Nutrient removal and microbial granulation in an anaerobic process treating inorganic and organic nitrogenous wastewater

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Abstract The sustainable anaerobic nitrogen removal and microbial granulation were investigated by using a laboratory anaerobic granular sludge bed reactor, treating synthetic (inorganic and organic) wastewater and piggery waste. From inorganic synthetic wastewater, lithoautotrophic ammonium oxidation to nitrite/nitrate was observed by an addition of hydroxylamine. Also, the results revealed that the Anammox intermediates (particularly, hydrazine) contents in the substrate would be one of the important parameters for success of the anaerobic nitrogen removal process. The results from organic synthetic wastewater show that if the Anammox organism were not great enough in the startup of the process, denitrification and anaerobic ammonification would be a process prior to the Anammox reaction. The anaerobic ammonium removal from the piggery waste was performed successfully, probably due to the Anammox intermediates contained in the substrate. This reactor shows a complex performance including the Anammox reaction and HAP crystallization, as well as having partial denitrification occurring simultaneously. From the activity test, the maximum specific N conversion rate was 0.1 g NH4-N/g VSS/day (0.77 g T-N/g VSS/day), indicating that potential denitrification is quite high. The NO2-N/NH4-N ratio to Anammox is 1.17. The colour of the biomass treating the piggery waste changed from black to dark red. It was also observed that the red-colored granular sludge had a diameter of 1–2 mm. The settleability assessment of the granular sludge revealed that the granular sludge had a good settleability even though it was worse than that of seed granular sludge.

Keywords Anaerobic nitrogen removal; Anammox; denitrification; granulation; hydrazine; hydroxylamine; P crystallization

Introduction

The conventional nitrogen removal process consists principally of two sub processes, nitrification and denitrification, resulting in intensive cost requirements due to the need of an oxygen and a carbon source during the process. Recently, Anaerobic ammonium oxidation (Anammox) has been reported to be a powerful piece of technology and that its application for nitrogen removal could lead to significantly lower (90% reduction) operational cost (Jetten et al., 2001). Beside the Anammox, there are some reports on lithoautotrophic organisms contributing to nitrogen removal: anoxic ammonium oxidizers such as Nitrosomonas eutropha (Schmidt and Bock, 1997), denitrifying nitrifiers such as Nitrosomonas europaea (Poth and Focht, 1985), and sulfur utilizing denitrifiers such as Thiobacillus denitrificans (Batcheler and Lawrence, 1978). Also, Fdz Polanco et al. (2001) described simultaneous sulfate reduction and an Anammox reaction as a possible new process.

Even though there are still many unknown reactions, it appears that these organisms can contribute to sustainable nitrogen removal under anaerobic/or anoxic conditions, depending on the type of electron donor in the substrate. Because these lithoautotrophic organisms, as well as the Anammox bacteria, are characterized by a low maximum growth rate, a reactor with high biomass retention, such as immobilization or granulation process (biofilm or UASB) is required. However, the granular sludge reactor for higher nitrogen loading application has a disadvantage of a longer startup time.
The research focuses on the development of an anaerobic nitrogen removal process and microbial sludge granulation. The sustainable anaerobic nitrogen removal was investigated by operation of a lab-scale anaerobic granular sludge bed reactor treating synthetic (inorganic and organic) and real N-rich waste (piggery waste). The effect of hydroxylamine and hydrazine as the Anammox intermediates was also estimated.

Materials and methods

Laboratory reactor setup

Four lab-scale 1–1.5 L UASB glass reactors (internal diameter 6 cm) with a 0.5 L settling tank were operated at mesophilic (35°C) conditions. The reactor was operated using a semi continuous feeding system. The substrate was fed four times daily in a fill-and-draw mode. During the overall operating period, the reactor was constantly operated with a hydraulic retention time (HRT) of 5 days. The settled sludge in the settler was recycled into the bottom of the UASB reactor, resulting in the upflow rate of about 0.5–1Q. 0.7 L of granular sludge (18.6 g VS/L and 65% VS/TS) was inoculated as seed biomass. Originally, the granular sludge was collected from a full-scale UASB reactor treating brewery wastewater. It had been preserved in a laboratory storage tank for a long time.

