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The existence and role of ammonia-oxidizing archaea in full-scale wastewater treatment plants

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ABSTRACT

The study investigated the richness and occurrence of archaeal and bacterial amoA genes in activated sludge samples from ten industrial and six domestic wastewater treatment plants (WWTPs). Three different strains of ammonia-oxidizing archaea (AOA) were grown as enrichment cultures to determine the optimal growth of ammonium concentrations. AOA growth was greatly retarded at ~10 mM and inhibited at ~20 mM ammonia concentrations. ASAT 1 WWTP had the highest abundance of the AOA amoA gene (5.83×10^7), but the copy number of ammonia-oxidizing bacteria (AOB) amoA genes was 5.17×10^4. İSTAÇ leachate WWTP, which contained the highest ammonia level (104.6 mM) within the samples, had AOA amoA gene copy (1.39×10^4) less than four orders of AOB amoA gene copy (1.34×10^8). The results of this study indicated that AOA may participate ammonia oxidation in domestic WWTPs. Most of the sequences were closely clustered among a marine archaeon (Group 1.1a). Fewer sequences were affiliated with a soil archaeon (Group 1.1a).

Keywords: Ammonia-oxidizing bacteria; Ammonia-oxidizing archaea; Wastewater treatment; Quantitative PCR; Nitrification

1. Introduction

Ammonia is converted to nitrite by ammonia-oxidizing organisms and ammonia oxidation is the rate-limiting step of nitrification [1]. Ammonia monooxygenase (AMO) performs ammonia oxidation and the amoA gene is frequently used for DNA fingerprinting [2,3]. For a long time, it was known that only anaerobic ammonia-oxidizing bacteria (Anammox) and ammonia-oxidizing bacteria (AOB) oxidize ammonia. Thereafter, scientific evidence introduced ammonia-oxidizing archaea (AOA) as a new player for ammonia oxidation. Culture-dependent and independent studies showed that members of Thaumarchaeota phylum in the archaeal domain also performed ammonia oxidation. Recent evidence also showed that some *Nitrospira* species performed complete nitrification [4,5]. These findings changed our understanding about nitrification. The presence of AOA was first detected in a metagenomics based study of the Sargasso Sea [6]. *Nitrosopumilus maritimus* was the first strain of AOA isolated from a tropical marine aquarium tank [7]. The four AOA-affiliated groups were *Nitrosopumilus* (Thaumarchaeal marine archeaon I.1a), *Nitrososphaera* (Thaumarchaeal soil archeaon I.1b), *Nitrosocaldus* and *Nitrosotalea* cluster [8].

Studies indicated that high amounts of archaeal amoA gene copies were found in marine and soil environments [6,9]. Culture-independent surveys [7,10,11] and quantitative real-time polymerase chain reaction (PCR) studies [12–15] indicated that archaeal ammonia oxidizers are more abundant and shows higher activity than their bacterial counterparts in various environmental samples.

In addition to natural ecosystems, AOA also thought to contribute to ammonia oxidation at wastewater treatment plants (WWTPs) [3,16]. AOA occurrence was demonstrated

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for the first time in five WWTPs in the USA [3] and then in two WWTPs in China [16]. Recently, a few studies were published indicating the importance of AOA that contributed to nitrification in engineered systems [17–22]. Archaeal *amoA* sequence analysis of the microbial population in a marine filtration system showed that AOA was rich and phylogenetically diverse [23]. In addition, gene sequence analysis in nitrifying wastewater treatment bioreactors also indicated the presence of AOA in such systems [3,16–18]. The limited research on AOA activity in WWTPs indicated the importance of the operating conditions of the reactors [3,16]. Park et al. [3] showed that all the archaeal amoA gene positive samples were taken from bioreactors that had been operated with long retention times and in low dissolved oxygen (DO) concentrations. Whereas Zhang et al. [16] indicated that samples that contained AOA had significantly lower hydraulic retention time. Moreover, the AOA–Anammox membrane aerated biofilm reactor (MABR) system has higher nitrogen removal than AOB–Anammox MABR and needs lower oxygen supply [24].

