Inhibition kinetics and granular sludge in an ANAMMOX reactor treating mature landfill leachate

Li Yun, Zheng Zhaoming, Li Jun, Zhao Baihang, Bian Wei, Zhang Yanzhuo and Wang Xiujie

ABSTRACT

The present study reports the inhibition kinetics and granular sludge in an anaerobic ammonium oxidation (ANAMMOX) – up-flow anaerobic sludge blanket reactor fed with diluted mature landfill leachate. The activity of ANAMMOX bacteria was inhibited by addition of mature landfill leachate, but gradually adapted to the leachate. The system achieved efficient nitrogen removal during 65–75 d and the average removal efficiencies for NH$_4^+$-N, NO$_2^{-}$/C$_0$-N and total nitrogen (TN) were 96%, 95% and 87%, respectively. ANAMMOX was the main pathway of nitrogen removal in the system, and heterotrophic denitrification occurred simultaneously. In addition, aerobic ammonia oxidation and aerobic nitrite oxidation were active in this system. Inhibition kinetic experiments showed that the NH$_4^+$-N and NO$_2^{-}$/C$_0$-N inhibition concentration threshold of ANAMMOX were 489.03 mg/L and 192.36 mg/L, respectively. ANAMMOX was significantly inhibited by mature landfill leachate, and was completely inhibited when the leachate concentration was 1,450.69 mg/L (calculated in chemical oxygen demand). Thus, the inhibition concentration of substrate and landfill leachate should be considered when applying the ANAMMOX process to landfill leachate. The color of granular sludge ANAMMOX changed from brick-red into a reddish-brown. The particle size increased from small to large, with evident granulation of the ANAMMOX sludge.

Key words | ANAMMOX, granular sludge, inhibition kinetics, mature landfill leachate, nitrogen removal

INTRODUCTION

Landfill leachate has a high concentration of organic matter, ammonia nitrogen, salinity, and various heavy metals (Bodzek et al. 2006; Farah & Christopher 2012). Thus, the untreated leachate would cause harm to the surrounding surface and ground water. According to the time it spent in the landfill, landfill leachate is classified as early, middle and mature. The chemical oxygen demand (COD)/ammonia nitrogen (C/N) of early landfill leachate is higher, and the majority of the organic matter is biodegradable. The C/N of mature landfill leachate is lower, and most organic matter is not biodegradable. Technologies meant for leachate treatment can be classified as biological methods and chemical and physical methods, and the biological methods are the more efficient and cheaper processes to eliminate nitrogen from leachate (Wiszniewski et al. 2006). A lot of significant carbon source is required for nitrogen removal processes of conventional nitrification and denitrification, which results in cost increases (Iacovi et al. 2006). In addition, conventional nitrification and denitrification processes produce a large amount of sludge, which results in increase in the burden of sewage treatment plant. Thus, there is a current need for efficient and low consumption nitrogen removal for mature landfill leachate.

Anaerobic ammonia oxidation (ANAMMOX) is an emerging efficient energy-saving nitrogen removal process. ANAMMOX bacteria use ammonia nitrogen and nitrite nitrogen as the substrate and inorganic carbon as carbon source for autotrophic nitrogen removal (van de Graaf et al. 1995). At present, autotrophic nitrogen removal technology of nitritation combined with an aerobic ammonia oxidation is of great interest in high ammonia nitrogen and low carbon nitrogen ratio wastewater treatment. There are two combination methods used for nitritation and an aerobic ammonia oxidation. In the first method, NO$_2^{-}$-N accumulation is first performed and then is mixed with landfill leachate as $\rho$(NO$_2^{-}$/N)/$\rho$(NH$_4^+$-N) at 1:1.32 for an aerobic ammonia oxidation. The second
method, the control effluent $\rho(\text{NO}_2^-\text{N})/\rho(\text{NH}_4^+\text{N})$ is about 1:1.32 in the nitritation stage for an aerobic ammonia oxidation. ANAMMOX process would be appropriate for use in the nitrogen removal of mature landfill leachate (Shen et al. 2012). However, the growth of ANAMMOX bacteria is very slow (Tang et al. 2011; Gilbert et al. 2014). Sludge intercept is essential for the process to function in a stable operation, and granular sludge is the most commonly used method. Most studies have only focused on the effects of factors when landfill leachate was treated (Zhang & Zhou 2006; Liu et al. 2010), and have not studied the granular sludge properties that are essential for effective application of ANAMMOX in engineering applications. Moreover, an additional difficulty in application of this method is that ANAMMOX bacteria are inhibited by organic matter (Liang & Liu 2012; Alyne et al. 2014; Carlos et al. 2015), salinity (Dapena-Mora et al. 2010; Jin et al. 2015; Liu et al. 2014) and heavy metal (Bi et al. 2014; Zhang et al. 2016) in wastewater. The ANAMMOX bacterial activity is also inhibited by a high concentration of substrate (Tang et al. 2010a; Lotti et al. 2012; José et al. 2013; Li et al. 2014). However, little is known about the inhibition of the anammox activity by mature landfill leachate.

