Functional relationship between ammonia-oxidizing bacteria and ammonia-oxidizing archaea populations in the secondary treatment system of a full-scale municipal wastewater treatment plant

Golam M. Islam\textsuperscript{1}, Peter Vi\textsuperscript{1}, Kimberley Ann Gilbride\textsuperscript{1,2,*}

1. Department of Chemistry and Biology, Ryerson University, 350 Victoria Street, Toronto, Ontario M5B 2K3, Canada
2. Ryerson Urban Water, Ryerson University, 350 Victoria Street, Toronto, Ontario M5B 2K3, Canada

ARTICLE INFO

Article history:
Received 15 November 2018
Revised 24 April 2019
Accepted 30 April 2019
Available online 10 May 2019

Keywords:
Wastewater treatment
Topic:
Ammonia-oxidation
Ammonia-oxidizing bacteria (AOB)
Ammonia-oxidizing archaea (AOA)
PCR
Digital droplet polymerase chain reaction (ddPCR).

ABSTRACT

The abundance of ammonia-oxidizing bacteria and archaea and their \textit{amoA} genes from the aerobic activated sludge tanks, recycled sludge and anaerobic digesters of a full-scale wastewater treatment plant (WWTP) was determined. Polymerase chain reaction and denaturing gradient gel electrophoresis were used to generate diversity profiles, which showed that each population had a consistent profile although the abundance of individual members varied. In the aerobic tanks, the ammonia-oxidizing bacterial (AOB) population was more than 350 times more abundant than the ammonia-oxidizing archaeal (AOA) population, however in the digesters, the AOA population was more than 10 times more abundant. Measuring the activity of the \textit{amoA} gene expression of the two populations using RT-PCR also showed that the AOA \textit{amoA} gene was more active in the digesters than in the activated sludge tanks. Using batch reactors and ddPCR, \textit{amoA} activity could be measured and it was found that when the AOB \textit{amoA} activity was inhibited in the anoxic reactors, the expression of the AOA \textit{amoA} gene increased fourfold. This suggests that these two populations may have a cooperative relationship for the oxidation of ammonia.

© 2019 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

Published by Elsevier B.V.

Introduction

In wastewater treatment, the activated sludge system is an engineered system employing aerobic and anaerobic processes to reduce the biochemical oxygen demand and eliminate toxicity by removing excess nutrients such as carbon, nitrogen and phosphorus. Nitrogen is most commonly present in wastewater in the form of ammonia (NH\textsubscript{3}) as a result of microbial decomposition of proteins, amino acids, uric acids and other compounds. If ammonia concentrations are not lowered to regulatory levels, problems associated with eutrophication, toxicity and poor discharge quality may arise (Holeton et al., 2011). To avoid these environmental consequences, ammonia concentration is decreased through a microbial-mediated process called ammonia oxidation, the first and rate limiting step of nitrification. This step in ammonia oxidation is carried out by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) which convert ammonia to hydroxylamine which is a reaction catalyzed by the key enzyme ammonia monoxygenase (AMO) (Rotthauwe et al.,...
1997). It is followed by the oxidation of hydroxylamine to nitrite using the enzyme hydroxylamine oxidoreductase (HAO) (Kimochi et al., 1998). Ammonia oxidation can be prone to recurring inhibition in many wastewater treatment systems because AOBs and AOAs are slow growing microorganisms with doubling times between 8 hr and several days (Martens-Habbena et al., 2009).

In domestic wastewater, AOB were detected in higher numbers than AOA when the dissolved oxygen conditions (Park and Noguera, 2004) and the influent ammonium concentrations (36.1–422.3 mg/L nitrogen) (Kim et al., 2013) were high. Until now 16 different AOB species have been cultured such as Nitrosomonas europaea and Nitrosonomas oligotropha. Ammonia-oxidizing bacteria have garnered considerable attention because they were thought to be the only group capable of performing the first and the rate-limiting step of nitrification. This traditional assumption changed when the first ammonia-oxidizing archaea strain Nitroso-

Nitrosopumilis maritimus was isolated using culture independent studies from the rocky substratum of a tropical marine aquarium tank (Könneke et al., 2009). This member of the newly proposed phylum Thaumarchaeota was also capable of chemolithotropic ammonia oxidation and thus challenged the proposed mechanisms of biogeochemical cycling of nitrogen. In marine and terrestrial environments, the widespread distribution of the putative Archeal amoA gene was also capable of amoA gene expression analysis and quantitative PCR (qPCR) respectively. Since the presence of various stages of treatment will be measured using end-point digital drop PCR (ddPCR) to monitor AOA amoA gene expression in laboratory batch cultures. These results will collectively demonstrate the cooperation of the two groups at the various stages of wastewater treatment and the effect of an inhibitor on their activity.

