The metabolism of polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) in enhanced biological phosphorus removal (EBPR) system under the tropical climate

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**The metabolism of Polyphosphate Accumulating Organisms
(PAOs) and Glycogen Accumulating Organisms (GAOs) in
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WANG LI

Interdisciplinary Graduate School

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(PAOs) and Glycogen Accumulating Organisms (GAOs) in
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under the tropical climate**

Wang Li

Interdisciplinary Graduate School

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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

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Authorship Attribution Statement

This thesis contains material from 2 paper published in the following peer-reviewed journal(s) where I was the first author.

Chapter 4 is published as Wang, L., Shen, N., Oehmen, A. and Zhou, Y. The impact of temperature on the metabolism of volatile fatty acids by Polyphosphate Accumulating Organisms (PAOs). *Environmental Research*, 109729 (2020). DOI: <https://doi.org/10.1016/j.envres.2020.109729>

The contributions of the co-authors are as follows:

- Prof. Zhou Yan provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof. Adrian Oehmen.
- I co-designed the study with Dr. Shen Nan and performed all the laboratory work at the Interdisciplinary Graduate School and the Nanyang Environmental Water Research Institute. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing.

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The contributions of the co-authors are as follows:

- Prof. Zhou Yan helped to design the study and edited the manuscript drafts.

-
- I wrote the drafts of the manuscript. The manuscript was revised together with Prof. Adrian Oehmen and Liu Jianbo.
 - I performed all the reactor operation, collected samples and analysis the performance, carried and conducted data evaluation
 - Dr. Le Chencheng conducted the PHAs determinations.

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- Prof. Zhou Yan helped to design the study and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof. Adrian Oehmen.
- I performed all the laboratory work, collected samples and analyzed the data.
- Dr. Liu Jianbo helped to analyzed the microbial data.
- Dr. Le Chencheng assisted in the analysis of PHAs data.
- Dr. Geng Yikun helped collecting the samples.

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Abstract

Enhanced biological phosphorus removal (EBPR) system has been widely applied in wastewater plants due to the economy and sustainability virtue. However, high temperature ($>30\text{ }^{\circ}\text{C}$) enhanced biological phosphorus removal (EBPR) is challenging because glycogen accumulating organisms (GAOs) can easily outcompete polyphosphate accumulating organisms (PAOs) under high temperature conditions. The nature of the carbon source is also known to impact the PAO-GAO competition, though previous studies have not assessed how carbon source impacts PAO metabolism at high temperature. This study investigated the effects of different carbon sources on two acetate/propionate enriched *Accumulibacter* PAO cultures at high temperature and compared the performance of carbon transformation with low temperature EBPR cases reported in literature, and revealed several key metabolic differences. Besides the common volatile fatty acids (VFAs) such as acetate and propionate, PAOs also utilized butyrate and iso-butyrate, but hardly used valerate and its isomer, iso-valerate. When acetate and propionate are limited, butyrate and iso-butyrate could be used as supplementary carbon source for EBPR. In addition, PAOs, under high temperature, seemed to prefer propionate compared with other VFAs. Nevertheless, high aerobic glycogen replenishment was realized with propionate as the sole carbon source anaerobically, a trait not previously observed at low temperatures, which may be one of the reasons for EBPR failure in the long-term operation with propionate as feed. A combined substrate of acetate, propionate and perhaps butyrate seems to be a better carbon source choice since the total VFA uptake rate was the highest when they were

present together in the anaerobic phase, and this increased the aerobic P removal efficiency and reduced the glycogen recovery compared to propionate as sole substrate.

GAOs have been previously observed to be more competitive for anaerobic volatile fatty acid (VFA) uptake as compared to PAOs at high temperatures. However, previous evidence regarding the impact of temperature on GAOs has exclusively focused on only one group of GAOs, namely *Candidatus Competibacter phosphatis* (or *Competibacter*). *Defluviicoccus vanus* GAO are another very important GAO group that have been found in EBPR plants, however, their competitiveness at high temperature for anaerobic VFA uptake was previously unknown. In this thesis, *Defluviicoccus vanus* GAO were enriched in two reactors at high temperature (30°C) and fed with acetate or propionate as sole carbon source to investigate their competitiveness for anaerobic VFA uptake at high temperature. The uptake rate of acetate or propionate in both GAO reactors was much lower than those of *Candidatus Accumulibacter phosphatis* PAOs operated under similar conditions, showing that PAOs displayed a competitive advantage at high temperature, unlike previous observations with *Competibacter* GAOs. The capacity of *Defluviicoccus vanus* GAOs to utilize other VFAs such as butyrate, iso-butyrate and valerate was also assessed. This study provides new insight into our understanding of GAOs in EBPR processes, where the impact of temperature on the PAO-GAO competition is highly dependent on the phylogenetic identity of the GAO present in the system.

Butyrate, as the supplementary or the sole carbon source, could be a promising carbon source for EBPR under tropical climate. When the carbon source of PAO reactor was gradually changed from acetate to butyrate, the abundance of *Candidatus Accumulibacter phosphatis* first decreased from 37.4% to 8.4% and then recovered to 13.9% after acclimation. Besides, *Rhodocyclaceae* increased from 2.0% to 14.5% who also played an important role in P-removal. Thus, a relatively stable P removal performance was realized throughout the whole operation period. Nevertheless, butyrate had negative impact on GAOs. The biomass and microbial diversity continually decreased in GAO reactor, and *Candidatus Competibacter phosphatis* reduced from 27.3% to 6.2% and the dominant population was replaced by *Zoogloea*. With addition of butyrate as carbon source, the total amount of synthesized PHAs reduced in both PAO and GAO cultures and the composition of PHA was greatly changed. The presence of novel PHA (PHH) may disturb the microbial activity in aerobic phase, where GAO culture was more severely affected. Glycogen turnover seemed to be limited in both reactors. This could reduce the GAO metabolism in both cultures and avoid the metabolism switching from PAO's to GAO's. Furthermore, the biomass growth rate of PAO culture was higher than that of GAO when fed with butyrate, which also provides PAO competitive advantage. All above results indicate that butyrate could not be well metabolized by GAOs but could provide PAOs with competitive advantage. The anaerobic butyrate uptake models by PAO and GAO were established, and the differences of their metabolic pathways were also studied based on metagenomic genes. The results show anaerobic carbon transformation of PAO culture

could be predicted by the random model where the acetyl-CoA and butyryl-CoA are randomly combined while the prediction of the selective model is more consistent with the experimental results of GAO culture. A higher gene expression of carbon transformation was found in PAO culture than that in GAO culture which suggests butyrate is more easily metabolized and converted to PHB and PHH by PAO. New strategies to promote EBPR performance at high temperatures are proposed based on the results of this work.

List of Abbreviations

COD	Chemical oxygen demand
DO	Dissolved oxygen
DvGAOs	<i>Defluviicoccus vanus</i> GAOs
EBPR	Enhanced biological phosphorus removal
FISH	Fluorescence <i>in situ</i> hybridization
GAOs	Glycogen-accumulating organisms
GC	Gas chromatography
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solid
NADH	Nicotinamide adenine dinucleotide
PAOs	Polyphosphate-accumulating organisms
PBS	Phosphate-buffered saline
PH2MV	Poly- β -hydroxy-2-methylvalerate
PHAs	Polyhydroxyalkanoates
PHB	Poly- β -hydroxybutyrate
PHH	Poly- β -hydroxyhexanoate
PHV	Poly- β -hydroxyvalerate
Poly-p	Polyphosphate
SBR	Sequencing batch reactor
SRT	Sludge retention time
TCA	Tricarboxylic acid
VFAs	Volatile fatty acids
WAS	Waste activated sludge
WWTPs	Wastewater treatment plants

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List of Publications

1. **Wang Li**¹, Shen Nan¹, Oehmen Adrian and Zhou Yan. 2020. The impact of temperature on the metabolism of volatile fatty acids by Polyphosphate Accumulating Organisms (PAOs). *Environmental Research*, 109729.
2. **Wang Li**, Oehmen Adrian, Le Chencheng, Liu Jianbo and Zhou Yan. 2020. *Defluviicoccus vanus* Glycogen-Accumulating Organisms (DvGAOs) Are Less Competitive Than Polyphosphate-Accumulating Organisms (PAOs) at High Temperature. *ACS ES&T Water*.
3. **Wang Li**, Liu Jianbo, Oehmen Adrian, Le Chencheng, Geng Yikun and Zhou Yan. 2021. Butyrate can support PAOs but not GAOs under tropical climate. *Water Research*, 193, 116884.
4. **Wang Li**, Oehmen Adrian, Liu Jianbo and Zhou Yan. The anaerobic metabolism of butyrate by polyphosphate accumulating organisms and glycogen accumulating organisms under tropical climate. *Submitted*

Chapter 1 Introduction

1.1 Background

Phosphorus is a necessary nutrient for the growth of aquatic organisms. However, excessive discharge of phosphorus into the water body could result in eutrophication. A large number of the plants and animals are affected due to the reduced dissolved oxygen (DO) in water body caused by the eutrophication. Therefore, phosphorus removal technologies have been developed to reduce phosphorus level in the wastewater before being discharged. Enhanced biological phosphorus removal (EBPR) system has been widely applied in wastewater plants due to the economy and sustainability virtue.

EBPR can be achieved through recirculating polyphosphate-accumulating organisms (PAOs) under alternating anaerobic and aerobic circumstances. In anaerobic environment, PAOs are able to take up and convert carbon sources, mainly volatile fatty acids (VFAs) to polyhydroxyalkanoates (PHAs). In this process, intracellular polyphosphate (poly-p) and glycogen are consumed to generate energy (ATP) and reducing power in PAOs. Meanwhile, orthophosphate will be released from polyphosphate degradation into the bulk solution. Under aerobic conditions, PAOs consume PHAs in cells and take up excessive phosphate and restore poly-p and glycogen at the same time (Hesselmann et al. 2000). However, a main competitor – glycogen-accumulating organisms (GAOs) always coexist in EBPR system and frequently cause the disturbance or failure of EBPR. GAOs always compete organic

carbon substrate with PAOs without contributing to phosphorus removal. GAOs were observed to be more competitive for anaerobic VFA uptake as compared to PAOs when temperature is above 25 °C (Lopez-Vazquez et al. 2009b, Whang and Park 2006). Nevertheless, a few high-temperature EBPR processes have been successfully operated recently indicating that PAOs could outcompete GAOs under certain conditions at high temperature (Ong et al. 2013, Shen et al. 2017, Winkler et al. 2011). Most of the previous studies on the competition between PAOs and GAOs were conducted at lower temperatures (i.e. <20 °C), or only reported short-term effects of high temperatures. The long-term effects of high temperatures on the competition between PAOs and GAOs are unknown. Therefore, it is important to investigate the competition between PAOs and GAOs at high temperature and reveal potential operational strategies that can keep a stable and good-performance of EBPR system in tropical regions.

1.2 Thesis objectives

The main objectives of this thesis are:

- (1) To study the effects of different carbon sources on PAOs enriched at high temperature. The kinetic and stoichiometry of PAOs at high temperature are compared with low-temperature PAOs reported in literature in order to improve the knowledge on how to maintain successful EBPR operation in warm climates.
- (2) To investigate the impact of carbon sources on *Defluviicoccus vanus* GAOs (*Dv*GAOs) operated at high temperature. The competitiveness of *Dv*GAOs for anaerobic VFA uptake at high temperature is studied and compared with PAOs

operated under similar conditions in order to explore the impact of temperature on the PAO-GAO competition.

- (3) To elaborate whether butyrate could be used as sole carbon source to promote the EBPR system under the tropic climate. The long-term performance of EBPR system fed by butyrate is studied in order to determine whether butyrate could be used as supplementary carbon source for EBPR.
- (4) To study the anaerobic metabolic model of butyrate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms and also to validate the model predictions with the experimental results. The intracellular biochemical transformation of butyrate is investigated and the factors that may affect the competition between PAO and GAO are also identified.

1.3 Scope of thesis

This thesis consists of eight chapters:

- (1) Chapter 1 introduces a brief background on the biological phosphorus removal technology and the main objectives of this thesis.
- (2) Chapter 2 presents a comprehensive literature review by introducing the basic principles of the EBPR system and its development history, and summarizing the achievements and limitations of the previous research.
- (3) Chapter 3 describes the details of parent reactors operation and sample analysis methods.
- (4) Chapter 4 investigates the effects of different carbon sources on enriched

Accumulibacter PAO cultures at high temperature (30 °C), compares the carbon transformation with low temperature (20 °C) cases reported in literature, and reveals several key metabolic differences.

- (4) Chapter 5 provides the new insight into our understanding of *Deftuviicoccus vanus* GAOs enriched by acetate or propionate in EBPR processes under tropic climate. The capacity of *Deftuviicoccus vanus* GAOs to utilize other VFAs such as butyrate, iso-butyrate and valerate are also assessed.
- (5) Chapter 6 further elaborates whether butyrate could be used as sole carbon source to promote the EBPR system, and compares the difference with commonly used acetate, propionate and mixed carbon source.
- (6) Chapter 7 develops the anaerobic metabolic model of butyrate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms and also validates the model predictions by the experimental results.
- (7) Chapter 8 summarizes the main outcomes of this thesis and recommendations for future research in the EBPR field.

Chapter 2 Literature review

2.1 Enhanced Biological Phosphorus Removal Process

Srinath et al. (1959) accidentally discovered that activated sludge could uptake phosphorus in excess of normal metabolic requirements when they studied the feasibility of growing rice plants. And it was found that more than 90% of soluble phosphorus could be removed by activated sludge. Later on, Levin and Shapiro (1965) proposed a mechanism for biological excess phosphorus removal based on metabolic pathways. EBPR process achieves net-P removal through recirculating the activated sludge containing PAOs under alternating anaerobic and aerobic conditions. This can be performed in a sequencing batch reactor (SBR) or continuous activated sludge process (Figure 2.1) (Izadi et al. 2020, Lu et al. 2006, Mino 2000, Shen et al. 2017). GAOs survive under similar conditions without contributing P-removal. In the anaerobic phase, PAOs or/and GAOs would have selective advantage over ordinary heterotrophic organisms that can only take up VFAs in aerobic condition to compete for carbon source. They assimilate the influent VFAs that are low in wastewater in anaerobic phase, leaving very limited VFAs for the heterotrophic organisms in the following aerobic phase. Therefore, PAOs would be expected to become the dominant population in EBPR system, but GAOs have similar anaerobic carbon metabolism and would compete with PAOs for carbon source in anaerobic phase. The VFAs are transformed into the corresponding PHAs using the energy from poly-P and glycogen degradation. In aerobic phase, PAOs are able to take up excessive phosphorus and store

as poly-P within cells. PAOs use the intracellular PHAs to provide energy for the phosphorus uptake and meanwhile poly-P and glycogen are replenished. Net P-removal would be achieved through discharging the high poly-P content waste activated sludge.

Figure 2.1 The principle of EBPR process.

EBPR is considered as one of the environmentally sustainable processes for phosphorus removal. High efficiency and stable of P removal could be obtained by optimizing operating parameters of EBPR process. However, the fluctuated phosphorus removal performance or failure of EBPR still occurs in some plants. Many factors such as competition for limited carbon source, wastewater quality variation, changed operational conditions may result in the fluctuations of EBPR. Thus, it is important to investigate the factors that may cause deteriorated EBPR process. Optimal operation and management are required to achieve a stable and effective phosphorus removal system.

2.2 Metabolisms of EBPR

2.2.1 Metabolism of PAOs

Polyphosphate-accumulating organisms is the main functional organisms in EBPR process. As introduced in Section 2.1, net-P removal is achieved through recirculating the PAOs in EBPR process. The metabolism of PAOs have been extensively studied previously (Hesseltmann et al. 2000, Lanham et al. 2014, Oehmen et al. 2005d, Smolders et al. 1994a, b). The metabolism of PAOs under anaerobic phase and aerobic phase can be briefly described as below:

During the anaerobic phase, PAOs will take up carbon source and release phosphorus:

During the aerobic phase, PAOs will degrade PHAs and uptake excess phosphorus:

Some metabolic models have been developed to predict the metabolic pathways in terms of reaction stoichiometry occurring in the biochemical transformations (Arun et al. 1988, Comeau et al. 1986, Smolders et al. 1994a). As pure strains of PAOs have not been isolated so far, these models were generally developed from the metabolic pathways of relating microorganisms and validated by comparing these models with the observations in the enriched cultures of PAOs. Comeau-Wentzel model was developed based on acetate as feed carbon source which is one of the most common VFAs in the influent. They assumed that the reducing power (i.e. NADH) for converting

the VFAs into PHAs in anaerobic phase was generated from the tricarboxylic acid (TCA) cycle. Instead, Mino et al. (1987) supposed that NADH was generated from internal glycogen degradation and proved this theory by using the experimental evidence (Mino et al. 1998). Mino model is widely accepted since then (Zhou et al. 2010). Based on Mino model, Smolders et al. (1994a) developed a more detailed anaerobic metabolic model using acetate as carbon source (Figure 2.2a). In this model, PAOs use energy from poly-P and glycogen degradation to take up acetate and convert it into acetyl-CoA, which will be transformed to poly- β -hydroxybutyrate (PHB) with the assistance of NADH. This process also releases phosphate to bulk solution. Although these reactions occur inside cells, the model is developed based on the experimental evidences and biochemical stoichiometry. The main content of PHAs is PHB when acetate is used as the carbon source.

Besides the anaerobic model, Smolders et al. (1994b) also proposed an aerobic stoichiometric model (Figure 2.2b). Glycogen recovery, poly-P replenishment and cell growth were quantified through the oxygen consumption. Basically, PHB degradation and oxidative phosphorylation generate energy that will be used for cell growth, poly-P restore and glycogen recovery. First, PHB is oxidized to CO_2 . This process generates NADH which later is converted to energy through oxidative phosphorylation. The produced energy supports PAOs' growth, cell maintenance, phosphate accumulation and storage and glycogen replenishment. The whole process is empirically determined by three coefficients: α (energy required for acetate uptake), δ (ATP produced from NADH), K (energy required for biomass synthesis), and ε (energy for the transport of

phosphate).

Figure 2.2 Model of the anaerobic metabolism (a) and aerobic metabolism (b) of the PAOs fed with acetate (modified from Smolder et al. 1994a,b).

Similar to the anaerobic metabolism of acetate by PAOs, Oehmen et al. (2005d) proposed the anaerobic metabolism of propionate by PAOs. The difference between propionate and acetate model is the PHA composition. Acetate is mainly converted to PHB while propionate is synthesized into poly- β -hydroxyvalerate (PHV) and poly- β -hydroxy-2-methylvalerate (PH2MV).

A typical PAO phenotype includes VFA uptake, PHA synthesis, glycogen degradation, cleavage of poly-P and P-release in anaerobic phase. An opposite process in aerobic phase consists of P-uptake, poly-P restoration, PHA oxidation and glycogen replenishment.

2.2.2 Metabolism of GAOs

Cech and Hartman (1990) found that some microorganisms can take up carbon source without P release under anaerobic phase in the EBPR system. Their substrate utilization

pattern is similar as PAOs except phosphate release. These microorganisms were named as “G bacteria” (Cech and Hartman 1993). Later, Mino et al. (1995) found that the microorganisms can upset the performance of EBPR process, deteriorate the phosphate removal and were identified as glycogen accumulating organisms (GAOs). Comparing to PAOs, which degrade the poly-P to provide the main energy anaerobically and recover and store poly-P in the following aerobic phase, GAOs hydrolyze glycogen to generate energy in anaerobic phase and rebuild glycogen aerobically. A typical GAOs phenotype includes VFA uptake, PHA synthesis and glycogen hydrolysis in anaerobic phase, and glycogen replenishment through PHA oxidation in aerobic phase. Compared to PAOs, GAOs neither release phosphate nor synthesize poly-P. Therefore, the glycogen conversion in GAOs is greater than that in PAOs.

Figure 2.3 Model of the anaerobic metabolism of the GAOs fed with acetate (modified from Zeng et al. 2003a).

