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Improved biological phosphorus removal induced by an oxic/extended-idle process using glycerol and acetate at equal fractions†

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The Oxic/Extended-Idle (O/EI) regime is a promising technology for biological phosphorus removal (BPR) from wastewater, but the BPR efficiency might be affected by an insufficient amount of carbon source in raw wastewater. In this study, a novel strategy *i.e.*, using acetate and glycerol at equal fractions as the carbon source, to simultaneously significantly improve BPR efficiency and reduce biomass waste of glycerol was reported. Experimental results showed that BPR efficiency could be enhanced when glycerol was not a dominant substrate, and the best BPR efficiency was 96.6 ± 1.2% when acetate and glycerol were at equal fractions. However, deterioration of BPR was observed when glycerol was the dominant substrate, and the worst BPR efficiency was 58.1 ± 1.8% when glycerol served as the sole carbon source. Fluorescence *in situ* hybridization analysis demonstrated that more polyphosphate accumulating organisms but less glycogen accumulating organisms were cultured in the activated sludge using acetate and glycerol at equal fractions. Further mechanism investigations revealed that the transformations of polyhydroxyalkanoates and glycogen, and the activities of key enzymes responsible for P removal (such as exopolyphosphatase and polyphosphate kinase) were all affected by the ratio of acetate to glycerol. In addition, the BPR performances between O/EI reactors and the classical anaerobic/oxic (A/O) reactors employing acetate and glycerol at equal fractions and solely glycerol were compared, the results showed that the Gly-fed O/EI reactor could drive better BPR performances than the Gly-fed A/O reactor. These results suggested that glycerol at moderate levels improved BPR, and waste glycerol could be an economical sustainable alternative to avoid carbon source deficiency in raw wastewater.

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1. Introduction

Excess phosphorus (P) in effluent is a critical factor for eutrophication of water bodies and should be eliminated to keep the ecological balance. The enhanced biological phosphorus removal (EBPR) process is considered to be the most economical and environmentally friendly treatment technology for net-P removal.[1–3] EBPR can be achieved *via* an activated sludge process by alternating anaerobic and oxic (A/O) conditions, by which polyphosphate accumulating organisms (PAOs), the microorganisms responsible for biological phosphorus removal (BPR), can be accumulated under such conditions. PAOs take up carbon sources under anaerobic conditions and store them as poly-β-hydroxyalkanoates (PHAs) by decomposing the intracellular stored polyphosphate (poly-P) and glycogen for energy and reducing power, respectively.[4] Under the subsequent oxic conditions, PHAs are utilized to produce energy for luxury P uptake, cell growth, glycogen replenishment and poly-P accumulation, thus, EBPR could be achieved by wasting P-rich sludge at the end of the oxic phase.[5] Generally, the excellent performance of EBPR could be achieved *via* an A/O process if operated successfully. However, EBPR performance is prone to accidental and unpredictable upsets due to external disturbances such as high rainfall, excessive nitrate entering into the next anaerobic phase and so on. In addition, EBPR highly depends on the presence of preferred readily biodegradable organic substrates (*e.g.*, volatile fatty acids, VFAs).[6] However, the amounts of available VFAs are always insufficient in the raw wastewater especially in the south of China, and external additions of VFAs are required to avoid the deterioration of EBPR in real wastewater treatment plants (WWTPs). Considering the substantial amounts of wastewater treated daily, therefore, any improvement in EBPR technologies might bring tangible economic and environmentally friendly consequences.

Recently, a novel biological phosphorus removal (BPR) technique (*i.e.*, oxic/extended idle regime, O/EI) is reported to

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achieve desirable and stable BPR performances but with different inducing mechanisms.[3,7] Anaerobic period is canceled in O/EI regime but the idle phase is extended suitably (*e.g.*, 210–450 min) compared with the classical A/O regime. The innovated O/EI regime was demonstrated to enrich PAOs mainly *via* some specific metabolic bio-process (*e.g.* substantial quantities of idle phosphorus release and a low idle production of PHAs) occurred during the extended-idle phase, and the O/EI regime opens new possibilities for phosphorus removal and seems to have a promising future in practical application.[2,8,9]

It is well known that the efficiencies of EBPR strongly depend on the available VFAs that play the electron donor role.[10,11] It was reported that 6–9 mg VFA was required to remove 1 mg phosphorus.[12] Whereas, the levels of readily biodegradable carbon sources in the raw wastewater and high-tech industrial wastewater are usually insufficient, in which the ratio of carbon to phosphorus (C/P) is always much lower than the suitable value.[13,14] Additionally, the VFAs for BPR are also consumed by other organisms such as glycogen accumulating organisms (GAOs), which are considered as the competitors of PAOs and do not contribute to P removal.[15,16] Addition of suitable external carbon source is often considered as an efficient approach to solve this dilemma. In this way, VFAs (such as acetate, propionate, butyrate, valerate) and glucose serving as supplemental carbon source have been well tested to avoid EBPR deterioration.[3,14] However, the additions of those chemicals substantially increase the overall wastewater treatment costs. Hence, the choice of additional suitable external carbon must take both the EBPR performance and economical alternative condition into consideration for long-term operation and great quantities of wastewater treated daily in real WWTPs. Thus, the utilization of some waste materials which could be easily converted to degradable carbon is considered as a promising alternative in future.