Substrate

Table 1 represents the substrate compositions for each reactor. The reactor A-1 for the inorganic synthetic wastewater was operated with high (1,000 mg NH₄-N/L and 1,300 mg NO₂-N/L) nitrogen concentration and later the reactor was operated with lower nitrogen concentration (100 mg NH₄-N and 50 mg NO₂-N/L), in order to decrease the nitrite inhibition in the Anammox reaction (Reactor A-2). Hydroxylamine and hydrazine, which are intermediates in the Anammox process, were added to the inorganic substrate (Reactor A-1 and A-2) to induce the Anammox reaction. An organic carbon source for Reactor B was a diluted sucrose solution of 5 g/L. To avoid potential deficiencies in the trace element supply, the mineral medium solution (20 mL per L influent) was added to the synthetic substrate. The medium solution contained KH₂PO₄ 13.61 g/L, NH₄Cl 49.20 g/L, CaCl₂ 4.44 g/L, MgCl₂·6H₂O 8.13 g/L and 10 mL/L of a trace element solution. The trace salt stock solution was prepared according to Ahn et al. (2000). This medium solution was selected for the enhancement of microbial granulation.

A slurry-type piggery waste with high strength was used as the substrate for Reactor C. The waste had high ammonium content (about 90% of NH₄-N/T-N). Ammonium nitrogen loading of the UASB reactor was 0.43 kg NH₄-N/m³-day. To induce the Anammox reaction, nitrite stock solution (NaNO₂) with the same flowrate was fed into the separated feed line with the substrate. The NH₄-N:NO₂-N ratio was 0.8–1.2.

Analysis

The nitrogen (ammonium, nitrite and nitrate) concentration was measured colorimetrically.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Substrate composition for lab-scale anaerobic sludge bed reactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor</td>
<td>Carbon source (mg/L)</td>
</tr>
<tr>
<td>A-1</td>
<td>NaHCO₃ 7,000</td>
</tr>
<tr>
<td>A-2</td>
<td>NaHCO₃ 6,000</td>
</tr>
<tr>
<td>B</td>
<td>Sucrose 5,000*</td>
</tr>
<tr>
<td>C</td>
<td>Piggery waste 25,700*</td>
</tr>
</tbody>
</table>

Note: H.A., hydroxylamine (NH₂OH); H.Z., hydrazine (N₂H₄); * as soluble COD
by Standard Methods (1998). In the operation of the reactor, the pH (Orion 720, USA), bicarbonate alkalinity (BA) and gas production (Wet-test gas meter, Sinagawa Model W-NK-0.5A, Japan) were monitored daily. A gas chromatograph (Tremetrics Model 9000, USA) with a TCD detector and a Hayesap Q (80/100) column was utilized to measure gas composition \((\text{N}_2\text{O}, \text{N}_2, \text{CO}_2, \text{NH}_3, \text{and CH}_4)\). The temperature for the column was kept at 35°C, 120°C for the injector, and 120°C for the detector. The helium carrier gas had a flow rate of 30 mL/min. Data integration was accomplished using a Varian 4270 Integrator. The sludge granulation during the operation was examined by microscopic analysis (Olympus BX60F5, Japan) and by settleability assessment (Ahn and Speece, 2003). Hydroxylamine and hydrazine were measured as described by Frear and Burrell (1955) and Watt and Chrisp (1952), respectively.

Microbial activity test of Anammox sludge
To detect the Anammox microbial activity, a batch test was conducted using a 0.5 L batch reactor. The Anammox sludge (100 mL) cultivated using the piggery waste (Reactor C), was inoculated into the batch reactor. Initial ammonium and nitrite concentrations in the reactor were set to 1,280 mg/L and 2,070 mg/L using the influent mixture of the UASB reactor, resulting in an initial NO\(_2\)-N/NH\(_4\)-N ratio in the batch reactor of 1.6 (for a nitrite unlimited condition).

