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2017

Shen, N., Chen, Y., & Zhou, Y. (2017). Multi-cycle operation of enhanced biological phosphorus removal (EBPR) with different carbon sources under high temperature. *Water Research*, 114, 308-315.

<https://hdl.handle.net/10356/86510>

<https://doi.org/10.1016/j.watres.2017.02.051>

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Multi-cycle operation of Enhanced Biological Phosphorus Removal (EBPR)**with different carbon sources under high temperature**Nan Shen^{1,2}, Yun Chen¹, Yan Zhou^{1,2*}

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Abstract:

Many studies reported that it is challenging to apply enhanced biological phosphorus removal (EBPR) process at high temperature. Glycogen accumulating organisms (GAOs) could easily gain their dominance over poly-phosphate accumulating organisms (PAOs) when the operating temperature was in the range of 25°C to 30°C. However, a few successful EBPR processes operated at high temperature have been reported recently. This study aimed to have an in-depth understanding on the impact of feeding strategy and carbon source types on EBPR performance in tropical climate. P-removal performance of two EBPR systems was monitored through tracking effluent quality and cyclic studies. The results confirmed that EBPR was successfully obtained and maintained at high temperature with a multi-cycle strategy. More stable performance was observed with acetate as the sole carbon source compared to propionate. Stoichiometric ratios of phosphorus and carbon transformation during both anaerobic and aerobic phases were higher at high temperature than low temperature ($20\pm1^{\circ}\text{C}$) except anaerobic PHA/C ratios within most of the sub-cycles. Furthermore, the fractions of PHA and glycogen in biomass were lower compared with one-cycle pulse feed operation. The microbial community structure was more stable in acetate-fed sequencing batch reactor (C2-SBR) than that in propionate-fed reactor (C3-SBR). *Accumulibacter* Clade IIC was found to be highly abundant in both reactors.

Keywords: multi-cycle, EBPR, high temperature, PAO/GAO competition, low internal storage, high turnover rates

1. Introduction

Enhanced biological phosphorus removal (EBPR) process is mainly carried out by polyphosphate accumulating organisms (PAOs) under alternating anaerobic-aerobic conditions. Under anaerobic conditions, PAOs take up carbon sources by using energy generated from hydrolysis of intracellular polyphosphate (poly-P) and glycogen, and accumulate carbon sources as poly- β -hydroxyalkanoates (PHA). Aerobically, PAOs are capable of accumulating excessive amount of phosphate into cells by oxidizing PHA to gain energy (Oehmen et al. 2005e, Zhou et al. 2010, Zhou et al. 2012). Glycogen-accumulating organisms (GAOs) are able to perform carbon conversions in a similar way without contributing to phosphorus accumulation. Hence, they are recognized as the major competitors of PAOs that cause EBPR failure.

Previous research demonstrated that the employment of EBPR in tropical climate was challenging due to the proliferation of GAOs when the temperature was higher (25°C~30 °C) (Lopez-Vazquez et al. 2009a, Lopez-Vazquez et al. 2009b, Panswad et al. 2003, Ren et al. 2011, Whang and Park 2006). However, a few successful EBPR processes operated at high temperature have shone some light on the feasibility of high temperature EBPR (Freitas et al. 2009, Ong et al. 2014, Ong et al. 2013, Winkler et al. 2011). A SBR operated with short cycles under 30°C developed a robust and active biomass that was able to rapidly recover from the COD, P and N shock loads (Freitas et al. 2009). Winkler et al. (2011) reported a distinctive microbial community structure developed at 30°C in a granular sludge reactor, where considerably more PAOs existed in heavier granules compared to lighter granules

that were dominated by GAOs. By discharging the sludge from the top of the sludge bed, 100% P-removal efficiency was established (Winkler et al. 2011). Ong et al. (2013) demonstrated an effective EBPR system at 28-32 °C with acetate as the sole carbon source. In that study, a lower COD/P ratio ($C/P=3$) led to relatively higher P-removal rates as compared to C/P ratio of 10. Tu and Schuler (2013) reported that PAOs community and EBPR performance can be recovered from GAO dominated conditions by controlling the acetate feeding rate and maintaining low concentration of acetate in the reactor. The observation could be explained by higher acetate permease activity of *Accumulibacter* under the acetate-limited conditions (Burow et al. 2008). Therefore, it seems low carbon concentration in bulk liquid and/or low COD loading together with short alternating anaerobic/aerobic cycles may offer certain advantages to PAOs.

In this study, a multi-cycle strategy was proposed to provide rapid alternating anaerobic and aerobic conditions and lower carbon sources concentration after feeding. The short sub-cycle may also possess higher turnover rates of carbon and phosphorus transformation. Two types of carbon source, i.e. acetate and propionate, were used to compare P-removal performance and microbial communities in two sequencing batch reactor (SBR) systems with multi-cycle. This study aimed to have an in-depth understanding on the impact of feeding strategy and carbon source types on EBPR performance in tropical climate. This research contributes to a new alternative phosphorus removal operation configuration and helps to better understand the P and C turnover rates of EBPR at high temperature.

