Effect of anaerobic reaction time on denitrifying phosphorus removal and N$_2$O production

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**Abstract**

Nitrous oxide (N$_2$O) is a highly potent greenhouse gas; however, the characteristics of N$_2$O production during denitrification using poly-$	ext{b}$-hydroxyalkanoates (PHA) as a carbon source are not well understood. In this study, effects of anaerobic reaction time (AnRT) on PHA formation, denitrifying phosphorus removal and N$_2$O production were investigated using a laboratory-scale anaerobic/anoxic/oxic sequencing batch reactor (An/A/O SBR). The results showed that operation of the An/A/O SBR for 0.78 SRT (47 cycles) after the AnRT was shortened from 90 min to 60 min resulted in anaerobically synthesized PHA improving by 1.8 times. This improvement was accompanied by increased phosphorus removal efficiency and denitrification. Accordingly, the N$_2$O–N production was reduced by 6.7 times. Parallel batch experiments were also conducted with AnRTs of 60, 90 and 120 min. All results indicated that in addition to the amount of anaerobically synthesized PHA, the kinetics of PHA degradation also regulated denitrifying phosphorus removal and N$_2$O production.

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**1. Introduction**

Denitrifying phosphorus removal, which can achieve simultaneous denitrification and P removal, is a novel biological nutrient removal technology. Denitrifying phosphorus removal occurs due to the capacity of denitrifying phosphorus accumulating organisms (DPAOs) to use nitrate and/or nitrite as an electron acceptor for P removal instead of oxygen (Kuba et al., 1996a). When compared with conventional enhanced biological phosphorus removal (EBPR) processes, the denitrifying phosphorus removal process can reduce the need for aeration by 30%, as well as reduce sludge production and the demand for carbon sources by 50% (Kuba et al., 1996a).

N$_2$O is a significant greenhouse gas with an approximately 300-fold greater warming potential than CO$_2$ (IPCC, 2001). Numerous studies have reported that both nitrification and denitrification could lead to N$_2$O production in wastewater treatment systems (Colliver and Stephenson, 2000; Kampschreur et al., 2008). Recently, Kampschreur et al. (2009) reviewed 28 studies relevant to N$_2$O emissions from full-scale and laboratory-scale wastewater systems. They found that N$_2$O emissions reached 0–14.6% of the nitrogen load in full-scale wastewater systems, whereas N$_2$O emissions varied from 0% to 95% of the nitrogen load in laboratory-scale wastewater systems. Moreover, several studies revealed that N$_2$O rather than N$_2$ was the major denitrification end-product in simultaneous denitrification and phosphorus removal systems (Zeng et al., 2003b; Meyer et al., 2005; Lemaire et al., 2006). If so, this reduces the potential advantages of denitrifying phosphorus removal technology (Zhou et al., 2008a,b).

Denitrification for denitrifying phosphorus removal is conducted using poly-$	ext{b}$-hydroxyalkanoates (PHA) as a carbon source. Poly-$	ext{b}$-hydroxybutyrate (PHB) is the main part of PHA and is the rate-limiting substance in organisms that grow on PHB (Beun et al., 2002). The slower nature of PHB degradation can produce competition for electrons between denitrifying enzymes, resulting in a higher NO reduction rate compared to the N$_2$O reduction rate (Kampschreur et al., 2009). Indeed, Schalk-Otte et al. (2000) reported that N$_2$O accumulated as soon as the stored PHB became the growth substrate due to a shortage of carbon. Moreover, kinetic studies of the PHA degradation indicated that PHB degradation follows first-order kinetics (Beun et al., 2002; Third et al., 2003), and that a decrease in the rate of PHB availability can result in limited reducing power for denitrification, leading to increased N$_2$O production (Third et al., 2003). These results indicate that PHA is associated with denitrification and N$_2$O production.

However, most studies of N$_2$O production during denitrifying phosphorus removal have focused on the effects of nitrite or free nitrous acid (FNA) (Zhou et al., 2008a,b), and few have considered the impact of intracellular PHA. Therefore, the mechanism for N$_2$O production in the denitrifying phosphorus removal process may not have been described properly to date, specifically at the intracellular compound level.

The anaerobic reaction time (AnRT) is of primary importance for designing EBPR processes. The AnRT guides the process design required to achieve the desired treatment efficiency. Specifically, the
AnRT has a significant effect on PHA synthesis. A shorter AnRT may result in insufficient PHA synthesis, which finally leads to low P removal efficiency. Conversely, a longer AnRT may cause microorganisms to suffer a longer famine period, which also negatively affects PHA synthesis and the N and P removal efficiencies. More recently, Freitas et al. (2009) examined the effect of cycle length on biological nutrient removal in a sequencing batch reactor (SBR) and found that a short cycle was more favorable for nutrient removal. However, it is still unclear how and why AnRT or cycle length affects P removal. Particularly, the relationship between AnRT and P removal is not fully understood. Therefore, in this study, both a continuous experiment and batch tests were conducted to investigate the effect of AnRT on anaerobic PHA synthesis during denitrifying phosphorus removal and to determine if these effects were correlated with N2O production and denitrifying phosphorus removal efficiency.

