Enhanced biological phosphorus removal from wastewater in the tropics

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2018

Cokro, A. A. (2018). Enhanced biological phosphorus removal from wastewater in the tropics. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/75924>

<https://doi.org/10.32657/10356/75924>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**Enhanced Biological Phosphorus Removal from
Wastewater in the Tropics**

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2018

Enhanced Biological Phosphorus Removal from Wastewater in the Tropics

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

2018

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincerest gratitude to those who have supported me during my journey as a PhD student.

First and foremost, I would like to thank Prof. Stefan Wuertz for his continuous support and guidance. Thank you for giving me the chance to embark on this exciting PhD programme in the first place. Thank you for your constant encouragement so that I can continuously grow as a scientist. It is a great privilege for me to complete my PhD under your guidance, and I am extremely thankful for all the valuable lessons that I have learned.

I am grateful for the research grant, which is supported by the Singapore National Research Foundation under its Environment & Water Research Programme and administered by PUB, Singapore's national water agency, and the Ministry of Education under the Research Centre of Excellence Programme.

I would also like to express my sincerest appreciation to Dr. Law Yingyu for her guidance. Thank you for your feedback and support throughout these years, which helped me move forward on this journey. I would like to thank Dr. Rohan Williams for his input, especially on data analysis. I have gained a lot of insights from your feedback. Next, I would like to extend my deepest gratitude to Prof. Per Nielsen for his feedback and suggestions, especially pertaining to EBPR. I have learned a lot from your inputs throughout the years. I would also like to acknowledge Mr. Ezequiel Santillan who has guided me in the multivariate analyses and carefully reviewed my manuscript draft. I am truly grateful for your support. Additionally, I would like to give special acknowledgements to Prof. Liu Yu, A/Prof. Cao Bin, and A/Prof. Scott Rice who have provided valuable comments during my qualifying exams.

Thank you to all my SCELSE colleagues for your support as well. Thank you Dr. Thomas Seviour for your input, especially in the early stage of my PhD journey; and members of cluster 1 for all your support, encouragement, and inputs during my presentations. My

sincerest gratitude to Ms. Priyadharsani Thangavelu, Ms. Sara Swa Thee, and Mr. Egunathan Kaliyamoorthy who have helped me tremendously in the lab, especially in the nutrient sample testing and PHA/glycogen analyses; to Mr. Larry Liew who has helped me during the field sampling, sludge collection and also for extracting my DNA samples; to Ms. Anna Ngoc for her help in preparing my DNA samples and sending them for sequencing and for helping me with PCR and qPCR; to Dr. Cecilia Cruz for her input on PCR/qPCR; to A/Prof. Veronica Rajal for the knowledge and help during her PCR course; and to Dr. Irina Bessarab for her assistance when I needed to send my DNA samples for sequencing. I would also like to thank Mr. Rasmus Kirkegaard and Mr. Mikkel Stokholm Bjerregard from Aalborg University, Denmark for their help with my amplicon sequencing samples and glycogen tests. Thank you all so much for your help.

I would also like to thank SCELSE staff for their support, especially Ms. Chia Kar Ling for her assistance in running my IC samples and her valuable input on GC and HPLC; Ms. Wahyuna Bte Sulaiman and Ms. Chew Ley Bryan for their assistance in the logistics during my research; Ms. Chee Wey Yeeng, Ms. Kartini Bte Saharawe, and Ms. Deborah Tjin for their support in the procurement process; and Dr. Kraeger Koh for his input especially on my reactor design and procurement process. A special thank you to Ms. Ng Soo Ching from the graduate study office at School of Civil and Environmental Engineering at NTU for her patience in answering and accommodating my inquiries.

Last but certainly not least, I am indebted to my family for their faith and unrelenting support. To my father for his patience and wisdom that kept me going; to my elder sisters and their families who have always been there for me, who always makes me feel happy when I am sad; and to my twin sister who is my constant cheerleader and is always there to help me and lift me up when I am feeling down. I am forever thankful to have all of you in my life.

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ABBREVIATIONS

° C	Degree in Celsius
Acetyl-CoA	Acetyl Coenzyme A
AlPO ₄	Aluminium phosphate
ATP	Adenosine Triphosphate
ATPase	ATP synthase
C	Carbon
DGGE	Denaturing Gradient Gel Electrophoresis
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic Acid
DPAO	Denitrifying PAO
EBPR	Enhanced Biological Phosphorus Removal
FAD	Flavin Adenine Dinucleotide
FADH ₂	Reduced form of FAD
FePO ₄	Iron (III) phosphate
FISH	Fluorescence In-Situ Hybridization
GAO	Glycogen Accumulating Organism
ICL	Isocitrate lyase
KDPG	2-keto-3-deoxy-6-phosphogluconate
MLE	Modified Ludzack Ettinger
MCM	Methymalonyl-CoA mutase
N	Nitrogen
NAD ⁺	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of NAD ⁺
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate

NADPH	Reduced form of NADP ⁺
Non-DGAO	Non-denitrifying GAO
Non-DPAO	Non-denitrifying PAO
Non-PAO denitrifier	Heterotrophic denitrifier
P	Phosphorus
PAO	Polyphosphate Accumulating Organism
PH2MB	Polyhydroxy-2-methylbutyrate
PH2MV	Polyhydroxy-2-methylvalerate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PO ₄	Phosphate
Poly-P	Polyphosphate
<i>ppk1</i>	Polyphosphate kinase 1 gene
PST	Primary Settling Tank
RAS	Returned Activated Sludge
rRNA	Ribosomal Ribonucleic Acid
SBR	Sequential Batch Reactor
SRT	Sludge Retention Time
TCA	Tricarboxylic Acid
VFA	Volatile Fatty Acid
WAS	Wasted Activated Sludge

ABSTRACT

Enhanced biological phosphorus removal capitalizes on the ability of polyphosphate accumulating organisms (PAOs) to store phosphorus in excess of what is needed for growth. Conventionally, EBPR requires the presence of an anaerobic phase followed by an anoxic or aerobic phase. A separation of the availability of carbon source and electron acceptors like oxygen or nitrate/nitrite is thought to be crucial for successful EBPR. Additionally, higher temperatures are thought to be detrimental to EBPR. However, recent findings have challenged this paradigm. This dissertation explores EBPR at warm temperatures, under conditions when (i) carbon sources like volatile fatty acids (VFAs) and electron acceptors, such as nitrate and/or nitrite, are simultaneously present and (ii) no planned anaerobic phase is provided.

Batch experiments were conducted to determine the capacities of PAOs, especially those that cannot utilize nitrate and/or nitrite (that is, non-denitrifying PAOs or non-DPAOs), to perform anoxic/aerobic EBPR at an ambient temperature of 30 ± 1 °C, which is the temperature of wastewater in Singapore. I hypothesized that non-DPAOs were capable of performing EBPR under anoxic/aerobic cycling, due to their inability to utilize nitrate. Hence they performed EBPR as they would under anaerobic/aerobic conditions. Four laboratory-scale sequencing batch reactors (SBRs) were inoculated with freshly collected returned activated sludge (RAS) from a wastewater reclamation plant in Singapore. They were subjected to (i) anaerobic/aerobic cycling as control; (ii) anaerobic/anoxic/aerobic cycling to determine the presence and proportions of active non-DPAOs in the full-scale sludge; (iii) anoxic/aerobic cycling with nitrate as the electron acceptor as the main condition tested; or (iv) fully aerobic conditions with oxygen. Either synthetic wastewater, with acetate or propionate as external carbon source, or primary settling tank (PST) effluent was used as feed.

The batch experiments revealed that PAOs in the full-scale sludge could perform EBPR under anoxic/aerobic cycling, and Type II *Accumulibacter*, a group of non-DPAOs, predominated in sludge collected from the full-scale plant without a designated anaerobic phase in the aeration tanks (Modified Ludzack Ettinger configuration). With their inability to use nitrate, non-DPAOs recognized the anoxic/aerobic conditions as anaerobic/aerobic and carried out EBPR accordingly. Consequently, the hypothesis was confirmed with the implication that EBPR can be successful at warm temperatures in treatment plants with an anoxic/aerobic configuration, eliminating the requirement for an anaerobic stage.

After the batch experiments, I further tested the impact of prolonged continuous anoxic/aerobic cycling on PAOs at the same warm temperature. Additionally, the responses of glycogen accumulating organisms (GAOs) and heterotrophic denitrifiers were determined. GAOs can compete with PAOs for carbon, and their presence has often been attributed as cause of EBPR failure at higher temperatures. In this study, two laboratory-scale enrichment reactors were inoculated with sludge from a source reactor that had been exposed to anaerobic/aerobic cycling for 179 days at 30 ± 1 °C. These reactors were fed synthetic wastewater with acetate as external carbon source and subjected to a 57-day anaerobic/aerobic cycling before switching to planned anoxic/aerobic cycling via simultaneous addition of acetate and nitrate for 43 days (3 sludge retention times or SRTs). I hypothesized that, just like non-DPAOs, non-denitrifying GAOs could recognize the anoxic phase as anaerobic. Consequently, they would consume acetate and proliferate. The third hypothesis stated that some non-DPAOs would also survive and perform EBPR accordingly.

When acetate and nitrate were concurrently added to the laboratory-scale enrichment reactors, nitrite was produced, resulting in the simultaneous presence of acetate, nitrate and nitrite at the end of the feeding phase of the SBR cycle. These components were subsequently consumed within the first 65 minutes of the cycle, leaving approximately 65 minutes of carbon-

depleted anaerobic conditions before aeration started. Despite this occurrence, I did not observe any direct inhibitory effect on phosphorus (P) release or uptake activities at the early phase of planned anoxic/aerobic cycling. Additionally, there was no discernible activity from denitrifying PAOs (DPAOs), a group of PAOs that can use nitrate and/or nitrite to perform simultaneous P and nitrogen (N) uptake under anoxic conditions. Thus, EBPR was likely due to PAOs that could not use nitrate and nitrite, which partially supported my third hypothesis.

With time I observed declining activities in my reactors during anoxic/aerobic cycling, as evident from the lower amount of P that was released or consumed per gram of biomass, and the moles of P released for every mole of carbon (C) consumed. Similarly, *Accumulibacter*, the most dominant PAO in my reactors, decreased in abundance. The decrease in activities was likely due to proliferation of non-PAOs that removed carbon. This explanation was supported by the decline in the ratio of P release/acetate consumed (in moles P/moles C), suggesting that more acetate was consumed without contributing to the P release activities.

Concurrent with the decline in EBPR and *Accumulibacter*, some genera belonging to GAOs, namely *Defluviicoccus* cluster 2, *CPB_S60*, and *Plasticicumulans* increased, indicating that these GAOs were more positively impacted by the concomitant presence of acetate, nitrate and the produced nitrite. *Defluviicoccus* cluster 2, a GAO genus that can not use nitrate or nitrite as electron acceptors, increased most noticeably, supporting my second hypothesis. Some genera of non-PAO denitrifiers, most notably *Dechloromonas* and *Zoogloea*, also increased. It is known that while some *Dechloromonas* strains can contribute to EBPR, others can not. In conclusion, the concomitant presence of acetate, nitrate and microbially produced nitrite did not directly inhibit P release or uptake in reactors; however, prolonged exposure to this condition lead to proliferation of non-PAO organisms that consumed acetate without performing significant P uptake, creating a carbon limitation that affected the stability of EBPR. Thus, anoxic/aerobic EBPR does not seem feasible in the long run for systems

containing certain types of GAOs. Operational strategies that could ensure selection of PAOs over these GAOs, such as the application of a lower carbon feeding rate or supplying combined acetate and propionate, should be tested.

Lastly, I explored the potential of adding wasted activated sludge (WAS) to revive failed EBPR systems operated at warm temperatures. For this study, two bioreactors that were operated at 29-31 °C and no longer displayed EBPR after anoxic/aerobic cycling were utilized. Anaerobic/aerobic cycling was used throughout this study. WAS, which was regularly wasted from each reactor during the sludge wastage phase, was collected and stored at 4 °C prior to usage. I hypothesized that the addition of WAS could lead to recovery of EBPR due to an increase in PAOs.

Approximately one third of mixed liquor in the two reactors was replaced with the stored WAS. Activities increased and PAOs surged in both reactors. This could be due to the lower mesophilic range of PAOs compared to GAOs, which enabled them to survive at colder temperatures during storage. Previous studies have also reported that anaerobic starvation should not lead to significant decay for PAOs, and that their EBPR capacities are independent of the length of starvation. There was a positive association between the change in PAO abundance and EBPR performance in the reactors, supporting my fourth hypothesis. In contrast, the abundance of the majority of GAOs such as *Dechloromonas* and *CPB_S60* genera decreased. Surprisingly, the genus *Defluviicoccus* cluster 2 increased in abundance after WAS addition and was positively correlated with EBPR performance. This could be due to an ample supply of carbon, as supported by higher ratios of P released/acetate consumed, suggesting that more acetate consumption was linked to P release activities. With the positive effect of WAS addition on PAOs and EBPR performance in the reactors, I concluded that WAS addition could be developed as cheap alternative to revive failing EBPR systems.

Overall, this dissertation showed that non-DPAOs were capable of performing EBPR in the presence of both carbon sources and nitrate/nitrite at warm temperatures. However, long term stability of anoxic/aerobic EBPR was affected by the proliferation of certain types of GAOs or heterotrophic denitrifiers, which could compete for carbon with PAOs. Additionally, WAS addition showed promise to help recover declining EBPR systems.

CHAPTER 1

INTRODUCTION

1.1 Phosphorus

Phosphorus is an element that plays important roles in all living organisms. It is significantly present in nucleic acids⁽¹⁾⁽²⁾, a vital component in the genetic information transmission and storage⁽¹⁾. In the form of phospholipids, phosphorus is a major constituent of the mammalian plasma membrane, which protects the cells from the surrounding environment⁽¹⁾. As hydroxyapatite, a type of calcium phosphate salt, phosphorus is an integral structural constituent of the bone⁽¹⁾. Additionally, phosphorus is also involved in many vital biological activities. Phosphorylated compounds like adenosine triphosphate (ATP) are essential for energy production, while phosphorylation is also needed for the activation of some enzymes and hormones⁽¹⁾.

In natural water bodies, phosphorus usually exists as phosphate (PO_4^{3-}). In the water/aqueous environment, phosphate can exist in the form of orthophosphate, polyphosphate, or organic phosphate⁽³⁾. Orthophosphate, sometimes referred to as “reactive phosphorus,” is the inorganic phosphorus that is readily available for biological utilization (Metcalf 2003). Examples of orthophosphate compounds are PO_4^{3-} , HPO_4^{2-} , and H_2PO_4^- . Polyphosphate is another form of phosphate that contains two or more phosphorus atoms. In water, polyphosphate can hydrolyse to form orthophosphate. Moreover, organic phosphate is a type of phosphorus that is attached to organic material (Metcalf 2003). Even though phosphorus is important for living organisms, elevated levels of phosphorus in water bodies can lead to eutrophication (Ong et al. 2013).

1.2 Impacts of elevated phosphorus content in water bodies

Phosphorus, along with nitrogen, is a major nutrient for the growth of algae and various photosynthetic microorganisms like cyanobacteria (Oehmen et al. 2007). Eutrophication is a natural process that can be accelerated by human activities, whereby elevated amount of

nutrients, mainly nitrogen, and phosphorus, in a water body lead to algal bloom. Unwanted algal bloom can create aesthetic and odour problems that diminish the recreational values of a water body⁽⁵⁾. Additionally, consuming water with a high content of toxic cyanobacteria can also create adverse health effect or even death (Seviour et al. 2003). Thus, phosphorus removal via chemical precipitation or Enhanced Biological Phosphorus Removal (EBPR) has become an integral part in wastewater treatment processes.

1.3 Chemical precipitation as a phosphorus removal method

Chemical precipitation method utilizes lime, iron, or alum to precipitate and remove phosphorus from the bulk water⁽⁶⁾. Using lime, dissolved phosphate in the water will react with the lime to form solid hydroxyapatite. Precipitation of phosphorus with iron will form solid iron (III) phosphate (FePO_4), whereas precipitation with alum will produce solid aluminium phosphate (AlPO_4)⁽⁶⁾. Even though chemical precipitation is a reliable method, it incurs additional costs from chemical usage and the production of sludge that must be treated.

1.4 Enhanced Biological Phosphorus Removal (EBPR)

Enhanced biological phosphorus removal (EBPR) is an economical, sustainable, and environmentally friendly phosphorus removal method that has been widely implemented (Dai et al. 2007, Kristiansen et al. 2013, Nielsen et al. 2010, Oehmen et al. 2007). Unlike chemical precipitation, which relies on chemically-induced phosphorus precipitation, EBPR utilizes the abilities of organisms, collectively known as polyphosphate accumulating organisms (PAOs), to take up and intracellularly store phosphorus to achieve P removal from the surrounding water (Oehmen et al. 2007).

Fundamentally, an EBPR system cyclically undergoes the “feast” and “famine” phases. This strategy provides a selective advantage for PAOs that can produce and utilize their intracellular storage compounds (Nielsen et al. 2010, Oehmen et al. 2007, Seviour et al. 2003). As described by Oehmen et al. (2007), the “feast” phase occurs when electron donor is present

without any electron acceptor (e.g. oxygen, nitrate, or nitrite), usually under anaerobic conditions. During the “feast” period, carbon sources like volatile fatty acids (VFAs) are consumed by PAOs and converted to polyhydroxyalkanoates (PHAs). PHAs will serve as energy source in the subsequent “famine” phase. The synthesis of PHAs utilizes energy from the hydrolysis of intracellular polyphosphate that releases orthophosphate to the surrounding water (Figure 1A). Consequently, the P concentration in the bulk water increases (Fig. 2). Additionally, energy and reducing power are also derived from the degradation of intracellular glycogen and/or utilization of the tricarboxylic acid (TCA) cycle (Nguyen et al. 2015, Oehmen et al. 2007, Oyserman et al. 2015).

Figure 1. The basic metabolisms of PAOs during the “feast” (A) and “famine” (B) periods with acetate as the main carbon source. Under the “feast” condition, acetate is consumed and stored as PHA inside the cells. The energy needed for these activities are generated through the hydrolysis of PAO’s internal poly-P reserve, which releases orthophosphate to the surrounding water. Reducing power for the PHA synthesis is supplied through the TCA cycle and/or glycolysis. Under the “famine” condition, PAOs take up phosphorus from the surrounding water and store it as intracellular poly-P. Previously synthesized PHA is oxidized to provide energy for this P uptake and storage. The glycogen pool is also replenished during this phase. Redrawn from Seviour et al. (2003).

Figure 2. Typical profiles for P and carbon in the bulk water throughout the “feast” (anaerobic) and “famine” (aerobic) stages. During the “feast” phase, carbon is consumed whereas orthophosphate is released, resulting in the inverse trends. Once aerobic conditions set in, the “famine” phase begins. P is taken up from the bulk water, resulting in a decrease in P concentration.

In the subsequent “famine” phase, electron acceptors are present with little to no carbon availability. Under these conditions, PAOs utilize the previously synthesized PHAs for carbon and energy to take up an excessive amount of P from the surrounding water and replenish their internal polyphosphate storage, to replace their internal glycogen pool (Fig.1B), and for biomass growth (Nguyen et al. 2015, Oyserman et al. 2015). Ultimately, phosphorus removal is achieved through the wasting of P-enriched sludge from the system (Oehmen et al. 2007).

In an EBPR system, there are two major groups of organisms: the aforementioned polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs).

1.4.1. Polyphosphate Accumulating Organisms (PAOs)

1.4.1.1 Types of PAOs

As briefly mentioned before, polyphosphate accumulating organisms (PAOs) refers to a group of bacteria capable of carrying out EBPR due to their ability to perform “luxury uptake,” that is, taking up phosphorus in excess of what is needed for growth (Gebremariam et al. 2011). Among these organisms, a subset of PAOs can also utilize nitrate and/or nitrite as terminal electron acceptor besides oxygen, enabling them to perform simultaneous P removal and denitrification when the anaerobic condition is immediately followed by an anoxic condition. This group of PAOs is commonly referred to as denitrifying PAOs, or DPAOs (Oehmen et al. 2007).

Various studies have been conducted to identify and characterize potential PAOs. Before the advancement of the “culture-independent molecular tools,” such as *fluorescence in situ hybridization* (FISH), denaturing gradient gel electrophoresis (DGGE), or 16S rRNA gene sequencing, studies on the microbiology of EBPR relied on the “culture-dependent technique.” However, the latter was often critiqued for its bias towards organisms that could acclimate to the culture medium. Moreover, this technique often provided inadequate insights into the true diversity of EBPR microbial communities because it was restricted to cultivable bacteria (Gebremariam et al. 2011).

Using the “culture-dependent technique,” the earliest attempts to isolate putative PAOs initially suggested that bacteria belonging to the genus *Acinetobacter* of the Gammaproteobacteria as primary PAOs (Fuhs and Chen 1975). Among the identified isolates of *Acinetobacter* are *Acinetobacter junii*, *A. lwoffii*, and the most studied *A. johnsonii*. However, characteristic phenotypes of PAOs, such as acetate uptake and PHA synthesis are absent in these species even though some degree of P release is detected. Moreover, *Acinetobacter* is often not significantly present in EBPR systems (Crocetti et al. 2000) The

absence of these physiological characteristics called into question the role of *Acinetobacter* as PAO in an EBPR system (Seviour et al. 2003). Other than *Acinetobacter*, various other organisms have also been proposed as putative PAOs, including *Lamproedia* sp. (Stante et al. 1997). One of the isolates, *Lamproedia hyalina*, exhibits the characteristics of PAOs: anaerobic acetate consumption with concurrent PHA synthesis and P release (Stante et al. 1997, Stockholm-Bjerregaard et al. 2017).

Another putative PAO that has been proposed is *Microthrix phosphovorans* (Nakamura et al. 1995). *M. phosphovorans*, a gram positive actinobacterial coccus exhibits both the anaerobic P release and aerobic P uptake of PAOs (Nakamura et al. 1995, Stockholm-Bjerregaard et al. 2017), as well as polyphosphate storage capacity (Beer et al. 2006). While *M. phosphovorans* can take up glucose under anaerobic conditions, it is incapable of taking up acetate directly. Instead, this bacterium consumes glucose which is then fermented to acetate (Kawakoshi et al. 2012, Santos et al. 1999). However, glycogen cycling is not observed (Stockholm-Bjerregaard et al. 2017). Additionally, Kawakoshi et al. (2012) reported the absence of genes responsible for synthesis of PHA. Using a 9-year assessment of 18 Danish wastewater treatment plants, Stockholm-Bjerregaard et al. (2017) reported that *Microthrix* was abundant in some plants, suggesting potential involvement of this genus on the EBPR in several of the plants.

Progression of the “culture-independent molecular tools” has enabled the phylogenetic studies to conduct investigation *in situ* and on uncultured mixed systems. However, it is recognized that these molecular techniques have challenges of their own, such as the potential biases due to primer specificity that may result in preferential PCR amplification, chimera (i.e. DNA sequence resulting from two or more parents⁽⁷⁾) formation, or FISH primer specificities where a certain probe unintentionally binds to other than the targeted organisms. These biases

may lead to erroneous interpretation of the molecular analysis results (Gebremariam et al. 2011).

Yet, despite its shortcomings, utilizations of these “culture-independent molecular tools” have led to discoveries of various PAOs, such as *Candidatus Accumulibacter phosphatis* (i.e. *Accumulibacter*) (Crocetti et al. 2000, Hesselmann et al. 1999), or other species belonging to the bacterial group Actinobacter. *Accumulibacter* belongs to the Betaproteobacteria subgroup 2 that is closely related to *Rhodocyclus*, and they are known to exhibit PAO phenotypes like PHA and poly-P cycling (Crocetti et al. 2000, Hesselmann et al. 1999). This type of PAO has been detected in many full-scale plants and identified as the main group of PAOs in laboratory scale systems receiving acetate or propionate as the main carbon source (He and McMahon 2011). To date, *Accumulibacter* has become one of the most extensively studied PAOs (Oyserman et al. 2015). Deeper analysis on the finer scale of *Accumulibacter* lineage has utilized the gene *polyphosphate kinase* (*ppk1*) as genetic marker. *ppk1* is the gene that encodes for the enzyme PPK1, which catalyses the synthesis of poly P from ATP (He et al. 2007). With this method, *Accumulibacter* can be classified into two major groups: type I, which is further classified into Clade IA to IE; and type II that is grouped into Clade IIA to IIG (Flowers et al. 2009, He et al. 2007, Peterson et al. 2008, Zeng et al. 2016).

Studies on the existence of denitrifying PAOs within the *Accumulibacter* lineage using FISH have revealed the presence of two different types of DPAOs based on the observed morphotypes: the nitrate utilizing DPAOs that correspond to the rod-type *Accumulibacter*, or the nitrite-utilizing DPAOs that are associated with the coccus-type *Accumulibacter* (Carvalho et al. 2007). Among the different Clades of *Accumulibacter*, Clade IA has been found to utilize nitrate, while Clade IIA does not (Flowers et al. 2009, He et al. 2007, Zeng et al. 2016). Moreover, when a laboratory scale enrichment system is fed propionate, the enriched

Accumulibacter type I can utilize nitrate, nitrite, or oxygen as electron acceptor (Lanham et al. 2011, Zeng et al. 2016).

Even though previous studies have provided insights into the presence of *Accumulibacter* DPAOs, Zeng et al (2016) noted that those studies mainly used synthetic wastewater that potentially led to a much simplified community structure, depending on the enriched *Accumulibacter* that could utilize the substrate supplied with the synthetic feed. Consequently, this practice often led to enrichment of *Accumulibacter* Clades IA and IIA, overlooking other Clades of *Accumulibacter* and their potential as DPAOs. Moreover, many of the earlier studies omitted other genes that play significant roles in the denitrifying P removal capacities of *Accumulibacter*, such as *narG*, which encodes for nitrate reductase, or *nirS* and *nirK* that encode for nitrite reductase enzymes. Using combinations of these genes (i.e., *ppk1*, *narG*, *nirS*, and *nirK*) to determine the denitrifying capacity of other *Accumulibacter* clades, this study has reported that *Accumulibacter* Clades IIC and IIF are capable of performing denitrifying P removal with nitrite as electron acceptor (Zeng et al. 2016).

Besides *Accumulibacter*, actinobacterial PAOs have also been reported in various EBPR plants (Kong et al. 2005). In addition to *Microlunatus phosphovor*us, other actinobacterial species, such as those belonging to the genus *Tetrasphaera*, are also capable of taking up P under aerobic conditions following anaerobic organic substrate uptake. Analysis of the microbial communities in various full-scale plants has revealed a significant presence of this group of bacteria. For example, organisms belonging to the genus *Tetrasphaera* can account for up to 30% of the total biomass volume (Kong et al. 2005, Kristiansen et al. 2013, Nguyen et al. 2015). Among these organisms, there are some species that have been successfully isolated: *Tetrasphaera japonica* (strain T1-X7), *T. australiensis* strains Ben 109 and Ben 110 (Maszenan et al. 2000), *T. elongata* strain ASP12 (Onda and Takii 2002), *T. elongata* strain LP2 (Hanada et al. 2002), *T. jenkinsii*, *T. vanveenii*, and *T. veronensis*

(McKenzie et al. 2006). Unlike *Accumulibacter* PAOs, actinobacterial PAOs do not accumulate PHA (Kong et al. 2005, Nguyen et al. 2011).

Phylogenetic analysis of the fine scale population of *Tetrasphaera*-related organisms that belong to the family Intrasporangiaceae have revealed the existence of three clades: I, II, and III (Kristiansen et al. 2013, Nguyen et al. 2011). Isolates *T. elongata* and *T. duodecadis* are grouped into Clade I, while Clade II includes *T. jenkinsii*, *T. australiensis*, and *T. veronensis*. *T. japonica* is not related to any of the known clades (Kristiansen et al. 2013), whereas Clade III is not related to any of the cultured isolates (Nguyen et al. 2011). Within these clades, there are six different morphologies observed: branched rods, small cocci, tetrads-forming cocci, filaments, thin filaments, or short rods (Nguyen et al. 2011). *Tetrasphaera*-PAOs have the capacities to consume glucose, amino acids, and acetate (Kong et al. 2005, Kristiansen et al. 2013, Nguyen et al. 2011). Gene analyses to determine the denitrification capacities of *Tetrasphaera* indicate that four of the isolates used in the study, namely *T. australiensis*, *T. jenkinsii*, *T. elongata*, and *T. japonica* can utilize nitrate/nitrite as the electron acceptor, suggesting their potentials as denitrifying PAOs (Kristiansen et al. 2013).

Recent screening for other potential PAOs has revealed a novel group of *Halomonas*-PAOs belonging to the gammaproteobacterial Halomonadaceae family. These PAOs, referred to as the *Candidatus* *Halomonas phosphatis* can take up ethanol, in addition to the commonly utilized VFAs like acetate or propionate. Additionally, they are often present in higher abundance than *Accumulibacter*. Within *Halomonas*-PAOs, two different clades have been found and cell morphologies are restricted to short rods. Unlike *Accumulibacter*, *Halomonas*-PAOs cannot perform denitrification. Further phylogenetic analysis of this group of PAOs is needed (Nguyen et al. 2012).

1.4.1.2. Anaerobic metabolism of PAOs with acetate as the external carbon source

Several biochemical studies have investigated the metabolism of PAOs (Beer et al. 2006, Crocetti et al. 2000, Kristiansen et al. 2013, Martín et al. 2006, Nguyen et al. 2015, Zhou et al. 2009, Zilles et al. 2002). Generally, the earlier biochemical models for PAOs under anaerobic condition include the uptake of carbon source, normally VFAs like acetate or propionate, by PAOs at the expense of their internal pool of polyphosphate. This VFA uptake is accompanied by the formation of intracellular polyhydroxyalkanoate (PHA), utilizing the tricarboxylic acid (TCA) cycle and/or glycolysis to gain additional energy and reducing power (Nguyen et al. 2015). Energy from the intracellular polyphosphate can be generated via breaking of the phosphodiester bonds or through the involvement of ATP synthase (ATPase) (Martín et al. 2006).

