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1 The effect of poly- $\beta$ -hydroxyalkanoates  
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16 **Abstract:** Poly- $\beta$ -hydroxyalkanoates (PHAs) and free nitrous acid (FNA) have been  
17 revealed as significant factors causing nitrous oxide (N<sub>2</sub>O) production in denitrifying  
18 phosphorus removal systems. In this study, the effect of PHA degradation rate on N<sub>2</sub>O  
19 production was studied at low FNA levels. N<sub>2</sub>O production always maintained at  
20 approximately 40% of the amount of nitrite reduced independent of the PHA  
21 degradation rate. The electrons distributed to nitrite reduction were 1.6 times that to  
22 N<sub>2</sub>O reduction. This indicated that electron competition between these two steps was  
23 not affected by the PHA degradation rate. Continuous feed of nitrate was proposed,  
24 and demonstrated to reduce N<sub>2</sub>O accumulation by 75%. While being kept low, a  
25 possible compounding effect of a low-level FNA could not be ruled out. The sludge

1 used likely contained both polyphosphate- and glycogen-accumulating organisms, and  
2 the results could not be simply attributed to either group of organisms.

3 **Keywords:** nitrous oxide (N<sub>2</sub>O); denitrifying phosphorus removal;

4 Poly-β-hydroxyalkanoates (PHAs); nitrite

## 5 **1 Introduction**

6 The enhanced biological phosphorus removal process (EBPR) is widely used as  
7 one of the most economical and efficient processes for removing phosphorus (P) from  
8 wastewater. It is achieved by polyphosphate-accumulating organisms (PAOs), which  
9 take up P and store it intracellularly as poly-phosphate (poly-P) under alternate  
10 anaerobic and aerobic conditions (Kuba et al., 1996; Wentzel et al., 1988). In the  
11 anaerobic phase, PAOs take up volatile fatty acids (VFAs) in wastewater and store  
12 them internally in the form of poly-β-hydroxyalkanoates (PHAs). Concomitantly, the  
13 intracellular poly-P is degraded and released into the mixed liquor as ortho-phosphate  
14 (PO<sub>4</sub><sup>3-</sup>-P), to provide energy for the uptake of VFAs. In the subsequent aerobic stage,  
15 PHAs are oxidized by oxygen to provide energy for PAOs to take up PO<sub>4</sub><sup>3-</sup>-P to  
16 recover the poly-P level, in which oxygen acts as the terminal electron acceptor (Ahn  
17 et al., 2002; Zeng et al., 2003).

18 P uptake can also be accomplished under anoxic conditions, in which PAOs  
19 utilize intracellular PHAs as electron donors and nitrate / nitrite as terminal electron  
20 acceptors (Johwan Ahn, 2001; Ma et al., 2013b; Meinhold J, 1999; Oehmen et al.,  
21 2007; Zhou et al., 2008a). The PAOs with that particular characteristic are referred to  
22 as denitrifying polyphosphate-accumulating organisms (DPAOs) (Meinhold J, 1999),

1 and the process referred to as the denitrifying phosphorus removal process. This  
2 process enables the organic carbon accumulated under anaerobic conditions to be  
3 used for simultaneous denitrification and P removal. This significantly saves the  
4 carbon source required for nitrogen and phosphorus removal from wastewater.  
5 Moreover, this process also leads to reduced sludge production and aeration costs  
6 (Guo et al., 2013).

7 Unfortunately, nitrous oxide ( $N_2O$ ) often appeared to be a significant  
8 end-product of denitrification during denitrifying phosphorus removal (Kampschreur  
9 et al., 2008; Lemaire et al., 2006; Wang et al., 2011a; Zhou et al., 2008a; Zhou et al.,  
10 2008b). Although  $N_2O$  accounts for merely 0.03 percent of the total greenhouse gas  
11 emitted to the atmosphere, its global warming potential of approximately 300-fold  
12 that of carbon dioxide makes it a significant greenhouse gas in the atmosphere (IPCC,  
13 2001).  $N_2O$  production may therefore severely compromise the advantage of  
14 denitrifying phosphorus removal systems.  $N_2O$  is an intermediate of nitrate or nitrite  
15 reduction. The factors that result in  $N_2O$  accumulation need to be understood, to  
16 support the development of mitigation strategies.

17 The accumulation of nitrite or free nitric acid (FNA), an intermediate of nitrate  
18 reduction and also an electron acceptor, has previously been reported to induce  $N_2O$   
19 accumulation in the denitrifying phosphorus removal process. Nitrite has been found  
20 to inhibit  $N_2O$  reduction in this process (Zhou et al., 2008a; Lemaire et al., 2006;  
21 Itokawa et al., 2001; Li et al., 2013a). Zhou et al. (2008b) concluded that FNA rather  
22 than nitrite was the inhibitor and caused  $N_2O$  accumulation during nitrite reduction.

1 The activity of  $\text{N}_2\text{O}$  reductase was inhibited by 50% at an FNA concentration of  
2  $1.0 \times 10^{-3}$  mg  $\text{HNO}_2\text{-N/L}$ , whereas full inhibition occurred at FNA concentrations  
3 of  $>4.0 \times 10^{-3}$  mg  $\text{NHO}_2\text{-N/L}$ . These results confirmed that nitrite or FNA would  
4 negatively influence  $\text{N}_2\text{O}$  reduction in the denitrifying phosphorus removal process.

5 In the meantime, the slow degradation kinetics of the PHAs, as electron donors,  
6 has also been reported to influence  $\text{N}_2\text{O}$  accumulation in this process. Li et al. (2013b)  
7 found that the dissolved  $\text{N}_2\text{O}$  concentration was up to 0.85 mgN/L when PHAs were  
8 the sole carbon source, but decreased to 0.21 mgN/L when acetate was added  
9 simultaneously. The proposed explanation was that the PHA degradation was not  
10 capable of providing sufficient electrons for denitrification and caused electron  
11 competition between the  $\text{N}_2\text{O}$  reductase and other denitrifying enzymes. Similarly,  
12 Zhou et al. (2012) revealed that it was the degradation rate of PHAs that potentially  
13 limited the  $\text{N}_2\text{O}$  reduction rate. Wang et al. (2011 a) also reported that PHAs were the  
14 inducer of  $\text{N}_2\text{O}$  production in this process. Based on these studies, it can be  
15 hypothesised that the slow PHA degradation rate directly caused the decrease of  
16 electron supply rate during the denitrification process. According to Pan et al. (2013),  
17 the electron flux going to  $\text{N}_2\text{O}$  reductase was slowed down due to the declining  
18 external carbon loading rate. So it could be further deduced that  $\text{N}_2\text{O}$  production  
19 would be stimulated when the PHA degradation rate decreased gradually.

