Nitrogen removal and functional bacteria distribution of ANAMMOX at ambient temperature
Taotao Zeng, Dong Li, Wei Liao, Wenxin Qiu and Jie Zhang

ABSTRACT
In this study, an up-flow anaerobic biofilter (AF) was operated to investigate the efficiency of anaerobic ammonium oxidation (ANAMMOX) in treating low strength ammonia (46.5 mg/L) at ambient temperatures (20.3–23.2 °C). Microbial compositions and functional populations of the upper (140–190 cm), middle (40–140 cm), and lower (0–40 cm) parts of the biofilter were monitored using scanning electron microscopy, denaturing gradient gel electrophoresis (DGGE), clone and sequence. The results show that stable biofilter performance was achieved with an average nitrogen removal rate of 2.26 kg/(m³·d) and a total nitrogen removal efficiency of 75.9%. Approximately 67% of the ammonia and nitrite disappeared in the middle part of the biofilter. The spherical bacteria, similar to ANAMMOX bacteria, dominated the middle part of the biofilter. There were eight bacterial DGGE bands; clone and sequence results showed that they included Oxalibacterium sp., Ignavibacterium album, Bacterium r115, Candidatus Kuenenia stuttgartiensis, Hippea maritima, Thioprofundum lithotrophicum, and Rhodopseudomonas palustris. The genus of ANAMMOX bacterium remaining at constant levels in different parts of the biofilter was identified as Candidatus Kuenenia stuttgartiensis. The AF bioreactor maintained high activity due to the ANAMMOX bacteria’s ability to adapt to ambient temperature and low matrix influent conditions.

Key words | anaerobic ammonium oxidation (ANAMMOX), biofilter, DGGE, functional bacteria, nitrogen removal

INTRODUCTION
Removing nitrogen from wastewater is critical to prevent eutrophication in receiving water bodies. Compared with conventional nitrification/denitrification processes for nitrogen removal, the anaerobic ammonium-oxidizing (ANAMMOX) process is a promising new and sustainable technology (Laurieni et al. 2015). Under anoxic conditions, ammonium and nitrite are converted to dinitrogen gas (N₂) using autotrophic ANAMMOX bacteria (Jetten et al. 2009). This process could reduce aeration energy consumption by 50%, requiring less biomass production and avoiding organic matter consumption (Perez et al. 2015). Thus, applying this technology could result in energy-saving wastewater treatment plants (WWTP), rather than the current energy-consuming types.

Limited by stringent operating conditions and the extremely slow growth rate of ANAMMOX bacteria, ANAMMOX-based processes are currently implemented at several WWTPs to treat high strength wastewaters (>500 mg/L NH₄⁺-N), such as digester effluents and reject water, at mesophilic temperatures (30–40 °C) (Nishimura et al. 2012; Zekker et al. 2012). Lowering the temperature substantially decreases nitrogen removal activity (Isaka et al. 2008). Despite these challenges, laboratory-scale reactors with ANAMMOX processes have been successfully operated at temperatures of 20 °C or below (Vazquez-Padin et al. 2011; Hendrickx et al. 2014). In a recent study, ANAMMOX bacteria were successfully enriched at 12 °C in a
laboratory-scale sequencing batch reactor (SBR) with low influent ammonium concentrations (Hu et al. 2015).

In another study, another 4.2 L reactor was operated at 10°C, fed with 61 mg (NH₄+NO₂)-N/L, and inoculated with activated sludge from two selected municipal WWTPs. The cold-adapted ANAMMOX species Candidatus Brocadia fulgida dominated the enriched biomass; the specific activity was 30–44 mg N/(g VS d). Even with these studies, there is a lack of knowledge about the ability of biofilm reactors operating at ambient temperatures to treat low strength wastewater (Gilbert et al. 2015). In particular, little attention has been paid to how microbial communities respond at different bio-layers.

In this study, an up-flow ANAMMOX biofilter was operated at ambient temperatures of 20.3–23.2°C, receiving synthetic water of 46.5 mg NH₄⁺-N/L. Total and layer-specific nitrogen removal performances were monitored, and the microorganism community composition was investigated as it related to the ANAMMOX biofilter function. Microbial analysis was performed using scanning electron micrographs (SEM), polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE), and sequencing of 16S rRNA gene clone libraries.