In this work, we use ANAMMOX granular sludge that was cultivated in the laboratory under an inorganic environment to treat mature landfill leachate. The performance of nitrogen removal, the pathway of nitrogen transformation, and the sludge characteristics were determined. The inhibition of ANAMMOX granular sludge by substrate and landfill leachate were determined and inhibition kinetic models were established. These findings will serve as reference for future application of ANAMMOX to mature landfill leachate treatment.

**MATERIALS AND METHODS**

**Experimental set-up and solutions**

(1) Continuous-flow experiment: up-flow anaerobic sludge bed (UASB) reactor was adopted as shown in Figure 1(a). The effective volume was 10 L, black soft material was used to reduce light, one third of the upside reactor had spherical polyvinyl chloride filler added to reduce the loss of the sludge, the diameter of the spherical filler was 10 cm, and a fibrous annular braided belt was in the spherical filler. The control temperature was 25 ± 1 °C, the hydraulic retention time (HRT) was 1.2 h, and the pH was 7.5–8.0. The concentration of influent ammonia nitrogen was unchanged during reactor operation, and the concentration of COD was adjusted by addition of the mature landfill leachate. The nitrogen removal performance and the characteristics of ANAMMOX granular sludge were tested in the different stages of the system. The process parameters of continuous-flow in different stages are shown in Table 1.

(2) Sequencing batch measurements: 500 mL serum bottle was used as shown in Figure 1(b) to investigate the performance of ANAMMOX, nitrification, and denitrification.
Sequencing batch experiments were carried out using ANAMMOX granular sludge collected from UASB at the stable operation period (70 d). Deionized water and phosphate buffer solutions were used to wash the ANAMMOX granular sludge three times to remove residual ammonium. The control temperature was 25 ± 1 °C, the pH was 7.5–7.8, and the rotor speed of the magnetic stirring apparatus was 200 ± 10 r/min. High purity nitrogen (99.999%) was used to remove the dissolved oxygen (DO) from water and maintain an anaerobic environment when the activity tests of ANAMMOX and denitrification were performed. Sufficient oxygen was provided during the activity tests of nitrification.

(3) Inhibition kinetics measurements: the tests set-up is also shown in Figure 1(b). The ANAMMOX granular sludge was collected from UASB at 75 d and was cleaned as described above. The concentrations of NH₄⁺-N were 60–1,000 mg/L but the NO₂⁻-N concentrations were adjusted to about 100 mg/L when NH₄⁺-N was the single inhibition factor, and the concentrations of NO₂⁻-N were 80–500 mg/L but the NH₄⁺-N concentrations were adjusted to about 150 mg/L when NO₂⁻-N was the single inhibition factor. The concentrations of mature landfill leachate (calculated in COD) were 0–1,500 mg/L in the landfill leachate inhibition kinetics experiment, and the concentrations of NH₄⁺-N were adjusted with ammonium chloride to about 460 mg/L to eliminate the effect of substrate concentration, and the NO₂⁻-N concentrations were adjusted with sodium nitrite to about 100 mg/L. The alkalinity and pH were adjusted by addition of sodium bicarbonate and hydrochloric acid. The experiment was performed in the constant temperature incubator. Samples were removed every 1 h to calculate the NH₄⁺-N and NO₂⁻-N removal efficiencies. All tests were repeated three times.