Anaerobic metabolism model of GAOs (Filipe et al. 2001b, Zeng et al. 2003a) was proposed and shown in Figure 2.3. Energy that was generated by hydrolyzing glycogen

rather than poly-P was used to transport acetate into cell. It was believed that the conversion of glycogen to acetyl-CoA produced ATP and reducing power (NADH) through Embden-Meyerhof-Parnas pathway. Consequently, glycogen conversion efficiency in GAOs is much higher than PAOs. During this process, NADH was produced and a part of NADH was used to convert pyruvate to propionyl-CoA (Liu et al. 1994) to keep the reduction-oxidation balance. Thus, GAOs not only produce acetyl-CoA but also generate propionyl-CoA. Both are utilized to synthesize PHB and PHV. Compared to PAOs that mainly produce PHB with acetate (Mino et al. 1998, Satoh et al. 1992), GAOs are able to synthesize both PHB (around 75%) and PHV (approximately 25%) (Filipe et al. 2001b, Zeng et al. 2003a). Similar to the anaerobic metabolism of acetate by GAOs, Oehmen et al. (2004) also proposed the anaerobic metabolism of propionate by GAOs. Different to acetate, propionate was mainly synthesized into PHV and PH2MV by GAOs. The composition of PHA is mainly determined by the carbon source and the type of microorganisms. The different PHA composition synthesized by different carbon source and different microorganism are summarized in Table 2.1.

Table 2.1 Summary of carbon transformation during the anaerobic phase with different carbon sources by different organisms (C-mol/C-mol).

Culture	PHB/C	PHV/C	PH2MV/C	PHA/C
Acetate-PAO ^a	1.33	0	0	1.33
Propionate-PAO ^b	0	0.56	0.67	1.22
Acetate-GAO ^c	1.36	0.46	0.04	1.86
Propionate-GAO ^d	0	0.83	0.67	1.50

a: Smolders et al. (1994), b: Oehmen et al. (2005b), c: Zeng et al. (2003), d: Oehmen et

al. (2004)

In the GAO aerobic metabolism model (Filipe et al. 2001a, Zeng et al. 2003a), PHB and PHV are oxidized to CO₂ generating energy used for biomass growth, glycogen recovery and cell maintenance. The difference in the aerobic metabolism between PAOs and GAOs is the absence of poly-P accumulation in GAOs.

GAOs compete with PAOs for the limited VFA in the wastewater influent without any involvement of P removal, which always deteriorate the performance of EBPR and even cause the failure of EBPR systems. It is important to investigate the competition between PAOs and GAOs to optimize the operation conditions favoring PAOs.

2.3 Microbial identification

To date, no pure cultures that demonstrate the physiology of PAO or GAO have been successfully isolated in EBPR system (Oehmen et al. 2007, Seviour et al. 2003, Kotlyarov et al. 2019, Tarayre et al. 2016). Thus, culture-independent methods have been applied to identify which microorganisms are responsible for phosphorus removal or detrimental to EBPR system. Using ribosomal RNA (rRNA) to analysis the microbiology of activated sludge including EBPR has been widely applied in the past decades, because the cultivation of microorganisms in pure culture is not necessary. The specific probes to target the corresponding microorganisms could be designed through the phylogenetic information of cloned 16S rRNA gene sequences. Particular microorganisms could be visualized and quantified by fluorescence *in situ* hybridization (FISH) probes. FISH was a prevailing pool to identify and quantify PAOs and GAOs in EBPR process (Seviour et al. 2003). The rapid development and

popularization of molecular biology technology in recent years has enabled microbiological identification to get rid of the limitations of traditional biochemical identification methods and enter a new historical process. It is now only needed to extract genomic DNA of sludge, and carry out 16S rRNA Amplicon Sequencing to know the species of bacteria. Generally, the sequence similarity above 99% can be regarded as the same species of bacteria.

Bond et al. (1999) demonstrated that *Betaproteobacteria* was the dominant bacteria in a lab-scale EBPR system with good P removal performance through 16S rRNA and FISH analysis, and Hesselmann et al. (1999) showed that the *Betaproteobacteria* '*Candidatus Accumulibacter phosphoatis*' (also known as *Rhodocyclus*-related bacteria) are PAOs. Subsequent studies confirmed that *Accumulibacter* were ubiquitous PAOs in EBPR processes worldwide (Crocetti et al. 2000, He et al. 2007, He and McMahon 2011). Furthermore, Carvalho et al. (2007) observed two different morphologies of *Accumulibacter* with distinct physiological states which indicated that there were different population within *Accumulibacter*. They found that coccus-morphotype *Accumulibacter* fed by acetate could not utilize nitrate as an electron acceptor whereas rod-morphotype *Accumulibacter* fed by propionate was able to use both nitrate and nitrite. Thus, some genetic markers like polyphosphate kinase 1 (*ppkI*) (He et al. 2007), 16S–23S rRNA internal transcribed spacer (ITS) region (He et al. 2006) and PHA synthase (*phaC*) (Wang et al. 2008) have been used to distinguish the subgroups (i.e. Types or clades) of *Accumulibacter*. He et al. (2007) developed specific *ppkI* primers to target the five clades of *Accumulibacter* (clade I, IIA, IIB, IIC and IID) and conducted

on three laboratory-scale and nine full-scale EBPR systems. Ong et al. (2014) has designed the *ppk1* primers for *Accumulibacter* clade IIF and hypothesized that this specific clade was able to perform the high-temperature EBPR system. Zhang et al. (2016) developed novel *ppk1* primer sets which could target nine individual *Accumulibacter* clades (clades IA, IB, IC, ID and IE of Type I and for clades IIE, IIG, IIH and II-I of Type II). With the development of modern biology, more and more unidentified *Accumulibacter* could be recognized.

Two major groups of GAOs: ‘*Candidatus Competibacter phosphatis*’ (refer as *Competibacter*) and *Alphaproteobacteria* GAOs were identified (Crocetti et al. 2002, Meyer et al. 2006, Wong et al. 2004). A group of *Gammaproteobacteria* was enriched in a poor P removal performance EBPR system and exhibited the GAO phenotype which was further named ‘*Candidatus Competibacter phosphatis*’ (Crocetti et al. 2002). Similar to *Accumulibacter*, *Competibacter* was also found to consist of distinct subgroups. Kong et al. (2006) found six different subgroups of *Competibacter* which were always coexisted with PAOs in full-scale EBPR plants. These subgroups presented different physiological characteristics, but all could deteriorate the P-removal performance. McIlroy et al. (2014) obtained two complete genomes from *Competibacter* through metagenomics and recognized these two genomes were affiliated with *Competibacter*-lineage subgroups 1 and 5 through phylogenetic analysis. *Alphaproteobacteria* related to *Defluviicoccus vanus* was identified as another GAOs in lab-scale EBPR reactors. Wong et al. (2004) identified the *Alphaproteobacteria* in a deteriorated lab-scale EBPR reactor which was affiliated with ‘*Defluvicoccus vanus*’

through 16S rRNA gene clone library and designed the FISH probes to target this subgroup 1 of the *Defluvicoccus vanus*. Furthermore, Meyer et al. (2006) identified subgroup 2 of the *Defluvicoccus vanus* in both lab-scale and full-scale EBPR process and designed the specific FISH probes to target cluster 2 of *Defluvicoccus vanus*. Burow et al. (2007) studied the distribution, abundance and ecophysiology of *Defluvicoccus vanus* GAO(DvGAO) in full-scale EBPR plants and confirmed they could compete carbon with PAOs and *Competibacter*.

2.4 Factors affecting EBPR performance

2.4.1 Carbon source

Volatile fatty acids (VFAs) are highly important carbon sources that drive EBPR in wastewater treatment plants. While a range of VFAs (acetate, propionate, butyrate, valerate, etc.) are found in wastewater and fermented liquors used as carbon sources for EBPR, acetate and propionate are the most commonly studied, due to their high abundance and important impact on the competition between PAOs and GAOs (Shen et al. 2017, Tu and Schuler 2013, Zeng et al. 2003b). *Accumulibacter* PAOs could be easily enriched by acetate as carbon source. A stable phosphorus removal performance achieved with acetate as the sole carbon source at high temperature and *Accumulibacter* Clade IIC was found to be highly abundant in that EBPR system (Shen et al. 2017). Tu and Schuler (2013) demonstrated that low acetate concentration could provide *Accumulibacter* PAOs competitive advantage over *Defluviicoccus vanus* GAOs. Propionate is another favorable VFA for EBPR system and it is the second most

dominant VFA in septic municipal wastewater (Lv et al. 2014). Pre-fermentation of wastewater may produce large amount of propionate as well (Thomas et al. 2003). Propionate has been believed to be a more favorable VFA than acetate for a better performance of EBPR in some studies (Carvalho et al. 2014, Chen et al. 2004, Pijuan et al. 2004b). Chen et al. (2004) compared two lab-scale SBRs provided with different carbon sources and found that a higher molar ratio of propionic to acetic acid led to a better phosphorus uptake capacity. *Accumulibacter* PAOs can easily take up propionate while *Competibacter* GAOs hardly utilize it, but propionate can promote the growth of another microorganism- *Defluviicoccus vanus* GAOs (Oehmen et al. 2005c).

Glucose has also been widely studied in EBPR process. Some studies suggested that glucose may be fermented to VFA before being taken up by PAOs (Sudiana et al. 1999, Wentzel et al. 1991), but the glucose metabolism in PAOs is still controversial to date. PAOs fed by glucose mainly produced PHV as primary PHA (Wang et al. 2002) while those fed by acetate and propionate mainly produced PHB and PHV, PH2MV, respectively. Glucose can induce glycogen transformation that leads to a large PHV fraction. On the other hand, glycogen transformation is more favorable for GAOs metabolism. Therefore, glucose as sole carbon source may deteriorate the EBPR process.

In recent years, using the sludge fermented liquid to improve biological nutrients (N and P) removal has attracted considerable attention because of its economic and sustainable virtue (Cao et al. 2019, Tong and Chen 2007, Wang et al. 2016). Tong and Chen (2007) showed that fermented liquid was a superior carbon source than pure

acetate for EBPR system and Tong and Chen (2009) exhibited that adding fermented liquid could enhance the phosphorus and nitrogen removal due to the increasing of influent short-chain fatty acids (SCFA). The fermentation liquid contains large amounts of SCFA (acetate, propionate butyrate and valerate), and the amount of butyrate even could reach 40%-60% of total VFAs in fermentation liquid of winery wastewater or waste activated sludge (Esteban-Gutiérrez et al. 2018, Huang et al. 2016, Yuan et al. 2009). Begum and Batista (2014) reported that butyrate could enrich both *Accumulibacter* and *Defluviicoccus*, and a good P-removal was realized at the beginning of the experiment at room temperature (22 °C) but eventually deteriorated due to the high abundance of *Defluviicoccus*. Cai et al. (2019) displayed that butyrate was a preferable carbon source compared to valerate in a granular sludge system with more poly-P content and high abundance of *Rhodocyclus*-related bacteria and *Actinobacteria* in the butyrate-fed granules. However, it was unclear how butyrate affected the competition between PAO and GAO, and why *Defluviicoccus* could outcompete *Accumulibacter* in the long-term operation.

2.4.2 Temperature effect

Temperature is a very important factor that would influence the PAO-GAO competition. Lower temperature could reduce the rate of biochemical transformations in EBPR system (Brdjanovic et al. 1998). However, some studies found that lower temperatures might be beneficial for EBPR (Erdal et al. 2003, Lopez-Vazquez et al. 2009a, Ren et al. 2011, Whang and Park 2002). This phenomenon is reasonable because PAOs is

psychrophilic bacteria while GAOs is mesophilic. High temperature has been reported to easily enrich GAOs (Lopez-Vazquez et al. 2009b), and achieving stable and efficient high temperature EBPR is typically challenging (Ong et al. 2014). Panswad et al. (2003) showed that the dominant microbial population shifted from PAOs (47-70%) to GAOs (64-75%) when temperature gradually increased from 20 °C to 30 °C, however, this study did not identify the phylogenetic identity of the PAOs and GAOs present. Lopez-Vazquez (2009a) reported that high temperature favored the growth of *Candidatus Competibacter phosphatis* (or *Competibacter*) GAOs, which outcompeted the growth of *Candidatus Accumulibacter phosphatis* (or *Accumulibacter*) PAOs.

Nevertheless, a few high-temperature EBPR processes have been successfully operated recently (Ong et al. 2014, Ong et al. 2012, Shen et al. 2017, Winkler et al. 2011). If PAOs can outcompete GAOs in tropical climate, EBPR would be preferable for wastewater treatment plants (WWTPs) in tropical regions like Singapore and the WWTPs treating high-temperature industrial effluents. Ong et al. (2012) demonstrated a lab-scale EBPR process could be operated at a relatively high temperature of 28 °C. This study stated that high temperature EBPR process has a better phosphorus removal performance at a lower ratio of C/P=3 than a higher ratio of 10 at a suitable pH 7 or 8. Shen et al. (2017) also successfully operated two EBPR systems at 30°C. That study used multi-cycle operation to promote a faster carbon and phosphorus turnover rate so that to build a good-performance EBPR system under high temperature. Besides, a granular sludge reactor with distinguishing microbial community structure achieved 100 % phosphorus removal efficiency at 30 °C by discharging the top granules that

were dominated by GAOs (Winkler et al. 2011). All these studies have shown the feasibility of high temperature biological phosphorus removal. Considering the important impact of temperature on the PAO-GAO competition, this is a considerable research gap that limits our knowledge and understanding on how to operate EBPR plants effectively at high temperature.

2.4.3 Other factors

Other factors influencing the performance of EBPR process, include pH, water-soluble forms of nitrogen, solids retention time (SRT), carbon source loading rate etc. Besides the carbon source and temperature, pH is another important factor affecting the competition between PAOs and GAOs. Smolders et al. (1994a) found that the ratio of P release/acetate uptake changed from 0.25 to 0.75 P-mol / C-mol accompanied with the pH rising from 5.5 to 8.5. Filipe et al. (2001d) showed that the acetate uptake rate of GAOs was higher than that of PAOs when pH was lower than 7.25 while the opposite results were obtained when pH was higher than 7.5. A higher pH condition (≥ 7.5) provided PAOs competitive advantage over GAOs by the model prediction (Lopez-Vazquez et al. 2009b). Ahn et al. (2007) demonstrated that the metabolism of activated sludge would change from phosphate accumulating metabolism to glycogen accumulating metabolism with increasing organic loading rate (160 to 1020 gCOD/m³/cycle). The effluent phosphorus concentration started to fluctuate when the P storage capacity exceeded the maximum capacity which eventually promoted the proliferation of GAOs. Tu and Schuler (2013) demonstrated that low acetate

concentration could provide *Accumulibacter* PAOs competitive advantage over *Defluviicoccus vanus* GAOs. Ersu et al. (2010) found that the total phosphorus (TP) removal efficiency could reach around 80% at 50 d SRT while extending the SRT to 75 d decreased the TP removal efficiency to 60%. Li et al. (2016) demonstrated that the system could maintain a good P removal performance at long SRT (30 days) and low COD loading (200 mg/L) compared to the performance with long SRT and high COD loading (300 mg/L). Nevertheless, the reduction of SRT to 20 days at high COD loading resulted in the increase of PAO population and improved system performance. Whang and Park (2006) reported that PAOs were dominant in the EBPR system at 20°C and SRT of 10 days. GAOs outcompeted PAOs when temperature increased to 30°C at SRT of 10 days, whereas the EBPR performance was recovered when SRT reduced to 3 days.

2.5 Summary

EBPR processes have been widely implemented in WWTPs because of its economic and environmental advantages. Previous researches have studied many factors that could affect the EBPR performance. One important factor contributing to the deteriorated EBPR is the undesirable proliferation of GAOs that always compete with PAOs for the limited carbon source in wastewater but have no capacity of phosphorus removal. Competition between PAOs and GAOs has been modelled to understand their behaviors. Due to the complexity of the carbon source in wastewater, it is essential to understand how other available carbon sources, besides acetate and propionate, impact on PAO and GAO competition. This is particularly important when fermented liquor is

employed to support nutrient removal processes. GAOs have been previously observed to be more competitive for anaerobic volatile fatty acid (VFA) uptake as compared to PAOs at high temperatures. However, previous evidence regarding the impact of temperature on GAOs has exclusively focused on only one group of GAOs, namely *Candidatus Competibacter phosphatis* (or *Competibacter*). *Defluviicoccus vanus* GAO are another very important GAO group that have been found in EBPR plants, however, their competitiveness at high temperature for anaerobic VFA uptake was previously unknown. This thesis provides new insight into our understanding of GAOs in EBPR processes, where the impact of temperature on the PAO-GAO competition is highly dependent on the phylogenetic identity of the GAO present in the system. This thesis aims to build more detailed and validated models to study the carbon transformation inside the PAOs and GAOs cells separately at high temperature. Through the comparison and investigation on the pathways of carbon transformation under tropical climate, this thesis aims finding the way to control the GAOs population and promote the proliferation of PAOs at high temperature. New strategies to promote EBPR performance at high temperatures are proposed based on the results of this work.

Chapter 3 Materials and methods

3.1 Parent reactors operation

3.1.1 PAO reactors feed with acetate and propionate

Two 6 L sequencing batch reactors (SBRs) were operated under similar conditions. One was fed with acetate (C2-PAO) while the other was fed with propionate (C3-PAO) at 30 ± 1 °C with pH controlled between 7.0-8.2 using 0.5 M NaOH or 0.5 M HCl. This pH range has been suggested to be suitable for PAO growth over GAOs at high temperatures (Lopez-Vazquez et al. 2009b, Oehmen et al. 2005b, Ong et al. 2013). Hydraulic retention time (HRT) was 12 h and sludge retention time (SRT) was 7-8 days through discharging 200 ml sludge per cycle. The SBRs were operated with a multi-cycle feeding strategy, with each cycle containing three reaction loops. Each loop started with 2 min feeding followed by a 40 min anaerobic phase and a 60 min aerobic phase. The details can be found in Shen et al. (2017). Both reactors were fed with 400 mg COD/L and the phosphate concentration was 20 mg PO₄-P/L. The dosage of other trace nutrients was reported previously in Shen et al. (2017). Two reactors were operated for more than one year before the batch experiments were carried out.

3.1.2 GAO reactors feed with acetate and propionate

Two lab-scale sequencing batch reactors (SBRs) were operated for more than six months to enrich GAOs. The seed sludge was from two enriched PAO cultures that were fed with acetate or propionate, respectively. The cultures were transformed from

PAOs to GAOs directly through reducing the influent phosphorus concentration to 2 mg P- PO_4^{3-} /L. Both reactors, with a working volume of 6 L, were fed with 400 mg COD/L of synthetic wastewater under a multi-cycle feeding strategy (Shen et al. 2017). Briefly, one cycle consisted of three loops, one loop containing 2 min feeding, 40 min anaerobic phase and 60 min aerobic phase and then followed by settling and discharging. One reactor was fed with acetate as the sole carbon source (C2-GAO) while the other one was fed with propionate (C3-GAO). The composition of other nutrients can be found in Shen et al. (2017). The reactors were operated under $30 \pm 1^\circ\text{C}$ with pH between 7.0-8.2, hydraulic retention time (HRT) of 12 h and sludge retention time (SRT) of 7-8 days. Nitrogen gas was purged to maintain anaerobic conditions, while the compressed air was supplied to obtain a dissolved oxygen (DO) level around 2-2.5 mg/L under aerobic conditions. During the operation, cycle studies were conducted to monitor the reactors performance.

3.1.3 PAO and GAO reactors feed with butyrate

Two SBRs with a 6 L working volume were seeded from two lab-scale acetate-fed enriched PAO and GAO cultures, respectively. A 6-h cycle consisted of three loops, each loop contained 30 min continuous feeding, 10 min anaerobic period and 60 min aerobic period and then followed by one hour settling and decanting period. One liter of synthetic wastewater was fed with nitrogen purging in every loop. Both reactors were fed with total 400 mg COD/L, 20 mg PO_4^{3-} -P/L (PAO reactor) or 2 mg PO_4^{3-} -P/L (GAO reactor) and other necessary nutrients (Shen et al. 2017) during three stages operation.