20 However, in most studies reported to date, both the nitrite / FNA concentration  
21 and the PHA degradation rate varied concurrently, and hence the individual effect of  
22 PHA degradation rate on  $\text{N}_2\text{O}$  accumulation in a denitrifying phosphorus removal

1 system is not known at present. In this study, we investigate the effect of PHA  
2 degradation rate on  $N_2O$  accumulation in a denitrifying phosphorus removal system  
3 under controlled and low FNA concentration. The electron distribution between  
4 different denitrification steps is calculated based on the measured consumption rates  
5 of various electron acceptors, and correlated with the PHA consumption rate. Finally,  
6 a strategy for effectively reducing  $N_2O$  accumulation was proposed and demonstrated.

## 7 **2 Material and methods**

### 8 **2.1 Sludge source**

9 The sludge was withdrawn from a 10 L laboratory-scale sequencing batch reactor  
10 (called the parent SBR) fed with domestic wastewater, augmented with acetate. A  
11 cycle time of the SBR was 8 h, consisting of 90 min anaerobic reaction (including 9  
12 min feeding at the beginning of this phase), 180 min anoxic reaction, 60 min aerobic  
13 reaction (including 5 min wasting at the end of this phase), 120 min settling and 30  
14 min decanting phases. In the 9 min feeding period, 5 L wastewater (composition  
15 described below) was added to the reactor. At the end of the anaerobic stage, 20 ml  
16  $NaNO_3$  stock solution (121.4 g/L  $NaNO_3$ ) was pumped into the SBR in one minute,  
17 resulting in an initial nitrate concentration of 40 mgN/L. The 60 min aerobic phase  
18 was included to take up the P remained at the end of the anoxic phase completely with  
19 a constant air flow of 0.2 L/min, In the wasting period, 450 mL mixed liquor was  
20 discarded to keep the solids retention time (SRT) in the reactor at approximately 15 d.  
21 The hydraulic retention time (HRT) was 11 h. The mixed liquor suspended solids  
22 (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were

1 about 3500 mg/L and 2500 mg/L, respectively. The parent SBR was operated over six  
2 months and reached pseudo steady state (as evidenced by the stable MLSS and  
3 MLVSS concentrations and N and P removal performance).

## 4 **2.2 Wastewater**

5 Domestic wastewater drawn from a sewer line on campus was used as the reactor  
6 feed after sedimentation. The wastewater, which had low COD: N: P ratios  
7 (100:47:0.37) was supplemented with the acetate at a concentration of 200 mg/L COD  
8 in order to be able to achieve satisfactory N and P removal. The mean influent COD,  
9  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N,  $\text{NO}_3^-$ -N and  $\text{PO}_4^{3-}$ -P were 340.6 mg/L, 68.7 mg/L, 0.003 mg/L,  
10 0.45 mg/L, 5.24 mg/L, respectively, following acetate addition.

11 Synthetic wastewater used in the batch experiments described in 2.3 contained  
12 98.80 mg/L  $\text{KH}_2\text{PO}_4$ , 112.50 mg/L of  $\text{NH}_4\text{Cl}$ , 14 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 90mg/L  
13  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 300 mg/L  $\text{NaHCO}_3$  and 0.30 mL/L of trace elements solution. The  
14 trace elements solution was made up of 1.50 g/L of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.12 /g/L  
15  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.03g/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.15g/L of  $\text{H}_3\text{BO}_3$ , 0.18 g/L KI, 0.06 g/L  
16  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.15 g/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.12 g/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 10 g/L EDTA  
17 (Smolders et al., 1994).

## 18 **2.3 Batch experiments**

19 A 1.30 L sealed reactor was used for all the batch experiments. Magnetic stirrers  
20 were used to keep the sludge in suspension. In order to ensure completely anaerobic  
21 conditions, high-purity  $\text{N}_2$  was sparged into it for 10 min before each batch test. All  
22 the tests were conducted in an air-conditioned laboratory, with the temperature

1 maintained at 20-22°C.

2 **2.3.1 Investigating N<sub>2</sub>O production characteristics in the denitrifying phosphorus**  
3 **removal reactor with pulse feed of nitrate**

4 This experiment was carried out to investigate the N<sub>2</sub>O production when  
5 denitrification and P removal occurred concurrently (hereafter called Test 1). 1.20 L  
6 sludge was withdrawn from the parent SBR at the end of the aerobic stage and  
7 washed for three times by centrifugation to remove any remaining nitrate, nitrite, P  
8 and external carbon. Then it was transferred into the batch reactor and mixed with the  
9 same domestic wastewater as used by the parent SBR forming a liquid volume of 1.20  
10 L and leaving a headspace of 0.1 L. The reactor was operated with a cycle time of 8 h,  
11 with a phase design identical to that used for the parent SBR. 3.20 ml nitrate stock  
12 solution (91.25 g/LNaNO<sub>3</sub>) was added as a pulse at the end of the anaerobic stage,  
13 resulting in an initial nitrate concentration of 40 mgN/L. During the aeration stage,  
14 DO was controlled at 2 mg/L. An N<sub>2</sub>O microsensor (Unisense, Denmark) was  
15 calibrated and then inserted into reactor to measure the dissolved N<sub>2</sub>O on-line during  
16 the 180 min anoxic reaction period. At the same time, off-gas sample were acquired  
17 with a microsyringe from the reactor headspace and analyzed immediately for N<sub>2</sub>O  
18 concentrations using the method to be further described in 2.4. Mixed liquor samples  
19 were taken every 30 min throughout the experiment. The parameters analyzed  
20 included PHAs, VFAs, glycogen, COD, nitrate, nitrite and PO<sub>4</sub><sup>3-</sup>-P.

21 **2.3.2 Studying N<sub>2</sub>O production during nitrite reduction under varying PHA**  
22 **degradation rates**



1           N<sub>2</sub>O accumulation would appear when the nitrite consumption rate is faster than  
2 the N<sub>2</sub>O reduction rate. In order to explore the reason for N<sub>2</sub>O accumulation in a  
3 denitrifying phosphorus removal system, this experiment was undertaken by using  
4 PHAs and nitrite as electron donors and electron acceptor, respectively (hereafter  
5 called Test 2). In each test, 1.3 L sludge was withdrawn from the parent SBR when its  
6 anaerobic reaction finished, and was washed for three times to remove the residual  
7 external carbon to ensure PHAs would be the primary carbon source. The batch  
8 reactor was filled with 1.30 L synthetic wastewater as described in 2.2. The resulting  
9 MLSS and MLVSS concentration were approximately 4300 mg/L and 3800 mg/L,  
10 respectively. During the experiment, nitrite in the reactor was kept relatively constant  
11 and non-rate limiting levels (between 20-25mg N/L) by manual addition of a stock  
12 nitrite solution (9.86 g/LNaNO<sub>2</sub>) every 30 min. pH was controlled at 8.00±0.05 by  
13 manually adding 0.50 M HCL and 0.50 M NaOH. pH was measured online using a  
14 pH meter (pH/oxi340i, WTW Company, Germany). FNA was calculated according to  
15 the formula:  $S_{NO_2-N}/(K_a \times 10^{pH})$ , with the  $K_a$  value determined by the formula  
16  $K_a = e^{-2300/(273+T)}$  for the measured temperature T(°C) (Anthonisen et al., 1976). In  
17 this case, the FNA was determined to be varying in a small range with a mean value  
18 of  $4.86 \times 10^{-4}$  mg HNO<sub>2</sub>-N/L.

