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Microbial population changes and metabolic shift of *candidatus accumulibacter* under low temperature and limiting polyphosphate

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ABSTRACT

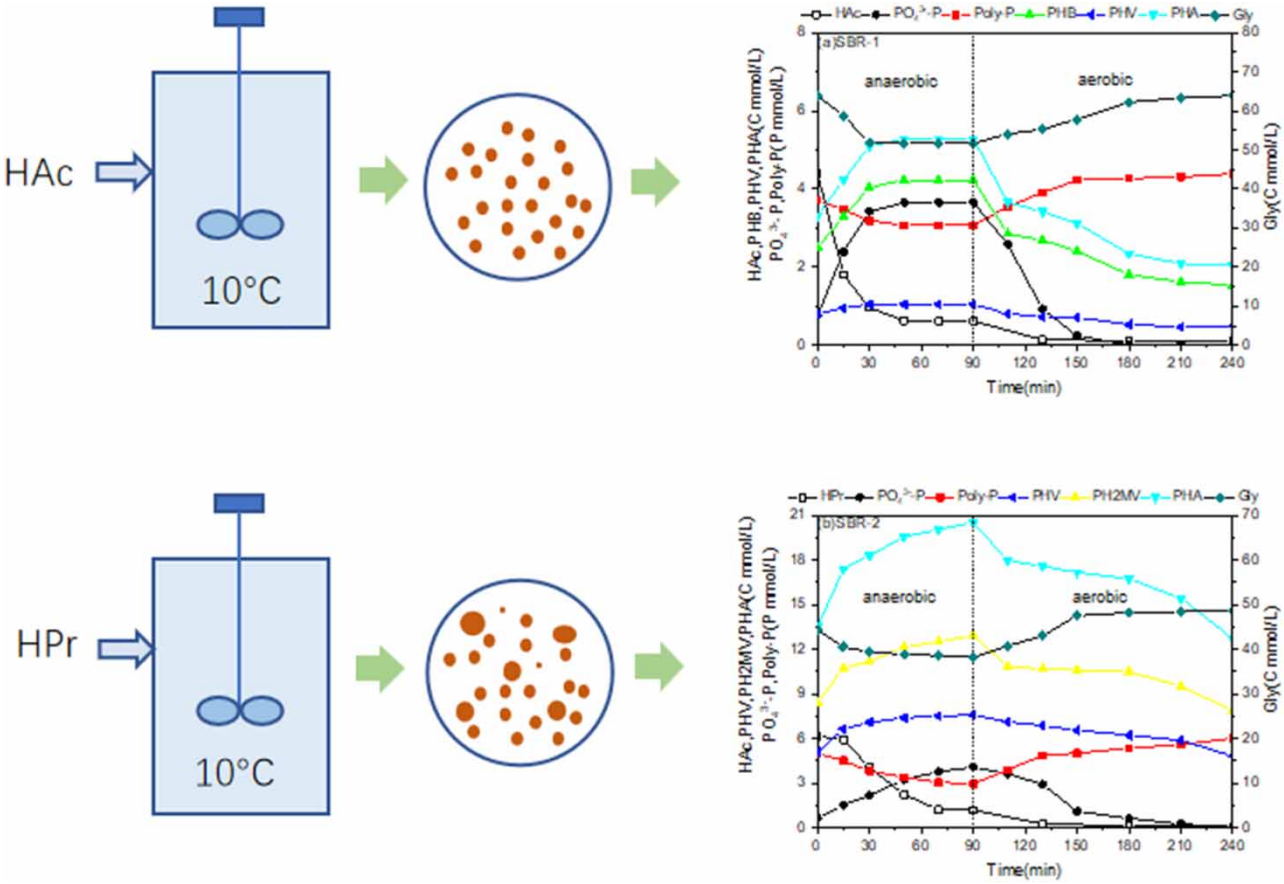
This study explored the microbial population dynamics of *Accumulibacter* (*Acc*) at low temperature and metabolic shift to limiting polyphosphate (Poly-P) in enhanced biological phosphorus removal (EBPR) system. The *Accumulibacter*-enriched EBPR systems, fed with acetate (HAc) and propionate (HPr) at 10 ± 1 °C respectively, were operated for 60 days in two identical SBR reactors (SBR-1 and SBR-2). The phosphorus removal performance in two systems was stable at 10 ± 1 °C, while the microbial community structure changed. Compared with the population structure in seed sludge, *Accumulibacter* clades reduced in the HAc system, while *Acc* I increased significantly in the HPr system. Low temperature was beneficial to the formation of granular sludge in the EBPR system, and the sludge granulation in the HAc system was more homogeneous than that in the HPr system. *Accumulibacter* in the HPr system can get ATP through glycogen accumulating metabolism (GAM) under limiting Poly-P condition at 10 ± 1 °C, while that in the HAc system cannot. This work suggests that poly-P levels can affect the metabolic pathway of *Accumulibacter* in EBPR systems under low temperature.

Key words: *Candidatus Accumulibacter phosphatis*, granular sludge, limiting polyphosphate, low temperature, metabolic shift

HIGHLIGHTS

- Low temperature was more beneficial to the proliferation of Acc I feeding with HPr.
- Sludge granulation was achieved in the EBPR system at 10 ± 1 °C.
- Acc in HPr system can behave as GAM under the limiting Poly-P at 10 ± 1 °C.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is one of the main processes for phosphorus removal in the activated sludge sewage treatment system. Polyphosphate accumulating organisms (PAOs) are the functional microorganism in the EBPR process. The process utilizes the metabolic characteristics of PAOs, which can release phosphate under anaerobic conditions and store polyphosphate (Poly-P) under aerobic or anoxic conditions. Through the discharge of Poly-P-enriched sludge, the removal of excess phosphorus in sewage is realized. Therefore, the activated sludge in the EBPR system enters the anaerobic environment firstly and then enters the aerobic or anoxic environment, alternately circulating. In the anaerobic phase, the Poly-P hydrolysis and glycogen degradation processes respectively provide ATP and reducing equivalent (nicotinamide adenine dinucleotide, NADH) for PAOs uptake of volatile fatty acid (VFA), and then VFA is transformed into polyhydroxyalkanoates (PHA) and stored in the cells (Mino *et al.* 1998). During the subsequent aerobic phase, PAOs degrade PHA to produce ATP, which is used to restore glycogen and Poly-P. In the EBPR system, glycogen accumulating organisms (GAOs) often exist, which have similar metabolic behavior to PAOs (Carvalheira *et al.* 2014). However, the GAOs only take glycogen as the source of ATP and NADH, and this process does not involve the degradation of Poly-P. Therefore, the proliferation of GAOs has adverse effects on the uptake of VFA by PAOs under anaerobic conditions. PAOs and GAOs often coexist in EBPR systems and the highly enriched PAOs culture is expected. Hence the competition between PAOs and GAOs has been paid extensive attention (Winkler *et al.* 2011; Shen & Zhou 2016; Wang *et al.* 2021; Ni *et al.* 2022). Schuler & Jenkins (2003) defined the metabolic patterns of PAOs and GAOs as polyphosphate accumulating metabolism (PAM) and glycogen accumulating metabolism (GAM), respectively.