In Stage 1 (0-30 days), acetate was fed as sole carbon for both reactors. In Stage 2 (30 – 160 days), carbon source was changed to a mixture of acetate and butyrate in pre-designed ratios, while acetate proportion was gradually reduced with the increased butyrate content. The acetate and butyrate mixing ratios were 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 during the 130 days operation. In Stage 3 (160 – 212 days), 100% of butyrate was fed as sole carbon source. The temperature was controlled at 30 ± 1 °C and pH was maintained between 7.0 - 8.0 by addition of 0.5 M HCl or 0.5 M NaOH. The hydraulic retention time (HRT) was 12 h and sludge retention time (SRT) was 7 – 14 days (more details are given in results section). Nitrogen was supplied into the reactors during feeding and anaerobic period while dissolved oxygen (DO) level was maintained between 1.0 – 2.0 mg/L during the aerobic period.

Table 3.1 The parent reactors operation for different cultures.

Cultures	Feed	COD (mg/L)	P (mg/L)	HRT (h)	SRT (days)
C2-PAO	Acetate	400	20	12	7-8
C3-PAO	Propionate	400	20	12	7-8
C2-GAO	Acetate	400	2	12	7-8
C3-GAO	Propionate	400	2	12	7-8
C4-PAO	Butyrate	400	20	12	7-14
C4-GAO	Butyrate	400	2	12	7-14

3.2 Sample analysis

Mixed liquor total suspended solids (MLTSS) and mixed liquor volatile suspended solid (MLVSS) were collected at the end of aerobic phase in cycle studies and batch tests, and determined according to standard methods (APHA, 2005). Liquid samples

from reactor cycle studies and batch experiments were immediately filtered by 0.45 μm membranes for volatile fatty acids (VFAs), phosphorus and ammonium analysis. VFAs were analyzed by gas chromatography (GC) with an Agilent DB-FFAP 30 m $\times$ 350 μm $\times$ 0.5 μm column. Phosphorus and ammonia were measured according to standard methods (APHA, 2005).

Solid samples were fixed by formaldehyde and then prepared for PHAs and glycogen extraction after freeze-drying. Glycogen analysis was carried out according to Zeng et al. (2003), and analyzed by high-performance liquid chromatography (Agilent 1200 series HPLC system) with a BC-Hi-Plex H, 300 $\times$ 7.7 mm column. PHAs were extracted from freeze-dried sludge according to Oehmen et al. (2005a). Gas chromatography-mass spectrometry (GC-MS) equipped with an RTX-5MS column was used to determine the PHA component and quantify it according to Le et al., (2021). Nine C₃-C₁₆ 3-hydroxycarboxylic acids standards, i.e. 3-hydroxypropanoic acid (3HP), 3-hydroxybutyric acid (3HB), 3-hydroxypentanoic acid (3HV), 3-hydroxyhexanoic acid (3HHx), 3-hydroxyoctanoic acid (3HO), 3-hydroxydecanoic acid (3HD), 3-hydroxydodecanoic acid (3HDOD), 3-hydroxytetradecanoic acid (3HTRD), and 3-hydroxyhexadecanoic acid (3HTED), were chosen for PHA calibration. The calibration parameters of these standards are presented in Table 3.1. The concentration of linear PHAs was directly determined through the calibration curve of each applicable standard. However, since there was no standard for branched-chain PHAs, their concentration was estimated from the calibration curve of the linear PHAs standard with the same number of carbons.

Ammonium consumption rate was calculated, and biomass growth rate was then determined through the formula of $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$. The carbon recovery ratio was calculated to assess the carbon balance according to Eq 3.1 (C-mol basis).

$$\text{Carbon recovery ratio} = \frac{\text{The amount of PHA synthesized}}{\text{The substrate taken up} + \text{the glycogen degraded}} \quad (3.1)$$

Table 3.2 Calibration parameters of GC-MS method for various PHA monomers.

Standards	regression coefficient	linearity range (ng)	R ² *
3-hydroxypropionic acid (3HP)	y=2E+8x-2E+6	10-1000	0.9982
3-hydroxybutyric acid (3HB)	y=9E+7x+6E+6	10-1000	0.9995
3-hydroxypentanoic acid (3HV)	y=9E+7x-6E+5	10-1000	0.9999
3-hydroxyhexanoic acid (3HHx)	y=1E+8x+4E+6	4-1000	0.9996
3-hydroxyoctanoic acid (3HO)	y=1E+8x+3E+6	2-1000	0.9998
3-hydroxydecanoic acid (3HD)	y=1E+8x+5E+6	4-1000	0.9998
3-hydroxydodecanoic acid (3HDOD)	y=1E+8x+4E+6	10-1000	0.9997
3-hydroxytetradecanoic acid (3HTRD)	y=7E+7x-2E+5	10-1000	0.9993
3-hydroxyhexadecanoic acid (3HTED)	y=5E+7x+8E+5	10-1000	0.9999

*Correlation coefficient, x:the amount of PHA monomer, y:area of the corresponding peaks.

3.3 Fluorescence in situ hybridization (FISH)

The population of PAOs and GAOs was analyzed by FISH according to Amann and Fuchs (2008). The EUBmix probes consisted of EUB338, EUB338II and EUB338 III, which target all bacteria (Daims et al. 1999). *Accumulibacter* PAO were targeted by PAOmix (PAO462, PAO651 and PAO846) (Crocetti et al. 2000). GAOQ431 and GAOQ989 were used to target *Competibacter* GAO (Crocetti et al. 2002) while DvGAOs Cluster 1 were determined by DF 218 and DF 618 and DvGAOs Cluster 2

was targeted by DF 988 and DF1020 (Meyer et al. 2006, Wong et al. 2004). A Nikon AIR confocal laser scanning microscope was used to visualize and quantify the microbial communities according to the procedures of Winkler et al. (2011). Twenty pictures were taken and analyzed for each sample to quantify specific communities.

3.4 Microbial community analysis

Sludge samples were collected from PAO and GAO reactors during the three stages for DNA analysis. Total genomic DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The extracted DNA was checked using 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The V4 region of 16S rRNA genes was amplified with primer pairs 515FmodF (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RmodR (5'-GGACTACNVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The Illumina Miseq sequencing service was provided by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Operational taxonomic units (OTU) with 3% dissimilarity cutoff were clustered using UPARSE (version 7.1, <http://drive5.com/uparse/>). The alpha diversity, including abundance-based coverage estimator (ACE), Chao1 richness estimator and Shannon diversity indices were calculated in the Mothur software program for each sample (https://mothur.org/wiki/download_mothur/). The beta diversity was determined by principal coordinates analysis (PCoA) based on the Bray-Curtis distance matrix (Zhang

et al. 2020). Microbial abundance at the genus level was visualized by the Circos online program (<http://circos.ca>).

Chapter 4 The impact of temperature on the metabolism of volatile fatty acids by Polyphosphate Accumulating Organisms (PAOs)

4.1 Introduction

It is known that carbon source and temperature are two highly crucial factors that influence the EBPR performance and stability (Lopez-Vazquez et al. 2009b, Lu et al. 2006, Whang and Park 2006). Pijuan et al. (2004a) and Carvalheira et al. (2014) demonstrated that propionate was the preferred carbon source to support EBPR systems at a temperature of 20 °C. Oehmen et al. (2004) also proved that propionate provided *Accumulibacter* PAOs with an advantage over glycogen accumulating organisms (GAOs) because *Accumulibacter* PAOs were able to utilize propionate while certain GAOs could not do so under similar conditions. Other studies showed that butyrate could also be taken up by *Accumulibacter* PAOs, though usually at lower rates as compared to acetate and propionate (Begum and Batista 2014, Oehmen et al. 2004, Pijuan et al. 2004a), and valerate was generally less preferred than butyrate (Cai et al. 2019).

VFAs are always limited in municipal wastewater, and it is often necessary to add additional carbon source to improve the nutrient removal performance of wastewater treatment plants. Compared to commercial carbon sources, such as methanol and acetate, VFAs produced from waste activated sludge (WAS) fermentation would be a more economical and sustainable alternative (Cao et al. 2019, Li et al. 2011, Tong and Chen 2007, Zheng et al. 2010). In the fermentation liquid, acetate and propionate are

the two most dominant VFAs, while butyrate, valerate and their isomers are also commonly present. It is not clear how such a VFA profile would affect the metabolic activities and behavior of *Accumulibacter* PAOs.

Many studies have reported that EBPR performance would fluctuate or even be deteriorated under high temperature conditions (Whang and Park 2002, Oehmen et al. 2007a, Panswad et al. 2003). This is because high temperature (30 °C) favors the growth of GAOs over PAOs (Lopez-Vazquez et al. 2008, Panswad et al. 2003). A stable EBPR performance was realized with a multi-cycle feeding strategy under high temperature (30 °C). Surprisingly, acetate was identified as the preferred carbon source to maintain system stability over propionate (Shen et al. 2017), unlike what is typically observed at low temperature (20 °C). Oehmen et al. (2005b) indicated that propionate could be used to minimize *Competibacter* which could provide a selective advantage for PAOs, and Thomas et al. (2008) even showed that propionate was the key factor providing competitive advantage for PAOs at low temperature. Nevertheless, Shen et al. (2017) was unable to establish why acetate might have led to a superior P removal performance as compared to propionate. We hypothesized that the behavior of *Accumulibacter* PAOs is different under low and high temperatures. The objective of the present work is to determine how the metabolism of VFA differs for *Accumulibacter* PAOs at high temperature as compared to low temperature, which has not previously been assessed. The impact of acetate, propionate and other VFA on *Accumulibacter* PAOs at high temperature is needed in order to understand if their metabolism is different under these conditions and how it is different. This will improve our capacity to optimize the

performance and better control EBPR systems operated in warm climates.

In this study, acetate, propionate, butyrate, valerate and their isomers were used as sole substrate or in mixtures to provide carbon source to two enriched *Accumulibacter* PAO cultures operated at 30 °C. The aims of this study were to (1) understand the response of different *Accumulibacter* PAO cultures to different carbon sources at high temperature (30 °C); (2) evaluate its impact on PAO metabolism and performance under anaerobic/aerobic conditions; (3) compare the performance of *Accumulibacter* PAO at high and low temperatures (previously established in literature) with the various carbon sources.

4.2 Experiment section

One liter of sludge was withdrawn from the C2-PAO and C3-PAO parent reactors at the end of aerobic phase to seed batch reactors. The sludge was washed with nutrient medium and 500 ml supernatant was discarded and replaced with chemical oxygen demand (COD) and P medium to start the anaerobic phase. The nutrient medium was made of trace nutrients fed to the reactors, which could be found in Shen et al. (2017). Eight batch reactors fed with different carbon sources were set-up for each EBPR culture (Table 4.1). Total COD concentration for each type of carbon source was fixed at 400 mg COD/L, and 20 mg PO_4^{3-} -P/L was also added at the beginning of the anaerobic phase. Trace nutrients were similar to the parent SBRs. The batch experiments were carried out in replicates with each type of carbon source on separate days, while batch 7 and 8 were carried out in single experiment. The temperature in the

batch tests was the same as the parent reactors, which was controlled at 30 ± 1 °C.

Table 4.1 Carbon source for the batch study.

Batch	Composition	Concentration (mg COD/L)
1	Acetate (C2)	400
2	Propionate (C3)	400
3	Butyrate (C4)	400
4	Iso-butyrate (ISO-C4)	400
5	Valerate (C5)	400
6	Acetate + Propionate	200+200
7	Acetate + Propionate + Butyrate + Valerate	100+100+100+100
8	Acetate + Propionate + Iso-butyrate + Iso-valerate	100+100+100+100

The batch tests started with a 2 h anaerobic phase followed by a washing step to remove any remaining COD at the end of the anaerobic phase. For non-parent VFAs like butyrate, iso-butyrate and valerate, the anaerobic phase was extended to 5 h. At the beginning of the aerobic phase, phosphorus was added back to the batch reactors according to the P concentration measured at the end of the anaerobic phase. The entire aerobic phase lasted for 3 h. Mixed liquor samples were filtered by 0.45 µm membrane filters and then analyzed for VFA and phosphorus concentrations. Solid samples were firstly fixed by formaldehyde for at least one hour, and then washed with Phosphate-buffered saline (PBS) buffer followed by freeze-drying. Freeze-dried sludge was used to analyze the polyhydroxyalkanoates (PHAs) and glycogen.

4.3 Results and discussion

4.3.1 SBR performance

Figure 4.1 Profiles of phosphorus, VFA, PHB, PHV, PH2MV during the anaerobic (A) and aerobic (O) phase in cycle study of the C2-PAO (a) and C3-PAO (b) bioreactors.

Fig. 4.1 shows the cycle study results of both reactors under steady state conditions, which exhibit the common PAO phenotype. FISH results confirmed that *Accumulibacter* PAOs were the dominant microorganisms in both reactors (Fig. 4.2). Quantitatively, there were 64.2% of *Accumulibacter* PAOs, 6.0% of *Defluviicoccus* GAOs and less than 1.0% of *Competibacter* GAOs in the C2-PAO reactor while the C3-PAO reactor consisted of 51.6% *Accumulibacter* PAOs and 8.3% *Defluviicoccus* GAOs, while *Competibacter* was not detected.

Figure 4.2 Bacterial population distribution in C2 reactor (a) and C3 reactor (b) by applying fluorescence in situ hybridization (purple: glycogen accumulating organisms, red: polyphosphate accumulating organisms and green: all bacterial).

For both reactors, VFA was consumed in the first ten minutes in anaerobic phases. The carbon uptake rate and P-release to VFA uptake ratio (P/VFA) of C3-PAO was found to be lower than those of C2-PAO (Table 4.2). The lower P/VFA ratio is likely due to the lower energetic requirement per C-mol of propionate uptake as compared to acetate uptake, which is consistent with literature findings at 20 °C (Oehmen et al. 2005c). The carbon uptake rate and the P/VFA ratio were found to be higher than those of low temperature lab-scale EBPR systems fed with acetate or propionate carbon sources (Oehmen et al. 2005b, Pijuan et al. 2004b). This may be due to the more active cultures with faster metabolism rates at higher temperature. Panswad et al. (2003) found that the specific phosphorus release rate of PAO increased with temperature changed from 20 °C to 35 °C. In this study, PAOs that were enriched at 30 °C and adapted to such high temperatures, showed higher activity than that of *Accumulibacter* PAOs enriched at low

temperature (20 °C). The PHA composition was similar to the studies reported at low temperature (Carvalheira et al. 2014, Hsu et al. 2013, Oehmen et al. 2005a, Schuler and Jenkins 2003).

During the aerobic phase, both cultures used PHA to uptake phosphorus and form glycogen. A higher phosphorus uptake rate was always found in C2-PAO compared to C3-PAO (Table 4.2), which may be because of the different PHA composition. Randall and Liu (2002) and (Liu et al. (2002)) showed that per C-mol unit of PHB could contribute to much higher phosphorus uptake than PHV. Thus, a much higher proportion of PHB with C2-PAO could result in a higher phosphorus uptake rate. The aerobic phosphorus uptake rate for both C2-PAO and C3-PAO was toward the high-end of the range observed at low-temperature (0.35-0.92 P-mmol/g VSS/h for C2-PAO and 0.25-0.72 P-mmol/g VSS/h for C3-PAO) (Carvalheira et al. 2014, Pijuan et al. 2004b). The aerobic phosphorus uptake rate may be insensitive to temperature within a certain range, and it depends more on the availability of PHA and the composition of PHA. Brdjanovic et al. (1997) reported the aerobic phosphorus uptake rate continuously increased with temperature from 5 °C to 30 °C but the rate did not change significantly within 20-30 °C (1.71 P-mmol/g VSS/h at 20 °C and 1.77 P-mmol/g VSS/h at 30 °C), which is in accordance with our results.

Table 4.2 Comparison of VFA uptake rate (C-mmol/g VSS/h), P-release to VFA uptake ratio (P-mol/C-mol), P aerobic uptake rate (P-mmol/g VSS/h), P-uptake to PHA consumption ratio (P-mol/C-mol), glycogen replenishment to PHA consumption ratio (C-mol/C-mol) between this study and low temperature EBPR studies.

	Acetate-PAO					Propionate-PAO				
	Anaerobic		Aerobic			Anaerobic		Aerobic		
	VFA uptake	P/C	P uptake	P/PHA	Gly/PHA	VFA uptake	P/C	P uptake	P/PHA	Gly/PHA
This study	4.92	0.51-0.77	0.67-0.89	0.76-0.90	0.22-0.37	4.50	0.45-0.65	0.41-0.60	0.63-0.80	0.30-0.52
Literature ^{a,b,c}	3.53-3.74	0.50	0.35-0.92	0.33-0.41	0.42-0.59	3.34-4.12	0.42	0.25-0.72	0.34-0.43	0.50

a: Oehmen et al. 2005b, b: Pijuan et al. 2004b, c: Carvalheira et al. 2014 (all cultures were pulse-fed at 20 °C).

In general, the aerobic P-uptake rate and P/PHA ratio of C2-PAO are higher than those of C3-PAO, perhaps due to a higher favorability of PHB oxidation versus PHV and PH2MV oxidation for P uptake. This impact was explored in more details during the batch tests (see below). P/PHA ratios reported in previous low temperature studies were all substantially lower than the values found in this study and the ratios were similar for both C2 and C3 cultures.

In fact, Glycogen/PHA (Gly/PHA) ratios were indeed higher under lower temperature with more glycogen production (Table 4.2). The ratios for both C2 and C3 cultures were comparable. However, a much higher glycogen replenishment to PHA degradation ratio was found in C3-PAO compared to C2-PAO at high temperature. This suggests that C3-PAO possessed more active glycogen metabolism at high temperature, which may gradually shift to GAO-like metabolism after long term operation.

4.3.2 Batch experiment results

4.3.2.1 Anaerobic metabolism

4.3.2.1.1 Anaerobic VFA uptake and P-release kinetics

Fig. 4.3 shows the VFA uptake profiles during the anaerobic phase for both cultures. Acetate and propionate were taken up completely in a short period. The COD concentration was converted from the measured VFA concentration. The consumption rates of the two carbon sources for the C2-PAO culture were slightly lower than those for C3-PAO (Table 4.3). Both cultures could utilize butyrate and iso-butyrate at lower uptake rates (Table 4.3) and the valerate uptake rate was even slower.

Figure 4.3 Anaerobic COD uptake in C2-PAO (a: single carbon substrate; b: mixed carbon substrate) and C3-PAO (c: single carbon substrate; d: mixed carbon substrate).

For mixed carbon source tests, the individual carbon consumption is described in Figure 4.4. The maximum specific VFA uptake rate of each individual carbon in the mixed carbon sources tests was also calculated and displayed in Table 4.3 while the total VFA uptake rates are shown in Table 4.4. Interestingly, the rates of propionate consumption were much higher than that of acetate, even for C2-PAO, usually by a factor of 2. Propionate was always observed to be the preferred carbon source in all mixed carbon source batch tests (Fig. 4.4 and Table 4.4). All the evidence suggests that both cultures prefer propionate as carbon source regardless of the presence of other substrates and

the parent feed. Carvalheira et al. (2014) also found that propionate was depleted more quickly when both acetate and propionate were present under low temperature conditions. PAOs require less energy per C-mol of propionate uptake as compared to acetate (Oehmen et al. 2005c).

Figure 4.4 The individual carbon consumption in mixed carbon sources tests in C2-

PAO (a:C2+C3, c:C2+C3+C4+C5 and e:C2+C3+iso-C4+iso-C5) and C3-PAO (b:C2+C3, d:C2+C3+C4+C5 and f:C2+C3+iso-C4+iso-C5).

The highest average substrate uptake rate for both cultures was the combined substrate of C2+C3 (Table 4.4). Remarkably, the total uptake rates of C2+C3 (C2-PAO: 8.90 C-mmol/g VSS/h and C3-PAO: 9.21 C-mmol/g VSS/h) were higher than that of all other single or combined substrates. The acetate uptake rate in the mixed C2+C3 substrate case was reduced as compared with acetate addition as single substrate, likely due to the substrate competition with propionate. However, the uptake rate of propionate in the mixed C2+C3 substrate test even increased as compared to the single substrate case (Table 4.3). The result confirms that *Accumulibacter* PAOs prefer propionate at high temperature.

Table 4.4 The total VFA uptake rate of mixed carbon source (C-mmol/g VSS/h) in C2-PAO and C3-PAO.

Substrate	Acetate-PAO	Propionate-PAO
C2+C3	8.90±0.12	9.21±0.18
C2+C3+C4+C5	4.51	5.25
C2+C3+iso-C4+iso-C5	4.09	4.88

Table 4.3 Specific VFA uptake rates (C-mmol/g VSS/h) of acetate-PAO and propionate-PAO and the ratio of P release to VFA uptake (P-mmol/C-mmol).