2. Methods

2.1. Process setup and operation

A sealed laboratory-scale SBR with a working volume of 7.5 L and overhead space of 1 L was operated at 22 ± 2 °C. The SBR was fed with synthetic wastewater and operated under alternating anaerobic–anoxic–aerobic conditions. At the end of the cycle, 125 mL of sludge was removed to achieve a solids retention time (SRT) of approximately 20 days and a mixed liquor suspended solid (MLSS) level of 3.7–4.7 g/L. The SBR was inoculated with activated sludge from the Quyang Sewage Treatment Plant (Shanghai, China) and a laboratory-scale plug-flow anaerobic–aerobic (A/O) reactor in which polyphosphate-accumulating organisms (PAOs) were successfully enriched (Wang et al., 2010). After the SBR was operated over 60 days, PAOs were enriched as reflected by the constant P removals and biomass concentrations. Thereafter, the experiments reported below were conducted. The SBR operation consisted of the following four phases:

Phase I (cycles 1–126): the SBR was operated with three 8 h-cycles per day. Specifically, each cycle consisted of 15 min feeding, 90 min anaerobic reaction, 210 min anoxic reaction, 30 min aerobic reaction followed by 45 min settling, 15 min decanting and 75 min idle time. During the first 15 min feeding period, 5.5 L of synthetic wastewater was pumped into the reactor, while 100 mL of KNO3 solution was pulse added into the reactor at the end of the anaerobic period, giving an initial NO3-N concentration of 40 mg/L.

Phase II (cycles 127–182): the operating mode was similar to that of phase I, except that the AnRT for the SBR was reduced to 60 min; accordingly, the idle period was extended to 105 min. The HRT of the SBR for this phase was 10.9 h.

Phase III (cycles 183–228): the operating conditions were similar to that of phase II, while the initial NO3-N concentration was increased to 50–60 mg N/L.

Phase IV (cycles 229–399): the operating conditions were similar to that of phase I, except that the initial NO3-N concentration was maintained at 50 mg N/L.

The liquid and solid-phase samples were taken for chemical analysis with sampling intervals of 10 min during the first 30 min of the anaerobic period and anoxic period and each 30 min afterwards. Gas samples were collected using gas-tight collecting bags and syringes and then analyzed immediately.

2.2. Synthetic wastewater

Synthetic wastewater used in this study contained (per liter): 512.53 mg of CH3COONa (400 mg of COD); 32.9 mg of KH2PO4 (7.5 mg of P); 42 mg of KH2PO4 (7.5 mg of P); 57.4 mg of NH4Cl; 85 mg of MgSO4·7H2O; 10 mg of CaCl2; 110 mg of NaHCO3. Additionally, the trace salt solution (0.3 mL/L) described by Wang et al. (2010) was added.

2.3. Batch experiments

Three series batch experiments were conducted to characterize the effect of AnRT on denitrifying phosphorus removal and N2O production. For Experiments 1 and 2, a 2.5 L sealed reactor with a working volume of 2.4 L and overhead space of 0.1 L was used. For Experiment 3, a 0.5 L sealed reactor without overhead space was used. The temperature for all tests was controlled at 22 ± 2 °C. The pH values in Experiments 1 and 2 were maintained at 7.5 ± 0.1 by adding 0.3 M HCl or 0.3 M NaOH; for Experiment 3, there was no pH control.

2.3.1. Effect of AnRT on denitrifying phosphorus removal and N2O production (Experiment 1)

The sludge (2 L) for the batch tests was withdrawn from the An/A/O SBR at the end of the decanting phase during phase III. After being washed three times, the sludge was evenly divided into three parts and then transferred into three batch reactors. Next, 1.7 L of synthetic wastewater was rapidly added to each reactor at the beginning of the cycle, which resulted in mixed liquid volatile suspended solids (MLVSS) levels of about 2.6 g/L in each bioreactor. The anoxic reaction time for all reactors was 210 min, while the AnRTs for three reactors were controlled at 60 min (R1), 90 min (R2) or 120 min (R3). The anoxic reaction was initiated by adding KNO3 solution to give an initial NO3-N concentration of 55 mg N/L.

2.3.2. Denitrification rates of nitrate and nitrite with PHA as a carbon source (Experiment 2)

The sludge for Experiment 2 was withdrawn from the An/A/O SBR at the end of the anaerobic phase during phase IV. The washed sludge was evenly divided into two parts and then transferred into two reactors. Next, 1.7 L of synthetic wastewater was added to the two reactors to give an initial P concentration of 40 mg/L. One reactor was then amended with KNO3 solution to give an initial NO3-N concentration of 40 mg/L, while the other received NaN02 solution to provide an initial N2O – N concentration of 30 mg/L. The anoxic reaction lasted for 210 min. The MLVSS for these two reactors were both approximately 3.0 g/L. The tests are conducted in triplicate.

