Effects of substrates on N\textsubscript{2}O emissions in an anaerobic ammonium oxidation (anammox) reactor

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Background

Nitrogen removal (NR) is an important component of wastewater treatment. Biological nitrogen removal (BNR) is often preferred to other non-biologic processes due to its high efficiency and energy conservation characteristics. Even so, the traditional BNR process has several disadvantages, such as excessive oxygen being consumed during the nitration period and the requirement of additional organic carbon for denitrification. In 1995, the biological anaerobic ammonium oxidation (anammox) reaction was first reported in an up-flow reactor (Mulder et al. 1995). The anammox process operates under anaerobic conditions where nitrite is used as an electron acceptor by anammox bacteria for oxidation of ammonia to nitrogen gas (N\textsubscript{2}) (Kuenen 2008). By using this new technology, only 50% of the source ammonium needs to be oxidized to nitrite. This means that the oxygen requirement is reduced to about 75% of the traditional BNR process. Anammox bacteria are autotrophic microorganisms, therefore, additional carbon input is also eliminated. The anammox process has demonstrated potential over the traditional BNR process, thus considerable research has been carried out from bench-scale to pilot-scale as the technology has proceeded to full-scale applications (Kartal et al. 2010).

N\textsubscript{2}O is a potent greenhouse gas, whose warming effect is 200–300 times that of CO\textsubscript{2} and 4–12 times greater than CH\textsubscript{4}. Many studies have shown that standard sewage denitrification
processes are a critical source of atmospheric N₂O (Kampschreur et al. 2009a; Wunderlin et al. 2013; Shaw and Koh 2012). In addition, research has generally shown that N₂ is the end product of the anammox process (Jetten et al. 2005); however, high N₂O emission from Anammox processes have also been reported (Kampschreur et al. 2009b). Thus, there is an urgent need to investigate the production of N₂O in the anammox process and develop methods of controlling and decreasing the greenhouse emissions from the anammox process.

In this study, an anammox reactor was used to study the effects of substrate concentrations on the emission of N₂O in an anammox process. The relationship between substrate concentrations and N₂O emissions was studied by changing the influent NH₄-N concentration. Furthermore, genetic analysis using the 16S rRNA gene was employed to characterize the microbial population of the anammox granules.

Results and discussion
Reactor performance
The removal performance of ammonia and nitrite is shown in Fig. 1a, b. Whenever the effluent NO₂⁻-N concentration fell below 10 mg L⁻¹, the nitrogen loading rate (NLR) was increased by adjusting the influent nitrogen concentration while maintaining a constant HRT of 8 h. During start-up period, with the influent NH₄-N and NO₂⁻-N concentrations set at 73.2 and 88.3 mg L⁻¹, respectively, effluent NH₄-N and NO₂⁻-N concentrations below 7 and 2 mg L⁻¹ were obtained, with the TN removal rate >80 %. Subsequently, at a constant HRT, the influent NH₄-N and NO₂⁻-N concentrations were further increased to 100.4 and 124.8 mg L⁻¹, respectively, and effluent NH₄-N and NO₂⁻-N concentrations initially were a little higher, but both soon decreased to below 8 mg L⁻¹ over a 3-day period. These results indicated that the seed anammox sludge could adapt quickly to changes in NLR. On day 24, the influent NH₄-N and NO₂⁻-N concentrations were increased to 145.0 and 176.4 mg L⁻¹, respectively, which were the highest levels used in this study. Under these conditions, the effluent NH₄-N and NO₂⁻-N concentrations were 27.0 and 14.8 mg L⁻¹, respectively. Accordingly, influent NH₄-N and NO₂⁻-N concentrations were decreased to 120 and 150 mg L⁻¹, respectively, and effluent NH₄-N and NO₂⁻-N concentrations were then maintained below 18.6 and 9.9 mg L⁻¹. Overall, the reactor could operate with a stable nitrogen removal rate of over 81 %.

