Mónica Isabel Gonçalves Carvalheira

Mestre em Engenharia Química e Bioquímica

The effect of key process operational conditions on enhanced biological phosphorus removal from wastewater

Dissertação para obtenção do Grau de Doutor em
Química Sustentável

Orientador: Maria d'Ascensão Miranda Reis, Professora Catedrática, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Co-orientadores: Adrian Michael Oehmen, Investigador Auxiliar, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Gilda de Sousa Carvalho, Investigador Pós-Doutoramento, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Júri:

Presidente: Prof. Doutora Ana Isabel Nobre Martins Aguiar de Oliveira Ricardo

Arguentes: Prof. Doutor Eugénio Manuel de Faria Campos Ferreira

Doutor António Manuel Pedro Martins

Vogais: Prof. Doutora Maria d'Ascensão Carvalho Fernandes de Miranda Reis

Doutora Nídia Dana Mariano Lourenço de Almeida

Doutor Adrian Michael Oehmen

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Dezembro, 2014

The effect of key process operational conditions on enhanced biological phosphorus removal from wastewater

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer à minha orientadora, Maria Ascensão Reis, pela oportunidade de realizar este trabalho, por todo o seu apoio, dedicação, partilha de conhecimento e entusiasmo transmitido durante todos estes anos.

Aos meus co-orientadores, Adrian Oehmen e Gilda Carvalho, agradeço toda a disponibilidade em ouvirem as minhas dúvidas e questões (e que não foram poucas), por todo o apoio e dedicação que me prestaram durante todo o trabalho, por todas as discussões e partilha de conhecimento que me motivaram cada vez mais, e principalmente pela amizade e preocupação que sempre demonstraram. Muito Obrigada!

Aos meus colegas do grupo BPEG agradeço a boa disposição, a entreajuda, as criticas e sugestões que me enriqueceram bastante ao longo destes anos e que me ajudaram a concretizar este trabalho. Em especial quero agradecer ao Ricardo Marques, pela amizade e ajuda nos momentos mais trabalhosos do meu doutoramento. À Luisa Neves, Carla Daniel, Cristiana Torres, Joana Pais, Catarina Oliveira, Christophe Roca e Filipe Ataíde pelos momentos de boa disposição, descontração, apoio e principalmente pela amizade! Muito Obrigada a todos! Sem vocês tinha sido muito complicado.

Ao meu "grupo de almoço" (Ricardo, Rui, Filipe, Catarina, Sílvia, Rodolfo, Margarida) quero agradecer pelos momentos de descontração e de boa disposição que me ajudaram a ultrapassar os piores momentos no laboratório.

Aos meus amigos Raquel Viveiros, Henrique Marçal, Joana Dias, Marco Araújo e Franklin Nóbrega muito obrigada pelo apoio incondicional durante estes anos, pela vossa presença nos piores momentos (mesmo estando mais longe) e principalmente pela vossa amizade! Muito Obrigada por tudo!

À minha mãe, irmão, tios, avós e primos, queria agradecer por todo o apoio, incentivo, carinho e motivação que me deram durante estes anos. E, principalmente, por me aturarem nos momentos mais complicados do trabalho. Muito Obrigada a todos!

À Fundação para a Ciência e Tecnologia agradeço o financiamento da bolsa.

ABSTRACT

Enhanced biological phosphorus removal (EBPR) is the most economic and sustainable option used in wastewater treatment plants (WWTPs) for phosphorus removal. In this process it is important to control the competition between polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), since EBPR deterioration or failure can be related with the proliferation of GAOs over PAOs. This thesis is focused on the effect of operational conditions (volatile fatty acid (VFA) composition, dissolved oxygen (DO) concentration and organic carbon loading) on PAO and GAO metabolism. The knowledge about the effect of these operational conditions on EBPR metabolism is very important, since they represent key factors that impact WWTPs performance and sustainability. Substrate competition between the anaerobic uptake of acetate and propionate (the main VFAs present in WWTPs) was shown in this work to be a relevant factor affecting PAO metabolism, and a metabolic model was developed that successfully describes this effect. Interestingly, the aerobic metabolism of PAOs was not affected by different VFA compositions, since the aerobic kinetic parameters for phosphorus uptake, polyhydroxyalkanoates (PHAs) degradation and glycogen production were relatively independent of acetate or propionate concentration. This is very relevant for WWTPs, since it will simplify the calibration procedure for metabolic models, facilitating their use for full-scale systems. The DO concentration and aerobic hydraulic retention time (HRT) affected the PAO-GAO competition, where low DO levels or lower aerobic HRT was more favourable for PAOs than GAOs. Indeed, the oxygen affinity coefficient was significantly higher for GAOs than PAOs, showing that PAOs were far superior at scavenging for the often limited oxygen levels in WWTPs. The operation of WWTPs with low aeration is of high importance for full-scale systems, since it decreases the energetic costs and can potentially improve WWTP sustainability. Extended periods of low organic carbon load, which are the most common conditions that exist in full-scale WWTPs, also had an impact on PAO and GAO activity. GAOs exhibited a substantially higher biomass decay rate as compared to PAOs under these conditions, which revealed a higher survival capacity for PAOs, representing an advantage for PAOs in EBPR processes. This superior survival capacity of PAOs under conditions more closely resembling a full-scale environment was linked with their ability to maintain a residual level of PHA reserves for longer than GAOs, providing them with an effective energy source for aerobic maintenance processes. Overall, this work shows that each of these key operational

conditions play an important role in the PAO-GAO competition and should be considered in WWTP models in order to improve EBPR processes.

KEYWORDS: Enhanced biological phosphorus removal; polyphosphate accumulating organisms; glycogen accumulating organisms; substrate competition; dissolved oxygen concentration; organic carbon load.

RESUMO

A remoção biológica de fosforo (EBPR) é a opção mais económica e sustentável usada nas estações de tratamento de águas residuais (ETARs) para a remoção de fósforo. Neste processo é importante controlar a competição entre os organismos acumuladores de fósforo (PAOs) e os organismos acumuladores de glicogénio (GAOs), uma vez que a deterioração ou falha do EBPR pode estar relacionada com a proliferação dos GAOs em relação aos PAOs. Esta tese tem como objectivo estudar o efeito das condições operacionais (composição dos ácidos gordos voláteis (AGV), concentração de oxigénio dissolvido (OD) e a carga de carbono orgânico) no metabolismo dos PAOs e GAOs. O conhecimento sobre o efeito destas condições operacionais no metabolismo do EBPR é muito importante, uma vez que representam fatores-chave com impacto no desempenho e sustentabilidade das ETARs. A competição pelo substrato entre acetato e propionato (os principais AVG presentes nas ETARs) em anaeróbiose, mostrou ser um fator relevante que afeta o metabolismo dos PAOs, e foi desenvolvido um modelo metabólico que descreve com sucesso este efeito. Os resultados indicaram que o metabolismo aeróbio dos PAOs não foi afetado pelas diferentes composições dos AGV, uma vez que os parâmetros cinéticos aeróbios para a absorção de fósforo, degradação de polihidroxialcanoatos (PHA) e produção de glicogénio foram relativamente independentes da concentração de acetato e propionato. Este aspecto permitirá simplificar os procedimentos de calibração dos modelos metabólicos, facilitando o seu uso em ETARs de grande escala. A concentração de OD e o tempo de retenção hidráulico (TRH) aeróbio afetam a competição entre PAO e GAO, onde baixos níveis de OD ou menores TRH aeróbio foram mais favoráveis para os PAOs do que para os GAOs. De facto, o coeficiente de afinidade para o oxigénio foi significativamente maior para os GAOs do que para os PAOs, mostrando que os PAOs têm vantagem competitiva na utilização de oxigénio, mesmo para os níveis limitantes de oxigénio que frequentemente ocorrem em ETARs. A operação das ETARs com baixos caudais de arejamento é muito importante para os sistemas de grande escala, uma vez que diminui os custos energéticos e pode, potencialmente, melhorar a sustentabilidade das ETARs. Longos períodos de operação com baixa carga de carbono orgânico, que são as condições mais comuns existentes em ETARs, também têm impacto na atividade dos PAOs e GAOs. Os GAOs exibiram uma taxa de decaimento de biomassa substancialmente maior do que os PAOs, sob estas condições, revelando a elevada capacidade de sobrevivência dos PAOs, e consequentemente vantagem em processos EBPR. Esta capacidade de

sobrevivência dos PAOs, em condições que se assemelham às condições em grande escala, foi relacionada com a sua capacidade de manter um nível residual de reservas de PHA durante mais tempo que os GAOs, proporcionando-lhes uma fonte eficiente de energia para os processos de manutenção aeróbios. Em geral, este trabalho mostra que cada uma das condições operacionais estudadas desempenha um papel importante na competição entre PAO e GAO e deve ser considerada nos modelos das ETARs, a fim de melhorar os processos EBPR.

PALAVRAS-CHAVE: Remoção biológica de fósforo; organismos acumuladores de fósforo; organismos acumuladores de glicogénio; competição de substrato; concentração de oxigénio dissolvido; carga de carbono orgânico.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOTATIONS AND ABBREVIATIONS

ATP	Adenosine Triphosphate Molecule
ASM	Activated Sludge Models
COD	Chemical Oxygen Demand
DNA	Desoxyribonucleic Acid
DO	Dissolved Oxygen
Dv-GAOs	*Defluviicoccus vanus-related* GAOs
EBPR	Enhanced Biological Phosphorus Removal
F/M	Food to Microorganism Ratio
FISH	Fluorescence *in situ* Hybridisation
GAO	Glycogen Accumulating Organisms
HAc	Acetate
HPr	Propionate
HRT	Hydraulic Retention Time
K_{O2}	Oxygen Affinity Constant
m_{ATP}	Aerobic Maintenance Coefficient
$m_{Glycogen}$	Aerobic Maintenance Coefficient for Glycogen Consumption
m_{PHA}	Aerobic Maintenance Coefficient for PHA Degradation
N_2O	Nitrous Oxide
NRMSD	Normalized Root Mean Squared Deviation
P	Phosphorus
PAO	Polyphosphate Accumulating Organisms
PH_2MB	Polyhydroxy-2-methylbutyrate
PH_2MV	Polyhydroxy-2-methylvalerate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PO_4^{-3}-P	Orthophosphate

PP	Polyphosphate
SBR	Sequencing Batch Reactor
SRT	Solid Retention Time
TCA	Tricarboxylic acid
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plants

SPECIFIC TERMS IN CHAPTER 3

f_{PAO_PHA}	Fraction of PHA in PAO
f_{PHA_max}	Maximum PHA content
$K_{S,Gly}$	Half-Saturation Coefficient for glycogen
$K_{S,fPHA}$	Half-saturation coefficient for the fraction of PHA in biomass
$K_{S,PP}$	Half-Saturation Coefficient for Polyphosphate (poly-P)
$K_{S\_HAc\_PAO}$	Half-Saturation Coefficient for Acetate Uptake
$K_{S\_HPr\_PAO}$	Half-Saturation Coefficient for Propionate Uptake
$q_{Glycogen}$	Glycogen production rate
q_{HAc}	Acetate uptake rate
q_{HPr}	Propionate uptake rate
q_{PHA}	PHA degradation rate
$q_{PO4\text{-}PP}$	Phosphorus uptake and Poly-P formation rate
$q_{smax\_PAO\_HAc}$	Maximum acetate uptake rate
$q_{smax\_PAO\_HPr}$	Maximum propionate uptake rate
$q_{smax\_PAO\_VFA}$	Maximum total VFA uptake rate
q_{VFA}	VFA uptake rate
S_{HAc}	Acetate concentration
S_{HPr}	Propionate concentration

X_{PAO}	Biomass concentration of PAOs
$X_{PAO,Gly}$	Glycogen concentration in PAO
$X_{PAO,PP}$	Polyphosphate concentration in PAO

1

MOTIVATION AND THESIS OUTLINE

1.1 MOTIVATION

The concern with water quality problems worldwide has led to the improvement of wastewater treatment processes, such as the enhanced biological phosphorus removal (EBPR) process. This process has been used to remove phosphorus from wastewater and, consequently, control eutrophication.

In EBPR process it is necessary to control the competition between polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), since GAOs are linked to the instability and deterioration of the EBPR process. Thus, it is important to find strategies which favour the proliferation of PAOs and suppress the growth of GAOs. Although, the impacts of several environmental and operational factors on this competition have been studied during the last years, the effect of other critical factors on PAO and GAO metabolism are still unknown. Since a combined acetate/propionate feed, low DO levels, high aerobic retention times (HRT) and low organic loading represent situations commonly encountered in wastewater treatment plants (WWTPs), the impact of these factors on the PAO-GAO competition were addressed in this work for the first time.

The main goals of this thesis were to:

1. Study the effect of different ratios of acetate and propionate on the anaerobic and aerobic kinetics of PAOs.
2. Study the effect of different DO levels on the PAO - GAO metabolism through short- and long-term tests, and the impact of extended aerobic HRTs on the microbial community in an EBPR system.
3. Assessment of the effect of a prolonged low carbon loading (mimicking a full-scale situation) on PAO and GAO metabolism and the impact of this factor on PAO and GAO activity and survival.

This knowledge is important to improve the prediction capability of the impact of these important parameters on EBPR performance, and optimise process activity and stability.

1.2 THESIS OUTLINE

The thesis includes six chapters, describing the work developed during this PhD project.

In the current chapter (chapter 1) the motivation and objectives of this thesis is presented. In chapter 2 a brief review concerning the EBPR process and PAO-GAO competition is provided. The research work performed during this PhD thesis has resulted in three scientific articles, presented in Chapters 3, 4 and 5, respectively. Each of these chapters is constituted by introduction, description of materials and methods, discussion of the results obtained and main conclusions.

Chapter 3 presents the study regarding the effect of a combined acetate/propionate feed on PAO metabolism. In this chapter, the effect of different ratios of acetate and propionate on anaerobic and aerobic kinetics of PAOs was addressed in order to better describe PAO activity through metabolic modelling.

Chapter 4 and 5 describe the studies performed to assess the effect of two critical operational conditions on the PAO-GAO competition. The aim of chapter 4 is to assess the impact of aeration on this competition. The effect of different DO concentrations on PAO and GAO aerobic metabolism, as well as the impact of increased aerobic retention time (from 3h to 9h) was studied. In chapter 5, the difference between anaerobic and aerobic activity of PAOs and GAOs, as well as their survival, under a prolonged low carbon load is discussed.

In chapter 6 the main conclusions of this PhD project are presented, as well as some suggestions for future work.

2

STATE OF THE ART

The sustainability of wastewater treatment plants (WWTPs) is an important issue and depends on the economic, social and environmental factors, where the selection and interpretation of these factors depends on the geographic area and demographic situation (Molinos-Senante et al., 2012; Muga and Mihelcic, 2008). The environmental effects in WWTPs, which can be positive or negative, are related with the energy consumption, utilisation of chemical reagents, sludge generation and emission of atmospheric pollutants (Molinos-Senante et al., 2012). Thus, in WWTPs the energy inputs necessary to achieve high quality wastewater effluent, as well as potentially negative environmental side-effects of the process (e.g. greenhouse gas production), should be minimised to improve the WWTPs sustainability.

The nutrients or contaminants removal from wastewater can be done through physical, chemical or biological processes or by combination of those. Comparing all, the biological process is the most economic, sustainable and efficient, so the use of this process to remove the organic matter, phosphorus and nitrogen from wastewater presents an advantage over the other options.

Phosphorus is an essential nutrient for the living organisms since it is part of adenosine triphosphate molecule (ATP) (molecule responsible for energy source in cellular processes), desoxyribonucleic acid (DNA) (biological molecule that contain the genetic material information), and the phospholipids in the cell membrane and the skeleton of all vertebrates (Filippelli, 2008). However, the excess of phosphorus in water systems promotes the proliferation of algae and other photosynthetic microorganisms, a process known as eutrophication (Seviour et al., 2003). This phenomenon creates one of the largest water quality problems worldwide. Eutrophication increases the water purification costs, interferes in the recreational and conservational value of impoundments, is harmful to aquatic life, and eutrophic water supplies cannot be used for drinking purposes, since it is sub-lethal for humans due to the presence of algal toxins (Mullan et al., 2002). For these reasons, it is important and necessary to control eutrophication in water systems, and phosphorus removal from wastewater is considered to be the best strategy (Wang et al., 2013). As phosphorus is the limiting nutrient for algae growth (Mullan et al., 2002), eutrophication is more affected by high phosphorus levels than high nitrogen levels. Thus, there is a growing awareness worldwide to minimise the emissions of phosphorus to water systems and, consequently, improve wastewater treatment.

2.1 ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR)

Enhanced biological phosphorus removal (EBPR) is a biological process used for phosphorus removal from wastewater without the use of chemical precipitants, becoming a sustainable and economic option for wastewater treatment (Lopez-Vazquez et al., 2009b; Oehmen et al., 2007). In this process, the activated sludge is recirculated through anaerobic and aerobic/anoxic conditions to promote the enrichment of polyphosphate accumulating organisms (PAOs), which are the bacteria responsible for the EBPR process (Figure 2.1). One of the most important groups of PAOs is *Candidatus* Accumulibacter phosphatis, as these microorganisms are able to store P as intracellular polyphosphate. Phosphorus removal from wastewater is obtained through the harvesting of PAOs containing polyphosphate in waste activated sludge (Oehmen et al., 2007).

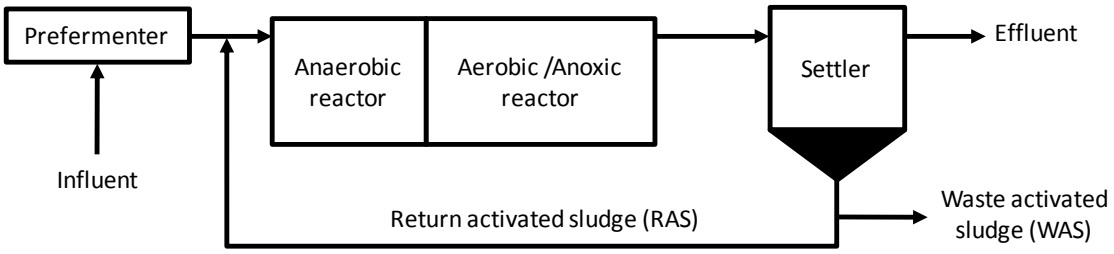


Figure 2.1 – Conceptual design of the EBPR process.

These PAOs, contrarily to other ordinary aerobic heterotrophs, have the ability to take up the volatile fatty acids (VFAs) in anaerobic conditions, and store them as polyhydroxyalkanoates (PHAs). The energy required for VFA uptake is provided by hydrolysis of intracellular polyphosphate, which results in phosphorus release into the bulk medium. The reducing power necessary for PHA formation and storage is largely produced by the glycolysis of glycogen (Figure 2.2), although recent studies have increasingly recognized the role of the tricarboxylic acid (TCA) cycle anaerobically in the generation of reducing equivalents (Lanham et al., 2013; Majed et al., 2012; Pijuan et al., 2008; Zhou et al., 2009). A decrease in glycogen concentration and polyphosphate content is observed anaerobically, along with an increase in phosphorus and PHA concentrations (Figure 2.3). In aerobic conditions, PAOs use the stored PHA as energy source for phosphorus uptake and polyphosphate storage, glycogen production and biomass growth (Figure 2.2), leading to the decrease of PHA

content and the increase of glycogen and polyphosphate content (Figure 2.3) (Oehmen et al., 2007). The major fraction of phosphorus removal achieved by the EBPR process is obtained through aerobic conditions. However, in anoxic conditions, some PAOs (i.e. denitrifying PAOs) are able to remove phosphorus from wastewater using nitrate or nitrite, instead oxygen, as electron acceptors.