19           With the potential effect of FNA on N<sub>2</sub>O production kept constant through the  
20 above experimental design, this experiment could investigate the impact of PHAs  
21 variation on N<sub>2</sub>O production under low FNA conditions. The activated sludge samples  
22 were taken every 30 min for the analysis of PHAs and nitrite. The dissolved and

1 off-gas  $N_2O$  concentrations were measured as described in Test 1. The experiments  
2 lasted for 4 h until nitrite reduction ceased.

### 3 **2.3.3 Controlling $N_2O$ production in the denitrifying phosphorus removal reactor** 4 **via continuous feed of nitrate**

5 With the aim to control the  $N_2O$  production during denitrifying phosphorus  
6 removal, this experiment was conducted (hereafter called Test 3). Test 3 was similar  
7 to Test 1 except that nitrate was added continuously rather than as a pulse. 60 ml  
8  $NaNO_3$  stock solution (4.86g/L $NaNO_3$ ) was continuously fed into reactor during the  
9 anoxic reaction period with a peristaltic pump, which ensured that the total amount of  
10 nitrate was equal to that added in Test 1.

### 11 **2.4 Analytical methods**

12 Mixed liquor samples were filtered through 0.45  $\mu m$  filter paper for the analysis  
13 of COD,  $NO_2^-$ -N,  $NO_3^-$ -N,  $PO_4^{3-}$ -P, and VFAs. VFAs were measured with gas  
14 chromatography according to Su et al. (2013). COD,  $NO_2^-$ -N,  $NO_3^-$ -N,  $PO_4^{3-}$ -P, MLSS  
15 and MLVSS were measured according to the standard methods (American Public  
16 Health Association (APHA), 1995). The solid-phase samples were used to measure  
17 PHAs and glycogen after freeze-drying. The total PHAs stored in the DPAOs were  
18 determined by the sum of poly- $\beta$ -hydroxybutyrate (PHB) and poly- $\beta$ -hydroxyvalerate  
19 (PHV), and both of them were analyzed according to Oehmen et al. (2007). Glycogen  
20 was measured with the method of colorimetry of sulfuric acid-anthrone (Frølund et al.,  
21 1996). The off-gas  $N_2O$  was measured via gas chromatograph (GC, Agilent 6890N,  
22 U.S.) (Yang et al., 2009), and the dissolved  $N_2O$  was continuously measured with an

1 N<sub>2</sub>O microsensor (with a detection limit of 3  $\mu\text{molN}_2\text{O-N/L}$  in water, Unisense,  
2 Denmark). The sensor was calibrated before each experiment.

3 The PHA, nitrite, N<sub>2</sub>O consumption rates were determined through linear  
4 regression of their profiles ( $R^2 > 0.90$ ), with accumulation represented as negative  
5 consumption. Since nitric oxide (NO), the precursor of N<sub>2</sub>O, usually does not  
6 accumulate during denitrification due to its toxicity on microorganism (Richardson et  
7 al., 2009), its reduction rate was assumed to be equal to the nitrite reduction rate. The  
8 biomass specific PHA degradation rate was determined by dividing its consumption  
9 rate by MLVSS. The specific reduction rate of nitrogen oxides, electron consumption  
10 rate of denitrifying enzymes and electron distribution percentages during  
11 denitrification were calculated according to Pan et al. (2013).

### 12 **3. Results and discussion**

#### 13 **3.1 N<sub>2</sub>O production characteristic in the denitrifying phosphorus removal** 14 **process with pulse feed of nitrate**

15 Test 1 was undertaken to investigate the N<sub>2</sub>O accumulation characteristic in the  
16 denitrifying phosphorus removal process. Fig.1(a) and Fig.1(b) show the cycle  
17 profiles of various parameters measured in this process. The phenotype of DPAOs  
18 was observed. In the anaerobic period with external carbon (COD) being available  
19 (feast period), microbial respiration proceeded immediately when nitrate was added.  
20 The COD and VFAs (calculated as COD) decreased from 233 and 119 mg/L to 80  
21 and 4 mg/L, respectively. Concurrently, the release of PO<sub>4</sub><sup>3-</sup>-P was observed, which  
22 reached 13 mg/L. The relatively low P release to VFA uptake ratio (0.1 molP/molC)

1 suggests the likely presence of glycogen-accumulating organisms (GAOs), which  
2 would compete for organic carbon with PAOs anaerobically (Lopez-Vazquez et al.,  
3 2007). In the subsequent period of no external carbon (famine period), the anoxic  
4 nitrate concentration decreased from the initial 40 mg N/L to 0.27 mg N/L at 210 min,  
5 and approximately 90% of the  $\text{PO}_4^{3-}\text{-P}$  was removed. In the aerobic stage, the  
6 remaining  $\text{PO}_4^{3-}\text{-P}$  (1.56 mg/L) was successfully taken up with oxygen as the terminal  
7 electron acceptor. At the end of the aerobic period,  $\text{PO}_4^{3-}\text{-P}$  and PHAs reached  
8 approximately zero and glycogen recovered to 10.7 mMC/gVSS.

9 The nitrite and  $\text{N}_2\text{O}$  production during the test are shown in Fig.1 (b). Nitrite  
10 gradually increased, reaching 7.0 mg N/L at 210 min, corresponding to an FNA level  
11 of  $0.06 \times 10^{-3}$  mg  $\text{HNO}_2\text{-N}$  /L, when nitrate depleted, The dissolved  $\text{N}_2\text{O}$  also increased,  
12 reaching approximately 0.15 mg N/L at this time. Accumulation of  $\text{N}_2\text{O}$  in the gas  
13 phase was also observed, giving an overall (liquid + gas)  $\text{N}_2\text{O}$  accumulation of about  
14 0.20 mg N/L.