### 1. Methods and materials

#### 1.1. Wastewater samples and characteristics

Samples from the wastewater treatment process were collected using sterile containers from the aerobic, returned sludge and the secondary digester stages of the full-scale activated sludge system of the Humber wastewater treatment plant (WWTP) in Toronto. The wastewater treatment plant contains a total of eight aeration tanks, each equipped with ceramic fine bubble dome diffusers. Each of the eight aeration tanks (ATS) are in series, fed from one common primary effluent channel in the north, and one common primary effluent channel in the south, although some mixing of the flow from the north and south primary effluent channels is possible and common in order to balance flows. Mixed liquor from the aeration tanks flows to large quiescent final clarification tank where the activated sludge is allowed to settle. A controlled quantity of this sludge (labeled returned sludge) is returned to the aeration tanks in order to maintain a sufficient volume of biomass. The excess is removed as waste activated sludge and is either thickened or anaerobically digested prior to transfer to another plant for further treatment.

Primary sludge, scum and thickened waste activated sludge are fed into eight primary digesters. Sludge from the primary digesters is transferred into the two secondary digesters. The resulting anaerobically digested sludge (biosolids) is subsequently transferred to another plant for further treatment (City of Toronto, 2016).

For the experiments, seven 1 L samples were obtained from aeration tanks 2, 4, 6 and 8, the recycled sludge flow path and anaerobic secondary digesters tanks 1 and 2. The samples were collected and transported on ice (4°C) in sterilized 1-L plastic bottles and used immediately in the lab scale batch reactors. Samples for nucleic acid extractions were collected by centrifugation of 2 mL aliquots of each sample at 13,000 × g for 2 min. The pellets were stored at −20°C for subsequent DNA extraction or at −80°C for subsequent RNA extraction.

Monitoring of the parameters of the sampled effluent of the wastewater was not performed at the time of sampling, however, the yearly averages for the operational parameters of the activated sludge samples were obtained directly from the annual report published by the North Toronto and Humber wastewater treatment plants (City of Toronto, 2016) and are listed in Table 1.

#### 1.2. Reactor set-up

Eight 500 mL bioreactors were setup in 2 L containers. The reactors were seeded with either composite aerated tank or digester samples and subjected to two aeration conditions by
either continuous bubbling through an aquarium diffuser to maintain an aerated state, or placing an 1-inch thick layer of mineral oil on top of the sample to inhibit atmospheric oxygen from entering the system. A second identical set of reactors was also set up with the presence of allylthiourea (5 μg/mL) (Sigma–Aldrich, Canada) (Pogue and Gilbride, 2007), an AOB AmoA inhibitor. Table 2 lists the conditions in each of the reactors. The batch reactors were maintained for 48 hr with 1.5 mL samples collected initially and after 24 and 48 hr. Samples were centrifuged at 13,000 × g for 2 min and the pellets frozen at −20°C or −80°C for DNA and RNA isolation respectively.

1.3. DNA extraction

DNA was extracted from the frozen pellets containing the equivalent of 0.25 g of dry sludge using the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) following the manufacturer’s instructions. The extracted DNA was suspended in 50 μL of DNA free water. One microliter of extracted DNA was used to measure the concentration by Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA), and the remaining DNA was stored at −20°C. The presence of DNA was confirmed by gel electrophoresis on a 1.5% agarose gel.

1.4. End-point PCR

For detection of the AOB and AOA presence in the wastewater samples, end-point PCR was used. The 25 μL final volume PCR reaction included 12.5 μL of 2x PCR mastermix (Thermo Fisher Scientific, USA) (containing 0.4 mmol/L dNTPs, 0.05 unit of Taq DNA Polymerase and 4 mmol/L MgCl2), 0.5 μL of the appropriate forward and reverse primer (10 μmol/L), 1.25 μL of DMSO (Sigma–Aldrich, Canada), 0.343 μL of bovine albumin serum (BSA) (Promega, Canada) and 8.9 μL of ultra pure Millipore water. One hundred ng of template DNA was added.