Recent studies have found that PAOs showed metabolic shift linking GAO behaviors when intracellular Poly-P was in a limiting level (Zhang *et al.* 2015; Welles *et al.* 2016a; Acevedo *et al.* 2017), with VFA taken-up and glycogen consumed

but without the release and absorption of phosphate. Limiting Poly-P means that, in the anaerobic phase, Poly-P is not sufficient to generate ATP for PAOs to uptake VFAs from liquid. For example, decreasing the influent P concentration (Acevedo *et al.* 2017) or increasing the Ca^{2+} concentration (Zhang *et al.* 2015) both caused the reduction of the available Poly-P in PAOs cells, resulting in the metabolic shift of PAOs from PAM to GAM. The metabolic process of PAOs can be indicated by a number of stoichiometric ratios, and the changes in stoichiometry could directly reflect the metabolic shift of PAOs from PAM to GAM. The typical GAM showed that the $P_{release}/HAc_{uptake}$ ratio of PAOs was almost zero, accompanied by higher $PHA_{synthesized}/HAc_{uptake}$ ratio and $Gly_{degraded}/HAc_{uptake}$ ratio and so on. Combined with microbial population analysis, it was found that the metabolic characteristics of PAO clades were different under the limiting Poly-P conditions. *Candidatus Accumulibacter phosphatis* (hereinafter referred to as *Accumulibacter* or *Acc*) is currently recognized as playing a leading role in PAOs, which can be divided into two main clades of *Accumulibacter* I type and II type (as *Acc* I and *Acc* II) based on gene 16SrRNA and polyphosphate kinase genes (ppk1) (Peterson *et al.* 2008; Kolakovic *et al.* 2021).

When intracellular Poly-P was depleted in the short term, the metabolic pathway of *Accumulibacter* would be changed. At the same time, the population structure of PAOs would be affected by limiting Poly-P level. However, there are different observations about PAOs microbial structure variation in literature, when Poly-P storage was kept in a limited level. Acevedo *et al.* found that *Acc* clades changed from *Acc* I to *Acc* II as Poly-P decreased (Acevedo *et al.* 2012). Welles *et al.* (2015) observed that the HAc-uptake of *Acc* II was higher than *Acc* I when *Accumulibacter* metabolized by GAM. It seemed to suggest that *Acc* II exhibited higher adaptability than *Acc* I under Poly-P deficiency. However, some scholars found that GAOs did not proliferate and *Acc* I could maintain GAM in a long period under lower Poly-P level (Acevedo *et al.* 2017). Welles *et al.* (2016b) detected *Acc* II and *Competibacter* coexisted in a low-phosphorus enrichment system, indicating that *Acc* II could behave as GAM for a long time under limiting Poly-P. Even in some studies, *Accumulibacter* was washed out and GAOs became prevailing with Poly-P restricted (Lv *et al.* 2014). Therefore, further researches are needed to explore the *Acc* clades dynamics under limiting Poly-P.

Temperature and carbon source are known as two key factors for EBPR performance and PAOs microbial community structure (Wang *et al.* 2020a; Ni *et al.* 2021). Temperature can influence the metabolic pathway of PAOs and competition between PAOs and GAOs (Winkler *et al.* 2011). An EBPR system operating at SRT = 5 d showed that the phosphorus removal activity was lost when the temperature dropped to 10 °C (Chan *et al.* 2020). Nevertheless, efficient EBPR was successfully achieved at 10 ± 1 °C (Wang *et al.* 2020b). It was found that temperature was also an important factor affecting the metabolism characteristics of *Accumulibacter* with Poly-P limitation. Under the restriction of Poly-P, *Acc* I did not seem to switch to GAM at low temperature (Tian *et al.* 2013). However, there are few studies on the metabolism shift of *Accumulibacter* at low temperature, especially for a long time. Moreover, the longer-term effect of low temperature on the population structure and metabolic behavior of *Accumulibacter* is not well understood.

The main objective of this research is to investigate the microbial community dynamics of *Accumulibacter* at low temperature and the metabolic shift to limiting Poly-P. The results of this research could help for in-depth understanding of *Accumulibacter* metabolism and activity variation at lower temperature under Poly-P limitation.

2. MATERIALS AND METHODS

2.1. Reactor setup and operation

Two identical SBR reactors (Figure 1) with 12 L capacity were operated for 60 days at 10 ± 1 °C, recorded as SBR-1 and SBR-2 respectively. The temperature was controlled by a water bath. The two systems have operated for 180 days at 20 ± 1 °C and enriched with *Accumulibacter*, which were fed with sodium acetate (SBR-1, HAc system) and sodium propionate (SBR-2, HPr system) as the sole carbon source, respectively. Two constant temperature control devices were set up to ensure that the temperature in both reactors was 10 ± 1 °C. The sludge was discharged at the end of every aerobic phase, controlling the sludge retention time (SRT) at 12 days. The dissolved oxygen (DO) in the aerobic phase varied between 2.5 and 3.0 mg/L. The initial pH for each cycle was 7.5 ± 1 and ranged from 7.0 to 8.8 during each cycle. The reactors were operated for four cycles per day with a weekly period of 6 h including 5 min for filling, 90 min for stirring to simulate the anaerobic condition, 150 min for aerating, 80 min for settling and withdrawing, and the rest of the time for static. Each cycle was flooded with 6 L synthesized wastewater, which were the same except for the carbon sources. The main influent components were as follows (Wang *et al.* 2020b): 400 mg COD/L (512.5 mg/L NaAc for SBR-1 and 342.86 mg/L NaPr for SBR-2), 25 mg P/L (109.68 mg/L KH_2PO_4), 20 mg N/L (76.43 mg/L NH_4Cl), 153.75 mg/L $MgSO_4{\cdot}7H_2O$ and 15 mg/L Ca^{2+}