15 Significantly, nitrite and  $\text{N}_2\text{O}$  reached their highest levels almost at the same  
16 time. The dissolved  $\text{N}_2\text{O}$  then rapidly dropped to 0 mg N/L when the accumulated  
17 nitrite decreased to the lowest level. These profiles indicate that the accumulated FNA  
18 could have been an inducer of  $\text{N}_2\text{O}$  accumulation in this system. Although Zhou et al.  
19 (2008b) revealed the inhibitory effect of FNA at a concentration of  $0.06 \times 10^{-3}$  mg  
20  $\text{HNO}_2\text{-N}$  /L on DPAOs was negligible, this conclusion may not be directly applicable  
21 to our sludge. The microbial community in our sludge, which likely contained a  
22 significant amount of GAOs, was different from that studied in Zhou et al. (2008b),

1 which contained a negligible GAO population according to Zhou et al. (2008a).  
2 GAOs have previously reported to be a major contributor to N<sub>2</sub>O production in  
3 denitrifying phosphorus removal systems (Zeng et al., 2003; Zhu et al., 2011).

4 Equally significantly, the maximum N<sub>2</sub>O accumulation also appeared to coincide  
5 with slow PHA degradation (Fig.1(a) and Fig.1(b)), suggesting that the PHA  
6 degradation rate could also be a contributor. This is in agreement with previous  
7 studies. Zhou et al. (2012) also suggested that the N<sub>2</sub>O accumulation could be  
8 attributed to the shortage of electrons provided by PHA oxidation. Li et al. (2013b)  
9 reported that the accumulated N<sub>2</sub>O during denitrification with PHAs decreased sharply  
10 with acetate addition. According to Pan et al. (2013), Nos has a lower affinity for  
11 electron carriers than Nir. All these supported that lower PHA degradation rates would  
12 lead to N<sub>2</sub>O accumulation during denitrification with PHAs as the sole carbon source.

13 Therefore, Test 2 reported below was designed to reveal the effect of the PHA  
14 degradation rate on N<sub>2</sub>O production by the sludge. Its effect is separated from that of  
15 FNA by keeping FNA at a constant and low level.

### 16 **3.2 The effect of PHA degradation rate on N<sub>2</sub>O production during denitrifying** 17 **phosphorus removal at a constant and low FNA level**

18 With the aim to investigate the individual effect of PHA degradation rate on N<sub>2</sub>O  
19 production in the denitrifying phosphorus removal system, nitrite concentration was  
20 controlled at a relatively constant level. pH was controlled at a constant level of 8.0.  
21 Fig. 2 shows the variations of nitrite, N<sub>2</sub>O and PHAs during nitrite reduction. The  
22 nitrite level was successfully controlled within the range of 20-25 mg N/L in the

1 whole test, and the FNA was thus controlled at a relatively constant and low level of  
2  $4.86 \times 10^{-4}$  mg  $\text{HNO}_2\text{-N/L}$ . The produced  $\text{N}_2\text{O}$  kept rising until nitrite reduction almost  
3 ceased. PHAs decreased gradually from 3.0 to 1.5 mMC/gVSS, and it degraded more  
4 slowly at the later stage of this test. Zhou et al. (2012) and Miao et al. (2014) also  
5 observed  $\text{N}_2\text{O}$  accumulation when nitrite reduction was supported with internal  
6 carbon sources (i.e. PHAs). By replenishing the reduced nitrite every 30 min (Fig. 2),  
7 the total reduced nitrite was 20.9 mg N/L. In comparison, the produced  $\text{N}_2\text{O}$  reached  
8 8.4 mg N/L, accounting for 40% of the reduced nitrite.

9 Interestingly, Fig.3 (a) shows that all the  $\text{N}_2\text{O}$  production percentages (the  
10 percentages of  $\text{N}_2\text{O}$  produced accounting for the total reduced nitrite) in every 30 min  
11 interval fluctuated around the average value of about 40%. Fig.3 (b) and (c) show the  
12 nitrite and  $\text{N}_2\text{O}$  specific reduction rates within 240 min obtained from linear  
13 regression of nitrite and  $\text{N}_2\text{O}$  concentrations in every 30 min period, respectively. It  
14 was found that both the nitrite and  $\text{N}_2\text{O}$  reduction rates decreased with time, which  
15 can be described with the exponential model. Comparing the parameters of the  
16 functions revealed that nitrite dropped more quickly than  $\text{N}_2\text{O}$ . This suggests that the  
17  $\text{N}_2\text{O}$  production rate was above its reduction rate during nitrite reduction, resulting in  
18 the apparent  $\text{N}_2\text{O}$  accumulation all the time.

19 Fig.3 (d) shows that the PHA degradation rates declined from  $0.15 \times 10^{-1}$  to  
20  $0.01 \times 10^{-1}$  mMC/(gVSS $\times$ min), implying that the electron supply rate varied during  
21 nitrite reduction. The prediction of PHA degradation rate was close to the exponential  
22 model as shown in Fig. 3(d). Beun et al. (2000) also observed that the PHB

1 degradation rate become lower when denitrification proceeded.

2 Pan et al. (2013) demonstrated that the Nir would obtain more electrons  
3 compared to Nos when electron competition was intensified by the slower electron  
4 supply rate, resulting in more severe inhibition with the N<sub>2</sub>O reduction. Consequently,  
5 the disparity of electron consumption rates between Nir and Nos would become much  
6 greater. However, Fig.3 (d) and Fig.4 (a) showed that both the Nir and Nos electron  
7 consumption rates descended simultaneously with the decreased PHA degradation  
8 rate (determining the electron supplying rate). The electron consumption rate for Nir  
9 fell from  $0.05 \times 10^{-1}$  to  $0.02 \times 10^{-1}$  m mole / (gVSS×min), and the rate for Nos fell from  
10  $0.03 \times 10^{-1}$  to  $0.01 \times 10^{-1}$  m mole / (gVSS×min). This suggests that the electron  
11 competition between Nir and Nos did not get more intensified when carbon was  
12 degraded more slowly in denitrification with PHAs.

13 The electron distribution is shown in Fig.4 (b). The percentages of electrons  
14 distributed to Nir and Nos remained approximately constant irrespective of the change  
15 in the PHA oxidation rate, which were about 40% and 25% of the total electrons  
16 consumed, respectively. This result was significant, for it could potentially account for  
17 the constant N<sub>2</sub>O accumulation during nitrite reduction under varying PHA degradation  
18 rates. In this case, the nitrite reduction rate seemed to be always 1.6 times that of N<sub>2</sub>O.  
19 This result is different from the electron competition observed in the  
20 methanol-utilizing denitrification process (Pan et al., 2013). During the denitrification  
21 with external carbon sources, the electrons were mainly used for microbial respiration  
22 and biomass growth. However, the electrons were also utilized for glycogen

1 replenishment besides those with the sludge used in this study. Whether this  
2 contributed to this difference remains to be further investigated.

