Multi-cycle operation of Enhanced Biological Phosphorus Removal (EBPR) with different carbon sources under high temperature

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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±1°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^{o}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^{o}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^{o}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.

2. Materials and methods

2.1 SBR Setup

Seed sludge for two SBRs was collected from a local water reclamation plant (WRP) in Singapore. Working volume of two SBRs was 6 L, and they were operated under identical operating conditions. The 6 hours cycle time consisted of 3 sub-cycles of 2 minutes feeding and 100 minutes alternating anaerobic/aerobic phase (Table 1), as well as 4 minutes sludge discharge, 25 minutes settling, and 25 minutes effluent discharge at the end of the cycle (Fig. S1). Briefly, stage 1 had 40 min of anaerobic phase and 60 min of aerobic phase while stage 2 had 35 min of anaerobic phase and 65 min of aerobic phase and stage 3 was operated the same as stage 1. In each cycle, 3 liters of synthetic wastewater was evenly distributed into the 3 sub-cycles during feeding phases. The process was controlled at a hydraulic retention time (HRT) of 12 h and solid retention time (SRT) of 7.5 days. Dissolved oxygen (DO) in the aerobic phase was controlled between 2-3 mg/L. Operating temperature was maintained at 30-32°C. pH was controlled between 7.2 and 8.0. The synthetic wastewater contained the following composition (mg/L): NH_4Cl, 100; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 30 and 0.5 mL trace element. The trace element contained the following composition (mg/L): $FeCl_3 \cdot 6H_2O$, 1500; H_3BO_3, 150; $CuSO_4 \cdot 5H_2O$, 30; KI, 30; $MnCl_2 \cdot 4H_2O$, 120; $Na_2Mo_4 \cdot 2H_2O$, 60; $ZnSO_4 \cdot 7H_2O$, 120 and $CoCl_2 \cdot 6H_2O$, 150. COD and $P\text{-}PO_4^{3-}$ concentrations were about 400 mg COD/L and 20-22 mg $P\text{-}PO_4^{3-}$/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 0.45μm 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×0.25 mm×0.5μm 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).

127 **2.3 DNA extraction and Illumina high-throughput sequencing**

128 Sludge samples from C2-SBR and C3- SBR during steady state were stored for
129 microbial community analysis. Improved Griffiths method was adopted for DNA
130 extraction (Towe et al. 2011). Briefly, frozen sludge pellet harvested from 2 ml
131 mixed liquor was mechanically lysed by bead-beating with Lysing Matrix E
132 (MPBiomedicals, CA, USA) followed by phenol-chloroform extraction and ethanol
133 precipitation. DNA was digested with RNase If (NEB, MA, USA) to remove
134 contaminated RNA, followed by quantification with Picogreen assays (Life
135 Technologies, Singapore). The isolated DNA was stored at -20^{o}C until further use.
136 Bacterial universal primers Nobar 341F (CCTACGGGNGGCWGCAG) and Nobar
137 805R (GACTACHVGGGTATCTAATCC) were used to amplify the V3 ~ V4 region
138 of bacterial 16S rDNA. The DNA samples were sequenced for bacterial communities
139 on an Illumina Miseq by Macrogen (Seoul, Korea).
140 The pairs of reads were merged with FLASH software and then assigned to each
141 sample. Quality control (QC) assessment was done to remove low-quality sequences
142 and some artificial replicate sequences, and all QC-passed sequences were analyzed
143 using RDP classifier to cluster them into relative species.

144 **2.4 qPCR and Fluorescence in situ hybridization (FISH)**

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

149 EUB338 II and EUB338 III) targeting all bacteria, PAOmix (equimolar of PAO462,
150 PAO651 and PAO846) and PAO651 targeting *Accumulibacter*-type PAO (Crocetti et
151 al. 2000), Acc-I-444 targeting *Accumulibacter* clade IA and type I, Acc-II-444
152 targeting *Accumulibacter* clade IIA, IIC and IID, GAOmix (equimolar of GAOQ431
153 and GAOQ989) targeting Competibacter-type GAO (Crocetti et al. 2002), DFI_mix
154 (equimolar TFO_DF218 and TFO_DF618) targeting Cluster I Defluvicoccus-type
155 GAO (Wong et al. 2004) and DFII_mix (equimolar of DEF988 and DF1020)
156 targeting Cluster II Defluvicoccus-type GAO (Meyer et al. 2006). All probes were
157 hybridized at 35% formamide. Images were visualized with Nikon A1R confocal
158 laser scanning microscope and analyzed with NIS Elements v4.10 by thresholding.
159 Quantification of the microbial communities was conducted by FISH visualization
160 following the procedures described by Winkler et al. (2011).
161