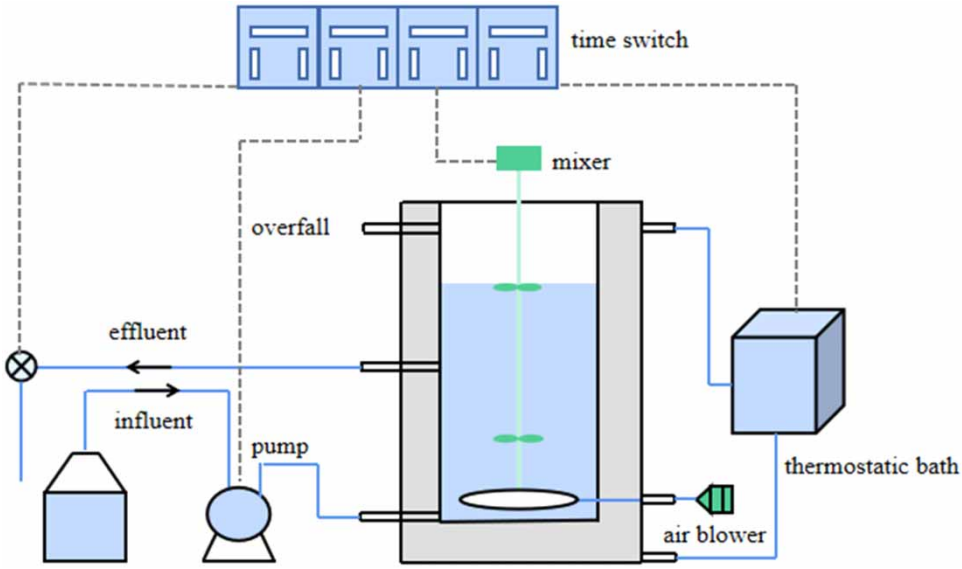


Figure 1 | The schematic diagram of the SBR used in this study.

supplied by tap water. In addition, 0.5 mL trace element solution was added to the influent per liter, which was prepared as described by Smolders *et al.* (1994). The trace element solution included (in g/L): KI (0.18), H_3BO_3 (0.15), $CoCl_2{\cdot}6H_2O$ (0.15), $MnCl_2{\cdot}4H_2O$ (0.12), $Na_2MoO_4{\cdot}2\ H_2O$ (0.06), $ZnSO_4{\cdot}7H_2O$ (0.12), $CuSO_4{\cdot}5H_2O$ (0.03), $FeCl_3{\cdot}6H_2O$ (1.50) and EDTA (6.00).

2.2. Experimental design

DO and pH were conducted to ensure SBR-1 and SBR-2 in the stable conditions after changing the temperature during the operation. The other routine monitoring parameters included orthophosphate (PO_4^{3-}-P), COD, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS). The reactor operated for 60 days (5 SRT) at 10 $\pm$ 1 °C. It was considered that the period was long enough to reflect the influence of low temperature on the microbial community structures and metabolic shift characteristics of the EBPR systems. After stable operation for 60 days, the population identification and cycle metabolic characterization (Cycle 1) for SBR-1 and SBR-2 were carried out, calculating the related biological kinetic rates and stoichiometry during the conversions. Cycle 1 represented the metabolic characteristics of SBR-1 and SBR-2 under high Poly-P condition at 10 $\pm$ 1 °C. And then an extraction process was added at the end of the anaerobic phase during each operation cycle. After the reactors were static for 30 min, the supernatant was removed and the synthesized wastewater was replaced without VFA and P. The extraction process was followed by Acevedo *et al.* (2017). After five extraction cycles, Cycle 2 was conducted to characterize the metabolic characteristics of *Accumulibacter* under short term limiting Poly-P condition. In Cycle 2, there is no phosphorus in influent and other components were the same as before. The measured parameters included PO_4^{3-}-P, VFA, PHA, Poly-P, glycogen during the whole of the cycles. In addition, MLSS and MLVSS at the initial and end of the aerobic phase and end of the anaerobic phase were determined.

2.3. Chemical analyses

The determinations of MLSS, MLVSS, PO_4^{3-}-P and COD were carried out according to Standard Methods (APHA 1998). PHA were determined by gas chromatography, according to the methods of Oehmen *et al.* (2005). The method of glycogen determination was modified as described in Oehmen *et al.* (2004). A certain amount of freeze-dried solid was taken and digested for 5 h at 100 °C after adding 5 mL 0.6 mol/L HCl solution. The content of Poly-P was calculated on the basis of TP and MLVSS (Acevedo *et al.* 2015). The DO, pH, and temperature were measured using DO and pH probes (Multi 340i, WTW company, Germany).

2.4. Identification of microbial population and characteristics of biomass morphology

Fluorescence *in situ* hybridization (FISH) technique was used to identify the population structures of *Accumulibacter* and GAOs in SBR-1 and SBR-2, following the procedure described by Winkler *et al.* (2011). The population structures of

inoculated sludge were also analyzed to investigate the influence of temperature on the population structure of the EBPR system. The 'all bacteria' corresponded to the EUB mix probe, which was a mixture of the EUB, EUB I and EUB II probes. *Acc* I and *Acc* II were targeted by the probes *Acc*-1-444 and *Acc*-2-444, respectively. The GAO mix probe (mixture of probes GAOQ431 and GAOQ989) targeted '*Candidatus Competibacter phosphatis*' (belonging to GAOs). The formamide concentration of all probes was 35%.

After hybridization, 10 randomly selected fluorescent images were analyzed by images software. The abundances of *Acc* I, *Acc* II and GAOs were expressed as the proportion of relative probes to the all bacteria probe based on the optical density calculation. The standard error of the mean (SEM) is calculated as the standard deviation divided by the square root of the number of images. The particle size distribution was identified, combined the wet sieving method (Laguna *et al.* 1999).

3. RESULTS AND DISCUSSION

3.1. Phosphorus removal performance at 10 ± 1 °C

The SBR-1 and SBR-2 reactors were operated with sodium acetate and sodium propionate at 10 °C for 60 days. Figure 2 represented the influent and effluent COD, PO_4^{3-}-P, MLSS and VSS/SS in two systems.

