Active ammonia-oxidizing bacteria and archaia in wastewater treatment systems

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Abstract
Ammonia-oxidizing bacteria (AOB) and archaia (AOA) are two microbial groups mediating nitrification, yet little is presently known about their abundances and community structures at the transcriptional level in wastewater treatment systems (WWTSs). This is a significant issue, as the numerical abundance of AOA or AOB at the gene level may not necessarily represent their functional role in ammonia oxidation. Using amoA genes as molecular markers, this study investigated the transcriptional abundance and community structure of active AOA and AOB in 14 WWTSs. Quantitative PCR results indicated that the transcriptional abundances of AOB amoA (averaged: 1.6 × 10⁸ copies g⁻¹ dry sludge) were higher than those of AOA (averaged: 3.4 × 10⁷ copies g⁻¹ dry sludge) in all WWTSs despite several higher abundances of AOA amoA at the gene level. Moreover, phylogenetic analysis demonstrated that Nitrosomonas europaea and unknown clusters accounted for 37.66% and 49.96% of the total AOB amoA transcripts, respectively, suggesting their dominant role in driving ammonia oxidation. Meanwhile, AOA amoA transcripts were only successfully retrieved from 3 samples, and the Nitrosospira sister cluster dominated, accounting for 83.46%. Finally, the substrate utilization kinetics of different AOA and AOB species might play a fundamental role in shaping their niche differentiation, community composition, and functional activity. This study provides a basis for evaluating the relative contributions of ammonia-oxidizing microorganisms (AOMs) to nitrogen conversions in WWTSs.

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Introduction
Nitrogen transformation is one of the most basic biogeochemical cycles and plays a primary role in human life and ecological environments (Galloway et al., 2004; Zhao et al., 2019). Microbe-mediated nitrification is the core component of nitrogen transformation in wastewater treatment systems (WWTSs) and includes ammonia oxidation to nitrite catalyzed by ammonia-oxidizing microorganisms (AOMs) and nitrite oxidation to nitrate conducted by nitrite-oxidizing bacteria (NOB) (Zumft, 1997). In particular, ammonia oxidation, depending on the catalysis of ammonia monoxy-
genase (AMO) harbored by AOMs, is not only the first and rate-determining step of nitrogen removal but also a crucial process for the stable operation of entire WWTSs (Kowalchuk and Stephen, 2001). Ammonia-oxidizing bacteria (AOB) have been considered as the sole AOM harboring the amoA gene encoding AMO since they were first isolated in 1890 (Winogradsky, 1890). In 2004 and 2005, the archaeal amoA gene was successively detected in marine (Venter et al., 2004) and soil genomes (Treusch et al., 2005), which sparked a great deal of interest about whether archaea can also participate in the ammonia oxidation process. Subsequently, in 2005, the first archaeal strain harboring a distinct amoA gene, Nitrosopumilus maritimus, was successfully isolated (Könneke et al., 2005). Afterward, a series of molecular studies confirmed the widespread existence of ammonia-oxidizing archaea (AOA) and their significant role in the ammonia-oxidation process in various ecosystems (Damashek et al., 2014; Walker et al., 2010; Zhao et al., 2020).

The coexistence of AOB and AOA has been confirmed in WWTSs, although which is more dominant remains a matter of debate. In most municipal and industrial wastewater treatment plants (WWTPs), AOB has an abundance at least two to four orders of magnitude higher than AOA (Gao et al., 2014; Wells et al., 2009; Zhang et al., 2015). In contrast, numerical dominance of AOA over AOB has been found in some WWTPs under extreme conditions, such as moderate toxicity or low temperature, as well as in nitrifying trickling filters and moving bed bioreactors with lower levels of influent ammonia (5.6–11.0 mg N L⁻¹) (Bai et al., 2012; Roy et al., 2017; Srithep et al., 2018). The reason behind this finding is that differences in process parameters and water conditions lead to a competitive relationship and different niches between AOB and AOA in different WWTPs, which impact the prevalence of AOA or AOB (Castellano-Hinojosa et al., 2018; Zhang et al., 2015).