Several models have been developed to explain the anaerobic transport of VFAs into the cells, such as passive transport (Comeau et al. 1986, Wentzel et al. 1986), active transport (Mino et al. 1987, Smolders et al. 1994), or secondary active transport (Saunders et al. 2007). One of the most common VFAs in wastewater treatment plant is acetate. As elaborated by Oehmen et.al. (2007), consumed acetate is converted to acetyl-CoA. Acetoacetyl-CoA is then formed when two acetyl-CoA molecules condense. Reduction of Acetoacetyl-CoA yields 3-hydroxybutyryl-CoA that polymerizes to form polyhydroxybutyrate or PHB (Fig. 3). The formation of 3-hydroxybutyryl-CoA involves NADH as an electron donor. NADH is the higher energy form of nicotinamide adenine dinucleotide (NAD^+), a significant coenzyme in energy metabolism⁽¹³⁾. NADH is produced when one hydrogen atom (H^+) and two electrons (e^-), generated when enzyme dehydrogenase removes two H^+ and two e^- from the substrates, are taken by $\text{NAD}^{+(11)}$. PHB, polyhydroxyvalerate (PHV), polyhydroxy-2-methylbutyrate (PH2MB) and polyhydroxy-2-methylvalerate (PH2MV) are types of PHAs commonly detected in EBPR systems (Oehmen et al. 2005).

Figure 3. Metabolic pathway for PHB synthesis. Enzyme β -ketothiolase (PhaA) catalyzes the formation of acetoacetyl-CoA from two condensed acetyl-CoA molecules. Enzyme Acetoacetyl-CoA-reductase (PhaB) catalyzes the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA. Polymerization of (R)-3-hydroxybutyryl-CoA to polyhydroxybutyrate (PHB) is catalyzed by enzyme PHB synthase (PhaC). HSCoA represents Coenzyme A that negatively regulates enzyme β -ketothiolase (PhaA). Source: (Kessler and Witholt 2001).

Passive transport was initially proposed in 1986 by Comeau et.al. and Wentzel et.al (i.e. Comeau/Wentzel model) as the mechanism used by PAOs to take up acetate and store it intracellularly (Comeau et al. 1986, Wentzel et al. 1986). In general, passive transport is a

mechanism by which molecules move across the cell membrane from the high concentration to the low concentration compartment. This mode of transport mechanism does not require energy input⁽⁸⁾⁽⁹⁾. On the contrary, Mino et.al. (1987) and Smolders et.al. (1994) suggested that PAOs consumed available acetate through an active transport mechanism (Mino et al. 1987, Oehmen et al. 2007, Smolders et al. 1994). Fundamentally, active transport is the movement of molecules across the cell membrane against a concentration gradient⁽⁹⁾. In the case of acetate uptake via the primary active transport mechanism, transport is directly coupled with polyphosphate hydrolysis that produces ATPs⁽¹⁰⁾.

Another type of active transport mechanism proposed is secondary active transport (Oehmen et al. 2007, Saunders 2006). Secondary active transport is a type of transport where trans-membrane movement of a molecule against the electrochemical gradient can occur due to the energy produced by the transmembrane movement of other molecules/ions down the electrochemical gradient. This type of transmembrane transport relies on the energy contained in an electrochemical gradient known as the proton motive force, or the PMF (Bahadur et al. 2015, Oehmen et al. 2007). PMF occurs due to the transport of H^+ across the inner membrane that causes proton accumulation on one side of the membrane⁽¹¹⁾. Consequently, the side becomes more positively charged, while the other side is negatively charged. PMF can provide energy to catalyse the formation of ATP by enzyme ATP synthase⁽¹¹⁾, which serves as energy source for substrate storage (i.e., PHA synthesis) under anaerobic conditions (Comeau et.al., 1986). Utilizing sludge that was highly enriched with *Accumulibacter*, Saunders et al.(2007) showed that *Accumulibacter* PAOs could fulfil some of the ATP requirements by utilizing the PMF. PMF is produced when internally stored polyphosphate is degraded and released from cells, along with protons (H^+), via the inorganic phosphate transport system known as Pit (Oehmen et al. 2007, Saunders et al. 2007).

To date, glycolysis and/or the TCA cycle have been proposed as the source of reducing power in the anaerobic metabolism of PAOs (Mino et al. 1998, Pereira et al. 1996, Zhou et al. 2009). Although glycolysis has been accepted as one potential source of reducing power, there are differing conclusions on whether glycolysis occurred via the Embden-Meyerhoff-Parnas (EMP) pathway (Mino et al. 1987) or the Entner-Doudoroff (ED) pathway (Wentzel et al. 1991). EMP and ED pathways convert glucose to pyruvate (i.e., glycolysis pathway)^(14,15). Both EMP and ED pathways involve the phosphorylation and cleavage of C6 sugar to two C3 intermediates⁽¹⁶⁾. The difference lies in the formation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate when fructose- 1,6-biphosphate is cleaved in the EMP pathway. This metabolism does not occur in the ED pathway. Instead, production of glyceraldehyde-3-phosphate and pyruvate occurs when 2-keto-3-deoxy-6-phosphogluconate (KDPG) is cleaved in the ED pathway⁽¹⁶⁾. Further confirmation of the involvement of the EMP pathway is presented by Martin et.al. (2006). Their study revealed the presence of the complete EMP pathway in sludge samples from two different treatment plants in the United States and Australia that were dominated by *Accumulibacter*. At the same time, they also found that genes involved in the ED pathway and typical enzymes that entered this pathway were not present. These findings indicate that the EMP pathway is the functioning metabolic pathway in glycogen degradation. These contrasting results on which pathways are active may be due to the possibilities that different communities are present in the sludge samples. Consequently, different species than those observed in the experiment may utilize the ED pathway (Martín et al. 2006). Involvement of the EMP pathway is further confirmed by Zhou et al. (2009) in an experiment that utilized sludge enriched with *Accumulibacter* and was significantly depleted in glycogen.

Besides glycogen degradation, the TCA cycle has also been reported to generate the reducing power needed for PHA synthesis. In principle, the TCA cycle, also referred to as the

Kreb's Cycle, is a chain of chemical reactions organisms (aerobes and facultative anaerobes—organisms that can thrive in both anaerobic and aerobic conditions) can derive most of their energy from⁽¹⁷⁾. According to the Comeau/Wentzel model, the reducing power is generated through the full TCA cycle (Comeau et al. 1986, Wentzel et al. 1986). Involvement of this type of TCA cycle was also suggested by Pereira et al. (1996) who observed production of dissolved bicarbonate from labelled acetate. Additionally, the reducing power generated by glycolysis alone cannot provide the reducing equivalents required for the observed PHA generation (Pereira et al. 1996). Involvement of glycolysis and full TCA cycle are illustrated by Fig. 4A. However, oxidation of succinate to fumarate by succinate dehydrogenase in the full TCA cycle produces the FADH₂ that will need to be re-oxidized to FAD⁺ (Zhou et al. 2009). Re-oxidation of FADH₂ can occur by transporting its electron to a terminal electron acceptor known as the iron-sulfur complex⁽¹⁸⁾. The process of re-oxidizing FADH₂ to FAD⁺ cannot occur under anaerobic conditions. As an alternative, the partial TCA cycle through the glyoxylate shunt (Louie et al. 2000, Oehmen et al. 2007) and the split TCA cycles (Hesselmann et al. 2000, Kortstee et al. 2000, Oehmen et al. 2007) have been proposed.

The partial TCA cycle with the involvement of a glyoxylate shunt converts isocitrate from the TCA cycle to malate and succinate through a glyoxylate shunt that bypasses some intermediates and CO₂ production (Fig. 4B). This results in a lower amount of reducing power production (Louie et al. 2000). However, like the full TCA cycle, oxidation of succinate to fumarate by succinate dehydrogenase produces FADH₂, and how FADH₂ can be re-oxidized to FAD⁺ under anaerobic conditions is still not fully understood (Oehmen et al. 2007, Zhou et al. 2009). Alternatively, the split TCA cycles (Fig. 4C), another type of TCA cycle where the right branch can be used for oxidative conversion of citrate to succinyl-CoA and the left branch for reduction of oxaloacetate to succinyl-CoA, might be involved (Hesselmann et al. 2000, Kortstee et al. 2000, Oehmen et al. 2007, Pramanik et al. 1999).

Figure 4. Schematic of the involvement of glycolysis in conjunction with (A) full TCA cycle with acetate or propionate as the external carbon source; (B) partial TCA with glyoxylate shunt; or (C) split TCA cycle, in the production of the required reducing power in PHAs synthesis. Conversion of isocitrate to malate and succinate through the glyoxylate shunt bypasses the production of some intermediates (α -Ketoglutarate and succinyl-CoA) and CO₂. Moreover, utilization of this shunt reduces the amount of reducing power NADH produced. In these diagram, [H] = NADH (Oehmen et al. 2007).

The involvement of glycolysis together with the split or full TCA cycle has been suggested due to the insufficient NAD(P)H produced by glycolysis alone (Martín et al. 2006). Similar to NADH, NADPH is the reduced form of NADP^+ that can serve as reducing factor due to the usable energy in its bonds⁽¹⁹⁾⁽²⁰⁾. The presence of fumarate reductase suggests the potential involvement of the split TCA cycle as an alternative. With this type of TCA cycle, a fraction of PHV can be accrued through the involvement of methylmalonyl-CoA. The PHV accumulation can also be observed in the acetate-fed EBPR system, where a small amount of PHV can be detected (Martín et al. 2006). This study also suggests the involvement of the full TCA cycle. In order to anaerobically re-oxidize hydroquinones, FADH_2 (the reduced form of quinones) produced by succinate dehydrogenase, a new form of cytochrome *b/b6*, is proposed (Martín et al. 2006). This novel cytochrome is a fusion protein containing a *b/b6* cytochrome with five transmembrane helices and a binding domain for NAD(P) and flavin (Martín et al. 2006). However, the role of this type of cytochrome needs to be confirmed (Zhou et al. 2009).

1.4.1.3. Aerobic metabolism of PAOs

Under the subsequent aerobic conditions, the anaerobically synthesized PHAs are degraded. Throughout the TCA cycle, catabolism (metabolic activities that break down molecules to smaller components and produce energy) occurs. Acetyl-CoA and propionyl-CoA, which are involved in biomass growth as carbon and energy sources, are produced when PHB and PHV, respectively, are degraded. Some of the ATPs are utilized by PAOs to fulfil the energy requirement for phosphate uptake and conversion to polyphosphate. Additionally, glycogen is also replenished using some of the carbon and energy (Oehmen et al. 2007). In the case of aerobic P uptake, the involvement of two phosphate transport systems, the Pit and Pst systems, has been suggested. Martin et al. (2006) postulated the involvement of Pit systems at the start of aerobic conditions when high levels of P were present in the surrounding environment. As the level of P decreased near the end of the aerobic phase, the Pst system was

activated (Martín et al. 2006). However, Burow et al. (2008) found that both Pit and Pst were likely to be involved from the start of the aerobic phase when high levels of P were present. Further analysis of the involvement of inorganic P transport systems is still needed to confirm the roles of Pit and Pst systems in the aerobic P uptake in *Accumulibacter* PAOs (Burow et al. 2008).

1.4.1.4 Recent findings on the anaerobic and/or aerobic metabolism of PAOs

Skenner et al (2015) reported the presence of conserved genes for the metabolism of carbon and phosphorus in all *Accumulibacter* genomes sequenced in the study, namely, *Accumulibacter* Clades IIF, IIC, IA, and IC. For example, all these *Accumulibacter* genomes possess the same genes that are involved in the synthesis and degradation of polyphosphate (*ppk1* and *ppx* genes, respectively). They also possess the low-affinity (PitA) and high-affinity (PstABC) P transporters, and share the same pathways for PHA synthesis, TCA cycle, and glycolysis. The sequenced *Accumulibacter* genomes support the involvement of the EMP pathway instead of ED to perform anaerobic glycolysis. Moreover, a possible anaerobic involvement of the full TCA cycle is supported by the presence of homologues for the novel cytochrome b/b7, first proposed by Martín et al (2006), that can potentially enable the anaerobic re-oxidation of reduced quinones (Martín et al. 2006) in six of the sequenced genomes (Skenner et al. 2015). It is noted that the absence of such homologues in two of the sequenced genomes might be due to incomplete genome assemblies (Skenner et al. 2015). Possible involvement of other TCA cycle types is also supported by the presence of the key enzymes ICL (isocitrate lyase) and MCM (methylmalonyl-CoA mutase) that are involved in the glyoxylate shunt or split TCA cycle, respectively (Skenner et al. 2015). Employing multiple lines of evidence, including metagenomic and metatranscriptomic analysis on the microbial communities of a tropical plant exhibiting EBPR trends, it is suggested that glycolysis and TCA cycles are of equal importance in the anaerobic metabolism of

Accumulibacter PAOs (Law et al. 2016). Despite the conserved metabolism, there are also several differences observed in the carbon and nitrogen metabolism within *Accumulibacter*. While most *Accumulibacter* taxa are constrained to utilize low molecular weight substrates (such as VFA), *Accumulibacter* Clade IIF is also capable of utilizing ethanol (Skenner et al. 2015).

Among known PAOs, *Accumulibacter* and *Candidatus Halomonas phosphatis* usually exhibit anaerobic and aerobic metabolism as predicted by the biochemical models described previously to generally include: VFA (acetate or propionate) uptake and conversion to PHA; degradation of polyphosphate for energy; utilization of glycolysis and/or TCA cycles for more energy and reducing power for anaerobic metabolism; and PHA oxidation for carbon and energy for cell growth, P uptake and polyphosphate replenishment, and replacement of the glycogen pool for aerobic metabolism (Nguyen et al. 2015). In contrast, *Tetrasphaera*-PAO displays some metabolic activities that are distinct from the existing biochemical models of PAOs. For example, despite their ability for aerobic P uptake and polyphosphate formation, *Tetrasphaera*-PAO is unable to synthesize PHA (Nguyen et al. 2011) and can perform fermentation to gain energy (Kristiansen et al. 2013). Moreover, *Tetrasphaera*-PAOs consumes mainly glucose and some amino acids, while acetate or propionate are the main substrates for *Accumulibacter* (Nguyen et al. 2015).

Based on the analysis of four *Tetrasphaera* isolates (*T. australiensis*, *T. japonica*, *T. jenkinsii*, and *T. elongata*), Kristiansen et al (2013) postulated a potential model to describe the metabolism of *Tetrasphaera*-PAOs, using glucose as the main substrate. These four isolates possess various genes that are involved in polyphosphate metabolism, such as the degradation and formation of polyphosphate, and cross-membrane transportation of phosphate via the low-affinity Pit and high-affinity Pst transporter systems. Similar to *Accumulibacter*, it is proposed that *Tetrasphaera*-PAOs also degrade polyphosphate and release inorganic phosphate for

energy to anaerobically consume available substrate and convert it into storage materials. Under aerobic conditions, inorganic phosphate is taken up to replenish the intracellular polyphosphate. Additionally, these isolates also possess genes that are involved in the forward TCA cycle. However, unlike *Accumulibacter*, these *Tetrasphaera* isolates do not have the genes involved in the glyoxylate shunt. Additionally, genes involved in the glycolysis and gluconeogenesis steps are also detected in all four isolates, whereas genes involved in the PHA synthesis are only detected in *T. japonica* (Kristiansen et al. 2013). Previous studies revealed the abilities of *Tetrasphaera* species to ferment (Kong et al. 2005, Nguyen et al. 2011). As expected, the genes necessary to ferment glucose are detected in all four isolates (Kristiansen et al. 2013). It is postulated that under anaerobic conditions, *Tetrasphaera*-PAOs can consume glucose. This consumed glucose is stored as glycogen or fermented to succinate, lactate, acetate, or alanine. Degradation of polyphosphate and fermentation of glucose can provide energy to synthesize intracellular glycogen. In the subsequent aerobic phase, the previously synthesized glycogen is broken down to provide energy for growth and polyphosphate synthesis (Kristiansen et al. 2013). Recently, amino acid (glycine) consumption and its intracellular accumulation was proposed as a novel strategy for growth of *Tetrasphaera*-PAOs under the cyclic feast/famine conditions (Nguyen et al. 2015). In an experiment utilizing *T. elongata* as the model organism, Nguyen et al. (2015) found that among the amino acids tested, glycine induced the most anaerobic P release activity, and this type of amino acid was used neither by *Accumulibacter* nor by *Candidatus Halomonas phosphatis*. Under anaerobic conditions, *T. elongata* consumes and accumulates glycine and concurrently releases inorganic phosphate. Due to its reliance on the polyphosphate availability for energy, the glycine uptake by *T. elongata* and other *Tetrasphaera*-related bacteria will cease once the intracellular polyphosphate is depleted (Nguyen et al. 2015). As *T. elongata* consumes glycine, it can ferment portions of this substrate to glutamine, alanine, and serine. Additionally, *T. elongata*

has been shown to accumulate glutamate. This accumulated glutamate is likely the product of protein degradation or other unknown internal metabolites that have not yet been explored (Nguyen et al. 2015). Following the glycine uptake and accumulation, *T. elongata* has been observed to release fermentative products like acetate, alanine, and succinate to the surrounding water. The metabolic capacities of *Tetrasphaera*-PAOs include the ability to intracellularly store amino acids and polyphosphate, to ferment glucose and glycine that can provide additional energy for their survival and proliferation in highly dynamic full-scale EBPR plants, and to utilize oxygen or nitrite/nitrate enabling simultaneous P and N removal (Nguyen et al. 2015).

1.4.2. Glycogen Accumulating Organisms (GAOs)

Besides PAOs, another group of organisms that is often detected in EBPR systems are the glycogen accumulating organisms (GAOs). First identified by Mino et al. (1995), GAOs refers to organisms that can use glycogen under anaerobic conditions and subsequently replenish glycogen under aerobic conditions. According to models developed for putative GAOs, typical phenotypes of GAOs include the anaerobic utilization of glycogen for energy to take up organic carbon and synthesize PHAs (Mino et al. 1995), and aerobic oxidation of the synthesized PHAs for growth and replenishment of the depleted glycogen (Oehmen et al. 2007). One of the major differences between PAOs and GAOs lies in the fate of intracellular polyphosphate, where GAOs do not degrade or accumulate polyphosphate like PAOs (Gebremariam et al. 2011, Liu et al. 1996, Satoh et al. 1992). Consequently, the dominance of GAOs instead of PAOs can lead to the failure of EBPR systems.

1.4.2.1.Types of GAOs

The presence of organisms that can anaerobically take up carbon without performing significant P uptake has been reported in deteriorated EBPR systems (Cech and Hartman 1990, Fukase et al. 1985, Tsai and Liu 2002). Initially, these organisms were thought to be the tetrad

forming organisms (TFOs) (Tsai and Liu 2002). Tetrad is a type of bacterial morphology formed by a group of four cocci (i.e., spherical/oval shaped bacteria) in a square formation^(21,22). Early attempts to identify the putative GAOs utilizing the “culture-dependent” method led to the isolation of some of the TFOs, namely, *Amaricoccus kaplicensis* (Maszenan et al. 1997), also referred to as *Tetracoccus cechii* (Blackall et al. 1997), and *Micropruina glycogenica* (Shintani et al. 2000). However, the actual classification of these isolates as GAOs remains inconclusive due to their insignificant presence in either full-scale or laboratory scale system (Seviour et al. 2003), and lack of insights on whether these isolates exhibit the typical GAO phenotypes (Oehmen et al. 2007).

As “culture independent methods” developed, other types of GAOs have been identified. Using techniques like DGGE on 16S rRNA genes amplified with PCR and FISH, Nielsen et al. (1999) and Crocetti et al.(2002) identifies a new group belonging to the Gammaproteobacteria that exhibits the phenotypes of GAOs and is often detected in both laboratory and full-scale EBPR systems (Crocetti et al. 2002, Nielsen et al. 1999). It belongs to the genus *Competibacter* (also denoted as GB group) and comprises of at least seven subgroups (Kong et al. 2002, Kong et al. 2006, Nielsen et al. 1999, Nielsen et al. 2009a, Wong and Liu 2006). GB subgroup 6 can potentially denitrify using nitrate and nitrite, whereas subgroups 1, 4, and 5 can potentially denitrify nitrate to nitrite (Kong et al. 2006). Rods and coccobacilli are two morphotypes of *Competibacter*-GAOs that have been detected. *Competibacter*-GAO cells are generally large and oval, and these organisms can be present as microcolonies or tetrads (Nielsen et al. 2009a). Among the commonly enriched GB bacteria exhibiting GAO phenotypes is the *Candidatus Competibacter phosphatis*, also referred to as *Competibacter* (Crocetti et al. 2002). *Competibacter* has been shown to form the GB subgroups 1 and 3 (Kong et al. 2002, Kong et al. 2006). Further analysis of the *Competibacteraceae* family revealed the presence of two other species, namely *Candidatus Competibacter denitrificans*

that belongs to *Competibacter* subgroup 1 and possesses the ability to denitrify, and *Candidatus* *Contendobacter odensis* that belongs to *Competibacter* subgroup 5 (McIlroy et al. 2014).

Other groups of GAOs that have been identified are subgroups of Alphaproteobacteria related to *Sphingomonadales* (Beer et al. 2004) and *Defluviicoccus*, namely, *Defluviicoccus vanus* (Meyer et al. 2006, Wong et al. 2004). Beer et al. (2004) applied DGGE and FISH to analyse the microbial communities in anaerobic/aerobic sequencing batch reactors (SBRs) with poor EBPR activities and found a significant presence of alphaproteobacterial organisms belonging to the genus *Sphingomonas*. This group of organisms exhibits the expected phenotype of GAOs, such as anaerobic build-up of PHA and aerobic accumulation of glycogen instead of polyphosphate (Beer et al. 2004). Another putative GAO belonging to the Alphaproteobacteria is closely related to *Defluviicoccus vanus*. These *D. vanus*-related GAOs belong to two distinct subgroups of the *Defluviicoccus*-GAOs: clusters 1 and 2 (Meyer et al. 2006, Oehmen et al. 2007, Wong et al. 2004). The cells of *Defluviicoccus*-GAOs are rods or cocci, and they tend to exhibit the tetrad-forming morphology (Nielsen et al. 2009a). Even though this group of GAOs has been observed in laboratory-scale systems, they are not abundantly present in full-scale EBPR systems (Nielsen et al. 2009a, Oehmen et al. 2007, Wong et al. 2004). Additionally, *Defluviicoccus*-GAO cluster 2 cannot denitrify when acetate is present as the carbon source (Burow et al. 2007). Further studies on the phylogenetic diversity of *Defluviicoccus*-GAOs revealed the presence of two additional clusters (McIlroy and Seviour 2009). The third proposed cluster of *Defluviicoccus*-GAO includes the filamentous morphotypes, and one species belonging to this cluster is *Candidatus* *Monilibacter batavus* (McIlroy and Seviour 2009). *Defluviicoccus*-GAOs in the fourth cluster exhibit the typical phenotypes and the tetrad-forming morphotype that are also commonly detected in *Defluviicoccus*-GAO clusters 1 and 2. Each of these *Defluviicoccus*-GAO clusters has been detected at varying abundance in either full-scale and/or laboratory scale systems. For example,

cluster 1 and 2 are commonly detected in lab-scale EBPR systems, while clusters 2 and 3 are usually detected in full-scale EBPR plants (Nobu et al. 2014).

1.4.2.2 Anaerobic metabolism of GAOs with acetate as the carbon source

Typical anaerobic metabolisms of GAOs consist of carbon uptake and synthesis of PHA at the expense of glycogen as the source of energy and reducing power. VFAs, in this case acetate, are transported into cells via secondary active transport that is energized by the PMF. The latter is generated by the efflux of protons through the ATPase, consuming ATP from the storage polymer for energy (McIlroy et al. 2014, Oehmen et al. 2007, Saunders et al. 2007). In the case of acetate consumption and PHA formation by GAOs, both acetyl-CoA and propionyl-CoA are formed. Propionyl-CoA is produced to achieve the reduction-oxidation (redox) balance because of excessive production of reducing equivalents by GAOs. To fulfil the energy and reducing power requirements for the carbon uptake (acetate) and conversion to PHA, GAOs have to rely on glycogen hydrolysis. Consequently, GAOs produce more reducing equivalents than necessary for the production of PHA (McIlroy et al. 2014). To keep the redox balance, GAOs will consume NADH by reducing pyruvate, one of the intermediates in the glycolysis of glycogen, to succinyl-CoA and eventually propionyl-CoA via the left reductive branch of the TCA cycle (Liu et al. 1994, Oehmen et al. 2007, Satoh et al. 1994). The formed propionyl-CoA will condense with acetyl-CoA to produce PHV (Oehmen et al. 2007). In addition to the involvement of the reductive branch of the TCA cycle, involvement of the right (oxidative) branch of TCA cycle in acetate catabolism has also been reported (Lemos et al. 2007).

One of the identified GAOs belonging to the Competibacteraceae family, *Candidatus* Competibacter denitrificans, has been suggested to potentially assimilate and/or ferment glucose to lactate, which can provide an alternative strategy to consume the additional reducing

equivalents. Moreover, it has also been suggested that *Ca. denitrificans* can obtain additional energy when the fermented lactate is excreted in symport with a cation (McIlroy et al. 2014).

Glycolysis of glycogen via the EMP pathway has been suggested to produce the reducing equivalents and energy for the anaerobic metabolism of GAOs (Sato et al. 1994). This hypothesis is further supported when one enzyme in the ED pathway, the glucose-6-phosphate dehydrogenase, has been observed to be inactive. This enzyme is needed to catalyse the starting reaction of the ED pathway (Filipe et al. 2001b, Lemos et al. 2007). However, possible involvement of ED pathway was observed by Lemos et al. (2007) when *in vivo* NMR analysis using ^{13}C -labeled acetate was applied to a GAO enrichment culture (81.2% enrichment). This culture contains *Ca. competibacter*, and *Defluviicoccus*-GAO clusters 1 and 2 that are related to *Defluviicoccus vanus*. Involvement of the ED pathway is further observed at increased temperature (Lemos et al. 2007). Recent genomic analysis of two genomes from the Comptibacteraceae family (i.e. *Ca. denitrificans* and *Ca. odensis*) reveals the presence of the EMP pathway genes, corroborating the potential involvement of this glycolysis pathway. However, only *Ca. denitrificans* contains the genes for the ED pathway. Thus, it is suggested that GAOs with genes for both pathways might shift from EMP to ED pathways when subjected to certain conditions (e.g., higher temperature). Alternatively, observation of the ED pathway when the temperature is high might be due to the selection of certain GAOs that favour the ED pathway over the EMP pathway (McIlroy et al. 2014).

Analysis of the ecophysiology of *Defluviicoccus*-GAOs cluster 1 and 2, including *Defluviicoccus vanus*, in full-scale plants reveals that both cluster 1 and 2 can anaerobically or aerobically take up glucose, acetate, propionate, and pyruvate. Cluster 2 can also take up a mixture of amino acids (Burow et al. 2007). When acetate is present, *Defluviicoccus*-GAOs cluster 2 can also take up leucine or thymidine anaerobically. While *Defluviicoccus*-GAOs cluster 2 can rely on glycolysis to produce the required energy and reducing power for the

acetate uptake and synthesis of PHA under anaerobic conditions, an involvement of the TCA cycle remains inconclusive (Burow et al. 2007).

Wong and Liu (2007) examined the ecophysiology of another novel group of *Defluviicoccus*-GAOs belonging to cluster 1 that was enriched in a deteriorating EBPR membrane bioreactor operated under anaerobic/aerobic condition. They reported the ability of this group of *Defluviicoccus*-GAOs to anaerobically consume acetate, propionate, lactate, and pyruvate and store them as PHA. This group of GAOs can also take up these carbon sources under aerobic conditions. As expected from the GAO metabolic model, this group of *Defluviicoccus*-GAOs can utilize glycogen anaerobically and restore the glycogen pool aerobically without any anaerobic Pi release or aerobic P uptake. Additionally, PHA is also used aerobically. However, unlike the trends observed by Burow et al. (2007), this group of GAOs cannot consume glucose or convert it to PHA (Wong and Liu 2007). Further metagenomic analysis of one strain of this group of *Defluviicoccus*-GAOs, referred to as *Candidatus Defluviicoccus tetraformis* (strain TFO71), reveals the presence of the genes for the EMP pathway and the TCA cycle, suggesting their potential involvement in this GAO's metabolism (Nobu et al. 2014)

1.4.2.3 Aerobic metabolism of GAOs with acetate as the carbon source

Under aerobic conditions, GAOs degrade the previously synthesized PHA (i.e., PHB and PHV). This PHA degradation produces the acetyl-CoA and propionyl-CoA that will be utilized for biomass growth and cell maintenance (Zeng et al. 2003). Moreover, the depleted pool of glycogen will also be renewed via gluconeogenesis. Gluconeogenesis is the conversion of non-carbohydrate precursors to sugars, namely glucose, which are used for catabolic reactions⁽¹⁷⁾. Utilizing the NMR analysis with ¹³C labelled acetate, Lemos et al. (2007) reported the involvement of ED pathways in the production of glucose under aerobic conditions (Lemos et al. 2007, Oehmen et al. 2007). As elaborated by Lemos et al. (2007), the synthesized PHB

is broken down to produce acetyl-CoA. When acetyl-CoA is metabolised via the TCA cycle or through the glyoxylate cycle, oxaloacetate is formed. Oxaloacetate will form phosphoenolpyruvate (PEP) in the first step of the gluconeogenesis pathway. PEP will be converted to glyceraldehyde-3-phosphate (Gly-3P) that will condense with dihydroxyacetone phosphate (DHAP), forming fructose-1,6-biphosphate^(23,24). This fructose-1,6-biphosphate will eventually form glycogen. Simultaneously, involvement of the ED pathway is also detected, where the glucose-6-phosphate will enter the ED pathway and form 6-phosphogluconate. This 6-phosphogluconate will then form 2-keto-3-deoxy-6-phosphogluconate (KDPG) that will be catabolized to pyruvate and gly-3P. Pyruvate carboxylase enzyme will convert pyruvate to oxaloacetate, which will enter the gluconeogenesis pathway (Lemos et al. 2007).

1.4.3. Competition between PAOs and GAOs

PAOs and GAOs possess highly similar metabolic activities. However, despite the similar abilities of both PAOs and GAOs to consume carbon in the absence of usable electron acceptor, GAOs do not significantly take up P but glycogen (Gebremariam et al. 2011, Oehmen et al. 2007). Hence, it is alleged that the presence of GAOs provides competitions for PAOs for the available carbon source, which can lead to deterioration of EBPR activities in the system (Oehmen et al. 2007, Tu and Schuler 2013). Several factors have been reported to affect PAO-GAO interactions, namely temperature, carbon source, pH, dissolved oxygen level, and presence of NO_x compounds like nitrate or nitrite/free nitrous acid.

1.4.3.1. Effect of high temperature

Temperature has been reported to have a significant impact on PAO-GAO competition, where lower temperatures seem to favour PAOs over GAOs. Comparison of EBPR systems operated at 5 °C and 20 °C reveals that PAOs can outcompete GAOs at lower temperature, as shown by higher EBPR activities at 5 °C than at 20 °C (Brdjanovic et al. 1998a, Erdal et al.