It was observed that the P removal performance tended to a stable state when SBR-1 and SBR-2 operated for 8 days. The average PO_4^{3-}-P concentration of effluent was 0.887 and 0.148 mg/L, and COD concentration was 0.254 and 0.414 C mmol/L, respectively. During the whole operation, the average MLSS of SBR-1 and SBR-2 was 4,200 ± 341 mg/L

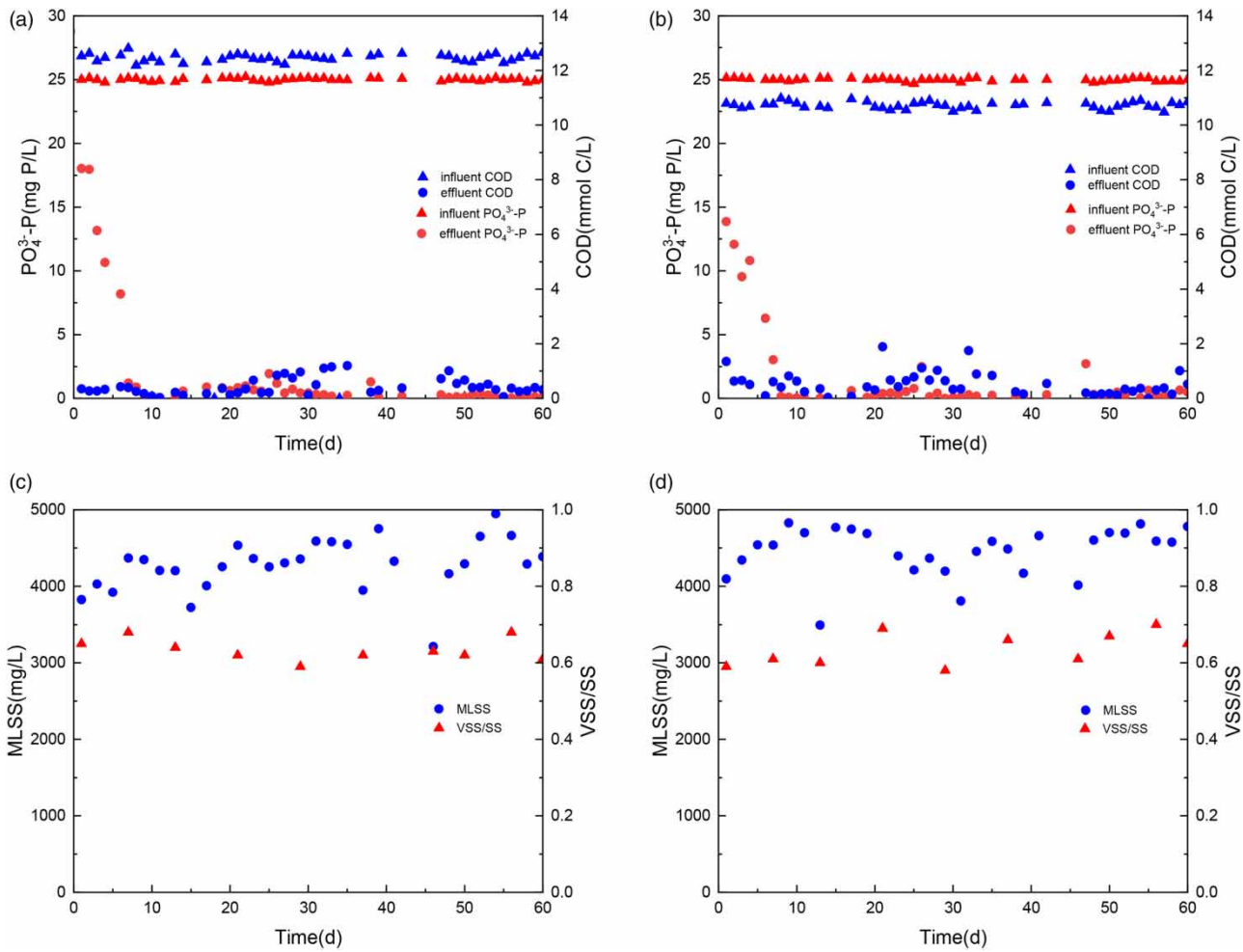


Figure 2 | Variation of COD, PO_4^{3-}-P, MLSS, and VSS/SS for SBR-1(a and c) and SBR-2(b and d).

and 4,500 ± 362 mg/L respectively and VSS/SS kept between 0.60–0.70 mg/mg, indicating that microorganisms in the systems could maintain good microbial activity and phosphorus removal ability at 10 ± 1 °C.

3.2. Microbial community and morphology

Low temperature environment could affect the specific microorganisms with phosphorus removal ability (Li *et al.* 2019). FISH was used to estimate the abundances of *Accumulibacter* and GAOs (Figure 3). The abundances of *Acc* I, *Acc* II and GAOs were represented as the proportions of relative probes to the all bacteria probe, as shown in Table 1. After running for 60 days at 10 ± 1 °C, the abundance of *Acc* I and *Acc* II in SBR-1 were 10 ± 2% and 16 ± 4%, and that in SBR-2 were 49 ± 11% and 26 ± 3% respectively. Compared with the population structure in the seed sludge, *Accumulibacter* clades decreased in SBR-1, while *Accumulibacter* in SBR-2 still remained at the original level and *Acc* I become the dominant population in the HPr system. Tian *et al.* (2013) only detected the presence of *Acc* I in *Accumulibacter* culture (NaAc·3H_2O as carbon source, SRT = 16d, 36d at different phases) enriched at 10 ± 1 °C. Combined with the results of this study, it seemed that low temperature and HPr with a SRT of 12d were more beneficial to the enrichment of *Acc* I. Previous studies suggested that low temperature would reduce the aerobic metabolic rate of PAOs and then influence minimal SRT for PAOs growth (Chan *et al.* 2020). Therefore, low temperature and HPr can be used as the key factors to enrich *Acc* I with a proper SRT. The abundance of GAOs became lower at 10 ± 1 °C, which verified the previous opinion that low temperature was not conducive to GAOs (Tian *et al.* 2013). Besides the effect of GAOs proliferation could be neglected when exploring the characteristics of GAM shift in the systems.

In addition, it was found that the biomass morphology of the systems changed significantly during the low-temperature operation, gradually forming granular sludge. Figure 4(a) showed the particle size distribution of the sludge particles in SBR-1 and SBR-2. The particle size of the sludge in SBR-1 was relatively small, and the maximum particle size was between 1.0 and 1.6 mm, accounting for 16.1% (/TSS). The proportion of particles of 0.5–1.0 mm was highest, accounting for 53%. However, the distribution of sludge particles in SBR-2 was inhomogeneous, and the diameter of certain sludge particles could reach 4.0 mm. Although the proportion of particles below 0.5 mm was highest (46.4%), the particles (38.4%) larger than 1.0 mm increased significantly compared with the original seed sludge in SBR-2. Figure 4(b) showed the sludge particles with diameters of 2.5–4.0 mm in SBR-2. It can be seen that during the low-temperature operation, more large size sludge