Substrate	Acetate-PAO		Propionate-PAO	
	VFA uptake	P/C	VFA uptake	P/C
C2	6.13±0.11	0.51±0.00	7.16±0.11	0.57±0.03
C3	6.30±0.07	0.49±0.01	9.17±0.14	0.47±0.02
C4	0.85±0.03	0.43±0.01	0.55±0.01	0.43±0.01
ISO-C4	0.50±0.02	0.33±0.01	0.78±0.02	0.44±0.01
C5	0.33±0.01	0.42±0.01	0.36±0.02	0.39±0.01
C2+C3	4.22+7.32	0.54±0.10	4.79+9.59	0.52±0.04
C2+C3+C4+C5	1.62+3.10+0.80+0.32	0.63	1.83+6.08+1.38+0.33	0.49
C2+C3+iso-C4+iso-C5	2.35+4.27+0.64+0.02	0.61	1.23+4.36+1.01+0.03	0.37

Addition of other VFAs like butyrate, valerate and their isomers reduced the total VFA uptake rate of the mixed carbon sources. The decline of total uptake rate may be due to the reduced concentration of preferred carbon sources, or the slow uptake rate of the exotic carbon sources. Interestingly, the uptake rates of butyrate and its isomer in the mixed carbon feeding test were significantly increased compared to its individual uptake rate in C3-PAO (Table 4.3) and were only 20-25% lower than the corresponding acetate uptake rate in the mixed carbon. Nevertheless, the butyrate and iso-butyrate uptake rates in mixed carbon feeding were comparable to the single carbon cases when C2-PAO was fed with mixed carbon source. Perhaps the acclimation to a longer chain carbon source (propionate vs acetate) favored the capacity of *Accumulibacter* PAOs to take up butyrate when fed in the presence of the other VFAs.

Anaerobic P-release profiles are displayed in Fig. 4.5. The ratios of P-release versus VFA uptake (P/VFA) are shown in Table 4.3. In general, the P-release and P/VFA ratio tended to decline with an increase in the chain length of the VFA added. The C4 and C5 carbon sources should require even less energy for active transport across the cell membrane as compared to C2 and C3 per C-mol of VFA uptake, as highlighted by Wang et al. (2018) for PHA producing mixed microbial cultures. In the case of *Accumulibacter* PAOs, similar results are obtained. It appears that less polyphosphate is needed to be hydrolyzed per C-mol of C4 and C5 to provide the energy necessary for transport and conversion to an acyl-CoA compared to C2 and C3.

Figure 4.5 Anaerobic phosphorus release and aerobic phosphorus uptake in C2-PAO (a: single carbon substrate; b: mixed carbon substrate) and C3-PAO (c: single carbon substrate; d: mixed carbon substrate). The dotted line is used to distinguish between the anaerobic phase and aerobic phase: the first dotted line is the boundary for acetate and propionate uptake while the second dotted line is the boundary for butyrate, iso-butyrate and valerate uptake in (a) and (c).

4.3.2.1.2 Anaerobic carbon transformation

The carbon transformation with different carbon sources during the anaerobic phase are presented in Table 4.5. Less glycogen was consumed in the C2-PAO with acetate as feed, as compared to low temperature EBPR models (glycogen/C: 0.5 C-mol/C-mol)

(Smolders et al. 1994a), while the glycogen consumption with propionate as feed was comparable to the model value (glycogen/C: 0.33 C-mol/C-mol) (Oehmen et al. 2005c). On the other hand, glycogen consumption with acetate and propionate as feed in C3-PAO were comparable to the model values in the current study. It is likely that part of the reducing power for C2-PAO fed with acetate could be generated from the tricarboxylic acid (TCA) cycle at high temperature, and C2-PAO fed with propionate as well as C3-PAO seemed to rely more on the hydrolysis of glycogen to provide reducing power. This may be because the acetyl-CoA produced from acetate uptake is a metabolite that can enter directly into the TCA cycle, while the propionyl-CoA produced from propionate uptake is not. Therefore, propionate fed culture or propionate as feed may encourage the glycogen recycling metabolism under high temperature.

Table 4.5 Summary of carbon transformation during the anaerobic phase (C-mol/C-mol).

Substrate	C2 PAO						C3 PAO					
	PHB/C	PHV/C	PH2MV/C	PHA/C	Gly/C	C %*	PHB/C	PHV/C	PH2MV/C	PHA/C	Gly/C	C %*
	C											
C2	1.33±0.03	0.21±0.04	0	1.54±0.07	0.37±0.10	1.14±0.13	1.21±0.28	0.30±0.08	0.07±0.06	1.57±0.30	0.42±0.11	1.08±0.10
C3	0	0.46±0.01	0.88±0.02	1.34±0.03	0.32±0.00	1.02±0.02	0.02±0.01	0.54±0.14	0.65±0.17	1.21±0.31	0.26±0.07	0.95±0.19
C4	0.64±0.00	0.06±0.02	0.04±0.01	0.74±0.03	0.27±0.04	0.58±0.01	0.57±0.03	0.20±0.05	0.04±0.03	0.80±0.07	0.40±0.00	0.57±0.05
ISO-C4	0.76±0.04	0.07±0.00	0.06±0.01	0.88±0.04	0.23±0.02	0.72±0.05	0.58±0.01	0.14±0.03	0.05±0.01	0.77±0.05	0.42±0.01	0.54±0.04
C5	0.11±0.11	1.08±0.14	0.16±0.11	1.35±0.14	0.33±0.02	1.01±0.09	0.04±0.04	1.08±0.09	0.34±0.05	1.47±0.01	0.74±0.00	0.84±0.00
C2+C3	0.51±0.06	0.71±0.04	0.20±0.01	1.42±0.03	0.28±0.01	1.10±0.02	0.36±0.07	0.73±0.03	0.20±0.06	1.29±0.10	0.37±0.03	0.94±0.06
C2345	0.48	0.76	0.18	1.42	0.36	1.04	0.43	0.35	0.09	0.88	0.16	0.75
ISOC2345	0.34	0.61	0.15	1.10	0.33	0.83	0.67	0.38	0.05	1.10	0.35	0.82

*carbon recovery: (PHA synthesized)/(substrate taken up+glycogen degraded)

C2345: C2+C3+C4+C5 and ISOC2345: C2+C3+iso-C4+iso-C5

For other carbon sources, the glycogen consumption per VFA consumed showed some variations, but was typically similar to the level observed for propionate uptake. While glycogen utilization was observed to be an important mechanism for nicotinamide adenine dinucleotide (NADH) generation with all carbon sources tested, the TCA cycle appears to be a more relevant means of generating reducing power for acetate uptake as compared to propionate uptake regardless of the temperature. Further study is required to assess the relevance (if any) of the TCA cycle in the transformation of other carbon sources.

Overall, the total amount of synthesized PHA and its composition from acetate or propionate fed batch tests (Table 4.5 and Fig. 4.6) are consistent with the models and some other low temperature EBPR systems (Carvalheira et al. 2014, Hsu et al. 2013, Schuler and Jenkins 2003, Smolders et al. 1994a). The major component of PHA synthesized with acetate was PHB and a minor proportion of PHV was detected. Meanwhile, propionate was mainly converted to PHV and PH2MV for both cultures. When both cultures were fed with a mixed carbon of C2+C3, three types of PHAs were generated, and most of which was PHV, followed by PHB and PH2MV. This result is consistent with a study under lower temperature where the main PHA component was found to be PHV when the reactor was fed with the same amount (in terms of COD) of acetate and propionate (Carvalheira et al. 2014). In general, the stoichiometry of the EBPR reactions (i.e. P/VFA, GLY/VFA and PHA/VFA) with acetate and/or propionate as feed at high temperature is highly similar as that at the low temperature, and temperature likely only affected the reaction kinetics.

Figure 4.6 PHA degradation rates of (a) C2-PAO and (b) C3-PAO under aerobic conditions.

PHB was the main PHA component fed with butyrate and iso-butyrate while valerate mainly converted to PHV. The amount of PHAs synthesized by utilizing butyrate and iso-butyrate was notably low (Table 4.5). When both cultures were fed with acetate and/or propionate, the carbon recovery ratio is close to or slightly higher than 1. This shows that although there was around 10% carbon loss through CO_2 production or measurement error, the main portion of the carbon input was generally conserved during the tests. However, the carbon recovery ratio for butyrate and iso-butyrate was much lower than 1. It is speculated that those VFA led to the formation of some unrecognized PHA fractions. During the PHA analysis, some unrecognized peaks were found when the cultures were fed with butyrate and iso-butyrate, and the peak area gradually increased with time during the anaerobic phase. These unidentified peaks were likely different PHA fractions. In a previous study, an unidentified monomer was also found when butyrate was used as carbon source (Pijuan et al. 2004a). Thus, the

low PHA content produced by butyrate and iso-butyrate was likely because of the underestimation of the amount of PHA produced.

It should also be noted that PAOs or other organisms in the sludge may not take up butyrate, iso-butyrate and valerate and store them as PHA directly, but ferment these carbon sources to acetate and/or propionate first, releasing CO₂ in the fermentation process. This could also contribute to the C recovery ratio being less than 1 in certain cases when fed with these carbon sources as single substrates or in combination. A small portion of CO₂ production in addition to the PHA formation is also predicted by the metabolic models, which also impacts this recovery ratio.

4.3.2.2 Aerobic metabolism

P-removal (%), P-uptake rates and glycogen recovery to PHA consumption ratios (glycogen/PHA) are shown in Table 4.6. The highest P-uptake rate was found in the acetate fed cases. Interestingly, P-uptake rate with butyrate and iso-butyrate as feed were generally higher or comparable with propionate cases. The main PHA component synthesized was PHB when acetate, butyrate and iso-butyrate were used as feed, while PHV and PH2MV were the major PHA component when propionate and valerate were used (Fig. 4.5). It seemed PHB was always the preferred PHA with fast degradation rate (Fig. 4.6). This led to higher P-uptake rates in those with high PHB levels. However, at 20 °C it was observed that PHA degradation and P-uptake kinetics were relatively independent of the PHA fractionation caused by feeding different ratios of acetate and propionate (Carvalheira et al 2014). It was further observed in the present study that a

much greater quantity of PHA was driven towards glycogen replenishment in the case of propionate addition to either culture. This is consistent with the observation in parent reactors. However, this is also unlike the study of Carvalheira et al. (2014) at 20°C.

Table 4.6 The aerobic P removal efficiency (%), P uptake rate (P-mmol/g VSS/h) and glycogen replenishment to PHA degradation ratio (C-mol/C-mol).

Substrate	Acetate-PAO			Propionate-PAO		
	P removal	P uptake	Gly/PHA	P removal	P uptake	Gly/PHA
C2	73.24	0.59	0.27	78.19	0.65	0.27
C3	59.53	0.36	0.78	66.53	0.37	0.98
C4	72.44	0.58	0.09	69.27	0.45	0.53
ISO-C4	77.72	0.43	0	49.90	0.30	0.29
C5	66.71	0.36	0.26	71.05	0.31	0.44
C2+C3	72.60	0.53	0.34	82.41	0.62	0.36
C2345	77.65	0.37	0.27	77.63	0.43	0.29
ISO-C2345	57.47	0.39	0.27	93.65	0.47	0.18

Overall, while high temperature clearly impacts anaerobic and aerobic *Accumulibacter* PAO kinetics, it also appears to impact their preference towards P uptake vs glycogen replenishment in ways that have not been previously observed at low temperature. It is clear that the higher glycogen/PHA ratio and lower P removal is unfavorable for PAOs and EBPR. Since more glycogen turnover and less P-uptake could transform the PAO metabolism (polyphosphate as main energy source) into GAO metabolism (glycogen as primary energy source), eventually it would deteriorate the EBPR performance (Erdal et al. 2008, Zhou et al. 2008). Shen et al. (2017) operated a high temperature EBPR system and found that EBPR performance was more stable with acetate as carbon source, while the performance of the other EBPR system fed with propionate always

fluctuated. The transformation of metabolism from PAO to GAO may be one of the reasons for its failure. While propionate seemed to be the preferred carbon source for both cultures under anaerobic conditions (Table 4.3), this did not translate into optimal aerobic P uptake performance. Therefore, propionate is not recommended as the sole carbon source to be added to stimulate PAOs activity at high temperature.

The phosphorus removal efficiency of the mixed carbon tests for both cultures revealed that when acetate and propionate were simultaneously fed to both cultures, the associated P-uptake rate was quite similar to the acetate tests, despite the additional PHV and PH2MV oxidized. This is further supported by the lower glycogen production per PHA oxidized as compared to the propionate tests. This suggests that the addition of mixed carbon sources can lead to the anaerobic kinetic advantages described above for *Accumulibacter* PAOs without sacrifice to the aerobic P uptake efficiency, when compared to the use of single substrates at high temperature. The aerobic kinetics of the mixtures including butyrate, valerate or its isomers did not accelerate P uptake, however. It may be that acclimation to alternative PHA fractions is necessary for *Accumulibacter* PAOs to perform efficient aerobic P uptake, as suggested previously in tests conducted at 20 °C (Oehmen et al 2004).

4.3.3 Impact of the study on EBPR operation at high temperature

Through the present study focused on the evaluation of carbon source on anaerobic and aerobic *Accumulibacter* PAO metabolism, high temperature conditions could affect their kinetics, including the VFA consumption rate, anaerobic phosphorus release and

aerobic phosphorus uptake rate, without significant changes to its anaerobic stoichiometry. Similar to low temperature EBPR, propionate seems to be a preferred carbon source for *Accumulibacter* PAOs as compared to acetate because less energy is required for its uptake. However, lower P uptake and higher glycogen replenishment with propionate as feed was found in both parent reactor and batch tests, which may negatively impact EBPR performance in long-term operation. Propionate was not the better carbon source providing competitive advantage for PAOs at high temperature.

While the other carbon sources tested presented lower uptake rates as compared to propionate, butyrate uptake was still significant, in some cases approaching the acetate uptake rate in mixed feeds. A combined carbon source of acetate, propionate and perhaps butyrate may be a better substrate combination for EBPR, since this led to high anaerobic VFA uptake rates coupled with higher aerobic P uptake rates and lower glycogen production rates. Therefore, WAS fermentation liquors that usually contain significant quantities of acetate, propionate and butyrate may be an effective alternative carbon source for EBPR at high temperature. It should be noted that this study focuses on the responses of *Accumulibacter* PAOs at high temperature towards changes in carbon sources, and it is not clear how GAO populations would respond to similar carbon source changes. As such, further study is required to investigate the effect of different carbon sources on GAO metabolism at high temperature to better understand the PAO-GAO competition under these conditions.

4.4 Conclusions

Propionate was always the preferred carbon source by *Accumulibacter* PAOs enriched with acetate or propionate at high temperature, but the addition of this carbon source alone led to high aerobic glycogen replenishment instead of high P uptake, contrary to what has been observed at low temperature. Thus, propionate addition alone as a supplemental carbon source is not an ideal strategy to promote EBPR at high temperature. Nevertheless, when propionate is fed in combination with acetate, high anaerobic VFA uptake rates and high aerobic P uptake kinetics were found. Mixed carbon source feeds are therefore recommended in order to maximize the EBPR activity of PAOs at high temperatures, which may improve P removal performance in warm climates.

Chapter 5 *Defluviicoccus vanus* glycogen accumulating organisms (DvGAOs) are less competitive than polyphosphate accumulating organisms (PAOs) at high temperature

5.1 Introduction

High temperature has been reported to easily enrich GAOs (Lopez-Vazquez et al. 2009b), and achieving stable and efficient high temperature EBPR is typically challenging (Ong et al. 2014). The type of carbon source can also impact the competition between PAOs and GAOs, though this has typically been addressed at lower temperatures (i.e. 20 °C). While *Accumulibacter* PAOs take up acetate and propionate at comparable rates, *Competibacter* GAOs prefer acetate, since they have been observed to take up propionate at negligible rates (Oehmen et al. 2004, Oehmen et al. 2005c). However, another group of GAOs, belonging to *Defluviicoccus vanus* (DvGAOs), are able to take up propionate at rates comparable to *Accumulibacter* PAOs at low temperature, but have been found to take up acetate at slower rates (Oehmen et al. 2006a, Oehmen et al. 2005c, Oehmen et al. 2006b). While DvGAOs, like *Competibacter* GAOs, have been observed to be present in full-scale EBPR plants (Crocetti et al. 2002, Meyer et al. 2006, Saunders et al. 2003), the impact of high temperature on the behavior of DvGAOs in comparison to PAOs has not previously been assessed. Considering the important impact of temperature on the PAO-GAO competition, this is a considerable research gap that limits our knowledge and understanding of how to operate EBPR plants effectively at high temperature. It is

possible that the stoichiometric and kinetic behavior of *Dv*GAOs at high temperature differs from *Competibacter* GAOs, unlike what is typically assumed in literature (Lopez-Vazquez et al. 2009b).

This study investigates the impact of carbon source on two enriched cultures of *Dv*GAOs operated at high temperature (30°C), where either acetate or propionate was used as the carbon source. The work compares the stoichiometry and kinetics observed with different VFAs (acetate, propionate, butyrate, valerate) fed as sole carbon substrate or in combination to understand their impact on *Dv*GAO metabolism at high temperature. These results are then compared with literature studies in order to evaluate how temperature impacts the carbon source competition between *Accumulibacter* PAOs, *Competibacter* GAOs and *Dv*GAOs. The aim is to reveal potential operational strategies that can be used to minimize the growth of GAOs at high temperature and better achieve efficient P removal performance, where high temperatures can limit EBPR effectiveness.

5.2 Experiment section

VFAs that are commonly found in wastewater were chosen as carbon sources for GAOs in a series of batch tests. The VFAs, namely, acetate, propionate, butyrate, iso-butyrate and valerate, was used either as sole or mixed carbon sources in the batch experiments. The applied concentration and compositions of the VFAs can be found in Table 4.1. For each batch test, one liter of sludge was withdrawn from the C2-GAO and C3-GAO reactors at the end of the aerobic phase and transferred into the batch reactor. The sludge

was then washed with PBS to remove any remaining substrate. The anaerobic phase of the batch tests was initiated with the addition of 400 mg COD/L VFAs, 2 mg PO₄-P/L and trace nutrients. Two hours of anaerobic phase was applied for acetate, propionate or mixed carbon sources, while five hours of anaerobic phase was carried out for butyrate, iso-butyrate or valerate. Three hours of aerobic phase was then implemented for all batch tests. Nitrogen and compressed air were purged to maintain anaerobic and aerobic conditions, respectively. Temperature and pH were similar to the parent reactors. During the batch tests, mixed liquor samples were collected at fixed time points for analysis. The batch experiments were carried out in replicates with each type of carbon source on separate days.

5.3 Results and discussion

5.3.1 Long-term SBRs operation

5.3.1.1 Enrichment of glycogen accumulating organisms (GAOs)

After 6 months operation under P limited conditions, the two reactors, namely, C2-GAO and C3-GAO reactors, achieved steady-state. Based on the FISH results (Fig. 5.1), *Defluviicoccus vanus* related GAOs (*Dv*GAOs) were dominant in both the C2-GAO reactor and C3-GAO reactor. In the C2-GAO reactor, Cluster 1 occupied 62.4 ± 3 %, Cluster 2 occupied 21.8 ± 4 %, while PAO and *Competibacter* were both less than 1 %. In the C3-GAO reactor, it contained 50.9 ± 2 % of Cluster 1, 29.7 ± 5 % of Cluster 2, 2.4 ± 1 % of PAO and 4 ± 1 % of *Competibacter*. At low temperatures (i.e. 20 °C), it is commonly found that *Competibacter* GAO was the dominant microorganism when

acetate was used as sole carbon source while *Dv*GAOs was the main population in propionate fed reactors under P-limited conditions (Crocetti et al. 2002, Filipe et al. 2001, Oehmen et al. 2006b, Zeng et al. 2003). However, *Dv*GAOs were found to be the main microorganism in both reactors at high temperature in this study.

Figure 5.1 Bacterial population distribution in C2-GAO (a) and C3-GAO (b) by applying fluorescence in situ hybridization (The first three pictures show the distribution of three bacteria separately. Purple is glycogen accumulating organisms, red is polyphosphate accumulating organisms and green is all bacterial. The last picture (lower right corner) merges the distribution pictures of the three bacteria on one picture at the same time).

