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Enhanced biological nutrient removal in sequencing batch reactors operated as static/oxic/anoxic (SOA) process

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HIGHLIGHTS

• Substantial phosphate (P) released in the 1-h static phase without stirring.

• Simultaneous nitrification-denitrification was found in aerobic stage of R1, R2.

• Rate of denitrification driven by glycogen was higher than endogenous decay rate.

• Denitrifying P removal was determined in post-anoxic stage of R2.

• Excellent nutrient removal could be achieved via static/aerobic/anoxic regime.

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ABSTRACT

An innovative static/oxic/anoxic (SOA) activated sludge process characterized by static phase as a substitute for conventional anaerobic stage was developed to enhance biological nutrient removal (BNR) with influent ammonia of 20 and 40 mg/L in R1 and R2 reactors, respectively. The results demonstrated that static phase could function as conventional anaerobic stage. In R1 lower influent ammonia concentration facilitated more polyphosphate accumulating organisms (PAOs) growth, but secondary phosphorus release occurred due to NO_x^- depletion during post-anoxic period. In R2, however, denitrifying phosphorus removal proceeded with sufficient NO_x^- . Both R1 and R2 saw simultaneous nitrification–denitrification. Glycogen was utilized to drive post-denitrification with denitrification rates in excess of typical endogenous decay rates. The anoxic stirring duration could be shortened from 3 to 1.5 h to avoid secondary phosphorus replease in R1 and little adverse impact was found on nutrients removal in R2.

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1. Introduction

Nitrogen (N) and phosphorus (P) are two key elements accelerating water eutrophication. Therefore both N and P removal is of vital significance for water pollution control. Biological nutrient removal (BNR) is considered to be the most economical and sustainable technique to meet increasingly rigorous discharge requirements. Polyphosphate accumulating organisms (PAOs) are enriched by recirculating the activated sludge through anaerobic and anoxic/aerobic conditions. PAOs take up carbon sources, particularly volatile fatty acids (VFAs) anaerobically and store them as poly- β -hydroxyalkanoates (PHA) via polyphosphate (poly-P) hydrolysis and glycolysis (Carvalho et al., 2007). Subsequently, PAOs oxidize PHA via the TCA cycle to provide energy for growth, glycogen replenishment, P uptake, and poly-P storage (Smolders

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, China. Tel.: +86 731 88823967; fax: +86 731 88822829. et al., 1995). Biological N removal is achieved through nitrification and denitrification. In the nitrification process, ammonium is converted to nitrite by ammonium oxidizing bacteria (AOB, nitritation) and nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB, nitritation). Denitrification is the process of nitrate reduction into nitrite and then into molecular nitrogen, which is performed by a functional group of heterotrophs that use oxidized nitrogen as the electron acceptor in respiration (Zhou et al., 2011).

Generally, Anoxic stage is located upstream of the aerobic zone in most BNR wastewater treatment plants (WWTPs), e.g., anaerobic/anoxic/oxic (A²/O) process. High mixed liquor recycle (MLR) rates are required to bring nitrate and/or nitrite to the anoxic zone since denitrification depends on aerobic ammonia oxidation. High denitrification rates (DNRs) can be achieved with the pre-anoxic regime given the supply of readily biodegradable carbon, which, however, is accompanied with such disadvantages as higher energy costs from MLR flows (Coats et al., 2011), dissolved oxygen (DO) return from the aerobic, and dilution of influent carbon (Winkler et al., 2011).





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Post-anoxic denitrification, with anoxic zone located downstream of aerobic zone thus eliminating the MLR pumping, has been one of the research hotspots recently. Post-anoxic sequencing batch reactors (SBRs) employed by Coats et al. (2011) and Winkler et al. (2011) and continuous post-anoxic membrane bio-reactors (MBRs) operated by Bracklow et al. (2010) and Vocks et al. (2005) exhibited excellent BNR performance. Furthermore, external carbon source was not needed to add in these post-anoxic processes, since post-anoxic denitrification could be driven by both PHA and/or glycogen (Vocks et al., 2005). Such a configuration would eliminate the need for external carbon augmentation and also potentially improve overall total nitrogen removal (Coats et al., 2011). Moreover, Xu et al. (2011) transferred part of the anaerobic mixed liquor to the post-anoxic zone for providing carbon source thus enhancing denitrifying P removal, and N was removed partially in the aerobic zone via simultaneous nitrification-denitrification (SND). Although promising results could be obtained in this modified post-anoxic configuration, the additional underflow pumping required increased operational costs.

Nitrate/nitrite existing in anaerobic phase could inhibit P release (Kuba et al., 1994). Free nitrous acid (FNA) generated from nitrite accumulated in aerobic phase could impair the metabolism of PAOs, thus inhibiting aerobic P uptake (Pijuan et al., 2010; Saito et al., 2004; Zhou et al., 2011). In addition, secondary P release might occur in post-anoxic period due to nitrite/nitrate depletion (Winkler et al., 2011). Hence, differing influent ammonia concentrations (transformed to different nitrite/nitrate contents) may have impacts on P elimination efficiencies, which, nonetheless, were hardly reported specifically in post-anoxic denitrification researches.