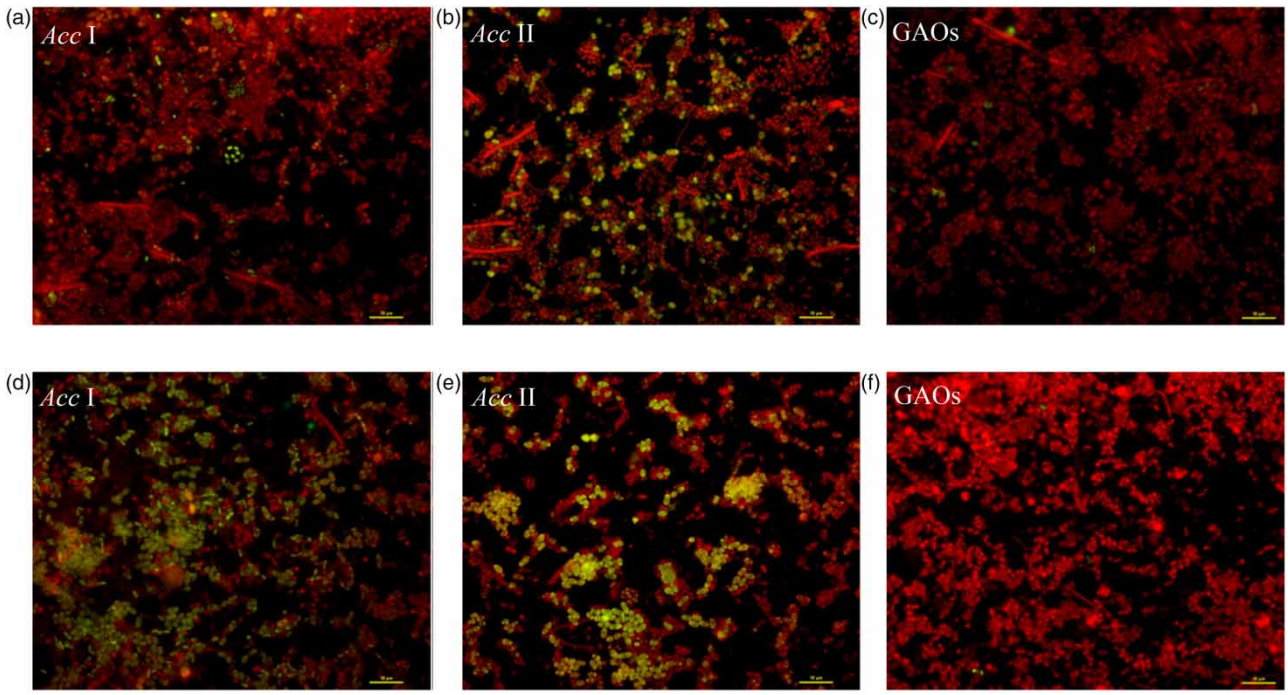


Figure 3 | Representative FISH images of the relative microbial population in SBR-1(a–c) and SBR-2(d–f). (samples taken on day 60) *Accumulibacteria*(*Acc*-I and *Acc* II) and GAOs are shown in yellow and all other bacteria are shown in red. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.036.

Table 1 | Comparison of population abundance between seed sludge and the enriched cultures running for 60 days in SBR-1 and SBR-2

		HAc		HPr	
		Seed sludge	SBR-1	Seed sludge	SBR-2
Acc I	%	19 ± 5	10 ± 2	37 ± 11	49 ± 11
Acc II	%	26 ± 13	16 ± 4	35 ± 4	26 ± 3
GAO	%	8 ± 3	3 ± 2	2 ± 1	1 ± 0

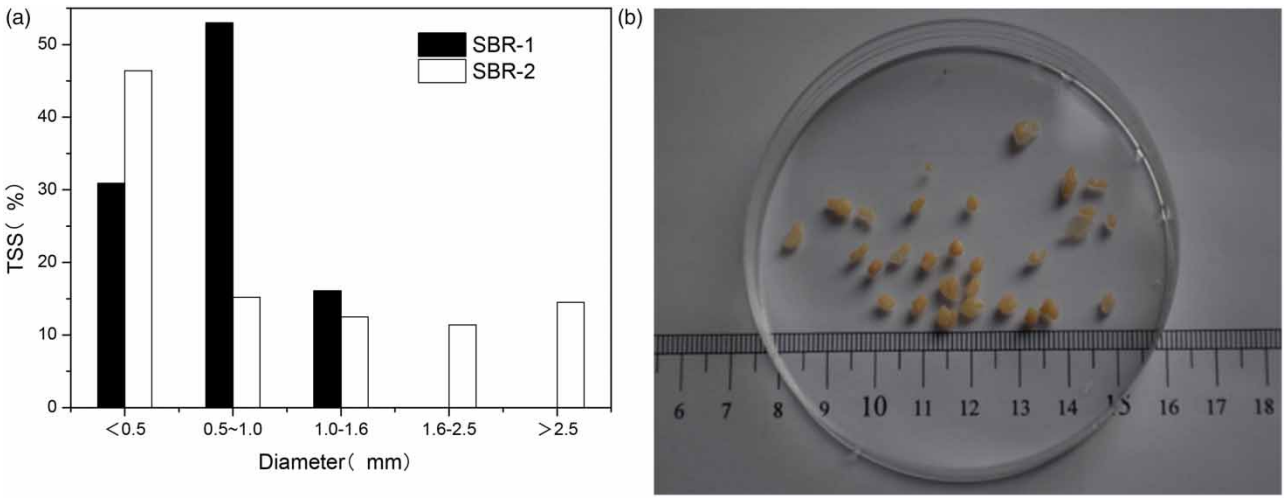


Figure 4 | Particle size distribution of sludge particles in SBR-1 and SBR-2 (a) and camera picture of the sludge particles with diameter of 2.5–4.0 mm in SBR-2 (b).

particles were formed in SBR-2. Although fine particles in SBR-1 was not as obvious as in SBR-2, the change of the average particle size of SBR-1 in the process could not be ignored. Compared with parent systems, temperature was the crucial factor that was changed in both reactors. Therefore, it can be concluded that low temperature was beneficial to the sludge granulation in the EBPR system, which was consistent with previous researches (Tian *et al.* 2017; Wang *et al.* 2020b). It probably resulted from the formation of alginate-like exopolysaccharides (ALE) at low temperature, which could promote the granulation of sludge (Lin *et al.* 2013; Tian *et al.* 2017). Interestingly, there were some differences of sludge granulation in the two EBPR systems at low temperature. The sludge granule size in HAc system was more homogeneous than that in HPr system, while the proportion of larger size (>1.0 mm) granules in HPr system was higher than that in HAc system. The different organic substrate was probable the main reason which led to different granulation, and further research is needed to investigate the mechanisms (Cai *et al.* 2019). The proportion of particles below 0.5 mm was highest (46.4%) in SBR 2, and that in 0.5–1.0 mm was highest (53%) in SBR 1, which indicated that the granule in SBR1 was more prominent than in SBR 2. In this study, larger size granules were achieved by feeding with HPr, which was a little bit different from the observation of Cai *et al.* (2016). Cai *et al.* (2016) observed that the larger and more stable granular sludge was achieved by mainly feeding HAc. This may be due to the different temperatures (the operating temperature of Cai *et al.* (2016) was 25 ± 2 °C). And the other possibility was that the *Accumulibacter* abundance of the HPr system was significantly higher than that of the HAc system in this study, which was more conducive to the formation of ALE.