162 **3. Results and discussion**

163 **3.1 SBRs' performance**

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

171 culture in C2-SBR was fully acclimated to the set operating conditions. The stable
172 performance lasted for more than 110 days till system faulty happened at the later
173 period of stage 2 (Fig. 1). However, good performance was only realized from day
174 178 onwards in C3 reactor, which was 78 days later than C2 reactor, and this
175 indicated the culture would need longer time to C3 feeding. Good P-removal
176 performance with P concentration below 2.50 mg/L lasted for 40 days before system
177 faulty for C3-SBR.
178 Due to system faulty (air supply blockage), P was accumulated in both reactors. The
179 problem was realized one week later. When oxygen supply of the system was limited,
180 effective aerobic phases were shortened that led to incomplete P-uptake and limited
181 glycogen replenishment. During the anaerobic phases, P was further released with
182 PHA accumulation. In this case, poly-P pool was gradually reduced and carbon
183 transformation was seriously disrupted. It has been reported that EBPR failure
184 caused by low DO was not reversible in short-term (Ma et al. 2015). In order to
185 recover the performance, the wastewater loading was reduced to 75% of original
186 loading by decreasing the feeding volume for both reactors from day 220 to 260.
187 C2-SBR gradually recovered with the effluent P concentration below 1 mg/L within
188 30 days. However, C3-SBR was not able to recover during the same period and the
189 effluent P concentration increased to influent level (14.68 mg/L).
190 At the beginning of stage 3, half of the C3-SBR reactor volume was replaced with
191 fresh sludge for further recovery. In order to compare the performance, 50% of
192 C2-SBR was also replaced with fresh sludge. The operating conditions were

193 resumed to that of stage 1. After 30 days operation, P-removal efficiency of C2-SBR
194 reached 95.63% and the good performance lasted till the end of experiment.
195 Meantime, the best P-removal efficiency in C3-SBR was only 52%, and it slowly
196 recovered after another 19 days. The good performance of C3-SBR only lasted for
197 about 31 days during the stage 3 when the effluent P concentration unexpectedly
198 increased from 3.27 to 14.87 mg/L within 3 days (not shown in the Fig. 1B). The
199 sudden increase of effluent P concentration implied the EBPR performance was
200 unstable with propionate as the carbon source.
201 Comparison of two reactors suggests that a faster and stable EBPR performance
202 could be obtained with acetate as carbon source. With acetate as carbon source, the
203 system can also recover more rapidly when system upset occurred. Cai et al. (2016)
204 also reported that larger and more stable EBPR granules were obtained by feeding
205 acetate rather than propionate under lower temperature. However, our findings are
206 different from some other studies under lower temperature where propionate is the
207 preferred carbon source for EBPR (Carvalheira et al. 2014, Pijuan et al. 2004a). It is
208 known that only one particular type of GAOs is able to compete for propionate (i.e.,
209 *Alphaproteobacteria* GAOs) (Oehmen et al. 2005c). A better P-removal performance
210 is often observed in propionate-fed EBPR systems. The abundance of
211 *Accumulibacter* PAOs is typically higher in propionate-fed EBPR system than that in
212 acetate-fed system, correspondingly GAOs generally present in a lower number
213 (Oehmen et al. 2006). The different observation from this study may be due to
214 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±1^{o}C) (Carvalheira 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^{o}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^{o}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^{o}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^{o}C was lower than 24^{o}C (0.20 vs 0.24 mmol/g-VSS/h). It is likely due to the proliferation of GAO under 32^{o}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-NO_3^- 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 NOx 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^{o}C and 30^{o}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^{o}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