### Inoculation sludge

The inoculation sludge was ANAMMOX granular sludge that was cultivated under an inorganic environment in a 50 L UASB reactor that had been in stable operation for 2–3 years. The color of the ANAMMOX granular sludge was brick-red. The main species of ANAMMOX bacteria present in the reactor was Candidatus Brocadia fulgida (JX852965-JX852969). The inoculation sludge concentration (mixed liquor volatile suspended solids, MLVSS) was about 5 g/L.

### Water quality of mature landfill leachate

The mature landfill leachate (more than 5 years old) was taken from Gao An Tun municipal landfill, which was sealed in a plastic drum after retrieval from the landfill and renewed approximately once a month. The water quality was as follows: NH₄⁺-N, 900–1,500 mg/L; NO₂⁻-N, 0–2 mg/L; NO₃⁻-N, 0–8 mg/L; COD, 2,000–4,000 mg/L; pH, 7.5–8.5; and alkalinity, 6,000–10,000 mg/L. The mature landfill leachate was diluted to the required concentration of ammonia nitrogen and moderate amounts of sodium nitrite were added to serve as an electron acceptor for anaerobic ammonia oxidation.

### Analytical methods

NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, total nitrogen (TN), COD, mixed liquor suspended solids (MLSS) and MLVSS were analyzed according to Standard Methods (APHA 2005). Temperature, DO, and pH values were determined using a WTW/Multi 3420 multiparameter device. For scanning electron microscopy (SEM), a Hitachi S-4300 instrument was used.

Inhibition kinetics of substrate would be described by the Haldane model, the equation as the following (Sheintuch et al. 1995; Surmacz-Gorska et al. 1996):

\[
\nu = \frac{v_{\text{max}}}{1 + \frac{k_s}{S} + \frac{S}{k_h}}
\]

where \( \nu \) is the substrate conversion rate, mg/(mg d); \( v_{\text{max}} \) is the maximum conversion rate, mg/(mg d); \( S \) is the substrate concentration, mg/L; \( k_s \) is the half-saturation constant, mg/L; and \( k_h \) is the half-inhibition constant, mg/L.

### Table 1

<table>
<thead>
<tr>
<th>Items</th>
<th>time/d</th>
<th>( \rho(\text{NH}_4^+\text{-N})/\text{mg/L} )</th>
<th>( \rho(\text{NO}_2^-\text{-N})/\text{mg/L} )</th>
<th>( \rho(\text{NO}_3^-\text{-N})/\rho(\text{NH}_4^+\text{-N}) )</th>
<th>TN volume loading kg/(m³ d)</th>
<th>COD volume loading kg/(m³ d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–7</td>
<td>50–60</td>
<td>50–70</td>
<td>4–8</td>
<td>1.23</td>
<td>2.1–2.7</td>
</tr>
<tr>
<td>2</td>
<td>8–24</td>
<td>50–60</td>
<td>50–70</td>
<td>3–8</td>
<td>1.11</td>
<td>2.1–2.7</td>
</tr>
<tr>
<td>3</td>
<td>25–47</td>
<td>50–60</td>
<td>50–70</td>
<td>3–9</td>
<td>1.13</td>
<td>2.1–2.7</td>
</tr>
<tr>
<td>4</td>
<td>48–75</td>
<td>50–60</td>
<td>70–90</td>
<td>3–13</td>
<td>1.42</td>
<td>2.7–3.2</td>
</tr>
</tbody>
</table>
concentration, mg/L; $k_s$ is half-saturation constant, mg/L; $k_i$ is Haldane inhibiting kinetics constant, mg/L.