Figure 1c shows the ratios of influent NO₂⁻-N/NH₄⁻-N, effluent NO₂⁻-N removal/NH₄⁻-N removal, and effluent NO₃⁻-N production/NH₄⁻-N removal. At the start-up period, influent NO₂⁻-N/NH₄⁻-N was set 1.2. Accordingly, effluent NO₂⁻-N removal/NH₄⁻-N removal, and effluent NO₃⁻-N production/NH₄⁻-N removal were 1.3 and 0.3, respectively, which were close to values reported by others (Strous et al. 1998). In order to investigate the effect of influent NH₄-N on N₂O emission, on day 12 influent NO₂⁻-N/NH₄⁻-N was changed to 1.09. As a result, effluent NO₂⁻-N removal/NH₄⁻-N removal increased to 1.55. Conversely, effluent NO₃⁻-N production/NH₄⁻-N removal decreased to 0.2. The same results were once more affirmed on day 31. Denitrification was considered to be the main reason for the additional NO₂⁻-N or NO₃⁻-N removal.

(See figure on next page.)

**Fig. 1** Reactor performance during the study. a Changes in nitrogen concentrations; b changes in NLR and NLRR; c ratios of inf. NO₂⁻-N/NH₄⁻-N, eff. NO₂⁻-N removal/NH₄⁻-N removal, and eff. NO₃⁻-N production/NH₄⁻-N removal. Inf. influent, Eff. effluent
**Figure a**

Concentrations (mg/L) vs. Time (days) for Inf.NH4-N, Inf NO2-N, Eff.NH4-N, Eff.NO2-N, and Eff.NO3-N.

**Figure b**

NLRR (g/L/d) vs. NLR (g/L/d) with the equation $y = 0.8127x + 0.0113$ and $R^2 = 0.9595$.

**Figure c**

Ratio vs. Time (days) for Inf.NO2-N/NH4-N, Eff.NO2-N removal/NH4-N removal, and Eff.NO3-N production/NH4-N removal.
$\text{N}_2\text{O}$ emission
$\text{N}_2\text{O}$ emissions over the course of the study are shown in Fig. 2. The conversion ratio of $\text{N}_2\text{O}$ was calculated from the removed nitrogen. On the first day, about 0.6–0.64 % $\text{N}_2\text{O}$ content was detected in the emission gas. On day 2, the influent pipe became blocked, thus only 0.34 % $\text{N}_2\text{O}$ was detected in the emission gas. However, this value increased to 0.54 % over the following 3 days and by day 6 the $\text{N}_2\text{O}$ concentration had reached 0.93 %, accompanied with a high effluent NH$_4$-N residual. On days 11–13 and 30–32, the effluent NH$_4$-N remained at 32–34 and 37–42 mg L$^{-1}$, respectively. Under these conditions, the $\text{N}_2\text{O}$ emissions were found to be significantly higher than the values associated with low effluent NH$_4$-N concentrations. Over the course of the study, $\text{N}_2\text{O}$ levels were determined to be 0.6–1.0 % in the off-gas.

Effects of influent NH$_4$-N, NO$_2$-N, NO$_3$-N and nitrogen removal rate on $\text{N}_2\text{O}$ emission
Effects of inlet NH$_4$-N, NO$_2$-N, NO$_3$-N and nitrogen removal rate on $\text{N}_2\text{O}$ production are shown in Fig. 3. The EGSB reactor used in this study was operated with a high recycle rate. Thus, influent NH$_4$-N, NO$_2$-N and NO$_3$-N were calculated by using the following equation.

$$x = a + n \times \frac{b}{n + 1}$$  \hspace{1cm} (1)

where $x$ is the concentration of inlet NH$_4$-N, NO$_2$-N, NO$_3$-N, $a$ is the influent concentration of NH$_4$-N, NO$_2$-N, NO$_3$-N, $n$ is the ratio of recycle rate to influent flow rate, $b$ is the effluent concentration of NH$_4$-N, NO$_2$-N, NO$_3$-N. According to the equation, inlet
Inlet NO₃-N concentration (mg/L)

N₂O content (%) = 0.0008x² - 0.0387x + 1.039

R² = 0.9704

Inlet NH₄-N concentration (mg/L)

Inlet NO₂-N concentration (mg/L)

Inlet NO₃-N concentration (mg/L)
NH₄⁺-N, NO₂⁻-N and NO₃⁻-N were determined by two factors: changing influent concentrations or different recycle rate. In order to observe the effects of inlet NH₄⁺-N, NO₂⁻-N and NO₃⁻-N, only the recycle rate was changed while the nitrogen loading rate was set with the same value 0.5 kg m⁻³ day⁻¹ (Fig. 3a–c). Also, the effects of nitrogen removal rate were evaluated with the same influent nitrogen concentrations.