Results and discussion
Nitrogen removal from inorganic synthetic wastewater
Figure 1(a) represents the operating results of Reactor A-1, which operated with constant nitrogen loading of 0.5 g T-N/L-day. At the end of phase I (about Day 40), the reactor shows that a little ammonium removal and a high nitrite accumulation occurred gradually. The reactor was discontinued on Day 43. However, 20 days later (Day 63 – phase II), it could be observed that somewhat of a decrease in both ammonium and nitrite occurred, resulting in a nitrogen conversion and removal of 0.02 g N/L-d and 18%, respectively. Based on the result of the addition of the Anammox intermediates (Strous et al., 1999), a trace amount (3.2 mg/L) of hydroxylamine was injected on Day 63. As shown in the figure, this resulted in a faster decrease of ammonium, whereas nitrite and nitrate increased at the same rate. Contrary to the results of the literature, a net nitrogen conversion in phase III was almost zero. This means that lithoautotrophic ammonium oxidation to nitrite/nitrate could occur by an addition of hydroxylamine, as described by Böttcher and Koops (1994). Even though the oxygen source under anoxic conditions is unknown, nitrite, NO\(_2\), and NO could theoretically have been used as the oxygen source (Schmidt and Bock, 1997).

The operating conditions of this reactor were switched to much lower influent nitrogen concentration (100 mg NH\(_4\)-N and 50 mg NO\(_2\)-N /L- Reactor A-2). For Reactor A-2, hydroxylamine and hydrazine were constantly added to the influent. As shown in Figure 1(b), the ammonium over 90% was removed during the operating period, and the nitrite showed higher concentration than that of the influent. The results show a similar trend to Reactor A-1. However, this reactor reveals that the nitrite reduction occurred gradually, meaning that microbial acclimation occurred as well. Particularly, the nitrite and nitrate decreased faster at the end of phase II, when the Anammox intermediates (10 mg/L of hydroxylamine and 9 mg/L of hydrazine) were added.

When the addition rate of the intermediates increased (phase III), the nitrite and nitrate temporarily increased, but were removed faster in phase II. The concentration of the Anammox intermediates was very low compared with those of the influent, and the
Hydrazine concentration gradually decreased according to the operation time. Particularly, the nitrite and hydrazine were dramatically reduced during the batch operation (phase IV). These results reveal that the Anammox intermediates (particularly, hydrazine) are important parameters for the success of the anaerobic nitrogen removal process. Table 2 represents the summarized results in each phase. The nitrogen loading and conversion rate were 0.03 g/N-d and 0.01–0.02 g N/L-d, respectively. The total nitrogen removal was 31–63.4%.

Figure 2 and Table 3 represent the results of the secondary operation for Reactor A-2. As seen in the figure, the results clearly show that ammonium oxidation and nitrite accumulation occurred between Day 130 to 175, resulting in a total N and ammonium removal of 48% and 92%, respectively. In addition, double ammonium loading resulted in a rapid nitrite reduction for the next 10 days. After Day 190, the reactor showed a stable performance, showing that the total N and ammonium removal was 30% and 57%, respectively. However, the nitrogen conversion rate was similar throughout the entire period (0.02 g N/L-d).