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84 **2. Materials and methods**85 **2.1 SBR Setup**

86 Seed sludge for two SBRs was collected from a local water reclamation plant (WRP)
87 in Singapore. Working volume of two SBRs was 6 L, and they were operated under
88 identical operating conditions. The 6 hours cycle time consisted of 3 sub-cycles of 2
89 minutes feeding and 100 minutes alternating anaerobic/aerobic phase (Table 1), as
90 well as 4 minutes sludge discharge, 25 minutes settling, and 25 minutes effluent
91 discharge at the end of the cycle (Fig. S1). Briefly, stage 1 had 40 min of anaerobic
92 phase and 60 min of aerobic phase while stage 2 had 35 min of anaerobic phase and
93 65 min of aerobic phase and stage 3 was operated the same as stage 1. In each cycle,
94 3 liters of synthetic wastewater was evenly distributed into the 3 sub-cycles during
95 feeding phases. The process was controlled at a hydraulic retention time (HRT) of 12
96 h and solid retention time (SRT) of 7.5 days. Dissolved oxygen (DO) in the aerobic
97 phase was controlled between 2-3 mg/L. Operating temperature was maintained at
98 30-32°C. pH was controlled between 7.2 and 8.0. The synthetic wastewater
99 contained the following composition (mg/L): NH_4Cl , 100; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200;
100 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 30 and 0.5 mL trace element. The trace element contained the
101 following composition (mg/L): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1500; H_3BO_3 , 150; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 30;
102 KI , 30; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 120; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 60; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 120 and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$,
103 150. COD and P-PO_4^{3-} concentrations were about 400 mg COD/L and 20-22 mg
104 P-PO_4^{3-} /L respectively in the feed. The concentration of allylthiourea (ATU) was 2-5

mg/L. Carbon sources for the two SBRs were acetate and propionate respectively. Hence, the two SBRs were named as C2-SBR and C3-SBR.

2.2 Analytical methods

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solid (MLVSS) were measured according to standard methods (APHA 1998). Liquid samples from the reactors were immediately filtered through 0.45 μ m membrane for acetate, propionate, and PO_4^{3-} -P analysis.

Acetate and propionate were analyzed using gas chromatography (GC) with flame ionization detector and equipped with a 30 m $\times$ 0.25 mm $\times$ 0.5 μ m DB-FFAP fused-silica capillary column. PO_4^{3-} -P concentration was analyzed using Vanadomolybdophosphoric Acid Colorimetric Method. Glycogen was extracted according to the method of Zeng et al. (2003). Briefly, 5ml of 0.6M HCl was added to freeze-dried biomass then heated at 100°C. After 6 hours, the glucose concentration in the supernatant was measured using Agilent 1200 series HPLC system (Agilent Technologies, Inc., Germany). Poly- β -hydroxybutyrate (PHB), poly- β -hydroxyvalerate (PHV) and poly- β -hydroxy-2-methylvalerate (PH2MV) were quantified as PHA components in this study. PHA content was determined following the method of Oehmen et al. (2005a). Freeze-dried samples were suspended with 3% H_2SO_4 acidified methanol and chloroform mixture. After 20 hours heating at 100°C, deionized water was added to remove the impurities and the organic portion was analyzed with Agilent 7890A GC system (Agilent Technologies, Inc., USA).

2.3 DNA extraction and Illumina high-throughput sequencing

Sludge samples from C2-SBR and C3- SBR during steady state were stored for microbial community analysis. Improved Griffiths method was adopted for DNA extraction (Towe et al. 2011). Briefly, frozen sludge pellet harvested from 2 ml mixed liquor was mechanically lysed by bead-beating with Lysing Matrix E (MPBiomedicals, CA, USA) followed by phenol-chloroform extraction and ethanol precipitation. DNA was digested with RNase If (NEB, MA, USA) to remove contaminated RNA, followed by quantification with Picogreen assays (Life Technologies, Singapore). The isolated DNA was stored at -20°C until further use. Bacterial universal primers Nobar 341F (CCTACGGGNGGCWGCAG) and Nobar 805R (GACTACHVGGGTATCTAATCC) were used to amplify the V3 ~ V4 region of bacterial 16S rDNA. The DNA samples were sequenced for bacterial communities on an Illumina Miseq by Macrogen (Seoul, Korea).

The pairs of reads were merged with FLASH software and then assigned to each sample. Quality control (QC) assessment was done to remove low-quality sequences and some artificial replicate sequences, and all QC-passed sequences were analyzed using RDP classifier to cluster them into relative species.