The ubiquity of AOA in the variety of environments and their abundance over AOB is currently well established, only a few studies have focused on the presence and the role of AOA in different engineered systems. These studies were restricted to a limited number of WWTP types. Yet, despite the limited number of studies on wastewater treatment system slow DO requirement, and fast nitrification rate of AOA indicates their importance in nitrification processes [25,26].

Archaeal and bacterial amoA genes from activated sludge samples of six domestic and ten industrial WWTPs were quantitatively detected using real-time PCR in this study. In addition, three different strains of AOA enrichment cultures were grown and analyzed to determine optimal growth conditions for AOA in different ammonium concentrations. Cloning and sequencing of archaeal amoA gene fragments were performed to detect AOA diversity.

2. Materials and methods

2.1. Activated sludge sample collection from WWTPs

Activated sludge samples were collected from six domestic and ten industrial WWTPs, including oil and petroleum refineries, alcohol, food, and chemical industries, and landfill leachate treatment plants. Duplicate samples were taken at different locations from the aerobic pool of the WWTPs. All samples were kept at 4°C until DNA extraction, which was performed within 2 h of sampling. The details of the WWTPs and their operational data are summarized in Table 1.

2.2. DNA extraction

Duplicate samples were homogenized using a FastPrep instrument (QBiogene, Carlsbad, CA) with a setting of 5.5 for 30 s. DNA was extracted using Fastprep DNA extraction kit for soil (QBiogene) from 0.5 g filtered sludge samples following the manufacturer's protocol and then for each sample, duplicate sets of DNA samples were pooled. Qubit fluorimeter (Invitrogen, Carlsbad, CA) was used for measurement of DNA quantity and quality.

2.3. Real-time PCR quantification

Quantitative real-time PCR (Q-PCR) was performed in triplicate, using an iCycler iQ5 thermocycler (Bio-Rad, Berkeley, CA) real-time detection system. The primer list and sequences are given in Table 2. AmoAF and AmoAR primers [26] were used to target archaeal amoA genes. The annealing temperature of Arch-amoAF and Arch-amoAR primers was 61°C. The 25 μL PCR mixture contained 12.5 μL of the iQ Sybr green supermix (Bio-Rad), 1 μL of each primer (0.4 μM), 9.5 μL DNAse, RNAse free water, and 1 μL of the DNA sample (10 ng). The PCR conditions started with 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 61°C for 60 s, and polymerization at 72°C

Table 1
Operational parameters of full-scale wastewater treatment plants

	Samples	COD (mg/L)	BOD_5 (mg/L)	NH_4N (mg/L)	pH	Ammonia
E1	Baker's yeast producing (Pakmaya)	760	ND	1,496	7.67	101.7 mM
E2	İSTAÇ leachate treatment	22,230	2,900	1,810	8.15	104.6 mM
E3	Beer production Istanbul	2,492	1,744	1,172	7.00	67.7 mM
E4	Raki production	28,000	13,000	508	7.00	29.3 mM
E5	Paper raw material (pulp) production	500–1,500	300–800	946	7.00	54.7 mM
E6	Kırıkkale Petrolium refinery	452	236	5.98	ND	0.4 μM
E7	Beer production Ankara	2,000	1,700	963	7.00	55.6 mM
E8	Gum production	ND	ND	NA	7.00	
E9	İzmit Petrolium rafinery	ND	ND	207	8.00	12.2 mM
E10	Sunflower oil production	ND	224	213	ND	14 mM
M1	ASAT 1 municipal WWTP (Antalya)	388	340	43	7.4	2.9 mM
M2	ASAT 2 municipal WWTP (Antalya)	831	370	41	7.8	2.9 mM
M3	İSKİ WWTP	600	300	723	7.3	41.8 mM
M4	Eskişehir WWTP	1,050	390	54	ND	3.6 mM
M5	ESART A.Ş WWTP	2,856	1,400	45.5	7.6	3.1 mM
M6	Paşaköy advanced biological treatment plant	670	268	41	ND	1.44 mM