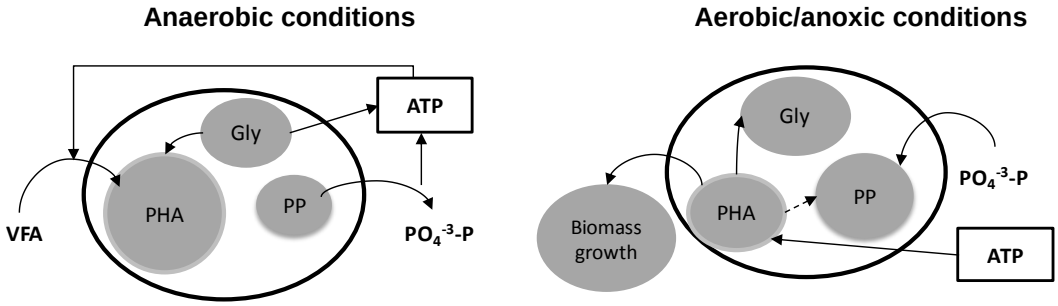


Figure 2.2 – Anaerobic and aerobic/anoxic metabolism of PAOs (Gly: Glycogen; PP: polyphosphate; PO_4^{-3}-P: orthophosphate).

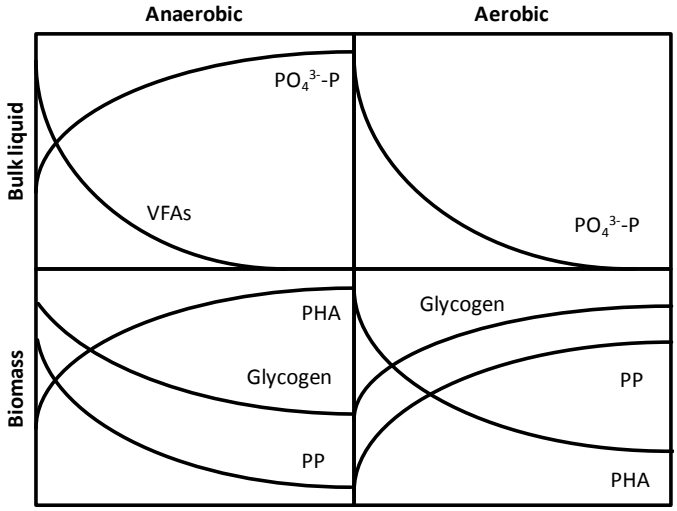


Figure 2.3 – Schematic representation of carbon and phosphorus transformation in the EBPR process (PP: polyphosphate; PO_4^{-3}-P: orthophosphate).

The EBPR process has several advantages comparing with conventional activated sludge treatment. One of them is that, when EBPR is operated successfully, it is possible to remove >90% of influent phosphorus, while using conventional activated sludge the removal lies between 20 – 40% (Mullan et al., 2002). Relatively to sludge production, the EBPR process is also advantageous, due to the fact that it

produces 20% less sludge when compared with chemical precipitation (Mullan et al., 2002; van Loosdrecht et al., 1997). Since it produces less sludge, the EBPR process is more cost-effective, achieving a reduction of 25% in energy costs (Mullan et al., 2002). Furthermore, EBPR is an environmentally friendly process, since the use of chemical precipitants is not necessary.

Nevertheless, the performance of this process is frequently represented as unstable and unreliable. In several EBPR plants process upsets were observed, leading to deteriorated performance and failure, resulting in violations relative to discharge regulations (Oehmen et al., 2007). Often, these deteriorations are compensated by the addition of chemical precipitants, to avoid excess P discharges, but this increases the costs and sludge production, as described above. These process upsets can be related with external factors, such as high rainfall, nutrient limitation or excessive aeration that affect the intracellular polymers (PHAs and glycogen) and, consequently, the phosphorus removal efficiency (Brdjanovic et al., 1998; Oehmen et al., 2007). The presence of nitrate in the anaerobic reactor can also affect the EBPR performance, since the presence of nitrate and VFAs simultaneously induces the proliferation of ordinary heterotrophic denitrifiers that grow faster than PAOs, decreasing the availability of VFAs for PAOs, and consequently, decreasing phosphorus removal (Puig et al., 2007). However, the deterioration, suboptimal operation or failure of EBPR process can also be related with the presence of another group of microorganisms, known as glycogen accumulating organisms (GAOs) (Lopez-Vazquez et al., 2009b). The most important group of GAOs is *Candidatus* Competibacter phosphatis, which belongs to the *Gammaproteobacteria*-GAOs group. Like PAOs, these organisms thrive under anaerobic and aerobic conditions, however they are not able to store polyphosphate (Oehmen et al., 2007). GAOs also have the ability to take up VFAs in anaerobic conditions and store them as PHAs. However, as GAOs are unable to accumulate polyphosphate, the energy and reducing power for VFA uptake and PHA formation comes from glycogen hydrolysis (Figure 2.4). For this reason it is observed a decrease in intracellular glycogen content and an increase in PHA concentration (Figure 2.5). Under aerobic conditions, GAOs utilise the PHA for cell growth and glycogen replenishment (Figure 2.4), resulting in an increase in glycogen concentration and consequent PHA decrease (Figure 2.5) (Dai et al., 2007; Oehmen et al., 2007). As GAOs compete with PAOs for the anaerobic uptake of VFAs without contributing to phosphorus removal, they are undesirable organisms in EBPR systems (Oehmen et al., 2007). This competition causes the increase of VFAs

requirements, so the minimisation of GAO growth in EBPR systems is very important because it increases the cost-effectiveness of this process (Oehmen et al., 2007).

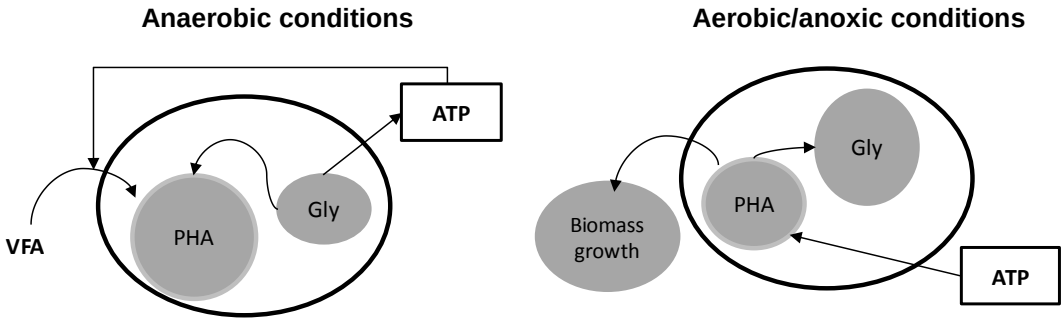


Figure 2.4 – Anaerobic and aerobic/anoxic metabolism of GAOs (Gly: Glycogen).

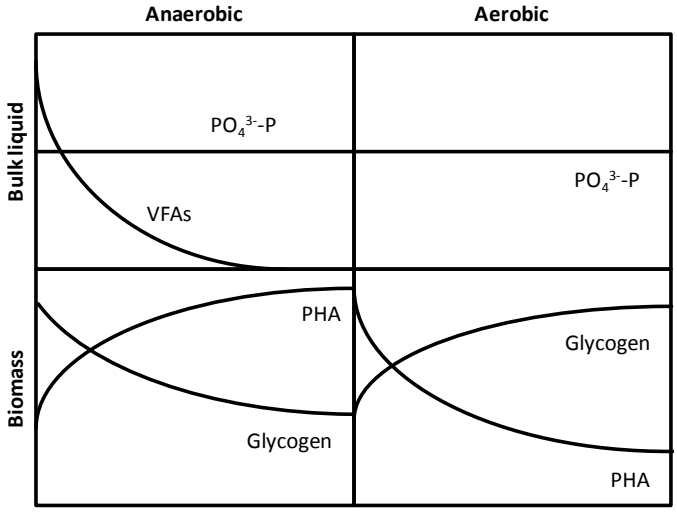


Figure 2.5 – Schematic representation of GAO carbon transformation in the EBPR process (PO_4^{-3}-P: orthophosphate).

Accumulibacter PAOs (Crocetti et al., 2000) and *Competibacter* GAOs (Crocetti et al., 2002; Kong et al., 2002) are both present in full scale plants. While the presence of *Accumulibacter* has been observed in most plants, the relative fraction of *Competibacter* varies with the type of WWTP (Lanham et al., 2013; Saunders et al., 2003; Zhang et al., 2011). Another group of GAOs, known as *Defluviicoccus vanus-related GAOs* (Dv-GAOs) and belonging to the *Alphaproteobacteria*-GAOs, was also detected in full-scale systems, however in low abundance (Burow et al., 2007; Meyer et al., 2006). Dv-GAOs comprise four clusters: cluster I and II (Meyer et al., 2006; Wong et al., 2004), which are the most studied, and cluster III (Nittami et al., 2009) and IV

(McIlroy and Seviour, 2009), which were discovered more recently. *Tetrasphaera* are another group of organisms that are present in WWTP, often at higher abundances than PAOs, and participate in EBPR (Nguyen et al., 2011). However, these organisms only have a partial PAO phenotype, since they take up phosphorus and store it as polyphosphate, but do not take up VFAs or store PHAs, thus they are still considered putative PAOs (Kristiansen et al., 2013; Nguyen et al., 2011). In EBPR processes identification of the microbial community, mainly which type of PAOs and GAOs are present, can be used to help optimise the wastewater treatment, since knowing the population present in the sludge, it is possible to operate the systems using the optimal operational conditions for this specific population and improve the performance of the wastewater treatment.

2.2 PAO-GAO COMPETITION: IMPACT FACTORS

In EBPR processes it is necessary to control the microbial competition between PAOs and GAOs in order to minimise the growth of GAOs and to achieve a high efficiency in phosphorus removal. Several environmental and operational factors have been often studied and identified as key factors in the PAO-GAO competition. These factors are: pH, temperature, VFA composition of the wastewater, sludge age, the ratio of phosphorus to organic carbon in the influent and the substrate feeding rate.

2.2.1 pH EFFECT

Several studies showed that a higher pH (> 7.25) is more beneficial for PAOs, improving the phosphorus removal efficiency and, consequently, the EBPR performance (Filipe et al., 2001; Oehmen et al., 2005a; Schuler and Jenkins, 2002). Filipe et al. (2001) observed that at pH of 7.25, the specific acetate uptake rate was the same for PAOs and GAOs. However when the pH in the anaerobic zone was maintained lower than 7.25, GAOs take up acetate faster than PAOs, while at pH higher than 7.5, PAOs removed acetate faster than GAOs. In this study, it was also observed that, for a complete phosphorus removal, it was necessary to assure a minimum pH value of 7.25 in the system. Thus, increasing the pH in the anaerobic zone promotes the improvement of phosphorus removal processes, since the conditions for an efficient use of the VFAs available for phosphorus removal are created. Oehmen et al. (2005a) observed that at a pH of 7.0, *Competibacter* was the

dominant population in an acetate-fed reactor, while *Alphaproteobacteria*-GAOs was the dominant population in a propionate-fed reactor. When the pH was increased to 8, the population of GAOs decreased in both reactors and the P removal increased, showing that PAOs have an advantage over GAOs at high pH. Zhang et al. (2005) observed a decrease in phosphorus removal and a shift in microbial community when the pH was changed from 7.0 to 6.5. At 7.0, the phosphorus removal efficiency was 99%, while after 14 days at pH 6.5, the phosphorus removal efficiency decreased to 17%. In the Lopez-Vazquez et al. (2009b) study it was observed that, when acetate or propionate was supplied as carbon source, PAOs only proliferated over GAOs at high pH (7.5). However, when acetate and propionate were present simultaneously, PAOs proliferated over GAOs independently of the pH applied. Fukushima et al. (2010), through the operation of three reactors, observed an *Accumulibacter* decline and a deterioration of EBPR activity after a pH reduction from 7.9 to 6.5 (in two reactors) and 6.5 to 6.0 (in one reactor) . However, the *Competibacter* population seemed to not be affected by either of these pH reductions. Weissbrodt et al. (2013) studied the effect of several factors on aerobic granular sludge and verified that, under alkaline conditions (pH > 7.3), *Accumulibacter* were selected, achieving a high phosphorus removal efficiency.

These studies showed that pH is an important factor in PAO-GAO competition, and a high pH (7.0 - 8.0) can promote an advantage for PAOs over GAOs, improving the phosphorus removal efficiency and, consequently, the EBPR performance. Nevertheless, the excessive pH can be detrimental for EBPR process and there is a pH limit where this control strategy will be not effective (Oehmen et al., 2007).

2.2.2 TEMPERATURE EFFECT

Several lab-scale studies showed that low temperatures improve the EBPR performance (Erdal et al., 2003; Panswad et al., 2003; Whang and Park, 2002, 2006). Whang et al. (2002, 2006) showed that, at 20ºC, PAOs were the dominant population due to their high specific anaerobic acetate uptake at this temperature. Nevertheless, GAOs were the dominant population at 30ºC due to a kinetic advantage in anaerobic acetate uptake, which can lead to eventual failure of EBPR systems. Panswad et al. (2003) observed a change in abundance from PAOs to GAOs when the temperature increased from 20 to 32.5ºC, which agrees with the increase of phosphorus concentration in the effluent and the decrease of phosphorus content in the sludge. In

this study, it was also found that, the specific phosphorus release rate increased with the temperature increase, while the specific phosphorus uptake rate decreased. Erdal et al. (2003) suggested that PAOs were favoured over GAOs at low temperatures, since a higher phosphorus removal and lower glycogen transformations were observed at 5ºC than at 20ºC. These authors also suggested that, at 5ºC, PAOs can dominate the EBPR system through changing their metabolic pathway. Lopez-Vazquez et al. (2009a) showed that GAOs were unable to maintain their activity at low temperatures even when subjected to low influent phosphorus concentrations, and at higher influent phosphorus concentrations PAOs consistently outcompeted GAOs. Through metabolic modelling predictions, Lopez-Vazquez et al. (2009b) also showed that PAOs were the dominant organisms at low temperatures (10ºC), independently of the carbon source and pH applied, since GAO metabolism was inhibited at this temperature. At high temperature (30ºC), GAOs (*Competibacter* and *Alphaproteobacteria*-GAO) were the dominant organisms. However, if a 75-25% acetate to propionate ratio and a pH value not lower than 7.0 are applied, it is possible to suppress the proliferation of GAOs at high temperatures. Ren et al. (2011) also investigated the impact of temperature variation on the PAO-GAO competition during EBPR acclimatisation. Under temperature variations from 22ºC to 29ºC and then to 14ºC, the domination of PAOs deteriorated, and the advantage of PAOs at low temperatures was not observed unlike other studies. *Alphaproteobacteria*-GAO and *Gammaproteobacteria*-GAO were more adaptable to the temperature changes and were the dominant organisms at the end of the study, even when the temperature was 14ºC. This study showed that the temperature variation during the enrichment process has a higher impact on PAOs than on GAOs. Using aerobic granular sludge, Weissbrodt et al. (2013) selected *Competibacter* organisms through higher mesophilic temperatures (>25ºC), while *Accumulibacter* were selected under low mesophilic temperatures (<20ºC).

These experimental evidences suggested that GAOs are able to outcompete PAOs under high temperatures. This implies that the PAO-GAO competition in EBPR systems may be more problematic in warm climates, and during the summer season.

2.2.3 VFA COMPOSITION AND SUBSTRATE FEEDING RATE EFFECT

The VFA composition has been shown to have an impact on the competition between PAOs and GAOs. The most common VFA present in the EBPR systems is acetate, though in systems where prefermenters exist, propionate is often present in

substantial quantities (Oehmen et al., 2007). Propionate also seems to be a more favourable carbon source to achieve successful EBPR (Chen et al., 2004; Oehmen et al., 2006; Wang et al., 2010)

Oehmen et al. (2005b), through short-term tests, observed that in a *Competibacter* GAO reactor enriched with acetate, GAOs were inefficient in taking up propionate, while *Accumulibacter* PAOs were able to take up acetate and propionate at similar rates. *Alphaproteobacteria*-GAOs dominated the reactor fed with propionate and were able to take up acetate at a rate that was 45% of their propionate uptake rate. Both types of GAOs showed a slower response than PAOs to carbon source changes, suggesting that the alternation between acetate and propionate can control the GAOs proliferation, as observed in the study of Lu et al. (2006). Through long-term studies, Oehmen et al. (2006) observed an unstable phosphorus removal in a reactor enriched with acetate, due to the competition between *Accumulibacter* and *Competibacter* for acetate uptake. However, a high and stable phosphorus removal and low phosphorus concentration in the effluent was observed when propionate was used as carbon source.

The metabolic model developed by Lopez-Vazquez et al. (2009b) showed that, when acetate and propionate are simultaneously supplied (75-25 and 50-50% acetate to propionate), PAOs were favoured over GAOs. However, when acetate or propionate is supplied as sole carbon source, PAOs only presented an advantaged when the pH was 7.5. Nevertheless, the real effect of a combined acetate/propionate feed on PAO metabolism is still unclear, even though it represents a common situation in most EBPR plants and a potentially effective way of minimising GAOs.

The substrate feeding rate is another factor that affects the competition between PAOs and GAOs. Tu and Schuler (2013) studied the effect of acetate feeding rate with high (>7.4) and low (6.4 – 7.0) pH. Under high substrate feeding rate and high pH, PAOs dominated the system. However, when the pH decreased to low values, GAOs dominated the system. Decreasing the acetate feeding rate (at low pH), but maintaining the total acetate load fed, PAOs recovered their activity and dominated the system. This suggests that the substrate loading rate is a more significant factor affecting the PAO-GAO competition than pH. Ahn et al. (2007) demonstrated that high organic loading rate promotes the proliferation of glycogen-accumulating metabolism organisms over PAOs. When the organic loading rate increased, the PAO population decreased (from 83.8 ± 4.9 to 32.2 ± 16.2%) and the phosphorus concentration in the effluent increased, suggesting an increase in GAO activity.