2.4 qPCR and Fluorescence in situ hybridization (FISH)

The abundance of target PAOs population was determined by SYBR Green based qPCR using the primers listed in Table 2. The presence of PAOs and GAOs in the sludge samples was also analyzed by FISH according to Amann and Fuchs (2008). The probes used for the hybridization are EUBMIX (equimolar of EUB338,

EUB338 II and EUB338 III) targeting all bacteria, PAOmix (equimolar of PAO462, PAO651 and PAO846) and PAO651 targeting *Accumulibacter*-type PAO (Crocetti et al. 2000), Acc-I-444 targeting *Accumulibacter* clade IA and type I, Acc-II-444 targeting *Accumulibacter* clade IIA, IIC and IID, GAOmix (equimolar of GAOQ431 and GAOQ989) targeting Competibacter-type GAO (Crocetti et al. 2002), DFI_mix (equimolar TFO_DF218 and TFO_DF618) targeting Cluster I Defluvicoccus-type GAO (Wong et al. 2004) and DFII_mix (equimolar of DEF988 and DF1020) targeting Cluster II Defluvicoccus-type GAO (Meyer et al. 2006). All probes were hybridized at 35% formamide. Images were visualized with Nikon A1R confocal laser scanning microscope and analyzed with NIS Elements v4.10 by thresholding. Quantification of the microbial communities was conducted by FISH visualization following the procedures described by Winkler et al. (2011).

3. Results and discussion

3.1 SBRs' performance

During the stage 1 acclimation period, performance in both reactors was fluctuated (Fig. 1). According to cyclic study result, P-uptake was not completed at the end of aerobic phase while the carbon sources were fully taken up within the first 10 minutes into anaerobic phase at the end of stage 1. Hence, aerobic phase was extended by 5 minutes during stage 2 from day 87 onwards to extend aerobic SRT from 4.5 days to 4.87 days. The performance of C2-SBR was gradually improved with P concentration in the effluent less than 0.3 mg/L. This indicated that the

culture in C2-SBR was fully acclimated to the set operating conditions. The stable performance lasted for more than 110 days till system faulty happened at the later period of stage 2 (Fig. 1). However, good performance was only realized from day 178 onwards in C3 reactor, which was 78 days later than C2 reactor, and this indicated the culture would need longer time to C3 feeding. Good P-removal performance with P concentration below 2.50 mg/L lasted for 40 days before system faulty for C3-SBR.

Due to system faulty (air supply blockage), P was accumulated in both reactors. The problem was realized one week later. When oxygen supply of the system was limited, effective aerobic phases were shortened that led to incomplete P-uptake and limited glycogen replenishment. During the anaerobic phases, P was further released with PHA accumulation. In this case, poly-P pool was gradually reduced and carbon transformation was seriously disrupted. It has been reported that EBPR failure caused by low DO was not reversible in short-term (Ma et al. 2015). In order to recover the performance, the wastewater loading was reduced to 75% of original loading by decreasing the feeding volume for both reactors from day 220 to 260. C2-SBR gradually recovered with the effluent P concentration below 1 mg/L within 30 days. However, C3-SBR was not able to recover during the same period and the effluent P concentration increased to influent level (14.68 mg/L).

At the beginning of stage 3, half of the C3-SBR reactor volume was replaced with fresh sludge for further recovery. In order to compare the performance, 50% of C2-SBR was also replaced with fresh sludge. The operating conditions were

resumed to that of stage 1. After 30 days operation, P-removal efficiency of C2-SBR reached 95.63% and the good performance lasted till the end of experiment. Meantime, the best P-removal efficiency in C3-SBR was only 52%, and it slowly recovered after another 19 days. The good performance of C3-SBR only lasted for about 31 days during the stage 3 when the effluent P concentration unexpectedly increased from 3.27 to 14.87 mg/L within 3 days (not shown in the Fig. 1B). The sudden increase of effluent P concentration implied the EBPR performance was unstable with propionate as the carbon source.

Comparison of two reactors suggests that a faster and stable EBPR performance could be obtained with acetate as carbon source. With acetate as carbon source, the system can also recover more rapidly when system upset occurred. Cai et al. (2016) also reported that larger and more stable EBPR granules were obtained by feeding acetate rather than propionate under lower temperature. However, our findings are different from some other studies under lower temperature where propionate is the preferred carbon source for EBPR (Carvalho et al. 2014, Pijuan et al. 2004a). It is known that only one particular type of GAOs is able to compete for propionate (i.e., *Alphaproteobacteria* GAOs) (Oehmen et al. 2005c). A better P-removal performance is often observed in propionate-fed EBPR systems. The abundance of *Accumulibacter* PAOs is typically higher in propionate-fed EBPR system than that in acetate-fed system, correspondingly GAOs generally present in a lower number (Oehmen et al. 2006). The different observation from this study may be due to different metabolic activity of PAOs and GAOs under high temperature.