Glycerol is inevitably produced with great amounts from biodiesel fuel production, and it is reported that 1 L of glycerol is produced per 10 L of biodiesel fuel.[17] Excess glycerol may become an environmental issue with associated disposal costs.[18] The substantial amounts of crude glycerol generated in the biodiesel industry would cause significant environmental issues if disposed improperly, and recently the effective utilization of waste glycerol as a renewable and low-cost feedstock has drawn much attention. It is documented that denitrification could be well driven by glycerol as a proper external carbon.[19,20] As for EBPR process, Yuan *et al.* showed although glycerol was substituted for acetate-fed A/O regime, the EBPR process deteriorated with phosphorus removal efficiency about 30%.[21] On the contrary, Guerrero *et al.* observed that glycerol-driven EBPR with a single-sludge A/O sequencing batch reactors (SBR) configuration is feasible if the anaerobic hydraulic retention time (HRT) is suitable.[22] However, till now, the feasibility of glycerol serving as additional carbon for enhanced biological phosphorus removal especially in O/EI regime has never been reported since the inducing mechanisms of O/EI were quite different from those in the conventional A/O regime, which makes the promising technology with blind area. In addition, the performances of BPR between the O/EI regime and classical A/O regime using the mixed glycerol and acetate have never been compared.

Therefore, the aim of this study is to evaluate the feasibility of glycerol serving as carbon source for BPR induced by O/EI regime and to investigate the mechanisms for the improved BPR at equal fraction of acetate and glycerol. First, performances of BPR in laboratory-scale O/EI regime fed with different ratios of acetate to glycerol were compared. As satisfactory BPR performance was achieved when acetate and glycerol were at equal fraction, then, the mechanisms for glycerol enhancing BPR were investigated by analysing the microbiology, biochemical transformations of intracellular biochemical transformations (mainly PHAs and glycogen), and the activities of key enzymes responsible for BPR. Finally, EBPR performances between O/EI and A/O regimes with acetate and glycerol at equal fraction and sole glycerol were compared as well.

2. Materials and methods

2.1. Comparison of BPR performance in O/EI regime with different ratios of acetate to glycerol

This experiment was divided into six Runs (1–6) with two replicate laboratory-scale sequencing batch reactors (SBR) operated in parallel. Synthetic wastewater (see 2.3 for details) was used and prepared daily in this study. One reactor (R1) was fed with acetate only serving as control throughout the experiment, because acetate is the most common VFA in raw wastewater. The other reactor (R2) was fed with mixture of acetate and glycerol in different ratios serving as a test reactor. During Run 1 (day 0–30), R2 was also only fed with sole acetate to obtain continued stable and efficient EBPR performance, soluble orthophosphate (SOP) removal efficiency was above 89% and lasted for consecutive days. Run 2–6, a acetate and glycerol mixture (in terms of chemical oxygen demand, COD) of 90/10% (Run 2, days 31–60), 70/30% (Run 3, days 61–90), 50/50% (Run 4, days 91–120), 20/80% (Run 5, days 121–150), 0/100% (Run 6, days 151–180) as carbon source was serially added into R2. Other conditions were identical in R1 and R2 without additional illustration.

Both reactors with a working volume of 2.0 L each were inoculated with activated sludge taken from a WWTP in Changsha, PR China, and operated with three 8 h cycles daily. Each 8 h cycle consisted of: (i) an initial 5 min of filling; (ii) a 235 min oxic phase; (iii) a 30 min settling and decanting phase; (iv) a 210 min of idle phase. During the oxic phase, air was supplied by air pump at a flow rate of 2.5 L min^{-1}, the influent pH was adjusted to 7.0 $\pm$ 0.1 by manually adding 4.0 M hydrogen chloride or 4.0 M sodium hydroxide, while pH was not controlled constantly during the entire experimental process. The HRT and nominal cell residence time in the two reactors were maintained at approximately 14 h and 12 d, respectively. The temperature was maintained at 20 °C in a temperature-controlled room. It should be pointed out that the sludge was gently mixed with a magnetic stirrer (150 rpm) to facilitate sampling during the cyclic tests, but magnetic stirrer was not performed in the routine operation.

2.2. Comparison of EBPR performances between the O/EI and the A/O reactors with acetate and glycerol at equal fraction and complete glycerol

Because O/EI reactor achieved its best BPR performance when the feed contained acetate and glycerol at equal fraction (defined as Ace/gly-fed) and its performance deteriorated when the organic carbon source in the feed was completely prepared with glycerol (defined as Gly-fed). The operational conditions and inducing mechanisms of O/EI were quite different from those in conventional A/O reactor, thus it is necessary to compare the EBPR performances between O/EI and A/O reactors when the feed were the above mentioned carbon sources (*i.e.*, acetate and glycerol at equal fraction, and complete glycerol). This batch tests were performed in four replicate reactors (Ace/gly-fed O/EI reactor, Gly-fed O/EI reactor; Ace/gly-fed A/O reactor, Gly-fed A/O reactor) with working volumes of 2.0 L each. The operation of O/EI reactors was the same as that depicted in the 2.1 Section except for the type of carbon source. As comparison, the conventional A/O reactors were also operated under cyclical anaerobic (120 min)–oxic (180 min)–settling/decanting (60 min)–idle (120 min) conditions according to the literatures.[1,4] During the anaerobic phase, the sludge was gently mixed with a mechanical stirrer (150 rpm), during oxic phase, air was constantly supplied into reactors at a flow rate of 2.5 L min^{-1}. Synthetic wastewater was also used for this batch test and prepared daily. The HRT and nominal cell residence time in the four reactors were also controlled at approximately 14 h and 12 d, respectively. Temperature was maintained at 20 °C in a temperature-controlled room, and pH was on-line controlled consistently at pre-designed set-point (pH = 7.0 ± 0.1) by a programmable logic controller.