Figure 1 Nitrogen removal from inorganic synthetic wastewater

Table 2 Summary of operating results from Reactor A-2

<table>
<thead>
<tr>
<th>Items</th>
<th>Inf. phase I</th>
<th>Inf. phase II</th>
<th>Inf. phase III</th>
<th>phase IV</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>day 35</td>
<td>day 37</td>
<td>day 81</td>
<td>day 88</td>
</tr>
<tr>
<td>NH₄-N (mg/L)</td>
<td>100</td>
<td>6</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>NO₂-N (mg/L)</td>
<td>50</td>
<td>85</td>
<td>50</td>
<td>98</td>
</tr>
<tr>
<td>NO₃-N (mg/L)</td>
<td>0</td>
<td>9.3</td>
<td>0</td>
<td>19.5</td>
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<tr>
<td>NH₂OH (mg N/L)</td>
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<td>0.03</td>
<td>4.2</td>
<td>0.013</td>
</tr>
<tr>
<td>N₂H₄ (mg N/L)</td>
<td>0</td>
<td>0.44</td>
<td>7.9</td>
<td>0.4</td>
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<tr>
<td>BA (mg/L as CaCO₃)</td>
<td>3,050</td>
<td>2,660</td>
<td>3,050</td>
<td>1,620</td>
</tr>
<tr>
<td>TIN (mg/L)</td>
<td>150.4</td>
<td>100.8</td>
<td>162.1</td>
<td>124.9</td>
</tr>
<tr>
<td>TIN removal (%)</td>
<td>33.0</td>
<td>31.3</td>
<td>52.0</td>
<td>51.1</td>
</tr>
<tr>
<td>N conversion rate</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Note: Inf., influent; TIN, total inorganic nitrogen; N conversion rate, g N/L-d; * based on the batch operation results with no feeding from Day 108 to 137
According to recent research, there are some results that the ammonium removal in the anaerobic (or anoxic) process treating inorganic wastewater, can be performed by anoxic ammonium oxidizers such as *Nitrosomonas eutropha* (Schmidt and Bock, 1997), lithoautotrophic denitrifying nitrifiers such as *Nitrosomonas europaea* (Poth and Focht, 1985), Anammox organisms with sulfate reduction bacteria (Fdz-Polanco *et al.*, 2001), as well as Anammox organisms such as *Brocadia anammoxidans* (Jetten *et al.*, 2001). The performance of the reactor in this study showed as that of the CANON process (Sliekers *et al.*, 2002). Even though microbial identification was not performed, the ammonium removal in this reactor could be performed by one of two organisms, an anoxic denitrifying autotrophic nitrifier or Anammox organism, due to substrate composition (and thus, absence of gaseous NO₂ or sulfate as electron donor) based on the results of the above literature.

### Nitrogen removal from organic synthetic wastewater

Figure 3 represents the performance of Reactor B, which was operated identically to the operating condition of Reactor A-1 except for the carbon source. As a result, from Day 10 to 20, there was a sign of Anammox activation (such as nitrate increase). After Day 20, ammonium was not removed at all, and denitrification (denitrification) was finally observed. Furthermore, it revealed a higher ammonium accumulation (approximately 50%) than that of the influent in the latter part of the operating period. Because the effluent contained organic materials (about 800 mg/L of COD), it could not be expected that the anaerobic ammonium accumulation was a result of the destruction of the anaerobic granule. The
granular sludge completely changed to dispersed sludge at the end of the period. The organic uptake and bicarbonate alkalinity production were respectively about 4 g SCOD/g NO₂-N reduction, and 5.3 g CaCO₃/g NO₂-N when the anaerobic ammonification was excluded. It was reported that the Anammox was less competitive with denitrification (Dong and Tollner, 2003; Ahn et al., 2004). However, this result indicates that if the Anammox organism was not enough, denitrification and anaerobic ammonification should be a process prior to the Anammox reaction in nitrogen removal from the organic N-rich waste.

Nitrogen removal from piggery waste
Contrary to the foregoing experiments for synthetic wastewater, the anaerobic ammonium removal from the really strong nitrogenous waste, piggery waste, was performed successfully as shown in Figure 4(a). Furthermore, the reaction quickly started in spite of the high ammonium concentration. The average nitrogen loading and nitrogen conversion were respectively about 1.0 g N/L-d and 0.6 g N/L-d. From the nitrogen and carbon mass balances, the NO₂⁻N/NH₄⁺-N removal ratio by the Anammox reaction was 1.48–1.79 (Ahn et al., 2004). As shown in Figure 4(a), the piggery waste used as a substrate contained 0.7–1.5 mg/L of hydroxylamine and 2.5–7 mg/L of hydrazine, depending on the sampling time at the piggery slurry storage pit. However, the concentrations of hydroxylamine and hydrazine in the effluent were quite a bit higher (about double) than those of the influent. Also, those concentrations in the effluent gradually showed a tendency to decrease according to the operating time, indicating process stability. The results indicate that hydroxylamine and hydrazine contents in the substrate would be an important parameter in anaerobic ammonium removal.