2.2.4 SLUDGE AGE EFFECT

The sludge age also appears to be an important operational factor affecting the PAO-GAO competition although different studies yielded contradictory results. The study of Li et al. (2008b) demonstrated that an increase in the sludge age promotes the reduction of phosphorus removal. Whang and Park (2006) observed that, at 30ºC and 10 days, GAOs were able to outcompete PAOs. Decreasing the sludge age for 5 days, GAOs coexisted with PAOs, displaying an unstable EBPR performance, although the EBPR efficiency improved. However, decreasing the sludge age to 3 days, the EBPR efficiency was improved and the EBPR performance was stable, possibly due to the higher anaerobic acetate uptake rate of PAOs. Therefore, when temperature is higher than 20ºC, it is possible to manipulate the sludge age to recover of EBPR efficiency when GAOs become the dominant population. Li et al. (2008b) found a higher phosphorus removal efficiency (>90%) at a sludge age of 8.3 days than a sludge age of 16.6 days (<85%). The sludge age also affected the sludge settleability, where the higher sludge age had a negative impact on the sludge settling.

Zhu et al. (2013) operated a humus soil sequencing batch reactor (HS-SBR) to study the effect of 3 different sludge ages (5, 10 and 15 days) on phosphorus removal efficiency. They observed a higher phosphorus removal (97.3%) at a sludge age of 10 and 15 days than that with a sludge age of 5 days (82.7%). Therefore, a short sludge age (<5 days) showed an adverse affect on phosphorus removal, whereas a sludge age between 10 and 15 days did not affect the phosphorus removal. This study showed that a relatively long sludge age exerted a useful effect on the phosphorus removal in the HS-SBR. Lee et al. (2007) also found that a high sludge age increased the phosphorus removal efficiency. With a sludge age of 20 days, they obtained a phosphorus removal efficiency of 93%, which was higher than that obtained with a sludge age of 15 days (78%). Further study would be useful to better establish the influence of sludge age on EBPR performance, and its impact on the PAO-GAO competition.

2.2.5 PHOSPHORUS TO ORGANIC CARBON RATIO EFFECT

The ratio of phosphorus to organic carbon (P/C) in the effluent is considered an important factor in the competition between PAOs and GAOs (Liu et al., 1997; Yagci et al., 2003). Liu et al. (1997) showed that using a high P/C ratio (20/100), PAOs could accumulate a high polyphosphate content, they have a faster acetate uptake rate and

thus they could outcompete GAOs. Reducing the ratio to 2/100, the polyphosphate content decreased, leading to an eventual proliferation of GAOs over PAOs. Both organisms could coexist if a median P/C ratio was applied, since the energy pool of PAOs was not enough for a complete acetate uptake, leaving acetate available for GAOs. Panswad et al. (2007) showed that a higher P/C ratio could promote the proliferation of PAOs. The increase of the P/C ratio from 0.02 to 0.16, promoted the increase of mass fraction of PAOs (from 0.1-0.15 to 0.47-0.71) and the decrease of mass fraction of GAOs (from 0.83-0.88 to 0.26-0.50). Hsu et al. (2013) studied the long-term effect of a high P/C ratio in acetate and propionate fed reactors. They observed that a phosphorus rich influent promoted the deterioration of phosphorus metabolism, being this deterioration affected by the carbon source used. Using acetate as carbon source, the high phosphorus loading suppressed PAOs, and GAOs became the dominant population. However, using propionate as carbon source, it was observed a proliferation of PAOs. These results showed that, with high phosphorus loading, propionate is a more suitable substrate to achieve a PAO enrichment. In the study of Zhu et al. (2013) a high P/C ratio (P/C = 0.1) promoted the proliferation of PAOs in the humus soil sequencing batch reactor. However, the phosphorus removal efficiency increased when the P/C ratio decreased from 0.1 to 0.0125. While high P/C ratios generally lead to higher PAO abundances, a balance must be achieved to ensure sufficient organic carbon to achieve phosphorus removal.

2.2.6 OTHER FACTORS

The effect of other factors on the PAO-GAO competition, such as dissolved oxygen (DO) concentration, aerobic hydraulic retention time (HRT) and prolonged low carbon loading, has never been systematically studied.

Several studies have shown that PAOs can be selected over GAOs at low (0.5 mg O_2/L) and high (> 3 mg O_2/L) DO concentrations (Lemaire et al., 2006; Oehmen et al., 2005b; Pijuan et al., 2004). Other studies were operated at very low DO concentrations (0.15 – 0.45 mg O_2/L), achieving a variable phosphorus removal efficiency (61% - 99%) (Li and Chen, 2011; Li et al., 2008a; Zheng et al., 2009). However, in these studies the effect of DO concentration on the microbial community and their metabolism was not addressed, thus it is still unknown how the DO level impacts the PAO-GAO competition and EBPR efficiency.

In Brdjanovic et al. (1998) it was observed that, in short-term tests, an excessive aeration promoted the deterioration of EBPR process, due to the high consumption of PHAs. However, the impact of a prolonged aerobic period on the competition between PAOs and GAOs and EBPR performance is still unknown.

Some studies showed that low organic loading periods, which occur mainly during weekends, can affect the EBPR performance, observing a decrease in phosphorus removal efficiency (Carucci et al., 1999; Temmink et al., 1996). However, the effect of prolonged low organic periods on the PAO-GAO competition was not yet addressed. Since low DO levels, high aerobic retention times and low organic loading represent situations commonly encountered in WWTPs, further study is necessary to assess their impact on the PAO-GAO competition and EBPR performance.

2.3 METABOLIC MODELLING OF THE PAO-GAO COMPETITION

Metabolic modelling of biological wastewater treatment is an interesting tool that can be used for optimisation and prediction of process performance, and as a supporting tool for design (Lopez-Vazquez et al., 2009b). Activated sludge models (ASM) and metabolic models have been used to describe EBPR processes, and their combination have been used to simulate the behaviour of full-scale EBPR plants (Oehmen et al., 2007). The ASM models only describe the bulk biochemical transformations of soluble and particulate compounds in the sludge (Oehmen et al., 2007), while the metabolic models use the metabolic pathways to describe the biochemical interactions that take place within the cells, providing a finer scale definition of the biological process and a more detailed description of the treatment systems (Oehmen et al., 2010). The key difference between these two models is the determination of the yield coefficients. While in ASM models the yield coefficients are determined experimentally, in metabolic models they are calculated theoretically through substrate, energy and reducing power balances (Oehmen et al., 2007). In order to understand the interactions and the competition between PAOs and GAOs, some authors developed metabolic models where the impact of different parameters was studied independently (Whang et al., 2007; Yagci et al., 2003, 2004, Zeng et al., 2003). Lopez-Vazquez et al. (2009b) studied the effect of 3 parameters (carbon source, pH and temperature) simultaneously and differentiated the biomass in 3 separate groups (*Accumulibacter*-PAO, *Competibacter*-GAO and *Alphaproteobacteria*-GAO). Using this model, which was calibrated and validated using data from enriched

PAO and GAO cultures, the metabolic activities associated with each culture was well described and enabled greater insight into how combinations of operational factors can influence the EBPR process. Oehmen et al. (2010) expanded the metabolic model developed by Lopez-Vazquez et al. (2009b) in order to incorporate the competition between PAOs and GAOs under sequential anaerobic/anoxic/aerobic conditions, which are representative of most full-scale EBPR plants.

Metabolic models may be an important, flexible and useful tool to understand the interactions between bacterial populations under different environmental and operational conditions, in order to optimise the EBPR process and improve the phosphorus removal efficiency. Furthermore, these metabolic models are useful to predict the behaviour of PAOs and GAOs depending on the operational conditions and wastewater characteristics. The effect of new factors on the PAO-GAO competition should be incorporated into metabolic models in order to further improve their prediction ability. As the metabolic models offer a better understanding of the complex microbial interactions that take place in activated sludge systems, their application to real WWTPs can be a useful tool to improve WWTP processes.

REFERENCES

Ahn, C.H., Park, H.D., Park, J.K., 2007. Enhanced biological phosphorus removal performance and microbial population changes at high organic loading rates. Journal of Environmental Engineering 133, 962–969.

Brdjanovic, D., Slamet, A., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998. Impact of excessive aeration on biological phosphorus removal from wastewater. Water Research 32 (1), 200–208.

Burow, L.C., Kong, Y., Nielsen, J.L., Blackall, L.L., Nielsen, P.H., 2007. Abundance and ecophysiology of Defluviicoccus spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. MICROBIOLOGY-SGM 153 (1), 178–185.

Carucci, A., Kühni, M., Brun, R., Carucci, G., Koch, G., Majone, M., Siegrist, H., 1999. Microbial competition for the organic substrates and its impact on EBPR systems under conditions of changing carbon feed. Water Science and Technology 39 (1), 75–85.

Chen, Y., Randall, A.A., McCue, T., 2004. The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. Water Research 38 (1), 27–36.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., Blackall, L.L., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology 148 (11), 3353–3364.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of Polyphosphate-Accumulating Organisms and Design of 16S rRNA-Directed Probes for Their Detection and Quantitation. Applied and Environmental Microbiology 66 (3), 1175–1182.

Dai, Y., Yuan, Z., Wang, X., Oehmen, A., Keller, J., 2007. Anaerobic metabolism of Defluviicoccus vanus related glycogen accumulating organisms (GAOs) with acetate and propionate as carbon sources. Water Research 41 (9), 1885–1896.

Erdal, U.G., Erdal, Z.K., Randall, C.W., 2003. The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on system performance. Water science and technology 47, 1–8.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001. pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. Water Environment Research 73 (2), 223–232.

Filippelli, G.M., 2008. The Global Phosphorus Cycle: Past, Present, and Future. Elements 4 (2), 89–95.

Fukushima, T., Onuki, M., Satoh, H., Mino, T., 2010. Effect of pH reduction on polyphosphate- and glycogen-accumulating organisms in enhanced biological phosphorus removal processes. Water Science & Technology 62 (6), 1432–1439.

Hsu, C.-H., Chang, W.-C., Chen, J.-J., Wu, J.-S., 2013. Comparing the long-term effect of high P/COD influent on enhancement of phosphate-accumulating organisms between acetate- and propionate-fed reactors. Journal of Chemical Technology & Biotechnology 88 (6), 1071–1079.

Kong, Y., Ong, S.L., Ng, W.J., Liu, W.-T., 2002. Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes. Environmental Microbiology 4 (11), 753–757.

Kristiansen, R., Nguyen, H.T.T., Saunders, A.M., Nielsen, J.L., Wimmer, R., Le, V.Q., McIlroy, S.J., Petrovski, S., Seviour, R.J., Calteau, A., Nielsen, K.L., Nielsen, P.H., 2013. A metabolic model for members of the genus Tetrasphaera involved in enhanced biological phosphorus removal. The ISME journal 7, 543–54.

Lanham, A.B., Oehmen, A., Saunders, A.M., Carvalho, G., Nielsen, P.H., Reis, M.A.M., 2013. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. Water Research 47 (19), 7032–7041.

Lee, D., Kim, M., Chung, J., 2007. Relationship between solid retention time and phosphorus removal in anaerobic-intermittent aeration process. Journal of Bioscience and Bioengineering 103 (4), 338–344.

Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z., 2006. Identifying causes for N2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. Journal of Biotechnology 122 (1), 62–72.

Li, H., Chen, Y., 2011. Research on polyhydroxyalkanoates and glycogen transformations: Key aspects to biologic nitrogen and phosphorus removal in low dissolved oxygen systems. Frontiers of Environmental Science & Engineering in China 5 (2), 283–290.

Li, H., Chen, Y., Gu, G., 2008a. The effect of propionic to acetic acid ratio on anaerobic–aerobic (low dissolved oxygen) biological phosphorus and nitrogen removal. Bioresource Technology 99 (10), 4400–4407.

Li, N., Wang, X., Ren, N., Zhang, K., Kang, H., You, S., 2008b. Effects of Solid Retention Time (SRT) on Sludge Characteristics in Enhanced Biological Phosphorus Removal (EBPR) Reactor. Chemical and Biochemical Engineering Quarterly 22 (4), 453–458.

Liu, W.-T., Nakamura, K., Matsuo, T., Mino, T., 1997. Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors—Effect of PC feeding ratio. Water Research 31 (6), 1430–1438.

Lopez-Vazquez, C.M., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., van Loosdrecht, M.C.M., 2009a. Temperature effects on glycogen accumulating organisms. Water Research 43, 2852–2864.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009b. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Research 43 (2), 450–462.

Lu, H., Oehmen, A., Virdis, B., Keller, J., Yuan, Z., 2006. Obtaining highly enriched cultures of Candidatus Accumulibacter phosphates through alternating carbon sources. Water Research 40 (20), 3838–3848.

Majed, N., Chernenko, T., Diem, M., Gu, A.Z., 2012. Identification of Functionally Relevant Populations in Enhanced Biological Phosphorus Removal Processes Based On Intracellular Polymers Profiles and Insights into the Metabolic Diversity and Heterogeneity. Environmental Science & Technology 46 (9), 5010–5017.

McIlroy, S., Seviour, R.J., 2009. Elucidating further phylogenetic diversity among the Defluviicoccus-related glycogen-accumulating organisms in activated sludge. Environmental Microbiology Reports 1 (6), 563–568.

Meyer, R.L., Saunders, A.M., Blackall, L.L., 2006. Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology 152 (2), 419–429.

Molinos-Senante, M., Hernández-sancho, F., Sala-garrido, R., Hernández-Sancho, F., Sala-Garrido, R., 2012. Economic feasibility study for new technological alternatives in wastewater treatment processes: a review. Water Science & Technology 65 (5), 898.

Muga, H.E., Mihelcic, J.R., 2008. Sustainability of wastewater treatment technologies. Journal of Environmental Management 88 (3), 437–447.

Mullan, A., Quinn, J.P., McGrath, J.W., 2002. Phosphate Removal from Wastewaters: A Novel Approach. Engineering in Life Sciences 2 (2), 63–66.

Nguyen, H.T.T., Le, V.Q., Hansen, A.A., Nielsen, J.L., Nielsen, P.H., 2011. High diversity and abundance of putative polyphosphate-accumulating Tetrasphaera-related bacteria in activated sludge systems. FEMS Microbiology Ecology 76 (2), 256–267.

Nittami, T., McIlroy, S., Seviour, E.M., Schroeder, S., Seviour, R.J., 2009. Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III Defluviicoccus. Systematic and Applied Microbiology 32 (7), 480–489.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Research 41 (11), 2271–2300.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms

relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Research 44 (15), 4473–4486.

Oehmen, A., Saunders, A.M., Vives, M.T., Yuan, Z., Keller, J., 2006. Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. Journal of Biotechnology 123 (1), 22–32.

Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J., 2005a. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Research 39 (15), 3727–3737.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2005b. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnology and Bioengineering 91 (2), 162–168.

Panswad, T., Doungchai, A., Anotai, J., 2003. Temperature effect on microbial community of enhanced biological phosphorus removal system. Water Research 37, 409–415.

Panswad, T., Tongkhammak, N., Anotai, J., 2007. Estimation of intracellular phosphorus content of phosphorus-accumulating organisms at different P:COD feeding ratios. Journal of Environmental Management 84, 141–145.

Pijuan, M., Oehmen, A., Baeza, J.A., Casas, C., Yuan, Z., 2008. Characterizing the biochemical activity of full-scale enhanced biological phosphorus removal systems: A comparison with metabolic models. Biotechnology and Bioengineering 99 (1), 170–179.

Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnology and Bioengineering 85 (1), 56–67.

Puig, S., Corominas, L., Balaguer, M.D., Colprim, J., 2007. Biological nutrient removal by applying SBR technology in small wastewater treatment plants: carbon source and C/N/P ratio effects. Water Science & Technology 55 (7), 135–141.

Ren, N., Kang, H., Wang, X., Li, N., 2011. Short-term effect of temperature variation on the competition between PAOs and GAOs during acclimation period of an EBPR system. Frontiers of Environmental Science & Engineering in China 5 (2), 277–282.

Saunders, A.M., Oehmen, A., Blackall, L.L., Yuan, Z., Keller, J., 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water Science and Technology 47 (11), 37–43.

Schuler, a J., Jenkins, D., 2002. Effects of pH on enhanced biological phosphorus removal metabolisms. Water Science and Technology 46, 171–178.

Seviour, R.J., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiology Reviews 27, 99–127.

Temmink, H., Petersen, B., Isaacs, S., Henze, M., 1996. Recovery of biological phosphorus removal after periods of low organic loading. Water Science and Technology 34 (1–2), 1–8.

Tu, Y., Schuler, A.J., 2013. Low Acetate Concentrations Favor Polyphosphate-Accumulating Organisms over Glycogen-Accumulating Organisms in Enhanced Biological Phosphorus Removal from Wastewater. Environmental Science & Technology 47 (8), 3816–3824.

Van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D., Heijnen, J.J., 1997. Biological phosphate removal processes. Applied Microbiology and Biotechnology 48 (3), 289–296.

Wang, D., Zheng, W., Liao, D., Li, X., Yang, Q., Zeng, G., 2013. Effect of initial pH control on biological phosphorus removal induced by the aerobic/extended-idle regime. Chemosphere 90 (8), 2279–2287.

Wang, Y., Jiang, F., Zhang, Z., Xing, M., Lu, Z., Wu, M., Yang, J., Peng, Y., 2010. The long-term effect of carbon source on the competition between polyphosphorus accumulating organisms and glycogen accumulating organism in a continuous plug-flow anaerobic/aerobic (A/O) process. Bioresource Technology 101 (1), 98–104.

Weissbrodt, D.G., Schneiter, G.S., Fürbringer, J.-M., Holliger, C., 2013. Identification of trigger factors selecting for polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge sequencing batch reactors. Water Research 47 (19), 7006–7018.

Whang, L.M., Filipe, C.D.M., Park, J.K., 2007. Model-based evaluation of competition between polyphosphate- and glycogen-accumulating organisms. Water Research 41 (6), 1312–1324.

Whang, L.M., Park, J.K., 2002. Competition between polyphosphate- and glycogen-accumulating organisms in biological phosphorus removal systems--effect of temperature. Water Science and Technology 46, 191–194.

Whang, L.-M., Park, J.K., 2006. Competition between Polyphosphate- and Glycogen-Accumulating Organisms in Enhanced-Biological-Phosphorus-Removal Systems: Effect of Temperature and Sludge Age. Water Environment Research 78 (1), 4–11.

Wong, M.-T., Tan, F.M., Ng, W.J., Liu, W.-T., 2004. Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic–aerobic activated sludge processes. Microbiology 150 (11), 3741–3748.

Yagci, N., Artan, N., Cokgor, E.U., Randall, C.W., Orhon, D., 2003. Metabolic model for acetate uptake by a mixed culture of phosphate- and glycogen-accumulating organisms under anaerobic conditions. Biotechnology and Bioengineering 84 (3), 359–373.

Yagci, N., Insel, G., Artan, N., Orhon, D., 2004. Modelling and calibration of phosphate and glycogen accumulating organism competition for acetate uptake in a sequencing batch reactor. Water Science and Technology 50 (6), 241–250.

Zeng, R.J., Yuan, Z., Keller, J., 2003. Model-based analysis of anaerobic acetate uptake by a mixed culture of polyphosphate-accumulating and glycogen-accumulating organisms. Biotechnology and Bioengineering 83 (3), 293–302.

Zhang, T., Liu, Y., Fang, H.H.P., 2005. Effect of pH change on the performance and microbial community of enhanced biological phosphate removal process. Biotechnology and Bioengineering 92, 173–182.