2.3. Synthetic wastewater

Synthetic wastewater was used throughout the investigation period. Total COD in influent was controlled at 300 mg L^{-1}, SOP concentration was maintained 15 mg L^{-1}, yielding theoretical influent 20 mg COD/(mg PO_4^{3-}–P), which was considered as being favorable for the growth of PAOs.[4] The other nutrient concentrations in the synthetic wastewater were described as below (per liter): 133.8 mg NH_4Cl, 1.0 mg $CaCl_2$, 1.0 mg $MgSO_4$, and 1.0 mL of a trace metals solution. The trace metals solution had been described in the literature.[21]

2.4. Chemical and microbial analyses

The liquor samples were taken with a syringe and immediately filtered through a Whatman GF/C glass microfiber filter (1.2 μm). The sludge samples were used to assay for mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solid (MLVSS), PHAs, and glycogen. The filtrate was used to measure the amount of COD, SOP, NH_4^+–N, and NO_x^-–N.

COD, SOP, NH_4^+–N, NO_x^-–N, MLSS and MLVSS were periodically measured according to procedures of detection in the literatures.[4,24] The analysis methods of the activities of key enzymes linked with BPR(such as exopolyphosphatase (PPX) and poly-phosphate kinase (PPK)) were the same as the description in the literature.[25] Sludge glycogen, poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV) and poly-3-hydroxy-2-methylvalerate (PH2MV) were measured according to the method detailed in Wang *et al.* (2012).[4] The total PHAs were calculated as the sum of the measured PHB, PHV and PH2MV.

Fluorescence *in situ* hybridization (FISH) study was conducted to quantify the abundances of PAOs and GAOs, and the measurements were the same as described in the literature.[2] Briefly, sludge samples were taken from reactors at the end of the oxic phase and fixed in 4% formaldehyde for 20 h at 4 °C and then subjected to freeze–thaw treatment to enhance the penetration of oligonucleotide probes. Cell samples were attached to poly-L-lysine coated slides and dehydrated with ethanol. The following hybridization and washing procedures were the same as that in the literature.[26] For quantitative analysis, 20 microscopic fields were analyzed for the hybridization of individual probes using a confocal scanning laser microscope (FV 500) with image database software (VideoTesT Album3.0). The oligonucleotide probes specific for PAOs, GAOs, and total bacteria, which were respectively labeled with 5′AMCA, 5′Cy3, and 5′FITC, were listed in Table S1 (ESI†).

3. Results and discussion

3.1. Comparison of the removal performances of COD and nitrogen among each Run in O/EI regime

The removal efficiencies of COD and nitrogen among each Run during steady operation were exhibited in Table 1. As shown in Table 1, low levels of NH_4^+–N, NO_2^-–N, COD but large amounts of NO_3^-–N were detected in effluent of each Run in O/EI reactor. The high concentrations of NO_3^-–N in effluent were ascribed to no specific anoxic period in O/EI regime. TN and COD removal efficiencies of each Run were respectively around 60% and 90%, suggesting TN and COD removal efficiencies were insensitive to the ratio of acetate to glycerol in feed.

3.2. Comparison of the removal performances of BPR among each Run in O/EI regime

Efficient and stable EBPR performance was quickly obtained during Run 1, the effluent SOP concentrations were in the range of 1.27–1.70 mg L^{-1}, and the corresponding phosphorus removal efficiencies were around 89–92%. Fig. 1 presents the profiles of the effluent SOP concentrations and their corresponding BPR efficiencies during Run 1–6. It was found that BPR efficiency slightly increased during Run 1–4, and it peaked at 97% during Run 4 (*i.e.*, 50% of the COD coming from glycerol), suggesting BPR was enhanced when the feed contained acetate and glycerol at equal fraction. However, further increase of the fraction of glycerol in feed from 50% to 100%, the efficiencies of BPR decreased from 97 ± 1.1% to 58 ± 1.3%, suggesting the percentage of glycerol exceed 50% posed an inhibitory impact on BPR. In recent A/O reactor studies, Yuan *et al.* (2010) also demonstrated that glycerol serving as the sole carbon in the feed resulted in the deterioration of EBPR process.[21] In a more recent study, Guerrero *et al.* (2012) showed

Table 1 Summary of the reactor performances of each run during stable operation in O/EI regime[a]

	COD		N			
	Effluent COD (mg L^{-1})	COD removal efficiency (%)	Effluent NH_4^+–N (mg L^{-1})	Effluent NO_2^-–N (mg L^{-1})	Effluent NO_3^-–N (mg L^{-1})	TN removal efficiency (%)
Run 1	24.5 ± 1.45	91.8 ± 2.4	4.23 ± 0.52	1.20 ± 0.17	7.48 ± 0.51	63.1 ± 0.51
Run 2	22.7 ± 2.31	92.4 ± 2.8	4.05 ± 0.56	1.15 ± 0.21	7.24 ± 0.48	64.5 ± 0.51
Run 3	21.2 ± 3.37	92.9 ± 3.1	3.84 ± 0.47	1.07 ± 0.24	7.16 ± 0.43	65.6 ± 0.51
Run 4	18.4 ± 1.23	93.8 ± 1.2	3.51 ± 0.59	0.92 ± 0.16	7.08 ± 0.42	67.2 ± 0.51
Run 5	26.4 ± 3.26	91.2 ± 1.5	4.86 ± 0.47	1.45 ± 0.27	8.09 ± 0.49	58.9 ± 0.51
Run 6	28.3 ± 2.15	90.6 ± 2.6	4.90 ± 0.43	1.59 ± 0.31	8.14 ± 0.47	58.2 ± 0.51

[a] Results are the averages and their standard deviations in triplicate tests.