Table 2
PCR primes that are used this experiment

Primer	Sequence	Target	Annealing	References
amoA-1f	GGGGTTTCTACTGGTGGT	AOB amoA gene		
amoA-2r	CCCCTCKGSAAAGCCTTCTTC		55	[27]
amoA-AF	ATGGTCTGGCTWAGACG	AOA amoA gene		
amoA-AR	GCCATCCATCTGTATGTCCA		61	[26]
amoA 104f	GCAGGAGACTAYATHTTCTA	AOA amoA gene		[28]
amoA 616r	GCCATCCATCTRTADGTCCA			[29]

for 30 s. Data capture was done for each cycle at 78°C for 15 s. The DNA standards were prepared in the range of 1×10^1 to 1×10^8 with *N. maritimus* clones, and melting curve analysis was performed after amplification. The quantification of bacterial amoA genes was performed using the primers of amoA 1F and amoA 2R [27]. The annealing temperature of amo1F-2R primers was 55°C. All the other PCR conditions were similar to the archaeal amoA genes except the annealing temperature as mentioned above. The standard DNA was prepared in the range of 1×10^1 to 1×10^8 with *Nitrosomonas* sp. and melting curve analysis was also performed similar to the analysis of archaeal Q-PCR. The Q-PCR analysis of archaeal amoA genes was done using the primers amoA 104f [28] and amoA 616r [29] in an Eppendorf instrument for culture experiment. *N. maritimus* clone was used for QPCR standard (10^8–10^1). Abundance of AOA in enrichment cultures were analysed from two independent replicates.

2.4. Enrichment cultures of AOA

Three different strains of AOA enrichment cultures, strain V (~98%), strain 123 (~96% purity), and *N. maritimus* (~98% purity) were cultivated in a range of ammonium concentrations (1, 3, 5, 10, and 20 mM) in order to assess optimum growth concentrations [30]. Enrichment cultures were made using modified synthetic *Crenarchaeota* medium supplemented with KH_2PO_4, NaCl, $MgCl_2$, $CaCl_2$, and KCl [7]. Cultures were kept at 37°C in the dark for 35 d. Triplicate samples from enrichment cultures were taken at 10 different time points both for colorimetric analyses [31] and for Q-PCR quantification.

2.5. Phylogenetic analyses of AOA communities

PCR amplification of the archaeal amoA gene for the cloning study was performed by using primers of Arch-amoAF and Arch-amoAR as mentioned above. Agarose (1%) gel electrophoresis was conducted to check the PCR products. PCR products were purified before performing the TOPO cloning reaction in order to increase cloning efficiency using the QIAquick PCR purification kit (Qiagen, Germany); 0.5 μL of TOPO vector, 3 μL fresh PCR products, and 0.5 μL of salt solution were mixed. After cloning, pCR4-TOPO constructs heat-shocked into the competent *E. coli* cells and 500 μL of SOC medium were added to the cells and later 100 μL from each transformation was spread onto prewarmed Luria-Bertani (LB) plates (containing 50 μg/mL kanamycin) for overnight incubation. Randomly selected colonies were transferred to new LB broth for overnight incubation. The plasmids were extracted using a QIAGEN MiniPrep Plasmid isolation kit (Qiagen Inc., Valencia, CA). Extracted DNA fragments were sequenced for nucleotide analyses. Big Dye terminator v3.1 premix (Applied Biosystems, CA, USA) was used for sequencing. Either M13 or T7 primer were used for one-strand amplification. In order to remove both the unincorporated dideoxynucleotides as well as salts from the reaction buffers, the products were cleaned prior to loading the sequencer (ABI, CA, USA). Phylogenetic and molecular evolutionary analyses of archaeal amoA sequences were performed using MEGA5 [32].

The sequences reported in this paper have been deposited into the GenBank database (accession numbers between KT236103–KT236111).

2.6. Statistical analysis

The number of amoA gene copies of archaea and bacteria in our samples were evaluated in correlation with the WWTPs operational parameters. Nonparametric Spearman's rank correlation coefficient (RS) calculation was performed. A two-tailed *t*-test was conducted to asses significance at p value of <0.05 using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA, USA; www.graphpad.com).

3. Results and discussion

Activated sludge samples from 16 full-scale domestic and industrial WWTPs were investigated using molecular tools to understand the diversity of ammonia-oxidizing microorganisms based on copy number of amoA genes of both AOA and AOB. Conventional parameters of the active sludge samples are shown in Table 1. Ammonia loads in domestic wastewater were lower than the industrial wastewater as expected. One of the industrial WWTPs treating mainly yeast production wastewater named Pakmaya (101.7 mM) and İSTAÇ one of the typical solid wastewater leachate treatment WWTP (104.6 mM) had the highest ammonia load. Ammonium concentrations were between 6.7 and 104.6 mM in industrial WWTPs, whereas they were 0.94–1.44 mM in domestic WWTPs.