The amplification of the AOB 16S RNA gene used primers CTO189f and CTO654r targeting a 465 bp fragment. The reaction was performed in a BIO-RAD T100 (Life Science Research Division, USA) PCR thermocycler with an initial denaturation step performed at 93°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 1 min, and elongation at 72°C for 45 sec. A final elongation was performed at 72°C for 5 min.

The amplification for the AOA 16S rRNA gene used primers Crenar771f and Crenar957r targeting a 186 bp fragment. The reaction was performed in a BIO-RAD T100 (Life Science Research Division, USA) PCR thermocycler with an initial denaturation step performed at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. A final elongation was performed at 72°C for 10 min.

Primers amoA-1F and amoA-2R (Rotthauwe et al., 1997) were used to amplify the 491 bp fragment of AOB amoA gene. The PCR amplification was performed using an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step at 72°C for 1 min.

The amoA gene fragment (635 bp) of AOA was PCR-amplified using the primers Arch-amoAF and Arch-amoAR (Francis et al., 2005). The PCR amplification protocol was as follows: a 10-min initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step at 72°C for 15 min.

The sequences of all primers used are listed in Table 3 and all PCR products were confirmed by visualization on a 1.5% agarose gel.

1.5. Quantitative PCR (qPCR)

Abundance information was determined using quantitative PCR (qPCR) on a Lightcycler 480 qPCR machine (Roche, Mannheim, Germany) to obtain Cp values with the total bacterial community and each of the ammonia-oxidizing populations using the specific primers.

For PCR targeting the 16S rRNA gene of the total heterotrophic population, the specific primer pair 341F and V3R was used (Muyzer et al., 1993). The 25 μL final volume PCR reaction included 12.5 μL of 2x PCR mastermix (Thermo Scientific, Canada) (containing 0.4 mmol/L dNTPs, 0.05 unit of Taq DNA Polymerase and 4 mmol/L MgCl2), 0.5 μL of forward and reverse primer (10 μmol/L), 1.25 μL of DMSO (Sigma–Aldrich, Canada), 0.343 μL of bovine albumin serum (BSA) (Promega, USA) and 8.9 μL of ultra pure Millipore water. Approximately 100 ng of template DNA was added. The amplification was performed in a BIO-RAD T100 (Life Science Research Division, USA) PCR thermocycler with an initial denaturation step performed at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C 1 min, and elongation at 72°C for 1 min. A final elongation was performed at 72°C for 2 min.
For each AOB and AOA qPCR was performed in triplicate sets. The qPCR reaction included a total volume of 20 μL, containing 2 μL (10×) SYBR green master mix (Roche, Mannheim, Germany), 0.2 μL of the specific forward and reverse primer at a concentration of 10 μmol/L, 2 μL BSA (Ambion Ultrapure BSA; 5 mg/mL), 2 μL of template DNA (100 ng) and 5.6 μL of water. The qPCR conditions were set as follows: 96°C for 2 min; 40 cycles of 5 sec at 96°C, 1 min at 53°C, and 1 min at 72°C.

Following the qPCR protocol, the LightCycler software was used to analyze the results and provide information such as amplification curves, melting curves, and Cp values for each sample. The average and standard deviation for Cp values of triplicate sets were calculated. The Cp value of each population was an indication of the abundance of that group. To estimate the relative abundance ratios of each of the target groups, the Cp values of each population were determined on a Nanophotometer (IMPLEN, Munich, Germany) and the copy numbers of target genes were 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. High efficiencies of 99% were obtained for both AOB amoA and AOA amoA gene amplification, with the R value ranging between 0.997 and 0.999. The Cp values obtained with the AOB and AOA primers using the wastewater samples could then be used to calculate their gene copy numbers in the samples.

### 1.7. RNA extraction, cDNA production and RT-qPCR

Total RNA was extracted from 0.25 g of wastewater sediment using the Trizol (Life Technologies Corp, CA, USA) method. One microliter of extracted RNA was used to measure the concentration by Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA), and the presence of the RNA was confirmed by gel electrophoresis on a 1% (W/V) agarose gel.