301 P-mol/C-mol was obtained by Ong et al. (2014) using acetate as the carbon source at
302 32^{o}C.
303 Anaerobic PHA/C ratios in each sub-cycle of C2-SBR were between 0.803 to 1.371
304 C-mol/C-mol with the average ratios lower than 1.33 C-mol/C-mol in HAc-fed
305 PAOs model developed by Smolders et al. (1994). Similarly, PHA/C ratios in each
306 sub-cycle of C3-SBR varied between 0.742 to 1.333 C-mol/C-mol with the average
307 ratios lower than 1.22 C-mol/C-mol in HPr-fed PAOs model developed by Oehmen
308 et al. (2005e) under lower temperature. Contin et al. (2000) observed increased ATP
309 concentrations per g microbial biomass for communities incubated at higher
310 temperatures. It is hence possible that PAOs utilize more energy on maintenance
311 respiration under higher temperature as stated above. In anaerobic phase, PAOs may
312 use PHA for maintenance when glycogen and poly-P level is low (Wang et al. 2011).
313 Multi-cycle in this study may cause much lower level of internal storage. It should
314 be noted that the PHA/C ratios of 2nd and 3rd sub-cycles were lower than 1st
315 sub-cycle in all the cyclic studies. There was probably carbon loss and/or oxygen
316 inhibition on anaerobic activity during the beginning of the feeding phase due to the
317 dissolved oxygen carried over from the last aerobic phase (Fig. S1). PHA/C ratios
318 between 0.83 to 2.04 C-mol/C-mol from different EBPR studies using acetate as the
319 carbon source were summarized by Schuler and Jenkins (2003). Those different
320 ratios could be due to the different operating conditions, e.g., system operating
321 strategy, SRT, operating temperature, etc..

322 The ratios of Gly/C in C2-SBR were between 0.118 to 0.531 C-mol/C-mol and only
323 limited sub-cycles Gly/C ratio was higher than 0.5 C-mol/C-mol (HAc-fed PAOs
324 model) (Smolders et al. 1994). The Gly/C ratio in C3-SBR was between 0.033 to
325 0.449 C-mol/C-mol while the ratio in HPr-fed PAOs model was 0.33 C-mol/C-mol
326 (Oehmen et al. 2005e). Carvalho et al. (2007) also reported the Gly/C ratio was lower
327 in propionate reactor than that in acetate reactor (0.32 vs. 0.69 C-mol/C-mol) at
328 lower temperature. Typically, the Gly/C ratio ranged from 0.3 to 1.2 (Schuler and
329 Jenkins 2003) while the ratio could be higher than 0.8 in a GAM-dominated culture
330 and less than 0.6 in a PAM-dominated culture. Both reactors in this study were
331 dominated by PAM during steady state.
332 The aerobic P-uptake rate ranged from 0.571 to 0.736 mmol/g-VSS/h in C2-SBR
333 and 0.457 to 0.537 mmol/g-VSS/h in C3-SBR. It is known that P-uptake rate was
334 lower with PHV than that with PHB (Lopez et al. 2006). The rates in both reactors
335 were comparable with the rates under lower temperature (0.23-0.92 mmol/g-VSS/h
336 in HAc-fed tests and 0.41-0.72 mmol/g-VSS/h in HPr-fed tests) (Pijuan et al. 2004b,
337 Shen and Zhou 2016). Interestingly, P/PHA ratios under high temperature were
338 higher than the results under lower temperature (0.686-1.056 vs 0.333 mol/C-mol
339 with C2 and 0.603-0.993 vs 0.435 mol/C-mol with C3) (Oehmen et al. 2005c). That
340 is, more phosphorus would be taken up per C-mol of PHA consumed. It seemed the
341 P-uptake efficiency using PHA was higher at high temperature. However, glycogen
342 replenishment was less, as evidenced by lower Gly/PHA ratios (i.e., 0.139-0.354
343 C-mol/C-mol with C2 and 0.127-0.401 C-mol/C-mol with C3). It is clear that

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

347 **3.3 Microbial community analysis**

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

366 the family of *Rhodocyclaceae*. Evidence is available that the family *Rhodocyclaceae*
367 belonging to *Betaproteobacteria* are important PAOs in the so far investigated EBPR
368 systems (Wagner et al. 2002). Datta and Goel (2010) also proved the ecophysiology
369 of PAOs in *Rhodocyclaceae* family employing dual staining and MAR-FISH.
370 *Candidatus* Competibacter phosphatis (*Competibacter*) under *Gammaproteobacteria*
371 is typically found in glucose or acetate-fed biosystems (Shen and Zhou 2016). The
372 *Rhodospirillaceae* genus *Defluviicoccus* in C3-SBR and *Gamaproteobacteria*
373 incertae sedis in both SBRs were possible GAOs.
374 The presence of *Accumulibacter* PAO and GAO population in both reactors was
375 confirmed and quantified by FISH (Fig. 3, Fig S3 and Table 4). Albertsen et al.
376 (2016) reported a novel Glycogen Accumulating Organism "*Candidatus*
377 *Propionivibrio aalborgensis*" that could be targeted by PAO462 and PAO846. To
378 avoid overestimation of *Accumulibacter* abundance, we also used PAO651 solely for
379 *Accumulibacter* quantification. The results for both types of quantification are
380 presented in the Table 4. At the end of stage 1, total bacterial population comprised
381 of 30.16% PAOs with PAOmix (15.02% with PAO651) and 25.75% GAOs in
382 C2-SBR while 20.19% PAOs with PAOmix (22.08% with PAO651) and 10.32%
383 GAOs were found in C3-SBR. The P-removal performance was not stable in both
384 reactors during that stage. From the microbial community analysis, it is clear that
385 PAO population was not dominant in stage 1. High abundance of GAO and other
386 potential dentirifiers and OHOs may occupy the major population and lead to carbon
387 sources competition. During stage 2, PAO increased to 74.65% with PAOmix (57.08%