3.2 Cyclic study of C2 and C3-SBRs

In normal pulse feed SBR operation, a typical cycle time can range from 4-6 hours with 1.5-2 hours for anaerobic phase and 2-3 hours for aerobic phase. In some studies, lower net PHA production and reduced PHA content was noted at the end of anaerobic phases, while P-release was found at the end of aerobic phases (Kong et al. 2002, Oehmen et al. 2005c, Wang et al. 2011). It is possible that prolonged anaerobic or aerobic phases may expose PAOs to endogenous starvation conditions. Therefore, a cycle with multiple sub-cycles may help to maintain the robustness of the microbial activity. However, too short cycles may also effect on complete PHA production. The cyclic studies demonstrated that both SBRs operated with acetate and propionate as the carbon sources exhibited typical metabolic transformations of carbon and phosphorus within every sub-cycle (Fig. 2). VFAs were completely taken up in the first 10 min into the anaerobic phase. PHA content detected at 20 min was nearly the same as the end of anaerobic phase in both reactors. Thus short anaerobic phase selected in this study would not affect the complete carbon transformation.

Carbon uptake rates in C2-SBR and C3-SBR were found to be 4.96 and 4.74 C-mmol/g-VSS/h, respectively. The values are slightly higher than 4.45 C-mmol/g-VSS/h that was reported in Whang and Park (2006) where pulse feed mode was applied to a SBR operated at 30 °C. These values are also higher than 3.534-3.744 C-mmol/g-VSS/h using acetate and 3.336-4.116 C-mmol/g-VSS/h using propionate in Pijuan et al. (2004b), where enriched EBPR culture was employed under lower temperature.

237 In anaerobic phase, PHB was the major PHA component in C2-SBR, which
238 accounted for 80.23-97.01% of total PHA. PH2MV was not detected in C2-SBR.
239 Meanwhile, the major PHA component produced with propionate were PHV
240 (57.20-68.67%) and PH2MV (26.95-40.81%) which were similar with the results
241 reported under lower temperature ($20\pm 1^{\circ}\text{C}$) (Carvalho et al. 2014, Hsu et al. 2013,
242 Oehmen et al. 2005b, Oehmen et al. 2005e, Vargas et al. 2011, Zeng et al. 2013).
243 However, PHA and glycogen content in biomass were significantly different with
244 low temperature studies. Under 20-25°C pulse feed conditions (acetate feed),
245 fraction of PHA in biomass was 0.1 C-mmol PHA/C-mmol active biomass
246 (C-mmol/C-mmol), while glycogen was 0.2 C-mmol glycogen/C-mmol active
247 biomass (C-mmol/C-mmol) (Kuba et al. 1997). In this study, PHA fractions were
248 0.026-0.050 C-mmol/C-mmol for C2-SBR and 0.029-0.054 C-mmol/C-mmol for
249 C3-SBR. Glycogen fractions were 0.010-0.044 C-mmol/C-mmol for C2-SBR and
250 0.025-0.044 C-mmol/C-mmol for C3-SBR. Such low carbon content is closely
251 related to the multi-cycle operation. Multi-cycle operation may increase the internal
252 carbon recycle flows, while it may also decrease internal carbon content. It has been
253 reported that PHA fraction could reach a very low level with the increase of
254 sub-cycle number with fixed HRT and SRT (Kuba et al. 1997). Notwithstanding the
255 low carbon content, P-removal performance was not affected. Glycogen fraction was
256 estimated to be 0.12 C-mmol/C-mmol in (Ong et al. 2014), which is significantly
257 higher than the value with multi-cycle in this study. At this stage, it is not clear if fast

carbon turnover and low carbon content (glycogen in particular) would favor PAO over GAO.

The P-release rates in C2 and C3-SBRs were 3.44-5.15 and 1.87-3.31 mmol/g-VSS/h, respectively. The rates were much higher than 0.20 mmol/g-VSS/h reported in Ong et al. (2014) where pulse feed SBR was operated under 32°C with acetate as carbon source. Under similar operating conditions as Ong et al. (2014), P-release rates were found to be 1.67-2.48 mmol/g-VSS/h at 30°C in Panswad et al. (2003). The values in this study were also higher than those at lower temperature (2.56 and 1.64 mmol/g-VSS/h) reported by Pijuan et al. (2004b). Interestingly, Ong et al. (2014) found that anaerobic P-release rate under 32°C was lower than 24°C (0.20 vs 0.24 mmol/g-VSS/h). It is likely due to the proliferation of GAO under 32°C in that study.

The transformation ratios of anaerobic P-release/C-uptake (P/C), PHA production/C-uptake (PHA/C), glycogen consumption/C-uptake (Gly/C) and aerobic P-uptake rates, P-uptake/PHA-consumption (P/PHA) and Gly-synthesis/PHA-consumption (Gly/PHA) during steady state are summarized in Table 3. It is noteworthy that nitrate was found in the effluent at the later stage of operation, although ATU was added. The total COD consumed by denitrifiers due to denitrification in anaerobic phases can be calculated by assuming 3.8 mg COD/mg N-NO₃⁻ and nitrite is equivalent to 3/5 nitrate (Beun et al. 2000). The ratios presented in Table 3 were corrected with the consideration of carbon consumption by denitrification. The NO_x concentrations within one cyclic study are shown in Fig. S2.