Zhang, Z., Li, H., Zhu, J., Weiping, L., Xin, X., 2011. Improvement strategy on enhanced biological phosphorus removal for municipal wastewater treatment plants: Full-scale operating parameters, sludge activities, and microbial features. Bioresource Technology 102 (7), 4646–4653.

Zheng, X., Tong, J., Li, H., Chen, Y., 2009. The investigation of effect of organic carbon sources addition in anaerobic–aerobic (low dissolved oxygen) sequencing batch reactor for nutrients removal from wastewaters. Bioresource Technology 100 (9), 2515–2520.

Zhou, Y., Pijuan, M., Zeng, R.J., Yuan, Z., 2009. Involvement of the TCA cycle in the anaerobic metabolism of polyphosphate accumulating organisms (PAOs). Water Research 43 (5), 1330–1340.

Zhu, R., Wu, M., Yang, J., 2013. Effect of sludge retention time and phosphorus to carbon ratio on biological phosphorus removal in HS-SBR process. Environmental technology 34 (4), 429—435.

3

THE EFFECT OF SUBSTRATE COMPETITION ON THE METABOLISM OF POLYPHOSPHATE ACCUMULATING ORGANISMS (PAOS)

SUMMARY: The type of carbon source present in the wastewater is one factor that affects the competition between polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) and therefore, the efficiency of the enhanced biological phosphorus removal (EBPR) process. This study investigated the impact of the carbon source composition on the anaerobic and aerobic kinetics of PAOs and the EBPR performance of an 85% PAO enrichment. When both acetate (HAc) and propionate (HPr) were present, propionate was depleted more quickly, with a constant uptake rate of 0.18 ± 0.02 C-mol/(C-mol biomass·h), while the acetate uptake rate decreased with an increase in propionate concentration, due to the substrate competition between acetate and propionate. The metabolic model for PAOs was modified to incorporate the anaerobic substrate competition effect. The aerobic rates for phosphorus (P) uptake, glycogen production and polyhydroxyalkanoates (PHA) degradation were within the same range for all tests, indicating that these rates are essentially independent of the acetate and propionate concentration, simplifying the calibration procedure for metabolic models. The metabolic model applied to describe the anaerobic and aerobic activity agreed well with the experimental data of HAc, HPr, P, PHA and biomass growth. The low glycogen consumption observed suggest that some reducing equivalents were generated anaerobically through the TCA cycle. The

results of this work suggest that the propionate uptake kinetics by PAOs can provide them an advantage over GAOs in EBPR systems, even when the propionate fraction of the influent is relatively low.

PUBLISHED AS: Mónica Carvalheira, Adrian Oehmen, Gilda Carvalho, Maria A.M. Reis. "The effect of substrate competition on the metabolism of polyphosphate accumulating organisms (PAOs)", Water Research, 64 (2014), 149–159.

3.1 INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is one of the most economical and sustainable processes used for the removal of phosphorus from wastewater. Phosphorus removal is achieved by recirculating the sludge through anaerobic and aerobic/anoxic conditions to promote the enrichment of activated sludge with polyphosphate accumulating organisms (PAO), the bacteria responsible for EBPR. One highly important group of PAOs in EBPR systems is *Candidatus* Accumulibacter Phosphatis. These PAOs are able to take up volatile fatty acids (VFAs) during the anaerobic phase and store them as polyhydroxyalkanoates (PHAs), which are consumed aerobically or anoxically to achieve P removal. However, there is another group of microorganisms, known as glycogen accumulating organisms (GAO) that compete with PAOs for the anaerobic uptake of VFAs without contributing to P removal (Oehmen et al., 2007). Thus, in EBPR processes, it is necessary to control the competition between PAOs and GAOs in order to optimise the phosphorus (P) removal efficiency.

The PAO-GAO competition can be influenced by several factors, such as, pH, temperature, the phosphorus (P) to VFA ratio, VFA loading rate and the VFA composition of the wastewater (Filipe et al., 2001; Liu et al., 1997; Lopez-Vazquez et al., 2009; Tu and Schuler, 2013). Acetate (HAc) (Chen et al., 2005; Hollender et al., 2002; Levantesi et al., 2002; Oehmen et al., 2005b; Onuki et al., 2002; Wang et al., 2010) and propionate (HPr) (Chen et al., 2005; Oehmen et al., 2005b, c, Pijuan et al., 2004; Wang et al., 2010) have been widely studied as carbon sources in EBPR processes, since they are the most abundant VFAs present in wastewater. However, the efficiency that PAOs and GAOs use these VFAs differs. Oehmen et al. (2005b) showed that the HPr uptake rate of *Competibacter*, an important group of GAOs, is very slow as compared to its HAc uptake rate, so a HPr feed can be used to control the growth of these GAOs in EBPR systems. Indeed, high *Accumulibacter* PAO fractions and good EBPR performance have been observed with HPr as the sole carbon source (Guisasola et al., 2007; Oehmen et al., 2005c; Pijuan et al., 2004). Nevertheless, another group of GAOs (*Defluviicoccus vanus*) has also been found to be competitive with *Accumulibacter* PAOs, particularly for HPr uptake (Lanham et al., 2008; Meyer et al., 2006; Oehmen et al., 2005a). Dai et al. (2007) have found that *Defluviicoccus vanus* GAOs prefer HPr to HAc when both substrates are present simultaneously, though it is still unclear if a similar situation exists for *Accumulibacter* PAOs.

Lopez-Vazquez et al. (2009) developed a metabolic model that incorporates HAc and HPr as carbon sources as well as their effect on PAO-GAO competition. In this model, the kinetic parameters were estimated from PAO and GAO enriched cultures, where HAc or HPr was fed as the sole organic substrate during culture enrichment. Through the calibrated model, the effect of different ratios of HAc to HPr (100-0, 75-25, 50-50, 0-100%) was evaluated through simulation studies. According to the simulation results, PAOs exhibit an advantage over GAOs when HAc and HPr are simultaneously supplied (75-25 and 50-50% HAc-HPr) as compared to cases where only HAc or HPr is fed. Nevertheless, the effect of a combined HAc-HPr feed on the metabolism of PAOs has not yet been studied in detail experimentally, even though it represents a common situation in most EBPR plants and a potentially effective way of minimising GAOs. Due to this limitation, it remains unclear how the effect of a combined VFA feed impacts the anaerobic and aerobic kinetic parameters and how any carbon source preferences that exist for PAOs should be modelled under these frequently encountered situations. This provided the motivation for the present study, which investigated the effect of a combined feed with different ratios of HAc to HPr, in order to 1) understand its impact on the anaerobic kinetics of PAOs, particularly with regards to substrate competition effects, 2) evaluate its impact on the kinetics of the subsequent aerobic phase, where significant differences have been suggested from enriched PAO cultures with either HAc or HPr, and 3) describe PAO activity with a combined HAc and HPr feed through metabolic modelling. It is expected that this study will improve the capacity of metabolic models to describe and predict the performance of EBPR processes.

3.2 MATERIALS AND METHODS

3.2.1 SBR ACCLIMATISATION WITH PAOS

A sequencing batch reactor (SBR - see Appendix H) with 2L of working volume was seeded from a lab-scale reactor enriched in PAOs. Each cycle consisted of 6 hours, with a 2 hour anaerobic period, 3 hour aerobic period and 1 hour settle/decant period. One liter of synthetic medium was fed during the first 5 minutes of the anaerobic phase in each cycle, resulting in an initial COD concentration in the reactor of 200 mg/L, with 75% acetate and 25% propionate, and a phosphate concentration of 20 mg/L. The hydraulic retention time (HRT) was 12 hours and the solid retention time (SRT) was 8 days. The 1L synthetic medium was composed of 250 mL of solution A

and 750 mL of solution B. Solution A contained per liter: 2.55 g $C_2H_3O_2Na.3H_2O$, 270µL $C_3H_6O_2$, 0.59 g NH_4Cl, 0.95 g $MgSO_4.7H_2O$, 0.44 g $CaCl_2.2H_2O$, 11.7 mg allyl-N thiourea (ATU, a nitrification inhibitor), 31.7 mg ethylene-diaminetetraacetic (EDTA) and 3.17 mL of a micronutrients solution. The micronutrient solution (based on Smolders et al. (1994)) contained per litre: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnCl_2.4H_2O$, 0.06 g $Na_2MoO.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$. Solution B contained per liter: 124.1 mg K_2HPO_4 and 96.8 mg KH_2PO_4.

To maintain anaerobic conditions, argon was bubbled into the reactor at a flow rate of approximately 15 mL/min. During the aerobic phase, the air flow rate was maintained at approximately 0.2 L/min. The temperature was controlled at 20 ± 1ºC and pH at a maximum of 7.5 and averaged 7.2 ± 0.3 in the anaerobic and aerobic zones, by automatic addition of 0.1M HCl.

3.2.2 BATCH EXPERIMENTS

Five batch tests were performed to study the effect of different ratios (100-0%, 75-25%, 50-50%, 25-75%, 0-100%) of acetate to propionate (HAc:HPr). The batch tests were operated under similar conditions to those in the SBR, where 300 mL of SBR sludge was taken at the end of the aerobic phase for each batch test. In the beginning of the anaerobic phase, 300 mL of synthetic medium was fed with the different HAc:HPr ratios, resulting in an initial VFA concentration of 200 mg COD/L. Each batch test consisted of a 2 hour anaerobic period and a 3 hour aerobic period.

Samples were obtained at various points throughout the cycle and analysed by the chemical methods described below. The determination of total suspended solids (TSS) and volatile suspended solids (VSS) was performed at the end of the aerobic period. Fluorescence *in situ* hybridisation (FISH) sampling was performed at the end of the anaerobic and aerobic periods.

3.2.3 CHEMICAL AND MICROBIAL ANALYSIS

Phosphate was determined by a colorimetric method implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands). For total phosphate, an acid digestion of a sample from the end of the aerobic period was performed with 0.3 M H_2SO_4 and 73 mg/L of $K_2S_2O_8$ and analysed using the flow

segmented analyser. To determine the poly-phosphate content, the supernatant phosphate concentration was subtracted from the total phosphate concentration obtained by the sample digestion. The concentration of VFAs in the supernatant was determined by liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad) and an IR detector. Sulphuric acid (0.005 M) was used as eluent at a flow rate of 0.6 mL/min and 50ºC operating temperature. Glycogen was determined as described by Lanham et al. (2012) (conditions: 2 mg biomass, HCl 0.9 M and 3h of digestion time), using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30ºC, with sodium hydroxide (NaOH 18 mM) as eluent, at a flow rate of 0.8 mL/min. PHAs were determined by gas chromatography (GC) using the method described by Lanham et al. (2013b) using a Bruker 430-GC gas chromatograph equipped with a FID detector and a BR-SWax column (60m, 0.53mm internal diameter, 1 µm film thickness, Bruker, USA). For TSS and VSS determination, standard methods were applied (APHA/AWWA, 1995). Fluorescence *in situ* hybridisation (FISH) was used to identify the population present in the sludge and was performed as specified in Amann (1995). The oligonucleotide probes used in FISH were: EUBMIX, comprising EUB338, EUB338-II and EUB338-III (Amann et al., 1990; Daims et al., 1999) to target all bacteria; PAOMIX (PAO 651, 462 and 846) to target *Accumulibacter* (Crocetti et al., 2000); ACC-I-444 and ACC-II-444 to target *Accumulibacter* type I and II, respectively (Flowers et al., 2009); GAOMIX (GAOQ989 and GB_G2) to target *Competibacter* (Crocetti et al., 2002; Kong et al., 2002) and *Defluviicoccus vanus* related GAOs were targeted through probes TFO_DF218 and TFO_DF618 for cluster I (Wong et al., 2004); DF988 and DF1020 for cluster II (Meyer et al., 2006); DF1013 and DF1004 for cluster III (Nittami et al., 2009) and DF181A and DF181B for cluster IV (McIlroy and Seviour, 2009). FISH quantification was performed by image analysis of 20 micrographs taken with a Zeiss LSM 510 Meta confocal laser scanning microscope. The biomass volume of *Accumulibacter* and *Competibacter* in relation to other Bacteria was calculated as the area covered by the specific probes divided by the area covered by EUBMIX. The standard error of the mean was calculated as the standard deviation divided by the square root of the number of images.

3.2.4 MODEL DESCRIPTION

The model used in this study was based on previous metabolic models developed by Lopez-Vazquez et al. (2009) and Oehmen et al. (2010). The substrate

competition that occurs when HAc and HPr are simultaneously present was incorporated into the model based on the experimental results, as shown later.

The stoichiometric matrix, anaerobic and aerobic stoichiometric parameters and kinetic expressions for PAOs are detailed in Appendix A–E. This model was implemented in Aquasim (Reichert, 1994) and defined the PAO activity in a sequencing batch reactor with alternating anaerobic-aerobic stages.

3.2.5 MODEL CALIBRATION AND VALIDATION

For the calibration of the model, four kinetic parameters were adjusted: the VFA uptake rate (q_{VFA}), glycogen production rate ($q_{Glycogen}$), PHA degradation rate (q_{PHA}) and poly-P formation rate ($q_{PO4\text{-}PP}$). The experimental data obtained from the five batch tests were used during model calibration, where the parameter estimation for the anaerobic and aerobic metabolism was performed separately in order to avoid error propagation. The initial concentration of VFA, PHA, P and glycogen was estimated simultaneously to each kinetic parameter in the anaerobic and aerobic phases. The initial polyphosphate concentration was determined as the steady-state polyphosphate concentration. The model validation was obtained through simulation of experimental results from all batch tests using the average of each kinetic parameter determined during calibration.

A long-term simulation was performed using the validated model developed in this study, in order to examine its ability to predict the steady-state activity. The initial concentrations of HAc, HPr, P, polyphosphate, PHA, glycogen and active biomass used in this simulation correspond to the initial concentrations from the 75-25% HAc-HPr batch test, which is similar to the operational conditions of the parent SBR. The simulation was executed for an operational period of 24 days (96 cycles), which corresponds to 3 SRT.

3.2.6 ERROR ANALYSIS

Percent error was calculated using the normalized root mean squared deviation (NRMSD), through the following equation:

$$NRMSD = \frac{\sqrt{\frac{\sum \left(x_{meas} - x_{pred}\right)^2}{n}}}{x_{meas,max} - x_{meas,min}} \qquad (3.1)$$

where x_{meas} and x_{pred} correspond to the measured and predicted concentrations for each parameter (VFA, P, PHA and glycogen) and $x_{meas,min}$ and $x_{meas,max}$ correspond to the minimum and maximum measured concentrations.

3.2.7 ANAEROBIC STOICHIOMETRIC CALCULATIONS

The anaerobic stoichiometry of each batch test was determined as the ratio of the maximum rates of P release, glycogen hydrolysis, polyhydroxybutyrate (PHB), polyhydroxy-2-methylbutyrate (PH_2MB), polyhydroxyvalerate (PHV), polyhydroxy-2-methylvalerate (PH_2MV) and PHA production, per VFA uptake. These rates and their respective standard deviations were determined through SigmaPlot 11 (Systat Software Inc.). The standard deviation of the anaerobic stoichiometry was determined through the calculation of error propagation for division using the following equation:

$$\Delta z = \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta y}{y}\right)^2} \times z \qquad (3.2)$$

where Δz corresponds to the error of the anaerobic stoichiometric ratio, Δx corresponds to the standard deviation of the P release, glycogen hydrolysis, PHB, PH_2MB, PHV, PH_2MV or PHA production rates, Δy corresponds to the standard deviation of the VFA uptake rate, x corresponds to the P release, glycogen hydrolysis, PHB, PH_2MB, PHV, PH_2MV or PHA production rates, y corresponds to the VFA uptake rate and z corresponds to the value of the anaerobic stoichiometric ratio.

ATP and redox balances were also performed on the experimental data. The ATP balance percentage was defined as the ratio of ATP production (through polyphosphate hydrolysis and glycogen degradation) per ATP consumption (for VFA uptake). The redox level of all reactants (VFA, glycogen and polyphosphate) and products (PHA and phosphate) were also calculated, and the redox balance

percentage was defined as the ratio of the products per reactants. These calculations were based on the equations proposed by Smolders et al. (1994) and Oehmen et al. (2005c), for acetate and propionate, respectively.

3.3 RESULTS AND DISCUSSION

3.3.1 SBR PERFORMANCE AND CULTURE CHARACTERISATION

The strategy of feeding acetate and propionate combined in a proportion of 75-25%, on a COD basis, successfully selected a PAO enrichment, as shown by FISH quantification. *Accumulibacter* PAOs were the dominant organisms present in the sludge, representing 85 ± 2% of all bacteria, although *Competibacter* GAOs were also present, but in much lower abundance (17 ± 7%) (Figure 3.1). *Defluviicoccus vanus* GAOs were not detected. This enrichment was attained after two months of SBR operation, when steady state conditions were achieved. A P removal efficiency of approximately 99% was observed, as well as a clear PAO phenotype regarding P, PHA and glycogen cycling. The average P concentration in the effluent was 0.64 ± 0.26 mg P/L. The P release and P uptake were 64.6 ± 10.8 mg P/L and 93.4 ± 9.7 mg P/L, respectively, during this period, with an average $P_{release}/VFA_{uptake}$ ratio of 0.35 ± 0.14 P-mol/C-mol.

This population structure is in agreement with Lopez-Vazquez simulations (2009) that predict an enrichment of about 85% of PAOs and about 15% for GAOs, when the carbon source is a mixture of 75-25% HAc-HPr. This is the first time that this result is experimentally validated, suggesting that the presence of propionate, even at only 25% of the COD fed, may have contributed to the enrichment of PAOs in detriment of GAOs. In order to investigate the kinetic impact of VFA mixtures, batch tests with different ratios of HAc to HPr were performed after this 2 month period of reactor steady state operation with good P removal.

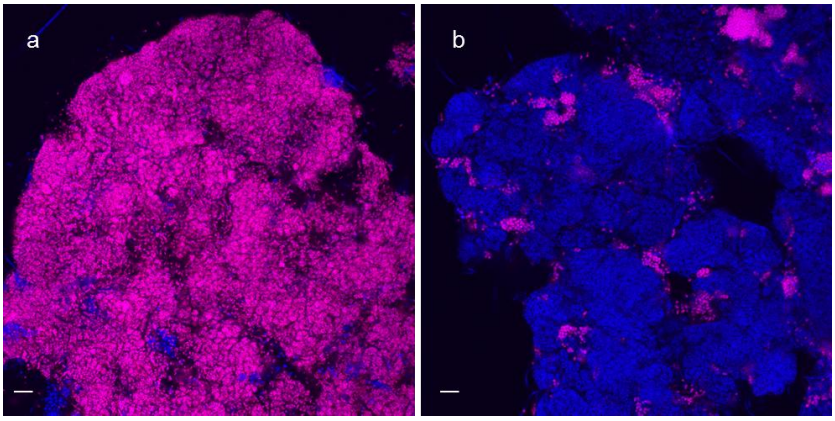


Figure 3.1 – Illustrative FISH image of the abundance of a) *Accumulibacter*, targeted by PAOmix and b) *Competibacter*, targeted by GAOmix. Specific populations are in magenta and other bacteria are in blue. Bar = 10 µm.

