACT

Different from ammonia-oxidizing bacteria (AOB), the influence of ammonia levels on ammonia-oxidizing archaea (AOA) in wastewater treatment systems was poorly understood. In this study, sludge contained AOA and AOB was enriched in three sequencing batch reactors (named as R1, R2, and R3) under different ammonia levels (14, 56, and 140 mg/L) using polymerase chain reaction (PCR), cloning, sequencing, and quantitative real-time PCR (qPCR) to evaluate the effect of ammonia levels on the diversity, abundance, and contribution of AOA and AOB to ammonia oxidation. Cloning results showed that there was only one dominant AOA species (*Nitrososphaera* cluster) in the reactors during 140-d operation. However, AOB communities varied significantly among the reactors. After 140-d enrichment, *Nitrosomonas ureae* cluster, the dominant AOB cluster in seed sludge, was also dominant in R1 and R2, while *Nitrosomonas europaea* cluster was enriched and dominated in R3. Diversity of AOB was higher than AOA under the three ammonia levels. Diversity of AOB under high ammonia concentration (in R3) was lower than that under low ammonia concentrations (in R1 and R2). QPCR results revealed that AOA abundance was almost unchanged under different ammonia levels during operational period. The ratio of AOB to AOA increased from 3.68×10^2 in seed sludge to 4.90×10^3, 1.25×10^5, and 3.77×10^5 in three reactors after 140-d running. This study suggested that AOB was much more competitive than AOA in high ammonia level environments and probably played a more important role than AOA in ammonia oxidation.

Keywords: Ammonia-oxidizing archaea (AOA); Ammonia-oxidizing bacteria (AOB); *AmoA* gene; Ammonia concentration; Wastewater

1. Introduction

Nitrification is an essential process in biogeochemical nitrogen cycle and biological nitrogen removal from wastewater. Ammonia oxidation to nitrite is the first and rate-limiting step of nitrification. For several decades, it was believed that aerobic ammonia oxidation was solely performed by ammonia-oxidizing bacteria (AOB) affiliated with the *beta-* and *gamma-proteobacteria* [1]. Members of the *beta-proteobacterial* genera

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Nitrosomonas and *Nitrosospira* are considered as the most important AOB in wastewater treatment plants (WWTPs). However, it was discovered that ammonia oxidation was driven by not only the domain *Bacteria* but also the domain *Archaea* [2]. Subsequently, ammonia-oxidizing archaea (AOA) was widely investigated in many natural environments, mainly focusing on its' abundance, diversity, and distribution [3–5]. Until now, two AOA pure cultures [2,6] and about 11 enrichment cultures [7,8] have been demonstrated. Compared with the studies of AOA in natural environments, the investigations of AOA in wastewater treatment systems were few. The presence of AOA in activated sludge was first reported in USA [9]. Later on, the abundance and diversity of AOA in WWTPs were investigated [4,10–14].

Until now, 16 named pure AOB cultures have been demonstrated and the links between environmental factors and AOB eco-physiological properties have been extensively studied [15]. However, different from AOB, the effects of environmental factors on AOA were poorly understood. Environmental factors, such as ammonia concentration, oxygen level, pH, and temperature, will affect the abundance and composition of AOA [8,16]. Among them, ammonia concentration is one of the principal factors affecting the community structure and abundance of AOA and AOB [8]. The "*Candidatus* Nitrosopumilus maritimus" strain SCM1, isolated from the gravel of a marine aquarium water, the first isolation of AOA, has been confirmed to exhibit high affinities to ammonia with low half-saturation constant (K_m = 0.133 μM), suggesting that SCM1 seems particularly well adapted to growth at ammonia levels prevailing in nutrient-limited open oceans [17]. The ammonia concentration value in the open ocean water is less than 0.03–1 μM and is more than 100-fold lower than the minimum concentration required for growth (>1 μM) of cultivated AOB [2,17]. The preference for low ammonia levels also had been reported for "*Ca.* Nitrososphaera gargensis" and "*Ca.* Nitrosotalea devanaterra" [18,19]. However, the soil AOA *Nitrososphaera viennensis* EN76 [6] and *Candidatus* Nitrosoarchaeum koreensis [20] were found to be adapted to higher ammonia concentrations (10–20 mM), and these ammonia concentrations are still much lower than the highest ammonia tolerance of AOB (50–1,000 mM). The K_m of AOB is higher than that of AOA. The results suggested that higher ammonia concentration is in favor of the growth of AOB.

However, there were no pure or enrichment AOA cultures originated from WWTPs. The relationship between ammonia concentration and the AOA and AOB in WWTPs is still unclear. Few studies have been undertaken to investigate this relationship in wastewater treatment. In three continuous flow reactors with different ammonia levels, AOA *amoA* gene abundance remained unchanged under low ammonia level (day 60, 0.06 ± 0.04 mg/L), while the numbers of AOA *amoA* gene reduced (day 60) and at last (day 360) disappeared in the reactors with higher ammonia level (0.25 ± 0.10 mg/L, 0.51 ± 0.33 mg/L) [21]. In a continuous nitrification reactor treating saline wastewater, the abundance of AOB in the reactor was ~40 times higher than that of AOA, and the ratio of AOB to AOA increased significantly up to ~2,000 to ~4,000 with the increase of ammonia loading from 0.26 to 0.52 kg N/(m^3 d) under low dissolved oxygen (DO) levels (0.15–0.5 mg/L) [22]. In a continuous stirred-tank reactor treating saline wastewater running under low DO levels (0.5 mg/L) and increasing ammonia loading (192, 384, and 576 mg N/(m^3 d)), the fluctuation of AOA abundance was <10-fold; however, the AOB *amoA* copy number fluctuated from 10^3 to 10^5 copies per ng DNA [23]. These results suggested that low ammonia-containing environments are in favor of AOA. However, the contrary results were also reported in previous studies. Fukushima et al. found high ammonia level repressed only archaeal mRNA expression [24]. AOA has also been detected in wastewater treatment systems with elevated ammonia levels [25], such as in anammox reactors with ammonia concentration varying from 74.9 to 241.6 mg/L. Overall, the influence of ammonia concentration on AOA remains unresolved and further investigation is still needed.