According to enhanced biological phosphorus removal (EBPR) theory, a conventional anaerobic phase was set in post-anoxic configurations mentioned above to promote P release regardless of sequencing or continuous reactors (Bracklow et al., 2010; Coats et al., 2011; Vocks et al., 2005; Winkler et al., 2011; Xu et al., 2011). In this study, however, considerable P release was observed in static phase. Compared to anaerobic phase, static phase means the absence of stirring. Excess P uptake was detected in subsequent aerobic phase, followed by which was anoxic phase. In this post-anoxic phase denitrification was found without exogenous carbon source addition.

Therefore, two static/oxic/anoxic (SOA) SBRs under different influent ammonia loads were investigated. This work focused on understanding of the potential BNR performance of the novel configuration under different influent ammonia concentrations with anaerobic stage replaced by static stage. The specific objectives were to: (1) speculate microbial metabolic mechanism in static phase, compared to traditional anaerobic phase; (2) evaluate P uptake rate with inhibitory effect of FNA and simultaneous nitrification-denitrification in aerobic phase; (3) confirm the internal carbon source utilized to drive post-denitrification rate; (4) assess the possible secondary P release or denitrifying P uptake due to different influent ammonia loadings and avoidance of secondary P release.

2. Methods

2.1. Experimental setup

Activated sludge obtained from a wastewater treatment plant in Changsha, which operates oxidation ditch process, was inoculated into two SBRs, R1 and R2, each with a working volume of 1.8 L. Each 8-h cycle of both reactors consisted of 1 h static phase, 2.5 h aerobic phase, 3 h anoxic phase, 0.5 h settling/decanting, and 1 h idle. At the beginning of each cycle, 1.2 L wastewater was fed to both reactors. SRT was maintained at 20 days by wasting 30 mL of mixed liquor at the end of anoxic phase of each cycle in each reactor. The pH was not controlled throughout the study. Synthetic wastewater was fed from the bottom of the reactor in the first 8 min of the static period. During slowly addition, the wastewater mixed with activated sludge. After addition, the activated sludge would settle slowly without stirring. The aeration was supplied at the bottom of the reactor by an air pump through diffusers, and the concentration of dissolved oxygen (DO) was maintained at 2–5 mg/L during aerobic stage. The anoxic mixing was accomplished by mechanical stirrers with stirring speed of 200 rpm. Both the aeration and anoxic mixing were automated using digital timers.

The reactors were started up as described above, and the two systems were gradually stabilized after the acclimatization of 22 days. Then the investigations were conducted on the two reactors over the steady-state periods.

2.2. Wastewater source and composition

Both reactors in the experiment were fed with synthetic wastewater. The main influent contents of the two reactors were both 350 mg/L COD, 12 mg/L PO_4^{3-} -P, 5 mg/L $MgSO_4$, and 5 mg/L $CaCl_2$ with the difference of 20 mg/L influent ammonia nitrogen (NH_4^+ -N) in R1 and 40 mg/L NH_4^+ -N in R2. Additionally, the trace elements were described in Wang et al. (2008).

2.3. Analytical methods

All samples were immediately filtered through a Whatmann GF/C glass microfiber filter ($1.2 \mu m$) to analyze NH₄⁺-N, nitrite nitrogen (NO₂⁻-N), nitrate nitrogen (NO₃⁻-N), total nitrogen (TN), soluble orthophosphate (SOP) and MLVSS according to standard methods (APHA, 1998). TOC was determined by using a TOC analyzer (Shimadzu TOC-500, Japan). The determinations of poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV), and poly-3-hydroxy-2-methylvalerate (PH2MV) were indicated in Wang et al. (2008), and the total PHA was calculated as the sum of measured PHB, PHV, and PH2MV. The examination of the presence of intracellular poly-P granules was carried out with 4, 6-diamidino-2-phenylindole (DAPI) according to the method described by Liu et al. (2001), and examined using a two-photon laser confocal microscope (FV1000). Poly-P granules are stained dark blue and DNA of microbial cells are stained light blue.

3. Results and discussion

3.1. The microbial metabolic mechanism in static period

Generally, stirring for 1–2 h after wastewater addition is operated as conventional anaerobic zone, where poly-P is hydrolyzed by PAOs and substantial SOP release into the system. However, comparable SOP release was encountered in the static stages of R1 and R2 (Fig. 1) with the release rates of 15.15 and 13.30 mg-P/(g-VSS h), respectively. Montil et al. (2005) found that anaerobic P release rates were 5–30 mg-P/(g-VSS h). P release rates in the static periods of R1 and R2 were advantageous compared to the variation ranges of that in conventional anaerobic zone. The results indicated that PAOs could take up VFAs quickly without stirring and exhibited a rapid P release, suggesting that static phase could still facilitate the favorable proliferation of PAOs. Intracellular poly-P was stained dark blue through DAPI staining. According to poly-P staining, there were large proportions of cells stained dark



Fig. 1. Variations of SOP, TOC, glycogen, PHA, NH⁺₄-N, NO⁻₂-N and NO⁻₃-N during a typical cycle in R1 (a, c) and R2 (b, d).

blue in activated sludge sampled at the end of aeration from R1 and R2 after DAPI staining, respectively, elucidating dominant poly-P containing cells in both systems. As noted, more dark blue area in poly-P staining of R1 was found than in R2, which suggested more PAOs in R1 than in R2 to some degree, consistent with higher P release rate in R1.