3.3. Metabolic conversion of substrate

3.3.1. Metabolic behavior of PAOs in high Poly-P cycle

Figure 5 (a) and 5(b) presented the metabolic conversions of SBR-1 and SBR-2 in high Poly-P cycle (Cycle 1) at 10 ± 1 °C. It can be seen that both systems conducted typical PAM. Under the anaerobic phase, *Accumulibacter* took up VFA to synthesize PHA, with degradation of Poly-P and glycogen. And then during the aerobic phase, *Accumulibacter* utilized ATP

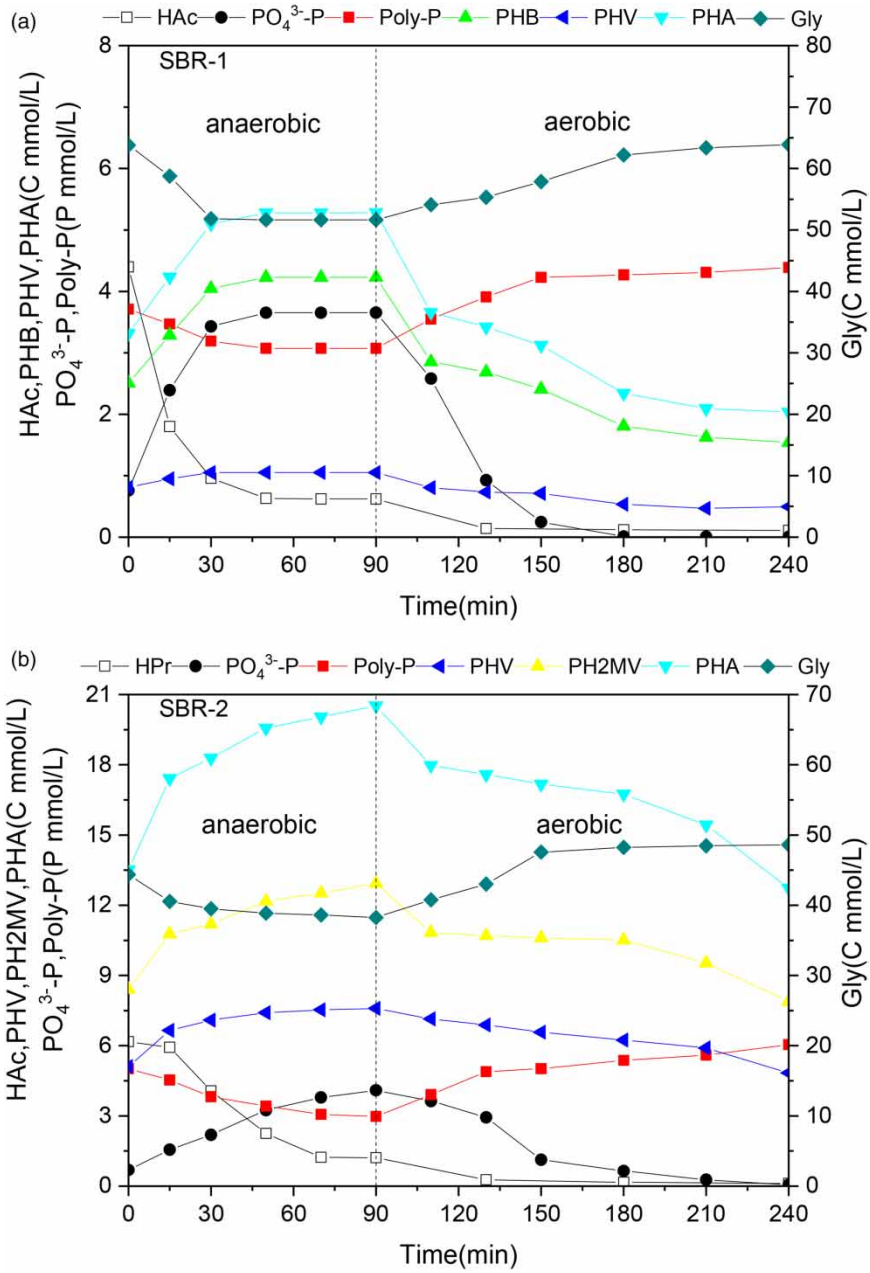


Figure 5 | Cycle metabolic conversions in SBR-1 (a) and SBR-2 (b) in high Poly-P cycle.

produced by PHA degradation to resynthesize Poly-P and glycogen. However, there were some differences of parameters in the cycles. The metabolism of SBR-1 tended to suspend after 50 min during the anaerobic phase, basically completing the process of phosphate release and HAc uptake. The release of phosphate in the anaerobic phase was 2.897 P mmol/L and the HAc uptake was 3.783 C mmol/L with the rate of 1.184 (C mol/mg VSS·h). The release of phosphate in SBR-2 lasted to the end of the anaerobic phase, and the content of PHA increased continuously. In the anaerobic process, the release of phosphate was 3.408 P mmol/L and the uptake of HPr was 4.953 C mmol/L with the rate of 0.910 (C mol/mg VSS·h). In the anaerobic phase, the degradation of glycogen and Poly-P in SBR-1 were 12.152 C mmol/L and 0.639 P mmol/L respectively and that in SBR-2 were 6.153 C mmol/L and 2.044 P mmol/L. Besides, this may be due to the diverse metabolic pathways of *Accumulibacter* utilizing different carbon sources, resulting in different components and quantities of PHA. The amount of PHA synthesized in the HPr system was greater. According to the stoichiometry of the anaerobic phase (Table 2), it

Table 2 | Comparison of anaerobic and aerobic stoichiometry in SBR-1 and SBR-2

					Anaerobic phase						Aerobic phase	
Cycle	VFA	Poly-P mg P/ Mg VSS	SRT d	T °C	P/VFA P mol/ C mol	PHA/ VFA C mol/ C mol	PHB/ VFA C mol/ C mol	PHV/ VFA C mol/ C mol	PH2MV/ VFA C mol/ C mol	Gly/ VFA C mol/ C mol	P/PHA P mol/ C mol	Gly/ PHA C mol/ C mol
1	HAc	0.036	12	10	0.765	0.520	0.456	0.064	–	3.125	1.124	3.770
2	HAc	0.013	12	10	0.286	0.305	0.257	0.048	–	1.528	0.785	2.478
1	HPr	0.043	12	10	0.685	1.413	–	0.500	0.912	1.236	0.518	1.332
2	HPr	0.017	12	10	0.259	0.632	–	0.309	0.323	2.466	0.449	2.970

was found that more glycogen and Poly-P degradation were needed in the HAc system than that in the HPr system for the synthesis of per PHA.