MATERIALS AND METHODS

Experimental setup and operational strategy

An up-flow anaerobic biofilter (AF), with a volume of 45 L, an internal diameter of 18.5 cm, and height of 200 cm, was used as an ANAMMOX reactor. An outlet was set at every 10 cm to collect effluent in the biofilter (Figure 1). Table 1 presents the characteristics of the volcanic stone used as carriers. Table 2 lists the characteristics of the synthetic wastewater, created using tap water mixed with different amounts of NaHCO₃, (NH₄)₂SO₄ and NaNO₂, which was pumped continuously from the bottom of the reactor and out of the upper outlet. The reactor was inoculated with mature ANAMMOX sludge and operated with stepwise decreases in influent ammonia concentration in previous reports (Taotao et al. 2015). To maintain the biofilter’s anaerobic conditions, no aeration equipment was used. In this study, the biofilter was run for 49 days at ambient temperature, with an influent ammonia concentration of 46.5 mg/L and hydraulic retention time (HRT) of 1.0 hours.

Analytical methods

Effluent samples were collected from every outlet for chemical analysis. NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were measured every day using Standard Methods (APHA 1998). Temperature, pH, and dissolved oxygen (DO) were measured using a WTW pH/DO meter (WTW Multi 340i, Germany). The nitrogen removal efficiency (NRE) was calculated as \( \left( \frac{N_{in} - N_{out}}{N_{in}} \right) \times 100 \), where \( N_{in} \) and \( N_{out} \) represent the nitrogen concentrations (mg/L) of the influents and effluents, respectively.
The nitrogen removal rate (NRR) (kg N·m⁻³·day⁻¹) was calculated as the daily nitrogen load removed (NLR) (daily flow rate × (N_in – N_out)) per reactor unit volume. Biofilm samples were collected from the reactor’s upper, middle, and lower parts for SEM and microbial population analysis.

**Sampling and SEM**

Volcanic filter samples were collected during reactor operations, and were stored in 50 mL sterile plastic test tubes at ~20 °C. The biofilm was removed from the volcanic filter with a sterile brush and placed into 10 mL sterile plastic test tubes. The upper, middle and lower parts of the biofilm were observed using SEM.

Samples were first washed twice in a 0.1 M phosphate buffer solution (pH 7.6) for 5 min each. The samples were then stored untouched for 2 hours in a 2.5% glutaraldehyde solution. Next, samples were washed in the buffer solution three times for 10 min each and then dehydrated for 10 min each in sequentially graded ethanol solutions at concentrations of 30, 50, 70, and 90%, combined with absolute alcohol. Then samples were substituted with a 1:1 ratio of absolute ethanol to isoamyl acetate, and left for 15 min. After being replaced again with isoamyl acetate, samples were dried using a vacuum spray. SEM observations were conducted using a scanning electron microscope (Hitachi S-4300, Japan).

**DNA extraction and PCR-DGGE**

Wet biomass samples of 0.2 g each were collected from the ANAMMOX biofilter and rinsed twice with a sodium phosphate buffer (PBS, 0.1 M, pH 8.0). The total community genomic DNA was extracted (Zeng et al. 2013), suspended in 50 µL of 1×TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and measured using a 0.8% (w/V) agarose gel electrophoresis. DNA concentration and quality were monitored using UV spectrophotometry.

PCR thermocycling was performed in a Takara PCR Thermal Cycler Dice. Bacterial universal primers GC-338F/518R directly amplified 16 s DNA V3 region from total DNA (Ovreas et al. 1997). ANAMMOX Primers Pla46 and Amx368 (with a GC-clamp) were used to amplify 16S rDNA of ANAMMOX bacteria for DGGE. Both protocols were as follows: samples were held at 94 °C for 5 min; followed by 30 cycles consisting of 94 °C for 40 s, 55 °C for 40 s, and then 72 °C for 60 s in each cycle; and 72 °C for 10 min. All products were purified with a TIANgel midi purification kit (Tiangen, China) using the manufacturer’s instructions.

The PCR products were loaded onto 8% polyacrylamide, with a linear denaturing gradient ranging from 30 to 60% (100% denaturing gradient contains 7 M urea and 40% formamide). DGGE was performed using the D-code System (Bio-Rad, USA) following the manufacturer’s instructions. Electrophoresis was run at a constant voltage of 120 V for 5 h at 60 °C. Gels were subsequently silver stained using the procedure described by Bassam & Gresshoff (2007); pictures were taken with a Gel Doc XR system (Bio-Rad).

**Cloning, sequencing and phylogenetic analysis**

Obvious DGGE bands in gel were excised and dissolved in 50 µL TE buffer at 4 °C overnight. A 2 µL TE solution was used as template, and reamplified PCR was performed using primers 338F/518R and Pla46/Amx368 without GC clamps. The products were purified using an Agarose Gel DNA Purification Kit (TIANGEN, China), ligated into the pMD19-T vector (TaKaRa) and then transformed into competent Escherichia coli DH5α (TaKaRa).