The P/C ratio was higher in each sub-cycle of C2-SBR than C3-SBR (Table 3). This result is reasonable that less energy is required for propionate uptake as compared to acetate (Carvalheira et al. 2014, Pijuan et al. 2004a). However, the P/C ratios in both reactors were higher than models (0.50 and 0.42 P-mol/C-mol) developed under lower temperature (Oehmen et al. 2005e, Smolders et al. 1994). Panswad et al. (2003) also observed that the specific phosphorus release rates increased with the increase of temperature. The higher P/C ratios should be partly due to higher maintenance energy required for PAO at high temperature. Brdjanovic et al. (1997) reported the ATP maintenance coefficients of PAOs in anaerobic phase were 0.00147 and 0.00363 mg-ATP/mg/h at 20°C and 30°C, respectively. Thus the net-P release excluding the P-release caused by maintenance or endogenous processes was also calculated in this study. The specific anaerobic maintenance coefficient was determined as 3.5 mgP/gVSS/h at 20°C in Oehmen et al. (2005d). In this study, about 2.44 mmol P in C2-SBR and 2.24 mmol P in C3-SBR were released for maintenance energy. The normalized P/C ratios were 0.540-0.637 mol/C-mol in C2-SBR and 0.249-0.448 mol/C-mol in C3-SBR after deducting the released P for maintenance (Table 3). Many studies used P/C and Gly/C ratios as indicative parameters for the extent of enrichment of PAOs, meanwhile the different stoichiometric ratios and kinetics were observed under different operating conditions (Schuler and Jenkins 2003, Welles et al. 2015). It has been reported anaerobic P/C ratios vary from 0.01 up to 0.93 P-mol/C-mol in Welles et al. (2015) and 0.38

P-mol/C-mol was obtained by Ong et al. (2014) using acetate as the carbon source at 32°C.

Anaerobic PHA/C ratios in each sub-cycle of C2-SBR were between 0.803 to 1.371 C-mol/C-mol with the average ratios lower than 1.33 C-mol/C-mol in HAc-fed PAOs model developed by Smolders et al. (1994). Similarly, PHA/C ratios in each sub-cycle of C3-SBR varied between 0.742 to 1.333 C-mol/C-mol with the average ratios lower than 1.22 C-mol/C-mol in HPr-fed PAOs model developed by Oehmen et al. (2005e) under lower temperature. Contin et al. (2000) observed increased ATP concentrations per g microbial biomass for communities incubated at higher temperatures. It is hence possible that PAOs utilize more energy on maintenance respiration under higher temperature as stated above. In anaerobic phase, PAOs may use PHA for maintenance when glycogen and poly-P level is low (Wang et al. 2011).

Multi-cycle in this study may cause much lower level of internal storage. It should be noted that the PHA/C ratios of 2nd and 3rd sub-cycles were lower than 1st sub-cycle in all the cyclic studies. There was probably carbon loss and/or oxygen inhibition on anaerobic activity during the beginning of the feeding phase due to the dissolved oxygen carried over from the last aerobic phase (Fig. S1). PHA/C ratios between 0.83 to 2.04 C-mol/C-mol from different EBPR studies using acetate as the carbon source were summarized by Schuler and Jenkins (2003). Those different ratios could be due to the different operating conditions, e.g., system operating strategy, SRT, operating temperature, etc..

The ratios of Gly/C in C2-SBR were between 0.118 to 0.531 C-mol/C-mol and only limited sub-cycles Gly/C ratio was higher than 0.5 C-mol/C-mol (HAc-fed PAOs model) (Smolders et al. 1994). The Gly/C ratio in C3-SBR was between 0.033 to 0.449 C-mol/C-mol while the ratio in HPr-fed PAOs model was 0.33 C-mol/C-mol (Oehmen et al. 2005e). Carvalho et al. (2007) also reported the Gly/C ratio was lower in propionate reactor than that in acetate reactor (0.32 vs. 0.69 C-mol/C-mol) at lower temperature. Typically, the Gly/C ratio ranged from 0.3 to 1.2 (Schuler and Jenkins 2003) while the ratio could be higher than 0.8 in a GAM-dominated culture and less than 0.6 in a PAM-dominated culture. Both reactors in this study were dominated by PAM during steady state.