2003). The negative effect of higher temperature on EBPR systems was further reported by Whang and Park (2002) who reported deteriorating EBPR activities in laboratory scale EBPR reactors operated at 30 °C, as compared to those operated at 20 °C. They observed that EBPR activities (P release and uptake) eventually ceased in the reactor operated at 30°C while VFA uptake, glycogen utilization, and PHAs synthesis were still observed under anaerobic conditions. Moreover, microscopic analysis reveals the dominance of cells that closely resembled the tetrad-forming morphology of GAOs (Whang and Park 2002). These findings suggest that GAOs can outcompete PAOs at higher temperature. Others observed a shift in the dominating communities from PAOs at 20 °C to GAOs when the temperature increased to 30 °C (Panswad et al. 2003). One possible explanation is that, as temperature increases, GAOs exhibit higher acetate uptake rates than PAOs. Consequently, this kinetic advantage enables GAOs to proliferate (Whang et al. 2007, Whang and Park 2002, 2006). In contrast, stable long-term P-removal (1 year) occurred in SBRs operated at 28 ± 1 °C (Ong et al. 2014a, Ong et al. 2013). When the microbial communities in reactors at 24 °C, 28 °C, and 32 °C were compared, there was a decrease in abundance of *Accumulibacter*-PAOs with increasing temperature. Quantification of *Accumulibacter*-PAOs using the real time quantitative polymerase chain reaction (qPCR) revealed that at 24 °C almost 64% of the total bacteria were *Accumulibacter*-PAOs. Their abundance dropped to 43% and 19% when temperature rose to 28 °C and 32 °C, respectively (Ong et al. 2014a, Ong et al. 2013). In contrast, the *Competibacter*-GAO population increased from less than 10% of the total population at 24 °C to 40% at 32 °C. However, unlike the previous findings, EBPR activities did not decrease regardless of the shift in the PAO-GAO dominance at higher temperatures. This may be due to a different metabolism of PAOs at higher temperatures rather than a shift in microbial population (Ong et al., 2013). Characterization of PAOs in the sludge revealed the presence of a single Clade IIF, suggesting its evolved capacity to carry out EBPR at high temperatures (Ong et al. 2014a). EBPR has also

been reported at a tropical full-scale treatment plant (Law et al. 2016). Analysis of the microbial community structure of this plant revealed the presence of *Accumulibacter*-related PAOs (<5% relative abundance), namely, *Accumulibacter* Clades IIA-C, in all of the samples collected, while Clade IIF was present in some samples. *Accumulibacter* Clade IIC was the most abundant PAO. *Accumulibacter* Clade I was present in lower numbers in all of the samples (Law et al. 2016). Unlike previous findings that reported higher GAO to PAO ratios, *Competibacter*-GAOs were detected at a much lower abundance than were *Accumulibacter*-PAOs at this particular plant. Another group of PAOs, *Tetrasphaera*-PAOs was present in low numbers (0.05% relative abundance), whereas *Defluviicoccus*-GAOs were not detected. These findings strongly suggest that EBPR in tropical full-scale plants is possible despite a higher prevailing water temperature (Law et al. 2016).

1.4.3.2. Effect of carbon source

The type of carbon source fed into the lab-scale reactor greatly affects PAO-GAO competition (Dai et al. 2007). Even though acetate, one of the most common VFAs in EBPR system, can lead to stable P-removal activities, this carbon source can also be utilized by GAOs for their growth. Consequently, the competition for acetate between PAOs and GAOs can cause the efficiency of EBPR to decrease. Another type of carbon source that is commonly present in EBPR systems is propionate. Propionate seems to favour PAOs over GAOs (Oehmen et al. 2006, Pijuan et al. 2004). Although some GAOs (i.e., alphaproteobacterial GAOs) can take up propionate, other known GAOs like *Competibacter* cannot. As a result, PAOs that are able to utilize propionate as well as acetate can outcompete GAOs (Oehmen et al. 2007). A recent study on carbon utilization by PAOs, using an enrichment system with *Accumulibacter* PAOs covering $85 \pm 2\%$ of the total bacterial population, revealed the preference of *Accumulibacter*-PAOs for propionate over acetate when both carbon sources are present (Carvalho et al. 2014b). In addition to VFAs like acetate and propionate, other groups of PAOs, namely,

Tetrasphaera-PAOs can utilize amino acids (e.g., glycine) as carbon source (Nguyen et al. 2015).

While *Competibacter*-GAOs cannot utilize propionate, alphaproteobacterial GAOs, especially those related to *D. vanus*, are capable of consuming both acetate and propionate (Lanham et al. 2008, Meyer et al. 2006). Similarly, Dai et al. (2007) analysed the capacity of GAOs related to *D. vanus* using an acetate-fed system that was highly enriched in *D. vanus*-related GAOs. The latter immediately consumed propionate as soon as acetate was switched to propionate. Moreover, this group of GAOs also exhibited a preference for propionate when both acetate and propionate were present (Dai et al. 2007). Besides VFAs, *Defluviicoccus*-related cluster 2 can also assimilate a mixture of amino acids (Burow et al. 2007). Similar to the carbon uptake capacities of Cluster 2, *Defluviicoccus*-GAOs belonging to cluster 1 have also exhibited the capacity to take up acetate and propionate (Burow et al. 2007).

Another type of carbon source commonly used in EBPR studies is glucose. However, failures are often observed over time. This is caused by the ability of external glucose to provide energy and reducing power for PHA synthesis. Consequently, poly-P dependence will decrease, and GAOs can outcompete PAOs (Mino et al. 1998). Although Kong et al. (2006) suggested that *Competibacter* is incapable of taking up glucose directly (Kong et al. 2006), other types of GAOs like those related to *Defluviicoccus* cluster 2 are able to assimilate glucose directly (Burow et al. 2007).

1.4.3.3. Effect of nitrate and nitrite/FNA

It has been previously reported that, ideally, the simultaneous presence of electron donor (i.e., carbon source) and electron acceptor (i.e., oxygen, nitrate or nitrite) should be avoided (Kuba et al. 1994). Yet, nitrate might be produced during nitrification in the subsequent aerobic stage, leading to possible recirculation of nitrate via the internal recirculation into the anaerobic stage. The presence of nitrate in what should be an anaerobic stage has been reported

to cause EBPR failure. The failure is attributed to the competition for carbon between PAOs and heterotrophic denitrifiers, or to the inhibitory effect caused by some intermediates resulting from denitrification steps like nitrite (Guerrero et al. 2011). However, a study on the effect of carbon source on the competition between PAO and heterotrophic denitrifiers under anoxic/aerobic cycling revealed that EBPR activities were not inhibited when the enrichment system, consisting of 72% PAOs and fed with a mixture of acetic acid, propionic acid and sucrose, was switched from anaerobic-anoxic-aerobic to anoxic-aerobic cycling (Guerrero et al. 2011). This switch created a condition where both electron donors (carbon sources) and acceptors (nitrate) are simultaneously present. Another NO_x compound often attributed to EBPR failure is nitrite, where the presence of nitrite has been reported to reduce aerobic (or anoxic) phosphorus uptake (Meinhold et al. 1999, Saito et al. 2004). However, analysis of the effect of nitrite on anoxic P uptake revealed that free nitrous acids (FNA) are potentially the true inhibitor instead of nitrite (Zhou et al. 2007).

FNA is a species of nitrite that inhibits anaerobic and aerobic metabolism of PAOs and GAOs, more so for PAOs (Pijuan et al. 2010, Ye et al. 2010, 2013). Anaerobically, one effect of the presence of FNA is its impact on the rates of acetate uptake by PAOs and GAOs. For PAOs, a small amount of FNA ($0.5 \mu\text{g/L HNO}_2\text{-N}$) decreases PAOs' uptake rate by 28%. As the FNA concentration increases to $22 \mu\text{g/L HNO}_2\text{-N}$, the acetate uptake rate of PAOs suffers almost 90% inhibition. Consequently, lower acetate consumption leads to lower energy consumption. Thus, an increase in FNA concentration would also reduce the P-release ratio (Ye et al. 2013). Possible effects of FNA on GAOs, namely, *Competibacter*-GAOs have also been reported. FNA is reported to inhibit the acetate uptake of GAOs, where up to 55% inhibition is detected when FNA concentration reaches $22 \mu\text{g/L HNO}_2\text{-N}$ (Ye et al. 2013). However, the negative impact of FNA is more severe for the anaerobic metabolism of PAOs than of GAOs (Ye et al. 2013). The differential inhibitory effects of FNA on PAOs and GAOs

might be due to varying acetate transport mechanisms that these two groups employ. While PAOs produce PMF when phosphate is pumped out of the cells, GAOs generate PMF when proton is exported by enzyme fumarate reductase and ATPase (Filipe et al. 2001b, d, Liu et al. 1994, Saunders et al. 2007). However, confirmation and details on how these different transport mechanisms can cause the differential FNA inhibition are still needed (Ye et al. 2013).

The presence of FNAs can also inhibit aerobic P-uptake by PAOs, where uptake activities are 50% inhibited when the FNA concentration is 0.52×10^{-3} mg/L $\text{HNO}_2\text{-N}$. Moreover, it is suggested that inhibitory effects by FNAs are reversible, even when the FNA concentration is in the range of 9.5 to 16.6×10^{-3} mg/L $\text{HNO}_2\text{-N}$ (Pijuan et al. 2010). Further analysis reveals that FNAs can affect all anabolic metabolisms of PAOs, a type of metabolism that consumes energy to synthesize organic compounds⁽²⁵⁾. It includes glycogen production where a 50% reduction is expected when the FNA concentration is approximately 0.48×10^{-3} mg/L $\text{HNO}_2\text{-N}$, and microbial growth where a 50% reduction can occur when the FNA concentration is 0.36×10^{-3} mg/L $\text{HNO}_2\text{-N}$ (Pijuan et al. 2010). Similar to its effect on anabolic metabolism, the presence of FNA can also inhibit catabolic metabolism in PAOs, which breaks down complex organics compounds to produce energy⁽²⁵⁾. Inhibition of PHA degradation by 40-50% is observed at FNA concentrations from 2 to 10×10^{-3} mg/L $\text{HNO}_2\text{-N}$ (Pijuan et al. 2010). In GAOs, the presence of FNA also affects aerobic metabolism, such as glycogen production, PHA degradation/ consumption, and bacterial growth. Utilizing sludge that is highly enriched in *Competibacter*, a direct inhibition of microbial growth of approximately 50% is observed when the concentration of FNA is approximately 1.5×10^{-3} mg/L $\text{HNO}_2\text{-N}$, while reduced production of glycogen and degradation of PHA occur when the FNA concentration exceeds 1.5×10^{-3} mg/L $\text{HNO}_2\text{-N}$. As it does for anaerobic metabolism in PAO and GAO, effects of FNA on aerobic metabolism are more severe in PAOs than in GAOs (Ye et al. 2010).

1.4.3.4. Effect of pH

Another important factor in the PAO-GAO competition is pH, with a higher pH favouring PAOs (Oehmen et al. 2007). This effect can be attributed to the higher energy requirement to consume acetate in this kind of environment, which can be accommodated by PAOs at the expense of their intracellular polyphosphates. That is, PAOs will utilize more poly P to provide for the higher energy demand (Oehmen et al. 2007). GAOs, on the other hand, do not have such luxury. As a result, a higher pH decreases the abilities of GAOs to take up acetate (Filipe et al. 2001b, Oehmen et al. 2007). One possible explanation for the higher energy requirement to consume VFAs at higher pH values was elaborated by Oehmen et al. (2007). Assuming that the intracellular pH is constant, an increase in the external pH will create a pH gradient across the cell membrane and lead to a higher difference between the electrical potential inside and outside the cells. Consequently, these conditions create the higher energy demand for VFA (e.g. acetate) uptake (Oehmen et al. 2007). Even though anaerobic metabolism of PAOs (i.e., rates of acetate uptake, PHAs synthesis, and degradation of glycogen) is not affected by pH between values of 6.5 and 8.0 (Filipe et al. 2001d), GAOs are able to take up acetate at higher rates than PAOs when the pH falls below 7.25. Thus, the anaerobic pH should be maintained above 7.25 as PAOs can outcompete GAOs in taking up available acetate (Filipe et al. 2001c). Similarly, a higher pH is also preferable for the aerobic metabolism of PAOs, with the optimum pH reported to range from 7.0 to 7.5 (Filipe et al. 2001a). Aerobic metabolism of PAOs is inhibited when the pH decreases below 6.0 (Oehmen et al. 2007).

The pH can also impact EBPR efficiency due to its effect on the free nitrous acid (FNA) production. Observing the detrimental effects of FNA on both anaerobic and aerobic metabolism in PAOs, it is crucial to avert accumulation of FNA in the system, and pH is one significant factor. It has been shown to affect the growth rates of NOBs or nitrite oxidizing

bacteria. When the pH falls below 6.5, NOB activities are inhibited (Jimenez et.al.,2011). If activities of NOBs are impaired due to a low pH, oxidation of nitrite will also be adversely affected, and accumulation of nitrite in the system can occur. Since protonation of nitrite produces nitrous acid, and an elevated level of free nitrous acid will negatively affect PAOs more severely than GAOs, a low pH can lead to failure of EBPR due to outcompetition of PAOs by GAOs.

1.4.3.5. Effect of dissolved oxygen

The concentration of dissolved oxygen (DO) has also been hypothesized to affect the competition, with lower DO concentration favouring PAOs over GAOs (Carvalho et al. 2014a, Law et al. 2016, Lemaire et al. 2006). When the level of DO reaches 4.5-5.0 mg/L, tetrad forming organisms (TFOs) proliferate and EBPR efficiency drops. PAOs seem to be more prolific when the level of DO ranges from 2.5 to 3.0 mg/L (Oehmen et al. 2007). Similarly, when an SBR system is operated at a very low DO concentration (approximately 0.5 mg O₂/L), a higher abundance of *Accumulibacter* and lower abundance of *Competibacter* is observed (Lemaire et al. 2006, Oehmen et al. 2007). Analysis of microbial communities in a tropical full-scale plant when oxygen was higher than 2.0 mg/L O₂ or lower than 1.5 mg/L O₂ confirmed the positive effect of low DO on PAOs, where abundance of *Accumulibacter*-PAOs increased when the DO level dropped (Law et al. 2016). Moreover, excessive aeration can lead to full depletion of intracellular PHAs. Since aerobic P uptake is reliant on the degradation of PHAs for energy, depletion of PHAs due to excessive aeration can impair P uptake by PAOs. Consequently, biological P-removal efficiency decreases (Brdjanovic et al. 1998b).

2. Motivation

Various studies on EBPR have been conducted to understand its underlying mechanisms, as well as to determine the effect of different operating conditions on activities and microbial communities, such as temperature or presence of nitrate and/or nitrite in the anaerobic zone.

While elevated temperature had been reported to be detrimental for PAOs and EBPR (Panswad et al. 2003, Whang and Park 2002, 2006), recent findings proved stable EBPR can occur at high ambient temperature (Law et al. 2016, Ong et al. 2016). Similarly, contrasting results have been reported regarding the effect of nitrate on the anaerobic compartment. Previous studies suggested an adverse effect of nitrate (Akin and Ugurlu 2004, Kuba et al. 1994, Patel and Nakhla 2006) or nitrite (Meinhold et al. 1999) on EBPR. However, Guerrero et al. (2011) observed successful EBPR when sludge enriched with PAOs was subjected to anoxic/aerobic cycling and fed with a mixture of acetic acid, propionic acid, and sucrose. These contradicting findings highlight a lack of complete understanding of EBPR. Additionally, a detailed study on the effect of the simultaneous presence of carbon and NO_x compounds at elevated temperatures is still lacking.

The simultaneous presence of carbon and nitrate/nitrite can occur in full-scale plants due to internal recirculation of sludge from the end of the aerobic to the beginning of the anaerobic tanks. Thus, understanding how this condition impacts EBPR and sludge microbial communities will be advantageous. This dissertation aims to explore this niche in an effort to gain a more complete knowledge of EBPR under anoxic/aerobic cycling at elevated temperature. Additionally, this thesis explores a strategy to resuscitate failing EBPR systems when the temperature is higher. With increasing temperature glycogen accumulating organisms (GAOs), a group of purported competitors of PAOs, can proliferate and outcompete PAOs for available carbon. Similarly, under anoxic/aerobic conditions, heterotrophic denitrifiers can also vie for carbon. This higher carbon competition can lead to EBPR failure. Thus, a readily available and cheaper mitigation approach will be beneficial to revive flagging EBPR systems.

To achieve these objectives, I aim to answer the following research questions:

1. Can PAOs in a full-scale sludge perform successful EBPR under strict anoxic/aerobic cycling at higher temperature?
2. Which group of PAOs is likely the important player in the anoxic/aerobic EBPR at higher temperature?
3. Can stable EBPR be maintained when sludge is continuously subjected to prolonged exposure to planned anoxic/aerobic cycling at higher temperature?
4. How does the PAO community react when being continuously subjected to prolonged exposure to the simultaneous presence of carbon and NO_x at elevated temperature?
5. How do GAOs and other organisms like heterotrophic denitrifiers respond when being continuously subjected to planned anoxic/aerobic cycling at elevated temperature?
6. Can the addition of wasted activated sludge (WAS) lead to increased activities and PAO abundance, to revive failing EBPR systems at elevated temperature?

Research questions 1 and 2 are explored in Chapter 2, where I utilized batch experiments using freshly collected sludge from a treatment plant in Singapore that exhibited EBPR despite the absence of a designed anaerobic compartment. I hypothesized that among the PAOs, those who could not use nitrate (i.e., non denitrifying PAOs or non-DPAOs) could perform EBPR under strict anoxic/aerobic cycling at elevated temperature, likely due to their inability to use nitrate. Consequently, they could recognize the condition as pseudo-anaerobic/aerobic and perform anaerobic/aerobic metabolisms accordingly.

Chapter 3 addresses Questions 3 to 5, where two laboratory scale bioreactors were continuously operated at elevated temperature and subjected to anaerobic/aerobic cycling before switching to planned anoxic/aerobic cycling. I hypothesized that like non-DPAOs, GAOs that did not use nitrate and/or nitrite could recognize the condition as pseudo-anaerobic/aerobic cycling, enabling them to perform their metabolism accordingly.

The last research question was addressed in Chapter 4, where I hypothesized the addition of WAS could lead to increased levels of PAOs in two failing EBPR reactors operated at elevated temperature. For this dissertation, I only tested this strategy to revive EBPR under basic anaerobic/aerobic cycling to determine its potential without possible biases that might be brought about by the simultaneous presence of carbon and NO_x compound on PAOs or GAOs.

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Chapter 2

Non-denitrifying Polyphosphate Accumulating Organisms Obviate

Requirement for Anaerobic Condition

Abstract

Enhanced biological phosphorus removal (EBPR) is a widely used process in wastewater treatment that requires anaerobic/aerobic or anaerobic/anoxic cycling. Surprisingly, phosphorus (P) release was observed in the presence of nitrate in the anoxic compartment of the activated sludge tank in a full-scale treatment plant with the Modified Ludzack Ettinger configuration. We therefore studied the potential of this full-scale activated sludge community to perform EBPR under anoxic/aerobic cycling. The polyphosphate accumulating organism (PAO) *Candidatus Accumulibacter* represented 3.3% of total bacteria based on 16S rRNA gene amplicon sequencing, and metagenome analysis suggested it was likely to be dominated by Clade IIC. Using acetate as the carbon source in batch experiments, active denitrifying organisms (DPAOs) were estimated to comprise 39-44% of the total PAO population in the sludge, with the remaining 56-61% unable to utilize nitrate. When propionate was provided as the organic carbon source, 95% of the PAO population was unable to denitrify. EBPR occurred under defined anoxic/aerobic conditions, despite the presence of DPAOs, when synthetic wastewater was supplemented with either acetate or propionate or when primary effluent was supplied. In addition, the P release and subsequent uptake rates under anoxic/aerobic conditions were comparable to those observed under anaerobic/aerobic conditions. In contrast, a significant reduction in P release rate was observed when acetate was provided under oxic conditions. We postulate that non-DPAOs that experience the anoxic condition as pseudo-anaerobic, a condition where non-usable electron acceptors are present, were the key players in anoxic/aerobic EBPR.

1. Introduction

Enhanced biological phosphorus removal (EBPR) is a microbial process that removes inorganic phosphorus from wastewater streams by intracellular accumulation. The process requires the cycling of biomass through the anaerobic/aerobic or denitrifying anaerobic/anoxic states. This mode of operation selects for organisms that can synthesize and utilize internal storage compounds in the form of polyhydroxyalkanoates (PHAs), along with glycogen and/or polyphosphate, from the assimilation of organic carbon substrates, mainly volatile fatty acids (VFAs) (Oehmen et al. 2007). In EBPR systems, polyphosphate accumulating organisms (PAOs) are capable of taking up P in excess of what is needed for cell growth. *Candidatus Accumulibacter phosphatis* (thereafter referred to as *Accumulibacter*) is the most studied PAO and has been widely enriched in lab-scale systems with acetate and/or propionate as substrates (Lu et al. 2006). Members of the actinobacterial genus *Tetrasphaera* have also been identified as putative PAOs (Kong et al. 2005, Maszenan et al. 2000) with a preference for amino acids or glucose as a substrate (Kristiansen et al. 2013, Nguyen et al. 2015).

Under anaerobic conditions, *Accumulibacter*-PAO generates energy and reducing equivalents required to take up organic carbon sources and to synthesize PHAs through hydrolysis of their internally stored poly P, as well as by glycolysis and/or the tricarboxylic acid (TCA) cycle (Comeau et al. 1986, Mino et al. 1987, Pereira et al. 1996, Zhou et al. 2009). This phase is followed by aerobic (or anoxic) conditions when the carbon source is depleted, and *Accumulibacter* oxidizes the previously synthesized PHAs, using oxygen or nitrate/nitrite as the terminal electron acceptor, to support P uptake and cell growth and to replenish its internal storage of glycogen (Oehmen et al. 2007).

A defined anaerobic phase, to separate the supply of electron donor (organic matter) and electron acceptor such as oxygen and nitrate, is assumed to be crucial for the stable

operation of EBPR. These conditions provide a selective advantage for PAOs over other heterotrophic organisms, due to their ability to take up organic carbon sources in the absence of an electron acceptor (Mino et al. 1998). However, several studies observed EBPR activity even when there was no physical or temporal separation of the supply of electron donor and acceptor (Ahn et al. 2002, Guisasola et al. 2004, Pijuan et al. 2005, Vargas et al. 2009). Under strictly aerobic conditions, simultaneous P release and acetate uptake coincided with PHA formation and glycogen degradation. Upon substrate depletion, P uptake activity proceeded concurrently with PHA degradation, glycogen formation and cell growth. Although the EBPR activity deteriorated after four days of completely aerobic operation in an acetate-fed enriched *Accumulibacter* culture (Pijuan et al. 2006), the EBPR activity was sustained throughout the 46-day aerobic operation in an enriched *Accumulibacter* culture fed with propionate (Vargas et al. 2009).

Guerrero et al. (2011) investigated the effect of nitrate and the type of carbon source on EBPR activity, by switching a PAO-enriched pilot plant that had been operated under anaerobic/anoxic/aerobic (A²O) conditions to anoxic/aerobic cycling (i.e., Modified Ludzack Ettinger (MLE) configuration). Both pilot plant studies and batch experiments showed that the presence of NO_x did not inhibit EBPR activities. Moreover, PAOs could outcompete heterotrophic denitrifiers for the available carbon sources, as long as volatile fatty acids in the form of acetate and propionate were supplied. EBPR failure was observed when a more complex carbon source (i.e. sucrose) was added under anoxic/aerobic conditions (Guerrero et al. 2011, Guerrero et al. 2012).

In this study, we report on P removal activities at a full-scale tropical water reclamation plant (WRP) with an MLE configuration for biological nitrogen and carbon removal; that is, the plant is not designed to achieve biological P removal. The P removal activities under anoxic/aerobic conditions may be due to the presence of anaerobic zones within flocs, or within

tanks, due to poor mixing, allowing conventional anaerobic EBPR activity to take place. Alternatively, we hypothesized that a fraction of PAOs in the sludge that cannot denitrify (non-DPAOs) is capable of performing EBPR under anoxic/aerobic conditions. Due to their inability to use nitrate as electron acceptor, non-DPAOs shift to anaerobic metabolisms. Hence, they experience the anoxic condition as anaerobic. It is known that PAOs can outcompete heterotrophic denitrifiers for organic carbon under anoxic conditions, using enriched EBPR sludge and *Accumulibacter* accounting for approximately 72% of the bacterial population (Guerrero et al. 2011). In contrast, in full-scale systems, *Accumulibacter* account for less than 10% of total bacteria, typically 3-6% (Lanham et al. 2013, Mielczarek et al. 2013, Pijuan et al. 2008)). To the best of our knowledge, no previous study has explored the potential of non-DPAOs in full-scale sludge to perform anoxic/aerobic EBPR. Thus, the objectives of this study were (i) to determine the proportion of non-DPAOs in a full-scale plant and (ii) to investigate the EBPR capacity of non-DPAOs in the full-scale sludge when exposed to anoxic/aerobic cycling in short-term batch experiments using acetate or propionate, two main types of VFAs detected in the influent wastewater, or real wastewater as organic carbon source. P removal activity was investigated in controlled batch experiments with freshly sourced sludge to represent as closely as possible the in situ activity at the plant.

2. Materials and Methods

2.1 Water reclamation plant characteristics and field sampling activity

The investigated Ulu Pandan Water Reclamation Plant-South Works (UPWRP-SW) in Singapore receives approximately 200 ML of wastewater per day with an operating temperature of $30 \pm 1^\circ\text{C}$ all year long. The plant consists of primary clarifiers and activated sludge treatment systems that adopt the Modified Ludzack Ettinger (MLE) configuration for biological nitrogen and carbon removal. There were six trains of biological treatment tanks in

the field, one of them being Tank 2B. Each train consisted of an anoxic followed by an aerobic compartment (Figure S1). The average hydraulic retention time (HRT) and solids retention time (SRT) are 8 h and 6 d, respectively. The mixed liquor of each activated sludge tank flows into corresponding secondary clarifiers. Internal recirculation brings the mixed liquor from the end of the aerobic compartment to the start of the anoxic compartment at twice the flow rate of the primary effluent, mixing it with the incoming wastewater and returned activated sludge (RAS). Chemical dosing was not in place for phosphorus removal.

Field sampling was conducted weekly from the 11th June to 23rd July, 2014, where grab samples were collected from specific locations of one of the activated sludge tanks (Tank 2B) to monitor the potential EBPR activity. The selected sampling points were primary effluent; the start, mid, and end sections of the anoxic compartment; and the start, mid, and end sections of the aerobic compartment. Samples were collected from all sampling locations for ammonium, nitrate, nitrite and phosphate analyses as well as volatile fatty acids (VFAs), total phosphorus (TP) and total chemical oxygen demand (TCOD) analyses for the primary effluent. Mixed liquor samples were collected for polyhydroxyalkanoates (PHAs) and glycogen analyses; concurrently, the pH, temperature, redox potential and dissolved oxygen (DO) concentration were measured with a portable DO/pH/EH/T meter (YSI Professional Plus, United States).

2.2. Laboratory scale batch experimental setup

All batch experiments were carried out in double jacketed sequencing batch reactors (SBRs) with freshly collected RAS from one of the activated sludge treatment trains (Tank 2B). Unless otherwise stated, the RAS was diluted with either primary effluent collected from UPWRP for each of the batch experiments, or with synthetic wastewater at a 1:1 (v/v) ratio to a mixed liquor suspended solids concentration of 1.0-2.1 g/L and total working volume of 4 L.

Primary effluent was collected on the same day of the batch studies, along with the RAS, and utilized upon return to the laboratory. The synthetic wastewater was prepared following the recipe by Pijuan et al. (2006) and Nittami et al. (2011) supplemented either with acetate or propionate as the external carbon source. Stock solutions were prepared as such: P-stock solution containing 2.46 g/L of KH_2PO_4 ; C-stock solution containing (g/L in milliQ water) peptone (0.5), NH_4Cl (0.84), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.8); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (3.2); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.84), yeast extract (0.4), allylthiourea (0.01), and nutrient solution (12 ml). This nutrient solution contained (in g/L) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.12), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.12), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.15), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.03), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.5), H_3BO_4 (0.15), KI (0.18), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.06), and EDTA (10) (Pijuan et.al., 2006; Nittami et.al., 2011). Each stock was added into a 2 L synthetic wastewater to achieve initial concentrations of 10-15 mg/l C and 40-45 mg/l P in each reactor. The SBRs were controlled with a programmable logic controller (PLC) linked to a SCADA interface for data visualization and storage.

Three sets of experiments were carried out to characterise the biochemical activity of PAOs in UPWRP sludge (see also Table 1):

- Set 1: Determining the proportions and activities of non-DPAOs in the full-scale sludge under anaerobic/anoxic/aerobic conditions.

The presence of non-DPAOs and DPAOs as well as their potential activities were established in batch tests. For this study, DPAOs refer to the PAOs that can utilize nitrate or nitrite to perform concurrent P uptake and denitrification to N_2 or an intermediate stage. Return activated sludge (RAS) collected from the plant was subjected to anaerobic/anoxic/aerobic cycling. Acetate was provided as the sole carbon source in all experiments, except for one of the batch experiments in July 2014 where the denitrifying activity of PAOs was also tested with propionate.

- Set 2: EBPR activity of non-DPAOs under anoxic/aerobic cycling with different organic carbon sources.

Once the presence and activities of non-DPAOs had been determined, their potential to perform EBPR under anoxic/aerobic cycling was tested. For this experiment, RAS was collected during the period of June and July 2014 and subjected to defined anoxic/aerobic cycling. Sludge was exposed either to synthetic wastewater supplemented with acetate or propionate or to primary effluent with a sludge to wastewater ratio of 1:1 (v/v). The nutrient profile of the primary effluent is shown in Table S1. At each tested condition, a positive control experiment was carried out by subjecting the sludge to anaerobic/aerobic cycling with synthetic wastewater supplemented with acetate. Throughout the experimental period for Set 2, weekly field sampling was conducted to monitor the EBPR activity in the full- scale plant as described in Section 2.1.

- Set 3: EBPR activity of non-DPAOs under completely aerobic conditions.

The hypothesis that non-DPAOs could perform anoxic/aerobic EBPR based on their inability to use nitrate was tested by comparing the anoxic/aerobic activities to those under fully aerobic conditions. Positive and negative control experiments were conducted concurrently in March 2015 by subjecting the sludge to anaerobic/aerobic cycling in the presence and absence of acetate, respectively.