To generate cDNA, the extracted RNA was treated using the Turbo DNA-free Kit (ABI, USA) to remove any DNA. The cDNA was produced using Superscript III reverse transcriptase (RT) kit (Invitrogen, New Zealand). Two negative controls were performed with all reactions. The first control contained soil RNA template and all DNAse/RT reagents, except for the final addition of the RT enzyme. A second control contained no template (water only) to ensure that all reagents were free from possible contaminants.

Once cDNA was produced the products could be amplified (RT-qPCR) as previously described for each population where the Cp values could be converted using the standard curve and to check the amplification efficiency. High efficiencies of 99% were obtained for both AOB amoA and AOA amoA gene amplification, with the R value ranging between 0.997 and 0.999. The Cp values obtained with the AOB and AOA primers using the wastewater samples could then be used to calculate their gene copy numbers in the samples.

### 1.8. Determination of activity of AOA in presence of AOB inhibitor with ddPCR

A stock allylthiourea solution (2 g/L) (Sigma–Aldrich, Canada) was prepared and filter sterilized through a syringe filter (pore

### Table 3 – Primer sets for 16S rRNA and amoA genes for eubacteria, AOB and AOA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
<th>Annealing temp</th>
<th>Amplicon size</th>
<th>Target group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>341F</td>
<td>CCT ACC GGA GGC ACC AG</td>
<td>60</td>
<td>193</td>
<td>Eubacteria (heterotrophs)</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td>534R</td>
<td>ATT ACC GGC GCT GCT GGC A</td>
<td>57</td>
<td>465</td>
<td>AOB</td>
<td>Kowalchuk and Stephen (2001)</td>
</tr>
<tr>
<td>CT0189f</td>
<td>AGG GRA AAG CAG GGC ATC G A</td>
<td>55</td>
<td>186</td>
<td>AOA</td>
<td>Ochsenreiter et al. (2003)</td>
</tr>
<tr>
<td>CT0654r</td>
<td>AGC C</td>
<td>53</td>
<td>491</td>
<td>AOB</td>
<td>Rotthauwe et al. (1997)</td>
</tr>
<tr>
<td>Crenar-771F</td>
<td>ACG GTG AGG GAT GAA AGC T</td>
<td>48–58</td>
<td>635</td>
<td>AOA</td>
<td>Francis et al. (2005)</td>
</tr>
<tr>
<td>amoA-1F</td>
<td>GCC CTC KGS AAA GCC TTC</td>
<td>48–58</td>
<td>635</td>
<td>AOA</td>
<td>Francis et al. (2005)</td>
</tr>
<tr>
<td>amoA-2R</td>
<td>STA ATG GTC TGG CTG AGA CG</td>
<td>48–58</td>
<td>635</td>
<td>AOA</td>
<td>Francis et al. (2005)</td>
</tr>
<tr>
<td>Arch-amoAF</td>
<td>STA ATG GTC TGG CTG AGA CG</td>
<td>48–58</td>
<td>635</td>
<td>AOA</td>
<td>Francis et al. (2005)</td>
</tr>
<tr>
<td>Arch-amoAR</td>
<td>GGC GCC ATC CAT CTG TAT GT</td>
<td>48–58</td>
<td>635</td>
<td>AOA</td>
<td>Francis et al. (2005)</td>
</tr>
</tbody>
</table>

* For DGGE analysis the following 40 bp GC clamp was added to the forward primers, 5′-GC CCG CCG GCC GGC GCC GGC GGC GGG GGG GGA CGG GGC GGG GGC GGG GGA CGG GGG G′-3′ (Muyzer et al., 1993).
size 0.2 mm). Allylthiourea was used at a final concentration of 5 μg/mL. Samples from reactors with and without the inhibitor, allylthiourea, were extracted for RNA as described above. Then, cDNA was produced and digital droplet PCR (ddPCR) was performed using the AOA amoA gene primer set. The reaction for the samples was prepared as follows: 22 μL total reaction containing 11 μL of EvaGreen Supermix (2×) (BioRad, Toronto, Canada), 0.550 μL each of 10 μmol/L AOA amoA forward and reverse primer, 5 μL of template (20 ng/μL) and 4.9 μL of DNase free water. The reactions were loaded into a 96-well plate and ran on a QX200 Droplet Digital ddPCR machine (BioRad, Toronto, Canada). The PCR protocol consisted of 35 cycles of denaturation at 95°C for 1 min (ramp rate 20°C/sec), annealing was done at 59°C for 1 min 30 sec (ramp rate 0.2°C/sec) and extension was done at 72°C for 1 min where the ramp rate for temperature was 20°C/sec. After the PCR, the data was analyzed using the Quantasoft software (Bio-Rad, Toronto, Canada).