In this study, the seed sludge taken from a municipal WWTP, which possessed AOA and AOB *amoA* genes, was enriched in three sequencing batch reactors (SBRs) with different ammonia concentrations: 14, 56, and 140 mg/L for 140 d. The abundance of AOA and AOB *amoA* genes in the seed sludge and enriched nitrifying-activated sludge was determined by quantitative real-time polymerase chain reaction (qPCR). And *amoA* gene clone libraries were constructed to evaluate the shifts of AOA and AOB populations. Spearman's rank correlation coefficients (SRCCs) were used to further elucidate the potential impacts of operational parameters on abundance and community structure of AOA and AOB.

2. Materials and methods

2.1. Seed sludge, wastewater, and reactors operation

Seed sludge was taken from YF WWTP, a Carrousel 3000 oxidation ditch process treating municipal wastewater in Beijing, China [12]. Table S1 (in Supplementary data) summarized operational parameters of the WWTP, including flow rate, mixed

liquid suspended solids (MLSS), sludge retention time (SRT), hydraulic retention time (HRT), DO, influent and effluent biochemical oxygen demand (BOD), chemical oxygen demand (COD), NH_4^+-N, and NO_x^--N (NO_2^--N + NO_3^--N). Seed sludge was enriched in three same SBRs, named as R1, R2, and R3. Working volume of the SBR was 5 L, with a volume exchange ratio of 60%. Each reactor received an inorganic synthetic wastewater containing different concentrations of influent ammonia: 14, 56, and 140 mg/L (calculated by N) in R1, R2, and R3, respectively. The concentrations of bicarbonate alkalinity were 280, 1,120, and 2,800 mg/L (calculated by $CaCO_3$), respectively. Each liter of the synthetic wastewater also contained 0.585 g NaCl, 0.075 g KCl, 0.147 g $CaCl_2{\cdot}2H_2O$, 0.049 g $MgSO_4{\cdot}7H_2O$, 0.054 g KH_2PO_4, and 1 mL trace solution. The trace solution per liter was consisted of the following compounds: 2.000 g of $FeCl_2{\cdot}4H_2O$; 4.300 g of disodium EDTA (Titriplex III); 0.100 g of $MnCl_2{\cdot}2H_2O$; 0.024 g of $NiCl_2{\cdot}6H_2O$; 0.024 g of $CoCl_2{\cdot}6H_2O$; 0.017 g of $CuCl_2{\cdot}2H_2O$; 0.068 g of $ZnCl_2$; .024 g of $Na_2MoO_4{\cdot}2H_2O$, 0.033 g $Na_2WO_4{\cdot}2H_2O$; and 0.062 g of H_3BO_3 [26]. The cycle of each SBR was scheduled as follows: (1) feeding phase for 2 min; (2) aerobic reaction phase; (3) settling phase for 30 min; (4) decanting phase for 2 min; and (5) idle phase. The aeration time for each reactor was adjusted according to the ammonia removal time. DO concentration was controlled above 2 mg/L during aerobic phase. pH was not adjusted, ranging between 7.8 and 8.3. Temperature of the reactors was not controlled, fluctuating between 15.1 and 29.5℃ during 140-d operation (from April to August).

2.2. Analytical methods

Sludge volume index (SVI), MLSS, NH_4^+-N, NO_2^--N, NO_3^--N, and alkalinity were analyzed in accordance with standard methods [27]. DO and pH were detected with the multifunctional water-quality tester (WTW Multi340i) equipped with a DO probe (WTW CellOx 325) and a pH probe (WTW SenTix41).

2.3. DNA extraction

Seed sludge and sludge samples periodically taken from the three SBRs were used for molecular analysis. Each fifty milliliters of activated sludge samples was freeze-dried using Labconco Freezone 1 L (Labconco, America) and stored at –20℃ until DNA extraction. Genomic DNA was extracted from 0.05 to 0.10 g of dry sludge sample using a FastDNA SPIN kit for soil (Qiagen, CA, USA), according to the manufacturer's instructions. The concentration and quality of DNA were determined by Nanodrop Spectrophotometer ND–1000 (Thermo Fisher Scientific, USA).

2.4. qPCR

qPCR was carried out in a Stratagene Mx3005P instrument (Agilent Technologies, USA) with a Fast Plus EvaGreen qPCR Master Mix (Biotium, USA). For each sample, quantification was performed in triplicate with a 20-μl mixture contained 10 μl of the qPCR Master Mix, 0.4 μl of each primer (0.2 μM), and 50–100 ng of each sample DNA. Primers named Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) [3] were used to amplify AOA *amoA* gene, which for AOB *amoA* gene was primers amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) [28]. Thermal cycling parameters for AOA *amoA* gene amplification were as follows: 96℃ for 2 min, followed by 40 cycles of 96℃ for 5 s, 53℃ for 1 min, and 72℃ for 1 min. AOB *amoA* gene amplification used the thermal cycling steps as follows: 96℃ for 2 min, followed by 40 cycles of 96℃ for 5 s, 57℃ for 1 min, and 72℃ for 1 min. The standard curves of AOA and AOB *amoA* genes were produced by ten-fold serial dilutions of plasmid DNA. For AOA, the plasmid DNA with an accession number of JQ277530 [12] was selected to be as the standard DNA, the copy numbers of which ranged from 7.21×10^1 to 7.21×10^8. Correlation coefficients (R^2) of AOA standard curve were 0.998, and the efficiency was 107.4%. For AOB, the copy numbers of standard DNA (with an accession number of JQ277552 [12]) were in the range of 5.46×10^1 to 5.46×10^8. R^2 for standard curve of AOB was 0.999, and the efficiency was 112.6%.

2.5. PCR, cloning, sequencing, and phylogenetic analyses

The primer sets used to amplify the AOA and AOB *amoA* genes were Arch-amoAF and Arch-amoAR, and amoA-1F and amoA-2R, respectively. For AOB *amoA* gene, the PCR amplification was performed in a 50-μl mixture contained 25 μl of GoTaq® Green Master Mix (Promega, Madison, USA), 1 μM of each primer, and 50–100 ng of each sample DNA. PCR conditions for AOB *amoA* gene were as follows: 94℃ for 30s, followed by 30 cycles at of 94℃ for 15 s, 55℃ for 20 s, 72℃ for 40 s, and a final extension step at 72℃ for 1 min [1]. For the amplification of AOA *amoA* gene, the PCR mixture was the same as the AOB *amoA* gene. PCR amplification was very difficult due to the low abundance of AOA *amoA* gene. Therefore, multi-

ple experiments were attempted to amplify the AOA *amoA* gene: (I) using different PCR mix (including TaKaRa Ex Taq®, GoTaq® Green Master Mix, and GoTaq® Hot Start Colorless Master Mix); (II) using different ratio of primer to sample DNA; (III) using gradient PCR (annealing temperature varying between 50 and 60 ℃). The PCR conditions for AOA *amoA* gene were 95℃ for 5 min, followed by 30 cycles of 45 s at 94℃, 1 min at annealing temperature, 1 min at 72℃, with a final extension at 72℃ for 15 min [3].