3.3.2 ANAEROBIC METABOLISM

Complete VFA uptake was observed in all batch tests, as can be observed in Figure 3.2. As observed in the studies of Oehmen et al. (2005b) and Pijuan et al. (2004), the maximum acetate and propionate uptake rates were similar when each substrate was fed individually (Table 3.1). However, when both substrates were fed, although HAc and HPr were taken up simultaneously (Figure 3.2b, c, d), HPr was depleted more quickly in each test. The maximum propionate uptake rate was similar in all batch tests where it was present (average 0.18 ± 0.02 C-mol/(C-mol biomass·h)). In contrast, the HAc uptake rate decreased from 0.21 ± 0.02 to 0.06 ± 0.01 C-mol/(C-mol biomass·h) as the initial HPr fraction increased (Table 3.1). This shows that PAOs exhibit a preference for HPr uptake as compared to HAc when both substrates are present simultaneously. A preference for HPr uptake could be related to the fact that PAOs require less energy from polyphosphate hydrolysis and glycogen degradation per C-mol of HPr uptake as compared to acetate (Table 3.2). Furthermore, PAOs could possess a higher substrate affinity for HPr as compared to HAc (i.e. lower Ks) through independent substrate transport mechanisms, or substrate competition could exist for a common transport mechanism for both HAc and HPr.

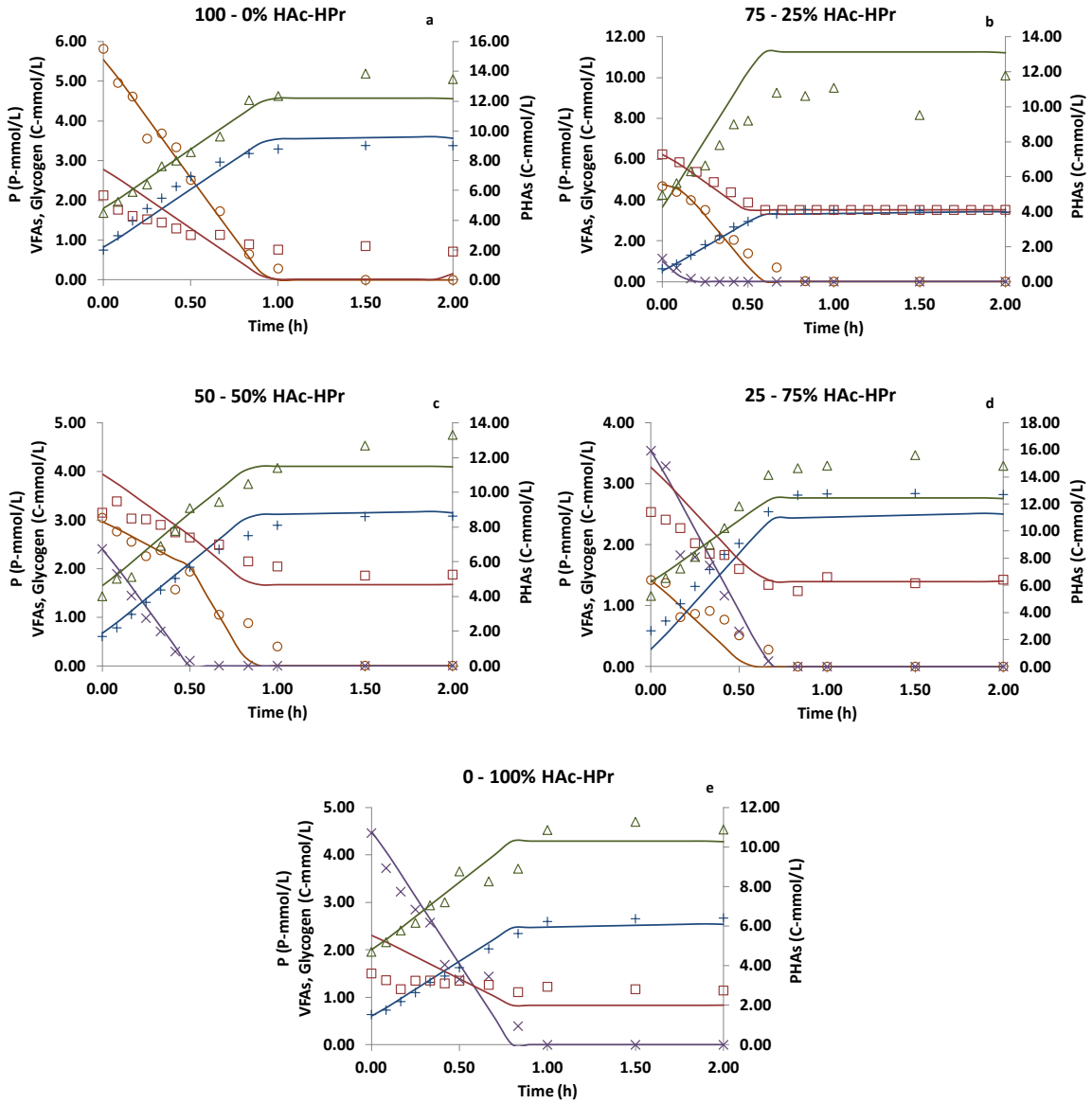


Figure 3.2 – Comparison of anaerobic experimental results (acetate (○), propionate (X), P (+), PHAs (Δ) and glycogen (□)) and model description (solid line) obtained in the five batch tests. Each batch test had a different proportion of acetate and propionate: a: 200 mg COD HAc/L; b: 150 mg COD HAc/L and 50 mg COD HPr/L; c: 100 mg COD HAc/L and 100 mg COD HPr/L; d: 50 mg COD HAc/L and 150 mg COD HPr/L; e: 200 mg COD HPr/L.

Table 3.1 – Acetate and propionate uptake rates for batch tests carried out with different acetate and propionate fractions.

	HAc:HPr **mg COD/L**	**% HAc-HPr**	**q_{HAc}** **C-mol/(C-mol biomass·h)**	**q_{HPr}** **C-mol/(C-mol biomass·h)**
a	200:0	100-0	0.21 ± 0.02	-
b	150:50	75-25	0.15 ± 0.01	n.d.
c	100:100	50-50	0.07 ± 0.01	0.17 ± 0.01
d	50:150	25-75	0.06 ± 0.01	0.20 ± 0.01
e	0:200	0-100	-	0.18 ± 0.01

n.d.: not determined, the number of data points was insufficient to calculate a rate due to rapid propionate depletion

T

		P VFA	Glycogen VFA	PHA VFA	PHB VFA	PH_2MB VFA	PHV VFA	PH_2MV VFA	%Acetyl -CoA	%Propionyl -CoA	ATP Balance (%)	Redox Balance (%)
Experimental	100-0% HAc-HPr	0.61 ± 0.07	0.29 ± 0.05	1.39 ± 0.14	1.20 ± 0.14	0.01 ± 0.00	0.17 ± 0.02	0.01 ± 0.02	91.4	8.6	94	122
	75-25% HAc-HPr	0.56 ± 0.03	0.29 ± 0.05	1.01 ± 0.10	0.67 ± 0.09	0.03 ± 0.00	0.28 ± 0.04	0.04 ± 0.01	77.9	22.1	97	85
	50-50% HAc-HPr	0.41 ± 0.04	0.14 ± 0.02	1.35 ± 0.12	0.13 ± 0.06	0.07 ± 0.01	0.89 ± 0.10	0.26 ± 0.03	38.1	61.9	87	132
	25-75% HAc-HPr	0.39 ± 0.03	0.24 ± 0.02	1.59 ± 0.11	0.08 ± 0.03	0.07 ± 0.01	1.08 ± 0.10	0.36 ± 0.04	34.0	66.0	89	141
	0-100% HAc-HPr	0.34 ± 0.02	0.05 ± 0.02	1.35 ± 0.10	0.15 ± 0.02	0.07 ± 0.00	0.56 ± 0.08	0.57 ± 0.05	29.5	70.5	74	135
Model	100% HAc (Smolders et al., 1994)	0.5	0.5	1.33	1.33	-	0	0	100	0	-	-
	100% HPr (Oehmen et al., 2005c)	0.42	0.33	1.22	0	-	0.56	0.67	18.2	81.8	-	-

Note: All data expressed in C-mol/C-mol except P/VFA which is express in P-mol/C-mol.

In order to better understand if the observed preference for HPr vs HAc uptake was due to different substrate affinities or to substrate competition, two additional anaerobic batch tests were performed, in parallel, with 50 mg COD/L of HAc and 150 mg COD/L of HPr (i.e. identical to the 25-75% HAc-HPr test) fed simultaneously, and 50 mg COD/L of HAc fed as single substrate. If the substrate affinity hypothesis was true, the HAc uptake rate would be the same in both tests, while if the substrate competition hypothesis was true the HAc uptake rate would be higher when HAc was fed alone. As can be observed in Figure 3.3, the acetate uptake rate was slower when HPr was present (0.07 C-mol/(C-mol biomass·h)) as compared to the test with acetate alone (0.17 C-mol/(C-mol biomass·h)). This result supports the validity of the substrate competition hypothesis where substrate competition was the main factor causing the decreased HAc uptake at higher HPr fractions, and not a difference between the affinity for HAc and HPr substrates.

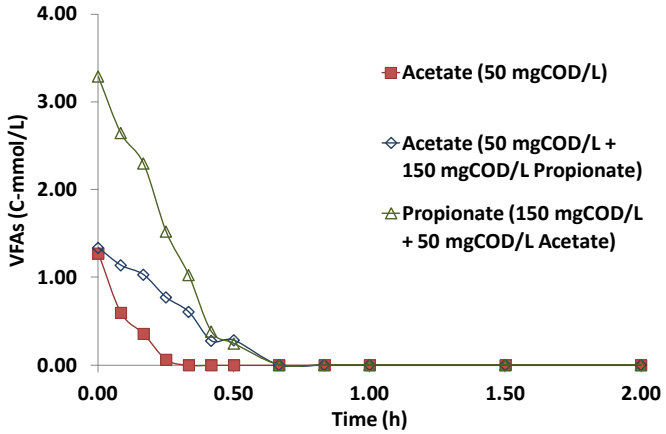


Figure 3.3 – VFA profiles in anaerobic batch tests to study the substrate competition and affinity hypotheses.

As the maximum propionate uptake rate was similar for all tests with HPr addition (Table 3.1), a constant value of $q_{smax\_PAO\_HPr}$ = 0.18 ± 0.02 C-mol/(C-mol·h) was implemented in the propionate uptake kinetic expression of the model (see Appendix D for full expression). In accordance with the experimental observations, the maximum acetate uptake parameter ($q_{smax\_PAO\_HAc}$) was a function of the propionate concentration (S_{HPr}), as defined below:

$S_{HPr} > K_{S\_HPr\_PAO}$: $q_{smax\_PAO\_VFA} - q_{smax\_PAO\_HPr} = q_{smax\_PAO\_HAc}$

$S_{HPr} \leq K_{S\_HPr\_PAO}$: 0.22 C-mol/(C-mol·h) = $q_{smax\_PAO\_HAc}$

where $K_{S\_HPr\_PAO}$ is the half-saturation coefficient for propionate uptake ($K_{S\_HPr\_PAO}$ = $K_{S\_HAc\_PAO}$ = 0.001 C-mmol/L) (Oehmen et al., 2010) and $q_{smax\_PAO\_VFA}$ is the maximum total VFA uptake rate, which was found to be 0.25 ± 0.02 C-mol/(C-mol·h) in this study. The acetate uptake parameter ($q_{smax\_PAO\_HAc}$) was used in the anaerobic acetate uptake equation, as shown below:

$$r_{HAc} = q_{smax\_PAO\_HAc} \cdot \frac{S_{HAc}}{S_{HAc} + K_{S,HAc}} \cdot X_{PAO} \cdot \frac{X_{PAO,PP}}{X_{PAO,PP} + K_{S,PP}} \cdot \frac{X_{PAO,GLY}}{X_{PAO,GLY} + K_{S,GLY}} \cdot \frac{f_{PHA,max} - f_{PAO,PHA}}{\left(f_{PHA,max} - f_{PAO,PHA}\right) + K_{S,fPHA}} \quad (3.3)$$

Interestingly, the maximum VFA uptake rate was higher when both HAc and HPr were present, as compared to when either substrate was present alone. This was also observed for *Defluviicoccus vanus* GAOs (Dai et al., 2007) and suggests that, although there is competition for the two substrates, some synergetic effects also occur. As observed in the present study, *Defluviicoccus vanus* GAOs prefer HPr to HAc (Dai et al., 2007), and their HPr uptake rate was higher than obtained for PAO culture. Thus, a mixture of acetate and propionate (75-25% HAc-HPr) can present an advantage for PAOs over both *Competibacter* and *Defluviicoccus vanus* GAOs.

The model was able to describe very well the anaerobic experimental results for acetate and propionate uptake, P release, PHA production and glycogen consumption, as shown in Figure 3.2. The NRMSD between the model predictions and experimental data was 7% for acetate, 7% for propionate, 7% for P, 9% for PHA and 11% for glycogen.

The anaerobic transformations associated with HAc and HPr uptake by PAOs (i.e. P release, glycogen consumption, PHA production) relative to 1 C-mmol of VFA uptake are presented in Table 3.2, where metabolic model predictions based on either HAc or HPr uptake are shown for comparison purposes. It can be observed that with a higher HPr fraction, less P is released, less glycogen is consumed and, in general, less PHA is produced, which is also the trend predicted by the metabolic models of HPr vs HAc. The glycogen transformations were generally lower than the metabolic model predictions, which could suggest that reducing equivalents may also be generated through the TCA cycle (Lanham et al., 2013a; Majed et al., 2012; Zhou et al., 2010). The ATP and redox balances of the experimental data are also presented in Table 3.2.

While the balances were reasonably closed in the case of 75-25% HAc-HPr (97% and 85% for the ATP and redox balance, respectively), some deviations were observed for the other tests, particularly the redox balance. This could be explained by the fact that these balances assume steady-state conditions, which is not necessarily a valid assumption after a short-term change in the carbon source composition. The % acetyl-CoA and % propionyl-CoA for each batch was also presented in Table 3.2 and were determined using equations 3.4 and 3.5 (Zeng et al., 2003):

$$\%\text{Acetyl}-\text{CoA} = \frac{\left(\frac{\text{PHB}}{\text{VFA}} + 0.4\times\left(\frac{\text{PH}_2\text{MB}}{\text{VFA}} + \frac{\text{PHV}}{\text{VFA}}\right)\right)}{\frac{\text{PHA}}{\text{VFA}}}\times 100 \qquad (3.4)$$

$$\%\text{Propionyl}-\text{CoA} = \frac{\left(\frac{\text{PH}_2\text{MV}}{\text{VFA}} + 0.6\times\left(\frac{\text{PH}_2\text{MB}}{\text{VFA}} + \frac{\text{PHV}}{\text{VFA}}\right)\right)}{\frac{\text{PHA}}{\text{VFA}}}\times 100 \qquad (3.5)$$

As can be observed by these results, the composition of PHAs is concordant with the change of carbon source, where an increase in the % propionyl-CoA was found with the increase of HPr concentration.

3.3.3 AEROBIC METABOLISM

The experimental data and model predictions for P, PHAs and glycogen obtained during the aerobic phase are represented in Figure 3.4. In all tests, P was removed to below 0.01 mg P/L at an average maximum rate of 0.048 ± 0.005 P-mol/(C-mol biomass·h). This result showed that the P uptake rate was independent of the carbon source ratio used and the PHA fractions were used with identical efficiency for P removal. The average ratio of VSS/TSS for the five batch tests was 0.71 ± 0.06 gVSS/gTSS, while the poly-phosphate content of the sludge was 12.3 ± 2.3%. Both data indicate the storage of polyphosphate by PAOs within the sludge, as also shown in previous studies (Oehmen et al., 2005a).

The kinetic parameters of the metabolic model that describe the P uptake and poly-P formation, PHA degradation and glycogen production are presented in Table 3.3. The kinetic rates for HAc are higher than the kinetic rates for HPr, as also observed in previous studies (Lopez-Vazquez et al., 2009). This result could be related

to the fact that less glycogen was consumed and less P was released during the preceding anaerobic period with HPr (Table 3.2), thus less glycogen was produced and there was less P to taken up in this experiment and less PHA was required for glycogen replenishment and P uptake. Nevertheless, the parameters obtained in the five experiments were within the same range (Table 3.3) and the parameters did not present a trend related with the variation of carbon source. Thus, the average of each kinetic parameter was used to validate the model, with the exception of the very low $q_{glycogen}$ with 100% HPr. The parameter for P uptake and poly-P formation ($q_{PO4\text{-}PP}$) was comparatively stable regardless of the carbon source used, unlike the predictions of the Lopez-Vazquez et al. (2009) model. This fact could be due to the enrichment of the PAO culture with a mixture of HAc and HPr (75-25% HAc-HPr), unlike other studies for model development and validation, that only used HAc or HPr as the sole carbon source. The fact that the $q_{PO4\text{-}PP}$ parameter was the most stable (Table 3.3), while the glycogen production and biomass growth rates varied slightly with the PHA degradation rate, suggests that PAOs tend to prioritise P uptake, while sacrificing glycogen production and cell growth, at lower intracellular PHA levels.

The carbon and phosphorus transformations can be well described by the kinetic parameters found in the model (Figure 3.4), with the exception of the batch test with only propionate as carbon source, where a high difference between the modelled and experimental glycogen data was observed, likely due to the low anaerobic glycogen consumption as described above. The NRMSD for the aerobic estimation of PHA, glycogen and P was 10%, 10% and 5%, respectively.

The results of this study suggest that the kinetic parameters for PHA, glycogen and P are independent of HAc and HPr concentration. This is of high relevance when modelling WWTPs, since the carbon source is usually a mixture of HAc and HPr (with a higher HAc fraction), facilitating the use of a constant set of kinetic parameters in most situations of WWTP operation.