Similar to traditional anaerobic period, the static phase also witnessed glycogen degradation and PHA synthesis with the P release (Fig. 1). The stoichiometries among P release (P_{rel}), VFA uptake (VFA_{up}), PHA syntheses (PHA_{syn}), glycogen degradation (Gly_{deg}) and the percentages of PHB, PHV and PH2MV in PHA reported by other researchers and in the present static phase were compared in Table 1. As described in Table 1, the stoichiometries calculated in static phase were comparable to that reported in traditional anaerobic phase, indicating that PAOs in static period exhibited similar metabolism way performed in conventional anaerobic stage and that static period functioned as anaerobic period. The ratios of glycogen degraded in static phase for reducing power to synthesized PHA were 0.46 and 0.58 C mmol per C mmol PHA in R1 and R2, respectively. Arun et al. (1988) and Carvalho et al. (2007) demonstrated that the ratio within or above a range of 0.28-0.36 would be indicated a successful enhanced biological phosphorus removal (EBPR). Nonetheless, a relatively more PHV accompanied with a bit PH2MV was determined in static stage with acetate as sole carbon source. Propionyl-CoA (polymerized with actetyl-CoA to yield PHV) could be synthesized via reductive TCA cycle (dark dash lines in Fig. 2) + succinate-propionate pathway (dark solid line in Fig. 2) by PAOs with acetate as carbon source (Kortstee et al., 2000). Some PAOs rely more on glycogen possibly, and reductive TCA cycle could consume excess reducing equivalents (Oehmen et al., 2010). As shown (Table 1), when P_{rel}/ VFA_{up} was lower (0.15–0.45) and Gly_{deg}/VFA_{up} (0.68–0.78) was

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Study	Gly _{deg} /VFA _{up} (mmol-C/mmol-C)	PHA _{syn} /VFA _{up} (mmol-C/mmol-C)	P _{rel} /VFA _{up} (mmol-P/mmol-C)	PHB (%)	PHV (%)	PH2MV (%
R1	0.68 ± 0.08	1.22 ± 0.09	0.36 ± 0.06	72.8 ± 2.39	25.5 ± 1.75	1.7 ± 0.74
R2	0.77 ± 0.06	1.14 ± 0.13	0.30 ± 0.07	71.5 ± 2.63	27.5 ± 1.84	1.2 ± 0.56
Pereira et al. (1996)	0.69	1.47	0.16	71.4	28.6	0
Winkler et al. (2011)	0.71	1.11	0.15	77.3	22.7	0
Liu et al. (1997)	0.78	1.47	0.45	75.8	20.0	<4.2
Yagci et al. (2003) ^b	0.51	1.20	0.91	88	7	5
Yagci et al. (2003) ^c	0.48	1.24	0.96	89	9	2
Smolders et al. (1994)	0.50	1.33	0.48~0.71	90	10	0
Lu et al. (2006)	0.46	1.26	0.62	94	6	0
Filipe et al. (2001)	0.53	1.30	0.57	88	12	0

Comparisons of stoichiometries and percentages of PHB, PHV and PH2MV in PHA between the static period and conventional anaerobic period (acetate as carbon source)^a.

^a Results of R1 and R2 are the averages and their standard deviations calculated from a long time operation.

^b Operated with COD/TP ratio (mg-COD/mg-P) of 11.4.

^c Operated with COD/TP ratio (mg-COD/mg-P) of 6.7.

Table 1



Fig. 2. The putative metabolic mechanism of PAOs in static phase.

higher, PHV/PHA was higher (20.0-28.6) (Liu et al., 1997; Pereira et al., 1996; Winkler et al., 2011). However, when P_{rel}/VFA_{up} was higher (0.48–0.96) and Gly_{deg}/VFA_{up} was lower (0.46–0.53), PHV/ PHA was lower (6-12). Therefore, it could be speculated that dominant PAOs in static phase relied more on glycolysis than on poly-P hydrolysis for energy, thus producing excessive reducing power, which, subsequently, was consumed to balance reductive potentials through reductive TCA cycle, and then propionyl-CoA was produced via succinate-propionate pathway. Contrasted to R2, lower Gly_{deg}/VFA_{up} and higher P_{rel}/VFA_{up} suggested more PAOs in R1, which was consistent with poly-P staining (more dark blue area in the sample from R1 than R2 through poly-P staining), resulting in relatively more poly-P hydrolysis than glycogen degradation. Additionally, the reason for higher PHA_{syn}/VFA_{up} in R1 than R2 might be that ordinary denitrifiers in R2 competed against PAOs for VFAs to reduce the remaining nitrate in static phase from the last cycle, leading to less carbon source available for PHA synthesis. The higher SOP release rate of R1 than R2 might be attributed to both more PAOs in R1 and inhibitory effect of remained nitrate in R2. Kuba et al. (1994) reported that the existing nitrate could inhibit anaerobic P release and thus EBPR activity, which only occurred after nitrate depletion (<1 mg/L). However, the inhibitory effect of remaining nitrate was limited, since only a small residue flowed into the next circulation.