The PCR products were then checked by electrophoresis using 1.5% agarose gel together with a DL1000 DNA marker (TaKaRa, Dalian, China). The target bands were excised and purified using Takara Agarose Gel DNA Purification Kit Ver. 2.0 (Takara, Dalian, China). The purified PCR products were ligated to pGEM®–T Easy vector (Promega, Madison, USA). Then, the recombinant plasmid was transformed into *E. coli* Competent Cells JM109 (TaKaRa, Dalian, China) following the instruction of the producer by heat-shock methods. White colonies were randomly picked to conduct whole cell PCR amplification with SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) primer set to screen positive clones. The positive clones were then sequenced by ABI 3730XL capillary sequencers (Applied Biosystems, Foster City, CA).

The similarity of the sequences was analyzed using Mothur 1.28 [29], and sequences were grouped as the same operational taxonomic unit (OTU) with 97% sequence similarity. Good's coverage, Chao1 richness estimator, and Shannon indices were then calculated based on the OTUs. The representative sequences retrieved in this study and the reference sequences obtained from the GenBank were aligned by MEGA 5.0 [30] to construct phylogenetic trees with the neighbor-joining (NJ) method (based on Jukes–Cantor corrected DNA distances). The phylogenetic trees were tested by bootstrap analysis with 1,000 replication.

2.6. Nucleotide sequence accession numbers

The gene sequences obtained in this study have been deposited in GenBank database under the accession numbers JX879926–JX879934 (AOA *amoA* gene) and JX879792–JX879925 (AOB *amoA* gene).

2.7. Statistical analysis

A nonparametric procedure, Spearman's rank correlation coefficient, was calculated to estimate the correlations between operational parameters and abundance and diversity of AOA and AOB. The statistical analysis was done using the SPSS Statistics software version 17.

3. Results and discussion

3.1. Reactors performance

In this study, together with the monitoring of NH_4^+-N, NO_2^--N, and NO_3^--N, the profiles of pH and DO during aerobic phase were recorded to determine the aeration time [31]. Because influent ammonia level of the three reactors was different, aeration time was set different. With the enrichment of nitrifying prokaryotes, aeration time decreased gradually. Operational parameters of each reactor such as aeration time, cycle numbers, average MLSS, and temperature could be found in Table 1. For each reactor, there were four phases, phases I, II, III, and IV (shown in Table 1).

In phase I (the startup phase), each reactor ran in an adaptive period. Tracking studies of the first cycle of each reactor are shown in Fig. S1 (See Supplementary data). Of the first cycle, the time for ammonia oxidation in R1, R2, and R3 was 4, 22, and 96 h, respectively. And nitrification rate was 0.92, 0.74, and 0.40 mg (NH_4^+-N)/[g (MLSS) h]. The influent ammonia concentration in R1 was lower than that of YF WWTP, so nitrifying prokaryotes in R1 acclimated quickly to the synthetic wastewater. The influent ammonia concentration of R2 and R3 was 2.24 and 5.60 times the influent of YF WWTP, the nitrifying prokaryotes acclimated slowly and nitrification rate in R2 and R3 was lower than that in R1. Another possible reason for the low nitrification rate may be the low temperature during phase I (15.1−17.9℃, shown in Table 1).

With adaption to the synthetic wastewater and the enrichment of nitrifying prokaryotes, in phase II, nitrification rate of the three reactors increased to 1.33, 3.31, and 1.93 mg (NH_4^+-N)/[g (MLSS) h], respectively. For R2 and R3 was 4 and 10 times the influent ammonia concentration of R1, nitrification rate in R2 and R3 increased quickly and was higher than R1. In phase III, the nitrification rate further increased to 1.66, 5.78, and 7.45 mg (NH_4^+-N)/[g (MLSS) h] in three reactors, respectively. At the end of phase IV (day 140), nitrification rate in the three reactors got to maximum value, 4.84, 8.03, and 10.35 mg (NH_4^+-N)/[g (MLSS) h], respectively. Tracking studies of the last cycle of each reactor are shown in Fig. S2 (See Supplementary data). After the nitrifying prokaryotes in R3 acclimated to the high ammonia concentration, nitrification rate in R3 was higher than R1 and R2 in phases III and IV. Another possible reason for the increasing of nitrification rate was due to the high temperature (27.9−29.5℃ in phase IV, shown in Table 1). Together with the seed

Table 1
Operational parameters of R1, R2, and R3

		R1				R2				R3			
Phase	T (°C)	Days (d)	Cycles	Aeration time (h)	MLSS (mg/L)	Days (d)	Cycles	Aeration time (h)	MLSS (mg/L)	Days (d)	Cycles	Aeration time (h)	MLSS (mg/L)
I	15.1–17.9	1–8	1–32	4	3,728	1–10	1–10	22	3,660	1–20	1–5	96	3,825
II	17.9–23.3	8–53	33–302	3	3,184	10–32	11–99	5	3,411	20–31	6–16	20	3,464
III	23.3–27.9	53–128	303–902	2	3,524	32–61	100–215	4	2,233	31–111	17–256	7	2,606
IV	27.9–29.5	128–140	903–998	1	2,823	61–140	216–690	3	2,257	111–140	257–373	5	2,541

Notes: MLSS: the average value.
pH: 7.8–8.3.

sludge (day 0 for phase I), there were 10 sludge samples (day 30 for phase II, day 60 for phase III, and day 140 for phase IV) from the three reactors using for analysis of the abundance and diversity of AOA and AOB based on *amoA* gene.

From Phase I to Phase IV, average MLSS in each reactor was 3,314, 2,890, and 3,109 mg/L, indicating that the biomass growth rate under these conditions was very low. One possible reason may be that the synthetic wastewater contained no organic substrate. Influent and effluent quality parameters reflecting the performance of R1, R2, and R3 during operational period are shown in Fig. 1. Since the aeration time was long enough for ammonia oxidation, effluent ammonia concentration of the three reactors was kept below 1.48, 2.93, and 2.84 mg/L, respectively. The average ammonia removal rate in R1, R2, and R3 was as high as 97.16% (91.67−100.00%), 98.84% (94.98−100.00%), and 99.30% (97.35−100.00%), respectively.