Inhibition kinetics by maturel and fill leachate would be described by the model of chlorophenol inhibition kinetics model when acetic acid was degraded (Kim et al. 1997). The equation used was as follows:

$$\nu = \frac{v_{\text{max}}[k_0]Sx}{k_s[k_1] + S}$$

(2)

where $\nu$ is the substrate conversion rate, mg/(mg d); $v_{\text{max}}$ is the maximum conversion rate, mg/(mg d); $S$ is the substrate concentration, mg/L; $k_s$ is half-saturation constant, mg/L; $k_0$ and $k_1$ are inhibiting constant.

$k_0$ and $k_1$ are calculated according to the following formula:

$$k_0 = \left[1 - \frac{\alpha}{\beta}\right]^{m}$$

(3)

$$k_1 = \left[1 - \frac{\alpha}{\beta}\right]^{n}$$

(4)

where $\alpha$ is toxic substance concentration, mg/L; $\beta$ is toxic substance fully inhibition concentration, mg/L; $m$ and $n$ are constant.

The above formula was revised by introduced speed ratio ($\lambda$), the revised equation as the follows:

$$\lambda = \frac{1 - \left[\frac{\alpha}{\beta}\right]^{n}}{1 + \left[\frac{\alpha}{\beta}\right]^{m}}$$

(5)

where $\lambda = \nu/\nu_0$, $\lambda$ is speed ratio, $\nu$ is the conversion rate under different maturel and fill leachate concentration, mg/(mg d); $\nu_0$ is the conversion rate of no maturel and fill leachate, mg/(mg d).

**RESULTS AND DISCUSSION**

**Operation performance of ANAMMOX system**

**Characteristics of nitrogen removal in ANAMMOX system**

Simulated inorganic wastewater was used during phase 1 (0–7 d). The removal efficiencies of NH$_4$-N, NO$_2$-N and TN were 85%, 92%, and 75%, respectively, at 1 d and increased to 95%, 99%, and 80% respectively, at 7 d. These increased rates indicated that the activity of inoculating ANAMMOX bacteria was robust, and the bacteria were able to quickly adapt to the new environment. The mature landfill leachate was added during phase 2 (8–24 d). The removal efficiencies of NH$_4$-N, NO$_2$-N and TN decreased to 72%, 79%, and 64%, respectively, at 11 d and returned to 86%, 99%, and 86%, respectively, at 18 d and then continuously operated for 7 d. The concentration of influent mature landfill leachate was increased at 25 d. The activity of the ANAMMOX bacteria was inhibited because of the increase of influent mature landfill leachate concentration, and the removal efficiencies of NH$_4$-N, NO$_2$-N and TN decreased to 34%, 48%, and 38%, respectively, at 33 d. The ANAMMOX bacteria begin to adapt to the new environment and showed gradually recovery of activity. The removal efficiencies of NH$_4$-N, NO$_2$-N, and TN reached 71%, 88%, and 73%, respectively, at 47 d. The average influent ratio $\rho$(NO$_2$-N)/$\rho$(NH$_4$-N) was 1.23, 1.11, and 1.13 during the above three phase, smaller than the theoretical ratio (1.32) of the ANAMMOX reaction (Strous et al. 1998). There were parts of NO$_2$-N that would be simultaneously dislodged due to denitrification in the system when landfill leachate was used as the influent, leading to insufficient NO$_2$-N levels. For this reason, the concentration of NO$_2$-N was increased to satisfy to provide sufficient ANAMMOX electron acceptor during phase 4. The average influent ratio $\rho$(NO$_2$-N)/$\rho$(NH$_4$-N) was 1.42, higher than the theoretical ratio of the ANAMMOX reaction. The average removal efficiencies of NH$_4$-N, NO$_2$-N and TN were 96%, 95% and 87%, respectively, during the 65–75 d period (Figure 2). This suggests that the ANAMMOX bacteria gradually adapted to the presence of the mature landfill leachate, and the system exhibited enhanced nitrogen removal.