2.3.3. Denitrification rate of N2O with PHA as a carbon source (Experiment 3)

The sludge mixture (–500 mL) for Experiment 3 was withdrawn from the An/A/O SBR at the end of the anaerobic phase in phase IV. An N2O solution was prepared in a 500 mL reactor by aerating 450 mL of synthetic wastewater with 10,000 ppm N2O gas for 15 min, after which the washed sludge (50 mL) was injected rapidly into the reactor. The anoxic reaction was 30 min. The experiment was repeated three times.

2.4. Analytical methods

The liquid samples were immediately filtered through Millipore filter units (0.45 μm pore size) for analysis of COD, NH4-N, NO3-N, NO2−N and PO4−P. NH4-N, NO3-N, NO2−N, PO4−P, MLSS and MLVSS were measured according to the Chinese State Environmental Protection Agency (SEPA) Standard Methods (Chinese SEPA, 2002). Merck COD reagents were used for the COD test (Merck, Germany).
Dissolved oxygen (DO), oxidation–reduction potential (ORP) and pH were measured online using oxygen, ORP and pH meters (oxi 3310 and pH 3310, WTW company, Germany), respectively. FNA-N was calculated according to Ma et al. (2010). Glycogen was determined using the method described by Jenkins et al. (2003).

The N\textsubscript{2}O concentrations in the gas samples were analyzed using a gas chromatograph (GC) (Agilent 7820, USA) equipped with an electron capture detector (ECD). The GC was equipped with a pre-column (Porapak Q 80/100 mesh, 1 m x 2 mm) and a main column (Porapak Q 80/100 mesh, 3 m x 2 mm). A 1 mL sample loop and a 10-port valve were used to inject the gas samples. The temperatures of the columns and the ECD were 100°C and 300°C, respectively. A mixture of 91% Ar + 9% CH\textsubscript{4} was used as the carrier gas. The dissolved N\textsubscript{2}O was measured by GC using a headspace method. The equilibrium temperature and time were 25°C and 3 h, respectively. N\textsubscript{2}O was quantified using the standard curves generated from certified standard gases (National Institute of Metrology, PR China).

Acetic acid (HAc) was measured using an Agilent 6890N gas chromatograph (GC) equipped with a 30 x 0.53 mm x 1 μm (length x ID x film) DB-WAXetr column and a flame ionization detector (FID) at 220°C. PHB, poly-hydroxyvalerate (PHV) and poly-3-hydroxy-2-methylvalerate (PH2MV) were measured according to a method described by Oehmen et al. (2005). The total PHA in the samples was calculated as the sum of the measured PHB, PHV and PH2MV.

3. Results and discussion

3.1. Typical cycles of the An/A/O SBR in phases I and II

During phases I and II, the mean effluent PO\textsubscript{4}\textsuperscript{3-}-P concentrations remained at 3.23 and 2.12 mg/L, respectively. For the effluent NO\textsubscript{2}-N concentration, it averaged at 11 and 3.28 mg/L during phases I and II, respectively. The effluent NO\textsubscript{3}-N concentrations were close to zero during both phases.

3.1.1. Variations of the nitrogen and phosphorus in typical cycles

In typical cycles of 77 (phase I), 119 (phase I), 149 (phase II) and 173 (phase II) for the An/A/O SBR, the PO\textsubscript{4}\textsuperscript{3-}-P concentrations increased remarkably during the anaerobic period due to the poly-phosphate hydrolysis to soluble ortho-phosphorus (SOP) and the release of phosphorus (Figs. 1b and e and 2b and e). However, anaerobic PO\textsubscript{4}\textsuperscript{3-}-P uptake occurred in all of these cycles. Specifically, the amounts of P absorbed anaerobically in cycles 77 (phase I), 119 (phase I), 149 (phase II) and 173 (phase II) were approximately 2.48, 18.32, 1.57 and 0.4 mg/L, respectively. Moreover, this absorption was significantly higher in phase I than in phase II (\textit{F}_{\text{observed}} = 8.04, \textit{F}_{\text{significance}} = 6.61, P (0.05) = 0.04 < 0.05). Similar anaerobic P uptake was also observed by Kong et al. (2002), but they did not provide relevant reasons. As the SBR was properly sealed and the overhead space was only 1 L, the DO level during the entire testing period was lower than 0.10 mg/L. Further studies...
are needed to fully determine the mechanisms of this anaerobic phosphate uptake.

In the anoxic phase, the denitrification efficiency of the An/A/O SBR increased after the AnRT was reduced from 90 min to 60 min (Table 1). Notably, the denitrification efficiency in cycle 173 was higher than that in cycle 149, even though the AnRTs for the two cycles were the same. Additionally, anoxic phosphorus uptake was observed in all typical cycles (Figs. 1b and e and 2b and e).