3.2. Abundance of AOA and AOB amoA genes

Fig. 2 showed the AOA and AOB *amoA* genes abundance of the seed sludge and enriched nitrifying-activated sludge of the three reactors in different phases. AOA *amoA* gene copies ($1.32 \times 10^4 \pm 4.02 \times 10^3$ copies/(g dry sludge)) occurred in the seed sludge were nearly two orders of magnitude lower than AOB *amoA* gene copies ($9.62 \times 10^6 \pm 2.73 \times 10^5$ copies/(g dry sludge)). The abundance of AOA *amoA* gene of seed sludge was consistent with the results of some industrial wastewater treatment systems [13], but it was lower than the sludge from a municipal WWTP [21]. The AOB *amoA* gene abundance of the seed sludge was higher than three domestic wastewater treatment systems [13]; however, it was lower than three industrial wastewater treatment systems [13].

Along the time of operation, shifts of the numbers of AOA and AOB *amoA* gene occurred in the three reactors. After 30-d operation, AOA copy numbers in R1 ($4.48 \times 10^4 \pm 3.66 \times 10^3$ copies/(g dry sludge)) were still in the same order of magnitude with that of seed sludge. While the numbers of AOA *amoA* gene in R2 and R3 decreased slightly ($1.34 \times 10^4 \pm 1.53 \times 10^3$ and $2.20 \times 10^4 \pm 7.31 \times 10^3$ copies/(g dry sludge)). As time going on (60 and 140 d), AOA *amoA* gene copy numbers of the three reactors were still in the same order of magnitude with that of seed sludge. While the abundance of AOB *amoA* gene significantly increased. When the reactors operation was prolonged to 140 days, AOB *amoA* gene copy numbers were more abundant ($2.69 \times 10^8 \pm 2.46 \times 10^7$, $8.1 \times 10^9 \pm 4.76 \times 10^7$ and $2.09 \times 10^{10} \pm 4.40 \times 10^9$ copies/(g dry sludge) for

R1, R2, and R3, respectively) than AOA *amoA* gene copy numbers. The ratio of AOB to AOA *amoA* gene for the seed sludge was 3.68×10^2, after running under different ammonia concentration for 140 d, the ratio increased to 4.90×10^2, 1.25×10^5, and 3.77×10^5 for R1, R2, and R3, respectively. In Sonthiphand's research, the numbers of AOA *amoA* gene were unchanged in the reactor with lower ammonia level (28 mg/L) after reactor running for 60 d, while in the reactors with higher ammonia levels (140 and 420 mg/L, respectively), the numbers of AOA *amoA* gene were deteriorated; after 360 d of reactor operation, AOA disappeared from the ammonia-oxidizing consortiums in all three reactors. While AOB enriched from 10^7 in seed sludge to 10^8 in reactors with ammonia concentration of 140 and 420 mg/L [21]. In Ye's study, the abundance of AOB in a continuous nitrification reactor was ~40 times larger than that of AOA, when increased ammonia loading, AOA decreased heavily (phase III), the ratio of AOB to AOA increased significantly up to ~2,000 to ~4,000 with the increase of ammonia loading (phase III and IV) [22]. In this study, as operation time went on, AOA was neither enriched nor vanished, its copy numbers remained unchanged in the three reactors with different ammonia levels, which was not agree with Sonthiphand's study. The copy numbers of AOB *amoA* gene in our study increased with time, from about 10^7 copies/(g dry sludge) in seed sludge to 10^8, 10^9, and 10^{10} copies/(g dry sludge) in 140 d operation for R1, R2, and R3, respectively, indicating the enrichment progress of this population, which was in accordance with the results of some studies [21,22].

After 140-d operation, the AOA abundance of the three SBRs was inhibited or decreased slightly under different levels of ammonia concentration, and for AOB, the abundance was significantly increased. Therefore, the successful enrichment of AOB was obtained. The ammonia and oxygen are the electron donor and acceptor for both AOA and AOB, these two types of microorganisms compete with one another for the substrates in WWTPs [7]. In this study, the DO was controlled and kept similar in three SBRs. Therefore, high ammonia concentration of the influent in the SBRs might be the main reason for the successful enrichment of AOB, as well as the failure enrichment of AOA. The K_m of AOA is much lower than AOB [17]. The high affinities to ammonia of AOA could well compete effectively with AOB under limiting ammonia concentrations; however, under the enrich ammonia concentrations, AOB with low ammonia affinity will comprise the majority of the ammonia-oxidizing microorganism [7]. The growth and activity of AOA were also found to be inhibited under certain ammonia concentrations, and these ammonia levels were much lower than that of AOB, based on the culture studies [7]. Therefore, the abundance of