3.2. Aerobic phosphorus uptake and simultaneous nitrification and denitrification (SND)

As shown (Table 2), glycogen was replenished with oxidation of PHA. The contents of glycogen regenerated and PHA oxidized were much higher than that reported in Coats et al. (2011) due to higher influent VFA loadings in R1 and R2. Conversely, lower glycogen synthesis per C mmol PHA oxidized may suggest more PAOs in the two reactors, resulting in more energy from PHA oxidation used for P uptake and poly-P storage in addition to glycogen replenishment. As noted, this ratio of R1 was lower than that of R2, indicating more PAOs proliferating in R1, consistent with the conclusions in static phase.

Aerobically, PAOs in R1, R2 took up SOP from wastewater rapidly, contributing to respective SOP of 0.72, 2.29 mg/L as aeration ceased although the SOP in R1, R2 was 50.10, 44.95 mg/L respectively when aeration was started up (Fig. 1). The SOP uptake rate of R1 was superior to that of R2 (6.65 vs 5.67 mg-P/(g-VSS h)), suggesting more dominant PAOs in R1 than R2. The reason for that was inhibitory effect of FNA probably, considering that the only discrepancy of test set between R1 and R2 lay in the difference of influent ammonia loadings, which would yield different FNA contents. FNA could impair ATP synthesis, enzyme expression and restrain activities of enzymes via nitric oxide (NO), product Table 2

Study	Aerobic period	d		Post-noxic period				
	Glycogen synthesized (mmol-C/g- VSS)	PHA oxidized (mmol-C/ g-VSS)	Glycogen synthesized/PHA oxidized (mmol-C/ mmol-C)	NO _x -N reduced (mg-N/g- VSS)	Glycogen required (mmol-C/g- VSS)	Glycogen utilized (mmol-C/g- VSS)	Glycogen required/ glycogen utilized (%)	Glycogen utilized/ NO _x -N reduced (mmol-C/mg-N)
R1	1.79 ± 0.14	1.66 ± 0.10	1.08 ± 0.06	1.85 ± 0.09	0.17 ± 0.01	0.92 ± 0.08	18.0 ± 0.93	0.50 ± 0.07
R2	1.89 ± 0.17	1.61 ± 0.12	1.17 ± 0.09	3.10 ± 0.14	0.26 ± 0.02	0.85 ± 0.07	30.3 ± 1.42	0.27 ± 0.04
Winkler et al. (2011)	-	-	-	0.32	0.03	0.17	17.0	0.54
Coats et al. (2011)	0.67	0.42	1.60	1.80	0.16	0.55	29.3	0.31

Aerobic metabolic	transformation	and interna	L carbon required	hand consumed	for post-denitrification	n in one cycle ^a
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^a Data of R1 and R2 are the averages and corresponding standard deviations calculated from a long time monitoring.

of FNA reduction, thus affecting the metabolism of PAOs (Zhou et al., 2011). FNA could generate from nitrite accumulated in aerobic phase and would have inhibitory effect on aerobic P uptake of PAOs (Pijuan et al., 2010; Saito et al., 2004). The FNA concentration was calculated using the formula $S_{N-NO_2}/(K_{\alpha} \times 10^{\text{pH}})$, with the K_{α} value determined using the formula $K_{\alpha} = e^{(-2300/(T + 273))}$ for a given temperature $T(^{\circ}C)$ (Anthonisen et al., 1976). The fluctuation of FNA during the whole cycle in R1 and R2 was illustrated in Fig. 3. Saito et al. (2004) concluded that 0.5×10^{-3} mg-HNO₂-N/L FNA could affect aerobic P uptake of PAOs severely, and Pijuan et al. (2010) found that 50% inhibition (IC₅₀) of the activity would be imposed on PAOs for aerobic P uptake when FNA reached 0.52×10^{-3} mg-HNO2-N/L. Fig. 3 shows that FNA fluctuated from 0.25 to 0.42×10^{-3} mg-HNO₂-N/L in the aerobic stage of R2 from 2nd h to 3rd h while FNA varied from 0.03 to 0.16×10^{-3} mg-HNO₂-N/L in R1, which indicated that PAOs in R2 suffered more inhibitory effect from FNA due to higher influent ammonia concentration.

Ammonia was oxidized to nitrate and/or nitrite with the aeration proceeding. As shown (Fig. 1), the amount of ammonia nitrogen reduced was much more than that of NO_{x}^{-} (nitrite + nitrate) produced. therefore simultaneous nitrification-denitrification (SND) was considered. In aerobic period, ammonia nitrogen would be mainly transformed to three forms: NO₂⁻ N₂ via SND and assimilated nitrogen (assimilated N), of which assimilated N was calculated according to cell formula (C₅H₇NO₂), where N percentage is accounted for 12% (Bruce and Perry, 2001). The sludge discharged was equivalent to the regenerated biomass in each cycle since the whole biomass in both systems maintained balanced, and the assimilated N in each cycle could be calculated in accordance with the discharged mixed liquor (30 mL), MLVSS and N percentage in cell formula. N₂ formed through simultaneous nitrification and denitrification was obtained by taking into account deduction of NO_x^- formed and assimilated N from ammonia nitrogen reduced. As summarized in Table 3, N removal through SND was 2.05 and 4.91 mg/L in R1, R2, corresponding to 14.7% and 17.8% TN removal,