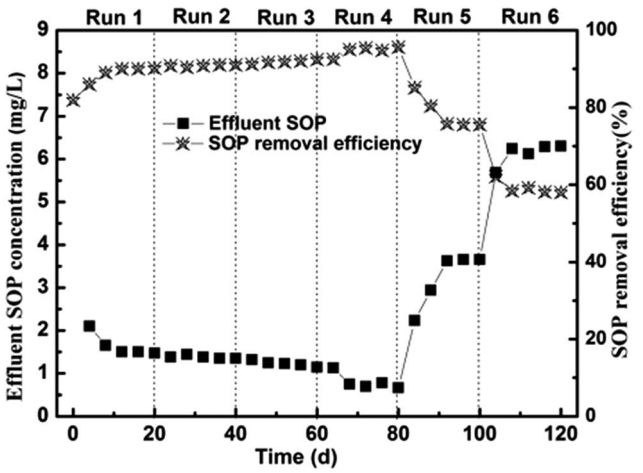

Fig. 1 Variations of effluent SOP concentration and SOP removal efficiency in O/EI reactor during long-term operation.

that the direct replacement of propionic acid by glycerol caused the loss of EBPR capability.[22] Those observations strongly indicated that the ratio of glycerol in feed was a key factor for BPR, and BPR was greatly improved when acetate and glycerol were at the equal fraction.

3.3. Variations of pH and dissolved oxygen (DO) during one cycle of each Run during stable operation

Previous studies have demonstrated that operational parameters (*e.g.*, pH and DO) could significantly affect the BPR performances.[27,28] Because pH and DO were not controlled constantly throughout experimental period. Therefore, it is necessary to verify that pH and DO were not significant variable factors affecting BPR performances in this study. The variations of pH and DO during each Run were periodically measured and shown in Fig. S1 (ESI†). It can be seen that the profiles of pH and DO during the Run 1–6 shown the similar trend, indicating pH and DO were not the affecting factors. For instance, during Run 4, DO remained almost unaltered during the initial 30 min though the air flow was at 2.5 L min^{-1} and then displayed a gradually increase trend and reached 5.2 mg L^{-1} at the end of oxic phase. During the subsequent extend-idle phase, DO decreased gradually to 0.5 mg L^{-1} during the initial 60 min, and then remained stable levels (0.1–0.3 mg L^{-1}) over the remainder idle phase. As for pH, pH increased sharply from 7.0 to 7.6 in the initial 30 min of oxic phase and then decreased slowly to 7.2 at the end of oxic phase, further dropped to the initial level of 7.0 during the extended idle phase. Those cyclic changes of pH and DO concentrations were similar to our previous observations in propionate-fed reactor.[3]

3.4. Mechanism of driving high BPR performance when feed contained acetate and glycerol at equal fraction

Results obtained in this study apparently showed that efficiency of BPR in O/EI reactor was greatly enhanced when the feed consisted of glycerol and acetate at equal fraction, however, BPR was deteriorated when the feed was complete glycerol. Because this is the first time to report BPR can be greatly improved when the carbon source included acetate and glycerol at equal fraction, therefore, it is necessary to explore the mechanisms for this phenomenon.

In general, the proliferation of GAOs was the primary reason for deterioration of EBPR system.[3,23] Therefore, FISH investigation was first conducted to quantify the abundances of PAOs and GAOs in the activated sludge and the quantitative analysis results were summarized in Table 2. As seen from Table 2, PAOs were the dominate microorganisms during Run 1–4, peaking at

Table 2 Bacterial populations and activities of PPX and PPK of each steady Run in O/EI reactor[a]

	Bacterial population[b]		Enzyme activities	
	PAO mix (%)	GAO mix (%)	PPX[c]	PPK[d]
Run 1	32 ± 3	14 ± 2	0.018 ± 0.004	0.216 ± 0.003
Run 2	35 ± 1	12 ± 2	0.021 ± 0.003	0.254 ± 0.004
Run 3	36 ± 4	11 ± 3	0.025 ± 0.002	0.261 ± 0.002
Run 4	40 ± 2	10 ± 3	0.032 ± 0.003	0.283 ± 0.005
Run 5	25 ± 2	19 ± 2	0.014 ± 0.002	0.164 ± 0.004
Run 6	18 ± 4	25 ± 3	0.007 ± 0.003	0.123 ± 0.002

[a] Results are the averages and their standard deviations in triplicate tests. [b] Percentage to all bacteria (EUB mix probe). [c] The unit is μmol *p*-nitrophenol per (min mg protein). [d] The unit is μmol NADPH per (min mg protein).