Notably, the high numerical abundance of AOA or AOB in an ecosystem might not necessarily reflect their predominant functional role in ammonia oxidation (Bahram et al., 2018; Mußmann et al., 2011). For instance, Jia and Conrad (2009) found that AOA amoA genes were more abundant than AOB amoA genes in cultivated soils, but with help of the DNA stable isotope probe method, they found that AOB, rather than AOA, played a dominant role in ammonia oxidation. Similarly, Di et al. (2009) observed that the abundance of AOA was almost equal to that of AOB, but as the ammonia nitrogen concentration increased, only the number of AOB increased by three to ten times. Ling et al. (2018) found that either AOB or AOA might actively participate in gene expression and carry out ammonia oxidation despite their low abundance at the DNA level in coral reef ecosystems with excess ammonia located in Sanya Bay and Yongxing Island, South China Sea. Chen et al. (2017) reported that the copy numbers of AOA and AOB amoA genes amplified by PCR using cDNA as a template were higher than those amplified using DNA. Furthermore, considering sensitivity and specificity, amoA mRNA has been characterized as a biomarker of ammonia oxidation activity in wastewater treatment processes (Aoi et al., 2004). Therefore, quantitative comparisons of the two AOMs based on the transcriptional level can provide a better interpretation of their functional activities and relative contributions to the nitrogen transformation process.

In the present study, the transcriptional abundance and diversity of AOB and AOA in 14 activated sludge samples were compared using reverse transcription, PCR amplification, and high-throughput sequencing of amoA genes. This study contributes to evaluating the relative contribution of active AOA and AOB to nitrification and promoting the management of nitrogen transformation processes in engineering systems.

### 1. Materials and methods

#### 1.1 Sample collection

Approximately 50 mL of activated sludge samples was collected from the aerobic tanks of fourteen full-scale nitrifying WWTSs, and 6 mL of activated sludge was collected from the aerobic tanks of seven WWTSs and stored in RNA storage reagent from September 2016 to October 2017. The samples were immediately transported to the laboratory in ice and kept frozen at -80°C until DNA extraction and RNA extraction. The performance profiles and operational parameters of the 14 WWTSs are listed in Table 1.

#### 1.2 DNA, RNA extraction and cDNA synthesis

Total DNA was extracted from 2.0 mL activated sludge using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer’s protocols. Total RNA was extracted using the E.Z.N.A.® Soil RNA Kit (Omega, USA), and cdNA was synthesized from total RNA using the FastQuant RT Kit (TianGen Biotech Co., Ltd., China). The potential genomic DNA contamination was removed with a 10 μL of gDNAse mixture containing 2 μL of 5 × gDNA Buffer, 2 μg of total RNA, and RNase-free ddH₂O. Reverse transcription reactions were performed in 10 μL mixture containing 2 μL of 10 × King RT Buffer, 1 μL of FastKing RT Enzyme Mix, 2 μL of FQ-RT Primer Mix, and 5 μL of RNase-free ddH₂O.

#### 1.3 Quantitative PCR and reverse transcriptional qPCR

To quantify the abundances of amoA genes in AOA and AOB, a quantitative polymerase chain reaction (qPCR) based on DNA templates was conducted with the specific primer set archamoA 19F/616R (Pester et al., 2012) and amoA 1F/2R (Rotthauwe et al., 1997). Likewise, the abundances of amoA gene transcripts in active AOA and AOB were quantified based on RNA reversed cDNA templates. Both qPCRs were performed in a 20 μL mixture containing 2 μL of DNA or cDNA templates, 10 μL of 2 × SuperReal PreMix Plus, 2 μL of 50 × ROX Reference Dye, 0.5 μL of each primer, and 5 μL of RNase-free ddH₂O. Amplification was performed under the following conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for AOA (58°C for AOB) for 30 sec, and extension at 72°C for 1 min for AOA (45 sec for AOB). Negative controls were carried out using pure water instead of PCR templates. Standard curves were generated using standard plasmids containing the gene fragments as templates. Finally, the abundances of amoA genes in AOA and AOB were normalized by copy number per gram of dry sludge.
Table 1 – Operational parameters and performance profiles of the 14 WWTSs. All the data were averaged values within one week before the sampling day (Wang et al., 2018).

<table>
<thead>
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<th>Process</th>
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<th>HRT (hr)</th>
<th>SRT (day)</th>
<th>DO (mg/L)</th>
<th>T (°C)</th>
<th>pH</th>
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HRT: hydraulic residence time; SRT: solid retention time; DO: dissolved oxygen; T: temperature; MLSS: mixed liquor suspended solids; COD: chemical oxygen demand; BOD: biological oxygen demand; NH₄-N: ammonium nitrogen; TN: total phosphorus; TP: total phosphorus; SS: suspended solids.
1.4. High-throughput sequencing and phylogenetic analysis