A C/P molar ratio of 3:1 was used in all experiments. The C/P of 3:1 was shown to be optimal for EBPR activity operated at $28 \pm 1^\circ\text{C}$ as reported by Ong et al. (2013). All tests and controls in the three sets of experiments were conducted in duplicate. Anaerobic conditions were achieved by continuously sparging the reactors with nitrogen during the first hour of the batch studies, while anoxic conditions were induced by adding sodium nitrate to an initial concentration of approximately 30 mg-N/L. Anaerobic/aerobic and anoxic/aerobic batch

reactors were operated for 7 hours, with an anaerobic (or anoxic) stage for the first 3 hours followed by a 4-hour aerobic stage. Similarly, fully aerobic reactors in Set 3 experiments were maintained for 7 hours, while anaerobic/anoxic/aerobic (Set 1) were operated for 9 hours, with each condition lasted for 3 hours. The length of each stage was selected to ensure that carbon had been consumed before aeration began. Mixed liquor samples were collected in 15-30 min intervals for ammonium, nitrate, nitrite, phosphate, VFA, PHA and glycogen analyses. The mixed liquor volatile suspended solids (MLVSS) concentration was measured at the end of each batch experiment. The temperature was kept at $30 \pm 1^\circ\text{C}$, while the dissolved oxygen concentration during the aerobic phase was maintained at 7-8 mg O₂/L (saturation level) using an on-off control. The pH was maintained at 7.0 ± 0.1 by automatic dosing of 0.5 M HCl or 0.5 M NaOH as required.

2.3 Chemical analysis

Nutrient profiles from each of the reactors were obtained to monitor the EBPR activity of the sludge under each of the prescribed conditions. Samples collected for phosphate, nitrate, nitrite, ammonium and VFA analyses were filtered immediately with disposable 0.2 µm sterile Acrodisc® Syringe filters with Supor® Membrane (Pall Life Science, USA). Phosphate, nitrate, nitrite and ammonium concentrations were analyzed using ion chromatography (Prominence, Shimadzu) fitted with Shim-pack IC-SA2 (250 mm length, 4.0 mm ID) for anions and Shim-pack IC-C4 (150 mm length, 4.6 mm ID) for cations. The same filtered samples were also subjected to acetate, propionate and butyrate analyses, using gas chromatography (Prominence, Shimadzu) equipped with a flame ionization detector (FID) fitted with a DB-FFAP (30 m length, 0.25 mm diameter, and 0.25 µm film) column (Agilent Technology, USA). TCOD, TP, MLSS and MLVSS analyses were carried out based on Standard Methods (APHA 1995). Mixed liquor samples collected for PHA and glycogen analyses were fixed with 10%

formaldehyde for at least one hour prior to washing and freeze-drying as described in Lanham et al. (2012). PHA analysis was done using gas chromatography (Prominence, Shimadzu) equipped with an FID detector and fitted with a DB-5MS Ultra Inert (30 m length, 0.250 mm diameter, and 0.25 μ m film) column (Agilent Technology, USA) as described in Oehmen et al. (2005a). Glycogen analysis was carried out by analysis of glucose after acid digestion as described by Kristiansen et al. (2013).

2.4 Calculations of active DPAOs or non-DPAOs fractions

From the anaerobic/anoxic/aerobic batch studies, the fractions of active DPAOs and non-DPAOs were calculated based on the observed anoxic and aerobic P uptake activities in the acetate-fed test reactors, using the modified total P uptake equation by Meinhold et al. (1999), as described by Oehmen et al. (2010) and Lanham et al. (2012), and represented by Equation 1. Meinhold et al. (1999) suggested considering the total amount of P consumed under anoxic or aerobic conditions within a defined time interval (Meinhold et al. 1999). However, the use of an arbitrary time interval to determine the total P consumed would prevent comparisons among various EBPR systems that adopt different residence times for anoxic and/or aerobic conditions, a potential problem reported by Oehmen et al. (2010). To remedy this issue, the authors suggested that the total P uptake method be used with the following conditions, originally proposed by Wachtmeister et al. (1997): (1) no simultaneous presence of electron acceptors and donors; (2) PHA not a limiting factor; (3) oxygen or nitrate not a limiting factor; and (4) no significant nitrite accumulation (Oehmen et al. 2010, Wachtmeister et al. 1997)

The equation was further modified to incorporate the correction factor of 1.85 ($\delta_{\text{anoxic}} = 1$; $\delta_{\text{aerobic}} = 1.85$), since nitrate, as a less efficient electron acceptor, yields less ATP compared to oxygen (Kuba et al. 1996, Murnleitner et al. 1997, Smolders et al. 1994).

$$f_{DPAO} = \frac{\Delta P_{anoxic}}{\Delta P_{aerobic}} \chi \frac{\delta_{aerobic}}{\delta_{anoxic}} \quad \text{Eq. 1a}$$

$$f_{DPAO} + f_{non-DPAO} = 1 \quad \text{Eq. 1b}$$

Equation 1a was modified to take into account the fact that the anoxic and aerobic conditions were applied to the same reactors (i.e., anaerobic/anoxic/aerobic conditions); hence the estimation of total PAOs should incorporate the observed activities in both the anoxic and the subsequent aerobic conditions (Equation 2). Additionally, since non-DPAOs are unable to utilize nitrate, the presence of an anoxic phase should not affect the activities of non-DPAOs in the subsequent aerobic phase.

$$f_{DPAO} = \frac{1.85 \Delta P_{anoxic}}{1.85 \Delta P_{anoxic} + \Delta P_{aerobic}} \quad \text{Eq. 2}$$

2.5 DNA extraction and sequencing

Representative DNA samples were collected for Set 2 and Set 3 experiments described in Section 2.2 (July, 2014 and March 2015) for 16S rRNA amplicon sequencing and metagenomics. Samples for DNA extraction were snap-frozen in liquid nitrogen and stored at -80°C. The DNA was extracted based on the FastDNATM 2 mL SPIN Kit for Soil (MP Biomedicals, USA) optimised for DNA extraction from activated sludge (Albertsen et al. 2015). The sludge samples were defrosted and subsequently homogenised with a Heidolph RZR 2020 overhead stirrer (Heidolph Instruments, DE) for 1 min at gearing II speed 9. Subsequently, 1.0 mL of the homogenised sample was transferred to a 2 mL tube and centrifuged at 21,100 x g for 5 min in a Sorvall Legend Micro 21 Microcentrifuge (Thermo Fischer Scientific, USA). The supernatant was discarded and the pellet was resuspended in 978 µL sodium phosphate buffer (pH 8). Resuspended cells were transferred to a Lysing matrix E tube and 122 µL MT buffer was added. The sample was then homogenized using a FastPrep FP120 Homogenizer (Thermo Savant, USA) for 4x40 seconds at speed 6, and the samples were

stored on ice for 2 min between each bead beating. After this step the manufacturer's protocol was followed.

16S rRNA gene amplicon sequencing was conducted to determine the microbial community structure of the activated sludge. Bacterial primers 27F (AGAGTTTGATCCTGGCTCAG, Lane 1991) and 534R (ATTACCGCGGCTGCTGG, (Muyzer et al. 1993)), were used to amplify an approximately 500 bp DNA fragment of the 16S rRNA gene (variable V1 to V3 regions). Amplification of PCR was done using the following conditions: 2 min of 95°C, 20 sec for 30 cycles of 95°C, 30 sec of 56°C, 60 sec of 72°C, and a 5 min elongation step at 72°C. This amplification utilized 1X Platinum® High Fidelity buffer, 2 mU Platinum® Taq DNA Polymerase High Fidelity, 400 pM dNTP, 1.5 mM MgSO₄, 5 uM V1-V3 adaptor mix (barcoded), and 10 ng of template DNA. Purification of PCR products was done using the Agencourt AmpureXP (Beckman Coulter Inc., U.S.A.) with 1.8 bead solution/PCR solution ratio. The QuantIT HS kit (Life Technologies, USA) was used to quantify the DNA concentration. Using Illumina MiSeq (Illumina Inc., USA), barcoded amplicons, which were pooled in equimolar amounts, were paired-end sequenced (2x250 bp).

For metagenomic DNA, sequencing library preparation was performed using a modified version of the Illumina TruSeq DNA Sample Preparation protocol: For each sample, 1 µg of the DNA was sheared on a Covaris S220 to approximately 300 bp, following the manufacturers' recommendation. Size selection was performed on a Sage Science Pippin Prep instrument, using a 2% EtBr agarose cassette and selecting for a tight peak around 400 bp. Each library was tagged with a TruSeq LT DNA barcode (Illumina) to allow for library pooling prior to sequencing. Library quantitation was performed using the Picogreen assay (Invitrogen) and the average library size was determined by running the libraries on a Bioanalyzer DNA 7500 chip (Agilent). Library concentrations were normalized to 4 nM and validated by qPCR on a

ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended by Illumina in their qPCR protocol, and the Illumina PhiX control library was used as a standard. Libraries were then combined in one pool, which was sequenced across two lanes of an Illumina HiSeq2500 sequencing run at a read-length of 151 bp paired-end.

2.6 Processing and analysis of amplicon sequencing data

The output from the MiSeq (Illumina Inc., San Diego, California, USA) was demultiplexed from the amplicon libraries in FASTQ-format for each sample in the composite library. Pre-processing of all amplicon libraries was performed according to Albertsen et al. (2015), and all sequenced sample libraries were subsampled to 10,000 raw reads. Taxonomy was assigned using MiDAS v.1.20 (McIlroy et al. 2015). The results were analysed in R (R Core Team, 2014) through the Rstudio IDE (<http://www.rstudio.com/>), using the R package *ampvis* (<https://github.com/MadsAlbertsen/ampvis>). Sequences were clustered into OTU at 97% level (species).

gDNA data was used to screen for the presence of 16S rRNA FISH probes targeting *Accumulibacter*, sourced from Nielsen et al. (2009) and defined *ppk1* primer sequences for identification of specific clades for this organism (refer to Table S2 for details of probe sequences detected in both samples). Relative abundance was calculated by normalizing the number of read counts for the specific FISH probe or *ppk1* primer to the universal EUB 338 probe targeting most bacteria (Daims et al. 1999).

2.7 Fluorescence in situ hybridization (FISH)

Samples from Set 2 and Set 3 experiments were fixed in paraformaldehyde for 2 h, washed twice with 1% phosphate-buffered saline solution and stored at -20°C in a 50:50 mixture of 1% phosphate-buffered saline solution and 100% ethanol. Target organisms were

analysed using EUBmix targeting all bacteria (a mixture of EUB338, EUB338II and EUB338III) (Daims et al. 1999) and PAOmix probes (PAO651, PAO462 and PAO846) targeting Accumulibacter–PAOs (Crocetti et al. 2000). For each analysis, a minimum of five images were collected to qualitatively demonstrate the presence of targeted organisms.

3. Results

3.1 EBPR activity of a full-scale MLE system

The effluent phosphorus concentration in the full-scale treatment plant was mostly below 1 mg P/L throughout the two-month weekly monitoring period, with an average of 0.3 ± 0.1 mg P/L, while the average P concentration in the PST effluent was 7.6 ± 0.2 mg P/L. Within this two-month period, the average P removal efficiency was $96.1 \pm 1.7\%$, indicating efficient biological phosphorus removal. In the presence of nitrate supplied by internal recirculation, P release was indeed observed at the start of the anoxic compartment of the sampled treatment train and was immediately followed by P uptake activity (Fig. 1). Statistically significant P uptake was consistently detected ($P=0.002$, $N = 9$) from the beginning to the middle of the anoxic tank. In addition, nitrate depletion occurred in the mid section of the anoxic tank compartment, resulting in an anaerobic zone from the middle to the end of the anoxic compartment. P release activity was also detected under this induced anaerobic condition. In the sequential aerobic compartment, P uptake was detected predominantly at the start to mid-section. P uptake activity was also present in the mid to end section of the aerobic compartment, although to a lower extent, when oxygen and nitrate were both present (Fig. 1). Nitrite was either absent or present at less than 1 mg N/L at all sampling locations.

3.2 Functional PAOs and GAOs in Ulu Pandan Sludge

Microbial community analysis using the 16S rRNA gene amplicon sequencing revealed the presence of both PAOs and GAOs in the investigated activated sludge, with the top 45 most abundant operational taxonomic units (OTUs) summarized in Table S3. *Accumulibacter* was detected as the predominant PAO on both sampling dates with a collective mean read abundance of 3.33% of total bacteria. Another putative PAO, *Tetrasphaera*, was not detected. FISH analysis also confirmed the presence of *Accumulibacter* (Fig. 2). The two main *Accumulibacter*-annotated OTUs were consistently detected on both occasions (July 2014 and March 2015), despite differences in relative abundance. The two OTUs accounted for 4.48% and 0.99%, and 0.91% and 0.26% for July and March, respectively (Table S3).

Figure 1. Average within-tank nutrient profiles obtained from sampling the full-scale Ulu Pandan water reclamation plant, conducted in June and July 2014 (N = 9; error bars refer to SEM). Mixed liquor suspended solids concentrations ranged from 1.0 to 1.8 g/L. Sampling locations were PST, primary settling tank effluent; Anx S, anoxic compartment start phase; Anx M, anoxic mid phase; Anx E, anoxic end phase; Aer S, aerobic compartment start phase; Aer M, aerobic mid phase; and Aer E, aerobic end phase. In some instances, error bars are contained within symbols.

Competibacter was the predominant GAO; it was less abundant than Accumulibacter in July 2014 and more abundant in March 2015. There were a total of 11 OTUs annotated to Competibacter with a collective mean read abundance of 2.7% across the two sampling dates. The two most abundant OTUs annotated to this taxon accounted for 0.70% and 0.68% of the reads (Table S3). In addition to functional PAOs and GAOs, a single OTU each was detected for the ammonia-oxidizing genus *Nitrosomonas* and the nitrite oxidizing genus *Nitrospira* among the top 45 OTUs (Table S3).

In agreement with the 16S rRNA gene amplicon sequencing data, analysis of FISH probe sequences detectable in metagenome reads confirmed that the overall PAO abundance on both sampling dates, as suggested by the total relative abundance corresponding to FISH probes PAO462, PAO652, and PAO846, was less than 7% of total bacteria, with a higher abundance detected in July 2014 (Fig. 3). Despite the difference in relative abundance in July and March, Accumulibacter Type II (targeted by probe Acc-II-444) was consistently more

Figure 2. FISH images of Ulu Pandan sludge sample collected in July 2014 (left) and March, 2015 (right) with probes EUBMIX (red) targeting most bacteria and PAOMIX (green) targeting *Accumulibacter* spp.

abundant than *Accumulibacter* Type I (targeted by Acc-I-444) (Fig. 3). Based on *ppkI* primer sequences, multiple strains were detected in Ulu Pandan sludge, with Clade IIC being the most abundant on both sampling dates. In addition, Clade IIB was detected on both sampling dates, clade IIA and IIF were only detected in July, and Clade IID was not detected in either sample. The *ppkI* primer sequence also showed the presence of *Accumulibacter* clade I but at lower levels than clade II primers (Fig. 3).

3.3 The proportion of active non-denitrifying and denitrifying PAOs

The capacity of functional PAOs in Ulu Pandan activated sludge to reduce nitrate was assessed by subjecting freshly collected sludge to anaerobic/anoxic/aerobic cycling. Under anaerobic conditions, P-release activity was detected when acetate (Fig. 4A) or propionate (Fig. 4B) was provided as the external organic carbon source, along with organic carbon consumption and PHA production. The observed P release activity ceased when the carbon source was still present, indicating that the P-release/C-uptake capacity was saturated. The carryover of residual organic carbon from the anaerobic phase resulted in some PHA production in the subsequent anoxic phase, likely by non-PAO heterotrophic organisms. The specific anaerobic P release rate was 27.3 ± 1.9 mg P/ g VSS·h and the acetate uptake rate was 12.6 ± 2.1 mg C/ g VSS·h (Table 1). In the same experiments, the P uptake rates under anoxic condition were significantly lower than those observed under aerobic conditions with a P uptake rate of 4.4 ± 0.4 mg P/ g VSS·h and 14.1 ± 1.2 mg P/ g VSS·h, respectively (Fig. 4A). Despite variations in EBPR activity a higher aerobic than anoxic P uptake rate was consistently observed (Table 1).

When propionate was fed as the organic carbon source, the anaerobic P release rate and propionate uptake rate were lower than when acetate was supplied with an average of 16.0 ± 0.1 mg P/ g·h and 6.6 ± 0.8 mg C/g VSS·h, respectively (Fig. 4B and Table 1). In addition,

there was no significant anoxic P uptake activity even when propionate was depleted (Fig. 4B). P uptake activity was mainly detected under aerobic conditions albeit at a significantly lower rate of 5.7 ± 0.7 mg P/ g VSS·h compared to that with acetate (Fig. 4B and Table 1).

The denitrifying activity of PAOs whenever acetate was the organic C source was confirmed in batch experiments. The fraction of active DPAOs was estimated to be 39-44% of the total PAOs, with the remaining 56-61% of the total PAOs unable to utilize nitrate as electron acceptor. Only 4.7% of the total PAOs were able to denitrify nitrate when propionate was provided as the organic carbon source.

Figure 3. Metagenomic read counts in full-scale sludge corresponding to targets of FISH probes (PAO462, PAO652, PAO846, Acc-I-444 and Acc-II-444) and primers (Acc-IIB-ppK1, ACC-IIC-ppk1, Acc-ppk1-763f, Acc-ppk1-1170r, Acc-ppk1-997r, Acc-ppk1-1002r, Acc-ppk1-460r and Acc-ppk1-600r) for polyphosphate accumulating organisms (PAOs), normalised to the EUB 338 universal probe (See Materials and Methods and Supplementary Table 1). The same PAOs can be targeted by different FISH probes and primers shown here and the overall PAO abundance was below 7% on both sampling dates.

3.4 EBPR activity under defined anoxic/aerobic cycling

EBPR activity was observed in batch experiments under anaerobic/aerobic conditions with acetate (Fig. 5A) or under defined anoxic/aerobic conditions when either acetate or propionate was provided as the organic carbon source, even in the absence of an intermediate anaerobic phase as observed in the field (Fig. 5B and C). When acetate served as the external carbon source, the average specific anoxic P release rate was 47.9 ± 2.3 mg P/g VSS·h (Fig. 5B and Table 1), while the anaerobic P release rate was 37 ± 0.8 mg P/g VSS·h (Fig. 5A and Table 1). The anoxic acetate uptake rate of 45.2 ± 6.1 mg C/g VSS·h was significantly higher than the anaerobic uptake rate (Table 1). Anoxic P release was immediately followed by anoxic P uptake at a rate of 5.4 ± 0.7 mg P/g VSS·h once acetate was depleted, with a concurrent decrease in nitrate concentration. The P uptake rate was similar to that observed in the anaerobic/anoxic/aerobic batch experiment (Table 1), indicating DPAO activity.

Consistent with experiments in Set 1, the majority of P uptake activity was observed during the aerobic phase, while only a fraction of P was taken up by DPAOs during the anoxic phase. The average aerobic P uptake rate of 17.6 ± 1.4 mg P/g VSS·h under anoxic/aerobic cycling was similar to the rate of 19.8 ± 2.3 mg P/g VSS·h under anaerobic/aerobic cycling (Table 1). When acetate was substituted with propionate, P release and uptake activities were also observed (Fig 5C). However, unlike the acetate-fed test reactors, anoxic P-uptake activity was not observed even after propionate was depleted, suggesting that the majority of nitrate reduction activity observed was from non-PAO heterotrophic denitrifiers. Similarly, anoxic/aerobic EBPR was also observed when synthetic wastewater was replaced with primary effluent, despite the lower amount of acetate present (Fig. 5D).

3.5 Comparison between anoxic/aerobic cycling and complete aerobic condition

Set 3 batch experiments were conducted in 2015 when the relative abundance of *Accumulibacter* had decreased, as shown by the metagenomic read counts on FISH probes

Figure 4. Average nutrient profiles ($\pm$ standard error of the mean) in anaerobic/anoxic/ aerobic batch experiments with Ulu Pandan sludge, conducted during June- and July 2014. Reactors were fed with (A) acetate (N = 4) or (B) propionate (N = 2) to determine the proportion of denitrifying and non-denitrifying polyphosphate accumulating organisms. In some instances, error bars are contained within symbols.

PAO462, PAO651 and PAO846 (Fig. 3). Despite the drop in abundance, EBPR still occurred when acetate-fed batch systems were subjected to anaerobic/aerobic (Fig. 6A) or anoxic/aerobic cycling (Fig. 6B). Although P release activity was also observed under complete aerobic conditions (Fig. 6C), the P release rate was significantly lower compared to the anoxic and anaerobic P release rate (Table 1). In addition, the aerobic P uptake activity commenced once acetate had been depleted (Fig. 6C). Consistent with findings reported above, the P release and uptake rates were comparable in both anaerobic/aerobic and anoxic/aerobic cycling experiments (Table 1). However, unlike experiments in Set 2, P uptake activity was not detected under anoxic conditions upon depletion of acetate (Fig. 6B), and despite an increase in nitrate reduction rate (Table 1). Residual EBPR activity was observed in the anaerobic/aerobic reactor when no acetate was added (Fig. 6D). This might be due to the

Table 1. Observed uptake, release and conversion rates for different experimental conditions.

Type of batch study	Date	Organic carbon source	Specific P release rate ¹ (mg P/g VSS·h)			Acetate uptake rate ¹ (mg C/g VSS·h)	Propionate uptake rate ¹ (mg C/g VSS·h)	Specific P uptake rate ¹ (mg P/g VSS·h)		Nitrate reduction rate ¹ (mg N/g VSS·h)	
			Anaerobic	Anoxic	Aerobic			Anoxic	Aerobic	External carbon**	Endogenous carbon***
Set 1											
Anaerobic/anoxic /aerobic	July, 2014	Acetate	27.3 (1.9)	-	-	12.6 (2.1)	-	4.4 (0.4)	14.1 (1.2)	-	10.6 (3.1)
Anaerobic/anoxic /aerobic	July, 2014	Propionate	16.0 (0.05)	-	-	-	6.6 (0.8)	0.21 (0.02)	5.7 (0.7)	-	5.7 (0.1)
Set 2											
Anaerobic/aerobic (Positive control)	June July, 2014	Acetate	37.0 (0.8)	-	-	20.3 (0.6)	-	-	19.8 (2.3)	-	-
Anoxic/aerobic	June, 2014	Acetate	-	47.9 (2.3)	-	45.2 (6.1)	-	5.4 (0.7)	17.6 (1.4)	13.5 (0.9)	3.7 (0.2)
Anoxic/aerobic	June, 2014	Propionate	-	35.7 (0.5)	-	-	24.7 (1.0)	nd*	13.2 (0.5)	9.6 (0.7)	2.9 (1.3)
Anoxic/aerobic	July, 2014	Primary Effluent (1:1 v/v)	-	39.4 (5.6)	-	21.4 (4.8)	-	1.1 (0.3)	5.9 (0.3)	9.5 (1.7)	4.9 (0.8)
Set 3											
Anaerobic/aerobic (Positive control)	March, 2015	Acetate	23.2 (1.2)	-	-	13.1 (0.5)	-	-	10.2 (1.5)	-	-
Anaerobic/aerobic (Negative control)	March, 2015	-	3.1 (0.3)	-	-	-	-	-	5.6 (0.9)	-	-
Anoxic/ aerobic	March, 2015	Acetate	-	23.7 (4.5)	-	36.8 (4.6)	-	nd	12.0	23.3(2.5)	nd
Complete aerobic	March, 2015	Acetate	-	-	9.1	58.8 (8.0)	-	-	5.3	-	-

* Not detected; ** Nitrate reduction rate determined in the presence of volatile fatty acids as external organic carbon source; *** Nitrate reduction rate determined after depletion of volatile fatty acids; **** Not applicable; ¹ Average (± Standard error of mean SEM).

Figure 5. Chemical transformations by Ulu Pandan activated sludge when subjected to anaerobic/aerobic cycling and fed with synthetic wastewater supplemented with acetate (A) or anoxic/aerobic cycling and fed with synthetic wastewater supplemented with acetate (B), propionate (C), or primary effluent at a sludge to wastewater volume ratio of 1:1 (D) in lab-scale reactors. Anoxic conditions in (B), (C), and (D) were achieved by manually adding sodium nitrate solution. Dissolved oxygen concentration under aerobic conditions was maintained at 7 mg O₂/L and pH was controlled at 7 ± 0.1 at all times. Due to lower concentrations of the nutrients in the PST effluent, the scales for y-axes in (D) are different from (A)-(C) to better showcase the activities occurring in the system, especially the occurrence of P release and uptake.

presence of a carbon source other than acetate that was still present in the mixed liquor or from hydrolysis of floc material. The primary effluent profile (Table S1) revealed the presence of

333 ± 88 mg/l COD, while the three main types of VFAs, acetate, propionate and butyrate, were detected at 36 ± 5.4, 9.2 ± 3.1, and 1.3 ± 0.4 mg/l COD, respectively. This suggested the presence of other carbon sources in the primary effluent, some of which may have been consumed by PAOs.

4. Discussion

We have demonstrated using repeated batch experiments that activated sludge from the full-scale Ulu Pandan water reclamation plant (WRP) was capable of performing EBPR under defined anoxic/aerobic cycling. Although glycogen accumulating organisms (GAOs) were observed to outcompete PAOs at temperatures above 25 °C in lab-scale systems (Whang and Park 2006), our previous study showed that this competitive dynamic was not observed at Ulu Pandan despite operational temperatures ranging from 29 to 31 °C (Law et al. 2016). *Accumulibacter* was the only known PAO detected in the sludge. In contrast, *Tetrasphaera* was significantly more prevalent (18-30% relative abundance) than *Accumulibacter* (3-5%) in full-scale treatment plants designed to perform EBPR (Eschenhagen et al. 2003, Nguyen et al. 2011). It is possible that a defined anaerobic zone, which would be unplanned in MLE systems, may be crucial for *Tetrasphaera* that can also grow by fermentation under anaerobic conditions (Kong et al. 2008).

In the present study, *Accumulibacter* PAOs were significantly less abundant (< 7% of the total bacteria) and non-PAO heterotrophic denitrifiers were likely much more abundant. While non-DPAOs are PAOs that cannot utilize nitrate or nitrite, non-PAO denitrifiers are heterotrophic denitrifiers that perform denitrification but do not have any known EBPR capacity. Some known non-PAO denitrifiers related to the genera *Aquaspirillum*, *Azoarcus* and *Thauera*, many of which are able to utilize VFAs as organic carbon source, can make up 17-40% of the biovolume of full-scale activated sludge plants (Thomsen et al. 2007). When fed with primary effluent under anoxic conditions, PAOs in the sludge were still able to release P

Figure 6. Comparison of chemical transformations by Ulu Pandan activated sludge during (A) anaerobic/aerobic (positive control); (B) anoxic/aerobic and (C) fully aerobic cycling, all with acetate as organic carbon source, with (D) anaerobic/aerobic cycling in the absence of added acetate (negative control). Anoxic conditions were achieved by manually adding sodium nitrate solution. Dissolved oxygen concentration under aerobic conditions was maintained at 7 mg O₂/L and pH controlled at 7 ± 0.1 at all times.

and produce PHA despite limited availability of VFAs. This suggests that *Accumulibacter* may have a higher affinity for VFAs than other non-PAO denitrifiers that were present in the sludge. The results contradict previous studies that have shown inhibition of P release by PAOs when nitrate is present and where the carryover of nitrate to anaerobic zones was thought to cause

failure to the EBPR process (Akin and Ugurlu 2004, Kuba et al. 1994, Patel and Nakhla 2006). P release could only take place when nitrate was depleted below 1 mg N/L (Akin and Ugurlu 2004, Patel and Nakhla 2006). In addition, Kuba et al. (1994) estimated that the presence of 15% DPAO in an enriched PAO culture could result in 20-30% deterioration in anaerobic phosphorus release when nitrate is added. In contrast, the presence of up to 47% of DPAOs relative to PAOs did not compromise the P release activity in the presence of nitrate in Ulu Pandan sludge. Consequently, EBPR activities were consistently detected in both sets of lab-scale anoxic/aerobic experiments (Fig. 5B, C, and D; Fig. 6B). EBPR activities were also detected in the anaerobic/aerobic batch experiments (Fig. 5A and 6A).

Based on the observed EBPR activities under anoxic/aerobic conditions experiments, lower gly/VFA and PHA/VFA ratios than those predicted by the PAO glycogen model suggested a higher reliance of PAOs on the TCA cycle. The TCA cycle was also likely utilized in the acetate-fed batch reactors under anaerobic/aerobic cycling, as indicated by the corresponding P/VFA and PHA/VFA ratios that were close to the values predicted by the anaerobic PAO-TCA model (Table 2, Set 2). Involvement of the TCA cycle was further supported by the production of some polyhydroxyvalerate (PHV) along with polyhydroxybutyrate (PHB) when acetate was provided as the sole organic carbon source (Fig. 5A and B). Acetate could be converted to both PHB and PHV through the TCA cycle, while PHB is predicted to be the sole PHA produced from acetate via glycogen (Gly) degradation (Hesselmann et al. 2000, Pereira et al. 1996, Pijuan et al. 2008). Although we did not perform a detailed analysis of the pathways involved in the TCA cycle, it is known that anaerobic operation of the TCA cycle in full or partially could occur via the glyoxylate pathway, a split TCA cycle, or with the involvement of novel cytochrome *b/b6*. (Hesselmann et al. 2000, Louie et al. 2000, Martín et al. 2006, Yagci et al. 2003).

The observed EBPR activity under anoxic/aerobic cycling can be attributed to the non-DPAO fraction in Ulu Pandan sludge, which is unable to utilize nitrate as electron acceptor. Participation of non-DPAOs was evident because EBPR was observed in the propionate-fed anoxic/aerobic batch reactors (Set 2) and DPAOs in the sludge did not use propionate (Set 1). This was supported by the lower EBPR activities, lack of anoxic P uptake, and drastically lower fraction of active DPAOs in the propionate-fed anaerobic/anoxic/aerobic batch systems (Fig. 4B; Table 1, Set 1). Hence the observed anoxic/aerobic EBPR in the propionate-fed reactor suggests the involvement of non-DPAOs. The non-DPAOs in Ulu Pandan sludge are also able to utilize acetate as an organic carbon source (Fig. 4A). When fed with acetate, comparable EBPR activities were detected in the anaerobic/aerobic (Fig. 6A) and anoxic/aerobic (Fig. 6B) Set 3 batch experiments (Table 1, Set 3). This supported our hypothesis that non-DPAOs could perform EBPR under anoxic/aerobic conditions, likely due to their inability to use nitrate. Consequently, non-DPAOs recognize the anoxic condition as pseudo-anaerobic. However, the activities were lower than those detected during June and July 2014 (Table 1, Set 2), which coincided with a lower abundance of *Accumulibacter* PAOs in 2015 (Fig. 3).