40 ± 2% during Run 4, whereby, GAOs were the predominant microorganisms during Run 6, accounting for 25 ± 3%. Hence, it can easily be understood that excellent BPR achieved during Run 4 whereas deterioration of BPR was achieved during Run 6. Now, another question was put forward that why the O/EI reactor could culture more PAOs during Run 4 when the fed was equally mixed acetate and glycerol?

It is well known that EBPR is closely related to the transformations of intracellular PHAs and glycogen, which are generally considered to be associated with the activities of PAOs and GAOs in the activated sludge. Variations of intracellular polymers can provide an indication to better understand the impact of different ratios of acetate to glycerol on the competition between PAOs and GAOs. The variations of intracellular

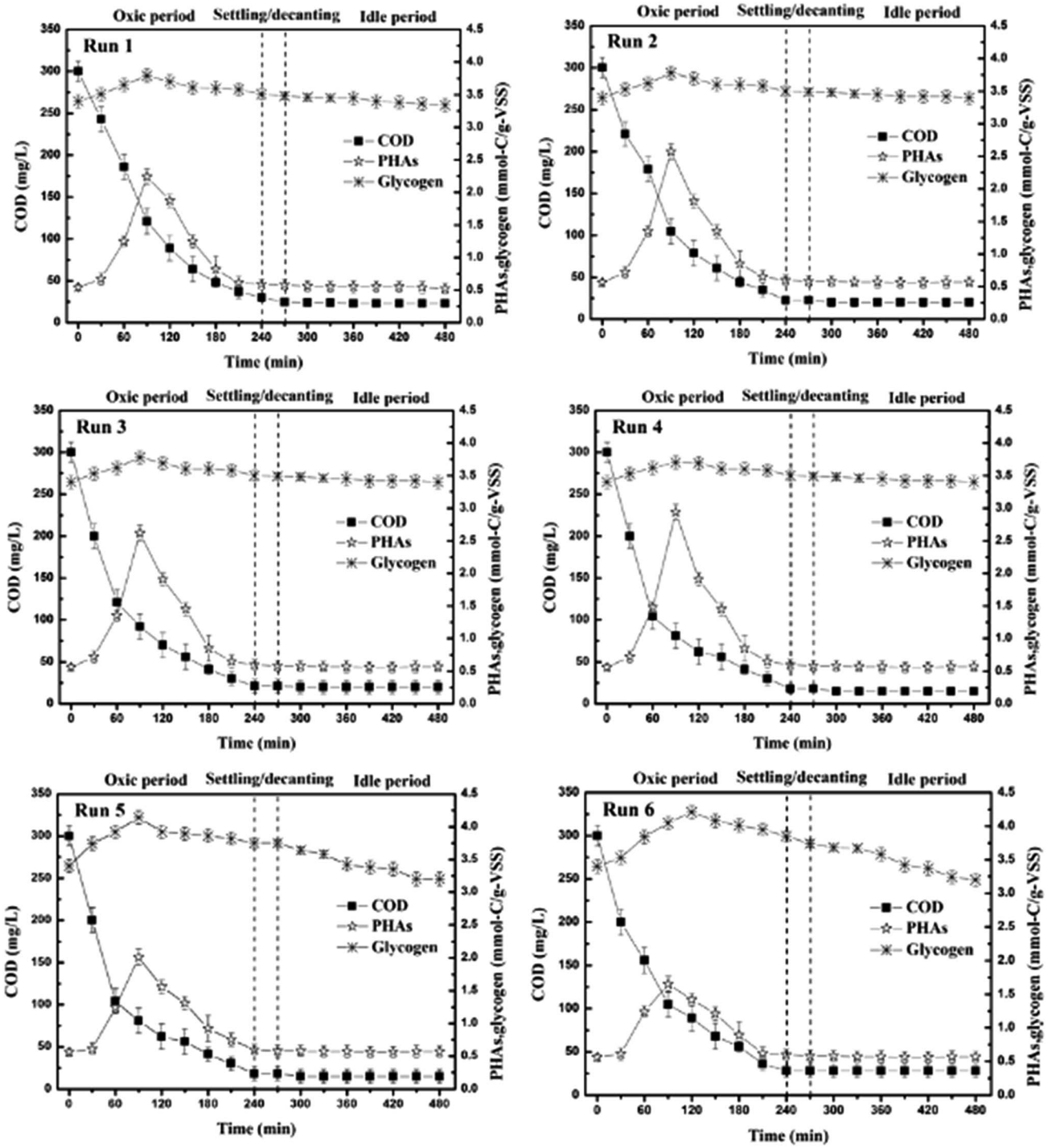

Fig. 2 Profiles of COD and intercellular polymers variations in one cycle during steady operation. Error bars represent standard deviations of triplicate tests.