To explore the phylogeny of amoA genes and their transcripts in AOA and AOB, purified PCR products obtained from DNA and cDNA templates were sequenced on the illumina MiSeq platform. Raw sequences were screened and filtered using QIIME software (Caporaso et al., 2010) and public scripts (Liu et al., 2020). Subsequently, the remaining clean reads were clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff. The diversity indices (i.e., Shannon and Simpson diversity) of the archaeal and bacterial amoA genes were calculated based on the OTU data (Wang et al., 2018). The representative sequences retrieved in this study were submitted to GenBank under accession numbers MT346380 - MT346449 for AOA amoA genes, MT346450 - MT346531 for AOB amoA genes, MT346532 - MT346539 for AOA amoA transcripts, and MT346540 - MT346578 for AOB amoA transcripts. Phylogenetic neighbor-joining trees were constructed using the representative sequence of each OTU and the reference sequences retrieved from the NCBI database using MEGA 6.0 (Kumar et al., 2016). The relative confidence of the tree topologies was estimated by bootstrap analysis based on 1,000 replicates.

1.5. Statistical analysis

Redundancy analyses (RDA) were performed to study the effects of operational parameters of the 14 WWTSSs on AOA and AOB amoA at the gene and transcript levels analyzed using R software.

2. Results and discussion

2.1. Abundances of AOA and AOB amoA at the gene and transcriptional levels

As shown in Fig. 1a, AOB amoA genes were detected in all 14 activated sludge samples with abundances varying from 1.74 × 10^6 to 2.65 × 10^9 copies g^-1 dry sludge. AOA amoA gene abundances were generally lower than AOB amoA gene abundances, ranging from 1.33 × 10^6 to 9.48 × 10^8 copies g^-1 dry sludge, and were below the detection limit in 3 samples (LF, XJH, and YC). However, the abundance of AOA amoA genes in 4 samples (JN, LY, BB, and YQ) reached 10^6 copies g^-1 dry sludge, which was numerically higher than that of AOB.

The transcriptional activities of AOA and AOB were quantified by using cDNA as a qPCR template in 7 samples (Fig. 1b). The results indicated that the abundance of AOB amoA transcripts ranged from 1.8 × 10^7 to 3.8 × 10^8 copies g^-1 dry sludge, with an average of 1.6 × 10^8 copies g^-1 dry sludge. In contrast, AOA amoA transcripts were lower in all 7 samples, with an average of 3.4 × 10^7 copies g^-1 dry sludge. The lowest abundance of AOA amoA transcript (1.9 × 10^6 copies g^-1 dry sludge) was present in sample YQ and the highest (1.1 × 10^9 copies g^-1) in sample LY.

There has been considerable controversy about the relative contributions of AOA and AOB to nitrification in various ecosystems. Consistent with previous studies based on DNA templates (Gao et al., 2013; Islam et al., 2019; Sinthusith et al., 2015), this study showed that the abundance of AOB was one to three orders of magnitude higher than that of AOA in ten out of fourteen WWTSs samples, and only in four samples was the abundance of AOA higher. However, quantitative analysis of the transcriptional activity of the two AOMs revealed that AOB amoA genes were always more abundant than AOA amoA genes, reflecting the predominance of AOB over AOA in nitrification. Although some studies have found that AOA primarily drive the nitrification activities rather than AOB, they may contribute differently to the nitrification process in different environments (Fan et al., 2018; Srithep et al., 2018; Zhang et al., 2012). On the one hand, as ammonia-rich environments, WWTSSs provide an advantage for AOB growth due to their lower affinity for ammonia than AOA; however, AOA tend to gain a competitive growth advantage in environments with low ammonia availability due to their k-type reproductive strategy (with relatively slow growth and the ability to degrade more complex substrates) (Zhou et al., 2016).
For instance, Zhang et al. (2015) reported that the metabolic activity of AOA was inhibited in systems with a high concentration of ammonia nitrogen (approximately 38.6 mg/L), while the growth of AOB was promoted. Dimitri Kils et al. (2017) documented that higher ammonia affinity could promote the advantage of AOA over AOB through kinetic analysis. On the other hand, as metabolically versatile microorganisms, AOA can utilize some organic matter, such as antibiotics or petroleum, suggesting that the relatively high abundance of AOA amoA in some WWTSs might result from their metabolic versatility instead of autotrophic growth (Mußmann et al., 2011; Zhang et al., 2015). In this study, redundancy analysis (Appendix A Fig. S3) showed that influent ammonia (NH$_4$-Niff) and other water parameters, such as temperature (T) and dissolved oxygen (DO), had negative effects on the abundance of AOA amoA genes. Meanwhile, NH$_4$-Niff and hydraulic residence time (HRT) were found to be negatively correlated with the activity of AOA amoA genes (Appendix A Fig. S4). Thus, it could be inferred that the ammonia-rich environment and complex wastewater compositions jointly regulated the relative abundances of AOB and AOA.