*Dv*GAOs enriched by propionate could also utilize acetate (Oehmen et al. 2006b, Wong et al. 2004), so it is possible to enrich *Dv*GAOs with acetate. Dai et al. (2007) successfully cultured *Dv*GAOs with acetate as the sole carbon source at low temperature (20 °C). It was speculated that DO concentration may influence the types

of GAOs and *Dv*GAOs could be enriched with low DO (< 1mg/L) vs *Competibacter* dominant GAOs enriched with DO of 3 mg/L when fed with acetate (Oehmen et al. 2005a, Oehmen et al. 2005b). Nevertheless, the high DO concentration of 2-2.5 mg/L in this study also enriched *Dv*GAOs. It is possible that the multi-cycle feeding strategy or relatively high pH range (7.0-8.2) employed caused the discrepancy with low temperature results. This is the first time that *Dv*GAOs were enriched at high temperature and the impact of temperature on the stoichiometry and kinetics of *Dv*GAOs will be discussed in the following sections.

5.3.1.2 Cycle studies of parent reactors

Cycle studies were carried out to study the GAOs metabolism when biomass was under steady-state conditions. One of the cycle study results is shown in Fig. 5.2. Both cultures exhibited the typical GAO phenotype. VFAs were completely consumed anaerobically and associated with glycogen consumption, while no significant phosphorus release was observed. At the same time, these VFAs were synthesized to corresponding PHAs (Table 5.2), and the major PHA component in C2-GAO and C3-GAO is consistent with literature findings at 20°C (Oehmen et al. 2006b, Zeng et al. 2003). Under aerobic conditions, PHA was degraded for glycogen replenishment, biomass maintenance and growth.

Both cultures could uptake their respective carbon source in the first 20 min of the anaerobic phases. The carbon uptake rates were found to be 4.04 and 4.01 C-mmol/g VSS/h respectively on average. It is worth noting that the carbon uptake rates of

*Dv*GAOs are lower than those of PAOs, which were operated under similar conditions except for the influent phosphorus concentration (Shen et al. 2017). This could be one of the reasons why PAOs could be successfully enriched at high temperature in the Shen et al. (2017) study, since the anaerobic substrate uptake is one of the important factors affecting the competition between PAOs and GAOs. PAOs with higher substrate uptake rates have a competitive advantage over *Dv*GAOs under such operational conditions. Lopez-Vazquez et al. (2009b) showed that *Competibacter* had a much higher acetate uptake rate than PAO at a temperature of 30°C (Table 5.1). Nevertheless, it is found in the present study that *Competibacter* did not survive under the present conditions. The *Dv*GAOs that were able to grow in the system seemed to be less competitive than PAO at high temperature, unlike what was previously hypothesized and typically incorporated into metabolic models of PAO and GAO (Lopez-Vazquez et al. 2009b).

Figure 5.2 Cycle profiles of VFA, PHB, PHV, PH2MV and glycogen in C2-GAO (a) and C3-GAO (b) during steady state.

Anaerobic carbon transformations in C2-GAO and C3-GAO is shown in Table 5.2. Although acetate enriched *Dv*GAO in this study, the anaerobic carbon conversions (Gly/C and PHA/C) and PHA composition were similar to the *Competibacter* dominant GAO cultivated by acetate at low (20°C) (Zeng et al. 2002) or high temperature (30°C) (Lopez-Vazquez et al. 2009a). Dai et al. (2007) also reported that the anaerobic metabolism of *Dv*GAOs (20°C) were in line with the existing metabolic model developed through *Competibacter* GAO. Moreover, the PHA content in C3-GAO was again similar to the model and other studies operated at lower temperatures (Dai et al. 2007, Oehmen et al. 2005a, Oehmen et al. 2006b, Zhang et al. 2008). Therefore, despite the GAO population and carbon source uptake kinetics being different, their stoichiometry maintained similar with the same carbon source.

Table 5.1 Comparison of VFA uptake rates (C-mmol/g VSS/h) between this study and other PAOs, *Competibacter* and *Dv*GAOs studies.

	Acetate-feed			Propionate-feed	
	PAOs	<i>Competibacter</i>	<i>Dv</i> GAOs	PAOs	<i>Dv</i> GAOs
30 °C	4.96±0.04 ^a	8.13 ^b	4.04±0.17 ^c	4.74±0.24 ^a	4.01±0.03 ^c

a: Shen et al. (2017), b: Lopez-Vazquez et al. (2009a), c: this study

Besides anaerobic substrate uptake rate, the aerobic biomass production is also another important factor affecting PAO-GAO competition (Zeng et al. 2003). Thus, the biological growth rate of *Dv*GAOs may play a key role in aerobic competition between PAO and GAO, which will be discussed below.

Table 5.2 Anaerobic (PHB/C, PHV/C, PH2MV/C and Gly/C) and aerobic (Gly/PHA) carbon transformation during the cycle studies in C2-GAO and C3-GAO (C-mol/C-mol).

	C2-GAO					C3-GAO				
	PHB/C	PHV/C	PH2MV/C	Gly/C	Gly/PHA	PHB/C	PHV/C	PH2MV/C	GLY/C	Gly/PHA
Cycle-1	0.96-1.61	0.40-0.66	0.00-0.12	0.77-1.77	0.79-1.02	0.00-0.08	0.48-0.64	0.54-1.07	0.64-1.10	0.39-0.96
Cycle-2	1.12-1.53	0.37-0.58	0.00-0.05	1.08-1.02	0.63-1.24	0.00-0.09	0.43-0.82	0.41-1.33	0.22-1.07	0.26-0.66
Cycle-3	1.20-1.95	0.38-0.71	0.00-0.05	0.36-1.09	0.39-1.26	0.00-0.10	0.43-0.56	0.46-0.92	0.37-1.00	0.32-0.68
Average	1.09-1.70	0.38-0.65	0.00-0.07	0.73-1.30	0.60-1.17	0.00-0.09	0.45-0.67	0.47-1.11	0.41-1.05	0.32-0.77
20°C Model^{a,b}	1.36	0.46	0.04	1.12	0.75	0.05	0.64	0.81	0.66	0.72
30°C Competibacter^c	1.54-1.60	0.50-0.74	NA	1.15-1.68	NA	NA	NA	NA	NA	NA
30°C PAOs^d	0.82-0.96	0.07-0.19	NA	0.24-0.41	0.21-0.28	0.03-0.05	0.49-0.71	0.27-0.43	0.16-0.30	0.19-0.26

a: Zeng et al. (2002), b: Oehmen et al. (2006b), c: Lopez-Vazquez et al. (2009a) and d: Shen et al. (2017)

5.3.2 Short-term batch experiments

5.3.2.1 Anaerobic carbon uptake kinetics

In order to determine the impact of carbon source on *Dv*GAOs at high temperature, a series of short-term batch experiments were conducted. The anaerobic carbon consumption kinetics for both cultures are shown in Fig. 5.3 and the specific maximum VFA uptake rates of *Dv*GAOs were calculated (Table 5.3). Acetate and propionate could be completely consumed within 2 hours for both cultures, but butyrate, iso-butyrate and valerate could not be completely consumed even when the anaerobic time was prolonged to 5 hours. The order of VFA uptake rate when fed with single carbon sources in C2-GAO was found to be acetate, followed by propionate > butyrate > iso-butyrate > valerate while the VFA uptake rate in C3-GAO followed the order of propionate > acetate > butyrate > iso-butyrate > valerate. The substrate uptake rates in the batch tests were also somewhat higher than that in the parent reactor, given the higher initial VFA concentration (through the pulse feed applied in the batch tests), which could increase the uptake rate.

Different from *Competibacter* GAO that could not anaerobically uptake propionate (Oehmen et al., 2004), *Dv*GAOs fed with acetate in this study (C2-GAO) consumed propionate at 70% of the acetate uptake rate at 30 °C. At 30 °C, C3-GAO were found to metabolize acetate at half of the propionate uptake rate, which is consistent with findings at 20 °C (Oehmen et al. 2005a). Although *Dv*GAOs in this study could easily utilize acetate and/or propionate, the uptake rates of these carbon sources were much

lower than those of PAOs under similar conditions (Table 5.3). This confirms that *Dv*GAOs are less competitive than PAO at high temperature due to their lower maximum anaerobic substrate uptake rate. Different to *Competibacter* GAO, which showed a much higher substrate uptake rate than PAO at a temperature of 30 °C (Lopez-Vazquez et al. 2009b), *Dv*GAOs would be outcompeted by PAO for anaerobic substrate uptake.

Figure 5.3 Anaerobic COD uptake in C2-GAO (a: single carbon substrate; b: mixed carbon substrate) and C3-GAO (c: single carbon substrate; d: mixed carbon substrate).

Table 5.3 The specific maximum VFA uptake rates (C-mmol/g VSS/h) of C2-GAO and C3-GAO in batch experiments compared to those of C2-PAO and C3-PAO under similar operation condition.

Substrate	Acetate-GAO	Propionate-GAO	Acetate-PAO*	Propionate-PAO*
C2	4.59 ± 0.37	2.76 ± 0.25	6.13 ± 0.11	7.16 ± 0.11
C3	3.30 ± 0.04	5.42 ± 0.35	6.30 ± 0.07	9.17 ± 0.14
C4	0.83 ± 0.03	0.76 ± 0.07	0.85 ± 0.03	0.55 ± 0.01
ISO-C4	0.51 ± 0.03	0.65 ± 0.01	0.50 ± 0.02	0.78 ± 0.02
C5	0.25 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	0.36 ± 0.02
C2+C3	3.10+4.91	1.69+6.85	4.22+7.32	4.79+9.59
C2+C3+C4+C5	2.10+5.15+1.25+0.35	1.05+6.49+0.72+0.29	1.62+3.10+0.80+0.32	1.83+6.08+1.38+0.33
C2+C3+ISO-C4+ISO-C5	1.94+4.74+1.21+0.07	1.19+6.22+0.91+0.05	2.35+4.27+0.64+0.02	1.23+4.36+1.01+0.03

* Data from Wang et al. (2020)

Both *Dv*GAO cultures could utilize a certain amount of butyrate, iso-butyrate and valerate, but at much slower uptake rates (Table 5.3). The uptake rates of butyrate, iso-butyrate and valerate by both GAOs are comparable to those of PAOs under similar operation conditions (Wang et al., 2020), suggesting that these VFAs as the sole carbon source would not provide a competitive advantage to PAOs over GAOs.

Table 5.3 and Fig. 5.4 display the individual VFA uptake performance with mixed carbon source feeds. The total carbon consumption rates with the mixed carbon sources is shown in Table 5.4. All carbon sources were consumed simultaneously except iso-valerate (Fig. 5.3). For both cultures, the overall VFA uptake rate of C2+C3 increased by 57 – 70% compared to their single parent substrate uptake rates. A similar observation was obtained for PAO cultures as well (Wang et al. 2020). Nevertheless, the VFA uptake rates of PAOs are much higher than those of GAOs, which may allow PAOs to win the competition with GAOs when C2 and C3 are used together at high temperature.

Table 5.4 The total VFA uptake rate of mixed carbon source (C-mmol/g VSS /h) in C2-GAO and C3-GAO.

Substrate	Acetate-GAO	Propionate-GAO	Acetate-PAO*	Propionate-PAO*
C2+C3	6.39 ± 0.01	4.14 ± 0.08	8.90 ± 0.12	9.21 ± 0.18
C2+C3+C4+C5	5.22	2.80 ± 0.01	4.51	5.25
C2+C3+Iso-C4+Iso-C5	4.65	2.80 ± 0.43	4.09	4.88

* Data from Wang et al. (2020)

In addition, when other longer chain VFAs (butyrate, iso-butyrate and valerate) are mixed with C2+C3, both the specific maximum uptake rates and total VFA uptake rates

of C2 and C3 were reduced. Such reduction was greater for PAOs than GAOs (Table 5.3 and Table 5.4). Indeed, the maximum propionate uptake rate of *Dv*GAOs was quite similar in single or mixed carbon cases, regardless of the presence of other VFAs, unlike the case of PAOs. This result suggests that the addition of higher order VFAs such as butyrate and valerate should be minimized in order to maximize PAOs' activity over GAOs' at high temperatures. EBPR can be supplemented with fermented feedstocks to provide sufficient carbon sources for P removal, where the fermentation processes should favor the production of acetate and propionate and avoid the accumulation of higher order VFAs.

Figure 5.4 The COD uptake of individual carbon in mixed carbon source by C2-GAO (a: C2+C3 c: C2+C3+C4+C5 and e: C2+C3+ISO-C4+ISO-C5) and C3-GAO (b: C2+C3 d: C2+C3+C4+C5 and f: C2+C3+ISO-C4+ISO-C5).

5.3.2.2 Anaerobic carbon transformation stoichiometry

The anaerobic carbon transformation stoichiometry with different substrates is summarized in Table 5.5 and Fig. 5.5. The anaerobic carbon stoichiometry produced by acetate and propionate as feed for both GAO cultures again matches low temperature GAOs metabolic models. As discussed before, high temperature would not affect the anaerobic carbon stoichiometry of GAOs, but primarily influence the substrate uptake rate in the anaerobic stage.

Figure 5.5 Anaerobically synthesized PHA composition in C2-GAO and C3-GAO.

The PHA composition using butyrate and iso-butyrate was quite distinct from the other feedstocks. Some uncommon PHAs, e.g. PH4MV, PHHx and PH2MHx were found by GC-MS. Table 5.5 summarizes the total amount of new PHAs and Table 5.6 shows the individual amount of new PHAs. It is worth noting that the new PHAs occupied a large proportion of total PHA produced and especially so in C3-GAO. It is believed that C4 and C5 may be converted by different metabolic pathways and thus synthesize new PHAs accordingly.

Table 5.5 Summary of carbon transformation during the anaerobic phase (C-mol/C-mol).

	Acetate - GAO							Propionate - GAO						
	PHB/C	PHV/C	PH2MV/C	NEW/C*	PHA/C	Gly/C	C %	PHB/C	PHV/C	PH2MV/C	New/C*	PHA/C	Gly/C	C %
C2	1.46±0.04	0.53±0.05	0.02±0.03	NA	2.02±0.12	1.29±0.02	0.88±0.06	1.56±0.08	0.50±0.11	0.03±0.00	NA	2.16±0.19	1.11±0.01	1.04±0.08
C3	0.16±0.02	0.74±0.04	0.68±0.07	NA	1.59±0.12	0.76±0.11	0.90±0.02	0.07±0.00	0.72±0.11	0.93±0.07	NA	1.72±0.18	0.81±0.02	0.95±0.90
C4	0.26±0.01	0.29±0.03	0.30±0.02	0.77±0.07	1.61±0.05	1.15±0.13	0.75±0.02	0.19±0.01	0.37±0.06	0.21±0.03	1.15±0.08	1.91±0.01	1.51±0.08	0.76±0.02
ISO-C4	0.03±0.03	0.45±0.01	0.36±0.01	0.53±0.01	1.36±0.04	0.80±0.02	0.76±0.01	0.20±0.04	0.35±0.07	0.28±0.05	1.03±0.05	1.85±0.10	1.01±0.11	0.92±0.00
C5	0.37±0.17	0.94±0.03	0.68±0.06	NA	1.99±0.08	1.58±0.03	0.77±0.04	0.23±0.04	0.78±0.08	0.58±0.04	NA	1.59±0.16	1.16±0.12	0.74±0.03
C2+C3	0.70±0.07	0.68±0.03	0.27±0.00	NA	1.65±0.10	0.67±0.11	0.99±0.00	0.70±0.5	0.90±0.04	0.60±0.01	NA	2.20±0.03	1.36±0.03	0.93±0.00
C2345	0.27±0.01	0.68±0.07	0.22±0.02	0.15±0.03	1.32±0.03	0.76±0.02	0.75±0.01	0.56±0.04	0.91±0.06	0.35±0.02	0.27±0.02	2.10±0.15	1.37±0.10	0.88±0.02
ISO-C2345	0.11±0.02	0.89±0.03	0.46±0.03	0.07±0.01	1.53±0.09	0.81±0.06	0.85±0.08	0.48±0.03	0.79±0.05	0.32±0.02	0.22±0.01	1.81±0.11	0.89±0.05	0.96±0.03

* New PHA includes PH4MV, PHHx and PH2MH

Table 5.6 The synthesized new PHA to VFA (C-mol/ C-mol) in C2-GAO and C3-GAO.

Substrate	Acetate-GAO			Propionate-GAO		
	PH4MV	PHHx	PH2MHx	PH4MV	PHHx	PH2MHx
C4	0.26	0.37	0.19	0.35	0.57	0.29
ISO-C4	0.42	0.08	0.02	0.78	0.23	0.09
C2+C3+C4+C5	0.02	0.11	0	0.07	0.18	0
C2+C3+Iso-C4+Iso-C5	0.06	0	0	0.21	0	0

It should be noted that the carbon recovery ratio was quite below 1 for the longer chain VFAs. GAOs may not be able to directly utilize longer chain VFA, e.g. butyrate and valerate, where butyrate and valerate can be converted into acetate and/or propionate by fermentation, causing a portion of the carbon to be lost as CO₂ during this process. Bengtsson et al. (2010) had shown that a portion of valerate was firstly broken down to acetyl-CoA and propionyl-CoA prior to the formation of PHB, PHV and PH2MV. Furthermore, PHHx was likely formed by the combination of acetyl-CoA and butyryl-CoA, which was not found with acetate and propionate as feed. These uncommon PHA may represent interesting polymers for WWTPs to recover, while they may also affect the aerobic biomass growth in WWTPs, since the form and availability of PHA is important in aerobic metabolic processes (Lopez-Vazquez et al. 2008). This will be discussed in the next section.

5.3.2.3 Aerobic carbon replenishment and biomass growth

The aerobic carbon conversion and biomass growth rate are shown in Table 5.7. Higher glycogen/PHA ratios were found with the parent carbon source in both C2-GAO and C3-GAO cultures. In contrast, a higher growth rate was always found with propionate

as feed as compared to acetate as feed. However, this was not found in our previous PAO study (Wang et al. 2020) where *Accumulibacter* PAO had a higher growth rate when fed with acetate as compared to propionate (6.76 vs 5.25 C-mmol/C-mol biomass/h). It is noteworthy that the growth rate of both C2 and C3 PAO are higher than *DvGAOs* with the same operating conditions. Compared to propionate, acetate could limit the growth of *DvGAOs*. A combined substrate of acetate and propionate encourages the highest growth rate of *DvGAO* (4.53 C-mmol/C-mol biomass/h), which is still lower than the growth rate of PAOs. In addition, lower glycogen/PHA ratios along with high growth rates with butyrate and iso-butyrate were found. It is likely that the degradation of the new PHA fractions identified may provide more energy to support biomass growth than glycogen replenishment.

Table 5.7 Aerobic carbon conversion from PHA to Glycogen (C-mol/C-mol) and biomass growth rate (C-mmol/C-mol biomass/h) in C2-GAO and C3-GAO.

Substrate	Acetate-GAO		Propionate-GAO	
	Gly/PHA	Growth	Gly/PHA	Growth
C2	0.75	2.97	0.56	2.71
C3	0.65	3.13	0.59	4.20
C4	0.49	3.68	0.41	3.89
ISO-C4	0.27	4.31	0.10	3.97
C5	0.49	2.93	0.55	2.47
C2+C3	0.48	4.53	0.56	4.33
C2+C3+C4+C5	0.88	3.89	0.67	3.60
C2+C3+ISO-C4+ISO-C5	0.62	2.56	0.31	2.93

5.3.3 Impact of high temperature on GAO metabolisms

As opposed to most studies previously reported for low-temperature GAOs, *Defluviicoccus vanus* GAOs were found to be the main GAOs enriched in both C2 and C3-GAO reactors. Although the population of GAOs was different, high temperature did not significantly change the corresponding stoichiometry of carbon conversion when acetate or propionate were used as carbon source. Nevertheless, kinetic behavior of *Dv*GAOs at high temperature totally differs from *Competibacter* GAOs, unlike in literature, where they are typically assumed to be the same.