1.9. AOB and AOA community profiles and identifications

A GC clamp (CCG CCG GGC GGC GGG CGG GGC GGG ACG GGG) was added to the 3′ end of 16S rRNA and amoA forward primers for AOB and AOA in order to perform PCR-DGGE. The PCR amplification for the DGGE amplicons for AOB amoA gene was 1 min at 93°C followed by 35 cycles for 30 sec at 92°C, 1 min at 57°C, and 45 sec at 68°C, followed by 5 min of final extension at 68°C. For the archaeal amoA gene amplicons, the PCR amplification was 5 min at 95°C followed by 34 cycles of 45 sec at 94°C, 1 min at 56°C, and 1 min at 72°C, followed by 10 min of final extension at 72°C.

Denaturing gradient gel electrophoresis was performed with 8% (W/V) acrylamide gel containing a linear chemical gradient ranging from 30% to 65% denaturant (100% denaturant, 7 mol/L urea and 40% (V/V) formamide) (Yang et al., 2000). Approximately 45 μL of each PCR product was loaded into individual lanes of the polyacrylamide gel. The gel was subjected to electrophoresis for 16 hr at 60°C at 100 V in 1x TAE buffer using the DCode universal mutation detection system (Bio Rad Laboratories, Hercules, CA, USA). After the electrophoresis, the gel was soaked in SYBR Gold nucleic acid gel stain solution (Thermo Fisher Scientific, USA) for 30 min and then de-stained in 1x TAE buffer for 15 min, and was photographed with Safe Imager™ 2.0 Blue Light Transilluminator (Thermo Fisher Scientific, USA).

1.10. DNA sequencing of PCR amplicons

Amplicons of the 16S rRNA and amoA genes were visualized with Safe Imager™ 2.0 Blue Light Transilluminator (Thermo-Fisher Scientific, USA) and select bands were excised using a sterilized scalpel. The excised bands were then placed into 1.5 mL Eppendorf microtubes. Thirty microliters of sterile ultra-pure water was added to each microcentrifuge tube, which was kept at 4°C overnight to enable the DNA to diffuse from the gel strips into the water phase. One microliter of the DNA solution was used as a template to re-amplify the DNA fragment with the appropriate primer sets for AOB and AOA used previously. The amplicons were then sent to ACTG Corp (Toronto, Canada) for Sanger sequencing. The resultant DNA sequences were inputted into the BLAST search program from NCBI to obtain possible matches.

2. Results

2.1. AOB and AOA presence in the activated sludge system

DNA was extracted from each of the wastewater samples and the AOB and AOA populations were targeted using end-point PCR and their specific 16S rRNA gene or their amoA gene primers. Although both groups were detected in all the samples, Fig. 1 shows that the AOB were more easily detected in the aerated tanks with both the 16S rRNA and the amoA gene primers while the AOA were often below the limit of detection in the aerated tanks but easily detected in the digester samples with their primer sets. Since AOA have been reported to prefer low oxygen conditions, their very low...
abundance in the aerated tanks was not surprising however their presence in the digesters was not anticipated since the tanks were considered to be anaerobic yet the ammonia oxidation reaction requires oxygen. In this case, the WWTP does not measure DO in the digesters due to the opaqueness of the samples and the design of the system however our results suggest that very low oxygen levels must exist in these units. Nonetheless, detection does not guarantee that the AOA are active and therefore further investigation was warranted.

2.2. Population diversity profiles for AOB and AOA

To examine the diversity of the AOA and AOB populations present in a full-scale WWTP, community profiles from seven locations during the treatment process were generated by amplifying the 16S rRNA and the amoA gene from each of the bacterial and archaeal populations, and running the amplicons on a DGGE gel. The profiles of each population did not vary significantly throughout the system. Furthermore, the DGGE community profile analysis (data not shown) suggests that the AOB and AOA populations were not very diverse yet the AOB populations were more prominent in the AT locations while the AOA population could only be amplified from the digesters and not the aerated tanks regardless of the primer set used.