The aerobic P-uptake rate ranged from 0.571 to 0.736 mmol/g-VSS/h in C2-SBR and 0.457 to 0.537 mmol/g-VSS/h in C3-SBR. It is known that P-uptake rate was lower with PHV than that with PHB (Lopez et al. 2006). The rates in both reactors were comparable with the rates under lower temperature (0.23-0.92 mmol/g-VSS/h in HAc-fed tests and 0.41-0.72 mmol/g-VSS/h in HPr-fed tests) (Pijuan et al. 2004b, Shen and Zhou 2016). Interestingly, P/PHA ratios under high temperature were higher than the results under lower temperature (0.686-1.056 vs 0.333 mol/C-mol with C2 and 0.603-0.993 vs 0.435 mol/C-mol with C3) (Oehmen et al. 2005c). That is, more phosphorus would be taken up per C-mol of PHA consumed. It seemed the P-uptake efficiency using PHA was higher at high temperature. However, glycogen replenishment was less, as evidenced by lower Gly/PHA ratios (i.e., 0.139-0.354 C-mol/C-mol with C2 and 0.127-0.401 C-mol/C-mol with C3). It is clear that

glycogen cycling pathways were limited under both anaerobic and aerobic phases in the system. Further, the results also confirmed that PAOs metabolic activities may be different at high temperature and low temperature.

3.3 Microbial community analysis

Illumina Miseq sequencing was applied to identify the microbial structure in both reactors during steady state. The results revealed that the families *Bacteroidetes incertae sedis*, *Flavobacteriaceae*, *Saprospiraceae*, *Chitinophagaceae*, *Planctomycetaceae*, *Rhodocyclaceae*, *Gammaproteobacteria incertae sedis* and *Verrucomicrobiaceae* were relatively dominant in both reactors, while *Ignavibacteriaceae* was only found in C2-SBR and *Rhodospirillaceae* genus *Defluviicoccus* was only found in C3-SBR. Kong et al. (2007) reported the bacterial group *Bacteroidetes incertae sedis* was found in 10 EBPR plants with the abundance of 9-19% and they pointed out that *Bacteroidetes* mostly probably have an important function in EBPR process. Hollender et al. (2002) reported that some strains within the *Flavobacteriaceae* were able to clearly show P-storage in the biomass and demonstrated P-release and uptake in the anaerobic and aerobic phases. The family *Saprospiraceae* belonging to phylum *Bacteroidetes* are strict aerobic gram-negative rods which specialist in protein hydrolysis and are capable of utilizing primarily amino acids as energy and carbon sources (Nielsen et al. 2012). PHA or polyphosphate granules were not found in *Saprospiraceae* (Nielsen et al. 2012). PAOs classified under *Betaproteobacteria* are tentatively named “*Candidatus Accumulibacter phosphatis*” and generally referred as *Accumulibacter*. It belongs to

the family of *Rhodocyclaceae*. Evidence is available that the family *Rhodocyclaceae* belonging to *Betaproteobacteria* are important PAOs in the so far investigated EBPR systems (Wagner et al. 2002). Datta and Goel (2010) also proved the ecophysiology of PAOs in *Rhodocyclaceae* family employing dual staining and MAR-FISH. *Candidatus Competibacter phosphatis* (*Competibacter*) under *Gammaproteobacteria* is typically found in glucose or acetate-fed biosystems (Shen and Zhou 2016). The *Rhodospirillaceae* genus *Defluviicoccus* in C3-SBR and *Gamaproteobacteria* incertae sedis in both SBRs were possible GAOs.

The presence of *Accumulibacter* PAO and GAO population in both reactors was confirmed and quantified by FISH (Fig. 3, Fig S3 and Table 4). Albertsen et al. (2016) reported a novel Glycogen Accumulating Organism “*Candidatus Propionivibrio aalborgensis*” that could be targeted by PAO462 and PAO846. To avoid overestimation of *Accumulibacter* abundance, we also used PAO651 solely for *Accumulibacter* quantification. The results for both types of quantification are presented in the Table 4. At the end of stage 1, total bacterial population comprised of 30.16% PAOs with PAOmix (15.02% with PAO651) and 25.75% GAOs in C2-SBR while 20.19% PAOs with PAOmix (22.08% with PAO651) and 10.32% GAOs were found in C3-SBR. The P-removal performance was not stable in both reactors during that stage. From the microbial community analysis, it is clear that PAO population was not dominant in stage 1. High abundance of GAO and other potential denitrifiers and OHOs may occupy the major population and lead to carbon sources competition. During stage 2, PAO increased to 74.65% with PAOmix (57.08%

with PAO651) and GAO decreased to 9.54% in C2-SBR at day 138 while 53.29% PAO with PAOmix (33.00% with PAO651) and 2.28% GAO were found in C3-SBR at day 157. The PAO proportion decreased to 52.51% with PAOmix (46.15% with PAO651) and GAO increased to 20.55% in C2-SBR at day 178 while PAO decreased to 31.57% with PAOmix (24.82% with PAO651) and GAO increased to 8.12% in C3-SBR at day 190. The P removal efficiency was more than 95% at day 138 and day 178 although the PAO population decreased in C2-SBR. On the other hand, P removal efficiency was more than 95% at day 190 while less than 50% at day 157 in C3-SBR. It seemed the reactor performance may not be directly linked to microbial community structure. In stage 3, half of the culture in both reactors was replaced with new sludge that had much lower PAO population (5.60%). PAO population increased to 63.92% with PAOmix (54.80% with PAO651) in C2-SBR at day 306 while it decreased to 11.04% with PAOmix (8.09% with PAO651) at day 319 in C3-SBR, although the P-removal efficiency was still more than 95% in both reactors on the sampling day.