polymers (*e.g.*, PHAs and glycogen) and COD during one typical cycle of each Run are illustrated in Fig. 2. It can be found that COD was rapidly consumed during the initial 90–120 min of oxic phase accompanied by substantial accumulations of PHAs and glycogen. After COD was depleted, oxidation of PHAs and luxury uptake of SOP occurred concurrently, and at the end of oxic phase, the contents of PHAs and glycogen came back to their initial levels. During the extended idle phase, COD remained almost unaltered, little amount of PHAs synthesis and glycogen degradation took place, simultaneously. Those changes were consistent with previous publications.[3,5] Apart from those similar observations among each Run, big differences that higher levels of intracellular PHAs synthesis but lower endogenous glycogen formation were observed during Run 4 compared with those in other Runs. It was reported that high transformation of endogenous glycogen indicated high activities of GAOs, because glycogen was the main energy storage material.[6] The low glycogen formation during Run 4 strongly suggested that few GAOs were cultured in the activated sludge, which was well in accordance with the results of FISH analysis. In addition, it is widely accepted that the energy generated from the oxidation of PHAs was used for cell growth, luxury SOP uptake and glycogen replenishment.[4] Cell growth was approximate in each Run (the change of VSS concentration was negligible throughout the experiment, data not shown), more PHAs degradation but less glycogen synthesis were detected when the feed consisted of acetate and glycerol at equal fraction, and this detection implied more energy generated from PHAs oxidation would be used for SOP uptake, and the P uptake (Fig. 3) during oxic phase further verified this hypothesis. The high synthesis of PHA was another reason for improved EBPR when the carbon source contained acetate and glycerol at equal fraction.

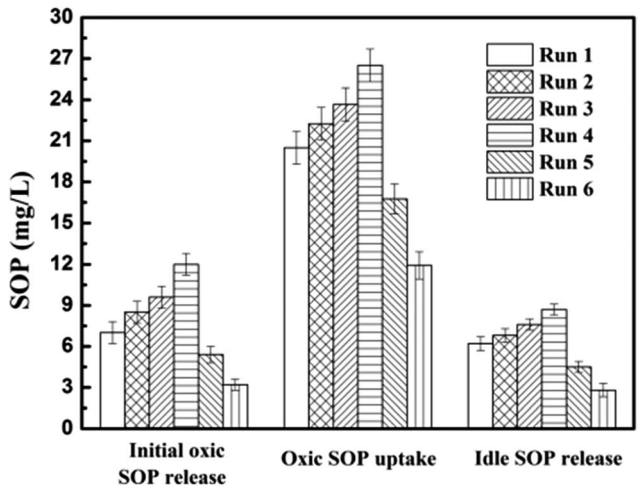

Fig. 3 Variations of SOP release and uptake of each Run during steady operation in O/EI reactor. Error bars represent standard deviations of triplicate tests.

The achievement of EBPR is directly dependent on phosphate release and uptake, which is related to the activities of key enzymes such as PPX and PPK.[4] Herein, the activities of PPX and PPK were measured and the results were shown in Table 2, it was found the activities of PPX and PPK during Run 4 exhibited the hightest specific activities. The amounts of SOP release during anaerobic phase and SOP uptake during oxic phase were displayed in Fig. 3. The highest anaerobic SOP release and oxic SOP uptake were observed during Run 4, which consisted well with the activities of PPX and PPK abovementioned. The type of carbon source can impact the activities of key enzymes. The results displayed in Table 2 clearly showed the feed included acetate and glycerol at equal fraction could achieve higher SOP release and uptake, and then higher SOP removal achievement could be obtained. Furthermore, more idle SOP release (around 8.7 mg L^{-1}) but negligible idle glycogen degradation (around 0.12 mmol-C per g-VSS) during Run 4 were measured. Those transformations of intermediate metabolites indicated that the energy for bacterial maintenance during the extended-idle phase was mainly provided *via* the hydrolysis of poly-P during Run 4, but seemed to be provided *via* both poly-P hydrolysis and glycogen degradation during Run 5 and Run 6. The relatively higher energy required from poly-P cleavage during Run 4 could enhance the role of intracellular poly-P playing in PAOs

Table 3 Summary of the reactor performances between O/EI- and A/O reactors during stable operation[a]

	A/O reactors		O/EI reactors	
Item	Ace/gly-fed reactor[b]	Gly-fed reactor[c]	Ace/gly-fed reactor[b]	Gly-fed reactor[c]
COD removal efficiency (%)	91 ± 2	89 ± 2	92 ± 2	90 ± 2
Effluent SOP (mg L^{-1})	1.35 ± 0.15	10.5 ± 0.48	0.50 ± 0.12	6.25 ± 0.27
SOP removal efficiency (%)	91 ± 1	30 ± 3.2	96 ± 0.8	58 ± 1.8
Effluent NH_4^+–N (mg L^{-1})	2.47 ± 0.4	3.12 ± 0.5	2.52 ± 0.52	3.08 ± 0.48
Effluent NO_2^-–N (mg L^{-1})	0.24 ± 3	0.28 ± 0.14	0.18 ± 0.18	0.23 ± 0.34
Effluent NO_3^-–N (mg L^{-1})	8.5 ± 0.5	8.9 ± 0.4	7.58 ± 0.54	8.14 ± 0.47
TN removal efficiency (%)	62.6 ± 1.2	59 ± 1.5	65.7 ± 0.51	61.8 ± 1.6
PAOs mix (%)[d]	38 ± 2	18 ± 2	40 ± 2	10 ± 2
GAOs mix (%)[d]	10 ± 2	23 ± 2	13 ± 2	26 ± 2

[a] Results are the averages and their standard deviations in triplicate tests. [b] Reactor employed acetate and glycerol at equal fraction as carbon resource. [c] Reactor employed glycerol as carbon resource. [d] Percentage to all bacteria (EUB mix probe).

metabolism. In other words, the intracellular biochemical transformations during Run 4 showed positive correlation with PAO metabolism, which might provide PAOs advantage over other populations.