Under fully aerobic conditions, both P release and PHA production were observed, indicating that PAOs could also perform EBPR in the presence of oxygen (Fig. 6C). Previous findings by Serafim et al. (2004), Pijuan et al. (2005, 2006), and Guisasola et al. (2004) suggested that PAOs could consume acetate in the presence of oxygen and store them as PHA, breaking down polyphosphate for energy and releasing P (Guisasola et al. 2004, Pijuan et al. 2005, 2006, Serafim et al. 2004). This explains the occurrence of aerobic P release and uptake in our study. Lower P release rates under aerobic than under anaerobic or anoxic conditions might be due to the presence of oxygen, an electron acceptor that can be used by non-DPAOs and DPAOs alike. While non-DPAOs could recognize anoxic conditions as pseudo-anaerobic and perform P release accordingly as we have hypothesized, the presence of available oxygen

Table 2. Comparison of biochemical stoichiometry for Ulu Pandan sludge under a feast phase in the presence of an external organic carbon source under anaerobic, anoxic or aerobic conditions and the subsequent famine phase under aerobic conditions after the depletion of carbon source with predictions using different metabolic models for PAOs and GAOs (values are molar ratios in C- or P-mol).

Experiment or model	Feast ¹			Famine ¹	
	P/VFA	Gly/VFA ^d	PHA ^e /VFA	P/PHA	Gly ^f /PHA
Set 2					
Anaerobic/Aerobic-acetate	0.7 (0.1)	0.2	0.9 (0.2)	1.2 (0.2)	0.4
Anoxic/Aerobic-acetate	0.5 (0.1)	0.2 (0.04)	0.5 (0.04)	1.3 (0.1)	1.0 (0.3)
Anoxic/aerobic-propionate	0.5 (0.02)	0.1	0.3 (0.1)	2.3 (0.03)	1.0
Anoxic/aerobic-primary effluent (1:1 v/v)	0.7 (0.1)	0.1	1.3 (0.3)	0.6 (0.1)	0.7
Set 3					
Anaerobic/Aerobic-acetate	0.8 (0.07)	0.5	0.9 (0.1)	0.6 (0.01)	0.6
Anoxic/Aerobic-acetate	0.2 (0.04)	0.1	0.6 (0.04)	0.5 (0.2)	0.3
Complete Aerobic	0.3(0.2)	0.05	0.5 (0.05)	0.3 (0.1)	0.2
Model					
Anaerobic PAO TCA model-acetate ^a	0.8	0	0.9		
Anaerobic PAO Glycogen model-acetate ^a	0.5	0.5	1.33		
Anaerobic PAO model-propionate ^b	0.4	0.33	1.22		
Aerobic PAO model ^a				0.41	0.42
GAO model ^c	0	1.12	1.86		0.65

^a Smolders et al. (1994); ^b Oehmen et al. (2005b); ^c Zeng et al. (2003)

^d Volatile fatty acids include both acetate and/or propionate.

^e Poly-hydroxyalkanoates; ^f Glycogen;

¹ Average ($\pm$ Standard error of mean SEM)

could prompt non-DPAOs to simultaneously release and take up P. Using oxygen as electron acceptor, non-DPAOs could immediately oxidize the increasingly available PHAs produced from the acetate consumption under aerobic condition, gaining energy to take up P. Consequently, non-DPAOs could perform P uptake and release activities concurrently, which reduced the net aerobic P release rates. Alternatively, lower P release rates under aerobic conditions could also be attributed to PAOs ability to perform oxidative metabolism. As observed by Pijuan et al. (2006), strict aerobic operation will lead to eventual depletion of poly P because it is no longer required as an energy source under completely aerobic conditions.

Besides non-DPAOs, the potential involvement of DPAOs in the anoxic/aerobic EBPR should not be overlooked. In anoxic/aerobic batch experiments (Set 2), we observed anoxic P uptake once acetate had been consumed (Fig. 5B), indicating that DPAOs were actively involved in taking up phosphate once acetate was depleted. It has been reported that under anoxic conditions almost 40% of the required energy for PHB synthesis is generated by polyphosphate degradation (Kuba et al. 1994). Consequently, DPAOs may have released P under anoxic conditions in the present study. While there was a possibility of simultaneous P release and uptake by DPAOs under anoxic conditions since they could potentially utilize nitrate to concurrently oxidize the synthesized PHA and consume P, the observed higher anoxic P release rates (Table 1, Set 2) do not make this explanation plausible. In addition, the potential involvement of the TCA cycle, as observed in the anoxic phase, agrees with a previous report that DPAOs may generate reducing power for PHB synthesis using the TCA cycle under anoxic conditions (Ahn et al. 2002). This would require DPAOs to release more P to generate enough energy for acetate uptake (Lanham et al. 2013a, Law et al. 2016, Zhou et al. 2009), supplying an explanation for the higher P release rate under anoxic conditions compared to anaerobic conditions in the present study. The lower anoxic P/VFA ratio could be due to VFA consumption by heterotrophic denitrifiers, which consume acetate without releasing P.

Throughout the experiments, non-DPAOs were detected at higher proportions than were DPAOs. Previously, the ability to couple P uptake with nitrate reduction is a metabolic trait attributed to Type I *Accumulibacter*, which is identified as a DPAO, whereas non-DPAOs are associated with Type II *Accumulibacter* that are unable to reduce nitrate but can reduce nitrite (Carvalho et al. 2007, Flowers et al. 2009). More recent finding by Skennerton et al. (2015) revealed the presence of the respiratory nitrate reductase gene in *Accumulibacter* Clade IIC, suggesting the ability to utilize nitrate as electron acceptor. However, lack of a complete denitrification pathway in one of the *Accumulibacter* Clade IIC strains analyzed in the study indicated that some Clade IIC *Accumulibacter* taxa might have a lower ability to use either nitrate or nitrite as electron acceptor (Skennerton et al. 2015). Additionally, studies have also suggested that some Type I PAOs are unable to utilize nitrate (Rubio-Rincón et al. 2017, Saad et al. 2016). Instead, this type of PAO likely relies on other organisms, such as *Competibacter* GAOs, to denitrify nitrate to nitrite first.

Our results indicate activities of non-DPAOs, as well as DPAOs, in anoxic/aerobic EBPR. Based on matching the FISH probes (PAO462, PAO652, PAO846, Acc-I-444 or Acc-II-444) and primers (see Materials and Methods and Supplementary Table 1 for details) with the corresponding target sequence in the sludge metagenome, Type II *Accumulibacter* predominate in Ulu Pandan sludge. Only nitrate was used to induce anoxic conditions in these experiments. It has been suggested that different PAO clades vary in metabolic stoichiometry and reaction rates, which will affect P removal efficiency (Welles et al. 2015). The P release rates observed in this study were substantially higher than those previously observed in various temperate wastewater treatment systems ranging from 11.0 to 19.2 mg P/g VSS·h with an estimated *Accumulibacter* PAO population of 18-40% based on qFISH analysis (He et al. 2008). This discrepancy was likely due to the presence of different PAO strains and/or higher operating temperatures in our system leading to higher reaction rates. However, exact

conditions that select for the different PAO clades remain poorly understood. Welles et al. (2015) observed that the influent P concentration determined the type of PAO enrichment, whereas Guerrero et al. (2012) demonstrated that reactor configuration had an impact on Type I and II enrichment. In our study, the non-DPAOs were able to utilize both acetate and propionate as organic carbon source, whereas DPAOs could only use acetate. This may provide Type II PAOs with a competitive advantage over Type I PAOs, resulting in their higher detected levels in Ulu Pandan sludge.

The establishment of DPAOs in a system is also thought to be influenced by the nitrate load entering the anoxic zone (Hu et al. 2002). If the nitrate load exceeds the denitrification potential of ordinary heterotrophic organisms (OHO), DPAOs can then utilize the excess nitrate (Hu et al. 2002). The presence of an anaerobic zone within the anoxic compartment of the plant studied here would suggest that the nitrate load was insufficient to support the activity of both OHO and DPAOs. Further work is needed to verify whether long-term exposure to high concentrations of nitrate under anoxic/aerobic cycling could lead to higher enrichment of DPAOs and how this would affect biological phosphorus removal.

5. Conclusions

From this study, we can conclude that:

- Type II non-denitrifying *Accumulibacter* clades predominated in a tropical full-scale activated sludge plant with MLE configuration. Non-DPAOs were most likely involved in the anoxic/aerobic EBPR activity observed in the full-scale plant and also in lab-scale reactors. This might be due to their inability to utilize nitrate. Thus, under anoxic/aerobic conditions, these PAOs could carry out EBPR as if the conditions were anaerobic/aerobic with comparable P release and uptake rates.

- Type II non-DPAOs are competitive at low concentrations of organic carbon and are capable of utilizing both acetate and propionate, whereas Type I DPAOs are only able to metabolize acetate. This may provide Type II non-DPAOs with an advantage under anoxic/ aerobic cycling.
- Understanding the conditions that select for specific *Accumulibacter* clades and anoxic/aerobic EBPR activity may facilitate improved design features, obviating the need for specified anaerobic zones.

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Supplementary Information

Non-denitrifying polyphosphate accumulating organisms obviate requirement for anaerobic condition

Table S1. Summary of the characteristics of primary effluent of Ulu Pandan Water Reclamation Plant.

Parameters	Concentration ¹
Chemical oxygen demand (mg/L)	333(88)
Total organic carbon (mg/L)	85 (13)
Total P (mg P/L)	7 (2)
Total Kjeldahl nitrogen (mg N/L)	46 (8)
Ammonium (mg N/L)	33 (4)
Acetate (mg/L)	33 (5)
Propionate (mg/L)	6 (2)
Butyrate (mg/L)	0.7 (0.2)
pH	6.7 (0.2)
Solids content (g VSS/L)	1.4 (0.2)
ORP (anoxic tank; mV)	-50 to -150
ORP (aerobic tank; mV)	50 to -50
Dissolved oxygen (mg O ₂ /L)	0.5 to 2.0

¹ Average ($\pm$ standard error of mean, SEM)

Table S2. 16S rRNA FISH probes and defined polyphosphate kinase 1 (ppk1) primer sequences for eFISH analysis. Annotations taken from Nielsen et al. (2009b) and Ong et al. (2014b).

<i>ProbeName</i>	<i>ProbeSequence (5'-3')</i>	<i>ProbeType</i>	<i>ProbeDescription</i>
EUB338	GCTGCCTCCCGTAGGAGT	General	Most Bacteria
EUB338-II	GCAGCCACCCGTAGGTGT	General	Planctomycetales
EUB338-III	GCTGCCACCCGTAGGTGT	General	Verrucomicrobiales
PAO462	CCGTCATCTACWCAGGGTATTAA C	PAO	Most Accumulibacter
PAO651	CCCTCTGCCAAACTCCAG	PAO	Most Accumulibacter
PAO846	GTTAGCTACGGCACTAAAAGG	PAO	Accumulibacter
Acc-I-444	CCCAAGCAATTTCTTCCCC	PAO	Clade IA
Acc-II-444	CCCGTGCAATTTCTTCCCC	PAO	Clade IIA
Acc-IIB-ppk1	GATGACCCAGTTCCTGCTCG	PAO	Clade IIB
Acc-IIC-ppk1	TCACCACCGACGGCAAGAC	PAO	Clade IIC
AccelIF-ppk1	CGAACTCGGCGAAAGCGAGTA	PAO	Clade IIF
Acc-ppk1-763f	GACGAAGAAGCGGTCAAG	PAO	Clade I
Acc-ppk1-1170r	AACGGTCATCTTGATGGC	PAO	Clade I
Acc-ppk1-893f	AGTTCAATCTCACCGAGAGC	PAO	Clade IIA
Acc-ppk1-997r	GGA ACTTCAGGTCGTTGC	PAO	Clade IIA
Acc-ppk1-1002r	CGGCACGAACTTCAGATCG	PAO	Clade IIB
Acc-ppk1-460r	CCGGCATGACTTCGCGGAAG	PAO	Clade IIC
Acc-ppk1-600r	ATCGCCTCCGAGCAACTGTTC	PAO	Clade IIF

Table S3. Mean relative read abundance (%) of the top 45 OTUs detected in activated sludge samples collected in July, 2014 and March, 2015.

OTU identifier	Jul-14	Mar-15	Mean	Annotation
OTU_10	6.72	1.71	4.22	k__Bacteria p__Actinobacteria c__Actinobacteria o__PeM15 f__PeM15 g__PeM15 s__
OTU_8	5.15	0.66	2.90	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Saprospiraceae g__MK04 s__ k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__Candidatus
OTU_12	4.48	0.91	2.70	Accumulibacter s__
OTU_6	1.69	2.96	2.32	k__Bacteria p__Nitrospirae c__Nitrospira o__Nitrospirales f__Nitrospiraceae g__Nitrospira s__sublineage1
OTU_24	1.96	2.50	2.23	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae
OTU_20	0.73	3.18	1.95	k__Bacteria p__Actinobacteria c__Actinobacteria o__Corynebacteriales f__Mycobacteriaceae g__Mycobacterium s__
OTU_4	2.68	1.10	1.89	k__Bacteria p__Chloroflexi c__Chloroflexia o__Chloroflexales f__Roseiflexaceae g__Kouleothrix s__
OTU_7	0.89	2.83	1.86	k__Bacteria p__Bacteroidetes c__Cytophagia o__Cytophagales f__Cytophagaceae g__uncultured s__
OTU_54	2.34	0.71	1.53	k__Bacteria p__Actinobacteria c__Actinobacteria o__Propionibacteriales f__Nocardoidaceae g__Marmoricola s__
OTU_16	1.49	1.56	1.52	k__Bacteria p__Actinobacteria c__Acidimicrobiia o__Acidimicrobiales f__Microthricaceae g__P58 s__
OTU_18	0.98	2.03	1.51	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__Azospira s__
OTU_39	0.86	2.00	1.43	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae g__Tahibacter s__
OTU_11	2.24	0.62	1.43	k__Bacteria p__Chloroflexi c__Chloroflexia o__Chloroflexales f__Roseiflexaceae g__Kouleothrix s__
OTU_15	1.16	1.69	1.43	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Saprospiraceae
OTU_28	1.29	1.45	1.37	k__Bacteria p__Actinobacteria c__Thermoleophilia o__Solirubrobacterales f__480-2 g__K2-78 s__
OTU_45	1.47	1.13	1.30	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Nordellaceae g__MNG7 s__
OTU_32	0.88	1.42	1.15	k__Bacteria p__Spirochaetae c__Spirochaetes o__Spirochaetales f__Leptospiraceae
OTU_29	1.33	0.73	1.03	k__Bacteria p__Chloroflexi c__Anaerolineae o__Anaerolineales f__Anaerolineaceae g__SBR1029 s__
OTU_72	0.99	1.01	1.00	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Chitinophagaceae g__uncultured s__
OTU_30	1.73	0.17	0.95	k__Bacteria p__Actinobacteria c__Actinobacteria o__Propionibacteriales f__Propionibacteriaceae g__B1-K2-141 s__
OTU_61	0.65	1.23	0.94	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae
OTU_36	1.11	0.77	0.94	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__PHOS-HE51 g__ s__
OTU_71	0.90	0.93	0.92	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__188up s__
OTU_25	0.75	1.03	0.89	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Bradyrhizobiaceae
OTU_49	0.28	1.45	0.87	k__Bacteria p__Actinobacteria c__Actinobacteria o__Corynebacteriales f__Mycobacteriaceae g__Mycobacterium s__

OTU_88	0.25	1.47	0.86	k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__Haliangiaceae g__Haliangium s__
OTU_27	0.87	0.80	0.83	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__A0839 g__ s__
OTU_37	1.09	0.56	0.83	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Saprospiraceae g__uncultured s__
OTU_33	0.48	1.15	0.81	k__Bacteria p__Spirochaetae c__Spirochaetes o__Spirochaetales f__Leptospiraceae g__Leptospira s__
OTU_99	0.70	0.86	0.78	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__uncultured s__
OTU_75	0.61	0.88	0.74	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Piscinibacter s__
OTU_43	0.95	0.53	0.74	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodobacterales f__Rhodobacteraceae g__Defluviimonas s__
OTU_1	0.59	0.80	0.70	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Competibacteraceae g__CPB_S18 s__
OTU_74	0.11	1.25	0.68	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Competibacteraceae g__Plasticicumulans s__
OTU_38	1.10	0.25	0.68	k__Bacteria p__Chloroflexi c__Chloroflexia o__Chloroflexales f__Roseiflexaceae g__ s__
OTU_132	0.57	0.77	0.67	k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptostreptococcaceae g__p-55-a5 s__
OTU_138	0.25	1.06	0.66	k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__Haliangiaceae g__Haliangium s__
OTU_66	0.61	0.66	0.63	k__Bacteria p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Saprospiraceae g__QEDR3BF09 s__
OTU_249	0.99	0.26	0.63	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__Candidatus Accumulibacter s__
OTU_93	0.68	0.55	0.61	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Hydrogenophilales f__Hydrogenophilaceae g__uncultured s__
OTU_65	0.58	0.59	0.59	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodospirillales f__Rhodospirillaceae g__Dongia s__
OTU_82	0.55	0.58	0.56	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Acidovorax s__
OTU_97	0.74	0.38	0.56	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__uncultured s__
OTU_90	0.39	0.72	0.56	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Nitrosomonadales f__Nitrosomonadaceae g__Nitrosomonas s__

OTU identifier: internal identifier for operational taxonomic units generated from analysis of 16S rRNA amplicon sequencing reads;

Figure S1. Typical configuration of the aeration tanks at UPWRP-SW, including Tank 2B from which the activated sludge used in this study was collected. PST, Primary Settling Tank; RAS, Returned Activated Sludge; FST, Final Settling Tank (Law et al. 2016).

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Chapter 3

Differential Responses of Non-denitrifying Glycogen and Polyphosphate Accumulating Organisms to Acetate, Nitrate, and Microbially Generated Nitrite at Warm Temperatures

Abstract

Enhanced biological phosphorus removal (EBPR) involves the uptake of phosphorus (P) from wastewater by polyphosphate accumulating organisms (PAOs), in excess of what is needed for microbial growth. Phosphorus is released by cells during anaerobic conditions when carbon (C) is provided and taken up in the absence of readily available C during aerobic or anoxic conditions. Carbon and nitrate/nitrite could be simultaneously present in the anaerobic tank in full-scale treatment plants due to internal recirculation of sludge. Previous works studying the effects of the presence of both acetate and nitrate yielded contradictory results. Additionally, no study has explored how this condition would affect the abundance of glycogen accumulating organisms (GAOs) and the resulting implication on PAOs. To determine how the concurrent presence of acetate, nitrate, and nitrite affected these communities, we investigated the response of non-denitrifying GAOs and PAOs to the simultaneous presence of acetate, nitrate and/or nitrite in a semi-batch system at 30 °C. We operated two replicate sequencing batch reactors at hydraulic and solids retention times (HRT and SRT) of 12 hours and 14 days, respectively. Both reactors were first subjected to anaerobic/aerobic cycling before switching to anoxic/aerobic cycling. The anaerobic/aerobic stage lasted 53 days and the anoxic/aerobic stage 43 days (three SRTs). During studies with anoxic/aerobic cycling, acetate, nitrate, and nitrite were consistently detected at the end of the short feed phase in both reactors. However, they were consumed within the first half of the anoxic phase, creating carbon-depleted anaerobic conditions prior to the aerobic phase.

In both reactors, P release and uptake rates at the beginning of the anoxic/aerobic stage were comparable to the rates observed toward the end of the anaerobic/aerobic stage, suggesting a lack of direct inhibitory effect of the concurrent presence of acetate, nitrate and nitrite on EBPR. A lack of denitrifying PAO activity indicated that P release and uptake were likely due to the activities of PAOs that could not use nitrite and nitrate. This observation supports the notion that planned anaerobic conditions are not strictly required during cycling for EBPR to occur. Activities in both reactors eventually dropped as the study progressed. The abundance of PAOs, especially *Accumulibacter* as the most prominent genus, also decreased under continuous exposure to anoxic/aerobic cycling. This decrease coincided with the proliferation of non-PAO organisms such as GAOs, resulting in reduced availability of carbon sources for PAOs. GAOs belonging to the genus *Defluviicoccus* (cluster 2) exhibited the most noticeable increase in the anoxic/aerobic stage. Since these taxa do not utilize nitrate or nitrite as terminal electron acceptors, they likely recognized the anoxic condition as pseudo-anaerobic and consumed acetate accordingly. Some non-PAO denitrifiers like *Zoogloea* and *Dechloromonas* also increased as the anoxic/aerobic stage progressed, further reducing the carbon availability for PAOs.

In conclusion, the simultaneous presence of acetate, nitrate, and nitrite did not negatively affect the activity of PAOs. However, it enabled the proliferation of GAOs and non-PAO denitrifiers, which competed for the same carbon sources as PAOs. Thus, long term exposure to the simultaneous presence of nitrate and/or nitrite and carbon sources may indirectly lead to reduction of PAOs, namely *Accumulibacter*, that could upset the stability of EBPR. This would be detrimental in full-scale plants where GAOs such as Cluster 2 *Defluviicoccus* are often present.

1. Introduction

Enhanced biological phosphorus removal (EBPR) is an economical and environmentally friendly approach to nutrient removal from wastewater. An EBPR system employs cycles of a carbon-rich “feast” phase, usually under anaerobic conditions, and a carbon-depleted “famine” phase, where electron acceptors like oxygen or nitrate/nitrite are present. This strategy selects for PAOs as well as glycogen accumulating organisms (GAOs), both of which can synthesize and utilize internal storage compounds. PAOs and GAOs that can utilize nitrite and/or nitrate as terminal electron acceptors are classified as denitrifying PAOs (DPAOs) and GAOs (DGAOs), respectively.

Unlike PAOs, GAOs do not take up much P and their presence is considered unfavourable for EBPR, since they can compete with PAOs for available carbon sources (Oehmen et al. 2007, Saunders et al. 2003). Several factors have been reported to affect the competition between PAOs and GAOs, including temperature, with higher temperatures favoring GAOs (Oehmen et al. 2007, Panswad et al. 2003, Whang and Park 2002). However, successful EBPR has been reported in a tropical plant (Law et al. 2016) and a lab-scale enrichment system operated at 28 ± 1 °C (Ong et al. 2013). Additionally, presence of nitrate at levels above 1 mg NO₃-N/L has been reported to inhibit P release (Akin and Ugurlu 2004, Kuba et al. 1994, Patel and Nakhla 2006), while nitrite above a critical concentration limits anoxic and aerobic P uptake (Meinhold et al. 1999, Saito et al. 2004).

The simultaneous presence of electron donors and acceptors such as acetate and nitrate, respectively, can occur in full-scale treatment plants due to the internal recirculation of sludge and did not inhibit EBPR in a laboratory study where PAOs could outcompete heterotrophic denitrifiers for the available volatile fatty acids (Guerrero et al. 2011). Further, PAOs

performed EBPR even under strict anoxic/aerobic conditions at 30 ± 1 °C in batch experiments using freshly sourced sludge from a full-scale plant (Cokro et al. 2017).

To the best of our knowledge, no study has explored the response of GAOs when subjected to the concomitant presence of nitrate and/or nitrite and acetate in a continuous system at higher ambient temperatures such as 30 °C. It is crucial to understand how this condition affects GAOs, because they are believed to compete with PAOs and may dominate PAOs in tropical regions. A better understanding of the dynamics of GAOs versus PAOs under such conditions could help ensure sustainable EBPR as well as shed light on the suitability of the simultaneous addition of NO_x and carbon (C) to improve EBPR at higher temperatures. Therefore, the objectives of this study were to determine (1) the dynamics of GAO and PAO communities in laboratory-scale reactors subjected to the simultaneous presence of NO_x and acetate for an extended period of 43 days (corresponding to three SRTs), (2) whether EBPR is stable under such conditions; and (3) the effect on other organisms, especially non-PAO denitrifiers that could potentially compete for carbon sources under anoxic conditions. We hypothesized that GAOs that could not utilize nitrate and/or nitrite may recognize anoxic conditions as pseudo-anaerobic, enabling these taxa to consume carbon sources and proliferate. We also hypothesized that a fraction of PAOs unable to use NO_x would survive and perform EBPR as was observed in a full-scale plant in Singapore (Law et al. 2016; Cokro et al. 2017).

2. Materials and Methods

2.1 Laboratory scale enrichment reactors

A double-jacketed sequencing batch reactor (R0) with a working volume of 5.4 L was inoculated on Day 0 with freshly collected return activated sludge (RAS) from a full-scale plant performing EBPR (Law et al. 2016) and operated at an ambient water temperature ranging from 29 to 31 °C. R0 was subjected to 6-h anaerobic/aerobic cycles consisting of the following

phases: 130 min anaerobic (including 5 min of feeding); 160 min aerobic; 1 min sludge discharge; 30 min settling; and 39 min of supernatant decant. Higher P concentrations at the end of the anaerobic stage relative to the start of the cycle and lower P concentrations at the end of subsequent aerobic stage suggested that EBPR was occurring consistently (Figure S1).

After 179 days the sludge was split evenly to inoculate two reactors, RA and RC. These were subjected to anaerobic/aerobic cycling for an additional 117 days before the cycling was switched to anoxic/aerobic conditions by the concurrent addition of nitrate and acetate (Stage IV). To create the anoxic condition, we added sodium nitrate in the feed. Like R0, these two reactors were subjected to the same 6-h cycles, but the anaerobic phase was replaced by an anoxic phase on Day 296. Hydraulic retention time (HRT) and sludge retention time (SRT) were maintained at 12 h and 14 d, respectively. These parameters were not reflective of the operating conditions at the source plant. Instead, longer SRT was selected to minimize the risk of PAO washout since source reactor R0 was inoculated using a 1:1 (v:v) sludge-to-feed ratio. HRT of 12 h was selected to capitalize on the reactor's design with minimal retrofitting necessary, since the supernatant discharge point was located in the middle of the reactors. This HRT did not adversely impact EBPR in the reactors. Additionally, HRT ranging from 10 h (Ong et al. 2014) to 24 h (Lu et al. 2006) have been used in previous studies using lab-scale enrichment reactors. Temperature, dissolved oxygen, and pH of the reactor were set at 30 ± 1 °C, 2.0 ± 0.1 mg/l, and 7.25 ± 0.25 , respectively, by addition of 0.5 M HCl or 0.5 M NaOH, as necessary. Synthetic wastewater was prepared following the recipe of Lu et al. (2006) with acetate as the external carbon source .

Several changes in the operating conditions were made to increase phosphorus removal in RA and RC (Table 1). To avoid bias that might have been caused by these changes, we only considered operational days from Day 239 onward for this study. During this period no other change but the cycling mode was applied to RA and RC. Both reactors were operated under

anaerobic/aerobic cycling on operational days 239 to 295. These operational days will be referred to as Stage III. The cycling mode was then switched to anoxic/aerobic from Day 296 to Day 339 and named Stage IV. The synthetic wastewater contained 40 mg PO₄-P/L, and the COD: P ratio was 18:1. Acetate was separated from non-acetate components in the feed, and mixing only occurred when the feed entered the reactors. Anoxic conditions were provided by adding sodium nitrate, yielding a nitrate concentration of 30 mg NO₃-N/L in the feed.

2.2. Sample collection

Cycle studies were regularly conducted where samples for physicochemical and microbial community analysis were collected. In every cycle study, nutrient samples were collected at six different sampling points at the beginning of the cycle ($t = 0$ min); end of feeding ($t = 5$ min); middle and end of anaerobic or anoxic ($t = 65$ and 130 min, respectively); and middle and end of aerobic ($t = 210$ and 290 min, respectively). For cycle studies conducted on Day 290 and Day 297, nutrient samples were collected at the beginning and end of the feeding regime, followed by a 10-min interval sampling until the end of the aerobic phase. Nutrient samples were immediately filtered upon collection using sterile 0.2- μ m Acrodisc® syringe filters with a Supor® membrane (Pall Life Science, USA). For most studies, solids samples were collected at the beginning and end of the cycle, while DNA samples were collected at the beginning of the cycle. DNA samples were immediately frozen in liquid nitrogen prior to storage at -80 °C.

2.3 Physicochemical analysis

Concentrations of anions (phosphate, nitrate, and nitrite) and acetate in the filtered nutrient samples were tested using Ion Chromatography and Gas Chromatography (Shimadzu, Prominence), respectively. Solids samples were analyzed using standard methods (APHA 1995). Unfiltered PHA/glycogen samples were fixed with 10% formaldehyde,

Table 1. Conditions applied to replicate reactors RA and RC after their inoculation on Day 179. The reactors were operated with anaerobic/aerobic cycling in Stages I, II, and III. This study used data from Stages III and IV (Day 242 onward).

Stage	Change applied	Day ^a	COD:P (mg/mg)
I	Sludge from reactor R0 was split to inoculate RA and RC ^{1,2}	179	20:1 ^b
II	COD:P ratio in RA and RC was increased to 25:1	216-235	25:1 ^b
	COD:P ratio changed to 18:1.	235	18:1 ^c
	Sludge from RA and RC was combined and re-distributed to aid reproducibility in reactors.	239	18:1 ^c
III	Anaerobic/aerobic cycling was maintained. No additional changes were applied.	239-296	18:1 ^c
IV	Anaerobic/aerobic cycling was replaced with simultaneous N and C/aerobic cycling. Other operating conditions described in Stage III remained unchanged.	296-339	18:1 ^c

¹ Each reactor received approximately 50% sludge from R0.

² Sludge discharge was disabled on Days 179 to 182 (10 cycles) to compensate for the mixed liquor reduction following the split of R0, and again on Days 199-200 (3 cycles).

^a The day when each change was applied, as counted from the day R0 was inoculated (Day 0). Some changes were maintained once applied.

^b Acetate and non-acetate components of the feed were mixed during preparation.

^c Acetate and non-acetate feed were prepared separately and only mixed immediately before the feed entered the system.

washed, and stored at -80 °C prior to freeze drying. PHA samples were extracted following the method described by Lanham et al. (2013b), while glycogen samples were prepared following the method reported by Lanham et al. (2012). Details on the materials and methods used for these measurements were elaborated in Chapter 2, subsection **2.3. Chemical Analysis** (pp. 60-61). Stoichiometric properties of RA and RC under feast phases were compared to reported PAO anaerobic metabolism models to determine whether the tricarboxylic acid (TCA) cycle (Comeau et al. 1986), glycolysis (Smolders et al. 1994), or both (Pereira et al. 1996) were utilized to generate the reducing equivalents necessary for PHA synthesis.