Fig. 3. Variation of FNA during the whole cycle of R1, R2.

respectively. The simultaneous nitrification-denitrification rates (SNDRs) were respective 0.27, 0.65 mg-N/(g-VSS h) in R1, R2. The diffusional limitation of oxygen in the sludge floc may explain the SND. Satoh et al. (2003) found that nitrification and denitrification could occur in different floc zones synchronously. Possibly there existed aerobic denitrifying bacteria. Noted that the concentrations of nitrite in both R1 and R2 reached maximum in the middle stages of aeration, which is consistent with that reported by Winkler et al. (2011). It was difficult to explicate that nitrite was further oxidized to nitrate or directly reduced to N₂ or both, which was a complicated process involved with ammonia oxidation bacterias (AOBs), nitrite oxidation bacterias (NOBs) and denitrifiers. AOBs and NOBs are primarily selected by concentrations of oxygen, ammonia, and nitrite, respectively, but factors affecting competitions of such interspecies as Nitrosomonas oligotropha and Nitrosospira are not clear (Nielsen et al., 2010).

3.3. Post-anoxic denitrification in two reactors

Fig. 1 shows that the concentrations of glycogen declined as the NO⁻_v concentrations decreased and that post-anoxic denitrification slowed down with the consumption of glycogen during anoxic period. Especially in the bottom half of the anoxic stirring, NO_v⁻ reduction was hardly driven with the balance of glycogen contents. Moreover, PHA stored in static phase was nearly balanced after 2.5 h aeration. Therefore, it could be proposed that glycogen, regenerated in aerobic phase, was later consumed for denitrification, consistent with that reported by Coats et al. (2011) and Winkler et al. (2011). In order to assess the availabilities of glycogen for post-denitrification, the utilization of glycogen for NO_v⁻ reduction was summarized in Table 2. 2.86 mg-COD/mg-NO₃⁻-N and no cells growth was assumed according to Winkler et al. (2011), and 1.14 mg-COD/mg-NO₂⁻-N was calculated. As described in Table 2, compared to glycogen required for NO_x⁻ reduction, less than half of glycogen was consumed to drive denitrification. The comparable NO_{x}^{-} was reduced in R1 and Coats et al. (2011) but the glycogen utilized in Coats et al. (2011) was nearly half that in R1. Note that the higher ratio of glycogen to NO_v⁻-N reduced, the less percentage of glycogen actually used for denitrification, since additional glycolysis was needed for energy to maintain and survive. Theoretically, denitrification was employed for energy supply. Lower NO_x⁻ reduction per g VSS provided insufficient energy, therefore more extra glycogen hydrolysis would be needed for survival and then lower percentage of glycogen for denitrification was calculated, which, however, could not explain the comparable percentage of glycogen for denitrification with 1.85 mg-NO_v⁻-N/g-VSS reduction in R1 compared to $0.32 \text{ mg-NO}_x^- \text{-N/g-VSS}$ quoted from Winkler et al. (2011), suggesting that denitrification was driven by complicated consortia with various denitrification capabilities under different environmental conditions, thus leading to distinctive results.

As shown (Table 4), the denitrification rates of R1, R2 were 0.92, 0.98 mg-N/(g-VSS h) (corrected to 20 °C, DNR₂₀) respectively in

Table 3						
Simultaneous	nitrification	and	denitrification	in	aerobic	phase ^a .

Reactor	MLVSS (mg/L)	Nitrogen distributio	n (mg/L)		SNDR (mg-N/(g-VSS h))	SND/TN (%)	
		Oxidized NH ₄ ⁺ -N	Formed NO _x ⁻ -N Assimilated N SND		SND		
R1 R2	2970 ± 29 3007 ± 23	13.47 ± 1.04 26.83 ± 1.55	5.50 ± 0.63 15.96 ± 1.27	5.94 ± 0.06 6.01 ± 0.05	2.05 ± 0.36 4.91 ± 0.58	0.27 ± 0.05 0.65 ± 0.08	14.7 ± 0.71 17.8 ± 0.87

^a Mean values of R1 and R2 are presented with standard deviations calculated from a long time monitoring.

Table 4

P uptake (release) rates and denitrification rates in anoxic stages of R1 and R2^a.

Reactor	Effluent SOP (mg/L)	SOP uptake (release) rate (mg-P/(g-VSS h))	Effluent nitrogen (mg/L)		Post-DNR(mg-N/(g-VSS h))			
			NH ₄ ⁺ -N	NO_2^N	NO_3^N	T (°C)	DNR	DNR ₂₀
R1 R2	3.98 ± 0.63 0.91 ± 0.06	(0.62 ± 0.05) 0.18 ± 0.02	0.57 ± 0.04 0.58 ± 0.07	0 0	0 6.03 ± 0.79	24.5 ± 0.6 24.5 ± 0.6	1.03 ± 0.08 1.10 ± 0.10	0.92 ± 0.06 0.98 ± 0.07

^a Results reported are the averages and corresponding standard deviations calculated from a long time monitoring.