**Stoichiometry relationship of ANAMMOX reaction in system**

Stoichiometry reflects the ratio relationship between substrate consumption and production in a system, and can be used to adjust the ratio of influent substrate concentration. The stoichiometry relationship of the ANAMMOX reaction in a system is shown in Figure 3. $\rho$(NO$_2$-N)/$\rho$(NH$_4$-N) was the average ratio of NO$_2$-N and NH$_4$-N consumed by the ANAMMOX reaction, $\rho$(NO$_3$-N)/$\rho$(NH$_4$-N) was the average ratio of NO$_3$-N that was produced and the NH$_4$-N that was consumed by ANAMMOX reaction. The ratio of $\rho$(NO$_2$-N)/$\rho$(NH$_4$-N) was 1.30 and the ratio of
\( \rho(\text{NO}_3^-/-\text{N})/\rho(\text{NH}_4^+/\text{N}) \) was 0.27 in phase 1 (0–7 d), consistent with the theoretical values. The ratio of \( \rho(\text{NO}_2^-/-\text{N})/\rho(\text{NH}_4^+/\text{N}) \) was 1.30 and the ratio of \( \rho(\text{NO}_3^-/-\text{N})/\rho(\text{NH}_4^+/\text{N}) \) was 0.19 in phase 2 (8–24 d), suggesting the synchronously-denitrification reaction reduced \( \text{NO}_3^-/-\text{N} \) in this phase. The ratio of \( \rho(\text{NO}_2^-/-\text{N})/\rho(\text{NH}_4^+/\text{N}) \) was 1.43 and the ratio of \( \rho(\text{NO}_3^-/-\text{N})/\rho(\text{NH}_4^+/\text{N}) \) was 0.13 in phase 3 (25–47 d), suggesting that the effect of the denitrification reaction increased with the increase in organic matter concentration.

### The activity of granular sludge and nitrogen transformation pathway

Heterotrophic denitrifying bacteria can grow in the presence of organic matters when the environment is anaerobic. Moreover, the DO of influent was 5–7 mg/L, and there were some aerobic nitrifying bacteria in the system at this time. The performance of ANAMMOX, nitrification, and denitrification were investigated during the stable operation period (65–75 d). The activity of ANAMMOX is shown in Figure 4(a). The degradation rates of \( \text{NH}_4^+/\text{N} \) and \( \text{NO}_2^-/-\text{N} \) were 0.128 and 0.184 g/(g d), and the production of \( \text{NO}_3^-/-\text{N} \) was 0.026 g/(g d); the measurements showed linear regression indicating that the rates were constant during the batch experiment, which reflect good data quality (R²-values were 0.987, 0.987 and 0.979). The activity of aerobic ammonia oxidation is shown in Figure 4(b), and the degradation rate of \( \text{NH}_4^+/\text{N} \) was 0.031 g/(g d). The activity of aerobic nitrite oxide is shown in Figure 4(c), and the degradation rate of \( \text{NO}_2^-/-\text{N} \) was 0.010 g/(g d). The activity of denitrification is shown in Figure 4(d) and the degradation rates of \( \text{NO}_3^-/-\text{N} \) and \( \text{NO}_2^-/-\text{N} \) were very similar, 0.026 g/(g d) and 0.028 g/(g d), respectively. The analyses of granular sludge activity during the stable operation period suggest that ANAMMOX was the major pathway of nitrogen removal, but heterotrophic denitrification occurred concurrently. Nitrogen removal performance was...
analyzed from the nitrogen transformation pathway: (i) there were two pathways of NH$_4^+$ removal, one was the ANAMMOX process, and the other was oxidized into NO$_2^-$ and NO$_3^-$; (ii) the main pathway of NO$_2^-$ removal was ANAMMOX, and some NO$_2^-$ was removed by denitrification and oxidation; (iii) NO$_3^-$ was mainly produced by ANAMMOX, and influent added NO$_3^-$ as well and the main removal pathway was heterotrophic denitrification.

### Inhibition kinetic characteristics of ANAMMOX

NH$_4^+$ and NO$_2^-$ are used as substrate by ANAMMOX bacteria at low concentration, but can inhibit at high concentrations. Most organic matter and heavy metals also act as inhibitors of ANAMMOX bacteria. Mature landfill leachate has characteristics such as high NH$_4^+$, high amounts of organic matter, and lots of heavy metals. We next investigated the inhibition of ANAMMOX granular sludge by substrate and landfill leachate.