2.4 DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from sludge samples using the FastDNATM 2 mL SPIN Kit for Soil (MP Biomedicals, USA) following a method that was optimized for activated sludge samples (Albertsen et al. 2015). Details were available in Chapter 2, subsections **2.5. DNA extraction and sequencing** and **2.6. Processing and analyses of amplicon sequencing data** (pp. 62-64) and in Supplementary Information (Appendix 1).

2.5 Statistical analysis

Multivariate analysis of the microbial community at either genera or species level was done using PRIMER 6 software with PERMANOVA+ (Clarke and Gorley 2006) to determine if the switch in condition led to a significant change in microbial communities, especially the GAO, PAO, and other non-PAO denitrifier populations. To better capture the trends in each of these communities, the data were standardized based on the total reads of OTUs or genera belonging to each bacterial group (GAOs, PAO, or denitrifiers) per sample, and the standardized data were square-root transformed to minimize the effects of the dominant species. A Bray-Curtis similarity matrix was then constructed. We used permutational multivariate analysis of variance (PERMANOVA) to check the null hypotheses that the communities in Stages III and IV were similar (Anderson and Walsh 2013). Homogeneity of variance in the two groups of samples was determined using PERMDISP analysis (Anderson 2006). For the aforementioned multivariate analysis, the number of permutations was set to 9999. Operating condition (stage) was used as a fixed factor.

In addition to the multivariate analysis, we also performed Wilcoxon signed-rank test to determine if the average abundance of selected genera, with respect to all bacteria changed significantly when we switched the operating condition. This test was generated using Real Statistics Resource Pack software (Release 4.3; Copyright (2013-2015) by Charles Zaiontz (<http://www.real-statistics.com>)).

3. Results

3.1. EBPR performance under anaerobic/aerobic cycling

During the anaerobic/aerobic cycling stages (I-III), P release and subsequent uptake were consistently detected in both reactors, RA and RC (Fig.1A for RA and Fig.1B for RC). Other phenotypes of EBPR, such as anaerobic PHA production with acetate and glycogen consumption followed by aerobic glycogen production and PHA utilization could be observed, as evident from the nutrient profiles on Day 290 for RA (Fig. 2A) and RC (Fig.2B). These trends confirmed that the microbial communities in RA and RC were capable of performing EBPR under typical anaerobic/aerobic cycling. Analysis of the stoichiometric properties of both reactors in Stage III revealed the molar ratios of P released per acetate consumed, glycogen utilized for every acetate consumed, and PHA produced relative to the amount of acetate consumed of 0.07 ± 0.01 (average $\pm$ standard error of the mean SEM), 0.7 ± 0.1 , and 1.4 ± 0.3 for RA; and 0.1 ± 0.02 , 0.8 ± 0.1 , and 1.02 ± 0.08 for RC, respectively (Table 2; Supplementary Table S2).

3.2. EBPR performance when NO_x and acetate were present simultaneously (Stage IV)

Anaerobic/aerobic cycling was replaced with anoxic/aerobic cycling from Day 296 to Day 339, and this phase was named Stage IV. In the cycle studies conducted throughout Stage IV, simultaneous presence of acetate and NO_x were consistently detected in reactors at the end of the feed phase (5 min from the start of the cycle), with nitrate concentrations ranging from 0.6-11.0 mg NO₃-N/L and 3.9-12.0 mg NO₃-N/L in RA and RC, respectively. Addition of nitrate in Stage IV led to the accumulation of nitrite in RA (Fig. 2C) and RC (Fig. 2D). Nitrite amounting to 6.0-11.5 mg NO₂-N/L in RA and 2.9 – 8.7 mg NO₂-N/L in RC was also detected at the end of the feed phase. The unintended nitrite accumulation was likely due to the slower nitrite denitrification rates in our reactors. Denitrification rates for nitrite were 1.7 mg N/gr VSS·h in RA and 2.9 mg N/gr VSS·h in RC, while nitrate consumption rates were detected

Figure 1. Phosphorus concentrations, in mg P/L, in the filtered nutrient samples collected during cycle studies in Stages I- IV. The COD:P ratio in reactors RA and RC was xxx in Stage I and xxx in Stage II, III and IV. Each graph contained data collected at the beginning of the stage, end of anaerobic, and end of aerobic in (A) Reactor RA, and (B) Reactor RC. Reactors were subjected to continuous anaerobic/aerobic cycling in Stages I-III and anoxic/aerobic cycling in Stage IV.

Figure 2. Nutrient profiles from cycle study conducted toward the end of Stage III (Day 290) in RA (A) and RC (B). Graph (C) referred to nutrient profile observed in RA after 24-hour of Stage IV (Day 297) while graph (D) referred to the profile in RC. Dashed lines represented the point when aeration began, 130 minutes after each cycle started.

Table 2. Stoichiometric values for RA and RC during the earlier phase (Day 246) and at the end (Day 295) of Stage III. Data for Stage IV included the values observed at the beginning (Day 297) and toward the end of this stage (Day 331). Glycogen and PHA samples were not collected in the last two cycle studies (Days 337 and 339). Average values $\pm$ SEM for each property in each stage were included. Values were in molar ratios (moles C or P).

RA/RC	Stage (Day)	FEAST		FAMINE		
		P released/acetate consumed	Glycogen consumed/acetate consumed	PHA produced/acetate consumed	P uptake/PHA consumed	Glycogen produced/PHA consumed
RA	Stage III (246)	0.1	0.6	0.8	0.2	0.5
	Stage III (295)	0.05	1.2	2.1	0.03	0.5
	Average ^a	0.07 (0.01)	0.7 (0.1)	1.4 (0.3)	0.1 (0.03)	0.6 (0.05)
	Stage IV (296)	0.06	0.7	1.04	0.06	0.4
	Stage IV (331)	0.03	0.9	0.9	0.05	0.8
	Average ^a	0.04 (0.004)	0.5 (0.1)	1.2 (0.2)	0.06 (0.01)	0.5 (0.1)
RC	Stage III (246)	0.1	0.7	0.8	0.3	0.5
	Stage III (295)	0.2	1.2	0.8	0.3	0.8
	Average ^a	0.1 (0.02)	0.8 (0.1)	1.02 (0.08)	0.2 (0.02)	0.6 (0.04)
	Stage IV (296)	0.3	0.7	0.7	0.4	0.9
	Stage IV (331)	0.07	0.2	0.4	0.3	0.6
	Average ^a	0.1 (0.03)	0.5 (0.07)	0.8 (0.2)	0.3 (0.05)	0.9 (0.1)
Model	PAO TCA+Glycogen ¹	0.16	0.7	1.48		0.44
	PAO TCA ²	0.5	0	0.9		
	PAO glycogen ³	0.5	0.5	1.33		
	Aerobic PAO ⁴				0.41	0.42
	GAO ⁵	0	1.12	1.86	0	0.65

¹ Pereira et al. (1996); ² Comeau et al. (1986); ^{3,4} Smolders et al. (1994); ⁵ Zeng et al. (2003); ^a Average ($\pm$ standard error of mean SEM).

at 6.4 mg N/g VSS·h and 4.8 mg N/g VSS·h for RA and RC, respectively (Table 3).

Although the duration of the anoxic phase was supposed to be 130 min, nitrate and nitrite in both reactors were depleted within the first 65 min of the cycle, except for RA on Days 296 and 297 when nitrite was depleted within 130 min of the planned anoxic phase. Additionally, acetate was also consumed within the first 65 min, creating an acetate-depleted anaerobic condition for the remainder of the intended anoxic phase. Regardless, acetate, nitrate, and the produced nitrite were simultaneously detected at the end of the feed phase before they were subsequently removed as the anoxic phase progressed.

Throughout Stage IV, typical EBPR features, namely, P release followed by P uptake, were detected in all cycle studies for RA (Fig. 1A) and in most studies for RC (Fig. 1B). Acetate consumption, as well as production and subsequent consumption of PHAs and glycogen were also observed in RA (Fig. 2C) and RC (Fig. 2D), confirming EBPR. Comparisons of activities detected on Day 290 (near the end of Stage III) and on Day 297 (one day after Stage IV commenced) yielded comparable P release and uptake rates (Table 3). On Day 297, following the 24-h exposure (that is, after four cycles) to planned anoxic/aerobic conditions, the specific P release rates detected in RA and RC were 12.5 mg P/g VSS·h and 50.0 mg P/g VSS·h, while in the subsequent aerobic condition, the specific P uptake rates were 2.6 mg P/g VSS·h in RA and 16.1 mg P/g VSS·h in RC (Table 3). The comparable P release and uptake rates suggested that during the earlier phase of Stage IV, EBPR activities were not impaired by the concomitant presence of acetate, nitrate and the produced nitrite (Table 3). Acetate consumption rates were 94.4 mg C/g VSS·h in RA and 106.1 mg C/g VSS·h in RC.

While direct inhibition on P release or uptake activities was not observed, as Stage IV progressed, net positive P removal was lost in RA in the last two cycle studies conducted on Days 337 and 339, even though P release and uptake still occurred (Fig.1A). EBPR was no

longer observed at the end of Stage IV for RC (Fig.1B). No apparent change in condition was observed prior to the failure. Diminishing EBPR activities in Stage IV were also evident by the generally lower amount of P release per gram of biomass (Fig. 3, Table S1) and P released/acetate consumed, in mole P/mole C in both reactors (Table 2, Fig.3, Table S2). The calculated P released/acetate consumed, glycogen/acetate consumed, and PHA produced/acetate consumed ratios (moles P or moles C) in Stage IV were 0.04 ± 0.004 , 0.5 ± 0.1 , 1.2 ± 0.2 for RA; and 0.1 ± 0.03 , 0.5 ± 0.07 , and 0.8 ± 0.2 for RC (Table 2, Supplementary Table S2).

3.3 Replicability of trends in PAO and GAO abundance in reactors RA and RC

Prior to microbial community analysis, we established whether the overall trends in GAO and PAO communities in each stage were replicated in both reactors. This was done by using multivariate analysis to compare the relative abundance of GAO or PAO populations in RA to these communities in RC. We calculated the relative abundance of the different PAO and GAO genera with respect to their own communities instead of the whole bacterial population detected. This was done to better capture the trends exhibited by these genera. GAO communities in RA and RC were not significantly different in Stage III even though they displayed a more heterogeneous dispersion (PERMDISP P-value = 0.0002). In Stage IV, GAO communities in both reactors were also statistically similar. Similarity of GAOs in RA and RC in Stage IV was reinforced by a higher PERMDISP P-value, which suggested a homogenous dispersion in the two communities (Table S3). Likewise, PAO communities in RA and RC did not significantly diverged from each other in Stage III and Stage IV, as suggested by higher PERMANOVA and PERMDISP P-values (Table S3). These observations were further supported by the nMDS plot which showed the lack of clear separation between the GAOs in the two reactors (Fig. S2A, B). A similar trend was also observed in the nMDS plot for PAOs (Fig. S2C, D). Hence the overall trends of GAOs and PAOs in RA were reproducible in RC.

Table 3. Uptake, release and denitrification rates in reactors RA and RC¹.

Day	Reactor	Anaerobic (or anoxic) specific P release rate (mg P/g VSS·h)	Aerobic specific P uptake rate (mg P/gVSS.h)	Specific acetate uptake rate (mg C/g VSS.h)	Nitrate denitrification rate (mg N/g VSS.h)	Nitrite denitrification rate (mg N/g VSS.h)
290	RA	17.1	2.5	89.6	NA	NA
	RC	56.3	15.7	97.1	NA	NA
297	RA	12.5	2.6	94.4	6.4	1.7
	RC	50.0	16.1	106.1	4.8	2.9

¹ These values were obtained on Day 290 as Stage III was ending, and on Day 297, one day after Stage IV was implemented. P release rates were calculated using the 20 min with the steepest P release slopes (4 data points), while aerobic P uptake rates were obtained using the steepest portion of the decreasing aerobic P uptake slopes. Acetate uptake rates were calculated using the amount of acetate added as the initial concentration instead of the highest detected value in the reactors at the end of feeding. This was due to the possibility that the acetate was taken up as soon as the feed entered the mixed liquor, which could lead to underestimation of the total acetate consumed. Acetate consumption rate calculations also took into account the steepest part of the linear acetate consumption slope.

Figure 3. Total amount of P released and consumed, in mg P/gr volatile suspended solids, throughout Stages III and IV (except for Days 337 and 339), and the amount of P released per mole of acetate consumed (moles P/moles C) for RA (A) and RC (B). No solid samples were collected on Days 337 and 339. Total P released was the difference in P concentrations at the end of anaerobic (or anoxic) and start of the cycle and normalized by the amount of biomass (VSS); while the amount of P uptake was calculated by subtracting the P concentration at the end of anaerobic (or anoxic) with the concentration at the end of aerobic phase, normalized by amount of VSS.

3.4 Abundance of GAOs and PAOs in the presence of acetate, nitrate, and nitrite

Once the reproducibility of overall trends for GAOs and PAOs in the two reactors had been established, we analysed the changes observed in the GAO and PAO populations during the transition from Stage III to Stage IV. The average relative abundance of all PAOs in Stage III was 3.3 ± 1.1 % of all bacteria (mean $\pm$ standard error of the means) in RA and 14.2 ± 4.0 % in RC. These abundances dropped to 1.9 ± 0.9 % for RA ($p = 0.2$; Wilcoxon signed rank test) and 6.3 ± 2.5 % for RC ($p = 1$; Wilcoxon signed rank test) in Stage IV (Table 4). Higher abundance of PAOs were detected in RC, which corroborated the higher activities observed in RC compared to RA. The decrease in PAO abundance in both reactors correlated with an increase in the abundance of competing organisms, such as certain GAO genera in both reactors as will be elaborated later, although the average abundance of GAOs with respect to overall community decreased in RC (Table 4, Fig. S3). In Stage III, GAOs made up on average 18.4 ± 9.1 % of the bacterial community in RA and 31.8 ± 7.2 % in RC. In Stage IV, the relative total GAO abundance in RA increased to 26.1 ± 6.6 % (Wilcoxon signed rank test p -value = 0.8; $N = 8$). In contrast, GAO abundance in RC decreased to 18.4 ± 7.6 % (Wilcoxon signed rank test p -value = 0.3, $N = 8$) (Table 4). The contrasting changes in the relative abundances of GAOs with respect to all bacteria might be related to changes in other bacteria not included in the analyses, which potentially affected the relative abundances of GAOs.

To further determine whether anoxic/aerobic cycling in Stage IV had any effect on GAOs and/or PAOs, we investigated their dynamics following the change in operating conditions. For this analysis, we focused on genera that exhibited replicable trends in both reactors. This was done to minimize the risks of overestimating trends exhibited by some genera that might not be caused by the switch in the cycling conditions since we did not have a control reactor where anaerobic/aerobic cycling was maintained.

Within the GAO community in Stage IV, *Defluviicoccus* cluster 2 increased the most, suggesting that this genus was affected by the simultaneous presence of the three compounds (Fig. 4A and B). In Stage III, the average relative abundance of all OTUs assigned to this genus was 0.4 ± 0.2 % of all bacteria in RA and 1.3 ± 0.5 % in RC. Their abundances increased to 11.2 ± 2.8 % (Wilcoxon signed-rank test p-value = 0.02; N = 8) and 6.0 ± 3.3 % (Wilcoxon signed-rank test p-value = 0.3; N = 8) for RA and RC, respectively, during Stage IV (Table 4). In addition to *Defluviicoccus* cluster 2, relative abundances of genera *CPB_S60* (Wilcoxon signed-rank test P values = 0.4 or 0.9 for RA or RC) and putative GAOs *Plasticicumulans* (Wilcoxon signed-rank test P-values = 0.04 or 0.1 for RA or RC) also increased in Stage IV for both RA and RC, suggesting that these genera benefited from the simultaneous presence of acetate and NO_x observed in the first half of the intended anoxic phase in Stage IV (Table 4). In contrast, during Stage III, the average abundance of the total *Accumulibacter* PAOs with respect to all bacteria was 3.3 ± 1.1 % and 14.2 ± 4.0 % in RA and RC, respectively (Table 4), decreasing to 1.8 ± 0.9 % in RA (Wilcoxon signed rank test P-value = 0.2) and 6.2 ± 2.5 % in RC (Wilcoxon signed rank test P-value = 0.1) in Stage IV (Table 4). Other PAO genera, such as *Candidatus* *Obscuribacter*, *Gemmatimonas*, and *Tetrasphaera* were detected at low abundances (< 0.5% of all bacteria in Stages III and IV).

We also analysed GAO and PAO genera with respect to the overall GAO or PAO community only. This was done to confirm whether the switch in the operating condition did indeed affect these communities. PERMANOVA analyses of the GAO community showed that the GAOs in Stage IV differed from the GAOs in Stage III confirming possible effects of the simultaneous presence of NO_x and acetate on the GAO population in Stage IV (Table S4). This notion was further supported by the PERMIDSP p-value which showed that dispersion in GAO communities in the two phases was homogenous. Hence, we could deduce that the differences between the GAOs in the two stages were caused by the changes in the cycling condition.

Table 4. Average total relative abundances of GAOs and PAOs for the reactors RA and RC in Stages III and IV. Average abundances for *Defluviicoccus* cluster 2, *CPB_S60*, and *Plasticicumulans* GAOs and *Accumulibacter* in Stages III and IV were also included. *Defluviicoccus* cluster 2 exhibited the most noticeable increase in both reactors throughout Stage IV. *Accumulibacter* were the most abundant PAOs detected in the systems. Relative abundances were % abundances with respect to all of the detected bacteria in RA or RC. Low abundance *Tetrasphaera* PAOs were detected throughout the study (< 0.5 % of all bacteria). Average values are presented with SEM.

Reactor	Stage	Relative abundance ¹ (%)						
		Total GAOs	Total PAOs	Total <i>Tetrasphaera</i>	<i>Defluviicoccus</i> cluster 2	<i>Plasticicumulans</i>	<i>CPB_S60</i>	<i>Accumulibacter</i>
RA	Stage III	18.4 (9.1)	3.3 (1.1)	0.01 (0.005)	0.4 (0.2)	0.09 (0.09)	1.7 (1.1)	3.3 (1.1)
RA	Stage IV	26.1 (6.6)	1.9 (0.9)	0.006 (0.004)	11.2 (2.8)	1.8 (0.7)	5.1 (2.0)	1.8 (0.9)
RC	Stage III	31.8 (7.2)	14.2 (4.0)	0.01 (0.005)	1.3 (0.5)	0.05 (0.02)	3.5 (1.0)	14.2 (4.0)
RC	Stage IV	18.4 (7.6)	6.3 (2.5)	0.008 (0.005)	6.0 (3.3)	1.0 (0.4)	4.1 (2.2)	6.2 (2.5)

¹ Average relative abundance compared to all bacteria ($\pm$ SEM)

Figure 4. Average relative abundances of GAO genera detected in Stage III and IV for RA (A) and RC (B), as well as PAO genera in both stages for RA (C) and RC (D). Relative abundances for each genus was calculated with respect to all bacteria. Error bars represented the standard error of means, and sometimes contained within symbol.

Multivariate statistical analysis of PAO genera with respect to its own group revealed not significantly affect the abundance of PAOs (Table S4). Although PERMANOVA analysis yielded a significant p-value for both RA and RC, the PERMDISP analysis revealed heterogeneous dispersions between the PAO communities in Stage III versus Stage IV in RA and RC (Table S4), suggesting that the differences observed were not caused by the change in condition from Stage III to Stage IV.

3.5. Abundance of other bacteria during simultaneous presence of acetate, nitrate, and nitrite

Besides GAOs and PAOs, we also examined how other groups of bacteria might be affected by switching the cycling conditions in Stage IV.

3.5.1. Changes of non-PAO denitrifiers in Stage IV

Due to the potential benefit of the simultaneous presence of acetate and NO_x for non-PAO denitrifiers, we extended our analysis to several known genera of denitrifiers, namely, *Rhodospirillum rubrum*, *Haliangium*, *Azoarcus*, *Dechloromonas*, *Sulfuritalea*, *Thauera*, *Zoogloea*, *Curvibacter*, and *Thermomonas*. The effects of prolonged exposure to anoxic/aerobic cycling in Stage IV on overall non-PAO denitrifier genera were not significant in RC as suggested by PERMANOVA p-value (Table S4). While the PERMANOVA p-value suggested a statistically significant difference in RA, PERMDISP analysis yielded a p-value that was less than 0.05, indicating that the observed significance was likely due to internal variation in the different sample groups rather than the change in conditions. Although overall non-PAO denitrifier abundance did not change significantly when reactors were switched to anoxic/aerobic cycling, the genera *Dechloromonas* and *Zoogloea* increased noticeably in both RA (Fig. 5A) and RC (Fig. 5B). Relative to all bacteria, *Dechloromonas* increased in RA and RC from 0.02 ± 0.007 % and 0.02 ± 0.007 % in Stage III to 0.7 ± 0.3 % (Wilcoxon signed rank P-value = 0.008) and

2.4 ± 1.6% (Wilcoxon signed rank P-value = 0.02), respectively, in Stage IV. *Zoogloea* in RA surged from 0.008 ± 0.004 % in Stage III to 1.1 ± 0.4 % (Wilcoxon signed rank P-value = 0.02) in Stage IV, while in RC, this genus increased from 0.01 ± 0.004 % in Stage III to 0.6 ± 0.2 % (Wilcoxon signed rank P-value = 0.008) in Stage IV (Table 5). The other denitrifiers, namely *Azoarcus*, *Curvibacter*, *Haliangium*, and *Thauera* also increased in Stage IV; however, their relative abundances were low (< 0.5% of all bacteria).

3.5.2. Other genera increasing in the presence of acetate and NO_x

Additionally, we also screened for other organisms whose abundances increased following the prolonged exposure to the simultaneous presence of acetate and NO_x. This because these organisms could benefit from the presence of anoxic/aerobic condition; hence, they could be denitrifiers whose proliferation potentially increased carbon competition for PAOs in the anoxic/aerobic systems. Of all the detected genera, *Azospira*, *K2-30-37*, and *MK04* increased in Stage IV. Relative abundance of *Azospira* increased in both reactors in Stage IV. In RA, this genus increased from 0.2 ± 0.09 % of all bacteria in Stage III to 5.1 ± 1.5 % (Wilcoxon signed rank P-value = 0.02) in Stage IV (Table 5). The abundance of this genus in RC increased from 0.3 ± 0.07 % of all bacteria in Stage III to 3.3 ± 1.0 % (Wilcoxon signed rank P-value = 0.08) in Stage IV. Genus *MK04* in RA increased from 0.01 ± 0.008 % of all bacteria to 1.8 ± 0.7 % (Wilcoxon signed rank P-value = 0.008), while it increased from 0.04 ± 0.02 % of all bacteria in Stage III to 2.4 ± 1.1 % (Wilcoxon signed rank P-value = 0.02) in Stage IV for RC. Additionally, genus *K2-30-37* also increased in both reactors, where abundance in RA increased from 0.2 ± 0.1 % to 0.9 ± 0.3 % of all bacteria (Wilcoxon signed rank P-value = 0.02) during Stage IV. In RC, the relative abundance for this genus increased from 0.08 ± 0.06 % to 1.4 ± 1.0 % of all bacteria (Wilcoxon signed rank P-value = 0.008) during this period (Table 5). Overall, the simultaneous presence of nitrate, nitrite, and acetate

Figure 5. Average relative abundances of non-PAO denitrifier genera in Stage III and IV for RA (A) and RC (B). Relative abundances were calculated with respect to all bacteria. Error bars represented the SEM and sometimes contained within symbols.

Table 5. Relative abundances of non-PAO denitrifiers, especially genera that increased in Stage IV, and other genera that might benefit from the simultaneous presence of acetate, nitrate and nitrite. Relative abundances were calculated with respect to all bacteria. Average values were presented with standard error of mean (SEM).

Reactor	Stage	Relative abundances ¹ (%)					
		Non-PAO denitrifiers				Others	
		Total denitrifiers	<i>Dechloromonas</i>	<i>Zoogloea</i>	<i>Azospira</i>	<i>MK04</i>	<i>K2-30-37</i>
RA	III	0.08 (0.02)	0.02 (0.007)	0.008 (0.004)	0.2 (0.09)	0.01 (0.008)	0.2 (0.1)
RA	IV	2.2 (0.8)	0.7 (0.3)	1.1 (0.4)	5.1 (1.5)	1.8 (0.7)	0.9 (0.3)
RC	III	0.07 (0.02)	0.02 (0.007)	0.01 (0.004)	0.3 (0.07)	0.04 (0.02)	0.08 (0.06)
RC	IV	3.3 (1.5)	2.4 (1.6)	0.6 (0.2)	3.3 (1.0)	2.4 (1.1)	1.4 (1.0)

¹ Average relative abundance compared to all bacteria ($\pm$ SEM)

led to an increase in other non-PAO organisms, which could compete with PAOs for the available carbon sources.

4. Discussion

In full-scale treatment plants, acetate and nitrate can be present at the same time due to the internal recirculation of sludge from the aerobic to the anaerobic zone. Previous studies have reported conflicting results about the effects of the concomitant presence of acetate and nitrate on the overall performance of an EBPR system (Cokro et al. 2017, Guerrero et al. 2011, Kuba et al. 1994, Patel and Nakhla 2006).

Earlier in Stage IV, only a slight decrease in specific P release rates was detected when P release and uptake rates were compared to those detected toward the end of Stage III. This suggested that EBPR was not directly impaired when acetate and nitrate were present at the same time, even though nitrite inadvertently accumulated. P release and uptake activities in RA and RC were not inhibited, as indicated by the calculated rates in RA and RC at the beginning of Stage IV, despite the exposure to more than 1 mg/L $\text{NO}_3\text{-N/L}$ of nitrate and more than 8 mg N/L of nitrite. This was in contrast to previous studies that reported inhibition of P release in the presence of more than 1 mg $\text{NO}_3\text{-N/L}$ of nitrate (Akin and Ugurlu 2004, Kuba et al. 1994, Patel and Nakhla 2006). Study results also contradicted the notion that the presence of more than 8 mg $\text{NO}_2\text{-N/L}$ of nitrite inhibit P release and that the negative effects could last several hours after exposure (Meinhold et al. 1999). Hence the critical threshold for nitrite was likely higher here, which could be due to different sludge characteristics in our sludge (Meinhold et al. 1999). For both Stages III and IV, comparisons of the stoichiometric properties to published stoichiometric models revealed the potential involvement of the TCA cycle and glycolysis in generating the reducing power for the acetate conversion to PHA in RA and RC.

DPAOs or denitrifying PAOs are those PAOs that can use nitrate or nitrite as terminal electron acceptor. Hence, this group of PAOs is capable of performing concurrent P uptake and denitrification under anoxic conditions (Oehmen et al. 2007). Here, we did not observe the occurrence of P uptake in the presence of nitrite, even when acetate had been depleted, suggesting a lack of discernible DPAO activities. Lack of DPAO activity showed that the observed P release and uptake trends in our reactors were likely due to the activities of PAOs that could not use nitrite and nitrate. This observation supports the notion that planned anaerobic conditions are not strictly required during cycling for EBPR to occur (Cokro et al. 2017).

When subjected to prolonged exposure to the simultaneous presence of acetate, nitrate, and nitrite, some genera of GAOs, especially genus *Defluviicoccus* Cluster 2, proliferated. As postulated, one possible reason for *Defluviicoccus* cluster 2 enrichment was its inability to denitrify with nitrate or nitrite as electron acceptor when acetate (Burow et al. 2007) or propionate (Tayà et al. 2013) was present as carbon source. The lack of denitrification capacity was likely due to the absence of a full set of genes that encode for the reductase enzyme necessary for denitrification (Wang et al. 2014). Since *Defluviicoccus* GAOs can utilize acetate as well as other carbon sources like propionate or pyruvate, these organisms could compete with *Accumulibacter* PAOs for available carbon sources (Burow et al. 2007). Another GAO that increased in Stage IV was *CPB_S60*. This genus is unable to utilize nitrite, but can use nitrate (Kong et al. 2006, McIlroy et al. 2015a). However, nitrate levels may have been insufficient to sustain this genus and it may have recognized the anoxic condition as virtually anaerobic, partially supporting our hypothesis. While the ability of *Plasticicumulans* to use nitrate and/or nitrite has not been reported, the increase in *Plasticicumulans* suggested a selective advantage of cycling conditions for this putative GAO.

The observed decline in PAOs, especially of *Accumulibacter*, compromised the long-term stability of EBPR under anoxic/aerobic conditions. This was in contrast to earlier work reporting EBPR in a full-scale plant with a Modified Ludzack Ettinger configuration and without a designed anaerobic stage (Law et al. 2016). The discrepancy might be due to the different GAOs that proliferated in the study. We observed high numbers of *Defluviicoccus* (cluster 2) organisms and a negligible presence of *Competibacter*-GAO (< 0.1% of all bacteria), while Law et al. (2016) reported the presence of *Competibacter*, with *Defluviicoccus*-GAO absent from all samples. *Competibacter* was suggested to co-exist, instead of compete with *Accumulibacter* (Law et al. 2016). On the contrary, *Defluviicoccus* could pose a more serious competitor for *Accumulibacter* than *Competibacter* (Seviour and Nielsen 2010a). While arguably the high acetate loading rate combined with the lower pH (7.0-7.5) used throughout Stage III and IV in our study could favour *Defluviicoccus* cluster 2, as also reported by Tu and Schuler (2013), it only proliferated in Stage IV (Figure S4), further corroborating that the presence of acetate, nitrate, and microbially produced nitrite potentially benefited *Defluviicoccus* cluster 2.

Besides GAOs and PAOs, we also analysed the abundances of some non-PAO denitrifiers. This was due to the potential of these denitrifiers to proliferate when nitrate/nitrite and acetate were present at the same time. The genera *Rhodoferax*, *Haliangium*, *Azoarcus*, *Dechloromonas*, *Sulfuritalea*, *Thauera*, *Zooglea*, and *Thermomonas* have been reported as denitrifiers and are found in full-scale plants (Hagman et al. 2008, McIlroy et al. 2016, Thomsen et al. 2007). These denitrifiers can take up acetate and/or propionate when nitrate and/or nitrite are present, posing a potential threat to PAOs.