3 Various carbon sources have been found to significantly influence  $N_2O$  production  
4 in denitrifying phosphorus removal systems. Wang et al., (2011b) showed that  $N_2O$   
5 production went up when the carbon source was switched from acetate to propionate.  
6 This could be related to the different amount and composition of PHAs produced  
7 anaerobically. Contradictorily, Li et al. (2013b) showed  $N_2O$  generation decreased by  
8 about 70 % by using propionate as the carbon source in place of acetate. Zhu & Chen  
9 (2011) found that the use of waste-activated sludge fermented under alkaline  
10 conditions as the carbon sources in an anaerobic – aerobic process decreased  $N_2O$   
11 production by 69% compared to acetate. They hypothesized that the presences of  $Cu_2^+$   
12 in the fermented products favored the proliferation of bacteria capable of reducing  
13  $N_2O$  to  $N_2$ .

### 14 **3.3 Controlling $N_2O$ production during denitrifying phosphorus removal by** 15 **limiting the electron supply rate via continuous feed of nitrate**

16 Based on the above results, this test was performed by limiting the electron  
17 acceptor supply rate to control  $N_2O$  production via continuous feed of nitrate (Fig.  
18 5(a)). VFAs were completely consumed anaerobically, resulting in PHA generation.  
19 PHAs were consumed with the continuous nitrate feed, declining from 7.9 to 3.3  
20 mMC/gVSS. PHAs degraded faster in the first 90 min of the anoxic period, consistent  
21 with Fig.1 (a). Efficient P removal and glycogen replenishment were achieved in the  
22 anoxic stage.  $PO_4^{3-}$ -P decreased from 11.7 to 0.67mg/L, and glycogen rose from 4.6 to  
23 8.6 mMC/gVSS.



1           The nitrate level was controlled below 1.5 mg N/L (Fig. 5(b)). Nitrite was less  
2 than 2.5 mg N/L at all time. The dissolved N<sub>2</sub>O accumulation of up to 0.04 mgN/L  
3 coincided with nitrite accumulated to 2.3 mgN/L, which was much lower than the  
4 nitrite accumulation in Test 1. More significantly, the maximum N<sub>2</sub>O production was  
5 0.05 mg N/L, which was 75% less than that in the denitrification process with pulse  
6 feed of nitrate. Therefore, the continuous feed of nitrate could efficiently reduce N<sub>2</sub>O  
7 accumulation in the denitrifying phosphorus removal system. With the slow feeding  
8 applied in Test 3, the electron supply resulting from PHA oxidation was meeting the  
9 electron demand of all steps of denitrification, with the rates of all denitrification steps  
10 limited by the nitrate supplying rate. Accordingly, the N<sub>2</sub>O production rate (nitrite  
11 reduction rate) was well balanced with its reduction rate, and hence most of the N<sub>2</sub>O  
12 produced was converted to N<sub>2</sub>. In addition, this method also attenuated the FNA  
13 inhibition on N<sub>2</sub>O reduction, for the highest FNA concentration decreased from  
14  $0.06 \times 10^{-3}$  mg HNO<sub>2</sub>-N to  $0.02 \times 10^{-3}$  mg HNO<sub>2</sub>-N.

## 15   **Conclusions**

16           N<sub>2</sub>O accumulation in a denitrifying phosphorus removal system was investigated.  
17 Lower PHA degradation rates did not stimulate higher N<sub>2</sub>O production. Electron  
18 distribution for Nos during nitrite reduction supported by PHAs did not vary with the  
19 PHA oxidation rate and remained at approximately 25%. As the sludge likely  
20 contained both PAOs and GAOs, the above observation cannot be uniquely attributed  
21 to either group. N<sub>2</sub>O accumulation can be reduced substantially by loading nitrate  
22 slowly in comparison to pulse feeding of nitrate.

## 1 Acknowledgements

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4 platform of Beijing Municipal Education Commission.

## 5 References

- 6 [1] Ahn, J., Daidou, T., Tsuneda, S., Hirata, A. 2002. Characterization of denitrifying  
7 phosphate-accumulating organisms cultivated under different electron acceptor  
8 conditions using polymerase chain reaction-denaturing gradient gel  
9 electrophoresis assay. *Water Res.*, 36, 403-412.
- 10 [2] American Public Health Association (APHA), A.W.W.A.A., Water Environment  
11 Federation (WEF). 1995. *Standard Methods for the Examination of Water and*  
12 *Wastewater*, Washington, DC.
- 13 [3] Anthonisen, A., Loehr, R., Prakasam, T., Srinath, E. 1976. Inhibition of  
14 nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control*  
15 *Federation)*, 835-852.
- 16 [4] Beun, J., Verhoef, E., Van Loosdrecht, M., Heijnen, J. 2000. Stoichiometry and  
17 kinetics of poly- $\beta$ -hydroxybutyrate metabolism under denitrifying conditions in  
18 activated sludge cultures. *Biotechnol. Bioeng.*, 68, 496-507.
- 19 [5] Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H. 1996. Extraction of  
20 extracellular polymers from activated sludge using a cation exchange resin. *Water*  
21 *Res.*, 30, 1749-1758.
- 22 [6] Guo, G., Wang, Y., Wang, C., Wang, H., Pan, M., Chen, S. 2013. Short-term

- 1 effects of excessive anaerobic reaction time on anaerobic metabolism of  
2 denitrifying polyphosphate-accumulating organisms linked to phosphorus removal  
3 and N<sub>2</sub>O production. *Front. Environ. Sci. Eng.*, 7, 616-624.
- 4 [7] Halet, D., Defoirdt, T., Van Damme, P., Vervaeren, H., Forrez, I., Van de Wiele, T.,  
5 Boon, N., Sorgeloos, P., Bossier, P., Verstraete, W. 2007. Poly- $\beta$ -hydroxybutyrate  
6 -accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic  
7 *Vibrio campbellii*. *FEMS Microbiol.Ecol.*, 60, 363-369.
- 8 [8] IPCC. 2001. *Climate Change 2001: the Scientific Basis*. Cambridge University  
9 Press.
- 10 [9] Itokawa, H., Hanaki, K., Matsuo, T. 2001. Nitrous oxide production in  
11 high-loading biological nitrogen removal process under low cod/n ratio condition.  
12 *Water Res.*, 35, 657-664.
- 13 [10] Johwan Ahn, T.D., Satoshi Tsuneda, Akira Hirata. 2001. Metabolic Behavior of  
14 Denitrifying Phosphate-Accumulating Organisms under Nitrate and Nitrite  
15 Electron Acceptor Conditions. *J. Biosci. Bioeng.*, 92, 442-446.
- 16 [11] Kampschreur, M.J., van der Star, W.R.L., Wielders, H.A., Mulder, J.W., Jetten,  
17 M.S.M., van Loosdrecht, M.C.M. 2008. Dynamics of nitric oxide and nitrous  
18 oxide emission during full-scale reject water treatment. *Water Res.*, 42, 812-826.
- 19 [12] Kuba, T., Van Loosdrecht, M., Heijnen, J. 1996. Phosphorus and nitrogen removal  
20 with minimal COD requirement by integration of denitrifying dephosphatation  
21 and nitrification in a two-sludge system. *Water Res.*, 30, 1702-1710.
- 22 [13] Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z. 2006.