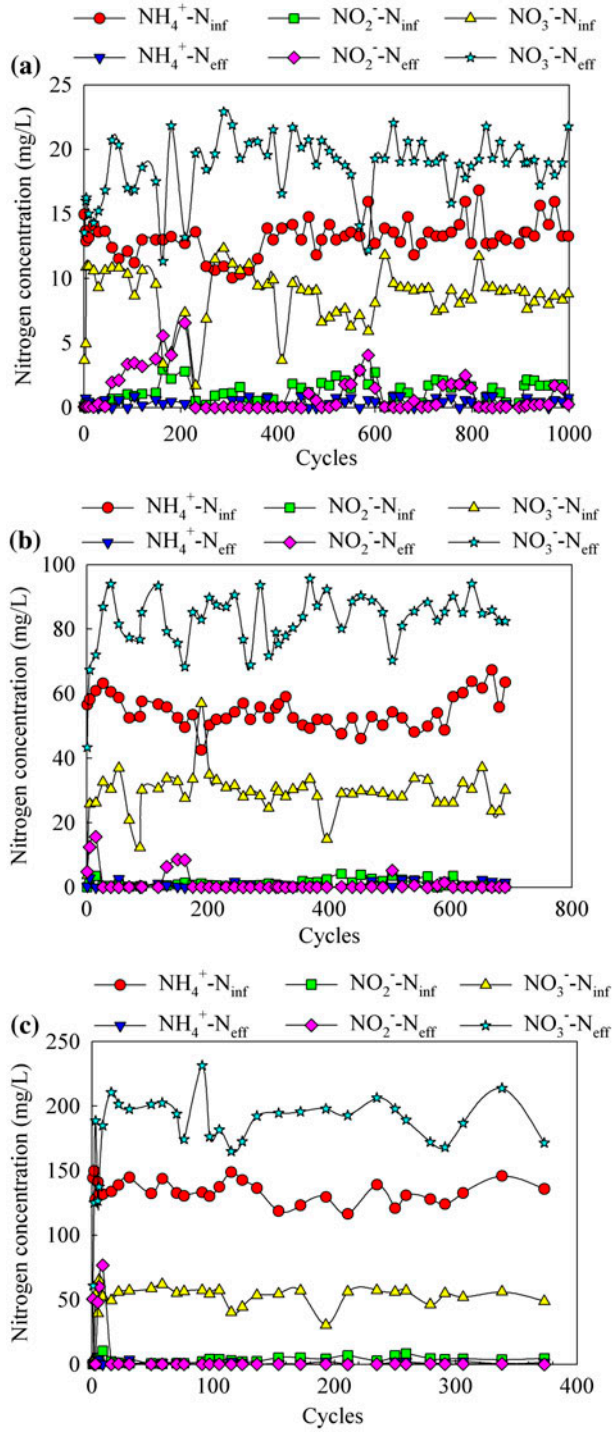

Fig. 1. Profiles of influent and effluent ammonia, nitrite and nitrate concentration during the 140-day enrichment of the nitrifying-activated sludge (a) R1, (b) R2, and (c) R3.

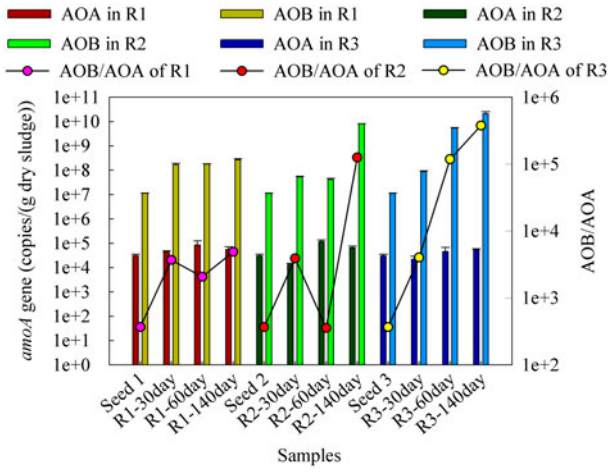

Fig. 2. Quantitative analysis of AOA and AOB *amoA* gene in seed sludge and enriched nitrifying-activated sludge samples from R1, R2, and R3 during 140-d operation.

AOA decreased slightly, and that of AOB significantly increased under the high ammonia levels in R2 and R3. Besides, the failure of AOA enrichment could be attributed to several other factors during reactor operation. One possible reason was a lack of organic carbon source for the growth of archaea, although AOA grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite [2], it might still need organic carbon source for growth. Besides, there were no vitamins and other trace elements necessary for the growth of archaea in the inorganic medium supplied [21]. On the other hand, the abundance of AOA *amoA* gene after 140-d operation was in the same order of magnitude with that of seed sludge, showing the synthetic wastewater used in this study was able to meet the growth of archaea to a certain extent.

3.3. *Diversity of AOB* amoA *gene*

Clone libraries of AOA and AOB *amoA* genes were constructed for each of the four activated sludge samples (seed sludge, R1–140 d, R2–140 d, R3–140 d). For each AOB clone library, 30–40 clones were randomly selected for sequencing. The Good's coverage values, Chao1 richness estimates, Shannon diversity index (Table 2), and rarefaction analysis (Fig. S3, in Supplementary data) of the four clone libraries were calculated. As shown in Table 2, the coverage of each clone library was high than 90%, indicating that the four clone libraries represented the majority of the AOB community in the four samples. The Shannon indices showed that the diversity of AOB *amoA* gene in seed sludge was lower than the enriched nitrifying-activated sludge samples. The ratios of OTU observed to OTU estimated by Chao1 estimator of the four samples were more than 80%, which also suggested that the four clone libraries represented the majority of the AOB community in the four samples.

Fig. 3(a) depicted the relative abundance and distribution of AOB *amoA* gene OTUs in different samples. A total of 23 OTUs were generated based on 134 AOB *amoA* gene sequences in the four clone libraries with a 3% distance cutoff. The seed sludge, R1–140, R2–140, and R3–140 contained 6, 10, 10, and 7 OTUs, respectively, suggesting that AOB *amoA* gene diversity in R1 and R2 was slightly higher than seed sludge and R3. Fig. 3(a) revealed that two OTUs (OTU1 and OTU3) were shared by three activated sludge samples (seed sludge, R1–140, and R2–140), and each of the six OTUs (OTU2, 5, 8, 13, 16, and 17) was distributed in two activated sludge samples, indicating that AOB *amoA* gene was diverse and widely disseminated. In seed sludge, R1–140, and R2–140, OTU1 was the main OTU, occupying 51.72, 44.74, and 43.24%, respectively. While in R3, 30% AOB *amoA* gene sequences belonged to OTU16. Phylogenetic tree (Fig. 4) showed OTU1 and OTU16 were affiliated to *Nitrosomonas ureae* cluster and *Nitrosomonas europaea* cluster, respectively, so the two clusters might be the main AOB species in the bioreactors.

AOB NJ tree (Fig. 4) showed that AOB *amoA* gene sequences recovered from the four samples fell into six clusters: *N. ureae* cluster (58 sequences belonging to 5 OTUs), *N. europaea* cluster (35 sequences belonging to 7 OTUs), *Nitrosomonas oligotropha* cluster (13 sequences belonging to 3 OTUs), *Nitrosomonas*-like cluster (12 sequences belonging to 3 OTUs), *Nitrosomonas marina* and *Nitrosomonas aestuarii* cluster (9 sequences belonging to 4 OTUs), and *Nitrosomonas communis* cluster (7 sequences belonging to 1 OTUs). *N. ureae* cluster and *N. europaea* cluster were the two dominant species, accounting for 43.28 and 26.12%, respectively.