Although switching of cycling conditions did not significantly affect overall non-PAO denitrifier abundance, the relative numbers of some genera increased. *Dechloromonas* was among the non PAO denitrifiers whose abundance was higher in Phase IV. *Dechloromonas* is

known to consume acetate under both anaerobic and anoxic conditions with nitrite as electron acceptor. Higher acetate consumption by this genus was observed when nitrite was present (McIlroy et al. 2016). While some members exhibited denitrifying PAO phenotypes (Kong et al. 2007), others did not (Ahn et al. 2007), indicating that not all members of *Dechloromonas* positively contributed in an EBPR system (McIlroy et al. 2016).

The prolonged exposure to the simultaneous presence of acetate, nitrate, and nitrite lead to enrichment of non-PAO denitrifiers like *Dechloromonas* and *Zoogloea* in our systems. This observation contradicted a previous finding by Guerrero et al. (2011) where the presence of acetate favoured PAOs over denitrifiers at room temperature. This discrepancy might be because PAO abundance in our reactors was much lower than in the previous study where up to 72% PAOs were present (Guerrero et al. 2011). In contrast, the highest amount of PAOs detected in our reactors throughout the study was 8.67% of all bacteria for RA and 32% for RC. Additionally, the increasing dominance of GAOs could have suppressed PAOs by outcompeting the latter for the available acetate, leaving a niche for *Dechloromonas* and *Zoogloea* to slowly increase as Stage IV progressed.

Among the other genera analysed, *Azospira*, *MK04*, *K2-30-37* noticeably increased in Stage IV in both reactors. *Azospira* fix nitrogen (Reinhold-Hurek et al. 1993), and one strain of this genus, *Azospira* sp. *OGA 24*, was capable of denitrifying nitrate with acetate as carbon source (Rossi et al. 2015). No physiological information for genera *MK04* or *K2-30-37* is currently available (McIlroy et al. 2015a); however, their presence in this study suggests potential denitrifying capacities of these genera.

5. Conclusions

Using two replicated lab-scale SBRs, we observed the population dynamics of GAOs and PAOs when exposed concurrently to acetate, nitrate, and nitrite that was microbially generated,

at higher temperature. Multivariate analyses revealed that the trends exhibited by GAOs or PAOs in both reactors were replicable. From this study, we conclude:

- GAO populations were more responsive than PAOs to the change from anaerobic/aerobic to anoxic/aerobic cycling.
- Multivariate analysis showed that the effect of prolonged exposure to anoxic/aerobic cycling on PAOs was likely minimal but it led to proliferation of possible non-PAO organisms competing for acetate, causing lower carbon availability for PAOs.
- Some GAOs, especially Cluster 2 *Defluviicoccus*, proliferated under these conditions, likely due to their inability to use nitrate or nitrite as electron acceptor. Thus, these organisms recognized the conditions as pseudo-anaerobic/aerobic and performed anaerobic and aerobic metabolisms accordingly.
- GAOs were able to outcompete PAOs for available carbon sources. This also provided a competitive advantage for some non-PAO denitrifiers such as *Zoogloea* and *Dechloromonas*. Consequently, the simultaneous presence of acetate, nitrate, and nitrite would not be ideal in the long run as stable EBPR was not achieved.
- Lack of DPAO activity suggested that EBPR was done by PAOs that could not use nitrite or nitrate. This observation supports the notion that planned anaerobic conditions are not strictly required during cycling for EBPR to occur. The challenge remains how to sustain their presence in the long run in the presence of specific competitors.

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Supplementary Information

Differential Responses of Non-denitrifying Glycogen and Polyphosphate Accumulating Organisms to Acetate, Nitrate, and Microbially Generated Nitrite at Warm Temperatures

APPENDIX 1

DNA extraction and 16S rRNA gene amplicon sequencing procedures

Defrosted DNA samples were homogenized using the Heidolph RZR 2020 overhead stirred (Heidolph Instruments, DE) at gearing II speed 9 for 1 minute. Subsequently, 1 ml of the samples were transferred to a 2 ml tube and centrifuged in a Sorvall Legend Micro 21 Microcentrifuge (Thermo Fisher Scientific, USA) set at 21,100 g for 5 minutes. The pellet was re-suspended in 978 μ L sodium phosphate buffer with pH = 8 and transferred to a Lysing matrix E tube where 122 μ L of MT buffer was then added. A FastPrep FP120 Homogenizer (Thermo Savant, USA) was utilized to homogenize the samples at speed 6 for 4x40 seconds. Samples were kept in ice for 2 minutes in between each bead beating process. Following the completion of the aforementioned step, manufacturer's protocol was followed.

The Polymerase Chain Reaction (PCR) amplification step consisted of 2 minutes of 95 °C; 30 cycles of 20 seconds of 95 °C, 30 seconds of 56 °C, and 60 seconds of 72 °C; and elongation stage at 72 °C for 5 minutes. For each of the amplification step, 10 ng of DNA template were mixed with 1X Platinum® High Fidelity buffer, 400 pM dNTP, 2 mU Platinum® Taq DNA Polymerase High Fidelity, 1.5 mM MgSO₄, and 5 μ M V1-V3 adapter (barcoded). Agencourt AmpureXP (Beckman Coulter Inc., USA) was utilized to purify the PCR products at a 1.8 bead solution/PCR solution ratio. Quantification of the DNA concentration was done using the QuantIT HS kit (Life Technologies, USA). Subsequently, barcoded amplicons were

pooled in equimolar concentrations, and paired-end sequenced at 2x250 bp with Illumina MiSeq (Illumina Inc., USA).

For each sample in the combined amplicon library, the sequencing output from the Illumina MiSeq (Illumina Inc., USA) was de-multiplexed from the amplicon libraries in FASTQ format. All sequenced sample libraries were subsampled to 10,000 raw reads, while all of the amplicon libraries were pre-processed according to Albertsen et al. (2015). MiDAS v.1.20 (McIlroy et al. 2015b) was utilized for assigning taxonomy. R package ampvis (<https://github.com/MadsAlbertsen/ampvis>) was used to analyse the results in R (R Core Team 2014) via the Rstudio Integrated Development Environment/IDE (<http://www.rstudio.com>). Sequences that fall within 97% similarity threshold were clustered into operational taxonomic units (OTUs). Samples for 16S rRNA amplicon sequencing were processed by the DNAsense Company in Aalborg, Denmark (<http://dnasense.com/>).

APPENDIX 2

Supplementary tables and figures

Table S1. EBPR capacities during Stage III and Stage IV in reactors RA and RC. The amount of P that was released or consumed was normalized to biomass (mg P/gr VSS).

Reactor	Stage	Days	Total P released (mg P/g VSS)	Total P uptake (mg P/g VSS)
RA	Stage III	242	7.1	8.9
	Stage III	246	11.0	21.2
	Stage III	255	13.5	17.4
	Stage III	263	14.3	19.3
	Stage III	267	7.5	19.7
	Stage III	277	5.8	6.0
	Stage III	282	4.9	6.7
	Stage III	290	5.2	5.6
	Stage III	295	4.6	6.8
	Stage IV	296	7.6	8.8
	Stage IV	297	6.1	7.2
	Stage IV	302	5.7	10.0
	Stage IV	311	4.7	5.7
	Stage IV	315	4.0	4.8
	Stage IV	325	2.8	7.0
	Stage IV	331	2.9	6.5
RC	Stage III	242	7.8	15.7
	Stage III	246	12.8	25.5
	Stage III	255	15.3	18.5
	Stage III	263	26.2	34.1
	Stage III	267	34.4	45.2
	Stage III	277	17.4	19.0
	Stage III	282	13.1	19.1
	Stage III	290	20.4	26.7
	Stage III	295	19.0	27.7
	Stage IV	296	41.3	49.2
	Stage IV	297	23.4	29.4
	Stage IV	302	33.9	46.9
	Stage IV	311	23.8	33.4
	Stage IV	315	21.7	32.6
	Stage IV	325	8.6	14.7
	Stage IV	331	8.0	14.2

Table S2. Stoichiometric ratios for reactors RA and RC based on cycle studies conducted under Stage III and IV. PHA and glycogen (gly)

samples were not collected on some of the cycle studies, such as studies conducted on Days 242, 333, 337, and 339.

Reactor	Stage	Days	FEAST			FAMINE	
			P released/Acetate consumed (moles P/moles C)	Glycogen (gly) consumed/Acetate consumed (moles C/moles C)	PHA produced/Acetate consumed (moles C/moles C)	P uptake/PHA consumed (moles P/moles C)	Glycogen produced/PHA consumed (mole C/mole C)
RA	Stage III	246	0.11	0.61	0.83	0.19	0.54
	Stage III	255	0.13	0.54	0.89	0.17	0.74
	Stage III	263	0.11	0.70	1.12	0.16	0.79
	Stage III	267	0.047	0.63	1.11	0.063	0.43
	Stage III	282	0.047	0.45	1.20	0.05	0.51
	Stage III	290	0.067	0.81	2.57	0.04	0.71
	Stage III	295	0.048	1.19	2.14	0.03	0.52
	Stage IV	296	0.058	0.68	1.04	0.061	0.44
	Stage IV	297	0.066	0.50	2.12	0.033	0.35
	Stage IV	302	0.034	0.49	0.84	0.068	0.25
	Stage IV	311	0.042	0.20	0.93	0.057	0.30
	Stage IV	315	0.039	0.58	1.20	0.045	0.99
	Stage IV	325	0.028	0.038	1.04	0.075	0.38
	Stage IV	331	0.025	0.93	0.92	0.047	0.81
	Stage IV	337	0.038	not collected (nc)*	nc	nc	nc
	Stage IV	339	0.041	nc	nc	nc	nc
Model			P rel/Acetate consumed	Gly consumed/Acetate consumed	PHA produced/Ace consumed	P uptake/PHA consumed	Gly produced/PHA consumed
PAO TCA+Glycogen ¹			0.16	0.7	1.48		0.44
PAO TCA Model ²			0.8	0	0.9		
PAO Glycogen ³			0.5	0.5	1.33		
Aerobic PAO ⁴						0.41	0.42
GAO Model ⁵			0	1.12	1.86		0.65

¹ Pereira et al. (1996); ² Comeau et al. (1986); ^{3 4} Smolders et al. (1994); ⁵Zeng et al. (2003)

* PHA/glycogen sample was not collected for this cycle study

Reactor	Stage	Days	FEAST			FAMINE	
			P released/Acetate consumed (moles P/moles C)	Glycogen (gly) consumed/Acetate consumed (moles C/moles C)	PHA produced/acetate consumed (moles C/moles C)	P uptake/PHA consumed (mole P/mole C)	Glycogen produced/PHA consumed (mole C/mole C)
RC	Stage III	246	0.13	0.66	0.77	0.26	0.48
	Stage III	255	0.12	0.65	1.20	0.13	0.60
	Stage III	263	0.16	0.34	0.98	0.22	0.56
	Stage III	267	0.19	0.43	0.99	0.28	0.64
	Stage III	282	0.14	0.83	1.04	0.21	0.59
	Stage III	290	0.24	1.22	1.35	0.24	0.78
	Stage III	295	0.18	1.18	0.83	0.26	0.78
	Stage IV	296	0.26	0.69	0.69	0.43	0.85
	Stage IV	297	0.23	0.67	1.74	0.17	0.60
	Stage IV	302	0.18	0.43	0.51	0.43	1.59
	Stage IV	311	0.18	0.32	0.62	0.53	0.91
	Stage IV	315	0.17	0.60	0.80	0.30	1.08
	Stage IV	325	0.079	0.37	0.88	0.18	0.87
	Stage IV	331	0.070	0.23	0.44	0.25	0.61
	Stage IV	333	0.092	not collected (nc)*	nc	nc	nc
	Stage IV	337	0.0069	nc	nc	nc	nc
	Stage IV	339	0.0029	nc	nc	nc	nc
Model			P rel/Acetate consumed	Gly consumed/Acetate consumed	PHA produced/Acetate consumed	P uptake/PHA consumed	Gly produced/PHA consumed
PAO TCA+Glycogen ¹			0.16	0.7	1.48		0.44
PAO TCA Model ²			0.8	0	0.9		
PAO Glycogen ³			0.5	0.5	1.33		
Aerobic PAO ⁴						0.41	0.42
GAO Model ⁵			0	1.12	1.86		0.65

¹ Pereira et al. (1996); ² Comeau et al. (1986); ^{3 4} Smolders et al. (1994); ⁵Zeng et al. (2003)

* PHA/glycogen sample was not collected for this cycle study

Table S3. Multivariate analysis to test reproducibility of GAOs or PAOs in both reactors. GAO or PAO communities in RA were compared to those in RC in Stage III and Stage IV to determine if the overall trends exhibited by GAOs or PAOs in RA were replicable in RC. Multivariate analysis was conducted with PRIMER 6 software. Raw data for each GAO or PAO genus was standardized by total counts of all GAOs or all PAOs, respectively. Square root transformation was done to standardized data to reduce the effects of dominant OTUs before Bray-Curtis similarities were constructed. Reactor served as factor for the analysis.

Community group	Stage	Reactors	PERMANOVA ¹		PERMDISP ²	
			P	Pseudo-F	P	F
GAO	III*	RA vs RC	0.3	1.1	0.0002	24.04
	IV**	RA vs RC	0.2	1.5	0.8	0.1
PAO	III*	RA vs RC	0.1	2.2	0.2	2.7
	IV**	RA vs RC	0.8	0.2	0.9	5.6

* n = 9 samples for each reactor; ** n = 8 samples for each reactor

¹ If $P < 0.05$, we rejected null hypothesis that there was no difference between GAO or PAO communities in RA and RC.

² If $P < 0.05$, dispersion of GAO or PAO communities in RA and RC were dissimilar (i.e. heterogenous dispersion)

Table S4. Comparison of OTUs belonging to GAOs, PAOs and non-PAO denitrifiers in Stage III versus Stage IV using multivariate analysis. Results were generated using PRIMER 6 software. Groups of OTUs belonging to GAOs, PAOs, or non-PAO denitrifiers in Stage III were compared to GAOs, PAOs, or denitrifiers in Stage IV to determine if change in conditions lead to statistically significant divergence in the GAOs, PAOs, or denitrifier. The two different stages served as factor for the analysis. Raw data for each OTU was standardized by total counts of all OTUs in each group (GAO, PAO, or non PAOs denitrifiers) in each sample. Square root transformation was applied to standardize data and minimize the effects of dominant OTUs before a Bray-Curtis similarity matrix was constructed.

Community group	Reactor	Stage*	PERMANOVA ¹		PERMDISP ²	
			P	Pseudo-F	P	F
GAO	RA	III vs. IV	0.001	7.1	0.2	2.8
GAO	RC	III vs. IV	0.0006	8.1	0.8	0.06
PAO	RA	III vs. IV	0.01	3.6	0.002	9.3
PAO	RC	III vs. IV	0.008	8.4	0.0001	32.5
Denitrifiers	RA	III vs. IV	0.009	3.6	0.004	15.6
Denitrifiers	RC	III vs. IV	0.9	0.4	0.3	2.1

*n = 9 samples per reactor for Stage 3; 8 samples per reactor for Stage 4;

¹ If P < 0.05, we rejected null hypothesis that there was no difference between GAO, PAO, or denitrifiers communities in Stages III and IV for each reactor.

² If P < 0.05, dispersion of GAO, PAO, or denitrifier communities in Stage III and Stage IV were heterogenous in each reactor.

Figure S1. Phosphorus concentrations, in mg/L P, in the filtered nutrient samples collected from reactor R0 during cycle studies conducted from Day 1 (1 day) after initial inoculation. R0 was subjected to continuous anaerobic/aerobic cycling. Each data point represented the P concentration detected at the beginning of cycle (Feed start), end of anaerobic, and end of aerobic phases.

Figure S2. nMDS plots to illustrate the replicability of GAO communities in RA and RC throughout Stage III (A) and Stage IV (B), as well as PAOs in both reactors in Stage III (C) and IV (D). Each data point represented the relative abundances of all GAO or PAO genera. Numbers represented days when DNA samples were collected. The relative abundance of each GAO or PAO genus was calculated with respect to all GAOs or all PAOs to better capture the dynamics of each community in RA and RC.

Figure S3. Total relative abundances of GAOs or PAOs in RA (A) and RC (C) throughout Stage III and IV. Each point represented the sum of relative abundances (% of all bacteria) of all OTUs that mapped to known GAOs or PAOs in each cycle study conducted. Dashed line represented the point where Stage III was switched to Stage 4.

Figure S4. Relative abundances of all OTUs belonging to *Defluviicoccus* cluster 2 and *Competibacter* detected in cycle studies conducted throughout Stage III and IV for RA (A) and RC (B). Dashed line represented the point when cycling condition was switched. *Competibacter* were consistently low throughout the study.

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Chapter 4

Potential of Wasted Activated Sludge (WAS) Addition to Reseed Failing EBPR Systems

Abstract

Enhanced biological phosphorus removal (EBPR) has been widely utilized in wastewater treatment. Despite extensive efforts to understand the underlying mechanisms of EBPR, unexpected failures still occur even in laboratory-scale bioreactors operating under controlled conditions that should theoretically be beneficial for EBPR. Thus, an affordable and simple strategy to resuscitate failing EBPR systems would be advantageous. In this study, we used stored wasted activated sludge (WAS) from two bioreactors to revive and/or increase EBPR activities in declining reactors operated at 30°C. Upon replacing a third of mixed liquor from each of two reactors that exhibited low to no EBPR activity with WAS that had been subjected to prolonged starvation for three weeks at 4 °C, we observed higher activities in both reactors, based on a higher ratio of P released/acetate consumed, that positively correlated with increased PAO abundance, *Candidatus Accumulibacter* outcompeted most glycogen accumulating organisms (GAOs), which do not perform significant P uptake, after WAS had been added to the reactors, despite the higher operating temperature that usually favors GAOs over PAOs. Here the lower storage temperature provided an advantage to PAOs due to their lower mesophilic range; in contrast, GAOs are mesophilic organisms that were more adversely affected at 4 °C. Consequently, PAOs could recover their activities faster than most genera of GAOs, enabling them to outcompete GAOs during bioaugmentation of reactors. One exception was *Defluviicoccus* cluster 2, which, surprisingly, increased in abundance while EBPR activities also increased. This phenomenon could be due to a lack of competition for carbon; as long as sufficient carbon was present, PAOs and *Defluviicoccus*

cluster 2 coexisted. Both PAO and GAO communities diverged from the pre-WAS period over time, as verified by next generation sequencing and multivariate analysis.. These differences were caused by the WAS addition instead of differences in the community dispersion between phases, as indicated by higher PERMDISP p-values. Based on 16S rRNA gene amplicon sequencing the relative abundance of *Ca. Accumulubacter* increased from 0.6 ± 0.3 % of all bacteria in one reactor to 9.5 ± 1.9 % and from 0.3 ± 0.1 % in the second reactor to 8.6 ± 2.1 %. In conclusion, the addition of WAS could be a simple strategy to revive failing EBPR systems leading to sustained higher PAO abundances and activities. Further studies should focus on combining this strategy with additional measures to control the proliferation of *Defluviicoccus* cluster 2 in light of competition for carbon in full-scale wastewater treatment plants.

1. Introduction

Enhanced biological phosphorus removal (EBPR) is a phosphorus (P) removal method that relies on capacities of polyphosphate accumulating organisms (PAOs) to accumulate more P than what is needed for growth (Gebremariam et al. 2011, Oehmen et al. 2007). An EBPR system typically consists of a carbon-rich “feast” phase, usually in the absence of electron acceptors, followed by a carbon-depleted “famine” phase with oxygen, nitrate, and/or nitrite as electron acceptors. Similarly, a group of bacteria known as glycogen accumulating organisms (GAOs) can also rely on their internal storage compounds (i.e., glycogen) for energy to consume carbon under anaerobic conditions. However, GAOs do not perform significant P uptake. Thus, the proliferation of GAOs has been linked to failure in EBPR systems due to competition with PAOs for available carbon (Oehmen et al. 2006).

Many efforts to understand the competition between PAOs and GAOs have utilized laboratory-scale batch or enrichment bioreactors because they are easily controlled and manipulated. Despite attempts to maintain favorable conditions for PAOs, a proliferation of GAOs accompanied by deteriorating EBPR activities is frequently observed in EBPR systems. Thus, exploring strategies to revive failing EBPR systems could be beneficial. One such alternative is the use of wasted activated sludge (WAS) that contains bacteria such as PAOs.

Activated sludge is regularly wasted from lab-scale and full-scale systems to maintain sludge retention times (SRTs) and to remove phosphorus from the system. In most cases, no external carbon is present in the WAS, since EBPR systems from which WAS originated have usually completed the “feed” and “famine” phases prior to sludge wasting. While this condition could lead to possible anaerobic starvation since stored WAS is neither aerated nor fed with additional external carbon sources, Lopez et al. (2006) found that EBPR was independent of the length of the starvation period under anaerobic conditions.

The applicability of adding WAS to revive and/or improve EBPR systems has not been explored. Thus, the objectives of this study were to study the effect of WAS addition on (1) EBPR and relative abundances of known PAOs and GAOs, and to (2) establish correlations, if any, between the changes in PAO and/or GAO communities and the observed activities following WAS addition at 30 °C. We hypothesized that stored WAS could lead to higher EBPR performance after an increase in PAO abundance and related activities.

2. Materials and methods

2.1 Laboratory scale bioreactors and source of WAS operation

Two laboratory-scale bioreactors with working volumes of 5.44 L, Reactor A (RA) and Reactor C (RC), were inoculated with sludge from a source reactor R0. The complete reactor operational history was recorded (Supplementary Material), and Day 0 in this study referred to the initial inoculation of R0. Operating parameters for RA and RC, including COD:P ratio, DO, T, pH, HRT, and SRT were elaborated in Chapter 3, subsection **2.1. Laboratory scale enrichment reactors** (pp. 99-101).

Following a previous study that necessitated the simultaneous addition of nitrate and acetate in both reactors for anoxic/aerobic cycling on Days 296-339, EBPR was lost in RC on Days 337 and 339, while low activity was still observed in RA (Supplementary Fig. S1). To explore the potential of WAS addition to revive failing EBPR reactors, our study started on Day 342, when both reactors were switched back to anaerobic/aerobic cycling, until Day 471, when the last DNA samples were collected. Throughout this period, RA and RC were subjected to continuous 6-h anaerobic/aerobic cycling at $30 \pm 1^\circ\text{C}$. Each cycle consisted of 130 min of anaerobic, including 5 min of feeding, followed by 160 min of aerobic phases; 1 min of sludge discharge; 30 min of settling; and 39 min of supernatant decant. It should be noted that the amount of yeast extract in RC was 5x times higher from Day 393 onwards,

except on Day 471 when no yeast extract was added to reactors. However, these changes did not have any bearing on system activities.

2.2. WAS collection, storage, and addition to reactors

WAS from RA and RC was collected during sludge wasting stage in each cycle. Two batches of WAS used in this study were collected during Days 284 to 296 (Batch 1), and during Days 316 to 337 (Batch 2). These batches were selected due to their relative freshness as compared to other available stored WAS and lack of noticeable algal growth. Most importantly, these batches were collected when source reactors still exhibited EBPR, indicating the presence of functional PAOs in the mixed liquor. The average MLVSS concentrations in RA during the collection period was 3.6 g VSS/L for Batch 1 and 3.3 g VSS/L for Batch 2. For RC, the average sludge content was 3.2 g VSS/L during collection of Batch 1 and 3.1 g VSS/L throughout collection of Batch 2. Upon collection, these batches of WAS were stored at low temperature (4 °C) before usage. No aeration was supplied, and no head space replacement occurred once WAS had been stored in the fridge.

A minor addition of WAS (400 mL) on Day 352 preceded the major reactor sludge replacement with WAS on Day 361, where approximately 1,900 mL of the mixed liquor from each reactor (approximately 35% of the working volume) was replaced with a mixture of 1,600 mL WAS of Batch 1 and 300 mL of Batch 2. Since the aforementioned minor WAS addition did not cause any discernible effects on activities, the period from Days 342-361 was considered as the pre-WAS addition phase, while the following period was categorized as the post-WAS addition phase.

2.3. Cycle studies and sample collection

In order to examine the activities and microbial communities before and after WAS addition on Day 361, cycle studies were periodically conducted in the pre-and post-WAS

addition periods, with the first cycle studies for the pre-WAS phase conducted on Day 345 and for the post-WAS on Day 368. For each cycle study, nutrient samples were collected at six time points: beginning of cycle (or feed start) at $t = 0$ min, end of feeding ($t = 5$ min), middle ($t = 65$ min) and end of anaerobic phase ($t = 130$ min), and middle ($t = 210$ min) and end of aerobic stage ($t = 290$ min). Upon collection, these samples were immediately filtered using $0.2\ \mu\text{m}$ Acrodisc® syringe filters (sterile) with Supor® membrane (Pall Life Science, USA). Solids samples were collected at the beginning of the cycle and end of the aerobic phase in most cycle studies. DNA samples were collected at the beginning of the cycle and immediately frozen in liquid nitrogen prior to storage at -80°C .

2.4. Physicochemical tests and analyses

Concentrations of phosphorus in the nutrient samples were measured via colorimetric methods using HACH Phosphorus TNT 843 (low range) and TNT 844 (high range) kits with HACH DR 6600 spectrophotometer (HACH, USA). Phosphorus trends were monitored to determine EBPR activities in RA and RC. To gauge the activities in each reactor throughout the experiment, the ratio of P released/acetate consumed under anaerobic conditions and the amount of P that was removed from the system (net P removal) by the end of aerobic phase were examined. The net P removal was calculated using Equation (1),

$$\left(\frac{P_{\text{Feed start}} - P_{\text{end of aerobic}}}{P_{\text{Feed start}}} \right) \times 100\% \quad (\text{Equation 1})$$

where $P_{\text{Feed start}}$ and $P_{\text{end of aerobic}}$ were the amount of P, in mg P/L, detected at the start of cycle and end of aerobic stage, respectively.

Volatile fatty acids (VFAs) were quantified using a Gas Chromatography (GC) system (Shimadzu, Prominence) as described in Chapter 2, subsection **2.3. Chemical Analysis** (pp. 60-61). Solids samples were analyzed following standard methods (APHA 1995).

2.5. DNA extraction

DNA from the sludge samples were extracted using FastDNA™ 2 mL SPIN kits for Soil (MP Biomedicals, USA). We followed the optimized method for activated sludge described by Albertsen et al. (2015), which was elaborated in Chapter 2, subsection **2.5. DNA extraction and sequencing** (pp. 62-64).

2.6. 16S rRNA gene amplicon sequencing

Polymerase Chain Reaction (PCR) was utilized to amplify approximately 500 bp DNA fragments from the V1 to V3 regions of 16S rRNA genes using bacterial primers 27F-AGAGTTTGATCCTGGCTCAG (Lane 1991) and 534R-ATTACCGCGGCTGCTGG (Muyzer et al. 1993). Details of the PCR amplification steps could be found in p. 63 of this thesis. The sequencing output was de-multiplexed in FASTQ format from the amplicon libraries. Sequenced samples library were subsampled to 10,000 raw reads, and all amplicon libraries were pre-processed following Albertsen et al. (2015). Taxonomy was assigned via MiDAS v.1.20 (McIlroy et al. 2015b), and results were analysed in R (R Core Team 2014) via the Rstudio Integrated Development Environment/IDE (<http://www.rstudio.com>) using the R package ampvis (<https://github.com/MadsAlbertsen/ampvis>). Sequences within 97% similarity threshold were grouped into operational taxonomic units (OTUs). 16S rRNA gene amplicon sequencing was done by DNAsense in Aalborg, Denmark (<http://dnasense.com/>).

2.7. Statistical analysis

Primer 6 software with PERMANOVA+ was utilized to perform multivariate analysis of the microbial communities in both reactors (Clarke and Gorley 2006). Data standardization was based on the total reads per samples. The standardized data were square-root transformed to reduce the effects of the dominant species prior to construction of a Bray Curtis distance matrix. Permutational multivariate analysis of variance (PERMANOVA) was conducted to

check the null hypothesis that the communities in RA and RC before and after substantial addition of WAS on Day 361 were similar (Anderson and Walsh 2013). PERMDISP analysis was used to determine the homogeneity of variance in the two sample groups (Anderson 2006). The number of permutations was set to 9999, and phases before and after WAS addition served as the fixed factor. Phase 1 included four samples collected prior to major WAS addition, (Days 345-359). Samples in these phases were compared to samples collected after WAS addition. To ensure a balanced number of samples in each group, the post-WAS addition period was further divided into three phases (Phases 2, 3, and 4). Phase 2 incorporated four samples collected at the beginning of the post-WAS period (Days 368-387), and Phases 3 and 4 encompassed samples collected at the later stage of the post-WAS period (Days 429 to 445 and Days 451 to 471, respectively) (Table 1).

Table 1. Experimental phases in reactors RA and RC. Each phase represents microbial community data from four samples, collected during cycle studies conducted on the days listed.

Phase	Duration (days) ¹	Description
1	345-359	Before portion of sludge in reactors was replaced with WAS (pre-WAS addition phase)
2	368-387	Post-WAS addition phase, early stage
3	429-445	Post-WAS addition phase, later stage
4	451-471	Post-WAS addition phase, last four samples before study ended

¹ Range of days when microbial community samples were collected that were included in the multivariate analysis. Days were counted from the initial inoculation of the source reactor (R0) from which RA and RC received their inoculum.

3. Results

3.1. EBPR in RA and RC before and after WAS addition

On Day 342, EBPR activities were low in reactors RA (Fig. 1A) and RC (Fig. 1B). An initial addition of 400 ml WAS to the reactors on Day 352 did not result in a measurable increase in activity in either reactor (Fig. 1). This was likely due to the small amount of WAS added in comparison to the working volume of reactors (5.44 L). Average P removal observed in the pre-WAS addition cycle studies was $26.0 \pm 13.1\%$ (average $\pm$ standard error of means or SEM) for RA and $4.8 \pm 2.4\%$ for RC (Fig. 2, Table 2). Low P release/acetate uptake was also detected in RA where ratios of P released per acetate consumed ranged from 0.01 to 0.06 moles P/moles C (Fig. 2A, Table 2) and in RC where it ranged from 0.01 to 0.02 moles P/moles C (Fig. 2B, Table 2).

After we replaced approximately 1,900 mL of sludge in RA and RC with the stored WAS, higher EBPR activities were observed in both reactors (Fig. 1 and 2). Ratios of P released/acetate consumed increased to 0.2-0.5 in RA (Fig. 2A, Table 2) and 0.03-0.4 in RC (Fig. 2B, Table 2). Higher P removal activities were also detected in both reactors, with % P removal averaging $58.4 \pm 6.0\%$ in RA and $43.7 \pm 6.3\%$ in RC (Fig. 2, Table 2).