#### Substrate inhibition and its dynamics

The effect of ANAMMOX inhibition by substrate concentration is shown in Table 2. NH$_4^+$ and NO$_2^-$ removal rate was increased at first and then decreased as the experimental substrate concentration increased. The highest NH$_4^+$ removal rate was 0.1540 mg/(mg d) when NH$_4^+$ concentration was 295.62 mg/L, and decreased to 0.1455 mg/(mg d) when the NH$_4^+$ concentration was increased to 930.51 mg/L. The NO$_2^-$ removal rate was 0.1649 mg/(mg d) when NO$_2^-$ concentration was 151.10 mg/L, and decreased to 0.1395 mg/(mg d) when the NO$_2^-$ concentration was increased to 497.82 mg/L. The inhibition of ANAMMOX by NH$_4^+$ and NO$_2^-$ is essentially by free ammonia (FA) and free nitrous acid (FNA) (Waki et al. 2007; Fernández et al. 2010). Ammonium has the equilibrium reaction in an aqueous solution: NH$_4^+ + OH^- \rightleftharpoons NH_3 + H_2O$. Similarly, nitrite has the equilibrium reaction in aqueous solution: NO$_2^- + H^+ \rightleftharpoons HNO_2$. Both dynamic balances will change as pH value changes. Unprotonated FA and FNA can penetrate the lipid membrane, but NH$_4^+$ and NO$_2^-$ penetrate less easily (Kadam & Boone 1996). Most reports have proposed that it is ammonia (NH$_3$) that serves as a substrate for microorganisms (Tang et al. 2010b).

The experimental results of substrate inhibition were nonlinearly fitted according to Equation (1) and using Origin 8.0 as shown in Figure 5. The correlation coefficients ($R^2$) were 0.9901 and 0.9985. This high correlation indicated that the Haldaone model well described the inhibition behavior of the two inhibitory factors well. The maximum ammonia oxidation rate ($V_{\text{max(NH}_4^-)}$) was 0.1893 mg/(mg d) and the Haldane inhibiting kinetics constant was 5,482.27 mg/L (FA was 151.16 mg/L) when ammonium was the single inhibiting factor. The maximum NO$_2^-$ removal rate ($V_{\text{max(NO}_2^-)}$) was 0.246 mg/(mg d) and Haldane inhibiting kinetics constant was 701.15 mg/L (FNA was 0.1056 mg/L) when nitrite was the single inhibiting factor. This indicates that ANAMMOX bacteria were more significantly inhibited by NO$_2^-$.

### Table 2 | Comparison of kinetic characteristics parameters

<table>
<thead>
<tr>
<th>Sludge</th>
<th>$K_m$(mg/L)</th>
<th>$K_s$(mg/L)</th>
<th>References</th>
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<tbody>
<tr>
<td></td>
<td>NH$_4^+$</td>
<td>NO$_2^-$</td>
<td>NH$_4^+$ N</td>
</tr>
<tr>
<td>HAG</td>
<td>17</td>
<td>19</td>
<td>11,679 735</td>
</tr>
<tr>
<td>FAS</td>
<td>25</td>
<td>21</td>
<td>9,016 179</td>
</tr>
<tr>
<td>IG</td>
<td>22</td>
<td>21</td>
<td>10,138 933</td>
</tr>
<tr>
<td>ANAMMOX granular</td>
<td>39</td>
<td>43</td>
<td>3,482 701</td>
</tr>
</tbody>
</table>

#### Mature landfill leachate inhibition and its dynamics

The composition of landfill leachate is complex, and includes a lot of toxic organic matter, salt ions and heavy metals that could act to inhibit ANAMMOX bacteria. ANAMMOX performance was investigated over a range of mature and fill leachate concentration of 0–1,262.37 mg/L (calculated in COD). The ammonia oxidation rate was reduced to 16.16% at the highest leachate concentration.

The experimental results of mature landfill leachate inhibition were nonlinearly fitted by Origin 8.0 as shown in Figure 6. The correlation coefficient ($R^2$) for the fit was 0.9714, indicating that a good fit of the observed data to
the inhibition model. The fully inhibitory concentration of mature landfill leachate was 1,450.69 mg/L (calculated in COD). Kinetic constants \( m \) and \( n \) were 2.49 and 0.99 respectively.