### 3.1.2. Transformations of PHA and glycogen in the typical cycles

As shown in Table 2, after the AnRT was shortened from 90 min to 60 min, the PHA synthesis and consumption as well as the glycogen degradation and synthesis all increased either during the anaerobic period or the anoxic period. Moreover, for phase II, the transformations of PHA and glycogen were enhanced gradually with the operation of the system (Table 2).

It should be noted that PHA degradation occurred in the latter part of the anaerobic phase along with glycogen degradation (Figs. 1b and e and 2b and e). Specifically, in cycles 77 (phase I), 119 (phase I), 149 (phase II) and 173 (phase II), over 0.49, 0.15, 0.12 and 0.21 mmol C/g-MLVSS of PHA, respectively, were degraded anaerobically. Moreover, from a long-term operation point of view, this reduction in the amount of PHA for phase I was greater than that of phase II.

### 3.1.3. N$_2$O production in the typical cycles

After the KNO$_3$ was pulse added at the beginning of the anoxic period, a remarkable increase in the N$_2$O production occurred in all typical cycles (Figs. 1c and f and 2c and f). Thereafter, the N$_2$O production rate decreased gradually as the amount of N$_2$O decreased. Indeed, the N$_2$O-N production showed an obvious and gradual decrease when the AnRT was shortened from 90 min to 60 min (Table 1).

### 3.2. Effect of AnRT on denitrifying phosphorus removal and N$_2$O production in batch Experiment 1

#### 3.2.1. Comparison of nitrogen and phosphorus removal in batch Experiment 1

Of R1, R2 and R3, which had AnRTs of 60, 90 and 120 min, respectively, the highest and lowest P release were observed in R2 and R1, respectively (Figs. 3b, 4b, 5b and 6a). Moreover, anaerobic P uptake occurred in R3 during the latter part of the anaerobic phase, which directly led to a reduced net P release when compared with that of R2. This occurred because a fraction of the released P was taken up again by DPAOs. It seems that an extension of AnRT itself cannot efficiently increase the release of phosphorus (Figs. 3b, 4b and 5b).

After the batch reactors were fed KNO$_3$ solution, denitrification and PO$_4^{3-}$-P uptake occurred simultaneously. The highest P removal was obtained in R2, while the lowest was observed in R1 (Fig. 6a). Moreover, the added NO$_3^-$-N was rapidly reduced in R1, R2 and R3, and this reduction was accompanied by the accumulation and reduction of NO$_2^-$-N (Figs. 3a, 4a and 5a), which resulted in corresponding denitrification efficiencies of 74%, 91% and 83%, respectively (Fig. 6a). These findings indicate that an AnRT of 90 min...
3.2.2. Effect of AnRT on transformations of HAc, PHA and glycogen in typical cycles of the An/A/O SBR.

Table 1
Comparison of nitrogen and phosphorus removals as well as N₂O production for typical cycles of the An/A/O SBR.

<table>
<thead>
<tr>
<th>Item</th>
<th>Phase I (AnRT = 90 min)</th>
<th>Phase II (AnRT = 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle</td>
<td>Cycle</td>
</tr>
<tr>
<td>P removal efficiency at the end of the anoxic phase (%)</td>
<td>77</td>
<td>119</td>
</tr>
<tr>
<td>P removal efficiency at the end of the post aerobic phase (%)</td>
<td>56</td>
<td>78</td>
</tr>
<tr>
<td>Denitrification efficiency (%)</td>
<td>6.74</td>
<td>7.85</td>
</tr>
<tr>
<td>NO₃-N concentration at the end of the anaerobic phase (mg/L)</td>
<td>19.44</td>
<td>12.16</td>
</tr>
<tr>
<td>NO₂-N concentration at the end of the anoxic phase (mg/L)</td>
<td>6.07</td>
<td>13.59</td>
</tr>
<tr>
<td>Net NO₃-N consumption rate (mg N/g-MLVSS h⁻¹)</td>
<td>0.62</td>
<td>1.05</td>
</tr>
<tr>
<td>Anoxic N₂O production (mg N/L)</td>
<td>6.09</td>
<td>7.52</td>
</tr>
<tr>
<td>Aerobic N₂O production (mg N/L)</td>
<td>0.66</td>
<td>0.33</td>
</tr>
<tr>
<td>Total N₂O production (mg N/L)</td>
<td>6.74</td>
<td>7.85</td>
</tr>
<tr>
<td>Ratio of anoxic N₂O-N production to denitrified nitrogen (%)</td>
<td>15.20</td>
<td>18.80</td>
</tr>
</tbody>
</table>

3.3. Effect of the AnRT on the anaerobic PHA synthesis by the phosphorus removal organisms

Table 2
Anaerobic and anoxic transformations of PHA and glycogen in typical cycles of the An/A/O SBR.