excess of typical endogenous decay rates (0.2-0.6 mg-N/(g-VSS h) reported by Kujawa and Klapwijk, 1999). Post-denitrification rates reported by other researchers were 0.31-0.95 (Winkler et al., 2011) and 0.67-0.88 mg-N/(g-VSS h) (Coats et al., 2011) (both corrected to 20 °C), both of which were lower than that in the present experiments. The reason might be more VFA taken up for PHA synthesis and thus more glycogen regenerated in aerobic periods of R1 and R2 (Table 2). The DNR of 2.2 mg-N/(g-VSS h) was found in the post-anoxic reactors (Vocks et al., 2005), where influent COD was up to 834 mg/L, much higher than in our reactors. Glycogen, driver of denitrification, was not depleted post-anoxically and remained steady before anoxic stirring ceased despite of remaining nitrate in R2, leading to halt of denitrification, which suggested that the anoxic phase was seemingly too long due to that glycogen in the bottom half of anoxic phase became nearly stable and denitrification tended to halt. This phenomenon may be attributed to the consortia's inability to completely utilize glycogen reserves. Lopez et al. (2006) and Lu et al. (2007) collectively observed that full depletion of glycogen did not occur even during starvation tests that lasted days. However, the very incomplete utilization of glycogen pools in anoxic period guaranteed sufficient glycolysis in static phase of next cycle for reducing equivalents or EBPR performance would deteriorate.

Denitrification is generally considered a 4-step process $(NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2)$. Reduction from nitrate to nitrite and reduction from nitrite to N₂ are often discussed in terms of denitrifying capabilities of denitrifiers. The denitrifiers in the EBPR plants belong to Betaproteobacteria and Gammaproteobacteria in the genera Thauera, Azoarcus, Zoogloea, Curvibacter, Accumulibacter and Competibacter, etc. (Nielsen et al., 2010), and different denitrifiers possessed different denitrifying capabilities. Type I Accumulibacter was capable of nitrite and nitrate reduction, but type II Accumulibacter was only able to reduce nitrite (Oehmen et al., 2010). Regarding to GAOs, Kong et al. (2006) and Burow et al. (2007) concluded that Competibacter sub-group 6 was capable of nitrate and nitrite reduction, while sub-groups 1, 4 and 5 were able to reduce nitrate only and Competibacter sub-groups 3 and 7 as well as Defluviicoccus Cluster II were unable to denitrify. Additionally, reduction abilities of these microorganisms were impacted by such environmental conditions as temperature, pH, carbon source, etc. Therefore, the evolution of nitrite was influenced by complex factors. Nitrite was firstly increased and subsequently declined in anoxic phase (Carvalho et al., 2007; Winkler et al., 2011). However, continuous decline of nitrite was found in anoxic period of R2, which suggested that the first-step reduction rate was lower than that of the second step but which specific microorganisms involved in the first or/and second step required further identification. Denitrifying PAOs was responsible for partial NO_x^- reduction in R2 because NO_x^- was reduced with P uptake. In anoxic stage of R1, however, post-denitrification was detected without P elimilation, consistent with the phenomena reported by Vocks et al. (2005), who supposed that the denitrification could not be linked directly to denitrifying PAOs and another species of bacteria was responsible for the observed phenomena, and this species could possibly be part of the PAO-group. Additionally, some sub-groups of *Competibacter* could participate in NO_x^- reduction of R1 and R2 (Burow et al., 2007; Kong et al., 2006).

3.4. Secondary P release and denitrifying P uptake

In the present research secondary P release was determined in R1 due to the depletion of NO_{x}^{-} before anoxic stirring ceased, resulting in 3.98 mg/L effluent SOP and 67.6% removal rate despite the SOP as low as 0.72 mg/L at the end of aeration. In R2, however, P uptake was encountered in anoxic period when NO⁻ was gradually reduced during the whole anoxic period, decreasing SOP from 2.29 mg/L at the beginning of stirring to 0.91 mg/L of SOP in the effluent, i.e. simultaneous N and P removal in anoxic phase. It is speculated that SOP was assimilated by the DNPAOs using NO_v as the electron acceptor, and denitrifying phosphorous removal occurred in the anoxic zone (Xu et al., 2011). Secondary P release occurred due to NO_{y}^{-} depletion in anoxic phase of R1 with the release rate of 0.62 mg-P/(g-VSS h) (Table 4), which was significantly lower than that in other reports (2-5 mg-P/(g-VSS h)) (Oehmen et al., 2005; Smolders et al., 1995; Wentzel et al., 1989). The reason was probably that PAOs also relied on glycolysis except poly-P hydrolysis for energy without external carbon source addition in anoxic phase because glycogen declined slightly anoxically with the secondary P release in R1 (Fig. 1), although substantial poly-P stained by DAPI could be available. The denitrifying P removal rate was 0.18 mg-P/(g-VSS h), substantial lower than that reported by Carvalho et al. (2007) (8.37 mg-P/(g-VSS h)). This discrepancy could not be solely attributed to the small amount of SOP entering the anoxic period adversely influencing denitrifying P uptake, because the location of anoxic stage was different. The higher denitrifying P uptake rates mentioned above were achieved through pre-anoxic stirring but the experiments herein were post-anoxic. One of advantages of the former was that the sufficient PHA with high reductive potential synthesized in anaerobic phase could be utilized as electronic donator to drive denitrifying P elimination directly. Nonetheless, relatively less internal carbon source was remained available in post-anoxic phase for denitrification since PHA in aerobic phase was used for P uptake, microorganism