As shown in Fig. 3a, average N₂O content was 0.6 % with an inlet NH₄⁺-N concentration of 27–28 mg L⁻¹. Increasing the inlet NH₄⁺-N concentration from 36 to 57 mg L⁻¹, N₂O increased from 0.65 to 1.4 %. Inlet NH₄⁺-N concentration and N₂O emission were simulated according to the current data by the following equation with P < 0.03.

\[
y = 0.0008x^2 - 0.0387x + 1.039
\]

where \( y \) is the N₂O emission, \( x \) is the inlet NH₄⁺-N concentration. In a word, increasing inlet NH₄⁺-N concentration tended to yield a higher N₂O concentration.

The influences of inlet NO₂⁻-N and NO₃⁻-N concentrations were also investigated during the study, though no obvious relationship was found with N₂O emissions (Fig. 3b, c).

**Bacteria community analysis**

Hierarchical cluster analysis was used to identify the differences of three bacterial community structures (Fig. 4). The three samples were sampled from the same reactor, showing obvious similarity of community structure. *Nitrosomonas*, which oxidizes ammonia to nitrite, was detected in all the three samples. In the anammox reactor, it is difficult to keep dissolved oxygen at zero. Thus, the anammox reactor provides the conditions for the growth of *Nitrosomonas*. However, *Nitrosomonas* is known to produce N₂O under low oxygen conditions (7). This was supported by the relationship between *Nitrosomonas* abundance and N₂O emission (Fig. 5).

In this study, N₂O emissions were found to be higher than the reported values. Okabe et al. reported that a N₂O emission of only 0.05–0.23 % was detected with a nitrogen removal rate of 7.5–15 kg N m⁻³ day⁻¹. However, the highest N₂O concentration of 1.67 % was quantified in this study, which is compared with other the results in Table 1. From Table 1, increasing nitrogen loading rates showed positive effect on decreasing N₂O concentrations. Longfei et al. (2015) also reported that the increase of nitrogen loading rate could reduce N₂O emission and they found it is more seasonable if compare the value of N₂O production per gram N removal (N₂O emission/nitrogen removal rate). Although higher nitrogen removal rate helps to reduce the footprint of the anammox system, it was difficult to maintain the stable running under high nitrogen removal rate due to floatation of anammox granules and pipe clogging. On the other hand, Kampirschreur et al. also found high N₂O concentrations with 0.6 % in one full-scale anammox reactor. Thus, increasing NLR is effective in reducing N₂O emission, but N₂O emissions

(See figure on next page.)

**Fig. 4** Hierarchical cluster analysis of 1, 2 and 3 bacterial communities. 1, N₂O emission 0.6 %, nitrogen removal rate 0.4 kg-N m⁻³ day⁻¹; 2, day 36, N₂O emission 0.7 %, nitrogen removal rate 0.73 kg-N m⁻³ day⁻¹; 3, day 50, N₂O emission 0.18 %, nitrogen removal rate 3 kg-N m⁻³ day⁻¹. The y-axis is the clustering of the 100 most abundant OTUs (3 % distance) in reads. The OTUs were ordered by genus. Sample communities were clustered based on complete linkage method. The color intensity of scale indicates relative abundance of each OUT read. Relative abundance was defined as the number of sequences affiliated with that OTU divided by the total number of sequences per sample.
are inevitable in an anammox reactor. Reducing N₂O emission is still a concern for anammox applications.

NO₂⁻N and NO₃⁻N are the substrates for denitrifiers. It is supposed that N₂O is produced as an intermediate of incomplete heterotrophic denitrification due to low COD/N ratio (Okabe et al. 2011). However, no relationship was found between NO₂⁻N, NO₃⁻N and N₂O emission in this study. Thus, it is difficult to explain the increasing N₂O emission during this study.