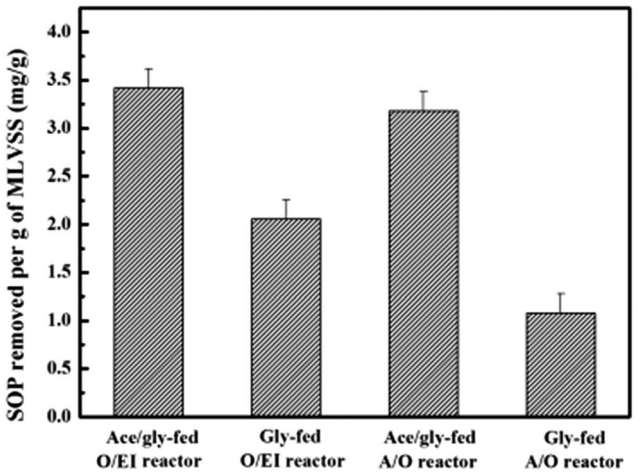

Fig. 4 SOP removed per g MLVSS during steady operation. The data are the averages and their standard deviations of three different measurements.

3.5. Comparison of EBPR performance between the O/EI and the classical A/O reactor

According to the above discussion, BPR performance could be further improved in O/EI reactor when the feed was glycerol and acetate at equal fraction. Because the operation mode and inducing mechanisms of phosphorus removal in O/EI regime were quite different from those in the classical A/O reactor, thus it is essential to compare the performances of EBPR between the reactors when fed with acetate and glycerol at equal fraction. In addition, deterioration of BPR was observed in O/EI reactor when glycerol served as the sole carbon source. It was reported that glycerol was also not suitable for EBPR in the literatures.[21,22] Therefore, it is also interesting to compare the BPR performances between the O/EI and A/O regimes fed with glycerol as sole carbon source.

Table 3 summarized the comparison results of the BPR performances between O/EI and A/O reactors during a 40 day period after stable operation. As shown in Table 3, the removal efficiencies of COD and TN were similar in all reactors, suggesting COD and nitrogen removal appealed to be unaffected by the operated regime. It should be noted that effluent SOP in O/EI reactors was much lower than those in A/O reactors with the same carbon source. Especially, the BPR efficiency was around