<table>
<thead>
<tr>
<th>Item</th>
<th>Phase I (AnRT = 90 min)</th>
<th>Phase II (AnRT = 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle</td>
<td>Cycle</td>
</tr>
<tr>
<td>Anaerobic PHB synthesis (mmol C/g-MLVSS)</td>
<td>1.83</td>
<td>1.85</td>
</tr>
<tr>
<td>Anaerobic PHV synthesis (mmol C/g-MLVSS)</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>PHA amount at the end of the anaerobic phase (mmol C/g-MLVSS)</td>
<td>2.13</td>
<td>2.18</td>
</tr>
<tr>
<td>PHA synthesis/HAc uptake (mmol C/mmol C)</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Anaerobic glycogen degradation (mmol C/mmol C)</td>
<td>1.07</td>
<td>1.02</td>
</tr>
<tr>
<td>Anoxic PHB degradation (mmol C/g-MLVSS)</td>
<td>1.76</td>
<td>1.73</td>
</tr>
<tr>
<td>Anoxic PHV degradation (mmol C/g-MLVSS)</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>Anoxic PHA degradation (mmol C/g-MLVSS)</td>
<td>2.11</td>
<td>2.08</td>
</tr>
<tr>
<td>Anoxic PHA degradation rate (mmol C/g-MLVSS h⁻¹)</td>
<td>1.71</td>
<td>1.83</td>
</tr>
<tr>
<td>Anoxic glycogen synthesis (mmol C/g-MLVSS)</td>
<td>1.12</td>
<td>1.20</td>
</tr>
</tbody>
</table>

3.3.2. Effect of AnRT on N₂O production in batch Experiment 1

Table 3 shows the gas production (N₂O, CH₄) and nitrification efficiency at the end of the aerobic stage. The nitrification efficiency (%) of the three reactors was consistent with the corresponding denitrification and phosphorus removal efficiencies (Figs. 3–5).

3.3.3. Effect of the short or long AnRT on PHA synthesis

Batch Experiment 1 confirmed that HAc could not be completely consumed if the AnRT was insufficient, which led to a reduction in the maximum amount of PHA synthesis that could be produced. Moreover, it should be noted that PHA synthesis still continued in some cases even though the HAc was close to depletion during the latter part of the anaerobic phase (Figs. 1e, 2e and 5b), indicating that PHA synthesis may lag behind HAc uptake. Therefore, it is likely that the apparent HAc was exhausted within the set AnRT, while complete PHA synthesis was not yet accomplished. This is likely to result in incomplete PHA synthesis and poor P removal if the set AnRT is too short.

In contrast, as shown in Table 2 and Fig. 6b, a long AnRT also lowered the net anaerobic PHA synthesis in the present study, which resulted in deteriorated phosphorus removal and denitrification. As the SBR was sealed, the lowered anaerobic PHA storage when with a long AnRT might be due primarily to the endogenous respiration for the maintenance of microbial cells. Indeed, Qin et al. (2005) found that anaerobic endogenous respiration of granular sludge occurred when external carbon and nitrate were not available, which subsequently led to a decrease in the PHB pool in microbial granules. In several studies a slight increase or decrease in the amount of PHA for the PAOs sludge was observed during the latter part of the anaerobic phase (Kong et al., 2002; Oehmen et al., 2005). This means that the increase in the PHA amount is not significant within the prolonged AnRT and instead a decrease in the PHA amount may occur. Therefore, an AnRT that is too long is not appropriate in terms of minimizing operating costs and ensuring good phosphorus removals.

Generally, during anaerobic endogenous period, poly-P hydrolysisis and glycogen degradation produce maintenance energy, and part of glycogen degraded can be converted to PHV which makes the overall amount of PHB not vary significantly (Lopez et al., 2006; Lu et al., 2007). Interestingly, our observations that a decline in PHA amount occurred during the latter part of the anaerobic phase are not in accordance with the work by Lopez et al. (2006) and Lu et al. (2007), who found that an increase in PHA content occurred when activated sludge used for phosphorus removal was subject to long-term anaerobic starvation. This is primarily be-
cause the biomass used in their experiments was withdrawn from the reactor at the end of the aerobic phase and initially with high levels of glycogen and poly-P and low level of PHA. However, when the levels of internal poly-P and glycogen were both lower, e.g. during the latter part of the anaerobic phase in the system evaluated here, microorganisms may also use PHA to generate maintenance energy. Indeed, the anaerobic decrease in PHA has been observed in some previous studies (Kong et al., 2002; Oehmen et al., 2005; Lopez et al., 2006). For example, observations of changes in PHA compositions by Lopez et al. (2006) showed that a decrease in the PHA amount occurred after 14 d of anaerobic starvation, although the long-term trend for the PHA amount (from day 0 to day 27.9) had increased. Further studies are required to confirm the findings.