Microbial activity of Anammox sludge
The result of the Anammox activity test using a batch reactor is shown in Figure 4(b). This figure shows that the Anammox reaction must be almost fully established within 1.5 days. According to these results, the maximum specific nitrogen conversion rate was 0.1 g NH₄⁺-N/
N/g VSS/day (0.77 g T-N/g VSS/day). Compared to the ammonium conversion rate, the maximum specific nitrite conversion rate was somewhat higher (0.67 g NO₂-N/g VSS/day), indicating that nitrite denitrification mainly occurred due to the unlimited condition of nitrite (initial NO₂-N/NH₄-N ratio = 1.6). In the batch test, 3,280 mg/L of COD was removed within 1.5 days, and the methane gas production was not observed. Literature reported that the organic requirement for unit nitrite removal (g COD/g NO₂-N) is 2.0 g COD for acetic acid and 2.8 g COD for lactic acid (Akunna et al., 1993). Also, Ho and Choi (2000) reported that 1.8–2.1 g COD/g NO₂-N and 2.1–2.6 g COD (as methanol)/g NO₂-N in the nitrite nitrification–denitrification process is necessary for piggery waste. If the organic requirement is assumed to be 2.1 g COD/g NO₂-N, then the NO₂-N/NH₄-N removal ratio by the Anammox is 1.17.

**Phosphorus removal in anaerobic reactor treating piggery waste**

In an anaerobic reactor treating piggery waste, it was observed that approximately 25% of the total phosphorus (T-P) was removed by phosphorus crystallization as shown in Figure 5. This must be due to a high pH and bicarbonate alkalinity, as were the operation conditions. It was found that about 135 mg/L of Ca²⁺ and 4 mg/L of Mg²⁺ were contained in the piggery waste used as the substrate. About 60 mg/L of T-P in the reactor was removed. Based on molecular weight, this amount was satisfactory for the HAP (hydroxyapatite) reaction ratio to occur (5Ca:3P = 2.1:1), and indicated that HAP crystallization could have occurred. However, this reaction cannot contribute to the ammonium removal such as struvite crystallization. This means that the Anammox reaction played a leading role for the ammonium removal in this experiment. In the reactor, the P removal was 12 mg P/day and according to the HAP reaction ratio (5.4 g HAP/g P), HAP crystals of 0.065 g/day could be produced. The result of this research shows that the complex performance, including the Anammox reaction and HAP crystallization as well as the partial denitritation effectively occurred in a single reactor.

**Microbial granulation and settleability**

At the bottom of the Reactor C treating the piggery waste, the colour of the biomass changed from black to dark red, which was accompanied by an increase in the content of
cytochrome (van de Graaf et al., 1996). By the microscopic analysis at the end of the experiment, it was also observed that the red-coloured granular sludge had a diameter of 1–2 mm at the lower part of the reactor, as shown in Figure 6. For Reactor A-2, the colour of the biomass changed from black to brown. The settleability assessment of the sludge in Reactor A-2 and Reactor C revealed that the granular sludge had a good settleability even though it was worse than that of the initial seed granular sludge, as shown in Figure 7.

**Conclusions**

- From the inorganic synthetic wastewater, lithoautotrophic ammonium oxidation to nitrite/nitrate was observed by hydroxylamine addition. The results reveal that the Anammox intermediates (particularly, hydrazine) are one of the important parameters for the success of the anaerobic nitrogen removal process.
- The results from the organic synthetic wastewater show that if the Anammox organisms were not enough at the startup of the reactor, denitrification and anaerobic ammonification would occur prior to the Anammox reaction in nitrogen removal from the organic N-rich waste.
Contrary to the foregoing experiments for synthetic wastewater, the anaerobic ammonium removal from the really strong nitrogenous waste, piggery waste, was performed successfully. It might be due to the hydroxylamine and hydrazine contents as Anammox intermediates in the substrate. In this reactor, a complex performance including the Anammox reaction and HAP crystallization as well as the partial denitrification were observed simultaneously in a single reactor.

From a microbial activity test of Anammox sludge, the maximum specific nitrogen conversion rate was 0.1 g NH₄-N/g VSS/day (0.77 g T-N/g VSS/day), indicating that potential denitrification is quite high. The NO₂⁻-N/NH₄⁺-N removal ratio by Anammox is 1.17. The colour of the biomass treating the piggery waste changed from black to dark red, which was accompanied by an increase in cytochrome content. It was also observed that the red-coloured granular sludge had a diameter of 1–2 mm at the lower part of the reactor. The settleability assessment of the sludge revealed that the granular sludge had a good settleability even though it was lower than that of the initial seed granular sludge.

References