3.1. Abundance of AOA and AOB amoA genes

The different domestic and industrial WWTPs studied herein had a wide range of characteristics; specifically, in terms of ammonium concentrations where it changes between 6.7 and 104.6 mM in industrial WWTPs, whereas 0.94–1.44 mM in domestic WWTPs. Q-PCR experiments

specific to archaeal and bacterial amoA genes were performed for all these different WWTPs. Q-PCR showed that also the copy number of AOA and AOB amoA genes varied between the samples (Fig. 1). ASAT 1 WWTP, which is a domestic plant with 1.44 mM ammonia concentration, had the highest AOA amoA gene abundance ($5.83 \times 10^7 \pm 1.31 \times 10^7$), whereas AOB amoA gene abundance was $5.17 \times 10^4 \pm 1.4 \times 10^4$). The highest ammonia level (104.6 mM) from İSTAÇ leachate WWTP had an AOA amoA gene copy number ($1.39 \times 10^4 \pm 9.89 \times 10^3$) four orders of magnitude less than AOB amoA gene copies ($1.34 \times 10^8 \pm 7.61 \times 10^7$). The ratio between AOB amoA and AOA amoA genes ranged between 8.7×10^{-4} and 9.64×10^3 in whole samples. The ratios in the domestic WWTP samples were between 1.4×10^4 and 8.7×10^4. However, the ratios varied significantly (4.32×10^3 to 9.64×10^3) in the samples from industrial WWTPs. Those high-level ammonia-containing WWTPs (E1; 101.7 mM and E2; 104.6 mM) showed at least three times more AOB amoA genes than AOA amoA genes (Table 1). Industrial WWTPs that contained medium levels of ammonium (E3; 67.7 mM and E5; 54.7 mM) possessed almost equal AOB and AOA amoA gene copies. Municipal WWTPs, which contain relatively low levels of ammonium (M2; 2.9 mM, M5; 3.1 mM), possessed more AOA amoA genes than AOB amoA genes by at least two orders of magnitude. Other municipal and industrial WWTPs possessed approximately equal amounts of both groups.

AOB occurrence was positively correlated to effluent chemical oxygen demand (COD; two-tailed nonparametric Spearman's rank correlation coefficient value of <0.05 [RS < 0.005]). However, the abundance of AOA amoA genes was negatively correlated to effluent COD (RS < 0.005). When COD increased, AOB also increased and AOA decreased. The abundance of AOB amoA genes was positively correlated to ammonia concentration (RS < 0.05), whereas the abundance of AOA amoA genes was not correlated to ammonia concentration (RS < 0.1933).

3.2. Enrichment cultures

In order to determine the optimal ammonia concentration for AOA growth, five different ammonium concentrations were tested (1, 3, 5, 10, and 20 mM). The results demonstrated that enriched AOA strains grew faster at 1 mM ammonium concentrations, whereas 20 mM of ammonium concentrations inhibited cell growth. Cells maintained growth at 3 and 5 mM ammonium concentrations, but were unable to consume all ammonia and with a relatively slower growth rate. At 3 mM ammonium concentration, up to 2.9 mM nitrite was produced, and up to 3.5 mM nitrite was produced at 5 mM ammonium concentration; and 10 mM ammonia concentrations greatly retarded cell growth. Only 8%–9% of ammonia was consumed during 35 d of incubation period. All results are shown in Fig. 2.

Growth of all three AOA enrichment cultures was greatly retarded at 10 mM ammonia concentrations, and only ~8% of ammonia was consumed, whereas 20 mM ammonia concentrations inhibited growth in all enrichment cultures. According to the growth assays, it is possible to conclude that AOA grows best at 1–3 nM ammonia concentrations. AOA also grew in around 5 mM ammonia concentrations; up to ~60% of ammonia was consumed and cell growth was monitored, yet with a respectively slower growth rate than at 1 and 3 mM concentrations. Enrichment strain V and enrichment strain 123 are soil archaeon, whereas *N. maritimus* is a marine archaeon; therefore, our results indicate that both marine and soil AOA have similar responses to different ammonia concentrations and cell growth is monitored best at ~1 mM.