3.3.2. Effect of AnRT on the activity of DPAOs linked to PHA synthesis

The PHA synthesis in the An/A/O SBR was much greater in phase II than in phase I, possibly due to a change in the microbial activities in the system. Lopez et al. (2006) found that the activity of PAOs dropped after long-term anaerobic starvation. Similar results were also reported by Pijuan et al. (2009). Therefore, it is likely that endogenous processes caused by a suboptimal AnRT affected the activities of DPAOs in the system evaluated here, which ultimately led to different capacities of PHA synthesis in DPAOs during phases I and II. Moreover, the lowered PHA storage due to the declined activities of the DPAOs in denitrifying phosphorus removal systems would cause incomplete denitrification, resulting in the accumulation of nitrite. The toxicity due to the accumulated nitrite or high FNA inhibited the activities of DPAOs, which in turn could lower PHA synthesis (Zhou et al., 2010).

It must be noted that after the An/A/O SBR was operated at an AnRT of 60 min, the maximum phosphate release rate was increased from 49.9 mg P/g-MLVSS/h for phase I to 68.6 mg P/g-MLVSS/h for phase II, and the maximum HAc uptake rate was also increased from 7.27 mmol C/g-MLVSS/h for phase I to 9.61 mmol C/g-MLVSS/h for phase II, indicating that the activity of the DPAOs was gradually increased. This directly resulted in the net PHA synthesis in one standard cycle increasing concurrently with a decrease in the nitrite accumulation (Table 2). Subsequently, the microbial activities were recovered smoothly due to the reduced nitrite inhibition.

Additionally, according to the proposed PAOs and glycogen accumulating organisms (GAOs) metabolism (Filipe et al., 2001), GAOs tend to produce more PHV than PAOs when fed with acetate, due to the partial glycogen hydrolysis through the succinate-propionate pathway. In our studied SBR, the PHB fractions during phase

Fig. 3. Variations in nitrogen, phosphorus, N2O, HAc, glycogen and PHA during one cycle in batch R1.
II and phase I contributed to 83% and 85% of PHA, respectively. Also, the P release/VFA-uptake (P mmol/C mmol) ratios during phases I and II were both around 0.24. All of these results indicate that the percentage of the GAOs in the SBR was remained constant during phases I and II. Therefore, the increase in the PHA synthesis capacity during phase II was due primarily to the increased activity of DPAOs.

3.4. Effect of AnRT on denitrification and phosphorus removal

3.4.1. Intracellular carbon flow during the anoxic reaction

To characterize the contribution of the internal carbon (PHA) and identify the mechanism of N2O production during the anoxic period, a simple intracellular carbon flow for anoxic reaction was proposed (Table 4). According to the metabolic model of the EBPR proposed by Kuba et al. (1996b), carbon supplied by PHA was used to replenish glycogen, biomass synthesis and oxidized to CO2. In the present study, nitrification was negligible during the anoxic period because the DO was below 0.2 mg/L. NO3/C03 /NO2/C02 ammonification is considered not to occur under these conditions, and the removal of NH4-N in the anoxic phase was only used for microbial cell synthesis. The biomass composition can be expressed as CH2.09O0.54N0.20P0.015 (Smolders et al., 1994); therefore, the carbon utilized for the biomass synthesis in an operating cycle can be calculated according to the reduced amount of NH4-N (Table 4). Theoretically, the denitrification reactions using PHB and PHV as the carbon source can be expressed as Eqs. (1) and (2), respectively.

\[
\begin{align*}
\text{CH}_3\text{C}_6\text{H}_5\text{O}_3\text{(PHB)} + 0.9\text{NO}_3^{-} & \rightarrow 0.45\text{N}_2 + 0.1\text{CO}_2 + 0.3\text{H}_2\text{O} + 0.9\text{HCO}_3^{-} & (1) \\
\text{CH}_3\text{C}_6\text{H}_4\text{O}_4\text{(PHV)} + 0.96\text{NO}_3^{-} & \rightarrow 0.48\text{N}_2 + 0.04\text{CO}_2 + 0.32\text{H}_2\text{O} + 0.96\text{HCO}_3^{-} & (2)
\end{align*}
\]

Accordingly, approximately 1.11 mol C of PHB or 1.04 mol C of PHV is required for a complete reduction of 1 mol NO3-N to N2.

3.4.2. Effect of AnRT on denitrification

According to the anoxic internal carbon flow in Table 4, the difference between the PHB or PHV amount demanded for the complete denitrification and the PHA amount used for denitrification for the An/A/O SBR was larger in phase I than in phase II. In other words, the An/A/O SBR during phase I lacked more amount of anaerobically synthesized PHA to meet the demands of complete denitrification when compared to phase II, which led to lower denitrification efficiencies for the SBR in phase I (Table 1). On the other hand, when the amount of the anaerobically synthesized PHA increased in the An/A/O SBR, the sequential anoxic degradation rate of PHA increased accordingly (Table 2). Furthermore, lower PHA degradation rates always corresponded with lower rates

![Fig. 4. Variations in nitrogen, phosphorus, N2O, HAc, glycogen and PHA during one cycle in batch R2.](image-url)
of NO$_3^-$-N denitrification and net NO$_2^-$-N consumption, as well as lower denitrification efficiencies (Tables 1 and 2). This was primarily because a low rate of PHB availability results in limitation of reducing power for denitrification, which may lead to competition for electrons between denitrifying enzymes and long-term accumulation of nitrite or FNA (Figs. 1a and d and 2a and d). Accordingly, denitrification efficiency decreased because nitrite or FNA has an inhibitory effect on denitrifiers (Zhou et al., 2007; Ma et al., 2010). Similar results were also obtained in batch Experiment 1 (Tables 3 and 4 and Fig. 6a). All of these results confirm that not only the amount of anaerobically synthesized PHA but the kinetics of PHA degradation has a significant effect on denitrification.