Okabe et al. indicated that denitrification by putative heterotrophic denitrifiers present in the inner part of the granule was considered the most probable cause of N₂O emission from the anammox reactor. In this study, inlet NH₄⁻N showed clear relation to N₂O emission (Fig. 3). Also, *Nitrosomonas* abundance increased with N₂O emission (Fig. 5). As shown in Fig. 5, only 0.025 g-N₂O emitted/100 g-N consumed was observed without *Nitrosomonas*. And the denitrifies were presumed to contribute the above N₂O emission. After that, *Nitrosomonas* abundance increased with N₂O emission. At last, 0.7 g-N₂O emitted/100 g-N consumed was observed, which was almost 30 times of the value without *Nitrosomonas*. The results got in this study showed that *Nitrosomonas* was the main cause of N₂O emission. *Nitrosomonas* competed with anammox bacteria for NH₄⁻N. Because anammox bacteria could not oxidize NH₄⁻N without NO₂⁻N, therefore, supplying enough NH₄⁻N is favorable for *Nitrosomonas*. While oxygen was always insufficient,

| Reaction type | Reactor volume (L) | Removal rate (kg m⁻³ day⁻¹) | N₂O emission (%) | References |
|--------------|--------------------|-----------------------------|------------------|------------|
| Granules-based | 70,000             | 7.14                        | 0.6              | Kampschreur et al. |
| Granules-based | 0.15               | 7.5–15                      | 0.05–0.23        | Okabe et al. |
| GAC-Granules-based | 10                | 0.8                         | 0.6–1.5          | Present work |

![Graph showing the relationship between Nitrosomonas abundance and N₂O emission.](image-url)
for NH$_4$-N oxidation in one anammox reactor, thus, N$_2$O produced due to NH$_2$OH oxidation (Wunderlin et al. 2013). The results of this study is partly consistent with the literature showing that NH$_2$OH oxidation by AOB was considered the most probable cause of N$_2$O production (0.6 % of the nitrogen load) in a full-scale Anammox reactor treating sludge reject water (Kampschreur et al. 2009b). Beyond that, this study could not exclude the possibility of N$_2$O emission by denitrifiers. Further study was needed to quantify N$_2$O emission contributed by denitrifiers and *Nitrosomonas* using real wastewater.

**Conclusions**

One anammox reactor was used to investigate the effect of substrate concentrations on N$_2$O emissions. The monitoring N$_2$O concentrations were determined as 0.6–1.0 % in the emission gas during this study. Increasing inlet NH$_4$-N concentration from 36 to 57 mg L$^{-1}$, N$_2$O increased from 0.65 to 1.4 %. Reduced inlet NH$_4$-N concentrations induced N$_2$O emission. The results got in this study suggested that in addition to denitrifiers, *Nitrosomonas* was also a significant cause of N$_2$O emissions.

**Methods**

**Anammox reactor and substrate**

The reactor had an inner diameter of 14 cm with a total liquid volume of 10 L including a reaction zone of 8 L and a settling zone of 2 L. The reactor was made of acrylic resin and had a water jacket for temperature control. Sampling ports were located at heights of 3, 17, 20 and 25 cm above the reactor bottom. Part of the effluent was collected in a 5-L container (with mixer and heater) for use as recycle (Fig. 6). The pH was adjusted by an online pH controller (TPH/T-10, Tengine, China) using 0.5 mol L$^{-1}$ H$_2$SO$_4$ (Yue et al. 2015). The reactor was enclosed in a black-vinyl sheet to prevent growth of photosynthetic bacteria and algae.

The reactor was operated in up-flow mode, with influent introduced at the bottom using a peristaltic pump (BT100-2J, LongerPump, China). A recirculation pump

![Fig. 6 Schematic view of the Anammox reactor system. GSS gas solid separator](image-url)
(BT600-2J, LongerPump, China) was used to dilute the influent (Fig. 6) with the treated wastewater in the 5-L recycle container.

The anammox seed sludge used in the reactor was taken from a pilot-scale anammox reactor (unpublished). The seed sludge was granule activated carbon (GAC)-based granules with settling velocity over 150 m h\(^{-1}\) (Wenjie et al. 2015). The initial seeding concentration (mass of mixed liquor suspended solids (MLSS) per liter) was set at 4 g MLSS L\(^{-1}\).