After 15 d of incubation, Q-PCR was used to calculate the number of cells in AOA cultures. Q-PCR efficiency for *N. maritimus* and enrichment culture strain V were 90% and 92%, respectively. Q-PCR results showed that 1.00×10^7 cells/mL were present at 28°C in defined medium containing 1 mM ammonium; and 2.05×10^7 cells/mL at 28°C in defined medium containing 3 mM ammonium. For the strain V enrichment culture, Q-PCR results showed that 9.79×10^6 cells/mL were present at 37°C in defined medium containing 1 mM ammonium, and 1.17×10^7 cells/mL at 37°C in defined medium containing 3 mM ammonium. Both enrichment strains of AOA grew best at 1–3 mM ammonium concentration.

AOB occurrence and the effective environmental factors have been well studied. However, there is very limited knowledge about the presence of AOA in WWTPs and how operational parameters affect AOA abundance in engineered

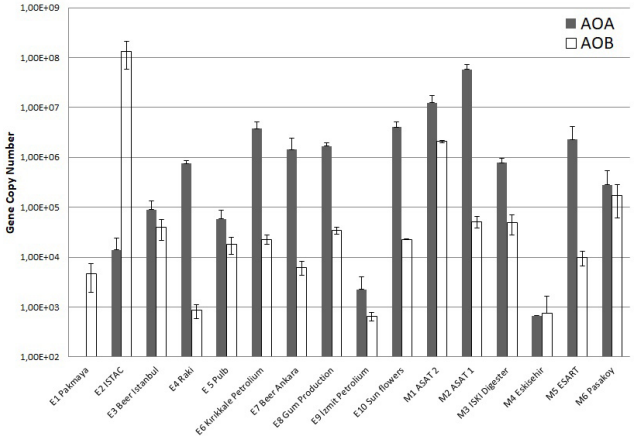

Fig. 1. Quantitative analysis of archaeal and bacterial amoA genes.

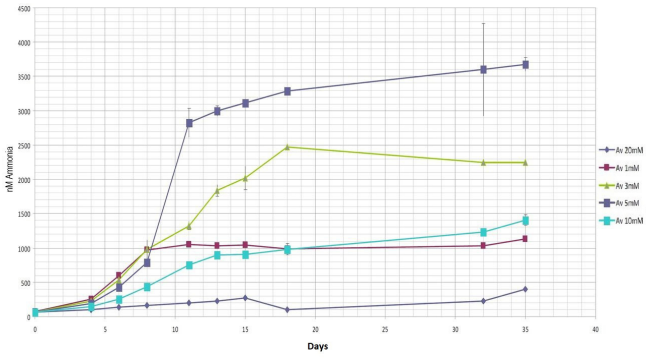

Fig. 2. Near-stoichiometric conversion of ammonia to nitrite by AOA enrichment culture (~96%) in archaeal enrichment media containing ammonium chloride and bicarbonate as sole energy and carbon sources, respectively. Nitrite production was checked in triplicate as described.

systems. Treatment plant environments are highly complex; and therefore, it is not easy to conclude which parameter mostly affects AOA occurrence [33].

The relative copy number of AOA and AOB amoA genes varied between the WWTPs with respect to ammonia concentrations. The archaeal amoA gene copy number per cell was 1, whereas its bacterial counterparts were 2.5 [7,34]. In that study, when these copy numbers were taken into account, the AOA amoA gene copy number outnumbered 13 WWTPs and AOB amoA gene copy number was greatest only at the highest ammonia-containing WWTPs such as İSTAÇ and Pakmaya. The AmoA gene copy number of both lineages was equal in Eskisehir WWTP. In accordance with our study, the abundance of AOA outnumbered AOB in low ammonia load in three domestic WWTPs [17], two municipal WWTPs [18], four industrials WWTPs [2], seven municipal WWTPs [35], and a municipal wastewater [19]. On the other hand, some studies revealed that AOB communities were dominant over AOA. Gao et al. [22] studied ten WTTPs, including three full-scale industrial WTTPs, four full-scale municipal WTTPs, and three lab-scale WTTPs, and they found that AOB communities were dominant over AOA. Similar results were also found by Wells et al. [36] and Zhang et al. [20] at a full-scale municipal WTTP and eight full-scale WTTPs, respectively. Bai et al. [17] and Gao et al. [21] reported that AOB was the dominant ammonia oxidizer at three industrial WTTPs and eight full-scale WTTPs, respectively. A lab-scale study of Jin et al. [37] also showed dominance of AOB. Archaeal AMO had higher affinity to ammonia than its bacterial counterpart [38]. Generally, AOA was more abundant in environments with low ammonia concentrations, which was consistent with our results.