### 2.2. Diversity and phylogeny of AOA and AOB amoA genes

High-throughput sequencing generated a total of 108,562 sequences of AOA amoA from 11 samples and 155,711 reads of AOB amoA from 13 samples. Sequencing results of the AOA

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**Fig. 2** – Heatmap displaying OTU numbers of AOA (a) and AOB (b) amoA genes.
The distribution of AOA OTUs in each WWTSs is shown in Fig. 2a. In the XT sample with the highest Shannon and Simpson diversity (Appendix A Table S1), 35 OTUs were detected and the abundance of OTUs was uniformly distributed. In the YQ sample with the smallest Shannon and Simpson diversity, only 6 OTUs were identified, and almost all the sequences were clustered into OTU1. Notably, 40.27% of all the sequences belonged to OTU1 affiliated with the Nitrosospaera sister cluster, which was also the most dominant OTU in the FX, YS, LH, and LY samples. The constructed phylogenetic tree of AOA amoA genes fell into four branches, namely, Nitrosospaera sister, Nitrosospaera, Nitrosotalea, and Nitrosopumilus clusters (Appendix A Fig. S1). As shown in Fig. 3a, a total of amoA gene in NJ, YC, YC1, and AOB amoA in LF were unavailable due to PCR amplification failure, which probably resulted from the low abundance of amoA genes or severe nonspecific amplification. These sequencing reads were classified into 70 operational taxonomic units (OTUs) for AOA amoA and 82 OTUs for AOB amoA at the 97% identity level. Coverage values higher than 99.95% in all samples indicated that the AOA and AOB amoA genes obtained by the current sequencing depth were close to saturation (Appendix A Table S1).
19 OTUs (including OTU1) containing 81.79% of the total sequences belonged to the Nitrososphaera sister cluster, which represented the highest abundance in most samples. A total of 44 OTUs, accounting for 12.11% of the total AOA amoA sequences, were assigned to the Nitrososphaera cluster. Although the Nitrosopumilus cluster contained 5.63% of the total AOA amoA sequences, more than half were distributed in the BB sample. In addition, a small number of the Nitrosotalea cluster were detected in the YS and XT samples.

As shown in Fig. 2b, there were significant differences in the distributions of AOB OTUs in various WWTSSs. The highest Shannon and Simpson diversity indices were observed in the YS sample, while the lowest indices were found in the LH sample (Appendix A Table S1). Among the 82 OTUs, OTU1 was the most abundant, accounting for 26.61% of the total number of AOB amoA sequences. Especially in the LY and YQ samples, the abundance of OTU1 reached as high as 80% of the total sequences. OTU2 was widely distributed in most samples but with low abundance, except for its predominance in the LH sample. The phylogenetic tree based on AOB amoA sequences displayed two branches (Appendix A Fig. S2), including Nitrosomonas (containing 65 OTUs) and Nitrosospira (17 OTUs). The Nitrosomonas cluster consisted of 6 subbranches, in which subcluster N. oligotropha including 32 OTUs was the only group widely distributed in all samples (Fig. 3b). In terms of abundance, the Nitrosomonas-like, N. oligotropha, and N. europaea clusters were the most dominant and the N. europaea cluster accounted for 99.71% of the total sequences in the YC1 sample.

Fig. 5 – Phylogenetic tree of reference sequences and OTU representative sequences retrieved from AOA (a) and AOB (b) amoA transcripts.
sample. Additionally, only 1.11% of the sequences were assigned to the Nitrosospira cluster, suggesting that it was not the main AOB species in WWTSs.

2.3. Diversity and phylogeny of AOA and AOB amoA transcripts

To explore the transcriptional profiles of the two AOMs, phylogenetic analysis of the active amoA genes was carried out in 7 WWTSs using high-throughput sequencing. Due to the low level of transcription of functional genes and unsuccessful amplification in some samples, 3 and 6 samples remained for subsequent analysis of AOA and AOB amoA sequences, respectively. Eight and 39 OTUs, respectively, were obtained from 31,947 AOA amoA transcript sequences and 71,999 AOB sequences based on a 97% similarity cutoff. Good's coverage values for each sample approaching 100% suggested that the active AOMs were well represented by the current sequencing results (Appendix A Table S2).