- 1 Identifying causes for N<sub>2</sub>O accumulation in a lab-scale sequencing batch reactor  
2 performing simultaneous nitrification, denitrification and phosphorus removal. *J.*  
3 *Biotechnol.*, 122, 62-72.
- 4 [14]Li, C., Liang, S., Zhang, J., Ngo, H.H., Guo, W., Zheng, N., Zou, Y. 2013a. N<sub>2</sub>O  
5 reduction during municipal wastewater treatment using a two-sludge SBR system  
6 acclimatized with propionate. *Chem.Eng. J.*, 222, 353-360.
- 7 [15]Li, C., Zhang, J., Liang, S., Ngo, H.H., Guo, W., Zhang, Y., Zou, Y. 2013b.  
8 Nitrous oxide generation in denitrifying phosphorus removal process: main causes  
9 and control measures. *Environ. Sci. Pollut. Res. Int.*, 20, 5353-5360.
- 10 [16]Lopez-Vazquez, C.M., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., van  
11 Loosdrecht, M.C.M. 2007. A Practical Method for Quantification of Phosphorus-  
12 and Glycogen-Accumulating Organism Populations in Activated Sludge Systems.  
13 *Water Environ Res*, 79, 2487-2498.
- 14 [17]Ma, B., Peng, Y., Zhang, S., Wang, J., Gan, Y., Chang, J., Wang, S., Wang, S., Zhu,  
15 G. 2013a. Performance of anammox UASB reactor treating low strength  
16 wastewater under moderate and low temperatures. *Bioresour. Technol.*, 129,  
17 606-611.
- 18 [18]Meinhold J, A.E., Isaacs S. . 1999. Effect of nitrite on anoxic phosphate uptake in  
19 biological phosphorus removal activated sludge. *Water Res.*, 33, 1871-1883.
- 20 [19]Miao, Z., Zeng, W., Wang, S., Peng, Y., Cao, G., Weng, D., Xue, G., Yang, Q.  
21 2014. Effect of temperature on anoxic metabolism of nitrites to nitrous oxide by  
22 polyphosphate accumulating organisms. *J. Environ. Sci. China*, 26, 264-273.

- 1 [20]Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis,  
2 M.A. 2007. Advances in enhanced biological phosphorus removal: from micro to  
3 macro scale. *Water Res.*, 41, 2271-2300.
- 4 [21]Pan, Y., Ni, B.J., Bond, P.L., Ye, L., Yuan, Z. 2013. Electron competition among  
5 nitrogen oxides reduction during methanol-utilizing denitrification in wastewater  
6 treatment. *Water Res.*, 47, 3273-3281.
- 7 [22]Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E. 2009.  
8 Mitigating release of the potent greenhouse gas N<sub>2</sub>O from the nitrogen cycle -  
9 could enzymic regulation hold the key? *Trends Biotechnol.*, 27, 388-397.
- 10 [23]Smolders, G., Van der Meij, J., Van Loosdrecht, M., Heijnen, J. 1994.  
11 Stoichiometric model of the aerobic metabolism of the biological phosphorus  
12 removal process. *Biotechnol.Bioeng.*,44, 837-848.
- 13 [24]Su, G., Huo, M., Yuan, Z., Wang, S., Peng, Y. 2013. Hydrolysis, acidification and  
14 dewaterability of waste activated sludge under alkaline conditions: combined  
15 effects of NaOH and Ca(OH)<sub>2</sub>. *Bioresour. Technol.*, 136, 237-243.
- 16 [25]Wang, Y., Geng, J., Ren, Z., He, W., Xing, M., Wu, M., Chen, S. 2011a. Effect of  
17 anaerobic reaction time on denitrifying phosphorus removal and N<sub>2</sub>O production.  
18 *Bioresour. Technol.*, 102, 5674-84.
- 19 [26]Wang, Y., Geng, J., Guo, G., Wang, C., Liu, S. 2011b. N<sub>2</sub>O production in  
20 anaerobic/anoxic denitrifying phosphorus removal process: The effects of carbon  
21 sources shock. *Chem Eng J*, 172, 999-1007.
- 22 [27]Wentzel, M., Loewenthal, R., Ekama, G., Marais, G. 1988. Enhanced

- 1 polyphosphate organism cultures in activated sludge systems- Part 1: Enhanced  
2 culture development. *Water S. A.*, 14, 81-92.
- 3 [28] Yang, Q., Liu, X., Peng, C., Wang, S., Sun, H., Peng, Y. 2009. N<sub>2</sub>O production  
4 during nitrogen removal via nitrite from domestic wastewater: main sources and  
5 control method. *Environ. Sci. Technol.*, 43, 9400-9406.
- 6 [29] Zeng, R.J., Yuan, Z., Keller, J. 2003. Enrichment of denitrifying glycogen-  
7 accumulating organisms in anaerobic/anoxic activated sludge system. *Biotechnol.*  
8 *Bioeng.*, 81, 397-404.
- 9 [30] Zhou, Y., Lim, M., Harjono, S., Ng, W.J. 2012. Nitrous oxide emission by  
10 denitrifying phosphorus removal culture using polyhydroxyalkanoates as carbon  
11 source. *J. Environ. Sci. China*, 24, 1616-1623.
- 12 [31] Zhou, Y., Pijuan, M., Yuan, Z. 2008a. Development of a 2-sludge, 3-stage system  
13 for nitrogen and phosphorous removal from nutrient-rich wastewater using  
14 granular sludge and biofilms. *Water Res.*, 42, 3207-17.
- 15 [32] Zhou, Y., Pijuan, M., Zeng, R.J., Yuan, Z. 2008b. Free Nitrous Acid Inhibition on  
16 Nitrous Oxide Reduction by a Denitrifying-Enhanced Biological Phosphorus  
17 Removal Sludge. *Environ. Sci. Technol.*, 42, 8260-8265.
- 18 [33] Zhu, X., Chen, Y. 2011. Reduction of N<sub>2</sub>O and NO generation in  
19 anaerobic-aerobic (low dissolved oxygen) biological wastewater treatment process  
20 by using sludge alkaline fermentation liquid. *Environ Sci Technol*, 45, 2137-43.