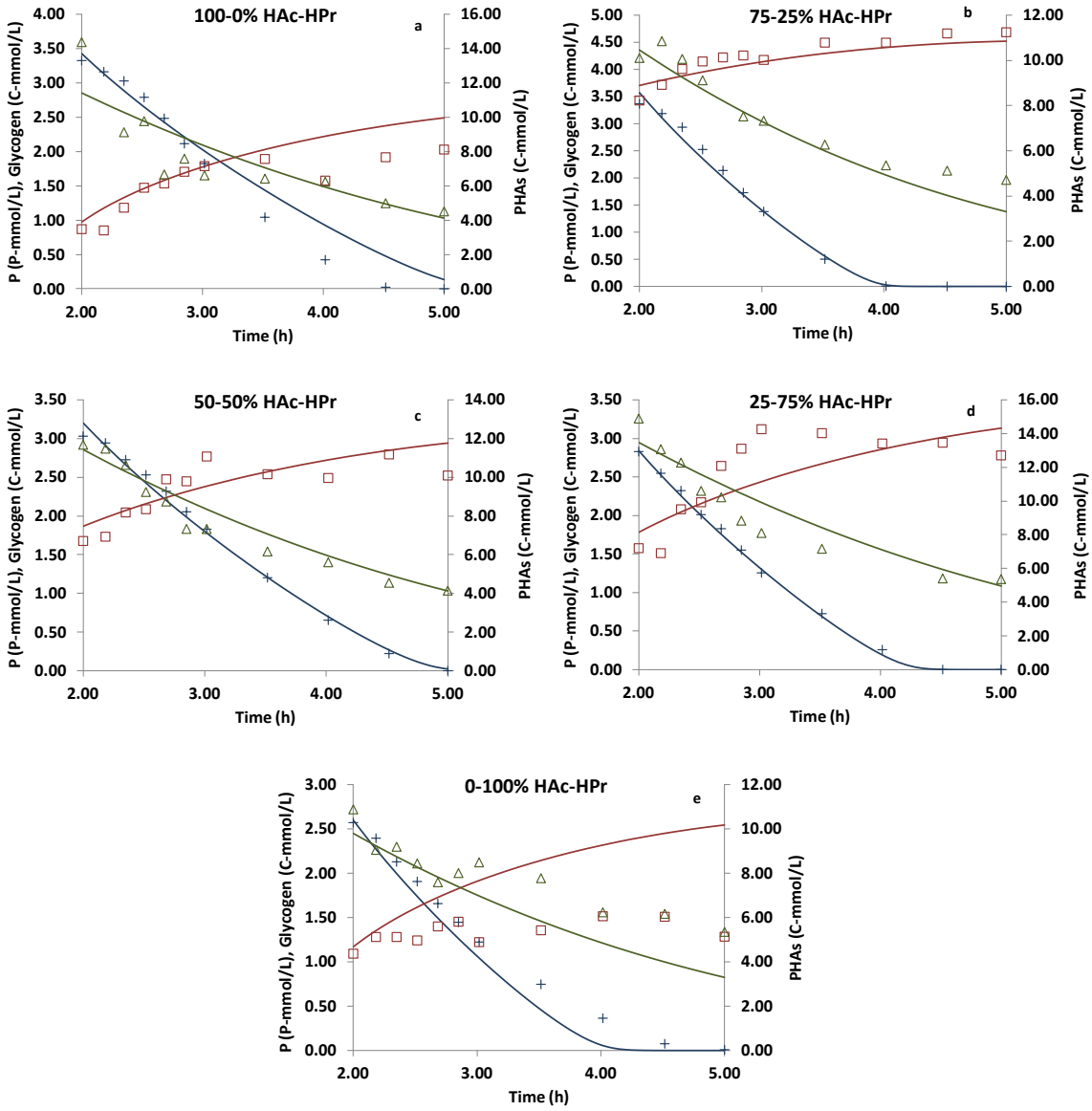


Figure 3.4 – Profile of aerobic P (+), PHAs (Δ) and glycogen (□) transformations and model predictions (solid line) for all batch tests. The feed solution for each test was: a) 200 mg COD HAc/L; b) 150 mg COD HAc/L and 50 mg COD HPr/L; c) 100 mg COD HAc/L and 100 mg COD HPr/L; d) 50 mg COD HAc/L and 150 mg COD HPr/L; e) 200 mg COD HPr/L. The model description was obtained through Aquasim simulations, using one parameter set to describe the PHA, P and glycogen profiles (Table 3.3).

Table 3.3 – Aerobic kinetic parameters of the model used in this study.

	q_{PHA}	$q_{Glycogen}$	$q_{PO4\text{-}PP}$	Biomass growth rate
	C-mol/(C-mol biomass·h)	**C-mol/(C-mol biomass·h)**	**P-mol/(C-mol biomass·h)**	**C-mol/(C-mol biomass·h)**
100-0% HAc-HPr	0.26 ± 0.02	0.0021 ± 0.0010	0.0087 ± 0.0020	0.060 ± 0.001
75-25% HAc-HPr	0.19 ± 0.01	0.0035 ± 0.0005	0.0072 ± 0.0011	0.023 ± 0.000
50-50% HAc-HPr	0.27 ± 0.01	0.0028 ± 0.0006	0.0072 ± 0.0014	0.069 ± 0.001
25-75% HAc-HPr	0.28 ± 0.01	0.0037 ± 0.0009	0.0071 ± 0.0026	0.072 ± 0.001
0-100% HAc-HPr	0.14 ± 0.01	0.0005 ± 0.0003	0.0061 ± 0.0014	0.028 ± 0.000
Model (This study)	0.23 ± 0.06	0.0030 ± 0.0007	0.0073 ± 0.0009	0.050 ± 0.023
100% HAc (Lopez-Vazquez et al., 2009)	0.800	0.015	0.020	-
100% HPr (Lopez-Vazquez et al., 2009)	0.330	0.008	0.002	-
100% HPr (Oehmen et al., 2010)	0.30	0.0150	0.0038	-

Note*:* Standard deviation for individual batch tests was determined through Aquasim, and the model standard deviation corresponds to the standard deviation of the average of each parameter.

3.3.4 LONG-TERM SIMULATION RESULTS

The long-term simulation results confirmed the model robustness, since after 3 SRT the VFA, PHA and P concentrations remained relatively well described and comparable to the experimental data (Figure 3.5). However, the glycogen concentration was observed to decrease after the 24 days of simulation. This could be related with the fact that the model did not incorporate reducing equivalents generated by the anaerobic TCA cycle, but only through glycogen degradation, leading to higher predicted glycogen consumption and, consequently, a decrease in glycogen concentration. The difficulty in achieving a consistently good description of glycogen dynamics in PAOs is well recognised in the literature (Brdjanovic et al., 2000; Lopez-Vazquez et al., 2009; Meijer et al., 2002; Van Veldhuizen et al., 1999). Recently, Lanham et al. (2014) proposed the incorporation of the anaerobic TCA cycle into the PAO model to describe their results using full-scale wastewater sludge, which could be considered in future studies. Nevertheless, in addition to the good description of the VFA, PHA and P, the biomass growth was also well described. After 96 cycles, the simulated biomass concentration remained relatively stable, varying less than 5%, with a concentration of 61.33 C-mmol/L and 59.47 C-mmol/L in the 1st and 96th cycle, respectively. Overall, the long-term simulation study supported the applicability of the estimated kinetic parameters of the model.

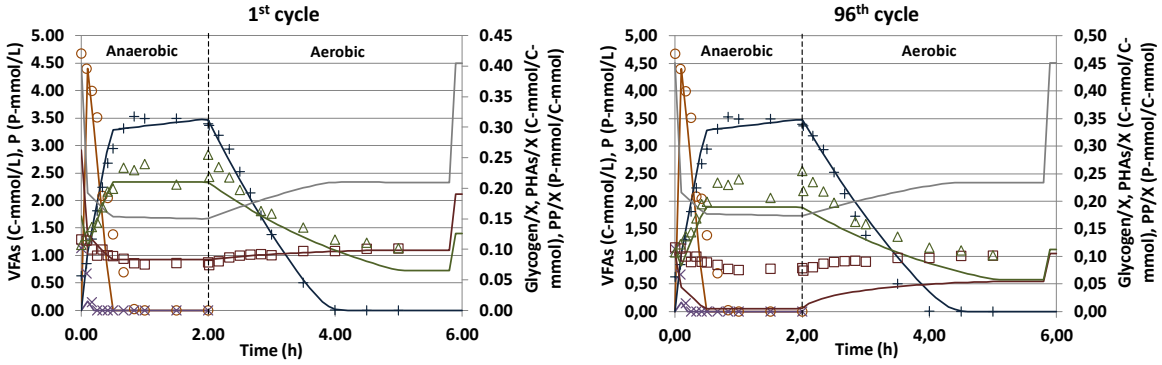


Figure 3.5 – Comparison between long-term simulation (solid lines) results obtained in the 1st and 96th cycle and the experimental data (points: acetate (○), propionate (X), P (+), PHA (Δ), glycogen (□), polyphosphate (PP) (grey line)).

3.3.5 IMPLICATIONS FOR FULL-SCALE SYSTEMS

Supplementation of the VFA content of the wastewater influent is often performed in WWTPs through prefermentation, and occasionally through external carbon dosing. The results of this study suggest that even relatively small increases in the propionate fraction can be advantageous for EBPR performance. Thomas et al. (2003) showed that the addition of molasses to the prefermenter, instead of direct supplementation with acetic acid, improved P removal, likely due to the increase in propionate fraction and elimination of GAOs. Molasses addition to prefermenters may be a more cost-effective solution to increase the propionate fraction of wastewater influents, due to the high costs associated with direct propionate supplementation.

The metabolic model of this study is more able to describe the behaviour of PAOs fed with mixed VFAs as compared to previous work. Indeed, while acetate is generally the most abundant VFA fraction present in most WWTPs, propionate is often present as well, which may have a significant impact on the microbial population selection and performance of the plant. Moreover, the finding that the aerobic kinetic parameters are insensitive to changes in carbon source implies a simpler calibration procedure when applying metabolic models to describe the behaviour of full-scale WWTPs. This suggests that plants employing occasional carbon dosing to the system (thereby changing the carbon source fractions) would not require re-calibration when accounting for the impact of this procedure on process performance.

3.4 CONCLUSIONS

An SBR was enriched with *Accumulibacter* PAO, using a combined feed of acetate and propionate (75-25% HAc-HPr), achieving good EBPR performance. The batch tests showed that propionate was depleted more quickly when both carbon sources are present, with an uptake rate similar for all batch tests (0.18 ± 0.02 C-mol/(C-mol biomass·h)), while the acetate uptake rate decreased with the increase of propionate concentration from 0.21 ± 0.02 to 0.06 ± 0.01 C-mol/(C-mol biomass·h). The difference between the acetate and propionate uptake rates for the PAO culture is due to the competition between these two substrates. The use of a combined carbon source feed (75% acetate and 25% propionate) seems more advantageous for PAOs over *Competibacter* and *Defluviicoccus vanus* GAOs, due to the high enrichment of PAOs (85 ± 2%) obtained in this study. The metabolic model obtained through the introduction of the anaerobic substrate competition effect described very well the

experimental data for carbon uptake. The aerobic kinetic parameters for PHA, glycogen and P were shown to be relatively independent of the concentration of acetate or propionate, showing that in WWTPs the metabolic model parameters do not need to be changed as a function of the VFA source.

REFERENCES

Amann, R.I., 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes, in: Akkermans van Elsas, J.D., de Bruijn, F.J., A.D.L. (Ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publications, Dordrecht, Holland, pp. 1–15.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56 (6), 1919–1925.

APHA/AWWA, 1995. Standard Methods for the Examination of Water and Wastewater. Port City Press. Balt.

Brdjanovic, D., Van Loosdrecht, M.C.M., Versteeg, P., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 2000. Modeling COD, N and P removal in a full-scale wwtp Haarlem Waarderpolder. Water Res. 34, 846–858.

Chen, Y., Liu, Y., Zhou, Q., Gu, G., 2005. Enhanced phosphorus biological removal from wastewater—effect of microorganism acclimatization with different ratios of short-chain fatty acids mixture. Biochem. Eng. J. 27 (1), 24–32.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., Blackall, L.L., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology 148 (11), 3353–3364.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of Polyphosphate-Accumulating Organisms and Design of 16S rRNA-Directed Probes for Their Detection and Quantitation. Appl. Environ. Microbiol. 66 (3), 1175–1182.

Dai, Y., Yuan, Z., Wang, X., Oehmen, A., Keller, J., 2007. Anaerobic metabolism of Defluviicoccus vanus related glycogen accumulating organisms (GAOs) with acetate and propionate as carbon sources. Water Res. 41 (9), 1885–1896.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. Syst. Appl. Microbiol. 22 (3), 434–444.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001. pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. Water Environ. Res. 73 (2), 223–232.

Flowers, J.J., He, S., Yilmaz, S., Noguera, D.R., McMahon, K.D., 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different "Candidatus Accumulibacter" clades. Environ. Microbiol. Rep. 1 (6), 583–588.

Guisasola, A., Vargas, M., Marcelino, M., Lafuente, J., Casas, C., Baeza, J.A., 2007. On-line monitoring of the enhanced biological phosphorus removal process using respirometry and titrimetry. Biochem. Eng. J. 35 (3), 371–379.

Hollender, J., van der Krol, D., Kornberger, L., Gierden, E., Dott, W., 2002. Effect of different carbon sources on the enhanced biological phosphorus removal in a sequencing batch reactor. World J. Microbiol. Biotechnol. 18 (4), 359–364.

Kong, Y., Ong, S.L., Ng, W.J., Liu, W.-T., 2002. Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes. Environ. Microbiol. 4 (11), 753–757.

Lanham, A., Oehmen, A., Saunders, A., Carvalho, G., Nielsen, P., Reis, M., 2014. Metabolic modelling of full-scale enhanced biological phosphorus removal sludge. Water Res. 66 (1), 283-295.

Lanham, A.B., Oehmen, A., Saunders, A.M., Carvalho, G., Nielsen, P.H., Reis, M.A.M., 2013a. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. Water Res. 47 (19), 7032–7041.

Lanham, A.B., Reis, M.A.M., Lemos, P.C., 2008. Kinetic and metabolic aspects of Defluviicoccus vanusrelated organisms as competitors in EBPR systems. Water Sci. Technol. 58 (8), 1693–1697.

Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013b. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. Process Biochem. 48 (11), 1626–1634.

Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2012. Optimisation of glycogen quantification in mixed microbial cultures. Bioresour. Technol. 118 (0), 518–525.

Levantesi, C., Serafim, L.S., Crocetti, G.R., Lemos, P.C., Rossetti, S., Blackall, L.L., Reis, M.A.M., Tandoi, V., 2002. Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. Environ. Microbiol. 4 (10), 559–569.

Liu, W.-T., Nakamura, K., Matsuo, T., Mino, T., 1997. Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors—Effect of PC feeding ratio. Water Res. 31 (6), 1430–1438.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Res. 43 (2), 450–462.

Majed, N., Chernenko, T., Diem, M., Gu, A.Z., 2012. Identification of Functionally Relevant Populations in Enhanced Biological Phosphorus Removal Processes Based On Intracellular Polymers Profiles and Insights into the Metabolic Diversity and Heterogeneity. Environ. Sci. Technol. 46 (9), 5010–5017.

McIlroy, S., Seviour, R.J., 2009. Elucidating further phylogenetic diversity among the Defluviicoccus-related glycogen-accumulating organisms in activated sludge. Environ. Microbiol. Rep. 1 (6), 563–568.

Meijer, S.C.F., Van Loosdrecht, M.C.M., Heijnen, J.J., 2002. Modelling the start-up of a full-scale biological phosphorous and nitrogen removing WWTP. Water Res. 36, 4667–4682.

Meyer, R.L., Saunders, A.M., Blackall, L.L., 2006. Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology 152 (2), 419–429.

Nittami, T., McIlroy, S., Seviour, E.M., Schroeder, S., Seviour, R.J., 2009. Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III Defluviicoccus. Syst. Appl. Microbiol. 32 (7), 480–489.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Res. 41 (11), 2271–2300.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res. 44 (15), 4473–4486.

Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J., 2005a. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Res. 39 (15), 3727–3737.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2005b. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol. Bioeng. 91 (2), 162–168.

Oehmen, A., Zeng, R.J., Yuan, Z., Keller, J., 2005c. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnol. Bioeng. 91 (1), 43–53.

Onuki, M., Satoh, H., Mino, T., 2002. Analysis of microbial community that performs enhanced biological phosphorus removal in activated sludge fed with acetate. Water Sci. Technol. 46 (1-2), 145–153.

Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnol. Bioeng. 85 (1), 56–67.

Reichert, P., 1994. AQUASIM - a tool for simulation and data analysis of aquatic systems. Water Sci. Technol. 30 (2), 21–30.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.

Thomas, M., Wright, P., Blackall, L., Urbain, V., Keller, J., 2003. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. Water Sci. Technol. 47, 141–148.

Tu, Y., Schuler, A.J., 2013. Low Acetate Concentrations Favor Polyphosphate-Accumulating Organisms over Glycogen-Accumulating Organisms in Enhanced Biological Phosphorus Removal from Wastewater. Environ. Sci. Technol. 47 (8), 3816–3824.

Van Veldhuizen, H.M., Van Loosdrecht, M.C.M., Heijnen, J.J., 1999. Modelling biological phosphorus and nitrogen removal in a full scale activated sludge process. Water Res. 33, 3459–3468.

Wang, Y., Jiang, F., Zhang, Z., Xing, M., Lu, Z., Wu, M., Yang, J., Peng, Y., 2010. The long-term effect of carbon source on the competition between polyphosphorus accumulating organisms and glycogen accumulating organism in a continuous plug-flow anaerobic/aerobic (A/O) process. Bioresour. Technol. 101 (1), 98–104.

Wong, M.-T., Tan, F.M., Ng, W.J., Liu, W.-T., 2004. Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic–aerobic activated sludge processes. Microbiology 150 (11), 3741–3748.

Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z., Keller, J., 2003. Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnol. Bioeng. 81 (1), 92–105.

Zhou, Y., Pijuan, M., Oehmen, A., Yuan, Z., 2010. The source of reducing power in the anaerobic metabolism of polyphosphate accumulating organisms (PAOs) - a mini-review. Water Sci. Technol. 61 (7), 1653–1662.

4

THE IMPACT OF AERATION ON THE COMPETITION BETWEEN POLYPHOSPHATE ACCUMULATING ORGANISMS AND GLYCOGEN ACCUMULATING ORGANISMS

SUMMARY: In wastewater treatment plants (WWTP), aeration is the major energetic cost, thus its minimisation will improve the cost-effectiveness of the process. This study shows that both the dissolved oxygen (DO) concentration and aerobic hydraulic retention time (HRT) affects the competition between polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs). At low DO levels, Accumulibacter PAOs were shown to have an advantage over Competibacter GAOs, as PAOs had a higher oxygen affinity and thus largely maintained their aerobic activity at low DO levels, while GAO activity decreased. Bioreactor operation at low DO levels was found to increase the PAO fraction of the sludge. Furthermore, an increase in aerobic HRT (at a DO level of 2 mg O_2/L), promoted the proliferation of GAOs over PAOs, decreasing the EBPR efficiency. Overall, this study shows that low aeration can be beneficial for EBPR performance through selecting for PAOs over GAOs, which should be incorporated into WWTP models in order to minimise energetic costs and improve WWTP sustainability.

PUBLISHED AS: Mónica Carvalheira, Adrian Oehmen, Gilda Carvalho, Mário Eusébio, Maria A.M. Reis. “The impact of aeration on the competition between polyphosphate accumulating organisms and glycogen accumulating organisms”, Water Research, 66 (2014), 296-307**.**

4.1 INTRODUCTION

In wastewater treatment plants (WWTPs), the economic, environmental and social factors should be considered in order to improve process sustainability (Molinos-Senante et al., 2012; Muga and Mihelcic, 2008). Improving the energetic efficiency of WWTPs is central to this theme, as minimising energetic inputs both lowers operational costs and reduces indirect greenhouse gas emissions. Several factors contribute towards WWTP energy consumption, where the aeration requirements are typically the most significant. Indeed, it has been estimated that the aeration requirements correspond to 45-75% of WWTP energetic costs (Rosso et al., 2008), so it is necessary to decrease the oxygen requirements in order to improve the cost-effectiveness of the WWTP.