During the aerobic phase, PHA degradation and resynthesis of Poly-P and glycogen could be observed. After 150 min, the Poly-P synthesis in SBR-1 was basically completed, but PHA continued to be consumed with glycogen increasing remarkably. In SBR-2, Poly-P was in an increasing trend, while glycogen synthesized slowly. This phenomenon indicated that *Accumulibacter* showed a preference for Poly-P synthesis at the aerobic phase. The ATP produced by PHA was firstly used for the synthesis of Poly-P, followed by glycogen regeneration. This was in accordance with Welles *et al.* (2016a) that Poly-P was the preferred storage polymer for PAOs.

3.3.2. Metabolic behavior of PAOs in limiting Poly-P cycle

Figure 6(a) and 6(b) show the metabolic conversions of SBR-1 and SBR-2 in limiting the Poly-P cycle (Cycle 2) at 10 ± 1 °C. After the extraction period, the intracellular Poly-P level of *Accumulibacter* decreased greatly. The P release in SBR-1 stopped at 70 min in the anaerobic phase, with the P release 0.772 P mmol/L and HAc uptake rate 0.883 (C mol/mg VSS·h). HAc was not completely uptaken, but glycogen was no longer consumed. Therefore, the surplus HAc entered the aerobic phase. The $P_{release}/VFA_{uptake}$ ratio of the anaerobic phase was 0.286 P mol/C mol, which was lower than that in Cycle 1. At the aerobic stage, *Accumulibacter* still favored Poly-P storage, increasing the synthesis of glycogen after Poly-P synthesis completed. The glycogen synthesis was 3.947 and 10.851 C mmol/L, respectively. Compared with 12.246 and 10.378 C mmol/L in Cycle 1, the glycogen synthesis of SBR-1 was drastically reduced in Cycle 2. After five extraction cycles, the glycogen level of SBR-1 was reduced from the initial 60 C mmol/L to 15 C mmol/L. On the contrary, under limiting Poly-P, the glycogen content in SBR-2 almost didn't decrease, and the uptake rate of HPr was not slowed down. The uptake of HPr was at a rate of 0.989 (C mol/mg VSS·h) with the P release of 1.143 P mmol/L and $P_{release}/VFA_{uptake}$ ratio of 0.259 P mol/C mol. It suggested that *Accumulibacter* in SBR-1 could not behave as GAM under the limiting Poly-P condition at 10 ± 1 °C, while that in SBR-2 could perform GAM. Previous study concluded that, under P-limiting conditions, both *Acc* I and II could shift to a GAM, while *Acc* II has a competitive advantage over *Acc* I (Welles *et al.* 2015). Moreover, it was believed that the metabolic pathway of *Accumulibacter* is dependent on the clade and environmental conditions. In this study, total abundance of *Acc* in the HPr system (SBR-2) was higher than that in the HAc system (SBR-1) with a similar abundance of *Acc* II for the two systems. However, the clades of two types (*Acc* I, II) were not identified in this study. So, probably, there are some clade differences for the two systems.

3.3.3. Effect of limiting Poly-P on the kinetic rate and stoichiometry

After five extraction cycles, the intracellular Poly-P of *Accumulibacter* in SBR-1 and SBR-2 decreased significantly from 0.036 and 0.043 mg P/mg VSS to 0.013 and 0.017 mg P/mg VSS, respectively (Table 3). Poly-P was the main component of sludge ash, which can reflect the proportion change of inorganic suspended solids (ISS) in total suspended solids (TSS). The Poly-P variation was in accordance with the variation of ISS/TSS ratio.

The kinetic rate and stoichiometry during the metabolic process of *Accumulibacter* can correspond to different metabolic patterns. Therefore, the changes in these stoichiometric parameters can be used as a method for estimating metabolic pathway (PAM or GAM) in an EBPR system. The difference between PAM and GAM is that the energy required for VFA absorption is supplied by Poly-P(PAM) or only by glycogen (GAM). The phosphate was released into the liquid during the Poly-P degradation. Therefore, the change trend of phosphate and glycogen is the key to investigation of the metabolic