388 with PAO651) and GAO decreased to 9.54% in C2-SBR at day 138 while 53.29%
389 PAO with PAOmix (33.00% with PAO651) and 2.28% GAO were found in C3-SBR
390 at day 157. The PAO proportion decreased to 52.51% with PAOmix (46.15% with
391 PAO651) and GAO increased to 20.55% in C2-SBR at day 178 while PAO
392 decreased to 31.57% with PAOmix (24.82% with PAO651) and GAO increased to
393 8.12% in C3-SBR at day 190. The P removal efficiency was more than 95% at day
394 138 and day 178 although the PAO population decreased in C2-SBR. On the other
395 hand, P removal efficiency was more than 95% at day 190 while less than 50% at
396 day 157 in C3-SBR. It seemed the reactor performance may not be directly linked to
397 microbial community structure. In stage 3, half of the culture in both reactors was
398 replaced with new sludge that had much lower PAO population (5.60%). PAO
399 population increased to 63.92% with PAOmix (54.80% with PAO651) in C2-SBR at
400 day 306 while it decreased to 11.04% with PAOmix (8.09% with PAO651) at day
401 319 in C3-SBR, although the P-removal efficiency was still more than 95% in both
402 reactors on the sampling day.
403 Thereafter, the performance of C3-SBR was quickly deteriorated 3 days after the
404 sampling date (day 319) with the P concentration increased from 3.27 to 14.87 mg/L.
405 It seemed the re-seeding was not able to help on C3-SBR recovery. The FISH results
406 show that PAO's community was more stable in C2-SBR than C3-SBR, while the
407 highly dynamic population change in C3-SBR could be the reason for its unstable
408 performance. It is noteworthy that the abundance of GAOs was much lower than
409 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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648 and aerobic conditions. Bioresource Technology 116, 340-347.

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651 **A list of Tables**

658

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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661

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

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

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664

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			

*Normalized stoichiometric ratios of 3 sub-cycles

667 **Table 4** The abundance of PAO and GAO during the operation period quantified by

668 FISH.

Stage	Time (day)	C2-SBR			Time (day)	C3-SBR		
		PAO		GAO		PAO		GAO
		PAOmix	PAO651			PAOmix	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%