Thereafter, the performance of C3-SBR was quickly deteriorated 3 days after the sampling date (day 319) with the P concentration increased from 3.27 to 14.87 mg/L. It seemed the re-seeding was not able to help on C3-SBR recovery. The FISH results show that PAO's community was more stable in C2-SBR than C3-SBR, while the highly dynamic population change in C3-SBR could be the reason for its unstable performance. It is noteworthy that the abundance of GAOs was much lower than PAOs during steady state. The low abundance of GAOs at high temperature, i.e.,

30°C, is unusual. In general, high temperature favors GAOs growth than PAOs, thus impose an adverse effect on phosphorus removal (Lopez-Vazquez et al. 2009c, Sayi-Ucar et al. 2015). Multi-cycle employed in this study may be helpful to maintain a stable performance at high temperature. More than 40% GAO was detected in Ong et al. (2014) with acetate as the carbon source in which pulsed feeding and a 10-day SRT were applied. Moreover, *Rhodospirillaceae* genus *Defluviicoccus* was not detected in C2-SBR and only a small amount of *Defluviicoccus* was found in C3-SBR (Fig. S4). Most of the GAO population in both reactors was *Competibacter*.

It was also noted that the morphology of PAOs in two reactors were distinctly different. In order to identify the specific PAO clades, PAO I (clade IA and other type I clades) and PAO II (clade IIA, IIC and IID) were verified by the probes Acc-1-444 and Acc-2-444, respectively. FISH quantification results suggested that number of PAO population stained with PAO II was highly close to that of PAOmix probe in both reactors (Fig. S5). PAO II was dominant in both reactors while a small amount of PAO I was also present in C3-SBR (Fig. S6).

The abundance of *Accumulibacter* clades was further investigated using qPCR (Fig. 4). PCR amplification of three ppk clade (clades IIB, IIC and IIF) were tested positive while no clear band was identified for the clade I, IIA and IID in both reactors (data not shown). Clade IIC was found to be the most abundant *Accumulibacter* clade in both reactors during stage 2. The results were different from the results in Ong et al. (2014) where Clade IIF was found to be dominant.

Respiratory nitrate reduction has been observed in reactors enriched with *Accumulibacter* type IIC (Kim et al. 2013), it is possible that the presence of nitrate in C2 and C3 SBRs may to certain extent alter the *Accumulibacter* population. The abundance of clade IIB, IIC and IIF was found to be highly different for the two SBRs, especially clade IIC (Fig. 4). The abundance of the three clades was much higher in C2-SBR than C3-SBR during stage 1 and 2 until the system failure at about 210 day when all the *Accumulibacter* population was seriously affected.

4. Conclusions

Multi-cycle operation could support a good EBPR performance under high temperature. Faster carbon and phosphorus turnover rates were realized in the multi-cycle system. PHA and glycogen content in biomass was low with multi-cycle operation while the low content did not affect the P-removal performance of the systems. Both acetate and propionate could be used as carbon source while a better and more stable EBPR performance can be maintained with acetate as feed under high temperature. The carbon uptake and P release rates were higher at high temperature than lower temperature. It was also found that under high temperature more phosphorus could be taken up by consuming per C-mol of PHA aerobically. PAO's community was found more stable in C2-SBR than C3-SBR. Moreover, *Accumulibacter* IIC was dominant in both reactors.

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A list of Tables

Table 1 Alternating anaerobic and aerobic phase under different stages.

Table 2 Primers for qPCR verification of *Accumulibacter* PAO.

Table 3 Summary of stoichiometric ratios of phosphorus and carbon transformation during the anaerobic and aerobic phases in stage 2.

Table 4 The abundance of PAO and GAO during the operation period quantified by FISH.

659 **Table 1** Alternating anaerobic and aerobic phase under different stages.

Time (min)	Stage 1	Stage 2	Stage 3
Days	0-86	87-263	264-345
Anaerobic phase	40	35	40
Aerobic phase	60	65	60

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662 **Table 2** Primers for qPCR verification of *Accumulibacter* PAO.

Primer	Gene	Target	Annealing Temp. (°C)	Reference
Acc-ppk1-763f/Acc-ppk1-1170r	<i>ppk1</i>	Acc-I PAO	61	(He et al. 2007)
Acc-ppk1-893f/Acc-ppk1-997r	<i>ppk1</i>	Acc-IIA PAO	61	(He et al. 2007)
Acc-ppk1-870f/Acc-ppk1-1002r	<i>ppk1</i>	Acc-IIB PAO	61	(He et al. 2007)
Acc-ppk1-254f/Acc-ppk1-460r	<i>ppk1</i>	Acc-IIC PAO	61	(He et al. 2007)
Acc-ppk1-375f/Acc-ppk1-522r	<i>ppk1</i>	Acc-IID PAO	61	(He et al. 2007)
Acc-ppk1-355f/Acc-ppk1-600r	<i>ppk1</i>	Acc-IIF PAO	61	(Ong et al. 2014)

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665 **Table 3** Summary of stoichiometric ratios of phosphorus and carbon transformation during the anaerobic and aerobic phases in stage 2.