2.3. Relative abundance of AOB and AOA in each stage of activated sludge system

The quantification of each population within the total bacterial community was determined using Cp values generated with qPCR of the 16S rRNA gene (Fig. 2). A general trend was observed where AOB were more prominent in the aeration tanks, and on average AOB abundance was 387 times greater than the AOA in the aerated tanks, and recycled sludge while the AOA population was more prominent in the digesters where the AOA population was 13 times more abundant in anaerobic digester 1 compared to the AOB. This confirmed the results seen with end-point PCR. Although both populations represent less than 1% of the total community (You et al., 2009), they are recognized as almost solely responsible for all the nitrification in the process which suggests they can be quite active even at low concentrations.

The 16S rRNA gene however does not directly quantify the number of AOB and AOA members since it can be present in variable copy numbers in bacterial genomes (Větrovský and Baldrian, 2013). In order to determine a more exact abundance estimate for AOB and AOA, qPCR using the amoA gene for each group was performed and the copy numbers of each population was determined (Fig. 3).

The AOB amoA gene was abundant in all aerated tanks and recycled sludge however decreased five times in number in anaerobic digester 1. In contrast, AOA amoA gene copy numbers were greater in the anaerobic digesters than in the aerated tanks and recycled sludge. The AOA amoA gene copy numbers varied from $5.5 \times 10^5$ to $10 \times 10^5$ copies, with AOA being present five fold higher in gene copy numbers in the anaerobic digester sludge 1 compared to all other locations. In comparison the AOB amoA gene copy numbers ranged from $4.1 \times 10^3$ to $1.7 \times 10^5$ copies and were higher than AOA in all samples tested.

Fig. 2 – Abundance ratios of AOB and AOA relative to the total heterotrophs within a full-scale activated sludge system using qPCR. AT: aeration tank, RS: recycled sludge; DIG: anaerobic digester.

Fig. 3 – The number of bacterial amoA gene copies (a) and the number of archaeal amoA gene copies (b) in the activated sludge system of a full-scale municipal wastewater treatment plant determined by RT-PCR. Error bars indicate one standard deviation of the average over 3 replicates.
Fewer copies of the amoA gene were detected in the system compared to 16S rRNA gene confirming the concept that some species have more than one copy of their rRNA gene and therefore the 16S rRNA copy number may not reflect the true cell numbers of the organisms. Using the amoA gene, the absolute abundance of the organisms and therefore their contribution to the ammonia oxidation process could be predicted (Wells et al., 2009), however, even this extrapolation is limited since cell numbers do not necessarily reflect the activity of the organism. We supplemented our results by examining the activity of the amoA gene using reverse transcriptase-qPCR (RT-qPCR) to measure the actual gene expression of the ammonia-oxidation gene in each population throughout the wastewater system.

2.4. AmoA gene expression for AOB and AOA

In the aerated activated sludge samples, the expression of the AOB amoA gene was significantly higher than in the recycled sludge or anaerobic digester samples (Fig. 4). This indicates that the activity of the AOB population was most prominent in the aeration tanks with aeration tank 6 displaying the highest activity. This expression represented about 33% (2.5 × 10^5 RNA transcripts/7.2 × 10^4 amoA gene copies) of the AOB activity while the expression of AOB in the digesters was less than 2%. On the other hand, AOA expression was approximately 20% in the aerated tanks but rose to close to 92% in digester 2. Although the gene copies numbers that were determined by qPCR provides a good representation of the quantity of the particular population at each step of the activated sludge system, monitoring of the gene expression by RT-qPCR confirmed the activity of each group in the system. Previous research on AOA indicated that the ammonia-oxidation process in these organisms is optimal at low oxygen and ammonia levels. However, the digesters in this WWTP had a substantial amount of ammonia yet still supported AOA activity. Since the structure of the microbial community in the digesters is in the form of flocs, it is possible that the AOA are contained in the interior of floc where the oxygen and ammonia levels may be lower and more consistent than on the surfaces or in suspension. Similar scenarios have been seen for nitrite-oxidizing bacteria in aerated lagoons where they have been found within flocs where microenvironments with low oxygen could be established (Daims et al., 2001; Pogue and Gilbride, 2007).