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671 **A list of Figures**

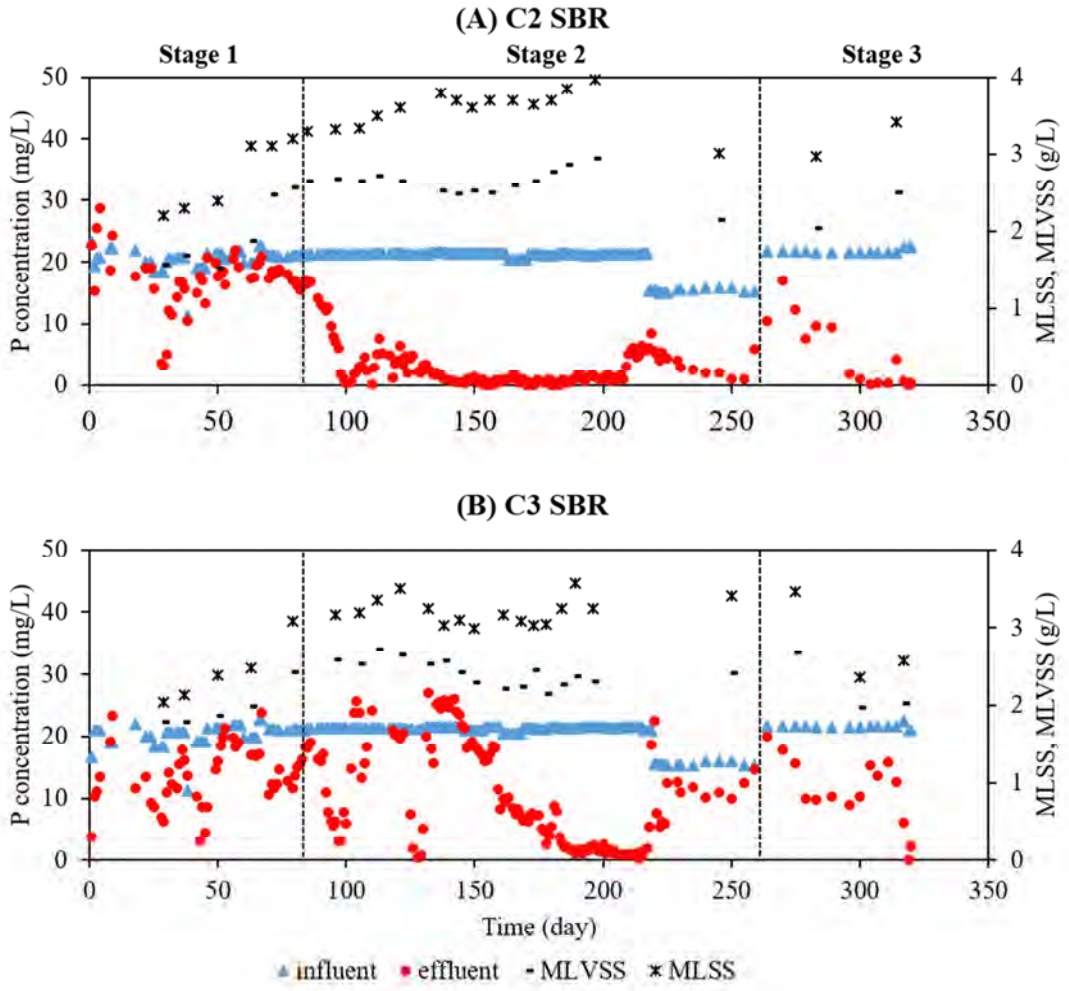
672 **Fig. 1** Historical profile of influent and effluent P, MLSS and MLVSS concentrations,
673 in (A) C2 and (B) C3-SBR. Different operating stages are separated by black dotted
674 lines.

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

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

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

686



687

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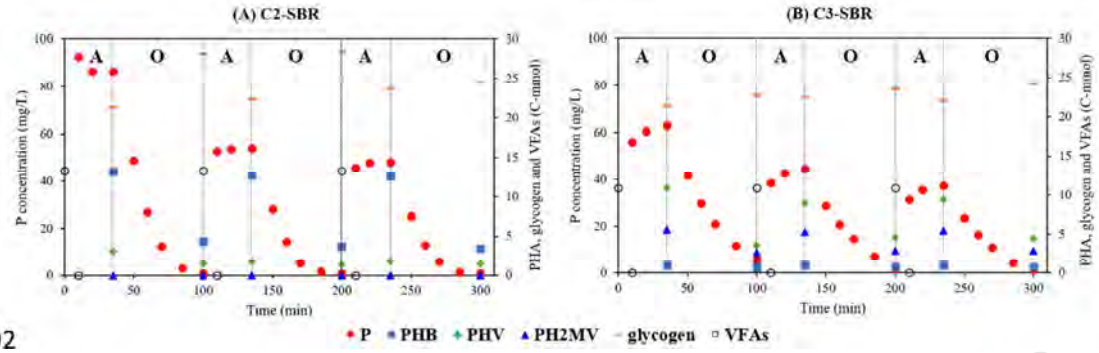
691



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693 **Fig. 2** Cyclic profiles of VFAs, phosphorus, PHA and glycogen during steady state of

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695 anaerobic phase, and 'O' represented aerobic phase. PHA, glycogen and VFAs