	C2-SBR								C3-SBR								
	Anaerobic phase					Aerobic phase			Anaerobic phase						Aerobic phase		
	P release/C P-mol/C-mol	Net-P release/C P-mol/C-mol	PHB/C C-mol/C-mol	PHV/C C-mol/C-mol	Gly/C C-mol/C-mol	P uptake mmol/g VSS h	P/PHA mol/C-mol	Gly/PHA C-mol/C-mol	P release/C P-mol/C-mol	Net-P release P-mol/C-mol	PHB/C C-mol/C-mol	PHV/C C-mol/C-mol	PH2MV/C C-mol/C-mol	Gly/C C-mol/C-mol	P uptake mmol/g VSS h	P/PHA mol/C-mol	Gly/PHA C-mol/C-mol
Sub-cycle 1	0.823-0.966	0.640-0.783	0.849-1.100	0.138-0.271	0.277-0.383	0.712-0.736	0.686-1.056	0.139-0.258	0.587-0.731	0.382-0.527	0.024-0.080	0.558-0.721	0.263-0.532	0.281-0.449	0.503-0.537	0.603-0.702	0.127-0.192
Sub-cycle 2	0.654-0.730	0.472-0.548	0.779-0.875	0.024-0.150	0.118-0.531	0.571-0.698	0.778-0.914	0.223-0.256	0.370-0.591	0.166-0.387	0.009-0.031	0.458-0.709	0.275-0.404	0.033-0.212	0.461-0.501	0.782-0.993	0.179-0.242
Sub-cycle 3	0.692-0.762	0.509-0.580	0.833-0.989	0.040-0.151	0.234-0.423	0.606-0.701	0.804-0.957	0.264-0.354	0.404-0.634	0.200-0.430	0.020-0.063	0.457-0.689	0.268-0.352	0.163-0.248	0.457-0.504	0.905-0.906	0.281-0.401
* Normalized value	0.723-0.819	0.540-0.637	0.821-0.964	0.069-0.189	0.244-0.407	0.639-0.709	0.751-0.979	0.214-0.277	0.454-0.652	0.249-0.448	0.025-0.050	0.491-0.706	0.268-0.429	0.162-0.303	0.474-0.514	0.737-0.844	0.189-0.262
Model	0.50		1.33		0.50				0.42		1.22			0.33			

666 *Normalized stoichiometric ratios of 3 sub-cycles

Table 4 The abundance of PAO and GAO during the operation period quantified by FISH.

Stage	Time (day)	C2-SBR			Time (day)	C3-SBR		
		PAO		GAO		PAO		GAO
		PAOm _{ix}	PAO651			PAOm _{ix}	PAO651	
Stage 1	71	30.16%	15.02%	25.75%	71	20.19%	22.08%	10.32%
Stage 2	138	74.65%	57.08%	9.54%	157	53.29%	33.00%	2.28%
	178	52.51%	46.15%	20.55%	190	31.57%	24.82%	8.12%
Stage 3	306	63.92%	54.80%	20.76%	319	11.04%	8.09%	35.91%

A list of Figures

Fig. 1 Historical profile of influent and effluent P, MLSS and MLVSS concentrations, in (A) C2 and (B) C3-SBR. Different operating stages are separated by black dotted lines.

Fig. 2 Cyclic profiles of VFAs, phosphorus, PHA and glycogen during steady state of SBRs operation in C2-SBR (A) and C3-SBR (B) during stage 2. 'A' represented anaerobic phase, and 'O' represented aerobic phase. PHA, glycogen and VFAs expressed in C-mmol due to the variation in concentrations cause by volume change.

Fig. 3 FISH quantification of sludge from (A) C2-SBR at day 138 and (B) C3-SBR at day 190. GAO hybridized with the probe GAOmix, DFI_mix and DFII_mix (purple), PAOs hybridised with the probe PAOmix (red) and All Bacteria hybridised with the general probe EUBmix (green), the lower right image was combined by the three kinds of probes.

Fig. 4 Gene abundance of clade Acc-IIB, Acc-IIC and Acc-IIF PAO in both reactors during stage 1 and 2.

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Fig. 4 Gene abundance of clade Acc-IIB, Acc-IIC and Acc-IIF PAO in both reactors during stage 1 and 2.

EBPR was successfully obtained and maintained at high temperature with multi-cycle strategy

More stable performance was observed with C2 as carbon source compared with C3

Faster C and P turnover rates were realized with multi-cycle than one-cycle pulse feeding

Accumulibacter Clade IIC was found to be highly abundant in both reactors