Sequencing was carried out on an ABI 3730 DNA sequencer by Sangon commercial services (China). The basic local alignment search tool (BLAST) was used to compare the experimental sequences with available sequences in the nucleotide collection database. Phylogenetic tree constructions were created using MEGA 5.0 and the neighbor-joining method with 1,000 bootstrap replicates.

**RESULTS AND DISCUSSION**

**ANAMMOX performance of biofilter**

Biofilter performance was monitored for 49 days, with a constant influent ammonia concentration of 46.5 mg/L (Figure 2). In this phase, a constant NLR of 2.98 kg/(m³·d) was obtained, with an HRT of 1.0 h and an ammonia concentration of 46.5 mg/L. Performance remained stable, with the effluent having an average NRR of 2.26 kg/(m³·d) and total nitrogen (TN) removal efficiency of 75.9%. In a related study,
Dosta et al. (2008) operated an SBR at different temperatures (from 30 to 15 °C) to determine ANAMMOX activities. They achieved an NRR of 0.29 kg N/(m³·d) with an HRT of 24 h at a temperature of 18 °C (Dosta et al. 2008). In this study, an NRR of 2.26 kg/(m³·d) was obtained with an average HRT of 1.0 h, demonstrating the high ANAMMOX activity of the AF for nitrogen removal.

In addition, during days 1–29, the average NLR and NRR were 2.49 kg/(m³·d) and 2.02 kg/(m³·d), respectively. However, during days 29–49, average NLR and NRR increased to 3.95 kg/(m³·d) and 2.92 kg/(m³·d), respectively. There was a jump in the NLR and NRR at day 29, which was due to HRT decreasing from 1.1 to 0.8 h.

ANAMMOX may be present in the biofilter for two reasons. First, the volcanic stone carriers may store ANAMMOX bacteria due to their high biological retention capacity, avoiding the impact of a high hydraulic load. Second, the genus of ANAMMOX bacteria could adapt to low substrate conditions after being domesticated, contributing to nitrogen removal in the biofilter. This indicates that ANAMMOX bacteria can effectively remove nitrogen from low strength wastewater at ambient temperature.

Figure 3 shows the results of effluent sampling from the outlet, and the layer-specific nitrogen removal performance. Ammonia and nitrite decreased proportionally with the chemical stoichiometry, indicating that the ANAMMOX process ran well in the biofilter. Total ammonia and nitrite removal efficiencies were 97.8% and 100%, respectively. Total nitrogen removal efficiency and total nitrogen removal rate were 89.3% and 2.5 kg/(m³·d), respectively. In the lower part of the biofilter (0–40 cm), TN removal of 25.1% was achieved. In the middle part of the biofilter (40–140 cm), 67% of the TN disappeared, indicating that most of the ANAMMOX bacteria were located in this part. These results indicated that there was more activity in the middle part than that of the low part. It is probably due to a little DO in the synthetic influent, which could inhibit the ANAMMOX bacteria activity, as no inert gas was added for crowding out DO. Only 3% of the TN disappeared after a height of 150 cm, which therefore defines a reasonable biofilter height to conserve consumption.
Morphology and SEM results of biofilm

Biofilm samples were collected from the upper, middle, and lower parts to observe morphology differences. The left hand photographs in Figure 4 show that the middle part of the biofilm was bright red, an indicator of ANAMMOX bacteria (Jenni et al. 2014). In the upper and lower parts of the biofilm, there were significantly lower proportions of red material, indicating a small amount of ANAMMOX bacteria (the full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wrd.2016.197). The right hand photographs in Figure 4 show the biofilm microscopic structure after being magnified by 10,000 times with SEM. In the middle section of the biofilm, ANAMMOX-like spherical bacteria were dominant, with some filaments present. This structure facilitates linking among cells, which is consistent with past studies (Yang et al. 2010). Rod-shaped bacteria dominated the upper part of the biofilm, with small numbers of spherical bacteria. Spherical, rod-shaped, and filament bacteria coexisted harmoniously in the lower part of the biofilm. The morphology and microscopic structure differed between the upper, middle and lower sections of the biofilm, corresponding with layer-specific nitrogen removal results (Figure 3).