The equations and parameters of the inhibition kinetics are shown in Table 3.

### Characteristics of granular sludge

#### Apparent characteristics

The particle size was small and the color of ANAMMOX granular sludge was brick-red at the start of the experiment (0 d; Figure 7(a)) due to the high concentration of ferroheme in the bacteria cells (Schalk et al. 2000; Shimasuura et al. 2007). The color of ANAMMOX granular sludge deepened to crimson and granular sludge particle size was increased at 35 d (Figure 7(b)). At 70 d, the color of ANAMMOX granular sludge was reddish-brown and there were filamentous bacteria on the external surface (Figure 7(c)). The explanation for the deepening color change maybe due to: (i) the covering of ANAMMOX bacteria by heterotrophic bacteria that grew on granular sludge surface; or (ii) adsorption of dark impurities from the landfill leachate on the granular sludge surface. The SEM photograph shows the granular sludge at 70 d. There are many filamentous bacteria evident on the surface of the granular sludge as shown in Figure 8(a), and the leachate organic matter may have stimulated growth of the filamentous heterotrophic bacteria. Under magnification, rod-shaped bacteria and spherical bacteria are evident (Figure 8(b)), possibly nitrobacteria. Nitrobacteria on the ANAMMOX granular sludge surface can relieve DO inhibition when present in the influent (Vázquez-Padín et al. 2009; Cho et al. 2011). ANAMMOX bacteria are spherical bacteria and the surface structure is crateriform (van de Graaf et al. 1996). Most of the ANAMMOX bacteria are located within the granular sludge and heterotrophic bacteria and nitrobacteria were on the surface. In addition, there were many holes in the sludge that would act to increase the mass-transfer effect of the sludge (An et al. 2013).

<table>
<thead>
<tr>
<th>Inhibition type</th>
<th>Fitted equation</th>
<th>Parameter 1</th>
<th>Parameter 2</th>
<th>Parameter 3</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NH}_4^+ - \text{N} )</td>
<td>( y = 0.1893\left(1 - 39.39/x + x/3,482.27\right) )</td>
<td>( \nu_{\text{max}} = 0.1893 )</td>
<td>( k_\alpha = 39.39 )</td>
<td>( k_h = 3,482.27 )</td>
<td>0.9901</td>
</tr>
<tr>
<td>( \text{NO}_2^- - \text{N} )</td>
<td>( y = 0.246\left(1 - 43.19/x + x/701.15\right) )</td>
<td>( \nu_{\text{max}} = 0.246 )</td>
<td>( k_\alpha = 43.19 )</td>
<td>( k_h = 701.15 )</td>
<td>0.9985</td>
</tr>
<tr>
<td>Mature landfill leachate</td>
<td>( y = \left[1 - (x/1,450.69)\right]^{2.49} / \left[1 + (x/1,450.69)\right]^{0.99} )</td>
<td>( \beta = 1,450.69 )</td>
<td>( m = 0.99 )</td>
<td>( n = 2.49 )</td>
<td>0.9714</td>
</tr>
</tbody>
</table>
holes may arise as N₂ is released after being produced by ANAMMOX bacteria inside the sludge (Bhunia & Ghangrekar 2007; Batstone & Keller 2001).

**Particle size distribution**

The granular sludge particle size distribution was characterized as the percentage of MLSS of the different particle size and total MLSS. As shown in Table 4, the difference in granular sludge particle size was smaller at 0 d than at 35 d and 70 d. At 0 d, 27.3% of the granular sludge particles were in the range of 0.5–1.0 mm, 24.4% were 1.5–2.0 mm (24.4%), and 14.2% were less than 0.5 mm. At 35 d, 43.4% were 1.5–2.0 mm in size and 25.2% were 2.0–2.5 mm. There was little change in the granular sludge particle size distribution at 70 d compared with that at 35 d, and the majority of the particles were in the range of 1.5–2.5 mm.