As depicted in Fig. 5, the seed sludge contained four AOB species, and *N. ureae* cluster was the most dominant species (58.62%), followed by *Nitrosomonas*-like cluster (20.69%), *N. oligotropha* cluster (13.79%), and *N. europaea* cluster (6.90%). After long-term cultures (140 d), the AOB species in R1 under low ammonia concentration (14 mg/L) were similar with the seed sludge, except the occurrence of new AOB cluster (*N. marina* and *N. aestuarii* cluster, 10.53%) and the disappearance of *N. europaea* cluster. For R2, the *Nitrosomonas*-like cluster disappeared under higher ammonia concentration (56 mg/L), and *N. ureae* cluster was also the dominant AOB species. The results suggested that *N. ureae* cluster was adapted to low

Table 2
Good's estimators of the AOB *amo*A clone libraries (3% distance cutoff)

Samples ID	AOB					
	Numbers of sequences	Numbers of OTUs	Chao 1	OTUs observed/OTUs estimated (%)	Good's coverage (%)	Shannon index
Seed	30	7	8.5	0.82	90.00	1.46
R1–140 d	40	9	10.5	0.86	92.50	1.73
R2–140 d	38	10	11	0.91	92.11	1.87
R3–140 d	30	7	7	1.00	96.67	1.70

ammonia concentration and also showed a wide tolerance of ammonia levels. Compared with the seed sludge, the *N. europaea* cluster was gradually enriched under higher ammonia levels, which account for 27.03 and 76.67% of the AOB in R2 and R3, respectively. Besides *N. europaea* cluster, a new AOB cluster *N. communis* cluster (23.33%) occurred in R3. The results indicated that *N. europaea* cluster and *N. communis* cluster maybe favorable for high ammonia concentration. Studies of isolated and mixed cultures showed that *N. europaea* exhibited the highest K_m values for ammonia (30−61 μM, 0.420−0.854 mg/L). In comparison, the K_m values for *N. communis*, *N. oligotropha*, and *N. ureae* were 14−43 μM (0.196−0.602 mg/L), 1.9−4.2 μM (0.027−0.059 mg/L), and 1.9−4.2 μM (0.027−0.059 mg/L), respectively, lower than those of *N. europaea* [36]. Consequently, *N. europaea* cluster and *N. communis* cluster were dominant in R3 with high ammonia level (140 mg/L), and *N. ureae* cluster was the dominant major clusters in seed sludge, R1, and R2 under low ammonia levels (25, 14, and 56 mg/L, respectively). However, the occurrence of *N. communis* cluster in R3 was contrary to the study of Limpiyakorn et al. [32], who found that *N. communis* cluster occurred almost exclusively in association with A^2O and AO systems receiving low influent ammonia concentrations (12−30 mg/L). *N. oligotropha* cluster and *Nitrosomonas*-like cluster disappeared in R3, suggested that high ammonia concentration did not facilitate the growth of these two AOB species. In the literature, AOB of *N. europaea* cluster is commonly found in WWTPs with high ammonia levels and members of the *N. oligotropha* cluster are majority strains existing under low ammonia levels [33].

Despite AOB *amoA* copy numbers increasing with time, AOB diversity decreased in R3 with high ammonia concentration, indicating that high ammonia concentration may be a selection pressure for AOB population. All AOB *amoA* gene sequences were affiliated to *Nitrosomonas* genus. *Nitrosospira* genus did not occur in any sludge samples, which was consistent with previous reports that *Nitrosomonas* instead of *Nitrosospira* genus was the dominant AOB species in nitrification bioreactors [34,25,4,12].

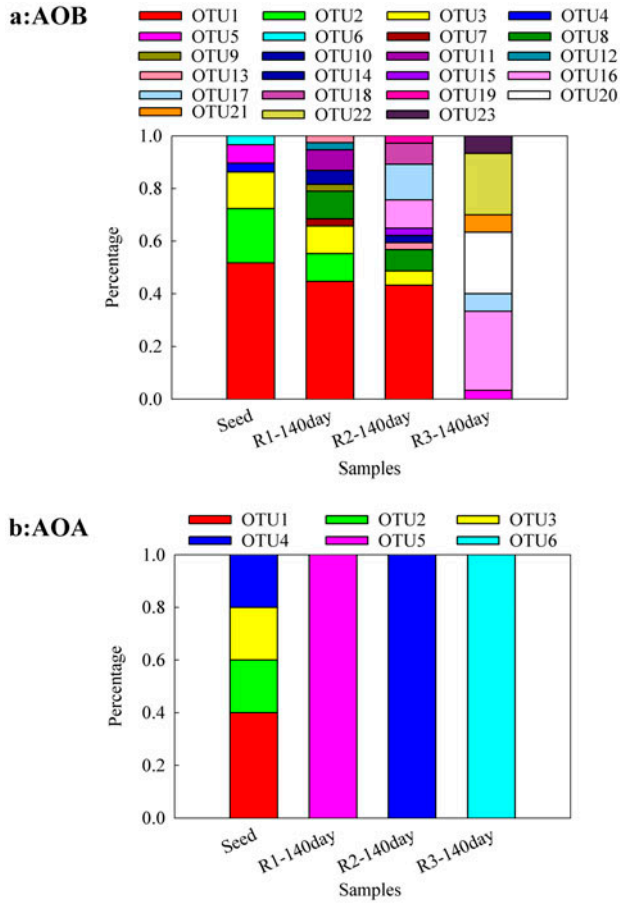

Fig. 3. Distribution and relative abundance of AOB and AOA *amoA* gene OTUs in seed sludge and enriched nitrifying-activated sludge samples from R1, R2, and R3 during 140-d operation (a) AOB and (b) AOA.