*Dv*GAOs could utilize acetate and/or propionate at a relatively high rate compared to other longer chain VFAs. Nevertheless, the uptake rates of acetate and/or propionate for both C2- and C3-GAOs at high temperature were lower than those of PAOs under similar operational conditions in both long-term operation and batch experiments. PAOs with higher anaerobic substrate uptake rates have a competitive advantage over GAOs under such operational conditions. In all mixed carbon batch tests, *Dv*GAOs show preference to propionate rather than acetate and other longer chain VFAs, even for C2-GAO that were acclimated to acetate. Besides the lower carbon source uptake rate, the growth of *Dv*GAOs with acetate as feed also seemed to be limited. Yet, acetate could easily enrich *Competibacter* GAOs in literature studies (Lopez-Vazquez et al. 2009a), which showed much higher acetate uptake rates than PAO at a temperature of 30 °C.

Hence, a combined substrate of acetate and propionate would be a better carbon source choice in high temperature EBPR systems. On one hand, the uptake rate of acetate and propionate for *Dv*GAOs at high temperature was lower than that of *Accumulibacter* PAOs, providing PAOs with a competitive advantage for anaerobic substrate uptake.

The biomass growth rate was also observed to be inferior for *Dv*GAOs in this scenario as compared to *Accumulibacter* PAOs. On the other hand, it could also lower the anaerobic substrate uptake rate by *Competibacter*, which are not able to take up propionate at significant rates. Thus, combined acetate and propionate feeds could avoid rapid proliferation of either *Dv*GAOs or *Competibacter*, even at high temperatures where these GAOs are more problematic.

Butyrate, iso-butyrate and valerate could not provide advantages to either PAOs or GAOs due to the comparable uptake rates when they are used as the sole carbon source. Addition of these carbon sources into acetate and/or propionate mixtures would reduce PAOs' competitive advantage in terms of VFA uptake rates. Some uncommon PHAs synthesized by butyrate and iso-butyrate may have an important role in PHA recovery or altering GAO's metabolic activity. Further studies need to investigate how these uncommon PHAs may affect the metabolism of GAOs.

5.4 Conclusions

Defulviicoccus vanus related GAOs (*Dv*GAOs) were enriched in both acetate and propionate fed reactors operated at high temperature. *Dv*GAOs could utilize acetate and/or propionate but could hardly metabolize other longer chain VFAs. However, *Dv*GAOs are less competitive than PAOs at high temperature due to their lower maximum anaerobic substrate uptake rate. In order to limit the proliferation of *Dv*GAOs and *Competibacter*, a combined carbon source substrate of acetate and propionate may be a better choice in high temperature EBPR systems. In addition, uncommon PHAs (PH4MV, PHHx and PH2MHx) were anaerobically synthesized in butyrate and iso-butyrate fed experiment. These uncommon PHAs may affect the metabolism of GAOs, and could be of interest for PHA recovery applications.

CHAPTER 6 Butyrate can support PAOs but not GAOs under tropical climate

6.1 Introduction

It is known that at higher temperatures, e.g. above 25°C, GAOs can outcompete PAOs (Lopez-Vazquez et al. 2009, Saunders et al. 2003, Thomas et al. (2003). Recently, a few studies using multistep feeding strategies or high pH with low C/P ratio successfully demonstrated that EBPR can be operated at high temperature (Ong et al. 2014, Shen et al. 2017). Nevertheless, the PAO-GAO competition requires study focused on suitable carbon sources to promote EBPR at high temperature (Qiu et al. 2019, Wang et al. 2020a).

While commercially-obtained carbon sources are commonly applied in wastewater treatment plants to enhance denitrification and EBPR, volatile fatty acids (VFAs) produced from sludge fermentation have been widely studied and implemented in EBPR systems (Cao et al. 2019, Ji et al. 2020, Thomas et al. 2003, Tong and Chen 2007). Acetate and propionate are two most common VFAs used to drive EBPR systems because they are abundant in wastewater and fermentation liquids. It should be noted that butyrate is another abundant VFA in fermentation liquids, which may be used to support EBPR systems as well. Yuan et al. (2009) showed that butyrate was the third most abundant VFA (12%-16% of total VFA) in fermented waste activated sludge, while Ahn and Speece (2006) also displayed that around 20% of butyrate could be formed during primary sludge fermentation. Esteban-Gutiérrez et al. (2018)

demonstrated that winery wastewater fermented at acidic pH could produce 60% of butyrate per total VFA, while Xie et al. (2014) showed that butyrate accounted for 10%-76% of the VFA species with different pH-adjustment strategies during fermentation of cassava thin stillage. Despite the potential significance of butyrate, EBPR studies have more widely focused on acetate and propionate, where knowledge about butyrate on the PAO-GAO competition at high temperature is particularly lacking.

Begum and Batista (2014) reported that butyrate could enrich more *Accumulibacter* than *Defluviicoccus*, with the potential for good P-removal at room temperature (22 °C). However, they also concluded that the uptake rate of butyrate was slow and P-release lasted the entire anaerobic phase (Begum and Batista 2014) with organic carbon entering the aerobic phase, which may have caused instability to the system. Levantesi et al. (2002) demonstrated a high-efficiency and stable EBPR with a mixture of acetate, propionate and butyrate, and *Accumulibacter* was enriched up to 53% of the total population, where the consumption rate of butyrate was comparable to that of acetate. Recently, Wang et al (2020b) indicated that the consumption rate of butyrate in mixed carbon sources including acetate and propionate was higher than its use as the sole carbon source by PAOs at high temperature. While butyrate has potential to be used as a supplemental carbon source to promote EBPR, there is no clear evidence of its impact on the PAO-GAO competition and on EBPR performance. The metabolism of PAO and GAO using butyrate is quite unclear currently, and must be understood in order to be able to assess the impact of this carbon source. This motivated the enrichment of PAO and GAO cultures in this study in order to be able to assess these factors for the

first time, which is of particular relevance at high temperatures, where GAOs display a higher level of metabolic activity.

Therefore, this study aimed to investigate how butyrate would affect the competition between PAOs and GAOs at high temperature. Two sequencing batch reactors (SBRs) were operated for seven months to enrich PAOs or GAOs. The carbon source of the two reactors was gradually shifted from acetate to butyrate to investigate the effects of butyrate on PAO and GAO cultures, in terms of system performance, microbial community selection and metabolic activities. This study provides insight into the impact of butyrate from sludge fermentation liquors on EBPR systems, providing a comparison of this important carbon source with acetate and propionate, which are much more commonly studied VFAs.

6.2 Experiment section

Two SBRs with a 6 L working volume were seeded from two lab-scale acetate-fed enriched PAO and GAO cultures, respectively (Wang et al., 2020a) and run three stages. In Stage 1, acetate was fed as the sole carbon source for both reactors. In Stage 2, carbon source was changed to a mixture of acetate and butyrate in pre-designed ratios, where the acetate proportion was gradually reduced and the butyrate content increased. In Stage 3, 100% butyrate was fed as the sole carbon source.

The batch tests were performed to study the butyrate competition between PAOs and GAOs. One liter of sludge was withdrawn from the C4-PAO and C4-GAO reactors at the end of aerobic phase during stage 3. The sludge was washed with PBS and

resuspended with 1L trace nutrients. To initiate anaerobic phase, 500 ml supernatant was discarded and replaced with synthetic wastewater. The synthetic wastewater consisted of 400 mg COD/L butyrate and other trace nutrients similar to the parent reactors. The batch tests consisted of 2 h anaerobic phase and 3 h aerobic phase. Sludge was washed with PBS to remove any remaining COD at the end of the anaerobic phase. The amount of phosphorus released was added back to the PAO culture at the beginning of the aerobic phase. The batch experiments were carried out in replicates. Other operation conditions such as DO, pH, and temperature were similar to the parent reactors.

6.3 Results and discussion

6.3.1 SBRs performance

A stable phosphorus removal performance was found during Stage 1 in the PAO reactor (Fig 1a). At the beginning of Stage 2 when butyrate was introduced, the effluent P concentration increased slightly, while both the VSS and active biomass concentration (Table 4 and Table S1) decreased. After a few days acclimation, the phosphorus removal performance and biomass concentration gradually recovered. During the periodic increases in the butyrate fraction, similar responses were occasionally noted (Figure 6.1a), particularly at a 50% butyrate fraction (day 92-109). During this time, the effluent P accumulated and the biomass concentration reduced sharply due to carbon source leakage to the aerobic phase. Thus, the SRT was prolonged from 7 to 10 days to prevent sludge washout. The performance gradually recovered, and a stable P removal

performance lasted for the rest of Stage 2. Butyrate was the sole carbon source for the PAO reactor in Stage 3, where stable P removal performance lasted more than two months till the end of the experiment. Overall, while butyrate may not be as efficient as acetate and propionate to support biomass growth, necessitating a higher SRT, it could be still utilized to maintain good EBPR performance at 30°C. Average P removal efficiency was $94.5\% \pm 3.5\%$, $93.3\% \pm 8.8\%$, $95.7\% \pm 3.1\%$ and average VSS/TSS was 0.53, 0.59 and 0.62 in Stages 1, 2 and 3, respectively.

The biomass concentration of the GAO reactor in Stage 1 was relatively stable. However, the active biomass concentration started to decrease with the addition of butyrate at the beginning of Stage 2 (Fig. 6.1b, Table 6.3). In order to avoid the sludge being washed out and retain the enrichment of GAOs, the SRT was also prolonged from 7 to 10 days from day 55. Although the biomass concentration stopped declining and remained constant throughout the rest of stage 2, it did not recover to the level of Stage 1, unlike the PAO reactor. In Stage 3, the biomass concentration reduced further when butyrate was used as the sole carbon source. The SRT was further prolonged to 14 days from day 170. Thus, a longer SRT was required to maintain a similar biomass concentration in the GAO reactor as compared to that of the PAO reactor. These results suggested that butyrate may not be as suitable to enrich for GAO growth under similar operational conditions.

Figure 6.1 Daily record of influent phosphorus concentration, effluent phosphorus concentration, MLTSS and MLVSS in (a) PAO reactor and (b) GAO reactor.

6.3.2 Microbial community

6.3.2.1 α diversity

The microbial population shift along the acclimation of the PAO and GAO reactors to a butyrate feed can be found in Fig. S1. In this section, one sample from each stage, i.e. 100% acetate (Stage 1), 50% acetate + 50% butyrate (Stage 2), and 100% butyrate (Stage 3), was chosen for comparison and discussion. The diversity index reports the Coverage index in all samples is around 99.8% (Table 6.1), indicating that the sequencing results are valid. When the carbon source in the two reactors was changed from acetate to butyrate, OTUs in the PAO reactor were not substantially affected, but decreased by 22% in the GAO reactor. And the ACE index decreased by 3.6% and 18.1% in the PAO and GAO reactors, respectively. This suggests that butyrate reduced the microbial diversity of both PAO and GAO cultures, and this impact was higher on the GAO culture.

Table 6.1 List of α diversity

Sample [*]	OTUs	Shannon	Ace	Coverage (%)
PA	398	3.86	494.12	99.85
PAB	381	3.72	473.67	99.88
PB	386	3.44	476.50	99.79
GA	461	3.49	557.88	99.84
GAB	462	3.89	584.91	99.84
GB	362	3.16	456.85	99.79

* PA: 100% Acetate for PAO reactor, PAB: 50% Acetate+50% Butyrate for PAO reactor, PB: 100% Butyrate for PAO reactor, GA: 100% Acetate for GAO reactor, GAB: 50% Acetate+50% Butyrate for GAO reactor, GB: 100% Butyrate for GAO reactor.

6.3.2.2 Microbial taxa

The genera with abundance greater than 5% in the PAO and GAO reactors were selected for Circos visualization (Fig. 6.2). *Candidatus Accumulibacter phosphatis* (or *Accumulibacter*) was the most abundant genus in the PAO reactor. The abundance of *Accumulibacter* in 100% acetate, 50% acetate + 50% butyrate, and 100% butyrate as feed samples were 37.4, 8.4 and 13.9%, respectively. Besides, the abundance of non-*Accumulibacter Rhodocyclaceae* was also substantial in the PAO reactor, at 2.0%, 12.3% and 14.5%. Some microorganisms belonging to the *Rhodocyclaceae* family, but not related to *Candidatus Accumulibacter phosphatis* could be potential PAOs performing EBPR (Datta and Goel 2010). Wu et al. (2013) also showed that *Rhodocyclaceae* had the capacity to perform P removal, and its presence ensured stable and efficient P-removal when the *Candidatus Accumulibacter phosphatis* abundance decreased. A

relatively stable phosphorus removal performance could be maintained in this study, which may be related to the enrichment of *Rhodocyclaceae*, when the *Accumulibacter* population was reduced.

In the GAO reactor, *Candidatus Competibacter phosphatis* (or *Competibacter*) was dominant when acetate existed, with an abundance of 27.3 and 19.4% in the samples of 100% acetate and 50% acetate + 50% butyrate as feed, respectively, but this population reduced to 6.2% with butyrate as the sole feed. This suggests that butyrate was less suitable to support the growth of *Competibacter* and the overall biomass in the GAO culture, which is evidenced by its reduced biomass concentration. Meanwhile, *Zoogloea* was enriched in the GAO reactor when the carbon source changed from acetate to butyrate, with an abundance of 0.2, 11.7 and 38.0%, respectively. *Zoogloea* have long been observed as floc-producing bacteria in activated sludge systems (Rosselló-Mora et al. 1995), and its enrichment may indicate carbon source leakage into the aerobic phase. During the turbulent period of PAO reactor operation, a small amount of *Zoogloea* was also enriched (Fig. 6.2). When the performance of the PAO reactor recovered, the population of *Zoogloea* greatly decreased. However, *Zoogloea* remained dominant in the GAO reactor till the end of Stage 3.

No *Defluviicoccus vanus* GAOs (*DvGAOs*) was detected in this study in either the PAO or GAO reactor. Wang et al. 2020a showed that *DvGAOs* were less competitive than PAOs at 30°C due to the lower maximum anaerobic substrate uptake rate. According to the microbial community analysis, overall, both *Accumulibacter* and *Competibacter* were negatively affected, but *Accumulibacter*, and potentially some other

Rhodocyclaceae-related PAOs, better adapted to butyrate than *Competibacter*.

Figure 6.2 Circles of the top 11 genus from (a)PAO reactor and (b) GAO reactor fed with 100% Butyrate, 50% Acetate+50% Butyrate and 100% Acetate, where line width respects abundance.

6.3.3 PAO/GAO reactors

6.3.3.1 Cycle studies

Cycle studies of the two reactors were performed at every carbon source mixing ratio in stages 1-3. Three cycle study results are reported here at acetate/butyrate ratios of 10:0 (stage 1), 5:5 (stage 2), and 0:10 (stage 3) to illustrate PAO and GAO performance. The PAO reactor displayed a clear PAO phenotype within every sub-cycle throughout the three stages regardless of the carbon mixing ratio (Fig. 6.4a, 6.4b and 6.4c). Carbon source was completely consumed with P-release during the feeding and anaerobic phase, and glycogen hydrolysis as well as PHAs synthesis were observed. In the aerobic phase, P was completely removed, accompanied by PHAs degradation and glycogen replenishment.

There was no clear trend of P-release to VFA uptake ratio (P/C ratio) among all the cycles, although a higher P/C ratio was found when the acetate to butyrate ratio was close to 1:1 (Table 6.2). It is noteworthy that in Stage 1, phosphorus uptake mainly occurred in the first 15 minutes of the aerobic phase (2.58 mmol/g VSS/h), while in the second and third stages, the phosphorus uptake slowed down (1.12 mmol/g VSS/h and 0.8 mmol/g VSS/h) (Figure 6.3a,b,c). A higher P-uptake rate was found when acetate was more dominant in the feed (Table 6.2). The slower P-uptake rate may be related to

the quantity and composition of PHA produced with butyrate feeding. With the increase of butyrate in the feed, the total PHA/C ratio was reduced by 34% compared to using acetate as sole carbon source (Table 6.2). Considering the decreased PHA storage, and that PHA degradation by PAOs is a 2/3 order kinetic reaction (Murnleitner et al. 1997), the 17% decrease in specific PHA storage per active biomass with an acetate feed (0.30 C-mmol/C-mmol) and a butyrate feed (0.25 C-mmol/C-mmol) would decrease the maximum PHA degradation rate by 11.4%. This decreased PHA degradation would, in turn, lower the ATP available for P uptake and other cell functions (i.e. biomass growth, glycogen replenishment, maintenance). Furthermore, the shift in PHA composition, where the PHH fraction was increased, may also have impacted the aerobic kinetics. Nevertheless, the net P-removal was comparable in all cycles, indicating that the lower PHA content only reduced the P-uptake rate and not the efficiency of P-uptake (Table 6.3). In addition, the anaerobic Gly/C and aerobic Gly/PHA ratios were relatively higher in stage 1 vs stage 2 and 3, indicating that glycogen turnover was limited with the addition of butyrate.

Similar to the PAO reactor, the PHA storage of the GAO reactor was also reduced with the addition of butyrate, though much more than as compared to PAOs, where a 58% reduction in PHA storage was found from Stage 1 to 3. Correspondingly, the maximum PHA degradation rate decreased by 57.2% due to the reduction in specific PHA storage per active biomass with an acetate feed (1.32 C-mmol/C-mmol) and a butyrate feed (0.37 C-mmol/C-mmol). This reduced PHA storage would clearly impact the PHA oxidation rate much more in the case of GAOs than PAOs. Furthermore, the PHA

content shifted in the GAO, producing much more PHH at the expense of PHB, and the glycogen degradation was also reduced by 61% (Table 6.2). The reduced PHA and glycogen cycling most likely reflects that butyrate is a less suitable substrate to support GAO's metabolism. The decline in glycogen turnover would be detrimental to GAOs, but PAOs would be able to rely on their polyphosphate for ATP production, while NADH generation could be provided by other pathways, including the TCA cycle or butyrate degradation to e.g. acetyl-CoA (Oehmen et al. 2010, Santos et al. 2020).

Figure 6.3 Cycle study of (a, b, c) PAO reactor and (d, e, f) GAO reactor on day 18, 105 and 170 where the corresponding feed was 100% acetate, 50% acetate+50% butyrate and 100% butyrate respectively.

Table 6.2 The anaerobic stoichiometric of PAO and GAO on day 18, 105 and 170.

Day	PAO							GAO				
	Anaerobic							Anaerobic				
	P/C	PHB/C	PHV/C	PH2MV	PHH/C	PHA/C	Gly/C	PHB/C	PHV/C	PHH/C	PHA/C	Gly/C
	P-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol
18	0.58±0.01	0.83±0.03	0.22±0.02	0	0	1.05±0.05	0.28±0.07	1.55±0.10	0.90±0.05	0	2.45±0.15	1.23±0.23
105	0.74±0.03	0.73±0.01	0.13±0.03	0.08±0.06	0.12±0.01	1.04±0.09	0.13±0.02	0.81±0.03	0.58±0.07	0.59±0.11	1.98±0.18	0.72±0.11
170	0.59±0.10	0.52±0.01	0	0	0.17±0.02	0.69±0.03	0.15±0.01	0.24±0.02	0.40±0.08	0.35±0.05	0.99±0.13	0.48±0.02

Table 6.3 The aerobic stoichiometric of PAO and GAO on day 18, 105 and 170.

Day	PAO				GAO	
	Aerobic				Aerobic	
	P uptake mmol/g VSS/h	Net P- %	Gly/PHA C-mol/ C-mol	Active biomass concentration g/L	Gly/PHA C-mol/ C-mol	Active biomass concentration g/L
18	2.58±0.53	96.4±2.4	0.33±0.08	1.33±0.10	0.55±0.07	0.79±0.04
105	1.12±0.19	98.7±0.1	0.17±0.04	1.07±0.00	0.34±0.06	0.68±0.11
170	0.80±0.30	99.2±0.3	0.24±0.09	1.25±0.01	0.54±0.05	1.01±0.02

6.3.4 Batch experiments

In order to further investigate the competition between PAO and GAO for butyrate, batch experiments were conducted and the results are shown in Fig. 6.4 and Table 6.4. Butyrate in the PAO reactor was completely consumed during the 2 hours anaerobic phase, while 35.1% of the added butyrate remained in the GAO reactor (Fig. 6.4). The change in feeding rate during the batch tests could result in incomplete substrate consumption at the end of the anaerobic phase. Nevertheless, the anaerobic specific maximum VFA uptake rate of PAO and GAO cultures is comparable given the lower active biomass concentration of the GAO culture (Table 6.4). Most of the PAO and GAO stoichiometric transformations observed in the batch tests were consistent with that observed in the cycle studies. Some parameters that differed include the PHA/C storage ratio and the PHA fractionation. The change in feeding rate in the PAO and GAO reactors may have led to reduced butyrate loss through fermentation and decarboxylation, impacting the quantity of PHA stored and its monomeric composition.