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697

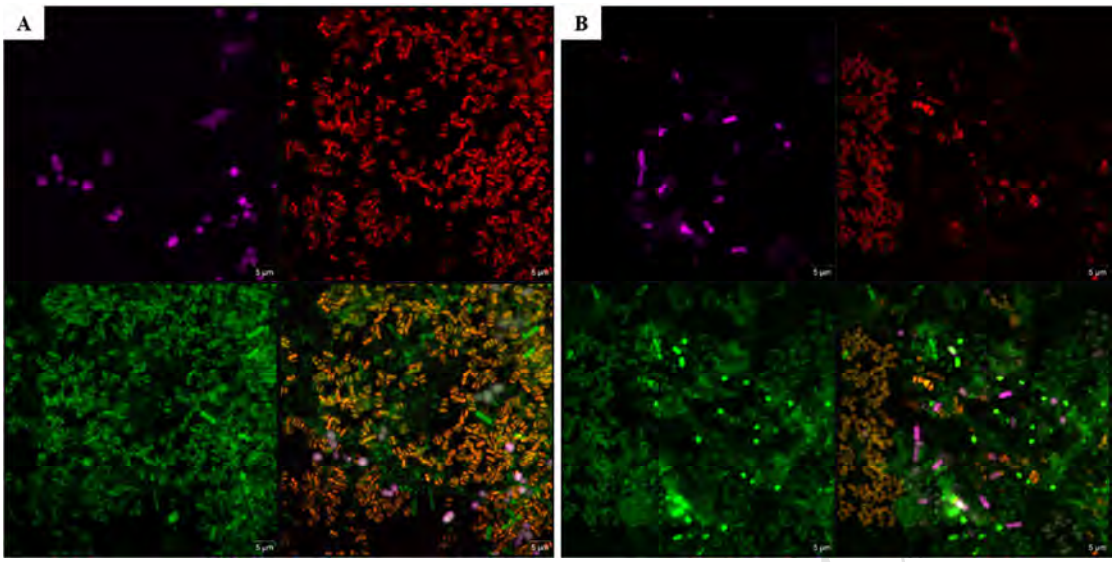
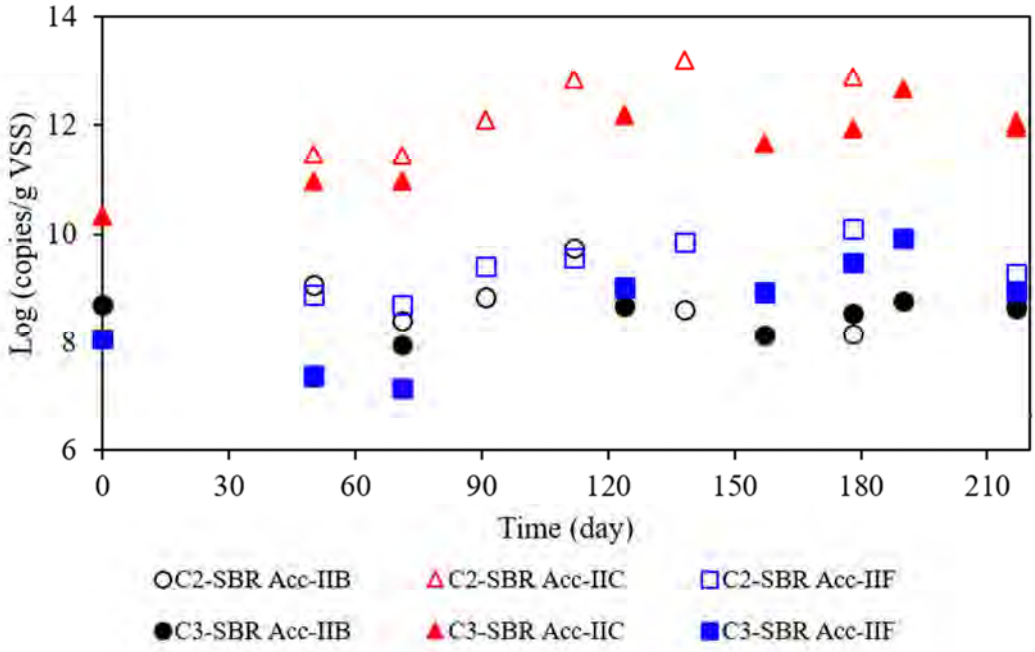


Fig. 3 FISH quantification of sludge from (A) C2-SBR at day 138 and (B) C3-SBR at day 190. GAO hybridized with the probe GAOmix, DFI_mix and DFII_mix (purple), PAOs hybridised with the probe PAOmix (red) and All Bacteria hybridised with the general probe EUBmix (green), the lower right image was combined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



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706 **Fig. 4** Gene abundance of clade Acc-IIB, Acc-IIC and Acc-IIF PAO in both reactors

707 during stage 1 and 2.

708

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

Graphic abstract