3.2. Impact of WAS addition on the microbial community, especially PAO and GAO populations

We investigated the effects of replacing approximately 1,900 ml of sludge from each reactor with stored WAS on the microbial community. Bacterial communities in RA and RC changed significantly after WAS addition as shown by the PERMANOVA p-values (Table S2). There were no significant differences when we compared bacteria in Phase 1 to those in Phase 2 in reactor RC, suggesting bacterial communities in Phase 2 had not yet fully responded to the WAS addition. In most of the comparisons, there was a homogenous

Figure 1. Phosphorus concentrations, in mg P/L, at the start of the cycle (feed start), end of anaerobic, and end of aerobic phases in RA (A) and RC (B) in the cycle studies conducted before and after WAS addition to each reactor to replace portions of the sludge. Dashed line indicates the day when WAS was added to reactors (Day 361). Days were counted from the initial inoculation of the source reactor (R0).

Figure 2. Activities observed in RA (A) and RC(B), as illustrated by the amount of P released/acetate consumed, in moles P/moles C; and the net amount of P that was removed from the system at the end of aerobic phase, in %. Zero net P removal indicated the absence of positive net P removal in the system at the particular cycle study, as the P concentration at the end of aerobic phase was higher than that at the beginning of the cycle. Dashed lines indicate the day when WAS was added to reactors (Day 361). Days were counted from the initial inoculation of the source reactor (R0).

Table 2. Average P removal activities in reactors RA and RC before and after major sludge replacement with WAS on Day 361.

Reactor	Pre/Post-WAS addition	P released/acetate consumed (moles P/moles C) ¹	% P removal ¹
RA	Pre-WAS addition	0.03 (0.02)	26 (13.1)
RA	Post-WAS addition	0.3 (0.02)	58.4 (6.0)
RC	Pre-WAS addition	0.02 (0.004)	4.8 (2.4)
RC	Post-WAS addition	0.2 (0.03)	43.7 (6.3)

¹ Mean ($\pm$ standard error of mean).

dispersion between the different phases (Table S2). As a result, the observed dissimilarities among communities before and after WAS addition could be attributed to the replacement of some sludge with the stored WAS.

We then performed a multivariate analysis comparing the abundances of PAOs and GAOs before (Phase 1) and after (Phases 2-4) the major WAS addition event. For this analysis, we calculated the relative abundance of each PAO genus with respect to the overall PAO community only. Similarly, the abundance of GAO genera was also calculated with respect to the overall GAO community. This was done to ensure that all relative changes in these important communities were captured.

PAOs in RC did not differ significantly between Phases 1 and 2 as supported by PERMANOVA (Table S3). As the experiment progressed into Phase 3, PAO communities started to diverge more significantly from Phase 1 (Table S3). The lower PERMDISP p-value suggested heterogeneous dispersion in these two communities. Consequently, the significant

difference in Phase 1 and 3 for PAO groups could be caused by the fact that one of the groups was more dispersed than the other. When comparing Phases 1 and 4 for reactors RA and RC, the differences between PAO communities in the two phases were statistically significant (Table S3). For both RA and RC, the dispersion of the communities in the two phases was homogenous (based on PERMDISP p-values) and any differences between these two phases were therefore due to the major WAS addition (Table S3).

Similar to the multivariate analysis done on PAOs, we also compared GAOs in the different phases (i.e., Phase 1 vs. Phases 2, 3, or 4), taking into account their relative abundances with respect to all known GAOs in the systems. Like in the case of PAOs, the GAO communities diverged more from the pre-WAS period in phase 4 of post-WAS addition than in the earlier phases 2 and 3. Likely, these differences were caused by the WAS addition instead of differences in the community dispersion between phases, as indicated by the higher PERMDISP p-values (Table S4).

We further examined each of the genera belonging to either group of organisms to assess if any genus displayed similar discernible changes in both reactors. Due to the lack of a control reactor, we focused our discussion on genera that exhibited similar changes in both RA and RC to minimize the risks of overestimating trends that might not be caused by the WAS addition.

Of the PAO genera summarized in Stokholm-Bjerregaard et al. (2017), *Accumulibacter*, *Obscuribacter*, *Gemmatimonas*, and *Tessaracoccus* were present in some or all samples from RA throughout the experiment. Additionally, *Pseudomonas* was also present in some samples from RC. Prior to the major WAS addition, the average relative abundance of these PAO genera with respect to all bacteria was 0.8 ± 0.3 % (average relative abundance $\pm$ standard error of mean) in RA and 0.3 ± 0.2 % in RC (Table 3). The abundance of PAOs

Table 3. Relative abundances of all PAOs, all GAOs, and specific genera that displayed similar trends in both reactors before and after WAS addition.

Reactor	Pre/Post-WAS	Relative abundance ¹ (%)						
		Total PAOs ¹	Total GAOs	Accumulibacter	<i>Tetrasphaera</i>	<i>Dechloromonas</i>	<i>Defluviicoccus</i> cluster 2	<i>CPB_S60</i>
RA	Pre-WAS	0.8 (0.3)	43.9 (4.5)	0.6 (0.3)	0.003 (0.002)	1.7 (0.3)	5.0 (3.3)	18.4 (2.9)
RA	Post-WAS	9.9 (2.0)	24.1 (4.2)	9.5 (1.9)	0.02 (0.006)	0.2 (0.1)	7.4 (1.5)	5.0 (1.2)
RC	Pre-WAS	0.3 (0.2)	12.7 (11.0)	0.3 (0.1)	0.01 (0.01)	6.1 (3.3)	1.0 (0.7)	6.1 (5.8)
RC	Post-WAS	8.7 (2.1)	28.2 (4.0)	8.6 (2.1)	0.01 (0.01)	0.1 (0.03)	11.5 (2.3)	2.6 (1.1)

¹ Relative abundance compared to all bacteria ($\pm$ SEM)

increased to 9.9 ± 2.0 % in RA and 8.7 ± 2.1 % in RC after the major sludge replacement (Table 3). The higher relative abundance of PAOs in RA compared to RC corroborated the higher activities observed in RA as compared to RC in the pre-and post-WAS periods (Table 2).

Accumulibacter was the most abundant PAO in both reactors with a relative abundance before the major WAS addition of 0.6 ± 0.3 % of all bacteria in RA and 0.3 ± 0.1 % in RC. Abundances increased to 9.5 ± 1.9 % in RA (Table 3, Fig. 3A, Fig. 4A) and 8.6 ± 2.1 % in RC (Table 3, Fig. 3B, Fig. 4A) after WAS addition. Other PAO genera were present at low abundances (< 1 % of all bacteria). Additionally, the genus *Tetrasphaera* was detected throughout the study but at a much lower abundance, while *Dechloromonas*, another putative PAO genus, decreased from 1.7 ± 0.3 % in RA and 6.1 ± 3.3 % in RC to 0.2 ± 0.1 % in the former and 0.1 ± 0.03 % in the latter (Table 3).

Among the known GAO genera detected in the reactors, genus *Defluviicoccus* cluster 2 increased in both RA and RC. Prior to the WAS addition, the average abundance of *Defluviicoccus* cluster 2 with respect to all bacteria was 5.0 ± 3.3 % in RA and 1.0 ± 0.7 % in RC. After WAS addition, the average relative abundance increased to 7.4 ± 1.5 % in RA (Table 3, Fig. 3A, Fig. 4B) and 11.5 ± 2.3 % in RC (Table 3, Fig. 3B, Fig. 4B). In contrast, *CPB_S60* decreased from 18.4 ± 2.9 % to 5.0 ± 1.2 % in RA (Table 3, Fig. 3A, Fig. 4C) and from 6.1 ± 5.8 % to 2.6 ± 1.1 % in RC (Table 3, Fig. 3B, Fig. 4C) after WAS addition.

It should be noted that the amount of yeast extract in the feed for reactor RC increased five times from Day 392. The concentration of yeast extract in RA remained the same. We did not observe any difference in EBPR activities in batch activity tests using sludge from RA and RC, supporting the notion that the additional yeast did not affect RC (data not shown). Thus, we concluded that the observed increase in both abundance of *Accumulibacter* PAOs and EBPR activity was due to the impact of WAS addition.

Figure 3. The average abundances of GAO and PAO genera detected in RA (A) and RC (B) throughout the pre-WAS and post-WAS addition periods. Each symbol represents the average relative abundance of each genus, in % of all bacteria $\pm$ SEM. GAO (f_*Competibacteraceae*) represents all OTUs belonging to family *Competibacteraceae* that were not assigned to any GAO genus. Error bars are sometimes contained within symbols. *Tetrasphaera* were present in low abundances, while *Dechloromonas* in the system likely did not perform EBPR, as indicated by a negative correlation between abundance and activities.

Figure 4. Temporal profiles for the relative abundance (% of all bacteria) in RA and RC during the pre-WAS and post-WAS periods for genera *Accumulibacter* (A), *Defluviicoccus cluster 2* (B), and *CPB_S60* (C). These genera exhibited similar increasing or decreasing trends in both reactors. Days were counted from the initial inoculation of source reactor 0. Dashed lines indicate the day when a portion of mixed liquor in RA and RC was replaced with WAS.

3.3. Correlation between known PAOs or GAOs and the observed change in EBPR

As postulated, increases in relative abundance of *Accumulibacter* in both reactors were positively correlated with a higher ratio of P released/acetate consumed, based on Spearman's correlation analysis ($\rho = 0.7$). Unexpectedly, *Defluviicoccus* cluster 2 in both reactors also correlated positively with increases in EBPR activities (Table S5). This might be because sufficient carbon sources for PAOs were present in the post-WAS period to allow co-existence. While some species of *Dechloromonas* can perform EBPR, the negative correlation between their relative abundances and the observed P released/acetate consumed (moles P/C) in RA (Spearman's correlation $\rho = -0.7$) and RC ($\rho = -0.8$) suggested that those species detected in our systems did not perform such activities (Table S5).

4. Discussions

In this study, we explored the possibility of using stored WAS to resuscitate failing EBPR systems at warm temperatures. Results supported our hypothesis since PAO abundances increased following a partial sludge replacement of one-third of the biomass with WAS and were positively correlated with the observed improvement in EBPR activities in reactors.

Although WAS was stored in the cold without addition of substrates to maintain PAOs, positive effects from the addition of WAS to the two reactors were still observed. Previous studies have found that anaerobic starvation yielded trivial decay rates for *Accumulibacter* PAOs (Lopez et al. 2006, Lu et al. 2007), and EBPR capacities of sludge after anaerobic starvation were independent of the length of starvation period (Lopez et al. 2006). Similarly, GAOs could also endure anaerobic starvation without significant biomass decay (Vargas et al. 2013). When external carbon source was limiting, organisms could enter dormancy where they maintained a low metabolic state to prolong their survival. Once carbon became available and the conditions became more favorable, these organisms could recover their activities (Jones and Lennon 2010, Kaprelyants et al. 1993). Thus, we hypothesized that the stored WAS contained some dormant yet functional PAOs and GAOs.

Indeed, when the stored WAS was returned to the reactors where carbon became available and the environment became more favourable, these dormant PAOs and GAOs could revert to their normal metabolic state. This could explain the eventual recovery of EBPR and an increase in number of PAOs, especially *Accumulibacter*, in both RA and RC following addition of stored WAS (Fig. 1, 2, 3, 4A), despite the length of WAS storage under apparent anaerobic starvation prior to actual usage. Additionally, the ability of GAOs to withstand starvation and recoup their activities once carbon was reintroduced, a phenomenon that was also observed by Vargas et al. (2013), was evident in *Defluviicoccus* cluster 2 which also proliferated in RA and RC in the post-WAS period (Figure 3, Figure 4B). While we did not characterize the stored WAS prior to the sludge replacement, the possible presence of internal glycogen and PHA might have contributed to the endurance of PAOs/GAOs in the stored WAS.

In addition to starvation, prolonged exposure to low temperature (4 °C) did not seem to adversely affect PAOs in the stored sludge. This could be due to the lower mesophilic range of PAOs (Lopez-Vazquez et al. 2007, Panswad et al. 2003). Indeed, lower temperatures have been reported to favor PAOs (Oehmen et al. 2007), where dominance of PAOs could be observed at 10 °C (Lopez-Vazquez et al. 2009). One possible reason for this was the lower maintenance requirement for this group of organisms at temperatures lower than 30°C (Lopez-Vazquez et al. 2007), which enabled them to be less inhibited at lower temperatures (Lopez-Vazquez et al. 2006). This trait could contribute to PAOs' ability to recover their EBPR capacities and flourish following the addition of WAS back into the reactors.

In contrast, GAOs are mesophilic organisms that thrive at warmer temperatures. Mesophilic organisms could suffer a 20 to 80 times reduction in activities when temperatures dropped from 37 °C to 0 °C (Struvay and Feller 2012). This could potentially explain the decrease in abundance of *CPB_S60* or lack of consistent increases in most GAO genera in both RA or RC when the stored WAS was returned to the reactors. Despite the high operating temperature that should have favored

GAOs over PAOs (Panswad et al. 2003, Whang and Park 2002, 2006), only *Defluviicoccus* cluster 2 increased in both reactors after WAS was added to the reactors. Proliferation of *Defluviicoccus* cluster 2 upon WAS addition suggested this genus was capable of surviving prolonged starvation and storage at temperatures as low as 4 °C.

Interestingly, the increase of this GAO genus was positively correlated with improved activities in RA and RC. It is possible that the carbon limitation, which could limit PAO activities, had not yet occurred. We observed that the amount of P released for every mole of acetate consumed after major WAS addition was close to the values obtained by PAO biochemical models, which were 0.2 moles P/mole C (Pereira et al. 1996), or 0.5 moles P/mole C (Comeau et al. 1986, Smolders et al. 1994). This result suggested that more acetate consumption was linked to P release, indicative of typical PAO metabolism under anaerobic conditions. Thus, the growth of *Defluviicoccus* cluster 2 did not interfere with EBPR as long as acetate concentrations were sufficient to support PAO activities.

While a positive effect of lower temperature on EBPR raises the possibility of forgoing the sludge replacement with WAS for a simpler step of cooling the failing system, it is imperative that the deteriorating system still contain PAOs. If failure occurs due to washout of PAOs, simply cooling the system might not be sufficient. Adding WAS from a healthy system, which still contains functional PAOs that benefit from storage at lower temperature, can ensure PAOs are present in the system and revive failing EBPR even when failure is caused by PAOs being washout from the system.

5. Conclusions

- (1) Stored WAS contained functional PAOs, even though the WAS had undergone long-term anaerobic starvation.

- (2) Storage at low temperature can benefit PAOs over some GAOs in the WAS, enabling them to recover their activities and even outcompete some GAOs when they are returned to EBPR systems where temperatures are higher.
- (3) While the addition of WAS lead to proliferation of *Defluviicoccus* cluster 2, this did not automatically lead to EBPR failure. However, this trend could be problematic in full-scale systems where carbon is often limiting. Further studies to control their proliferation are still needed to obtain an optimized WAS addition method for full-scale application.
- (4) Optimized WAS addition could be used as a cheap and simple strategy to resuscitate failing EBPR systems.

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Supplementary Information

Potential of wasted activated sludge (WAS) addition to reseed failing

EBPR systems

APPENDIX A

Reactors Operation History

A double-jacketed sequential batch reactor (SBR) with a working volume of 5.44 L was inoculated with freshly collected returned activated sludge (RAS) from a wastewater reclamation plant (WRP) at a 1:1 (v:v) sludge to synthetic wastewater ratio. This WRP is a plant with a designed Modified Ludzack Ettinger (MLE) configuration for their aeration tanks and the year-long operating temperature of 30 ± 1 °C. Despite the operational conditions that theoretically would not sustain EBPR, this plant consistently exhibited successful EBPR.

This SBR, hereby referred to as R0, was continuously subjected to six hour anaerobic/aerobic cycles, with each cycle consisted of 130 min of anaerobic stage, including the first 5 minutes of feeding; 160 minutes of aerobic stage; 1 minute of sludge discharge; 30 minutes of settling; and 39 minutes of supernatant decant. Hydraulic retention time (HRT) and sludge retention time (SRT) were maintained at 12 hours and 14 days, respectively. Temperature of the reactor was set at 30 ± 1 °C, and dissolved oxygen at 2.0 ± 0.1 mg/l O₂. The pH of the system was kept at 7.25 ± 0.25 by addition of 0.5M HCl or 0.5M NaOH, as necessary. Synthetic wastewater was prepared following the recipe by Lu et al. (2006), with acetate as the external carbon source. A COD:P ratio of approximately 20:1 (mg COD/mg P) was adopted throughout the operation of R0, which ran for 179 days before the sludge was evenly split to start two systems: Reactor A (RA) and Reactor C (RC).

Following their inoculation, RA and RC were still subjected to anaerobic/aerobic cycling with other operating conditions (pH, DO, temperature) maintained for an additional 117 days until Day 296, when anaerobic/aerobic was replaced by planned anoxic/aerobic cycling. Several changes have been deliberately applied to both reactors (Table S1). As an effort to minimize the risk of carbon limitation, the COD:P ratio was increased to approximately 25:1 (mg COD/mg P) on Days 216-235. Additionally, starting from Day 235, acetate and non-acetate components of the feed were separated during the preparation step and only mixed immediately before the feed entered the reactors in each of the feeding stage. This practice was adopted as a precautionary step against the risks of unwanted microbial growths in the feed. Due to an unintended underestimation of the amount of P in the feed, the COD:P ratio used after the acetate and non-acetate feed component segregation was 18:1 (mg COD/mg P), and this ratio was maintained ever since. On Day 239, sludge from RA and RC were mixed and re-split. This was done as an attempt to increase reproducibility. Days 239 (after the sludge mixing and re-splitting) to 296. On Day 296, anaerobic/aerobic cycling was replaced with the simultaneous presence of N and C/aerobic condition and maintained until Day 339. Following unexplained loss of activities in RC on Days 337-339, conditions in both reactors were switched back to anaerobic/aerobic cycling on Day 342, and pre-WAS addition period began until major sludge replacement was done on Day 361, marking the beginning of post-WAS addition period (Table S1).

Table S1. History of operational changes in reactors RA and RC from their inoculation with sludge from R0 on Day 179 until the end of this study (Day 471).

Day ^a	Change applied	COD:P (mg/mg)
179	Sludge from reactor R0 was split to inoculate RA and RC ^{1, 2}	20:1 ^b
216-235	COD:P ratio in RA and RC was increased to 25:1	25:1 ^b
235	COD:P ratio changed to 18:1.	18:1 ^c
239	Sludge from RA and RC was combined and re-distributed to aid reproducibility in reactors.	18:1 ^c
239-296	Anaerobic/aerobic cycling was maintained. No additional changes were applied.	18:1 ^c
296-339	Anaerobic/aerobic cycling was replaced with simultaneous N and C/aerobic cycling. Other operating conditions remained unchanged.	18:1 ^c
342	Cycling was returned to anaerobic/aerobic and maintained ever since. Pre-WAS addition period began (1 st cycle study conducted on Day 351).	18:1 ^c
352	Minor WAS addition (400 mL WAS) was conducted	18:1 ^c
361	Major sludge replacement with WAS; post-WAS addition period started (1 st cycle study conducted on Day 368)	18:1 ^c
471	Study completed	18:1 ^c

¹ Each reactor received approximately 50% sludge from R0.

² Sludge discharge was disabled on Days 179 to 182 (10 cycles) to compensate for the mixed liquor reduction following the split of R0, and again on Days 199-200 (3 cycles).

^a The day when each change was applied, as counted from the day R0 was inoculated (Day 0). Some changes were maintained once applied.

^b Acetate and non-acetate components of the feed were mixed during preparation.

^c Acetate and non-acetate feed were prepared separately and only mixed immediately before the feed entered the system.

Table S2. Multivariate analysis of whole bacterial communities detected in RA and RC in Phase 1 (pre-WAS addition) compared to those observed in Phases 2, 3, or 4 of the post-WAS period to determine if the microbial populations diverged after WAS addition. Four samples were included in each phase. Results were generated with Primer 6 software. Raw data were standardized by the total counts of all bacteria in each sample to account for variations in sequencing depth. Standardized data was square root transformed to minimize the effects of dominant OTUs, and Bray-Curtis similarity was then constructed.

Reactor	Phase	PERMANOVA ¹		PERMDISP ²	
		P(MC)	Pseudo-F	P	F
RA	1 vs. 2	0.02	4.3	0.1	4.2
RA	1 vs. 3	0.001	9.8	0.03	9.7
RA	1 vs. 4	0.005	6.9	0.2	1.9
RC	1 vs. 2	0.06	3.1	0.7	0.6
RC	1 vs. 3	0.003	8.4	0.3	1.6
RC	1 vs. 4	0.003	7.9	0.9	0.1

¹ Null hypothesis that there was no difference between the bacterial population in the compared phases were rejected if $p < 0.05$. P(MC) denoted p-value obtained using Monte Carlo test due to the small number of unique permutation for our samples (unique permutation = 35).

² Dispersion of the bacterial communities in each of the compared phases was homogenous when $p > 0.05$.

Table S3. Multivariate analysis of PAO communities detected in RA and RC before WAS addition (Phase 1) compared to Phases 2-4 of post-WAS period to determine if the PAOs populations diverged after WAS addition. Four samples were included in each phase. Results were generated with Primer 6 software. Raw data were standardized to the total counts of OTUs belonging to only PAOs in each sample to account for variations in sequencing depth. Standardized data were square root transformed to minimize the effects of dominant OTUs, and the Bray-Curtis similarity was then constructed.

Reactor	Community group	Phase	PERMANOVA ¹		PERMDISP ²	
			P(MC)	Pseudo-F	P	F
RA	PAO	1 vs. 2	0.04	6.0	0.5	0.3
RA	PAO	1 vs. 3	0.01	10.2	0.03	21.2
RA	PAO	1 vs. 4	0.03	7.04	0.5	1.8
RC	PAO	1 vs. 2	0.7	0.2	0.8	0.05
RC	PAO	1 vs. 3	0.01	10.6	0.03	9.1
RC	PAO	1 vs. 4	0.01	10.4	0.05	6.5

¹ Null hypothesis that there was no difference between the bacterial population in the compared phases were rejected if $p < 0.05$. P(MC) denoted p-value obtained using Monte Carlo test due to the small number of unique permutation for our samples (unique permutation = 35).

² Dispersion of the bacterial communities in each of the compared phases was homogenous when $p > 0.05$.

Table S4. Multivariate analysis of GAO communities detected in RA and RC in Phase 1 versus Phases 2, 3, or 4 to assess if GAOs community diverged after WAS addition. Four samples were included in each phase. Results were generated with Primer 6 software. Raw data was standardized by the total counts of OTUs belonging to GAOs community only in each sample to account for variations in sequencing depth. Standardized data was square root transformed to minimize the effects of dominant OTUs, and Bray-Curtis similarity was then constructed.

Reactor	Community group	Phase	PERMANOVA ¹		PERMDISP ²	
			P(MC)	Pseudo-F	P	F
RA	GAO	1 vs. 2	0.05	4.3	0.2	2.8
RA	GAO	1 vs. 3	0.004	12.4	0.1	4.6
RA	GAO	1 vs. 4	0.008	7.9	0.1	4.7
RC	GAO	1 vs. 2	0.3	1.1	0.5	0.4
RC	GAO	1 vs. 3	0.09	2.8	0.6	1.3
RC	GAO	1 vs. 4	0.006	6.5	0.5	2.3

¹ Null hypothesis that there was no difference between the bacterial population in the compared phases were rejected if $p < 0.05$. P(MC) denoted p-value obtained using Monte Carlo test due to the small number of unique permutation for our samples (unique permutation = 35).

² Dispersion of the bacterial communities in each of the compared phases was homogenous when $p > 0.05$.

Table S5. Spearman correlation analysis between total PAOs, total GAOs, or some of their genera that exhibited similar trends in both reactors, and the observed changes in activities in RA (N = 18) and RC (N = 16). Activities were represented by P released/acetate consumed, in moles P/moles C. Relative abundances for all PAOs, GAOs, or some of the genera included in the analysis were calculated with respect to all bacteria.

Reactor	Organisms	Spearman's ρ^1	p- value ²	Reactor	Organisms	Spearman's ρ^1	p- value ²
RA	All PAOs	0.7	0.001	RC	All PAOs	0.7	0.001
RA	All GAOs	-0.1	0.6	RC	All GAOs	-0.1	0.8
RA	<i>Accumulibacter</i>	0.7	0.001	RC	<i>Accumulibacter</i>	0.7	0.001
RA	<i>Defluviicoccus</i> cluster 2	0.5	0.05	RC	<i>Defluviicoccus</i> cluster 2	0.6	0.006
RA	<i>CPB_S60</i>	-0.4	0.1	RC	<i>CPB_S60</i>	-0.3	0.2
RA	<i>Dechloromonas</i>	-0.7	0.001	RC	<i>Dechloromonas</i>	-0.8	0.0001

¹ Negative value indicated a negative correlation between the relative abundances and the P released/acetate consumed.

² P-value > 0.05 suggested that the correlation was not statistically significant.

Figure S1. Summary of P concentrations (in mg P/L) detected at the beginning of cycle (feed start), end of anaerobic (or anoxic), and end of aerobic stage of the samples collected during cycle studies for RA and RC. This summary encompassed the period from RA and RC inoculation to the last study conducted (Day 339) before pre-WAS phase began on Day 342.

CHAPTER 5

CONCLUSIONS

5.1. Research Objectives

Enhanced biological phosphorus removal (EBPR) is an economical and environmentally friendly phosphorus removal method that has been widely used in wastewater treatment plants worldwide. In Chapter 1, I have reviewed information regarding types of organisms and metabolisms in an EBPR system, as well as the effect of several operating parameters on the activities and microbial communities of an EBPR system. Among these parameters were temperature and the presence of NO_x compounds. There have been contradictory observations on how these parameters impact an EBPR system and its communities, suggesting that there is still a gap in our understanding of the effects of these parameters on EBPR. Moreover, potential consequences brought on by the combination of these two factors (that is, simultaneous presence of carbon and nitrate and/or nitrite at warm temperature) have not been determined. Thus, the main objectives of this dissertation related to the feasibility of EBPR under anoxic/aerobic cycling when temperatures are higher. Additionally, a strategy to revive failing EBPR system operated at elevated temperature was also tested.

Closing that gap would be beneficial in tropical regions, especially if EBPR were to be incorporated into existing plants without designed anaerobic tank, since this could potentially eliminate the need to retrofit the biological treatment trains. Additionally, a readily available and affordable approach to quickly mitigate faltering EBPR systems could reduce their down time. This could be advantageous for lab-scale reactors that are used for various EBPR studies as well as for full-scale plants because it could minimize the risks of service disruptions.

5.2. Implications of Dissertation

The occurrence of EBPR in full-scale plants without designed anaerobic tank might be due to the presence of anaerobic zones in the supposedly anoxic compartment, or represent the

contribution of non-DPAOs. In Chapter 2, I have shown via batch experiments that PAOs, especially non-DPAOs in the full-scale sludge, could successfully carry out anoxic/aerobic EBPR based on their inability to respire nitrate, at 30 °C and at lower abundance of PAOs in the full-scale sludge than in many other studies utilizing PAO-enriched sludge. This finding challenged the notion that an anaerobic stage is strictly required for EBPR.

The potential of EBPR under anoxic/aerobic cycling at warm temperatures was further corroborated in Chapter 3, where I observed a lack of direct inhibitory effect of the simultaneous presence of acetate, nitrate, and produced nitrite on EBPR. However, decreased activities were observed as the study progressed. This was likely caused by carbon limitation due to the proliferation of competitors such as certain GAOs and non-PAO denitrifiers. In this study, the abundance of *Defluviicoccus* cluster 2 increased most distinctly while *Competibacter* remained low, suggesting a selective advantage for the former due to the concomitant presence of acetate, nitrate and produced nitrite.

In contrast, Law et al. (2016) reported a higher abundance of *Competibacter* while *Defluviicoccus*-related organisms were absent in a full-scale plant in Singapore with modified Ludzack Ettinger (MLE) configuration. Nitrite was low (< 1 mg/L N) in this particular plant (Cokro et al. 2017). Proliferation of *Competibacter* with negligible *Defluviicoccus*-GAOs was also reported by Ong et al. (2014a), where sequential batch reactors (SBRs) were operated with anaerobic/aerobic cycling at 28-32 °C. This result raised the possibility that the concomitant presence of nitrite and acetate, or lack thereof, might contribute to proliferation of different GAOs.

The discrepancy in the types of GAO that proliferated in a system suggested that the effect of anoxic/aerobic cycling at higher temperature might not be universal for all types of GAOs. Thus, it is crucial to determine which GAOs dominate in EBPR systems prior to the adoption of anoxic/aerobic cycling since different GAOs may interact differently with PAOs. For example,

Defluviicoccus-GAOs were stronger competitors for *Accumulibacter* PAOs under carbon limitation than was *Competibacter* (Seviour and Nielsen 2010b), which likely co-existed with PAOs in another study (Law et al. 2016).

In Chapter 4, I have shown the potential of adding wasted activated sludge (WAS) to resuscitate flagging EBPR systems at elevated temperature. In this dissertation, I confirmed the benefits of adding WAS to resuscitate failing EBPR system. Hence, this method could be considered as a readily available first aid strategy to revive PAOs and EBPR activities.

The overall findings of this dissertation have supported the notion that anoxic/aerobic EBPR is possible at warm temperature. However, the feasibility of this condition will likely depend on several things such as the community profiles (i.e., types of GAOs, PAOs, and other organisms that are present in the system) and substrate availabilities. Moreover, the behaviour of an EBPR system when subjected to prolonged continuous exposure to certain conditions might be different than when subjected to short-term exposure (batch experiments). Hence, it might not be sufficient to solely rely on short-term batch studies to infer the effects of certain conditions on EBPR.

5.3. Future Works

Future studies on anoxic/aerobic EBPR at warm temperatures should also be done using sludge where different types of PAOs, especially with different metabolisms than *Accumulibacter* (e.g., *Tetrasphaera*) dominate. Moreover, while my studies covered an extended period (43 days) of exposure to anoxic/aerobic cycling, longer enrichments should also be tested. Additionally, optimization studies need to be conducted to determine the strategy that selected PAOs over certain types of GAOs under anoxic/aerobic cycling at warm temperature, such as using a combination of acetate and propionate as carbon sources to select for PAOs (Lopez-Vazquez et al. 2009).

Future studies should also cover the applicability of WAS addition as mitigation strategy under anaerobic/anoxic/aerobic, or anoxic/aerobic cycling conditions to better understand whether

the addition of WAS could be adopted as a “one size fits all” strategy to resuscitate failing EBPR systems at warm temperatures.

Lastly, I hope the findings presented in this dissertation can provide insights into the potential of EBPR at elevated temperature, especially when there is no designed anaerobic stage in the plant design.

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