Thus, the ANAMMOX granular sludge particle size increased and then stabilized under the experimental condition. This could be due to the following reasons: ① the granulation of sludge could be related to up-flow velocity (O’Flaherty et al. 1997; Alves et al. 2000) or ② the growth of heterotrophic bacteria on the granular sludge surface could alter the particle size, the removal efficiencies of COD was 10–25% which could give a better indication on the heterotrophs’ rate. Kindaichi et al. (2007) proposed that ANAMMOX activity occurs in the 1 mm sized granular sludge particles, and larger sizes will suffer from low substrate concentration in the interior, reducing nitrogen removal performance. The ANAMMOX granular sludge

**Table 4** | Granule size distribution in ANAMMOX UASB reactor during different period

<table>
<thead>
<tr>
<th>Granule size (mm)</th>
<th>0 d</th>
<th>35 d</th>
<th>70 d</th>
</tr>
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<td>&lt;0.5</td>
<td>14.2</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5–1.0</td>
<td>27.3</td>
<td>7.8</td>
<td>7.1</td>
</tr>
<tr>
<td>1.0–1.5</td>
<td>15.2</td>
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<tr>
<td>1.5–2.0</td>
<td>24.4</td>
<td>43.4</td>
<td>40.8</td>
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<td>2.0–2.5</td>
<td>13.5</td>
<td>25.2</td>
<td>25.3</td>
</tr>
<tr>
<td>&gt;2.5</td>
<td>5.4</td>
<td>12.9</td>
<td>14.6</td>
</tr>
</tbody>
</table>
Particle size increased in this study although the smaller sludge particle size have the better mass transfer efficiency and the bigger specific surface area. Generally speaking, the larger size granular sludge is better able to resist adverse conditions. The inhibitors may be trapped on the granular sludge surface and the impact of internal ANAMMOX bacteria would be reduced. In a word, ANAMMOX granular sludge were mainly concentrated in 0.5–2.0 mm particles in the early stage and were mainly concentrated in 1.5–2.5 mm particles after domestication with matured and fill leachate.

**CONCLUSIONS**

The mature landfill leachate was used to domesticate the ANAMMOX bacteria cultivated under inorganic conditions. After 75 d of running, the system gradually adapted to implement efficient denitrification of landfill leachate. The system improved nitrogen removal during 65–75 d. During the stable operation period, the average removal efficiencies of NH$_4^+$-N, NO$_2^-$-N and TN were 96%, 95%, and 87%, respectively. ANAMMOX was still the main pathway of nitrogen removal in the system with concurrent heterotrophic denitrification. Aerobic ammonia oxidation and aerobic nitrite oxide showed activity of 0.031 g/(g d) and 0.010 g/(g d) in the system. ANAMMOX inhibition kinetics experiments showed that the inhibition concentration thresholds for NH$_4^+$-N and NO$_2^-$-N were 489.05 mg/L and 192.36 mg/L, respectively, the half-saturated constants were 39.39 mg/L and 43.19 mg/L, respectively, and the inhibiting kinetic constants were 3,482.27 mg/L and 701.15 mg/L, respectively. ANAMMOX was significantly inhibited by mature landfill leachate, and activity was almost completely inhibited at a leachate concentration of 1,450.69 mg/L (calculated in COD). The color of the granular sludge ANAMMOX changed from brick-red to a reddish-brown. The particle size grew from small to large and then stabilized. During the stable operation period, particles greater than 1.5 mm in size comprised 80.7% of the total, and there were large numbers of holes in the granular sludge that facilitate mass-transfer. Rod-shaped bacteria, spherical bacteria and filamentous bacteria were detectable on the surface. Thus, the inhibition concentration of substrate and landfill leachate must be considered in the design of strategies to use the ANAMMOX process for treatment of landfill leachate.

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**REFERENCES**


Dapena-Mora, A., Vázquez-Padín, J. R., Campos, J. L., Mosquera-Corral, A., Jetten, M. S. M. & Mendez, R. 2010 Monitoring the
stability of an Anammox reactor under high salinity conditions. 


ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142 (8), 2187–2196.