Enhanced biological phosphorus removal (EBPR) is an economic and sustainable process used to remove phosphorus (P) from wastewater. In this process, it is necessary to control the competition between polyphosphate accumulating organisms (PAOs) and another group of microorganisms known as glycogen accumulating organisms (GAOs) (Oehmen et al., 2007). Minimising the growth of GAOs improves the efficiency of organic carbon and oxygen utilisation for P removal, thus minimising P effluent concentrations.

The competition between PAOs and GAOs, and consequently the efficiency of EBPR, can be affected by several factors, such as pH (Filipe et al., 2001; Lopez-Vazquez et al., 2009; Weissbrodt et al., 2013; Zhang et al., 2007), temperature (Lopez-Vazquez et al., 2009; Weissbrodt et al., 2013; Whang and Park, 2006), volatile fatty acids (VFA) composition of the wastewater (Lopez-Vazquez et al., 2009; Oehmen et al., 2006), sludge age (Whang and Park, 2006) and the ratio of P to organic carbon in the influent (Liu et al., 1997). However, the effect of aeration on the PAO-GAO competition has never been systematically studied.

Previous studies have shown that PAOs can be selected over GAOs at dissolved oxygen (DO) concentrations of about 0.5 mg/L (Lemaire et al., 2006), and high DO concentrations > 3 mg/L (Oehmen et al., 2005; Pijuan et al., 2004), achieving successful EBPR operation. Griffiths et al. (2002) performed morphological observations of WWTP sludges and hypothesised that the DO concentration could affect the quantity of tetrad forming organisms thought to be GAOs. Numerous studies (Li and Chen, 2011; Li et al., 2008; Zheng et al., 2009), have operated EBPR systems at very low DO concentrations (0.15 - 0.45 mg/L), with variable P removal efficiency

achieved (61% - 99%). However, none of these studies have investigated the effect of DO concentration on the microbial community and their metabolism, thus it is still unknown how the DO level impacts the PAO-GAO competition and EBPR efficiency.

Furthermore, it is not only the DO concentration that can affect the success of EBPR, but also the length of the aerobic period. Brdjanovic et al. (1998) showed through a series of short-term tests that extended aerobic periods can promote the deterioration of the EBPR process through excessive consumption of their internal storage polymers. Since both PAOs and GAOs depend on the consumption of storage polymers such as polyhydroxyalkanoates (PHA) aerobically, it is unclear if increased PHA consumption would be more beneficial for PAO or GAO. Thus it is important to study the impact of aerobic hydraulic retention time (HRT) on the competition between PAOs and GAOs, since its effect on the microbial selection of PAOs vs GAOs is not yet known.

In this study, the effect of aeration on the competition between PAOs and GAOs was investigated for the first time. For this purpose, batch tests of enriched PAO and GAO cultures were performed at different DO levels, in order to determine how the aerobic kinetics of each population varies as a function of the DO concentration. Moreover, long-term tests were performed to assess the performance and microbial population of each system operated at different DO levels. Finally, the impact of the aerobic HRT on the microbial population and EBPR performance was also studied. This knowledge will contribute towards the understanding of the competition between PAOs and GAOs as a function of both the DO concentration and the aerobic HRT. This could lead to the development of strategies to minimise WWTP energetic costs, improving the EBPR efficiency, and enabling greater sustainability of these processes.

4.2 MATERIALS AND METHODS

4.2.1 SBR OPERATION

Two sequencing batch reactors (SBR - see Appendix H) with 2L of working volume were operated to obtain enriched cultures of PAOs and GAOs. The reactors were seeded with sludge from a wastewater treatment plant in Lisbon, Portugal. Each cycle consisted of 6 hours, with a 2 hour anaerobic period, 3 hour aerobic period and 1 hour settle/decant period. Both reactors were fed, during the first 5 minutes of the anaerobic phase, with one liter of synthetic medium and were operated with a hydraulic

retention time (HRT) of 12 hours and a solids retention time (SRT) of 8 days. The initial chemical oxygen demand (COD) concentration in each reactor was 200 mg/L, where the PAO reactor contained a mixture of acetate (HAc) and propionate (HPr) as carbon source (75-25% HAc-HPr), while the GAO reactor was fed with only HAc. The difference in carbon source fed to each system was based on a previous study (Lopez-Vazquez et al., 2009) that predicted that PAOs would proliferate with a 75-25% HAc-HPr ratio and GAOs would proliferate with only HAc. The 1L synthetic medium was composed of 250 mL of solution A and 750 mL of solution B. Solution A contained per liter, in both reactors: 0.59 g NH_4Cl, 0.95 g $MgSO_4.7H_2O$, 0.44 g $CaCl_2.2H_2O$, 11.7 mg allyl-N thiourea (ATU, a nitrification inhibitor), 31.7 mg ethylene-diaminetetraacetic (EDTA), 3.17 mL of a micronutrients, 2.55 g $C_2H_3O_2Na.3H_2O$ and 270µL $C_3H_6O_2$ in the PAO reactor or 3.40 g $C_2H_3O_2Na.3H_2O$ in the GAO reactor. The micronutrient solution (based on Smolders et al.(1994)) contained per litre: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnCl_2.4H_2O$, 0.06 g $Na_2MoO.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$. Solution B contained per liter: 124.1 mg K_2HPO_4 and 96.8 mg KH_2PO_4 in the PAO reactor and 7.50 mg K_2HPO_4 and 5.86 mg KH_2PO_4 in the GAO reactor. In both reactors, the temperature was controlled at 20 ± 1ºC. pH was controlled, by automatic addition of 0.1 M HCl, at 7.5 and 7.0 for PAO and GAO reactors, respectively, which is also consistent with previous predictions for the selection of PAOs and GAOs, respectively (Lopez-Vazquez et al., 2009). To ensure anaerobic conditions in the reactors, argon was bubbled at a flow rate of approximately 15 mL/min. In the aerobic phase, the dissolved oxygen was maintained at 8 ± 0.2 mg O_2/L (with continuous aeration) or controlled at 2 ± 0.2 mg O_2/L using an on/off control valve. Both reactors were operated for >3 SRT at the aforementioned conditions with continuous aeration before commencing DO control at 2 mg O_2/L. Operation with DO control continued for >3 SRT prior to performing the batch tests detailed below. The objective of this decrease in DO concentration to 2 mg O_2/L was to mimic the DO level often experienced in full-scale EBPR plants.

The SBRs were monitored through routine sampling of volatile fatty acids (VFAs), P, PHAs and glycogen along the anaerobic/aerobic cycles. Total suspended solids (TSS) and volatile suspended solids (VSS) samples were taken at the end of the aerobic phase, while fluorescence in situ hybridisation (FISH) samples were taken at the end of both the anaerobic and aerobic phases.

4.2.2 BATCH EXPERIMENTS AT DIFFERENT DO LEVELS

After the SBRs were operated at DO = 2 mg O_2/L for > 3 SRT, five aerobic batch tests were performed to study the effect of different DO concentrations (0.6 ± 0.1, 1.0 ± 0.2, 2.1 ± 0.2, 3.1 ± 0.1 and ~8 mg O_2/L, respectively) on each culture. In order to estimate the oxygen affinity coefficient, two additional batch tests were performed for the PAO culture at 0.3 ± 0.1 and 0.1 ± 0.0 mg O_2/L, respectively. Waste sludge (500 mL) was taken from the PAO or GAO reactor at the end of the anaerobic phase for these tests. The DO concentration was controlled using an on/off valve in all experiments except at a DO of ~8 mg O_2/L, which was achieved through continuous aeration. The pH was controlled at 7.5 and 7.0 for PAOs and GAOs, respectively.

Samples for PHA, glycogen, and phosphate (in the case of PAOs) quantification were taken at various points over time and analysed by the methods described below. Samples for TSS, VSS and FISH analysis were obtained at the end of the experiment.

4.2.3 EFFECT OF AEROBIC HYDRAULIC RETENTION TIME (HRT)

The effect of increasing the aeration period on the PAO SBR was also studied. The cycle time was increased from 6h to 12h in this reactor, consisting of a 2 hour anaerobic period, 9 hour aerobic period and 1 hour settle/decant period. The HRT and SRT were 1 and 16 days, respectively. The SRT was increased to 16 days in order to maintain a constant biomass concentration and avoid biomass washout, thereby maintaining constant the food to microorganism ratio (F/M) during the study, since this ratio can affect the EBPR stability (Ahn and Park, 2008). The DO concentration was controlled at 2 ± 0.2 mg O_2/L, while other operational conditions (e.g. pH and temperature), feed composition and sampling procedures were identical to those detailed in section 4.2.1.

4.2.4 CHEMICAL ANALYSIS

The TSS, VSS, PHA, glycogen, phosphate and total phosphate were determined as described in section 3.2.3 – Chapter 3.

Error bars represented in the figures correspond to the error of the PHA and glycogen method determined in Lanham et al. (2012, 2013) and the standard deviation of P and VFA analysis. The standard deviation of the anaerobic transformations and

VSS/TSS ratio were determined through the calculation of error propagation as described in section 3.2.7 - Chapter 3.

4.2.5 MICROBIAL ANALYSIS

Fluorescence *in situ* hybridisation (FISH) was performed as described in section 3.2.3 – Chapter 3. The probes used were: EUBMIX (EUB338, EUB338-II and EUB338-III), PAOMIX (PAO 651, 462 and 846); GAOMIX (GAOQ989 and GB_G2); *Defluviicoccus vanus-related GAOs* cluster I (TFO_DF218 and TFO_DF618), cluster II (DF988 and DF1020), cluster III (DF1013 and DF1004) and cluster IV (DF181A and DF181B).

4.2.6 PARAMETER ESTIMATION

To estimate the aerobic kinetic parameters (q_{PHA}, $q_{Glycogen}$ and q_{PP}) of PAOs and GAOs, a previously developed metabolic model (Lanham et al., (2014); Oehmen et al., 2010) was used. The equations describing the PHA degradation (equation 4.1), glycogen production (equation 4.2) and poly-P formation (equation 4.3) of PAOs, and the PHA degradation (equation 4.4) and glycogen production (equation 4.5) of GAOs are shown below (see Table 4.1 for definition of components and parameters):

$$r_{PHA,PAO} = q_{PAO,PHA,OX} \cdot f_{PAO,PHA}^{2/3} \cdot X_{PAO} \cdot \frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.1)$$

$$r_{GLY,PAO} = q_{PAO,GLY,OX} \cdot f_{PAO,PHA}^{2/3} \cdot \frac{1}{f_{PAO,GLY}} \cdot X_{PAO} \cdot \frac{f_{PAO,GLY,max} - f_{PAO,GLY}}{f_{PAO,GLY,max} - f_{PAO,GLY} + K_{S,GLY}} \cdot \frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.2)$$

$$r_{PP,PAO} = q_{PAO,PO4\_PP,OX} \cdot \frac{1}{f_{PAO,PP}} \cdot X_{PAO} \cdot \frac{f_{PAO,PP,max} - f_{PAO,PP}}{f_{PAO,PP,max} - f_{PAO,PP} + K_{S,PP}} \cdot \frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,PHA}} \cdot \frac{S_{PO4}}{S_{PO4} + K_{S,PO4}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.3)$$

where $f_{PAO,PHA} = \frac{X_{PAO,PHA}}{X_{PAO}}$, $f_{PAO,GLY} = \frac{X_{PAO,GLY}}{X_{PAO}}$, $f_{PAO,PP} = \frac{X_{PAO,PP}}{X_{PAO}}$

$$r_{PHA,GAO} = q_{GAO,PHA,OX} \cdot f_{GAO,PHA}^{2/3} \cdot X_{GAO} \cdot \frac{X_{GAO,PHA}}{X_{GAO,PHA} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.4)$$

$$r_{GLY,GAO} = q_{GAO,GLY,OX} \cdot f_{GAO,PHA}^{2/3} \cdot \frac{1}{f_{GAO,GLY}} \cdot X_{GAO} \cdot \frac{f_{GAO,GLY,max} - f_{GAO,GLY}}{f_{GAO,GLY,max} - f_{GAO,GLY} + K_{S,GLY}} \cdot \frac{X_{GAO,PHA}}{X_{GAO,PHA} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.5)$$

where $f_{GAO,PHA} = \frac{X_{GAO,PHA}}{X_{GAO}}$, $f_{GAO,GLY} = \frac{X_{GAO,GLY}}{X_{GAO}}$

Table 4.1 – Parameter description and kinetic coefficients for *Accumulibacter* PAO and *Competibacter* GAO (Lanham et al., (2014); Oehmen et al., 2010).

Parameter	Description	Value	Units
$q_{PAO,PHA,OX}$	PHA degradation rate for PAOs	-[a]	C-mol/(C-mol.h)
$q_{PAO,GLY,OX}$	Glycogen production rate for PAOs	-[a]	C-mol/(C-mol.h)
$q_{PAO,PO4\_PP,OX}$	P-uptake rate for PAOs	-[a]	C-mol/(C-mol.h)
$q_{GAO,PHA,OX}$	PHA degradation rate for GAOs	-[a]	C-mol/(C-mol.h)
$q_{GAO,GLY,OX}$	Glycogen production rate for GAOs	-[a]	C-mol/(C-mol.h)
X_{PAO}	Biomass concentration of PAOs	-	C-mmol/L
$X_{PAO,PHA}$	PHA concentration in PAO	-	C-mmol/L
$X_{PAO,Gly}$	Glycogen concentration in PAO	-	C-mmol/L
$X_{PAO,PP}$	Poly-phosphate concentration in PAO	-	C-mmol/L
X_{GAO}	Biomass concentration of GAOs	-	C-mmol/L
$X_{GAO,PHA}$	PHA concentration in GAO	-	C-mmol/L
$X_{GAO,Gly}$	Glycogen concentration in GAO	-	C-mmol/L
S_{PO4}	P concentration in the bulk liquid	-	P-mmol/L
S_{O2}	Oxygen concentration	-	mg/L
$f_{PAO,PHA}$	Fraction of PHA in PAO	-	C-mol/C-mol
$f_{PAO,GLY}$	Fraction of glycogen in PAO	-	C-mol/C-mol
$f_{PAO,,PP}$	Fraction of poly-P in PAO	-	C-mol/C-mol

$f_{GAO,PHA}$	Fraction of PHA in GAO	-	C-mol/C-mol
$f_{PAO,GLY}$	Fraction of glycogen in GAO	-	C-mol/C-mol
$f_{PAO,GLY,max}$	Maximum glycogen content per PAO biomass concentration	0.8	C-mol/C-mol
$f_{PAO,PP,max}$	Maximum poly-P content per PAO biomass concentration	0.30	P-mol/C-mol
$f_{GAO,GLY,max}$	Maximum glycogen content per GAO biomass concentration	0.5	C-mol/C-mol
$K_{S,PHA}$	Half-saturation coefficient for PHA	0.13 (PAO) 0.12 (GAO)	C-mmol/L
$K_{S,GLY}$	Half-saturation coefficient for glycogen	0.01	C-mmol/L
$K_{S,PP}$	Half-saturation coefficient for poly-phosphate (poly-P)	0.01	P-mmol/L
$K_{S,PO4}$	Half-saturation coefficient for orthophosphate	0.01	P-mmol/L
$K_{S,O2}$	Half-saturation coefficient for oxygen	0.01	O_2-mmol/L

[a]See Appendix F and G for estimation as a function of the DO concentration.

The parameter estimation tool of Aquasim (Reichert, 1994) was used to estimate the maximum aerobic PHA degradation, glycogen production and P uptake rates for PAOs and GAOs from each batch test., The initial concentration of PHA, P and glycogen in aerobic phase was estimated simultaneously to each kinetic parameter. The estimated rate at each DO concentration was used to evaluate the relationship between the DO concentration and the aerobic kinetics as explained in section 3.2. The $K_{S,PHA}$ parameter was also estimated in this study, but did not vary significantly between the different batch tests (0.13 ± 0.03 C-mmol/L for PAOs and 0.12 ± 0.03 C-mmol/L for GAOs). All other parameters were equivalent to those proposed in previous studies (Lanham et al., (2014); Oehmen et al., 2010). The percent error between the model predictions and experimental data was determined through the normalised root mean square deviation (NRMSD) as described in section 3.2.6 - Chapter 3.

4.3 RESULTS AND DISCUSSION

4.3.1 PAO AND GAO SBR CHARACTERISATION AT TWO DIFFERENT DO LEVELS

Each SBR was routinely monitored in order to characterise its performance with continuous aeration in the aerobic phase (DO ~8 mg O_2/L) and with the DO controlled at 2 mg O_2/L. Figure 4.1a (PAO) and 4.1b (GAO) present the SBR results where the DO concentration was maintained at 8 mg O_2/L, while Figure 4.1c (PAO) and 4.1d (GAO) present the results obtained when the DO concentration was controlled at 2 mg O_2/L. As expected, the VFAs were taken up in the anaerobic phase in each reactor and stored as PHA, with simultaneous glycogen degradation, while PHA degradation aerobically led to glycogen production. Anaerobic P release and aerobic P uptake was also observed in the PAO reactor. Interestingly, the quantity of P release and P uptake increased when the DO concentration decreased from 8 to 2 mg O_2/L (Table 4.2), although a high P removal efficiency of 99 ± 1 % was achieved at both DO concentrations. The VSS/TSS ratio decreased with DO concentration decrease, which reflected the simultaneous increase in the content of inorganic polyphosphate in the sludge (Table 4.2). FISH quantification revealed that the PAO reactor was highly enriched in *Accumulibater* at both DO concentrations, while *Competibacter* were present in lower abundance (Table 4.2). However, *Accumulibacter* increased from 71 to 90% and *Competibacter* decreased from 20% to <1% after the DO concentration was changed from 8 to 2 mg O_2/L. The FISH results along with the P removal and polyphosphate storage by the sludge showed that the decrease in DO concentration was more advantageous for PAOs as compared to GAOs in this system.