### 3.5. Identifying the causes of N$_2$O production during the denitrifying phosphorus removal

#### 3.5.1. Effect of the amount of anaerobically synthesized PHA on N$_2$O production

As shown in Table 4, the amounts of PHA available for the complete denitrification were not sufficient in all cases, which inevitably led to N$_2$O accumulation. Nevertheless, as the anaerobic PHA synthesis increased, the ratio of N$_2$O-N production to denitrified nitrogen decreased (Tables 1–3 and Fig. 6b). However, relatively more N$_2$O was produced in our experiment, even though the COD/N in the initial anoxic phase was higher than that observed in some studies in which COD/N impacts on N$_2$O production were examined during denitrification based on

![Figure 5. Variations in nitrogen, phosphorus, N$_2$O, HAc, glycogen and PHA during one cycle in batch R3.](image-url)
external carbon (Yang et al., 2009; Hwang et al., 2006). Hwang et al. (2006) demonstrated that a negligible amount of N\textsubscript{2}O was produced during denitrification when the COD/N was 3. Similarly, Yang et al. (2009) reported that when the COD/NO\textsubscript{3}^-N ratios were 4.7 and 3.8, nitrate was completely denitrified without N\textsubscript{2}O production, whereas when the COD/NO\textsubscript{3}^-N ratios were 1.5 and 0.4, N\textsubscript{2}O accumulation occurred. Indeed, the denitrification using intracellular PHB produces more N\textsubscript{2}O compared to that using the external soluble carbon.

It should also be noted that the amount of PHA at the beginning of the anoxic phase in R3 was higher than that in R1, but that the ratio of N\textsubscript{2}O production to denitrified nitrogen in R3 was greater than that obtained in R1. This may have been due to the fact that unconsumed HAc during the anaerobic period was residual to the anoxic phase in R1, and this was exhausted by ordinary denitrifying bacteria, which therefore improved the denitrification rate and reduced N\textsubscript{2}O emission.

3.5.2. Effect of the kinetics of PHA degradation and denitrification on N\textsubscript{2}O production

In addition to the amount of PHA, the kinetics of PHA degradation also had a significant effect on denitrification phosphorus removal efficiency as well as N\textsubscript{2}O production. A slower rate of PHA degradation can lead to competition for electrons between denitrifying enzymes, and even influence the expression of denitrifying enzymes (Kampschreur et al., 2009). Specifically, under certain conditions, e.g., with a limited carbon source or nondegradable carbon sources, nitrate reductase (Nar) may have a competitive advantage at capturing electrons when compared to nitrite reductase (Nir). This imbalance of the two reduction rates easily results in nitrite or FNA accumulation and N\textsubscript{2}O production (Zeng et al., 2003a,b; Lemaire et al., 2006; Zhou et al., 2008a,b). Moreover, the reduction of N\textsubscript{2}O is the last step of denitrification, which makes it more difficult for N\textsubscript{2}O reductase (Nos) to compete for electrons than other enzymes, particularly when the amount of the anaerobically synthesized PHA is low. Therefore, if more PHA is synthesized, more carbon sources are available for denitrification and the PHA degradation rate can be improved. This ultimately leads to a lower ratio of N\textsubscript{2}O-N production to denitrified nitrogen. Nevertheless, PHB is degraded 6–20 times slower than soluble COD (Third et al., 2003). Consequently, more N\textsubscript{2}O was produced in the present study by using of internal PHA for denitrification, even though the initial COD/N of the anoxic phase in our experiment was comparable to that of ordinary denitrifying systems (using external carbon).

The denitrification rates with the addition of nitrate, nitrite and N\textsubscript{2}O were evaluated in Experiments 2 and 3 based on PHA consumption. The NO\textsubscript{3}^-N denitrification rate was 13.43 ± 0.17 mg N/g-MLVSS h\textsuperscript{-1}, which was higher than the NO\textsubscript{2}^-N denitrification rate of 9.90 ± 0.15 mg N/g-MLVSS h\textsuperscript{-1}, while the lowest value of 4.23 ± 1.21 mg N/g-MLVSS h\textsuperscript{-1} was the N\textsubscript{2}O denitrification rate. These findings support our assumption that Nos was less competitive in obtaining electrons during denitrification, and also explain the fact that NO\textsubscript{3}^-N accumulation and N\textsubscript{2}O production occurred during the anoxic phase in both the continuous operation and batch tests.