Although the presence of both AOB and AOA was observed in all samples and probably simultaneously contributed to nitrification, AOA was dominant in the WWTPs with low ammonia concentration, whereas AOB was the dominant nitrifier in the WWTPs with high ammonia concentration. Our enrichment work showed that the AOA strains studied grew faster at 1 mM ammonium concentrations and that 20 mM of ammonium concentration inhibited cell growth. Cell growth was observed at 3 and 5 mM ammonium concentrations, albeit with a relatively slower growth rate, and they were unable to consume all the ammonia. These findings were consistent with studies of Bai et al. [17] and Limpiyakorn et al. [18,39].

These results could suggest that AOA may be adapted to low ammonia concentrations. Culture studies also showed that enriched AOA strains grew faster at 1 mM ammonium concentrations; a slower growth rate was observed at around 5 mM concentrations, whereas 20 mM of ammonium inhibited cell growth. Evidence from Sauder et al. [19] and Fukushima et al. [40] supports our enrichment studies. They reported that only archaeal amoA mRNA expression was repressed at high ammonium level. AOA could prefer low ammonia [41], and also AOA growth was depressed by high ammonium load [42], in concordance with our results. Significant AOA occurrence in all municipal WWTPs suggests that AOA may potentially contribute to ammonia oxidation in WWTPs with low ammonium content.

The results of this study may change our thoughts about nitrogen removal in WWTPs, especially domestic ones. In light of such information, AOA should be taken into consideration as a potential player ammonia oxidation in WWTPs, specifically where ammonia levels are low. These high-level ammonia-containing WWTPs (E1; 101.7 mM and E2; 104.6 mM) showed at least three times more AOB amoA genes than AOA amoA genes. One reason for AOB dominance over AOA in this study might be the relatively higher ammonia concentration in industrial WWTPs than in domestic plants (6.7–104.6 mM). Some studies reported a similar correlation for industrial bioreactors and WWTPs [17,18,20,36,43]. Contradictory to our study, Kayee et al. [35] reported that AOB amoA genes outnumbered AOA amoA genes in a plant with a lower concentration of ammonia (8.5–9.7 mg/L). Gao et al. [21] studied several WWTPs operating under low ammonia concentration and found different results. In view of these studies, the effect of ammonia load on the abundance of AOA is still unclear and more research must be conducted in order to understand the relationship between ammonia level and AOA abundance. Our studies showed that both marine and soil AOA have a similar response to different ammonia concentrations and cell growth is monitored best at ~1 mM. Similar to our study, Chen et al. [44] showed that elevated ammonia concentrations in soil could be unfavorable for AOA growth.

AOB amoA genes abundance was positively correlated to effluent COD ($p < 0.05$), but AOA amoA gene abundance was negatively correlated to effluent COD ($p < 0.05$). Park et al. [45] showed that low DO level might have facilitated the growth of *Thaumarchaeota* in WWTPs, but controversial results were also found in the study of Kayee et al. [35]. The authors found high numbers of amoA genes in WWTPs with elevated DO concentration of 3.25 mg/L. Sato et al. [46] showed that with the increase in NH_4 concentration in the pilot-scale membrane bioreactor, *Nitrosomonas*-related AOB drastically increased and became dominant, whereas in the initial sludge of the reactor, AOA was dominant, and the relative abundances of AOB were quite low.