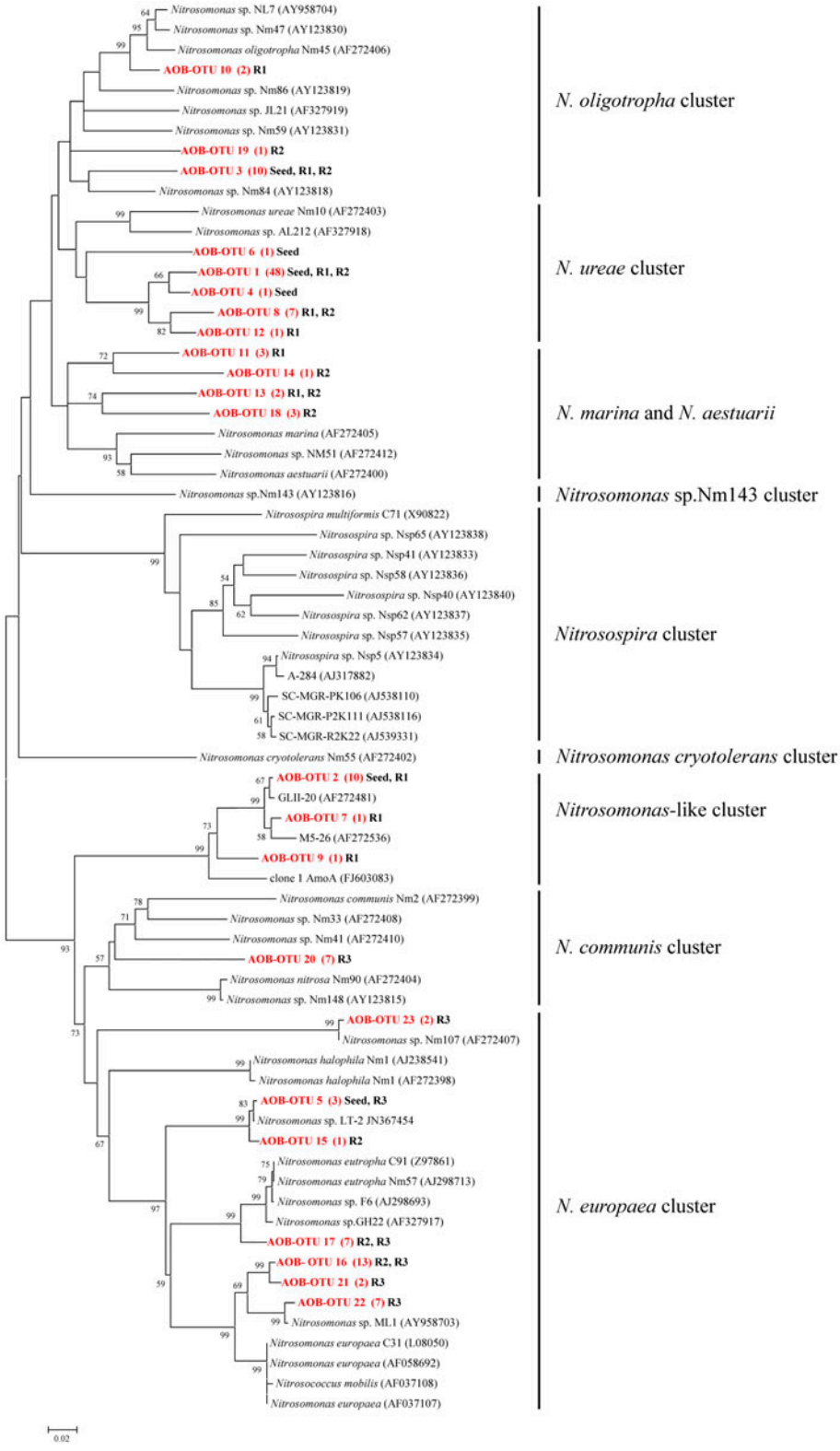

Fig. 4. NJ phylogenetic tree based on AOB *amoA* gene sequences. Sequences obtained in this study were named "AOB-OTU-" and in bold. Other sequences were obtained from GenBank. The sequences numbers of each OTU were displayed by the number in parentheses.

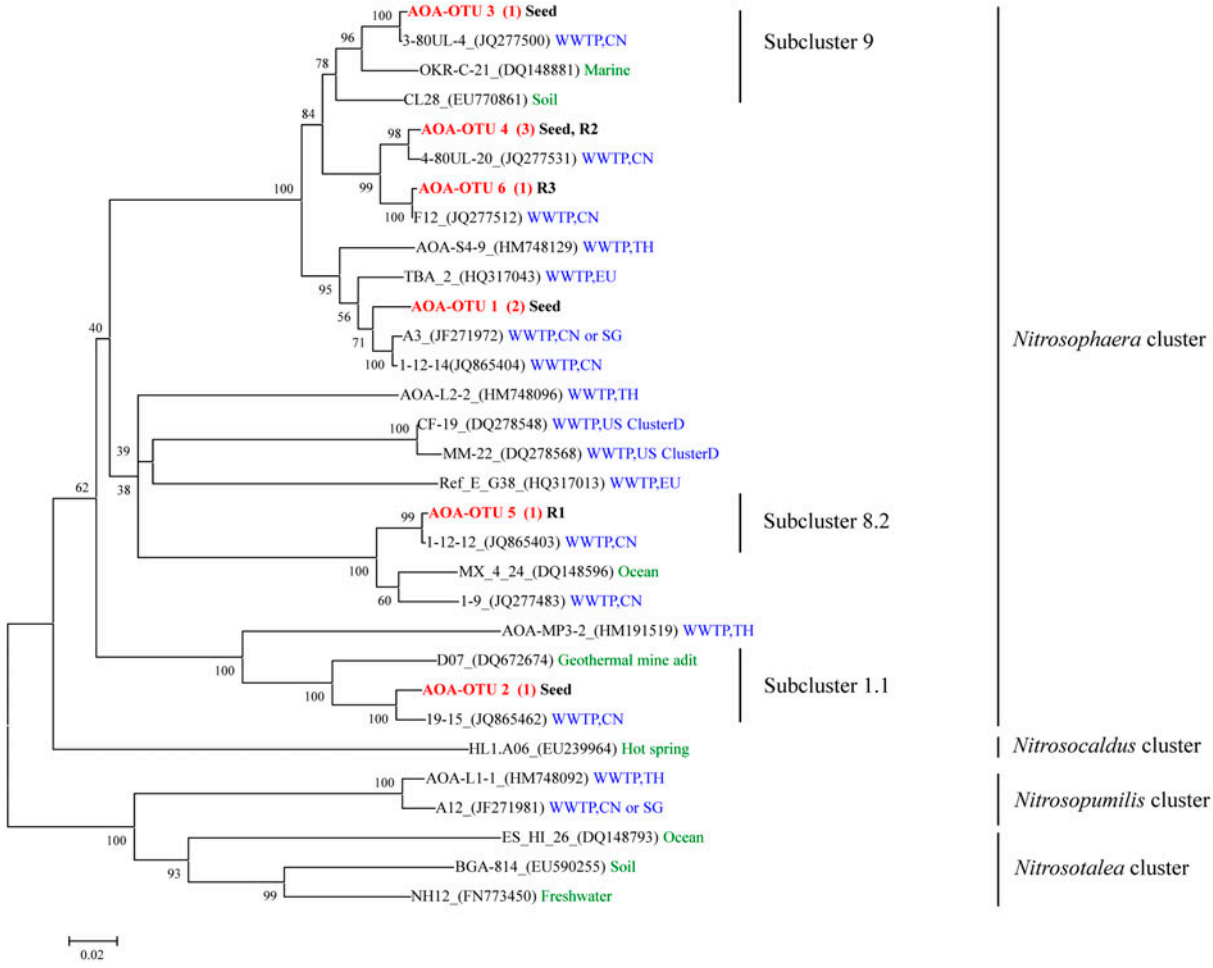

Fig. 5. NJ phylogenetic tree based on AOA *amoA* gene sequences. Sequences obtained in this study were named "AOA-OTU-" and in bold. Other sequences were obtained from GenBank. The sequences numbers of each OTU were displayed by the number in parentheses.