3.5.3. Effect of nitrite or FNA on N\textsubscript{2}O production

As shown in Figs. 1c, 1f, 2c, 2f, 3c, 4c and 5c, significant nitrite or FNA accumulation was always associated with a high ratio of N\textsubscript{2}O-N production to denitrified nitrogen. Therefore, nitrite or FNA had an important effect on N\textsubscript{2}O production.

In batch Experiment 2, nitrite addition to the DPAOs sludge stimulated the net N\textsubscript{2}O production rate by 4.7 times when compared with that obtained in the nitrate addition batch (data not shown). This finding is consistent with that of Lemaire et al. (2006), who reported that the addition of nitrite to the sludge led to a net N\textsubscript{2}O production rate that was five times higher than that obtained in response to nitrate addition. Similarly, Zeng et al. (2003a) demonstrated that once nitrite was close to 5 mg N/L, N\textsubscript{2}O was produced instead of N\textsubscript{2}. They later found that Nos was inhibited when the nitrite concentration reached approximately 1 mg N/L (Zeng et al., 2003b). Recently, Zhou et al. (2008a) reported that the N\textsubscript{2}O reduction activity decreased by 50% at FNA concentrations of 0.0007–0.001 mg H\textsubscript{2}NO\textsubscript{3}-N/L and was totally inhibited.

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**Table 3**

Comparison of denitrification rate, N\textsubscript{2}O production and the transformation of PHA in R1, R2 and R3.

<table>
<thead>
<tr>
<th>Item</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO\textsubscript{3}^-N denitrification rate (mg N/g-MLVSS h\textsuperscript{-1})</td>
<td>13.41</td>
<td>10.58</td>
<td>10.45</td>
</tr>
<tr>
<td>Net NO\textsubscript{2}^-N consumption rate (mg N/g-MLVSS h\textsuperscript{-1})</td>
<td>0.68</td>
<td>1.95</td>
<td>1.03</td>
</tr>
<tr>
<td>Anoxic N\textsubscript{2}O production (mg N/L)</td>
<td>1.97</td>
<td>2.25</td>
<td>2.93</td>
</tr>
<tr>
<td>Ratio of anoxic N\textsubscript{2}O-N production to denitrified nitrogen (%)</td>
<td>4.84</td>
<td>4.52</td>
<td>6.43</td>
</tr>
<tr>
<td>PHA amount at the end of the anaerobic phase (mmol C/g-MLVSS)</td>
<td>2.51</td>
<td>2.91</td>
<td>2.68</td>
</tr>
<tr>
<td>PHA synthesis/HAc uptake (mmol C/mmol C)</td>
<td>0.86</td>
<td>0.86</td>
<td>0.79</td>
</tr>
<tr>
<td>PHA degradation rate (mmol C/g-MLVSS h)</td>
<td>0.69</td>
<td>0.77</td>
<td>0.73</td>
</tr>
</tbody>
</table>

\* The mean rate of PHA degradation during the initial 60 min of anaerobic reaction.  
\* R1: AnRT = 60 min; R2: AnRT = 90 min; R3: AnRT = 120 min.
when the FNA concentration was greater than 0.004 mg HNO2-N/L. For our studied An/A/O SBR, the maximum FNA concentrations were 0.0004, 0.0003, 0.0005 and 0.0007 mg N/L, respectively, in cycles 77 (phase I), 119 (phase I), 149 (phase II) and 173 (phase II), and that they were 0.0010, 0.0009 and 0.0013 mg N/L, respectively, in R1, R2 and R3. This suggests that the inhibitory effect of FNA on N2O reduction activity also occurred in our studied systems.

In addition, for cycles 77 (phase I), 119 (phase I), 149 (phase II) and 173 (phase II) in the An/A/O SBR, as the nitrite concentrations increased at the end of the anoxic phase, the N2O production increased gradually in the postoxic period (Table 1), possibly via the denitrification pathway. Indeed, Kim et al. (2010) reported that N2O emission was greatly enhanced when nitrite was added to an NH4+-N oxidizing system, and they demonstrated that N2O was produced via the denitrification pathway by AOB.

4. Conclusions

AnRT significantly influenced anaerobic PHA synthesis. A reduction in AnRT from 90 min to 60 min in the An/A/O SBR resulted in an improvement in PHA synthesis of about 1.8 times. The PHA storage and the kinetics of PHA degradation are both key variables in controlling the N2O emission for anoxic P removal reactor. Particularly, a lower PHA storage leads to a lower PHA degradation rate, and the combined effects of lower PHA storage and degradation rate stimulate the N2O production during denitrification. Nitrite or FNA can inhibit the activity of denitrifying enzymes and phosphorus uptake, leading to poor denitrification efficiency and a large amount of N2O production.

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References