AOB amoA gene abundance was positively correlated to ammonia concentration (two-tailed nonparametric Spearman's rank correlation coefficient value of <0.05). However, AOA amoA gene abundance was not correlated to ammonia concentration (two-tailed nonparametric Spearman's rank correlation coefficient value of <0.1933). DNA-based Q-PCR studies of amoA gene gave only evidence about richness of AOA and AOB. In situ activity of AOA was in debate in engineered systems. Sole direct evidence of in situ activity of AOA in WWTPs was provided by Mussmann et al. [2]. They performed a nitrification model and concluded that the ammonia oxidation in the reactor could be enough to feed only 0.01%–1% of AOA found in the plant and would not be enough to support a population of autotrophic ammonia oxidizers. They speculated that other metabolic pathways of AOA could support AOA growth in plants. Therefore, whether the AOA gained most of their energy from chemoautotrophic ammonia oxidation in the plant is questionable and merits further study. Weber et al. [47] provided the first evidence for growth of *Thaumarchaeota* without ammonia oxidation in soil. Group 1.1c *Thaumarchaeota* was optimal at 30°C and amoA gene could not be detected with Q-PCR. Glutamate and casamino acids stimulated archaeal growth. Studies showed that ammonia and DO might be the most

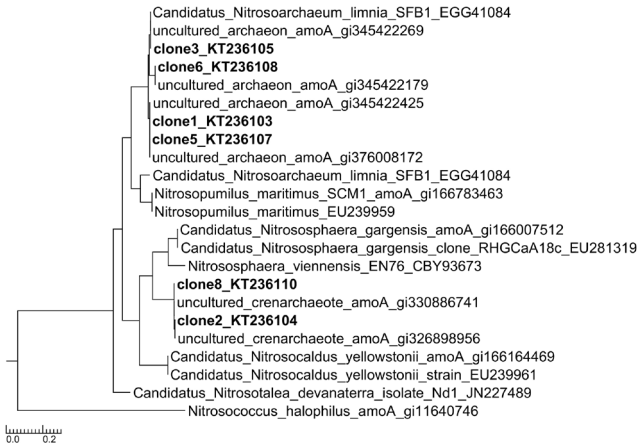

Fig. 3. A maximum likelihood method tree based on archaeal amoA gene sequence. The tree showed phylogenetic tree relationships among archaeal amoA sequences from ASAT 2 domestic WWTP. Phylogeny was reconstructed 9 clone sequences and reference sequences. *Nitrosococcus halophilus* (AOB) amoA sequence was used as outer group. The tree was constructed using MEGA version 5.

important parameters to affect the AOA community structure and abundance. However, ammonia and DO are used by AOB and AOA; these microorganisms could be in competition for the ammonia and oxygen in WWTPs [39].

3.3. Communities of ammonia-oxidizing archaea

AOA amoA gene of M2 (ASAT2 Lara) WWTP sample was chosen for phylogenetic analysis of AOA at a functional gene level. Archaeal amoA sequences affiliated to AOA amoA sequences were previously deposited in the GenBank database with 94%–100% identity. The MEGA program package version 5.0 [32] (http://www.megasoftware.net/) was used for the phylogenetic analysis. Nine AOA amoA sequences were aligned with reference sequences for the construction of the phylogenetic tree shown in Fig. 3. Most of the sequences were closely clustered among a marine archaeon (Group 1.1a). Few sequences were affiliated with a soil archaeon (Group 1.1a), but no sequence was found affiliated to thermophilic AOA archaeon and *Candidatus Nitrosotalea devanaterra* isolate were found. Clone 3 and 6 was affiliated with *Candidatus Nitrosoarchaeum limnia*, and Clone 2 and 8 was affiliated to *Candidatus Nitrososphaera gargensis* and *Nitrososphaera viennensis*. A novel AOA strain named *Candidatus Nitrosotenuis cloacae* with low-salinity adaptation and possibly enhanced cell-biofilm attachment ability was enriched from the sludge of a wastewater treatment system [24].

4. Conclusion

Occurrence and copy number of AOA and AOB varied among the WWTPs, especially depending on ammonia concentrations. The potential role of AOA on nitrification in domestic WWTPs, which have low ammonium content, was proposed because of the significant abundance of AOA amoA genes in these plants. The findings of this study and others could change our knowledge on ammonia oxidation in WWTPs. It is also considered that AOA could be new player involved in nitrification in WWTPs. To clarify the roles of AOA and AOB in WWTPs, further combinations of in situ activity investigations and molecular tools studies should be undertaken.

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Conflict of interest

The authors declare no conflict of interest.

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