Microbial community and phylogenetic analysis

Upper, middle, and lower parts of the biofilm were collected for bacterial DGGE analysis. Total DNA with a length of 23 kb was extracted, and 16 s rDNA V3 region was usually selected, resulting in DGGE profiles substantially different in terms of the number, resolution, and relative intensity of the amplification products (Yu & Morrison 2004). Electrophoresis was carried twice to verify the uniformity of the DGGE profiles. DGGE profiles (Figure 5(a)) show the abundance of microbial populations. There were eight bacterial DGGE bands, indicating the presence of eight dominant genera in the biofilter. This limited number of bands may be due to the use of synthetic...
influent. Special primers for ANAMMOX bacteria were used for PCR; Figure 5(b) shows the DGGE results. There were two bands in the upper, middle, and lower parts of the biofilm; each band was located at the same position in the DGGE gel. This suggests that the genus of ANAMMOX bacteria was the same in different parts of the biofilm. Cloning, sequencing, and phylogenetic analysis were conducted to gain further insights into the microbial community structure.

The eight distinct bands of bacterial DGGE were carefully excised for cloning and sequencing. The nucleotide sequences were compared to available sequences in GenBank database using BLASTN; Table 3 shows the results. Figure 6 presents the phylogenetic tree of total bacteria based on 16S rDNA sequences. Bands 4 and 5 were similar to Candidatus Kuenenia stuttgartiensis, with sequence homologies of 95% and 93%, respectively. Candidatus Kuenenia stuttgartiensis are functional ANAMMOX bacteria, contributing the most to nitrogen removal in the biofilter.

Other bacteria were also present in the biofilter. Band 1 was 93% homologous with Oxalicibacterium sp. (GU295961.1), belonging to Gram-negative betaproteobacteria. Band 2 displayed 84% similarities with Ignavibacterium album (AB478415.1), a strictly anaerobic Gram-negative bacteria (Iino et al. 2013); band 3 was affiliated with Bacterium rJ15 (AB021333.1) with a similarity level of 88%. Bands 6 and 7 were 95% and 91% homologous with Hippea maritima (AB072402.1) and Thioprofundum lithotrophica (AB468957.1), respectively. These two types of bacteria are anaerobic autotrophic bacteria, associated with sulfate reduction (Goevert & Conrad 2010; Tang et al. 2010). Band 8

Table 3 | Bacterial 16S rRNA sequences of ANAMMOX biofilter

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest match (GenBank accession numbers)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxalicibacterium sp. (GU295961.1)</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>Ignavibacterium album (AB478415.1)</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>Bacterium rJ15 (AB021333.1)</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>Candidatus Kuenenia stuttgartiensis (AF375995)</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>Candidatus Kuenenia stuttgartiensis (AF375995)</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>Hippea maritima (AB072402.1)</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>Thioprofundum lithotrophica (AB468957.1)</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>Rhodopseudomonas palustris (D12700.1)</td>
<td>93</td>
</tr>
</tbody>
</table>

Figure 6 | Phylogenetic tree of total bacteria based on 16S rDNA sequences.
had a 93% similarity with *Rhodopseudomonas palustris* (D12700.1); this is an autotrophic bacterium that can live well in anaerobic micro-environmental conditions. Sequencing results indicated that most bacteria were anaerobic autotrophic microorganisms, corresponding to the inorganic synthetic influent and the biofilter’s anaerobic environment.

ANAMMOX bands 1 and 2 (Figure 5(b)) were also excised for cloning and sequencing. The nucleotide sequences were submitted to the GenBank database, returning numbers of KF194202 (band 1) and KF194203 (band 2). Sequencing bands 1 and 2 revealed a 97% similarity with *Candidatus Kuenenia stuttgartiensis* (AF375995). Figure 7 shows the neighbor-joining phylogenetic tree of ANAMMOX bacteria. The genus of ANAMMOX was consistent with previous results from the bioreactor, operated in high strength ammonia conditions (Xu et al. 2014). *Candidatus Kuenenia stuttgartiensis* adapted to variable substrate concentrations, and could maintain a high activity level with low matrix influent, contributing to the AFs high NRR.

**CONCLUSIONS**

This study indicated that nitrogen could be effectively removed from low strength wastewater with ANAMMOX activities at ambient temperatures (20.3–23.2 °C). The NRR and efficiency levels were 2.26 kg/(m$^3$·d) and 75.9%, respectively. Approximately 67% of the ammonia and nitrite disappeared in the middle part of the biofilter. Morphology and SEM results showed ANAMMOX-like bacteria dominated the middle part of the biofilter, with eight different genus of bacteria cultured. The genus of ANAMMOX bacteria was constant across different parts of the biofilter, and was identified as *Candidatus Kuenenia stuttgartiensis*. The activity in the AF bioreactor remained high, due to the ANAMMOX bacteria's adaptation to ambient temperature and low matrix influent conditions.

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