3.4. *Diversity of AOA* amoA *gene*

The PCR amplification and clone library construction of AOA *amoA* gene were difficult due to its low abundance. In this study, multiple experiments were attempted to amplify AOA *amoA* gene from the four samples, details could be found in the material and methods. Finally, PCR amplifications were successful for all the four samples. Of the four clone libraries, 467 white colonies were tested and 119 positive clones were sequenced; however, only nine sequences were AOA *amoA* gene. As shown in Fig. 3(b), in total, 6 OTUs were generated based on the 9 AOA *amoA* gene sequences with a 3% distance cutoff. Four OTUs (OTU1, OTU2, OTU3, and OTU4) were recovered from six AOA sequences of the seed sludge. And only one OTU contained one or two AOA sequences were recovered from R1–140, R2–140, and R3–140, respectively. Fig. 3(b) showed that OTU4 was shared by seed sludge and R2–140. The results indicated that the diversity of AOA *amoA* gene in seed sludge was higher than enriched nitrifying-activated sludge.

Fig. 5 showed the AOA NJ tree. AOA *amoA* gene diversity analysis was carried out according to a new AOA nomenclature system provided by Pester et al. [5]. As shown in Fig. 5, 66.67% AOA *amoA* gene sequences belonged to general *Nitrososphaera* cluster. *Nitrososphaera* subcluster 1.1, *Nitrososphaera* subcluster 8.2, and *Nitrososphaera* subcluster 9 contained 11.11% AOA *amoA* gene sequences, respectively. The seed sludge contained members of general *Nitrososphaera* cluster (60.00%), *Nitrososphaera* subcluster 9 (20.00%), and *Nitrososphaera* subcluster 1.1 (20.00%). *Nitrososphaera* subcluster 1.1 and *Nitrososphaera* subcluster 9 disappeared in all three reactors after long-term enrichment, and only one AOA species was observed

in each reactor. *Nitrososphaera* subcluster 8.2 instead of general *Nitrososphaera* cluster become the only AOA species in R1. General *Nitrososphaera* cluster was the dominant AOA cluster in R2 and R3.

In this study, all retrieved AOA *amoA* sequences belonged to *Nitrososphaera* cluster. Fig. 5 also depicted that most sequences retrieved from other WWTPs were distributed in *Nitrososphaera* cluster, such as JQ277500 from China [12], HM748129 from Thailand [10], HQ317043 from Europe [11], and DQ278548 from the United States [9]. In Gao et al.'s study [12,25], most of the sequences retrieved from 8 to 10 wastewater treatment systems belonged to *Nitrososphaera* cluster, which was in agreement with this study. Although Park et al. suggested that AOA belonging to cluster D may be widespread in activated sludge bioreactors [9], in this study, there were no sequences belonging to cluster D. However, in Jin et al.'s study [23], Ye and Zhang study [22], and Wu et al. study [35], the dominant AOA in bioreactors treating saline wastewater belonged to *Nitrosopumilis* cluster, which was different from this study.

AOA (only four *Nitrososphaera* subclusters) was not as diverse as AOB in all the three reactors during 140-d operation. AOB community structure significantly shifted in different reactors, while AOA occurred in small fluctuations, indicating that AOB was more sensitive than AOA to high ammonia levels, and AOB played more important role than AOA. Compared with AOB ecotypes, the K_m value for ammonia of isolated and mixed AOA cultures, such as "*Ca*. Nitrosopumilus maritimus" strain SCM1 [17], AR enrichment culture [37], and strain MY1 [20], was 0.002, 0.009, and 0.010 mg/L, respectively, which was lower than those of AOB, indicating that some AOA ecotypes might be more suited to low ammonia levels. So, during operational period, AOA abundance of the three reactors was almost unchanged, while AOB abundance increased significantly.

3.5. Correlations between operational parameters and community structures

The SRCC were used to further elucidate the potential impacts of operational parameters on abundance and community structure of AOA and AOB. The operational parameters of the three reactors could determine the pollutant removal performance. Ammonia concentration, oxygen level, pH, temperature, organic carbon, salinity, and sulfide levels in WWTPs could affect the abundance and composition of AOA and AOB. Oxygen level and pH were similar in the three nitrifying bioreactors. Therefore, in this study, some water-quality indicators (including influent ammonia, nitrite and nitrate, and effluent ammonia, nitrite and nitrate, ammonia removal rate) and T were selected to investigate their relationships with the abundance and diversity of AOA and AOB, according to the aim of this study. The results of SRCC are shown in Table S2 (See Supplementary data). AOA and AOB abundance both had a positive correlation with temperature (SRCC = 0.698, $p = 0.025$ and SRCC = 0.810, $p = 0.004$). AOB abundance was positively correlated with effluent ammonia (SRCC = 0.763, $p = 0.010$). *N. europaea* was positively correlated with influent ammonia (SRCC = 1.000, $p < 0.010$); however, *N. oligotropha* was negatively correlated with influent ammonia (SRCC = 1.000, $p < 0.010$), indicating the two AOB species might be favorable for high and low ammonia concentrations, respectively. Both *Nitrososphaera* subcluster 1.1 and *Nitrososphaera* subcluster 9 had a negative correlation with temperature (SRCC = −1.000, $p < 0.010$ for both subcluster). *N. ureae* was positively correlated with effluent nitrite concentration (SRCC = −1.000, $p < 0.010$).

4. Conclusion

AOB *amoA* gene abundance was larger than that of AOA in all three reactors with different ammonia concentrations, and the ratio of AOB to AOA was significantly affected by ammonia level. All retrieved AOA and AOB belonged to genus *Nitrososphaera* and *Nitrosomonas*, respectively. After enrichment, *N. ureae* cluster (in R1, R2) and *N. europaea* cluster (in R3) were dominant under low and high ammonia level, respectively. The diversity of AOA was very low (only four *Nitrososphaera* subclusters) in all reactors during 140-d operation, and diversity of AOB was higher than AOA. AOB may play more important role than AOA in ammonia oxidation.

Supplementary material

The supplementary material for this paper is available online at 10.1080/19443994.2015.1123196.

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