1 **Table and Figure captions**

2 Fig. 1 Variations of COD, VFAs, PHAs,  $\text{PO}_4^{3-}\text{-P}$  and Glycogen (a) and nitrate, nitrite  
3 and  $\text{N}_2\text{O}$  (b) in a typical cycle during denitrifying phosphorus removal with pulse feed  
4 of nitrate

5 Fig.2 The nitrite and  $\text{N}_2\text{O}$  concentration profiles during nitrite reduction using PHAs  
6 as the carbon source

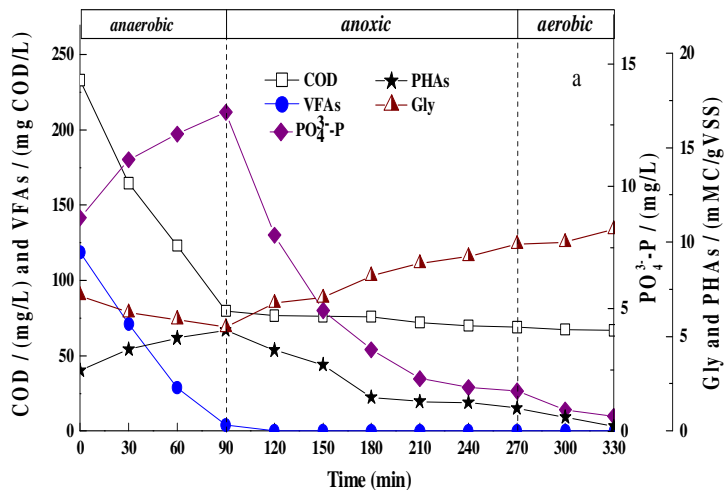
7 Fig.3  $\text{N}_2\text{O}$  production (a), specific nitrite reduction rate (b), specific  $\text{N}_2\text{O}$  reduction  
8 rate (c), specific PHA degradation rate (d) during nitrite reduction using PHAs as the  
9 carbon source

10 Fig.4 Electron consumption rate (a) and electron distribution (b) during nitrite  
11 reduction using PHAs as carbon source

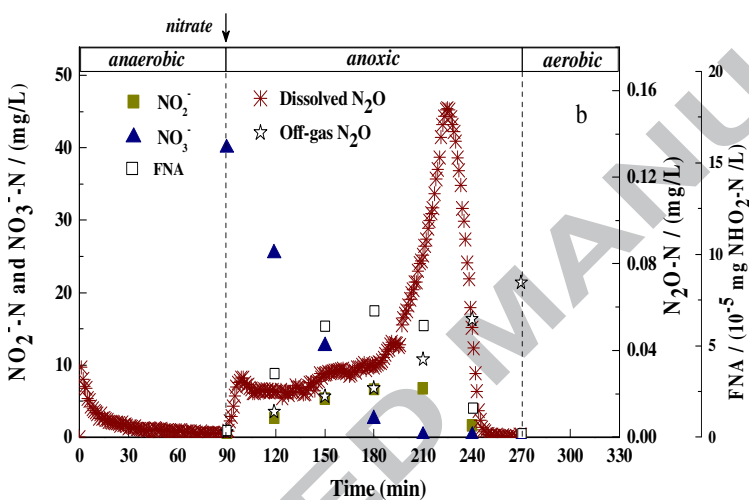
12 Fig. 5 Variations of COD, VFAs, PHAs,  $\text{PO}_4^{3-}\text{-P}$  and Glycogen (a) and nitrate, nitrite  
13 and  $\text{N}_2\text{O}$  (b) in a typical cycle during denitrifying phosphorus removal with  
14 continuous feed of nitrate

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3 Fig. 1 Variations of COD, VFAs, PHAs,  $\text{PO}_4^{3-}\text{-P}$  and Glycogen (a) and nitrate, nitrite and  $\text{N}_2\text{O}$  (b) in a typical

4 cycle during denitrifying phosphorus removal with pulse feed of nitrate

5



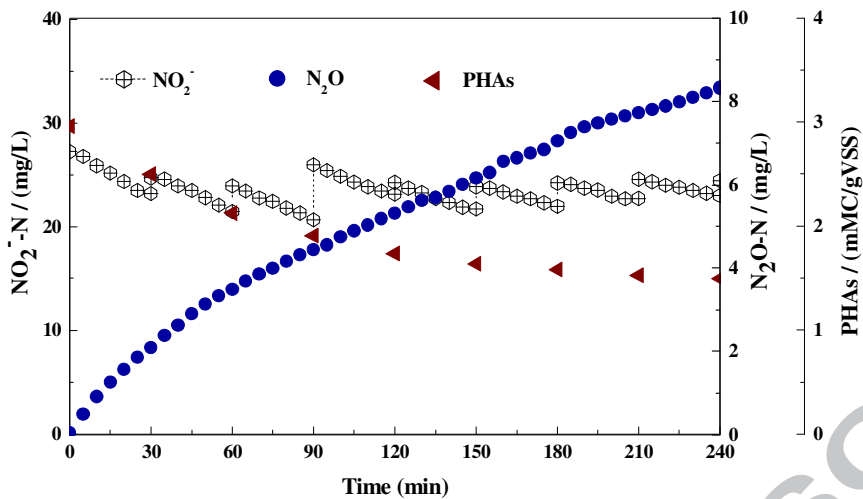
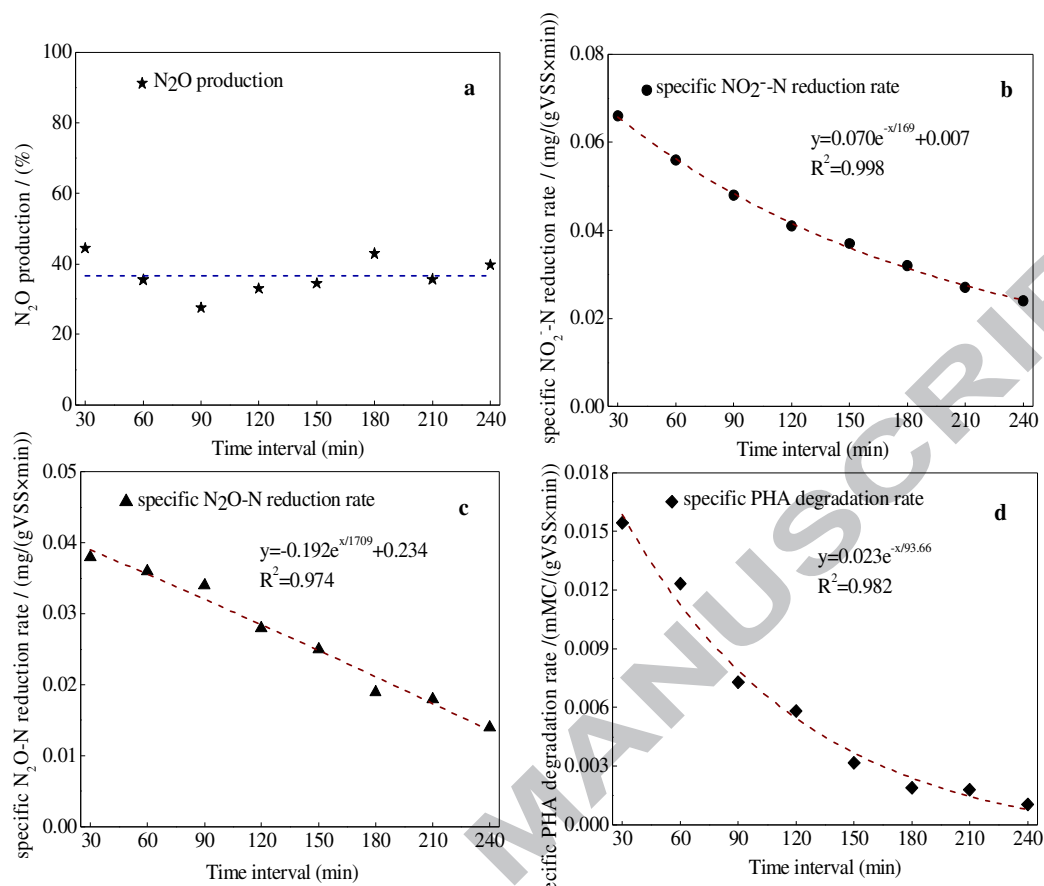