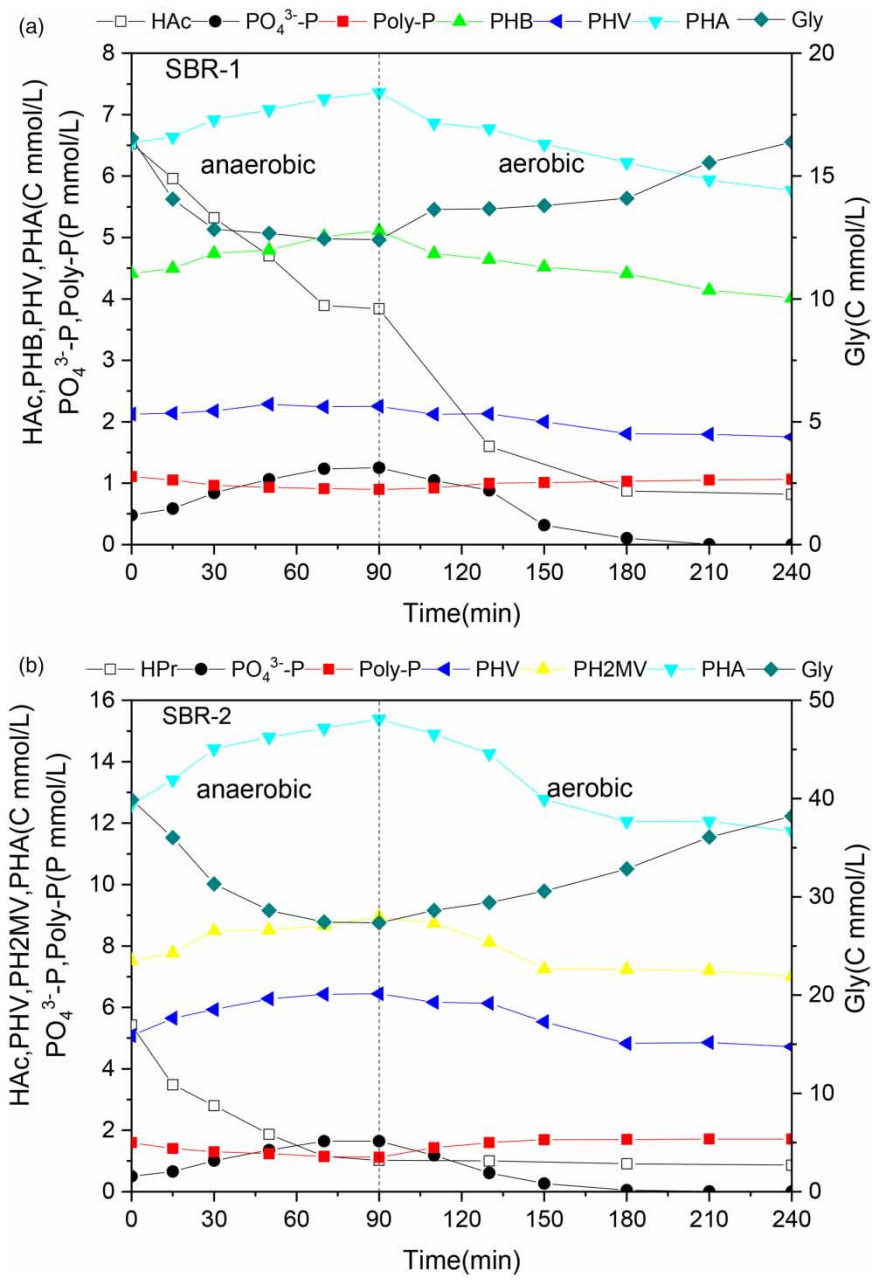


Figure 6 | Cycle metabolic conversions of SBR-1 (a) and SBR-2 (b) in limiting Poly-P at 10 ± 1 °C.

shift in the system. The metabolic shift of PAM to GAM will reduce the $P_{release}/VFA_{uptake}$ ratio. Besides, in the anaerobic phase, the utilization of glycogen will be increased, and more glycogen degradation will provide more ATP and NADH, showing $Gly_{degraded}/VFA_{uptake}$ ratio increasing, when PAM shifts to GAM. Combined with the stoichiometric parameters in Table 2, $P_{release}/VFA_{uptake}$ ratios of SBR-1 and SBR-2 were reduced from 0.765 and 0.685 P mol/C mol of Cycle 1 to 0.286 and 0.259 P mol/C mol, respectively. From higher to limiting Poly-P condition, $Gly_{degraded}/VFA_{uptake}$ ratio of SBR-2 increased from 1.236 to 2.466 C mol/C mol, while that of SBR-1 decreased from 3.125 to 1.528 C mol/C mol. It indicated that under Poly-P limitation, *Accumulibacter* in SBR-1 cannot get ATP from glycogen degradation.

The studies of metabolic shift for *Accumulibacter* were usually based on HAc (Acevedo *et al.* 2012; Acevedo *et al.* 2015; Welles *et al.* 2015; Acevedo *et al.* 2017), accompanied by the higher $PHA_{synthesized}/VFA_{uptake}$ ratio. However, in this study $PHA_{synthesized}/VFA_{uptake}$, $PHB_{synthesized}/VFA_{uptake}$ and $PHV_{synthesized}/VFA_{uptake}$ of the HAc system were reduced under the

Table 3 | Measures regarding MLSS, IS/SS and Poly-P of SBR-1 and SBR-2 in Cycle 1 and 2

		MLSS			ISS/TSS			Poly-P/VSS		
System	Cycle	a mg/L	b	c	a mg/mg	b	c	a mgP/mgVSS	Δanaerobic	Δaerobic
SBR-1	1	4663	4533	4497	0.32	0.31	0.36	0.036	0.006	0.014
	2	3203	3156	3328	0.18	0.17	0.21	0.013	0.002	0.002
SBR-2	1	5189	4439	5019	0.30	0.29	0.33	0.043	0.017	0.028
	2	3796	3887	3938	0.22	0.20	0.22	0.017	0.005	0.006

Note: a, b and c represent the early stage of anaerobic, the end stage of anaerobic and the end of the aerobic stage, respectively.

condition of Poly-P deficiency. The components of PHA were different in the HAc system and HPr system. Researches on metabolic shift using HPr as the carbon source are lacking. Although $PHA_{synthesized}/VFA_{uptake}$ ratio of SBR-2 during the anaerobic phase was also decreased in Cycle 2, the stoichiometric ratio of the aerobic phase could still reflect GAM occurrence in aerobic phase, $P_{uptake}/PHA_{degraded}$ ratio decreased and $Gly_{synthesized}/PHA_{degraded}$ ratio increased in SBR-2. This may be as a result of the low phosphate concentration in the liquid. The ATP used to synthesize Poly-P was reduced, and the excess ATP produced by PHA degradation promoted the synthesis of glycogen. Contrasting with SBR-2, the $Gly_{synthesized}/PHA_{degraded}$ ratio in SBR-1 decreased.

The changes mentioned above demonstrated that *Accumulibacter* in the HAc system cannot behave as GAM under limiting Poly-P condition at 10 ± 1 °C, but that in the HPr system it has the ability to get ATP through GAM, so as to avoid being in a disadvantaged position. Two *Accumulibacter* types (I and II) coexisted in SBR-1 and SBR-2. The difference between SBR-1 and SBR-2 is the carbon source. Maybe different conversion pathways of carbon sources lead to the distinction in the utilization of glycogen. Another possibility is that *Accumulibacter* has numerous subclades with diverse metabolic characteristics, and it is unclear whether the different subclades play different roles in the metabolic process (Kolakovic *et al.* 2021). Further research is required to elucidate that.

4. CONCLUSIONS

After operating for 60 days at 10 ± 1 °C, the abundance of *Accumulibacter* in the HAc system reduced, while that of *Acc* I in the HPr system increased significantly, becoming dominant PAOs. Phosphorus removal performance of both HAc and HPr systems was stable and granular sludge formed gradually at 10 ± 1 °C. The sludge granulation in the two systems was different and there were more larger granules in the HPr system. *Accumulibacter* in the HPr system has the ability to get ATP through GAM, while that in the HAc system cannot do this under limiting Poly-P condition at 10 ± 1 °C. This work provides a deep insight into the metabolic behavior of PAOs under limiting Poly-P at low temperature, which is meaningful for maintaining a stable performance of an EBPR system.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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