The reactor was fed with synthetic wastewater with a nitrite to ammonium molar ratio of 1.0–1.2. The detailed composition of the influent is shown in Table 2. The influent storage tank was flushed with nitrogen gas to maintain DO under 0.5 mg L\(^{-1}\), and Na\(_2\)SO\(_3\) was added to a concentration of 40 mg L\(^{-1}\) (shown to be harmless to Anammox bacteria, Wenjie et al. 2014) to keep the DO level close to zero.

**Analytical methods**

NO\(_2\)-N and NH\(_4\)-N were measured by the colorimetric method according to Standard Methods (APHA 1995). Total nitrogen (TN) was determined by the persulfate method using the UV spectrophotometric screening method (APHA 1995) for quantification of TN as NO\(_3\)-N (the oxidization product of the persulfate digestion). NO\(_3\)-N (of the original sample) was determined by calculation of the difference of TN and the sum of NO\(_2\)-N and NH\(_4\)-N. The pH was measured by using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured by using a DO meter (6010, Jenco, USA).

**Gas collection and analysis**

Gas was collected through the GSS (Fig. 1) and the volume was measured using an inverted cylinder containing tap water with the pH lowered to 3 using 1-N H\(_2\)SO\(_4\). Gas analyses were performed by using a GC-112A gas chromatograph (INESA INSTRUMENT, China).

**DNA extraction and high-throughput 16S rRNA gene pyrosequencing**

After 139 days of operation, the particle based granules were taken out from the Anammox reactor. A granular sludge sample was first ground with a pestle under liquid nitrogen. Meta-genomic DNA was extracted using the E.Z.N.A. Soil DNA Kit (OMEGA Biotec. D5625-01, USA) according to the manufacturer’s instructions. Amplification of the 16S

| Table 2  Composition of synthetic wastewater | Concentration (mg L\(^{-1}\)) |
|-----------------------------------------------|-------------------------------|
| (NH\(_4\))\(_2\)SO\(_4\), NaNO\(_2\) (as mg N L\(^{-1}\)) | 200–1000                     |
| KHCO\(_3\)                                             | 1000                          |
| KH\(_2\)PO\(_4\)                                       | 20–1300                      |
| CaCl\(_2\)·2H\(_2\)O                                    | 100                           |
| MgSO\(_4\)·7H\(_2\)O                                   | 200                           |
| Na\(_2\)SO\(_3\)                                       | 24.81                         |
| Trace element solution 1 (g L\(^{-1}\)): FeSO\(_4\)·7H\(_2\)O 10, C\(_{10}\)H\(_{14}\)N\(_2\)Na\(_2\)O\(_5\) 5.6 | 1 mL L\(^{-1}\)              |
| Trace element solution 2 (g L\(^{-1}\)): MnCl\(_2\)·4H\(_2\)O 0.352, CoCl\(_2\)·6H\(_2\)O 0.096, NiCl\(_2\)·6H\(_2\)O 0.08, CuSO\(_4\)·5H\(_2\)O 0.1, ZnSO\(_4\)·7H\(_2\)O 0.172, NaSeO\(_4\)·10H\(_2\)O 0.105, NaMoO\(_4\)·2H\(_2\)O 0.11, C\(_{10}\)H\(_{14}\)N\(_2\)Na\(_2\)O\(_5\) 5.0 | 1 mL L\(^{-1}\)              |
rRNA gene was performed using primers 27F (forward primer: 5′-AGAGTTTGATCCTG-GCTCAG-3′) and 533R (reverse primer: 5′-TTACCAGGCTGCTGGC-3′). PCR was carried out according to the following thermocycling parameters: 120 s initial denaturation at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, 5 min final elongation at 72 °C, 10 °C until halted by user. Duplicate PCR products were pooled and purified using the AXYGEN gel extraction kit (Axygen, USA) (Feng et al. 2012).

Pyrosequencing was carried out by a 454 Life Sciences Genome Sequencer FLX Titanium instrument (Roche). Sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97 % similarity) using the MOTHUR program.

Authors’ contributions
Yue Jin carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Dunqiu Wang participated in the design of the study and performed the statistical analysis. Wenjie Zhang conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests
The author(s) declare that they have no competing interests.

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