Fig. 4. Biological nutrients removal during stage 1, 2 of the two reactors.

growth and then glycogen replenishment. The contents of PHA leveled off in anoxic period of R1 and R2, suggesting PHA did not participate in post-denitrification. Thus smaller amounts of glycogen replenished aerobically were involved in post-denitrification. Therefore, denitrifying P uptake was driven slowly and incompletely. Especially when glycogen inclined to stable after 1.5 h stirring, denitrifying P removal ceased, consistent with NO_x⁻ reduction performance with the same reason. As noted, the corresponding pre-denitrification rate measured by Carvalho et al. (2007) above was 6.3 mg-N/(g-VSS h), much higher over that in this post-anoxic configuration. Regardless, the post-denitrification rates were enough to reduce NO_x⁻ to meet discharge requirements. Therefore, post-anoxic denitrification was advantageous over pre-anoxic denitrification because mixed liquor recycle was eliminated in the former format.

3.5. BNR performance of two reactors with shortened anoxic phase

Since secondary P release was attributed to NO_x^- depletion, could secondary P release be avoided if stopping anoxic stirring before NO_x^- was completely reduced in R1? Actually, the concentrations of TN, SOP were both very low with 1.5 h anoxic stirring in R2, and glycogen pools in the bottom half of anoxic stirring tended to steady, contributing to little P and N elimination. Therefore, duration of anoxic period of the two reactors was shortened to 1.5 h, but the static and aerobic periods were still conducted for 1 and 2.5 h, respectively. The two reactors was both operated for another 100 days (i.e. 121–220 days, equivalent to 5 SRTs), named as stage 2, and the initial run (1–120 days) was stage 1. The nutrients removal performances of the two reactors during stage 1 and 2 were described in Fig. 4.

As shown (Fig. 4), in stage 2 both reactors obtained excellent P and N removal efficiencies. The SOP effluent in R1 was as low as 0.72 mg/L, which suggested that secondary P release could be avoided until the remained nitrate was 0.92 mg/L at the end of stirring. The SOP effluent in R2 was 0.96 mg/L, comparable to 0.91 mg/L in stage 1 of R2 due to the nearly half of denitrifying P uptake in the bottom half of anoxic period. The effluent TN of R2 in stage 2 was 7.81 mg/L, a little higher than that in stage 1 (6.61 mg/L), corresponding to 80.7% TN removal rate. The results indicated that 1.5 h stirring was suitable for P and N removal performance. Actually, 3 h anoxic phase was operated for better insight into effects of influent ammonia loads on P removal and further interrogation of evolutions of internal carbon sources (i.e. PHA and glycogen) driving denitrification and denitrifying P uptake in post-anoxic period.

4. Conclusion

The investigation focused on understanding of a novel post-anoxic BNR process. Excellent nutrient removal could be achieved in this configuration. P release in static phase, aerobic P uptake, secondary P release or denitrifying P uptake in anoxic phase were impacted by influent ammonia loadings. SND was found in R1, R2. Post-denitrification was driven by glycogen, and denitrifying P uptake was found in R2. Anoxic duration was shortened from 3 to 1.5 h because secondary P release occurred due to NO_x^- depletion in R1 and denitrification nearly ceased in the bottom half of anoxic stirring in R2.

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References

- Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Shinath, E.G., 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Pollut. Control Fed. 48, 835– 852.
- APHA, 1998. Standard Methods for the Examination of Water and Wastewater. American Public Health Association.
- Arun, V., Mino, T., Matsuo, T., 1988. Biological mechanism of acetate uptake mediated by carbohydrate consumption in excess phosphorus removal systems. Water Res. 22, 565–570.
- Bracklow, U., Drews, A., Gnirss, R., Klamm, S., Lesjean, B., Stüber, J., Barjenbruch, M., Kraume, M., 2010. Influence of sludge loadings and types of substrates on nutrients removal in MBRs. Desalination 250, 734–739.
- Bruce, E.R., Perry, L.M., 2001. Environmental biotechnology: principles and applications[M]. Metcalf and Eddy, Inc. McGraw-Hill, New York.
- Burow, L.C., Kong, Y.H., Nielsen, J.L., Blackall, L.L., Nielsen, P.H., 2007. Abundance and ecophysiology of *Defluviicoccus* spp., glycogen accumulating organisms in fullscale wastewater treatment processes. Microbiology-SGM 153, 178–185.
- Carvalho, G., Lemos, P.C., Oehmen, A., Reis, M.A.M., 2007. Denitrifying phosphorus removal: linking the process performance with the microbial community structure. Water Res. 41, 4383–4396.
- Coats, E.R., Mockos, A., Loge, F.J., 2011. Post-anoxic denitrification driven by PHA and glycogen within enhanced biological phosphorus removal. Bioresour. Technol. 102, 1019–1027.
- Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001. Stoichiometry and kinetics of acetate uptake under anaerobic conditions by an enriched culture of phosphorus-accumulating organisms at different pHs. Biotechnol. Bioeng. 76, 32–43.
- Kong, Y.H., Xia, Y., Nielsen, J.L., Nielsen, P.H., 2006. Ecophysiology of a group of uncultured Gammaproteo bacterial glycogen-accumulating organisms in fullscale enhanced biological phosphorus removal wastewater treatment plants. Environ. Microbiol. 8, 479–489.
- Kortstee, G.J.J., Appeldoorn, K.J., Bonting, C.F.C., van Niel, E.W.J., van Veen, H.W., 2000. Recent developments in the biochemistry and ecology of enhanced biological phosphorus removal. Biochemistry (Moscow) 65, 332–340.