Importantly, the biomass growth rate of PAO was higher than that of GAO (Table 6.4).

Since the anaerobic substrate uptake rate is comparable between PAO and GAO, the aerobic biomass yield (PAO vs GAO: 0.13 vs 0.07 g VSS/g COD) likely plays a key role in the PAO-GAO competition with this carbon source. The slower growth rate of GAO may also explain the lower biomass concentration observed in the parent reactor, and why the SRT had to be extended more in the GAO reactor than the PAO reactor.

Figure 6.4 Phosphorus and carbon transformation of (a) PAO reactor and (b) GAO

reactor fed with butyrate in batch experiment.

Table 6.4 The anaerobic specific maximum VFA uptake rate, anaerobic stoichiometry, aerobic P-uptake rate and aerobic stoichiometry of PAO and GAO feeding with butyrate.

Microbe	Anaerobic							Aerobic			
	VFA uptake	P/C	PHB/C	PHV/C	PH4MV/C	PHH/C	Gly/C	P uptake rate	P/PHA	Gly/PHA	Growth rate
	C-mmol/g VSS/h	P-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	mmol/g VSS/h	P-mol/ C-mol	C-mol/ C-mol	C-mmol/C-mol biomass/h
C4-PAO	2.59±0.39	0.79±0.12	0.81±0.08	0.02±0.02	0.14±0.02	0.25±0.02	0.18±0.04	0.83±0.02	0.76±0.22	0.24±0.04	13.25±0.31
C4-GAO	2.90±0.58	NA	0.51±0.22	0.20±0.07	NA	0.42±0.08	0.34±0.11	NA	NA	0.49±0.09	10.17±0.55

6.3.5 Impact of butyrate on EBPR system

Through long-term reactor operation and short-term batch experiment results, it is concluded that butyrate is a promising carbon source for EBPR systems in tropical climates. A relatively stable P-removal performance was realized with butyrate in a PAO reactor throughout the whole operation process, whereas butyrate may not be suitable for GAOs under similar operational conditions. With addition of butyrate in the feed, the total synthesized PHA reduced and the composition of PHA greatly changed. Such changes likely disturbed the microbial activity in the aerobic phase, which severely affected the GAO culture. While both *Accumulibacter* and *Competibacter* were negatively affected to a certain extent, *Accumulibacter* and other Rhodocyclaceae PAOs appear to adapt better to butyrate than *Competibacter*.

Though PAO could completely consume butyrate after acclimation, the specific maximum uptake rate of butyrate (2.59 C-mmol/g VSS/h) in batch experiments was still much lower than those of acetate and propionate by PAO at 30°C (acetate: 6.30 and propionate: 9.17 C-mmol/g VSS/h) (Wang et al. 2020b) and led to a lower P uptake rate. EBPR plants with very high butyrate fractions would likely require longer HRTs and SRTs to achieve good P removal and system stability. Nevertheless, the abundance of butyrate in fermented liquors is typically less than acetate, reducing the risk of butyrate entering into the subsequent aerobic phase and de-stabilising the EBPR process. Despite the fact that the *Accumulibacter* enrichment with butyrate is not as abundant as that in acetate and/or propionate fed cultures, the enrichment of other non-

Accumulibacter Rhodocyclaceae in PAO reactor can greatly contribute to phosphorus removal. Butyrate is often the second or third most abundant VFA after acetate and propionate in the wastewater and fermented liquids. This study for the first time revealed the function of butyrate in the competition of PAO and GAO and reported that butyrate would inhibit GAO's metabolism by reducing glycogen and PHA turnover significantly. Thus, using butyrate in mixed VFA sludge fermentation liquors is likely beneficial to EBPR, reducing the competition from GAO at high temperature.

Through long-term reactor operation and short-term batch experiment results, it is concluded that butyrate is a promising carbon source for EBPR systems in tropical climates. A relatively stable P-removal performance was realized with butyrate in a PAO reactor throughout the whole operation process, whereas butyrate may not be suitable for GAOs under similar operation conditions. With addition of butyrate in the feed, the total synthesized PHA reduced and the composition of PHA greatly changed. Such change may disturb the microbial activity in aerobic phase, which severely affected the GAO culture. While both *Accumulibacter* and *Competibacter* were negatively affected to a certain extent, *Accumulibacter* and other *Rhodocyclaceae* PAOs can better adapt to butyrate than *Competibacter*.

6.4 Conclusions

A stable phosphorus removal performance could be realized using butyrate as mixed or sole carbon source under tropical climate. When the carbon source is gradually changed

from acetate to butyrate, both *Accumulibacter* and *Competibacter* are negatively affected, but *Accumulibacter* could better adapt to butyrate. The enriched *Rhodocyclaceae* in PAO reactor also assisted in P-removal. Besides, the net PHA production and the composition of PHA were greatly changed in both cultures, which affected their aerobic metabolisms with GAO culture being more severely affected. Glycogen turnover were limited in both reactors, which could provide competitive advantage for PAOs. Thus, butyrate could be used as carbon source to promote EBPR and also suppress the population of GAOs.

CHAPTER 7 The anaerobic metabolism of butyrate by polyphosphate accumulating organisms and glycogen accumulating organisms under tropical climate

7.1 Introduction

In the EBPR systems, glycogen accumulating organisms (GAOs) always coexist with PAOs and compete for the limiting carbon source without contributing to phosphorus removal, leading to the failure of EBPR (Crocetti et al. 2002, Lopez-Vazquez et al. 2009b, Whang and Park 2006). Attempting to reduce the proliferation of GAO and maintain a stable phosphorus removal performance (Lopez-Vazquez et al. 2009b, Oehmen et al. 2006, Oehmen et al. 2005b), considerable studies have investigated the competition between PAO and GAO over the past few decades. Metabolic models have been established to evaluate the dynamics of PAO and GAO populations and to better understand the factors that may affect their interactions (Dai et al. 2007, Filipe et al. 2001e, Lanham et al. 2014, Oehmen et al. 2005d, Smolders et al. 1994a). Most of these models were developed using acetate and/or propionate as carbon sources as they are the most abundant carbon sources in wastewater.

In recent years, using sludge fermented liquid to improve biological nutrients (N and P) removal has attracted increasing attention because of its economic and sustainable virtues (Cao et al. 2019, Tong and Chen 2007, Wang et al. 2016). The fermentation liquid contains large amount of short-chain VFAs (acetate, propionate butyrate and valerate). Amount of butyrate could even reach 40%-60% of total VFAs in fermentation

liquid of winery wastewater or waste activated sludge (Esteban-Gutiérrez et al. 2018, Huang et al. 2016, Yuan et al. 2009). Levantesi et al. (2002) successfully operated an EBPR reactor fed by a mixture of acetate, propionate and butyrate with a stable phosphorus removal performance. Cai et al. (2019) showed that butyrate could enrich bacteria with phosphorus removal function, such as *Rhodocyclus*-related bacteria and *Actinobacteria*, and the system exhibited more stable phosphorus removal performance than that enriched by valerate. By operating two PAO and GAO reactors with mixed acetate and butyrate or sole butyrate as carbon sources, Wang et al. (2020a) demonstrated that butyrate provides competitive advantage for PAOs by maintaining their activity and inducing *Rhodocyclus*-related bacteria's growth. All these studies confirm that butyrate could be used as a supplementary carbon source or the sole carbon source to support the EBPR system. However, the detailed metabolic activities and pathways of PAOs and GAOs using butyrate are unclear. Thus, this study developed and validated the anaerobic metabolic model of butyrate uptake by PAOs and GAOs. Through modeling and experimental data, this study described the intracellular biochemical transformation of butyrate and identified the factors that may affect the competition between PAO and GAO. Furthermore, the proposed metabolic pathways of butyrate uptake by PAO and GAO were verified by metagenomic sequencing technology to distinguish the differences of their metabolic pathways.

7.2 Materials and Methods

7.2.1 Metabolic model for butyrate uptake by PAOs and GAOs

Noting the similarities in the uptake and utilization of acetate, propionate and butyrate by both PAOs and GAOs, the corresponding anaerobic metabolism are proposed and displayed in Fig. 6.1 and Fig. 6.2, respectively. Butyrate is taken up and converted to butyryl-CoA through the energy derived from poly-phosphate (poly-P) and/or glycogen degradation. Butyryl-CoA with acetyl-CoA and/or propionyl-CoA from glycogen hydrolysis is synthesized into the corresponding PHAs. The detailed reactions are described below.

7.2.1.1 Anaerobic metabolism of butyrate uptake by PAOs

Reaction 1: Butyrate uptake and convert to butyryl-CoA

Similar to acetate and propionate, the energy required for butyrate uptake is α_{PAO} mol ATP/C-mol of butyrate which is related to the extracellular pH, and conversion of one mole butyrate to butyryl-CoA need one mole ATP (Oehmen et al. 2005d, Smolders et al. 1994a). The reaction of butyrate uptake and conversion is expressed below (mol basis):

Reaction 2: Poly-P degradation for ATP

The main energy used for butyrate uptake and conversion is from the poly-phosphate degradation (Oehmen et al. 2005).

Reaction 3: Glycogen hydrolysis for NADH₂ (EMP)

In addition to the energy provided by the degradation of poly-P, glycogen hydrolysis generates part of the energy, which also provides reducing power (NADH₂) to maintain the cellular redox balance. Glycogen could be hydrolysed through Entner-Doudoroff (ED) pathway or Embden-Meyerhof-Parnas (EMP) pathway (Hesselmann et al. 2000, Martín et al. 2006). Building upon the recent consensus that PAOs utilize glycogen through the EMP pathway (Oehmen et al. 2010), EMP glycogen hydrolysis pathway is used in this study. Glycogen is degraded to pyruvate and further transformed to acetyl-CoA.

Reaction 4 and 5: Activated Acetyl-CoA and Butyryl-CoA

Before forming the corresponding PHA monomer, acetyl-CoA and butyryl-CoA would be activated, and reducing power would be required for this process (Filipe et al. 2001c).

Determination of overall anaerobic stoichiometry for PAO:

All internal anaerobic metabolic reactions for PAOs are listed above (Reaction 1 to 5). Through linking the measurable parameters with internal reactions and stoichiometric balance, an integrated overall relationship could be obtained. All parameters are listed below:

$$\begin{aligned}
 r_{\text{butyrate}} &= -r_1 \\
 r_{\text{HPO}_3} &= -r_2 \\
 r_{\text{H}_3\text{PO}_4} &= r_2 \\
 r_{\text{glycogen}} &= -r_3 \\
 r_{\text{pyruvate}} &= 2r_3 - r_{3a} \\
 r_{\text{acetyl-CoA}} &= r_{3a} - r_4 \\
 r_{\text{acetyl-CoA}^*} &= r_4 \\
 r_{\text{butyryl-CoA}} &= r_1 - r_5 \\
 r_{\text{butyryl-CoA}^*} &= r_5 \\
 r_{\text{NADH}_2} &= 2r_3 + r_{3a} - 0.5r_4 - 0.5r_5 \\
 r_{\text{ATP}} &= -(1 + 4\alpha_{\text{PAO}}) r_1 + r_2 + 3r_3 \\
 r_{\text{CO}_2} &= r_{3a} \\
 r_{\text{H}_2\text{O}} &= r_1 - r_2 - r_3
 \end{aligned}$$

Assuming there is no accumulation of acetyl-CoA, butyryl-CoA, energy and reducing power in the cells: $r_{\text{acetyl-CoA}} = 0$, $r_{\text{butyryl-CoA}} = 0$, $r_{\text{NADH}_2} = 0$ and $r_{\text{ATP}} = 0$. Then, substituting these into the above equations and the overall stoichiometry is obtained below (mole basis):

$$\begin{aligned}
 & -\text{Butyrate} - \frac{1}{6} \text{glycogen} - \left(\frac{1}{2} + 4\alpha_{\text{PAO}}\right) \text{HPO}_3 + \left(\frac{1}{2} + 4\alpha_{\text{PAO}}\right) \text{H}_3\text{PO}_4 + \frac{1}{3} \text{acetyl-CoA}^* \\
 & + \text{butyryl-CoA}^* + \frac{1}{3} \text{CO}_2 - \left(4\alpha_{\text{PAO}} - \frac{1}{3}\right) \text{H}_2\text{O} = 0
 \end{aligned} \tag{6}$$

PHAs condensation

Two types of PHA could be produced from acetyl-CoA^{*} and butyryl-CoA^{*} (Le et al. 2020). Two acetyl-CoA^{*} molecules could form one 3-hydroxybutyrate (3HB) molecule, and two butyryl-CoA^{*} molecules could form two 3-hydroxybutyrate (3HB) molecules while one acetyl-CoA^{*} molecule and one butyryl-CoA^{*} molecule could form one 3-hydroxyhexanoate (3HH) molecule. These molecules are further synthesized into the corresponding polymers (PHB and PHH). According to Filipe et al. (2001c) and Oehmen et al. (2005d), these molecules could be condensed randomly or selectively, which would form different PHA composition. Both random and selective condensation

are considered and displayed below.

Acetyl-CoA^{*} and butyryl-CoA^{*} randomly condensed to PHB. PHH is calculated based on the probability described in Filipe et al. (2001c) and the overall reaction is shown below (C-mol basis):

For selective condensation model, acetyl-CoA^{*} will preferentially combine with butyryl-CoA^{*} to form PHH. The integrated reaction is shown below (C-mol basis):

Figure 7.1 Proposed anaerobic metabolism of butyrate by PAOs.

7.2.1.2 Anaerobic metabolism of butyrate by GAOs

Comparing to PAOs, the energy required for butyrate uptake and conversion by GAOs derives only from the glycogen hydrolysis. Pyruvate hydrolyzed from glycogen converts to not only acetyl-CoA (reaction 3a), but also propionate-CoA through propionate-succinate pathway (Filipe et al. 2001c). Propionate-CoA is also activated to propionate-CoA^{*}. The energy required for butyrate uptake in GAO is defined as α_{GAO} (mol ATP/C-mol of butyrate). The additional reactions are listed below:

Linking the all measurable parameters with internal reactions for GAOs:

$$r_{\text{butyrate}} = -r_1$$

$$r_{\text{glycogen}} = -r_3$$

$$r_{\text{CO}_2} = r_{3a}$$

$$r_{\text{Pyruvate}} = 2r_3 - r_{3a} - r_{3b}$$

$$r_{\text{H}_2\text{O}} = r_1 - r_2 - r_3 + 2r_{3b}$$

$$r_{\text{acetyl-CoA}} = r_{3a} - r_4$$

$$r_{\text{acetyl-CoA}^*} = r_4$$

$$r_{\text{propionyl-CoA}} = r_{3b} - r_9$$

$$r_{\text{propionyl-CoA}^*} = r_9$$

$$r_{\text{butyryl-CoA}} = r_1 - r_5$$

$$r_{\text{butyryl-CoA}^*} = r_5$$

$$r_{\text{NADH}_2} = 2r_3 + r_{3a} - 0.5r_4 - 0.5r_5 - 0.5r_9 - 2r_{3b}$$

$$r_{\text{ATP}} = -(1 + 4\alpha_{\text{GAO}})r_1 + 3r_3$$

Simliar to PAO metabolism, assuming there is no accumulation of acetyl-CoA, propionyl-CoA, butyryl-CoA, energy and reduing power in the cells: $r_{\text{acetyl-CoA}} = 0$, $r_{\text{propionyl-CoA}} = 0$, $r_{\text{butyryl-CoA}} = 0$, $r_{\text{NADH}_2} = 0$ and $r_{\text{ATP}} = 0$. Then, all internal reactions could incorporate into one relationship (mol basis):

$$- \text{Butyrate} - \left(\frac{1}{3} + \frac{4}{3}\alpha_{\text{GAO}} \right) \text{glycogen} + \left(\frac{1}{2} + \frac{4}{3}\alpha_{\text{GAO}} \right) \text{acetyl-CoA}^* + \left(\frac{1}{6} + \frac{4}{3}\alpha_{\text{GAO}} \right)$$

For random model, acetyl-CoA^{*}, propionyl-CoA^{*} and butyryl-CoA^{*} are condensed based on the probability described in (Filipe et al. 2001c). Two acetyl-CoA^{*} molecules could form one 3-hydroxybutyrate (3HB) molecule, two butyryl-CoA^{*} molecules could form two 3-hydroxybutyrate (3HB) molecules and they are added together to form total PHB. One acetyl-CoA^{*} molecule and one butyryl-CoA^{*} molecule could form one 3-hydroxyhexanoate (3HH) molecule or one 3-hydroxy-4-methylvalerate (3H4MV) molecule while two propionyl-CoA^{*} molecules could form one 3-hydroxy-2-methylvalerate (3H2MV) molecule. Since PH2MV, PH4MV and PHH are isomers of each other, they are added together and simplified as PHH. Furthermore, one acetyl-CoA^{*} molecule and propionyl-CoA^{*} molecule could form one 3-hydroxyvalerate (3HV) while propionyl-CoA^{*} molecule and one butyryl-CoA^{*} molecule could form one 3-hydroxy-2-methylhexanoate (3H2MH) molecule. The overall reaction is shown below (C-mol basis):

For selective condensation model, assuming acetyl-CoA^{*} preferentially combines with propionyl-CoA^{*} to form PHV and then combines with butyryl-CoA^{*} to form PHH. The remaining butyryl-CoA^{*} is condensed to PHB without PH2MH being produced.

The integrated reaction is shown below (C-mol basis):

$$\begin{aligned}
 & - \text{CH}_2\text{O}_{0.5} \text{ (butyrate)} - \left(\frac{1}{2} + 2\alpha_{\text{GAO}} \right) \text{CH}_{10/6}\text{O}_{5/6} \text{ (glycogen)} + \frac{2}{3} \text{CH}_{1.5}\text{O}_{0.5} \text{ (PHB)} \\
 & + \left(\frac{5}{24} + \frac{5}{3}\alpha_{\text{GAO}} \right) \text{CH}_{1.6}\text{O}_{0.4} \text{ (PHV)} + 0.5 \text{CH}_{5/3}\text{O}_{1/3} \text{ (PHH)} + \left(\frac{1}{12} + \frac{1}{3}\alpha_{\text{GAO}} \right) \text{CO}_2 - \\
 & \left(\frac{1}{4} + \frac{1}{3}\alpha_{\text{GAO}} \right) \text{H}_2\text{O} = 0
 \end{aligned} \tag{12}$$

Figure 7.2 Proposed anaerobic metabolism of butyrate by GAOs.

7.2.2 Experiments

The sludge was withdrawn from the parent reactors (C4-PAO and C4-GAO) to investigate the carbon transformation through batch experiments. The detailed experimental steps can be found in Wang et al. (2020a), and the results of batch experiments were used to compare with the proposed model prediction in this study.

7.2.3 Metagenome library construction and high-throughput sequencing

The genomic DNA were extracted from 0.2 g sludge that were obtained from the C4-PAO and C4-GAO reactors, respectively. DNA extraction procedure can be found in study of Wang et al. (2020a). Extracted DNA samples were sent for high-throughput

sequencing. The metagenomic library was constructed according to the Illumina operating instructions (www.mrdnalab.com, Shallowater, USA), and Paired-end sequencing was performed on Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using HiSeq 3000/4000 PE Cluster Kit and HiSeq 3000/4000 SBS Kits.

7.2.4 Bioinformatics analysis

Metagenome sequencing data were mainly assembled by IDBA-UD. Metagenomic nucleic acid sequence data sets were referred to the SOAP aligner (<http://soap.genomics.org.cn/>) to evaluate gene abundance. As for functional annotation, BLASTP (Version 2.2.28+, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied to compare non-redundant gene set sequences with KEGG's gene database (GENES). An e-value cutoff of $1e-5$ was used. According to the comparison results, KOBAS 2.0 (KEGG Orthology Based Annotation System) was used for functional annotation. Finally, the sum gene abundance of the corresponding KO (KEGG Orthology), Pathway, EC, and Module were showed as the abundance of this functional category.