The transcriptional abundances of the 8 OTUs retrieved from active AOA amoA genes are shown in Fig. 4a. The Simpson and Shannon diversity indices were found to be the highest in samples XJH and LY, respectively (Appendix A Table S2). OTU1, the most abundant OTU, containing 40.51% of the total number of sequences, was dominant in the LY (42.9%) and BB (72.33%) samples. OTU2, accounting for 13.04% of total transcripts, dominated in the LY sample. OTU3 occupied 85% of active AOA amoA gene sequences in the single XJH sample. As shown in the phylogenetic tree of active AOA amoA gene sequences (Fig. 5a), the OTUs fell into three groups, namely, Nitrososphaera (2 OTUs), Nitrososphaera sister (5 OTUs), and Nitrosopumilus clusters (1 OTU). The Nitrososphaera sister cluster was detected in all samples and constituted almost all the active AOA amoA gene sequences in the XJH sample, while the Nitrosopumilus cluster existed only in sample BB (Fig. 6a).

As shown in Fig. 4b, the distributions of the 39 OTUs of the active AOB amoA genes were significantly different in the 6 samples. The highest OTU number (21) and the highest diversity of active AOB amoA genes were found in the YC sample, while the lowest OTU number (8) was obtained from the NJ sample (Appendix A Table S2). Among the 39 OTUs, OTU4 accounted for the largest number (25%) of total sequences and was the only OTU that could be detected in all 6 samples. According to phylogenetic analysis (Fig. 5b), the 39 OTUs were branched into two groups, i.e., the Nitrosomonas cluster (35 OTUs) holding over 99%, and the Nitrosospira cluster (4 OTUs) containing less than 1% of AOB active amoA gene sequences. Furthermore, among the five subgroups of the Nitrosomonas cluster, the N. europaea and unknown subclusters were dominant overall, while N. oligotropha was abundant only in the LY sample and represented nearly half of the total sequences (Fig. 6b).

Phylogenetic analysis showed that the Nitrososphaera sister cluster was the dominant AOA species in WWTSs at both the gene and transcriptional levels. Similar observations have also been reported in previous studies on other terrestrial ecosystems, such as river sediments and sewage treatment plants, in which very few sequences were affiliated with the Nitrosopumilus and Nitrosotalea clusters (Chen et al., 2017; Wang et al., 2019). This phenomenon could be explained by the kinetic consideration that the Nitrososphaera sister cluster possesses a relatively low affinity for ammonia compared to its counterparts, implying that they may have a high tolerance for and adaptation to ammonia. An isolated strain affiliated with Nitrososphaera sister could still grow well at ammonia concentrations up to 15 mM (Sauder et al., 2017; Tournas et al., 2008).

Fig. 6 – Composition and distribution of AOA (a) and AOB (b) amoA transcripts at the species level in different activated sludge samples.
The composition of AOB exhibited inconsistent patterns with respect to the gene and transcript sequencing results. Among the two branches of AOB, the Nitrosomonas cluster predominated over Nitrosospira at the gene level, which could be attributed to the fact that Nitrosomonas has a growth advantage in environments with high ammonia concentrations, while Nitrosospira is more adapted to an oligotrophic environment (Li et al., 2020; Yang et al., 2020). Furthermore, our results also revealed that Nitrosomonas europaea and unknown were the most active, although Nitrosomonas-like, N. oligotropha, and N. europaea were the dominant AOB populations in WWTSs, consistent with previous studies that documented the dominance of N. europaea in bioreactors and WWTSs (Gao et al., 2014; Wells et al., 2009). This finding was in agreement with their ecophysiologic properties that Nitrosomonas europaea tends to play a leading role in environments with high ammonia levels due to its high Ks value for ammonia (Koops and Pommerening-Röser, 2001).

3. Conclusions

The numerical abundances and community structures of AOA and AOB obtained from amoA transcripts were distinct from those obtained from the gene level. The abundances of AOA amoA transcripts were found to be lower than those of AOB in all WWTSs despite several opposite results at the amoA gene level. The Nitrososphaera sister cluster was confirmed to be the dominant AOA species actively participating in ammonia oxidation. For active AOB, Nitrosomonas europaea and unknown clusters were the most active. The substrate utilization kinetics of different AOA and AOB species determined their niche differentiation, community composition, and functional activity. Taken together, the niche partition of AOA and AOB species played a fundamental role in determining their numerical dominance and shaping their community composition and functional activity. The investigation of AOMs based on transcriptional analysis can provide a complete perspective for evaluating their relative contributions to nitrogen transformation and the biogeochemical nitrogen cycle in various ecosystems.

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Appendix A Supplementary data


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