2.5. AOA amoA gene expression when AOB ammonia oxidation is inhibited

To explore the relationship between AOB and AOA in wastewater treatment, the inhibitor allylthiourea was added to the reactors as a selective inhibitor of the AOB ammonia-oxidation reaction (Surmacz-Gorska et al., 1996) and the RNA expression from the AOA amoA gene was measured using ddPCR. Since this experiment could not be conducted in the full-scale WWTP, lab batch reactors were employed. Inoculum from both the aerated tanks and the digesters was used to seed reactors that were then incubated either with bubbling to maintain an aerobic environment or under oil to prevent additional oxygen entering from the atmosphere and thereby reduce the oxygen levels. It can be noted that the inoculums from the two different sources in the plant (aerated tanks vs digesters) arrived in the lab with different levels of oxygen and ammonia levels (Fig. 5, initial concentrations) due to their sources (AT inoculum had 3.4 DO and 1.45 mg/L ammonia while the Dig inoculum had 0.6 DO and 53 mg/L ammonia). No adjustments to the inoculums were carried out so that subsequent reactions in the reactors were due to the activities of the authentic bacterial community. Within 24 hr the DO measurements of the reactors either rose or declined due to the reactor set up (aerated vs oil overlaid) (Fig. 5). After 48 hr the aerated reactors had DO levels of about 7–8 mg/L while the oil-overlaid reactors had fallen to 0.5 mg/L regardless of the inoculum used. Since the plant does not monitor DO measurements in their digesters, we had to assume that these measurements were in line with what they typically achieved in the digesters.

Fig. 4 – The expression of bacterial amoA gene (a) and the expression of archaeal amoA gene (b) in the activated sludge system of a full-scale municipal wastewater treatment plant determined by RT-PCR. Error bars indicate one standard deviation of the average over three replicates.
The ammonia levels in the reactors over the 48 hr period of the study in the reactors seeded with AT inoculum decreased from 1.45 to 0.38 mg/L when the reactors were aerated but rose from 1.45 to 1.8 mg/L without aeration. With inhibitor, the ammonia level in the AT reactor increased initially and then fell to initial concentrations when aerated while the ammonia level decreased slightly when the AT sample was not aerated. Taken together, it appeared that the ammonia-oxidizing community in the aerated tanks which are likely AOB continued to process ammonia when they were maintained in aerated conditions however when oxygen availability was low (oil overlaid reactors) their ability to process ammonia decreased resulting in higher ammonia levels.

With inhibitor, the initial increase in the ammonia levels implies that the AOB were responsible for ammonia removal, however after 24 hr the ammonia levels started to decline, which suggests that a second mechanism was initiated. We proposed that the second mechanism is the AOA population. The ammonia levels in the reactors seeded with DIG inoculum supports our suggestion that there is an alternative mechanism capable of oxidizing ammonia since inhibiting AOB did not cause an increase in the ammonia concentrations but instead a decrease regardless of the presence of the inhibitor or the aeration conditions. In this case, our observation that AOA are present in the digesters and are capable of ammonia oxidation reinforces the idea they are at least partially responsible for the ammonia removal in the digesters (Fig. 5). Additional, ammonia-oxidation may also be occurring in the digesters through the microbial process of anaerobic ammonia-oxidation (anammox). This process is known to directly oxidize ammonia to nitrogen gas using nitrite as an electron acceptor (Asadi et al., 2012, 2016). The detection and activity of anammox in the digesters will need to be followed to verify their contribution to the reduction of ammonia in this system.

To confirm the activity of the AOA, the expression of the AOA amoA gene in the reactors was measured using ddPCR. The ddPCR technology is based on the ability to partition the PCR sample into approximately 20,000 droplets, each of which undergoes standard PCR amplification. The number of positive droplets are determined and analyzed using Poisson statistics to determine the target DNA template concentration in the original sample (BioRad, Toronto, Canada).

Fig. 6 shows that the expression of the AOA amoA gene in each of the reactors. The expression of the AOA amoA gene decreased slightly over time in the reactors seeded with the AT inoculum. Since the major ammonia oxidizer in the AT inoculum was previously determined to be AOBs, it was not surprising that there was less expression of the AOA amoA gene over time. However, an exception was noted in the reactor overlaid with oil and containing the inhibitor. In this reactor, the expression increased suggesting that while AOB

---

**Fig. 5** – Parameter readings for the reactors seeded with AT inoculum for oxygen (a) and ammonia (b) and for the reactors seeded with DIG inoculum for oxygen (c) and ammonia (d). See Table 2 for reactor designations.
are selected against by both the inhibitor and the low oxygen levels, the expression of the AOA amoA gene was somehow activated.