7.3 Results and Discussion

7.3.1 Anaerobic stoichiometry

1.3.1.1 Anaerobic metabolism of PAO

The carbon transformation and P-release to butyrate uptake ratio (P/C) during the anaerobic phase is summarized in Table 7.1. The data was used to compare with the

random/selective model predictions. According to the model prediction, the P release to butyrate uptake ratio should be $0.13 + \alpha_{\text{PAO}}$ (P-mol/C-mol), where the α_{PAO} is related to the extracellular pH (Oehmen et al. 2005d, Smolders et al. 1994a). Smolders et al. (1994a) showed that when pH increased from 5.5 to 8.5, P/C varied between 0.25 to 0.75 P-mol/C-mol at 20 °C. The experiment result of P/C ratio was 0.79 P-mol/C-mol, giving the α_{PAO} value of 0.66. And the pH for this study was controlled between 7.0 - 8.0, where the α_{PAO} could be around 0.50 to 0.65 P-mol/C-mol according to (Smolders et al. 1994). Besides, a higher maintenance energy was required for PAO at high temperature which also could contribute to a higher P/C ratio (Brdjanovic et al. 1997, Shen et al. 2017). Therefore, the P/C ratio obtained by the experiment is in good agreement with the model prediction. In addition, the experiment results of anaerobic carbon transformation (PHA/C) are consistent with the values predicted by the random model (Table 7.1). Wang et al. (2020a) showed that the anaerobic stoichiometry of acetate-PAO and propionate-PAO at high temperature (30 °C) was comparable to model prediction developed at 20 °C indicating that temperature would not affect the stoichiometry. Thus, temperature would also not change the anaerobic stoichiometry of butyrate by PAO. This can be proven by the alignment of the experimental results at high temperature with the model prediction in this study. The butyrate was mainly converted into PHB (66.4% of total PHA) and PHH (32.0% of total PHA). This indicates that acetyl-CoA^{*} and butyryl-CoA^{*} are randomly combined. Nevertheless, Oehmen et al. (2005d) exhibited that there was greater binding affinity between acetyl-CoA^{*} and propionyl-CoA^{*} in propionate-fed PAO culture, evident from the

agreement between their experimental results and the selective model prediction. Therefore, different carbon sources would have different affinity, which leads to the synthesis of different PHA components. The good agreement between experimental results and random model prediction suggests that the proposed model accurately describes the anaerobic stoichiometry related to butyrate uptake by PAOs. The glycogen degradation to butyrate uptake ratio (glycogen/C) was slightly lower than the model prediction. It is also found that the PHA/C, Glycogen/C and P/C ratios of butyrate model prediction are all lower compared to the acetate and propionate models (Table 7.1). The required energy for per C-mol of VFA conversion to corresponding acyl-CoA would decrease as the amount of carbon in the VFA increases (Oehmen et al. 2005d, Wang et al. 2018). Thus, P/C ratio of butyrate model is lower than that of acetate and propionate model. When there is the same energy, more butyrate could be transported and converted to butyryl-CoA. Besides, different carbon sources will synthesize different PHA components which may further influence the P-uptake and glycogen recovery in aerobic phase. Randall and Liu (2002) and Lopez et al. (2006) showed that P-uptake rate was higher with PHB than that with PHV. Higher P-uptake rates and P removal efficiency were found with acetate-fed and butyrate-fed compared to that of propionate-fed in the short-term batch experiments (Wang et al. 2020a). This may be because the main component of PHA is PHB with acetate and butyrate. However, there is small amount of PHH synthesized by butyrate. Further study is needed to investigate how it would influence the PAO's aerobic metabolism.

Table 7.1 Comparison of model predictions with experiment results of PAO.

Stoichiometry	P/C P-mol/ C-mol	PHAs/C C-mol/ C-mol	PHB/C C-mol/ C-mol	PHV/C C-mol/ C-mol	PHH/C C-mol/ C-mol	Glycogen/C C-mol/ C-mol
Random model	$0.13+\alpha_{\text{PAO}}$	1.17	0.79	0	0.38	0.25
Selective model	$0.13+\alpha_{\text{PAO}}$	1.17	0.67	0	0.5	0.25
Experiment results ^a	0.79 ± 0.12	1.22 ± 0.12	0.81 ± 0.08	0.02 ± 0.02	0.39 ± 0.04	0.18 ± 0.04
Acetate model ^b	$0.33+\alpha_{\text{PAO}}$	1.33	1.33	0	0	0.50
Propionate model ^c	$0.22+\alpha_{\text{PAO}}$	1.23	0	0.56	0.67(PH2MV)	0.33

a: Wang et al. (2020a), b: Smolders et al. (1994), c: Oehmen et al. (2005b)

7.3.1.2 Anaerobic metabolism of GAO

Table 7.2 shows GAO's anaerobic carbon transformation and the comparison with the random/selective model developed. In previous proposed GAO model, the energy required for butyrate uptake was defined as α_{GAO} , which is related to the extracellular pH. Filipe et al. (2001c) estimated that α_{GAO} was around 0.06-0.12 when pH was between 7-8 for acetate enriched GAO. In order to simplify the calculation, α_{GAO} is assumed to be zero for butyrate enriched GAO in this study. Further study is needed to investigate the accurate α_{GAO} value for butyrate enriched GAO at different pH.

Table 7.2 Comparison of model predictions with experiment results of GAO.

Stoichiometry	PHA/C	PHB/C	PHV/C	PHH/C	PH2MH/C	Glycogen/C
Random model	1.38	0.68	0.06	0.46	0.18	0.50
Random composition	100%	49%	4%	34%	13%	NA
Selective model	1.38	0.67	0.21	0.50	0	0.50
Selective composition	100%	49%	15%	36%	0	NA
Experiment results ^a	1.13±0.12	0.51±0.22	0.20±0.07	0.42±0.08	0	0.34±0.11
Result composition	100%	45%	18%	37%	0	NA
Acetate model ^b	1.86	1.36	0.46	0.04(PH2MV)	0	1.12
Propionate model ^c	1.50	0.10	0.58	0.82(PH2MV)	0	0.67

a: Wang et al. (2020a), b: Filipe et al. (2001c), c: Oehmen et al. (2004)

Similar to PAO models, temperature would not change the stoichiometry of GAOs (Wang et al. 2020b). It is found that the total amount of PHA obtained from the experiment was lower than that predicted by both random and selective models. One of the possible reasons is that a small amount of butyrate may have been fermented, causing carbon loss. Another reason may be that α_{GAO} was not zero in actual operation, which causes a slight deviation between the experimental results and the model predictions. Thus, the proportion of individual PHA was calculated to compare different PHA components so as to ascertain the corresponding combined forms (Table 7.2). From the comparison, prediction by the selective model is more consistent with the experimental results. This suggests that acetyl-CoA^{*} preferentially combines with

propionyl-CoA^{*} forming PHV and then combines with butyryl-CoA^{*} forming PHH. The remaining butyryl-CoA^{*} is condensed to PHB, and no PH2MH is produced. Similar to the PAO culture, the glycogen/C ratio of GAO was also slightly lower than the model prediction.

It is worth noting that the experimental results of PAO are consistent with the prediction of the random model while GAO is closer to the prediction of the selective model. Oehmen et al. (2004) found that different PHA formation also existed between PAO (selective) and GAO (random) enriched by propionate. However, their results showed opposite model preference. Although the reasons for the opposite results remained unclear, PAO and GAO seem to take different metabolic pathways for PHA production. It seems that when propionyl-CoA^{*} exists, acetyl-CoA^{*} tends to combine with it to generate PHV. However, there is no propionyl-CoA^{*} in PAO culture, where acetyl-CoA^{*} and butyryl-CoA^{*} are randomly combined. It is precisely because of this different affinity that causes butyrate to synthesize different PHA components by PAO and GAO cultures. Further study may be needed to investigate how the different PHA components influence the competition between PAO and GAO.

7.3.2 Metabolic pathway analysis in KEGG

Metagenomic sequencing technology was also used in this study to verify the proposed metabolic pathways of butyrate uptake by PAO and GAO. Approximately 10 Gb of metagenomic sequences were yielded in the sample of PAO-C4 and GAO-C4, with a total number of predicted genes ranging from 29,881,090 to 21,956,328, respectively.

Microbiome information has been discussed in detail in our previously study (Wang et al. 2020a). The metabolic pathway based on metagenomic genes is studied and the results are displayed in Figure 7.3.

Figure 7.3 Variations of identified genes involved in PHAs synthesis pathway for PAO and GAO cultures.

By comparing the proposed metabolic model with experiment results, it is hypothesized that butyrate is converted to two PHAs (PHB and PHH) by PAO and three PHAs (PHB, PHV and PHH) by GAO. The functional gene changes and metabolites involved are discussed in this section. There are mainly two synthetic routes for PHB synthesis. One is that Acetyl-CoA generates Acetoacetyl-CoA by acetyl-CoA acyltransferase 2 (EC [2.3.1.16], including gene *fadA* and *ACAA2*) and acetyl-CoA C-acetyltransferase (EC [2.3.1.9], gene *atoB*), and then further convert to PHB by 3- hydroxyacyl-CoA dehydrogenase (EC [1.1.1.35], including gene *fadJ* and *fadN*). This is a widely reported pathway to synthesize PHB by acetate. The second is via Butyryl-CoA in acyl-CoA oxidase (EC [1.3.3.6], gene *ACOX*), butyryl-CoA dehydrogenase (EC[1.3.8.1], gene *bcd*), and acyl-CoA dehydrogenase (EC[1.3.8.7], gene *acd*; EC[1.3.99.-], gene *fadE*) to

generate Crotonoyl-CoA, followed by enoyl-CoA hydratase (EC[4.2.1.17], including genes *echA*, *fadB* and *fadJ*) catalyzing the Crotonoyl-CoA substrates to PHB with the concomitant release of CoA. Furthermore, it is found that the unusual PHA, i.e. PHH, is synthesized by Butyryl-CoA and Acetyl-CoA under combined promotion of acetyl-CoA acyltransferase2, acetyl-CoA C-acetyltransferase and 3- hydroxyacyl-CoA dehydrogenase. In the GAO culture, on top of the above-mentioned PHB and PHH, PHV was also produced. The synthesis imitated with the generation of 3-Oxovaleryl-CoA from Propionyl-CoA and Acetyl-CoA, followed by conversion to PHV.

A higher gene expression of *ACOX*, *bcd* and *acd* was showed in PAO culture (with abundance of 0.04‰, 0.10‰ and 0.89‰ respectively) than GAO culture (with abundance of 0.03‰, 0.04‰ and 0.33‰ respectively) (Fig. 4), which had the function of converting Butyryl-CoA to Crotonoyl-CoA. Besides, a higher gene expression of *echA* and *fadJ*, belonging to enoyl-CoA hydratase, was also showed in PAO culture than GAO culture (0.93‰ and 0.71‰ vs 0.62‰ and 0.38‰). The Crotonoyl-CoA could be further synthesized to PHB by enoyl-CoA hydratase. This suggests that butyrate could be more easily converted to PHB by PAO. A comparable abundance of gene *atoB* and *fadA* was showed in both PAO and GAO culture, however, gene *fadJ* and *fadN* had a higher gene expression in PAO culture. This shows that although butyrate and acetate could be converted to 3-Oxohexanoyl-CoA in both PAO and GAO, which could be more easily further synthesize to PHH in PAO culture. Perhaps because the gene expression of synthetic PHB and PHH was relatively low in GAO culture, forcing acetyl-CoA to preferentially combine with propionyl-CoA to generate PHV. All the

results indicate that butyrate is more easily converted to the PHB and PHH by PAO. As discussed before, total amount of PHAs obtained from the experiment was consistent with model prediction in PAO culture while total amount of PHAs was lower than that predicted by proposed model in GAO culture. The amount of usable PHAs would further affect the biological activity of the two cultures in the aerobic stage. Wang et al. (2020a) also discussed that butyrate could support PAO but not GAO under tropical climate in the long-term operation. To our knowledge, this is the first report that uses metagenomics to describe the metabolic pathways of butyrate in EBPR system.

7.4 Conclusions

The experiment results of anaerobic carbon transformation proved that butyrate was utilized by PAO through the random model while GAO's metabolism is more consistent with the prediction of the selective model. PAO and GAO seem to take different metabolic pathways. A higher gene expression of carbon transformation was found in PAO reactor than GAO reactor which may suggest that butyrate is more easily converted by PAO.

CHAPTER 8 Conclusions and recommendations

8.1 Conclusions

This thesis evaluated the impacts of carbon sources on both anaerobic and aerobic *Accumulibacter* PAO metabolism at high temperature. High temperature conditions could affect their kinetics, including the VFA consumption rate, anaerobic phosphorus release rate, and aerobic phosphorus uptake rate, without significantly changing its reaction stoichiometry. Similar to low temperature EBPR, propionate seems to be a preferred carbon source for *Accumulibacter* PAOs as compared to acetate because less energy is required for its uptake. However, lower P uptake and higher glycogen replenishment with propionate as feed was found in both parent reactor and batch tests, which may negatively impact EBPR performance in long-term operation. A combination of acetate, propionate and perhaps butyrate may be better substrates formula for EBPR, since this led to higher anaerobic VFA uptake rates coupled with higher aerobic P uptake rates and lower glycogen production rates.

Like PAO culture, various carbon sources were also tested on anaerobic and aerobic metabolism of *Defulviicoccus vanus* related GAOs. It is found that *Dv*GAOs could utilize acetate and/or propionate but could hardly metabolize other longer chain VFAs. However, *Dv*GAOs are less competitive than PAOs at high temperature due to their lower maximum anaerobic substrate uptake rate. In order to limit the proliferation of *Dv*GAOs and *Competibacter*, a combined substrate of acetate and propionate may be a better choice in high temperature EBPR systems.

Furthermore, a stable phosphorus removal performance was realized using butyrate as mixed or sole carbon source under tropical climate. When the carbon source was gradually changed from acetate to butyrate, both *Accumulibacter* and *Competibacter* are negatively affected, but *Accumulibacter* could better adapt to butyrate. The enriched *Rhodocyclaceae* in PAO reactor also assisted P removal. Besides, the net PHA production and the composition of PHA greatly changed in both cultures, affecting their aerobic metabolisms with GAO culture being more severely affected. Glycogen turnover were limited in both reactors, which could provide competitive advantage for PAOs. Thus, butyrate could be used as carbon source to promote EBPR and also suppress the population of GAOs. Therefore, WAS fermentation liquors that usually contain significant quantities of acetate, propionate and butyrate can be an effective alternative carbon source for EBPR at high temperature.

8.2 Recommendations

During the development of anaerobic metabolism of butyrate by GAOs, in order to simplify the calculation, α_{GAO} was assumed to be zero in this study. Further study is needed to investigate the accurate α_{GAO} value for butyrate enriched GAO at different pH, since pH will influence the PHA production and the composition of PHA.

Further study is also needed to investigate how the different PHA components influence the competition between PAOs and GAOs. Unusual PHH was synthesized with butyrate in both PAO and GAO cultures, its influence on PAO and GAO's aerobic metabolism is still unknown. It is speculated that PHH is not easily utilized and has a

greater adverse effect on GAO. Further research is needed to study the effect of PHH on the competition of PAO and GAO.

Other than the short-chain VFAs, fermentated liquid also contains other organic compounds, e.g. humic acid, amino acid, and polysaccharide. Whether these organic compounds can be utilized by PAO and GAO, or how it affects the competition between PAO and GAO is still unknown. Further study is also needed to investigate the effect of other organic compounds in fermented liquid on EBPR system.

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Supporting information

Figure S1 Percent of community abundance of PAO (a) and GAO (b) culture at Genus level.

Figure S2 Cycle study of PAO reactor at (a) C2:C4(9:1), (b) C2:C4(8:2), (c) C2:C4(7:3), (d) C2:C4(6:4), (e) C2:C4(4:6), (f) C2:C4(3:7), (g) C2:C4(2:8), (h) C2:C4(1:9).

Figure S3 Cycle study of GAO reactor at (a) C2:C4(9:1), (b) C2:C4(8:2), (c)

$C2:C4(7:3)$, (d) $C2:C4(6:4)$, (e) $C2:C4(4:6)$, (f) $C2:C4(3:7)$, (g) $C2:C4(2:8)$, (h)
 $C2:C4(1:9)$.

Table S1 The stoichiometry and kinetic of PAO during the anaerobic and aerobic phase.

Substrate	Anaerobic								Aerobic			
	P/C	Gly/C	PHB/C	PHV/C	PH2MV/C	PH4MV/C	PHH/C	PHA/C	P uptake	Net	Gly/PHA	Active
	P-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	mmol/ g VSS/h	P-removal %	C-mol/ C-mol	biomass concentration
C2:C4 (9:1)	0.50±0.01	0.25±0.09	0.62±0.10	0.11±0.03	0.13±0.08	0	0	0.86±0.06	3.61±0.68	93.7±1.5	0.27±0.03	1.14±0.04
C2:C4 (8:2)	0.61±0.07	0.23±0.03	0.63±0.01	0.02±0.02	0.01±0.01	0.01±0.01	0	0.66±0.04	1.89±0.48	98.7±0.4	0.30±0.04	1.06±0.05
C2:C4 (7:3)	0.68±0.02	0.21±0.01	0.64±0.05	0.15±0.02	0.10±0.06	0	0.04±0	0.93±0.06	2.05±0.37	99.8±0.1	0.25±0.04	1.22±0.19
C2:C4 (6:4)	0.52±0.04	0.20±0.02	0.54±0.01	0.15±0.02	0.16±0	0	0.05±0	0.90±0.02	1.69±0.28	99.3±0.7	0.27±0.08	0.88±0.06
C2:C4 (4:6)	0.84±0.06	0.35±0.05	0.86±0.05	0.06±0	0.07±0.01	0	0.34±0.07	1.32±0.14	1.13±0.26	91.6±0.6	0.30±0.01	1.18±0.03
C2:C4 (3:7)	0.56±0.02	0.20±0	0.63±0.03	0.15±0.03	0	0.08±0	0.18±0.02	1.04±0.08	0.93±0.21	99.2±0.4	0.26±0.04	1.17±0.06
C2:C4 (2:8)	0.49±0.04	0.14±0.02	0.57±0.09	0.12±0.04	0	0	0.15±0.02	0.84±0.13	1.17±0.28	99.6±0.1	0.33±0.15	1.22±0.17
C2:C4 (1:9)	0.50±0.08	0.13±0.02	0.62±0.07	0.06±0.05	0	0	0.19±0.04	0.87±0.12	0.83±0.22	99.7±0.1	0.18±0.01	1.52±0.06

Table S2 The stoichiometry of GAO during the anaerobic and aerobic phase.

Substrate	Anaerobic							Aerobic	
	Gly/C	PHB/C	PHV/C	PH2MV/C	PH4MV/C	PHH/C	PHA/C	Gly/PHA	Active biomass
	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	C-mol/ C-mol	concentration g/L
C2:C4 (9:1)	1.22±0.06	1.30±0.11	0.51±0.07	0	0	0.16±0.02	1.96±0.16	0.66±0.08	0.74±0.11
C2:C4 (8:2)	0.98±0.12	1.05±0.02	0.32±0	0	0.01±0.01	0.40±0.01	1.77±0.02	0.56±0.01	0.66±0.05
C2:C4 (7:3)	1.16±0.04	1.24±0.08	0.61±0.26	0	0	0.68±0.03	2.54±0.37	0.47±0.07	0.62±0.01
C2:C4 (6:4)	0.89±0.09	0.83±0.01	0.36±0.04	0	0.03±0.01	0.43±0.02	1.65±0.06	0.54±0.04	0.68±0.16
C2:C4 (4:6)	0.89±0.01	0.91±0.04	0.66±0.07	0.11±0.07	0	0.59±0.03	2.27±0.09	0.40±0.03	0.67±0.05
C2:C4 (3:7)	0.59±0.18	0.60±0.01	0.60±0.04	0	0	0.37±0.03	1.57±0.08	0.51±0.08	1.09±0.07
C2:C4 (2:8)	0.55±0.03	0.48±0.01	0.44±0.03	0	0	0.58±0.07	1.51±0.09	0.42±0.05	0.83±0.12
C2:C4 (1:9)	0.38±0.11	0.44±0.07	0.40±0.10	0	0	0.44±0.07	1.28±0.11	0.39±0.14	1.28±0.25