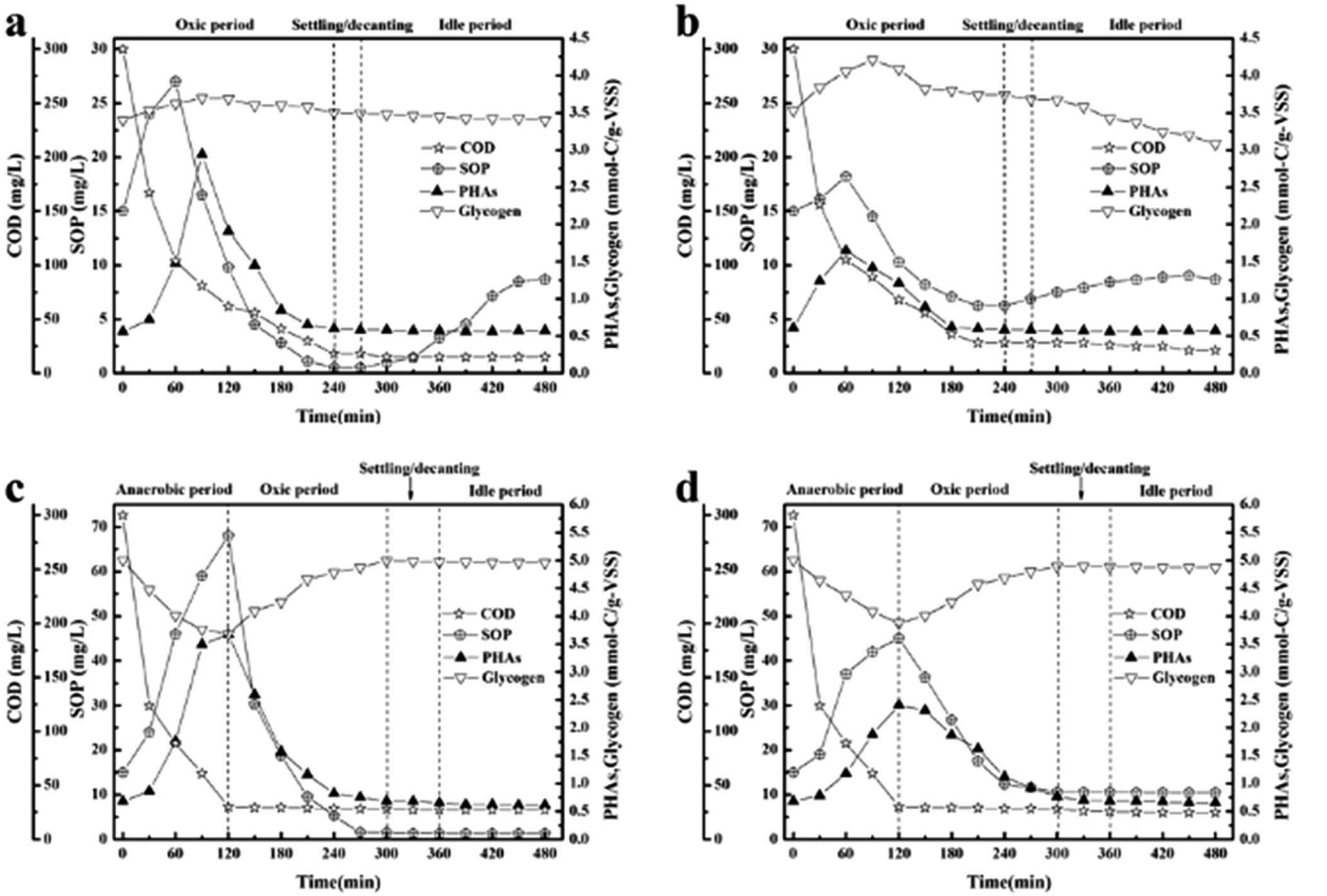

Fig. 5 Evolution of main compounds (SOP, COD, PHAs and glycogen) in one typical cycle of O/EI reactors (a: Ace/gly-fed reactor; b: Gly-fed reactor) and A/O reactors (c: Ace/gly-fed reactor; d: Gly-fed reactor).

30 ± 3.2% in the classical Gly-fed A/O reactor, which was consistent with the result obtained in Yuan *et al.* (2010),[19] however, the corresponding BPR efficiency was 58 ± 1.8% in Gly-fed O/EI reactor, which was 1.9-fold of that in A/O regime. The BPR efficiencies differences between Ace/gly-fed O/EI reactor and Ace/gly-fed-A/O reactor were negligible (95 ± 0.8% *versus* 91 ± 1%). The comparison results clearly suggested that the O/EI reactors could achieve better BPR performances than those in the widely accepted A/O reactors fed with respectively acetate and glycerol at the equal fraction and complete glycerol as carbon source.

Phosphorus removal per g MLVSS could more accurately express the phosphorus removal ability for it could eliminate the variations of MLVSS and MLSS. Fig. 4 showed the BPR capability to per g MLVSS in four reactors during the steady operations. It was found that SOP removed per g MLVSS in the Ace/gly-fed O/EI and Ace/gly-fed A/O reactors were 3.42 ± 0.2 mg g^{-1} and 3.18 ± 0.2 mg g^{-1}, respectively. However, the corresponding SOP removed in Gly-fed O/EI and A/O reactors were 2.06 ± 0.2 mg g^{-1} and 1.08 ± 0.2 mg g^{-1}. Obviously, the BPR capability in the Gly-fed O/EI reactor was approximately 1.9 time than that in Gly-fed A/O reactor.

Previous studies have shown that the type of carbon source is a critical factor affecting the competition of PAOs–GAOs, then further affecting BPR performance.[3,6] Bacterial populations were analyzed and the results were also exhibited in Table 3, it can be seen from Table 3, more PAOs but less GAOs were cultured in the activated sludge sample from O/EI reactors than those form A/O reactors, which were in agreement with the relative higher BPR achieved in O/EI reactors and were the main reason for O/EI reactor displaying better BPR performance.

The profiles of COD, SOP, PHAs and glycogen in one cycle of O/EI and A/O reactors during stable operations were illustrated in Fig. 5. As can be seen in Fig. 5, COD was rapidly consumed in anaerobic period in A/O reactors but in oxic period in O/EI reactors, which might sign obvious difference in metabolic mechanism of PAOs in two regimes. The PHAs productions in O/EI reactors were much lower than those in A/O reactors. For instance, the max PHAs accumulated in O/EI reactor with glycerol serving as the sole carbon source was only 1.65 mmol-C per g-VSS, much lower than 2.56 mmol-C per g-VSS in the corresponding A/O reactor. Previous studies have demonstrated anaerobic VFAs consumption and the subsequent PHAs production required adenosine triphosphate (ATP) and reducing power, which were generally considered to be supplied respectively with poly-P hydrolysis and glycogen degradation in EBPR.[3] Whereas, both ATP and NADH for PHAs production appeared to be provided *via* the tricarboxylic acid (TCA) cycle in O/EI reactors since negligible phosphorus release and glycogen degradation during PHAs accumulation, suggesting part of COD was consumed *via* TCA cycle to generate ATP and NADPH for PHAs formation, which might be the primary reason for O/EI reactors showing lower PHAs accumulation.

In addition, high levels of nitrate in effluent were detected of all reactors due to no strict anoxic zone, thereby, the accumulated nitrite can be recirculated or entered into the next anaerobic phase and then compromised the anaerobic metabolisms of PAOs in A/O reactors.[29] Our previous study, however, has shown that the O/EI regime could forbore higher level of nitrate than A/O regime.[3] This characteristic would be another reason for O/EI reactors showing better BPR performances.

4. Conclusions

In this study, the effect of ratio of acetate to glycerol on the performance of BPR induced by O/EI regime was investigated, and experimental results showed BPR could be greatly improved and the corresponding efficiency of BPR was 96.6 ± 1.2% when acetate and glycerol in the feed were at equal fraction, in which more abundance of PAOs but less GAOs were cultured. Further investigations showed that higher PHA was synthesized at equal fraction, which provided more energy for luxury-P uptake. Comparison of BPR efficiencies between O/EI and A/O reactors at equal fraction of acetate and glycerol and sole glycerol were also conducted. Experimental results suggested the BPR efficiencies between two regimes were similar when acetate and glycerol were at equal fraction, but higher BPR performance was obtained in Gly-fed O/EI reactor than in Gly-fed A/O reactor. The use of renewable waste substrates, glycerol, to enhance BPR is feasible.

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