In the case of the reactors seeded with the digester inoculum, the expression of the AOA population remained constant without the inhibitor but in the presence of inhibitor there was a large increase in the expression of the AOA amoA gene (Fig. 6). These results again suggest that AOA ammonia oxidation may be stimulated when the AOB are inhibited and therefore help to compensate for their inactivity.

3. Discussion

Although the presence of ammonia-oxidizing archaea in municipal wastewater treatment has been previously observed (Park et al., 2006; Zhang et al., 2009), the abundance of AOB and AOA populations and the contribution of each population to the ammonia-oxidation process under various operational parameters of the activated sludge system is still not resolved (Wells et al., 2009). The results from this study clearly show that AOB could be detected in all stages of the secondary treatment process however abundance was higher in both relative and absolute quantities in the aerated tanks. The decrease in the abundance of both the 16S rRNA and amoA gene numbers for AOB in the anaerobic digesters demonstrates that AOB prefer the conditions in the aerated tanks rather than the low DO of the anaerobic digesters.

On the other hand, the AOA were found in very small concentrations at all stages of the WWTP although the abundance of AOA throughout the system gradually increased until they were consistently above detection limits in the digesters. While the WWTP process assumes that the dissolved oxygen levels in the digesters approach zero, it appears that there is a sufficient amount of oxygen present for the maintenance of AOA. Using RT-qPCR we were able to demonstrate gene expression of the AOA amoA gene in the digesters, which confirms the participation of the AOA in the reduction of ammonia within the WWTS in that location.

It is interesting to note that although AOBs greatly outnumber the AOA in all samples of the activated sludge system, expression of the AOB and AOA amoA genes showed that the AOB abundance and activity decreased along the wastewater pathway while the AOA abundance and activity increased. This suggests a cooperative association between the two ammonia-oxidizing groups. Although the exact parameter data from the full-scale anaerobic digesters could not be obtained, it is expected that the concentration of dissolved oxygen is extremely low. While ammonia levels are high on entry into the primary digesters, the concentrations decrease rapidly and appear to be within the concentrations tolerated by AOA in the secondary digesters. The conditions may provide the ideal niche for the AOA to be present and active and not in direct competition with the AOB for ammonia.

The cooperation between the two groups is further supported by the reactor data. When allythiourea was added to the reactors to inhibit the AOB ammonia-oxidation reaction, the AOA amoA gene expression increased. This increase in expression was accompanied by a decrease in ammonia levels suggesting that the AOA are actively utilizing the ammonia when the AOB are inhibited. Similar results were seen by other researchers studying manure amended with sulfadiazine (SDZ) where they noted that ammonia oxidation was taken over by AOA when AOB were suppressed by the antibiotic (Schauss et al., 2009). Although AOB and AOA co-exist in environments, their relative distribution may be affected by the proximal environmental parameters as suggested by others (Chen et al., 2013; Wessén et al., 2010). The quantification of the expression from the AOA amoA gene in this study by RT-PCR and ddPCR supports earlier studies that were based on gene numbers alone (Gao et al., 2013; Li et al., 2013; Wessén et al., 2010).

A recent model of the interaction among AOB, AOA and anaerobic ammonium-oxidizing (anammox) bacteria proposes that more than 50% of ammonia oxidation being mediated by AOB happens at the initial stages of treatment while AOA are responsible for up to 90% of the ammonium oxidation.
removal afterwards (Pan et al., 2016). Our results appear to support the proposed model where AOB are the prominent ammonia oxidizers active in the aerated tanks followed by an increase in AOA activity in the digesters. Furthermore, alternative ammonia removal by anammox may also be active. We have previously detected anammox in the digester tank but their presence is extremely low and not consistently found with standard PCR techniques (data not shown). Recent literature suggests that AOA coupled with anammox may improve biological nitrogen removal from wastewater (Pan et al., 2016). Using sensitive diagnostic molecular methods, further experimental data are needed to confirm the functional relationship between the ammonia oxidation groups, including determining whether AOA activity is coupled with anammox activity in the digesters for biological nitrogen removal.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (RGPIN/227565-2013) to K.A.G.

REFERENCES