- Kuba, T., Wachtmeister, A., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Effect of nitrate on phosphorus release in biological phosphorus removal systems. Water Sci. Technol. 30, 263–269.
- Kujawa, K., Klapwijk, B., 1999. A method to estimate denitrification potential for predenitrification systems using NUR batch test. Water Res. 33, 2291– 2300.
- Liu, W.T., Nakamura, K., Matsuo, T., Mino, T., 1997. Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors-effect of P/C feeding ratio. Water Res. 31, 1430–1438.
- Liu, W.T., Nielsen, A.T., Wu, J.H., Tsai, C.S., Matsuo, Y., Molin, S., 2001. In situ identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. Environ. Microbiol. 3, 110–122.
- Lopez, C., Pons, M.N., Morgenroth, E., 2006. Endogenous processes during long-term starvation in activated sludge performing enhanced biological phosphorus removal. Water Res. 40, 1519–1530.
- Lu, H., Keller, J., Yuan, Z., 2007. Endogenous metabolism of Candidatus Accumulibacter phosphatis under various starvation conditions. Water Res. 41, 4646–4656.
- Lu, H., Oehmen, A., Virdis, B., Keller, J., Yuan, Z.G., 2006. Obtaining highly enriched cultures of *Candidatus Accumulibacter phosphatis* through alternating carbon sources. Water Res. 40, 3838–3848.
- Montil, A., Hall, E.R., van Loosdrecht, M.C.M., 2005. Kinetics of phosphorus release and uptake in a membrane-assisted biological phosphorus removal process. J. Environ. Eng. 133, 889–908.
- Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y.H., Aviaja, H.A., Vollertsen, J., 2010. A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. Water Res. 44, 5070–5088.
- Oehmen, A., Carvalho, G., Lopez-Vazquez, C.M., van Loosdrecht, M.C.M., Reis, M.A.M., 2010. Incorporating microbial ecology into the metabolic modeling of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Res. 44, 4992–5004.
- Oehmen, A., Zeng, R.J., Yuan, Z.G., Keller, J., 2005. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnol. Bioeng. 91, 43–53.

- Pereira, H., Lemas, P.C., Reis, M.A.M., Crespo, J.P.S.G., Carrondo, M.J.T., Santos, H., 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo C-NMR labelling experiments. Water Res. 30, 2128–2138.
- Pijuan, M., Ye, L., Yuan, Z.G., 2010. Free nitrous acid inhibition on the aerobic metabolism of poly-phosphate accumulating organisms. Water Res. 44, 6063– 6072.
- Saito, T., Brdjanovic, D., van Loosdrecht, M.C.M., 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. Water Res. 38, 3760–3768.
- Satoh, H., Nakamura, Y., Ono, H., Okabe, S., 2003. Effect of oxygen concentration on nitrification and denitrification in single activated sludge flocs. Biotechnol. Bioeng. 83, 604–607.
- Smolders, G.J.F., Vandermeij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal processstoichiometry and pH influence. Biotechnol. Bioeng. 43, 461–470.
- Smolders, G.J.F., Vandermeij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1995. A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol. Bioeng. 47, 277–287.
- Vocks, M., Adama, C., Lesjean, B., Gnirss, R., Kraume, M., 2005. Enhanced postdenitrification without addition of an external carbon source in membrane bioreactors. Water Res. 39, 3360–3368.
- Wang, D.B., Li, X.M., Yang, Q., Zeng, G.M., Liao, D.X., Zhang, J., 2008. Biological phosphorus removal in sequencing batch reactor with single-stage oxic process. Bioresour. Technol. 99, 5466–5473.
- Wentzel, M.C., Dold, P.L., Ekama, G.A., Marais, G.V.R., 1989. Enhanced polyphosphate organism cultures in activated sludge. Part III: Kinetic model. Water SA 15, 89–102.
- Winkler, M., Coats, E.R., Brinkman, C.K., 2011. Advancing post-anoxic denitrification for biological nutrient removal. Water Res. 45, 6119–6130.
- Xu, X.Y., Liu, G., Zhu, L., 2011. Enhanced denitrifying phosphorous removal in a novel anaerobic/aerobic/anoxic (AOA) process with the diversion of internal carbon source. Bioresour. Technol. 102, 10340–10345.
- Yagci, N., Artan, N., Cokg ör, E.U., Randall, C.W., Orhon, D., 2003. Metabolic model for acetate uptake by a mixed culture of phosphate- and glycogen-accumulating organisms under anaerobic conditions. Biotechnol. Bioeng. 84, 359–373.
- Zhou, Y., Oehmen, A., Lim, M., Vadivelu, V., Ng, W.J., 2011. The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. Water Res. 45, 4672–4682.