Fig.2 The nitrite and N<sub>2</sub>O concentration profiles during nitrite reduction using PHAs as the carbon source



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Fig.3 N<sub>2</sub>O production (a), specific nitrite reduction rate (b), specific N<sub>2</sub>O reduction rate (c), specific PHA

3

degradation rate (d) during nitrite reduction using PHAs as the carbon source

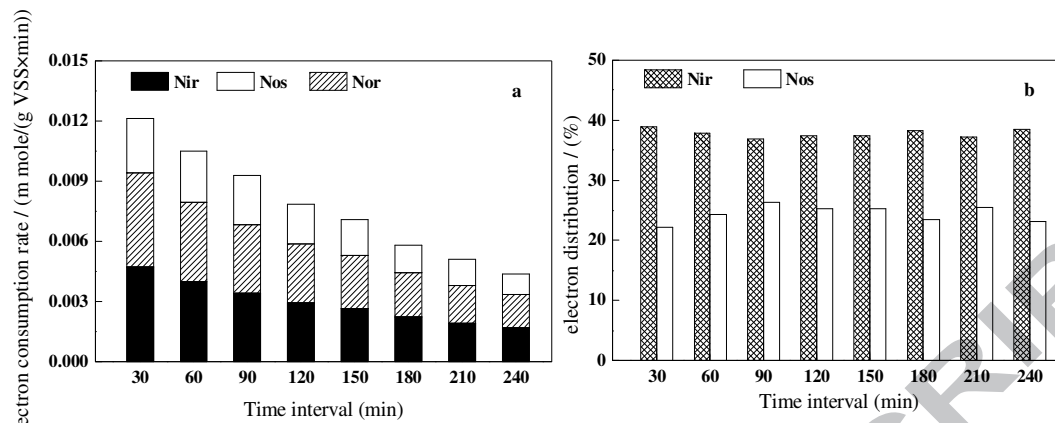
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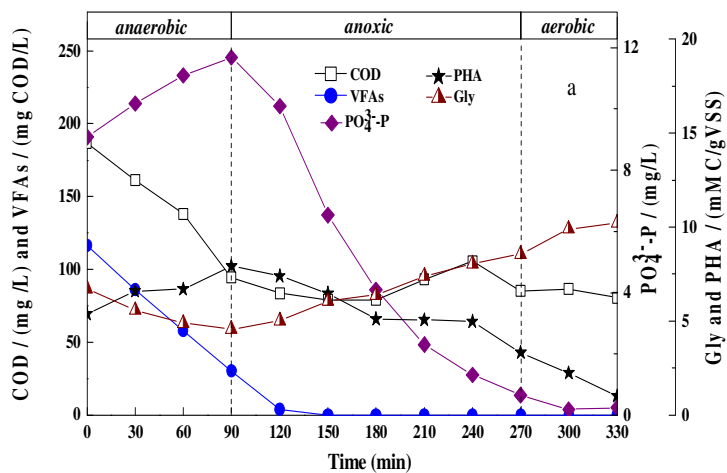


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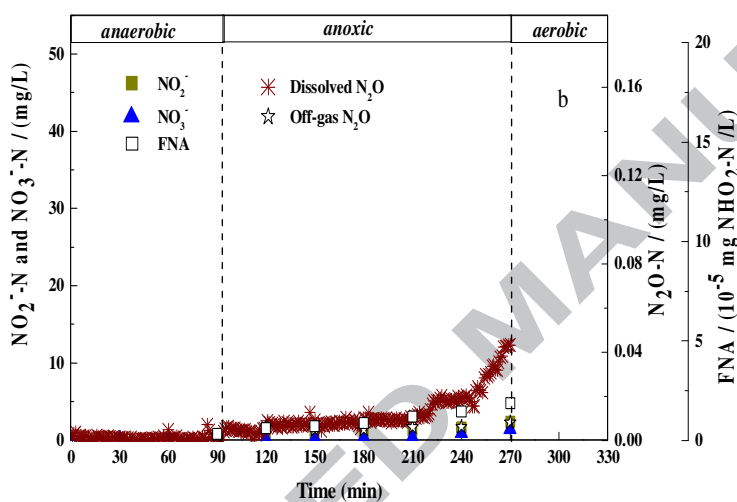
2 Fig.4 Electron consumption rate (a) and electron distribution (b) during nitrite reduction using PHAs as carbon

3 source

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3 Fig. 5 Variations of COD, VFAs, PHAs,  $\text{PO}_4^{3-}\text{-P}$  and Glycogen (a) and nitrate, nitrite and  $\text{N}_2\text{O}$  (b) in a typical

4 cycle during denitrifying phosphorus removal with continuous feed of nitrate

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## Research highlights

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- ✧  $\text{N}_2\text{O}$  production in denitrifying phosphorus removal process was studied.
- ✧ The decrease of PHAs degradation rate did not cause an increase in  $\text{N}_2\text{O}$  production.
- ✧ The electrons distributed to nitrite reductase were 1.6 times of that to  $\text{N}_2\text{O}$  reductase.
- ✧ Controlling nitrate supply rate via continuous feed could reduce  $\text{N}_2\text{O}$  production.

ACCEPTED MANUSCRIPT