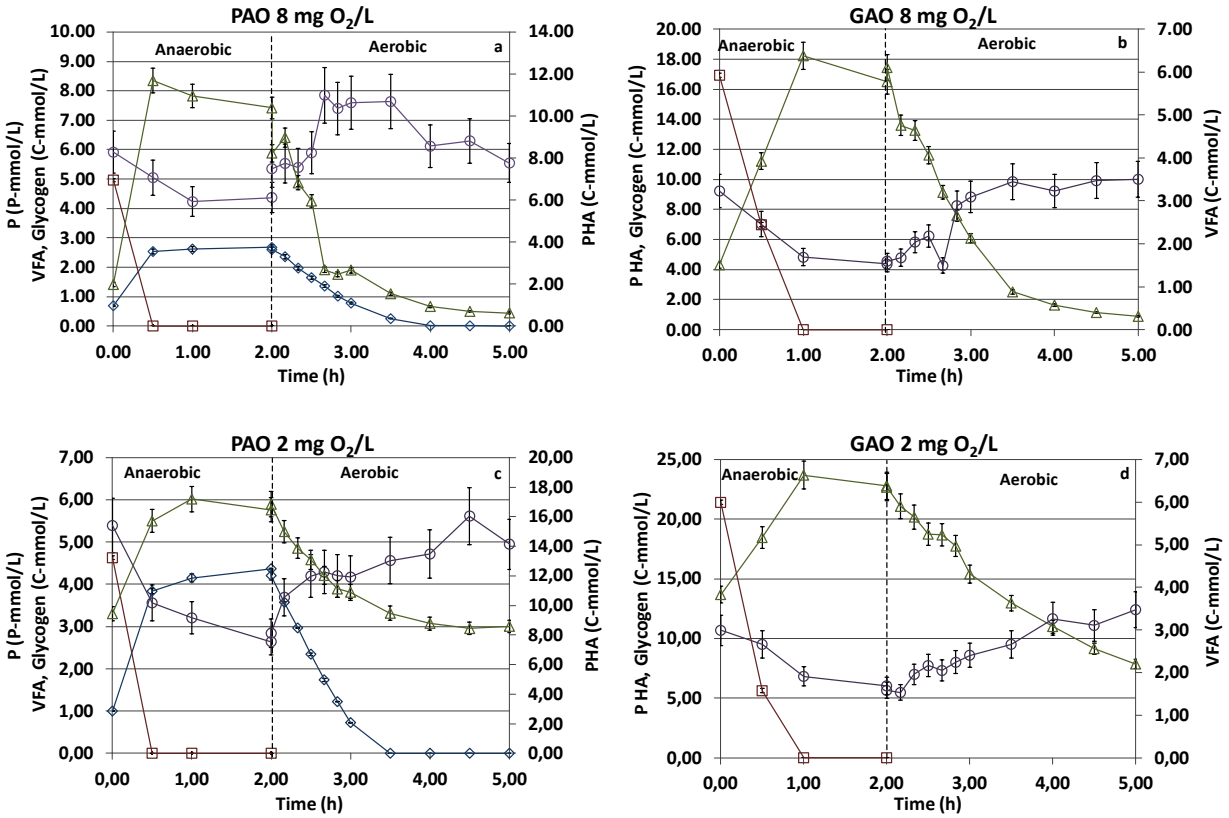


Figure 4.1 – Profile of P and carbon transformations in the PAO reactor at DO concentrations of 8 mgO_2/L (a) and 2 mgO_2/L (c) and carbon transformations in the GAO reactor at DO concentrations of 8 mgO_2/L (b) and 2 mgO_2/L (d) (VFA (□), PHA (Δ), P (◊), glycogen (○)).

Table 4.2 – Comparison of the SBR-PAO and SBR-GAO at 2 and 8 mg O_2/L.

	PAO		**GAO**	
	8 ± 0.2 mg O_2/L	**2 ± 0.2 mg O_2/L**	**8 ± 0.2 mg O_2/L**	**2 ± 0.2 mg O_2/L**
$\frac{VSS}{TSS}$ (gVSS/gTSS)	0.64 ± 0.09	0.57 ± 0.02	0.94 ± 0.08	0.96 ± 0.01
$P_{release}$ (mg P/L)	45 ± 7	66 ± 11	-	-
P_{uptake} (mg P/L)	78 ± 15	114 ± 15	-	-
% P	8.2 ± 2.8	15 ± 2	0.9 ± 0.4	1.0 ± 0.4
Accumulibacter (%)	71% (SEM: 9%)	90% (SEM: 1%)	<1%	<1%
Competibacter (%)	20% (SEM: 5%)	0.7% (SEM: 0.1%)	63% (SEM: 4%)	65% (SEM: 2%)

The anaerobic transformations in the PAO reactor (i.e. P release, glycogen consumption, PHA production) relative to 1 C-mmol of VFA uptake are presented in Table 4.3. The P release per VFA uptake ratio was observed to increase substantially after the decrease in DO concentration from 8 to 2 mg O_2/L, which agrees well with the increase in *Accumulibacter* PAOs at the expense of *Competibacter* GAOs. The total PHA production was observed to decrease slightly after changing the DO, while glycogen consumption increased, although both fractions were in the expected range for PAOs. The PHA composition (PHB, PHV, PH_2MB and PH_2MV) was also affected by the DO, along with the %Acetyl-CoA and %Propionyl-CoA (equations 4.7 and 4.8), where the lower DO concentration led to results that are more similar to those predicted by the PAO model with a 75-25% HAc:HPr carbon source fraction (Table 4.3). The higher propionyl-CoA fraction observed at a DO concentration of 8 mg O_2/L suggests greater activity by GAOs under this condition (Zeng et al., 2003), which agrees well with the FISH and performance results.

$$\%\text{Acetyl}-\text{CoA} = \frac{\left(\frac{PHB}{VFA} + 0.4 \times \left(\frac{PH_2MB}{VFA} + \frac{PHV}{VFA}\right)\right)}{\frac{PHA}{VFA}} \times 100 \qquad (4.7)$$

$$\%\text{Propionyl}-\text{CoA} = \frac{\left(\frac{PH_2MV}{VFA} + 0.6 \times \left(\frac{PH_2MB}{VFA} + \frac{PHV}{VFA}\right)\right)}{\frac{PHA}{VFA}} \times 100 \qquad (4.8)$$

In the GAO reactor, the VSS/TSS ratio remained stable at approximately 0.95 after the decrease of the DO concentration from 8 to 2 mg O_2/L (Table 4.2), which agrees well with the very small quantity of inorganic P contained in the sludge (~1% at each DO level). FISH quantification showed that *Competibacter* was highly abundant (Table 4.2) at each DO concentration, while the *Accumulibacter* population was negligible. It should be noted that *Defluviicoccus vanus*-related GAOs were not observed in either reactor. The change in DO concentration therefore did not appear to impact the GAO population, perhaps due to the limited P concentration in the feed, ensuring that PAOs were not able to outcompete GAOs. The anaerobic transformations for the GAO reactor (i.e. glycogen consumption and PHA production) per C-mmol of VFA uptake are presented in Table 4.3 and show that while the glycogen consumption was similar at both DO concentrations, the PHA production decreased with the decrease of DO concentration. A possible explanation for this result could be related to the fact that when the DO concentration decreased, the aerobic PHA degradation also decreased, such that at the end of the aerobic period the PHA concentration at 2 mg O_2/L was higher than at 8 mg O_2/L (Figure 4.1b, d). Consequently, in the following anaerobic period, lower PHA production may have been necessary to approach their maximum PHA content, thereby leading to a lower PHA/VFA ratio. Nevertheless, the percentages of each monomer (PHB and PHV) and corresponding Acetyl-CoA and Propionyl-CoA fractions (equations 4.7 and 4.8) were similar in both situations and agreed with the anaerobic metabolic model predictions for GAOs (Zeng et al., 2003). Overall, the results from the 2 SBRs showed that the lower DO concentration appeared to be more advantageous for PAOs then for GAOs.

Table 4.3 – Anaerobic carbon and phosphorus transformations for PAO and GAO metabolism at different DO levels

		PHA / VFA	Glycogen / VFA	P / VFA	%PHB	%PHV	%PH_2MB	%PH_2MV	%Acetyl-CoA	%Propionyl-CoA
PAO	8 ± 0.2 mg O_2/L	1.40 ± 0.09	0.26 ± 0.15	0.33 ± 0.02	57.7	38.7	1.7	1.9	73.9	26.1
	2 ± 0.2 mg O_2/L	1.17 ± 0.16	0.46 ± 0.12	0.56 ± 0.02	66.7	25.2	3.1	5.0	78	22
	Model (Oehmen et al., 2005b; Smolders et al., 1994)	1.30	0.46	0.48	-	-	-	-	80.7	19.3
GAO	8 ± 0.2 mg O_2/L	1.97 ± 0.14	0.79 ± 0.20	-	66.8	33.2	-	-	80.1	19.9
	2 ± 0.2 mg O_2/L	1.47 ± 0.21	0.75 ± 0.24	-	69.4	30.6	-	-	81.7	18.3
	Model (Zeng et al., 2003)	1.85	1.12	-	-	-	-	-	83.1	16.9

Note: PHA/VFA and Glycogen/VFA are expressed in C-mol/C-mol and P/VFA is expressed in P-mol/C-mol.

4.3.2 BATCH TESTS WITH DIFFERENT DO CONCENTRATIONS

After acclimatisation of each culture to a DO concentration of 2 mg O_2/L, which is a DO level routinely applied in WWTP, a series of aerobic batch tests were performed for each culture at different DO levels, in order to study the effect of DO concentration on the kinetics of PAO and GAO. The aerobic kinetic parameters (q_{PHA}, $q_{Glycogen}$ and q_P) were estimated according to the model equations presented in section 4.2.6. Figure 4.2 and 4.3 present the experimental data and model description of each parameter for PAOs and GAOs, respectively, for each of the batch tests conducted. The calibrated parameters (see Appendix F and G) were found to describe well the aerobic metabolism of PAOs and GAOs. The normalized root mean squared deviation (NRMSD) for the aerobic estimation of PHA consumption, glycogen production and P uptake for PAO culture was 8%, 8% and 4%, respectively. For GAO culture, the NRMSD for PHA consumption and glycogen production was 6% and 6%, respectively. In the PAO reactor, at a DO concentration of 0.1 mg O_2/L, the P uptake was not complete and glycogen was observed to be consumed during the cycle. This consumption can likely be explained by the necessity of energy generation for cell maintenance under this oxygen-limited condition. The energy for cell maintenance is typically generated aerobically through PHA degradation, as can be observed in the other experiments by the slow PHA degradation taking place once P uptake is completed and glycogen stabilises. Aerobic glycogen consumption for maintenance energy purposes has been previously observed in PAOs and GAOs (Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013), although typically after PHA reserves are depleted. Indeed, Lanham et al. (2014) modified the PAO-GAO metabolic model to incorporate this degradation into the aerobic maintenance processes of PAOs and GAOs. Another possible explanation is the presence of anaerobic micro-niches within the flocs at this very low DO level, leading to glycogen degradation for anaerobic maintenance energy generation.

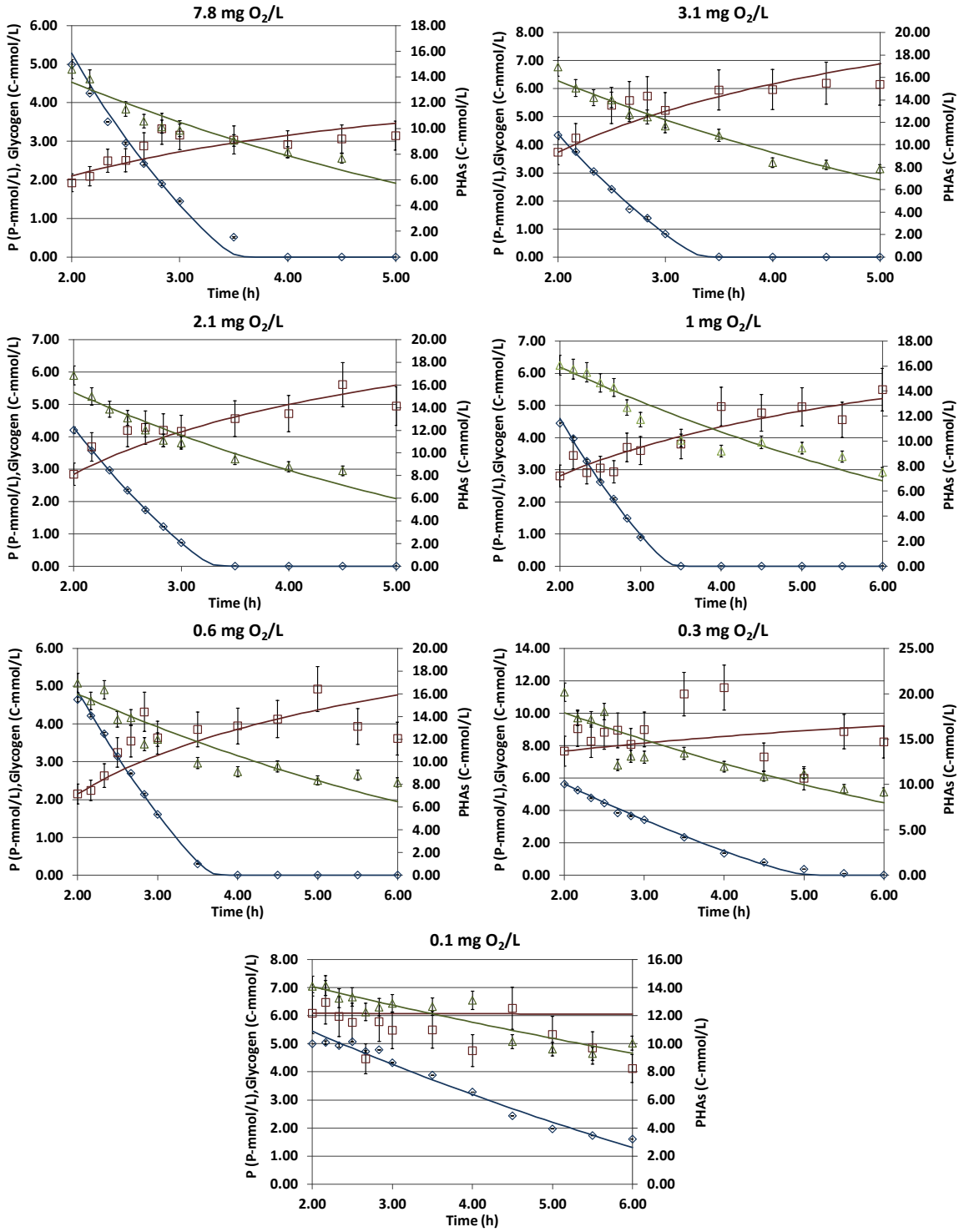


Figure 4.2 – Experimental results (PHA (green Δ), glycogen (red □) and P (blue ◊)) and model descriptions (solid lines) for PAOs at different DO concentrations.

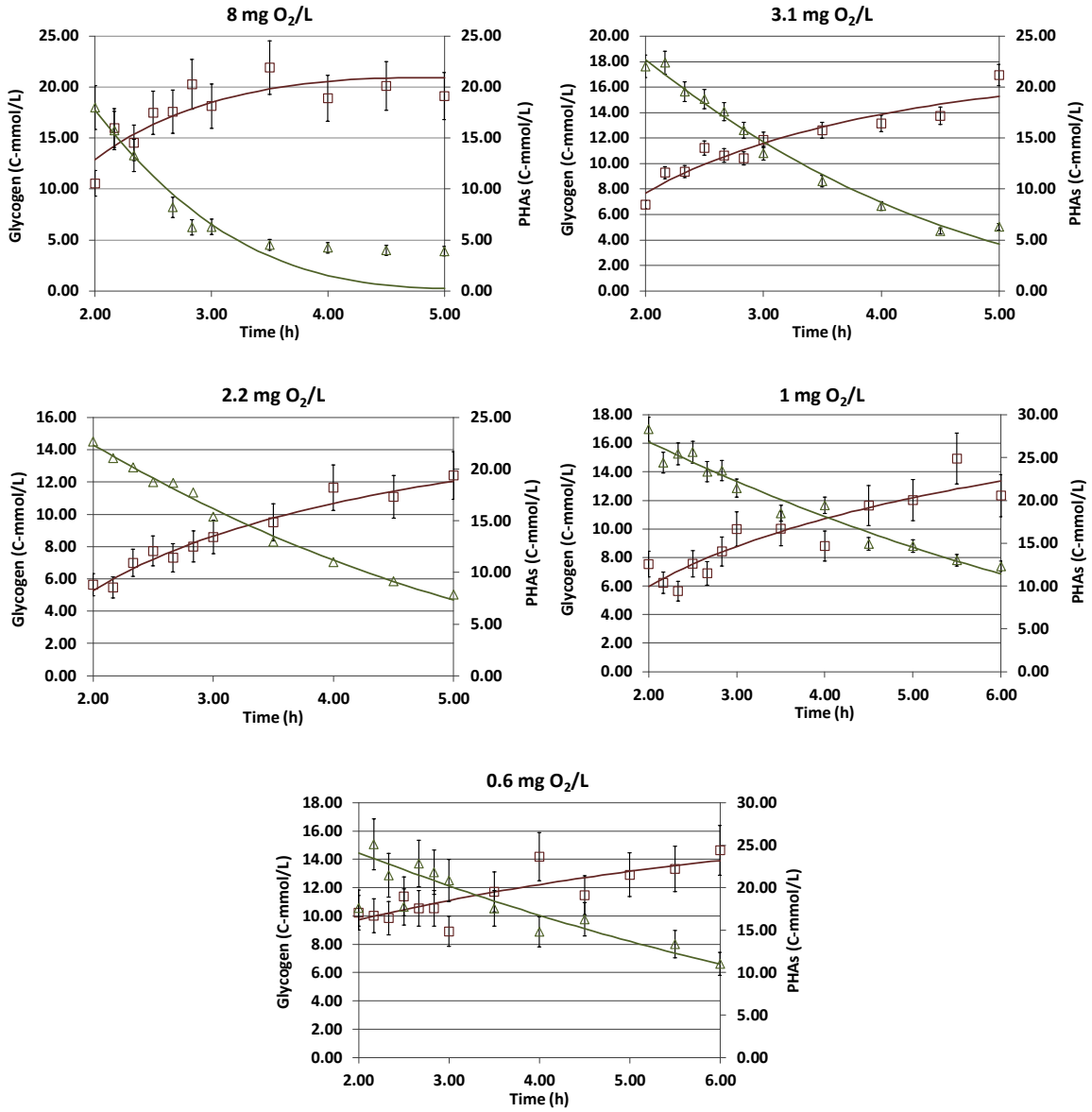


Figure 4.3 – Experimental results (PHA (green Δ) and glycogen (red □)) and model descriptions (solid lines) for GAOs at different DO concentrations.

Figure 4.4a and 4.4b present the aerobic kinetic parameters (q_{PHA}, $q_{Glycogen}$ and q_P) as a function of the DO concentration for PAOs and GAOs, respectively. The maximum specific aerobic metabolic rates of PHA consumption, P uptake and glycogen production for PAOs were relatively stable over a wide DO concentration range, decreasing more substantially in the 0.1 - 0.6 mg O_2/L range. From the results shown in Figure 4.4b the maximum specific aerobic rates of GAOs continually increased as a function of the DO concentration over the tested range (0.6 - 8.0 mg O_2/L). The testing of even higher DO levels (> 8 mg O_2/L) would necessitate the supply

of pure oxygen, which is not practically feasible in WWTPs. Assuming that the kinetic rates of PAOs and GAOs are at a maximum at the oxygen saturation level with aeration (~8 mg O_2/L), the P uptake rate of PAOs was reduced by 20% and the PHA consumption rate by 27% at a DO level of 0.6 mg O_2/L, while the PHA consumption and glycogen production rates of GAOs were reduced by 77% and 88%, respectively, at 0.6 mg O_2/L. This shows a distinct advantage for PAOs over GAOs at low DO levels, suggesting that EBPR systems should favour operation at low DO concentrations in order to minimise GAO activity.

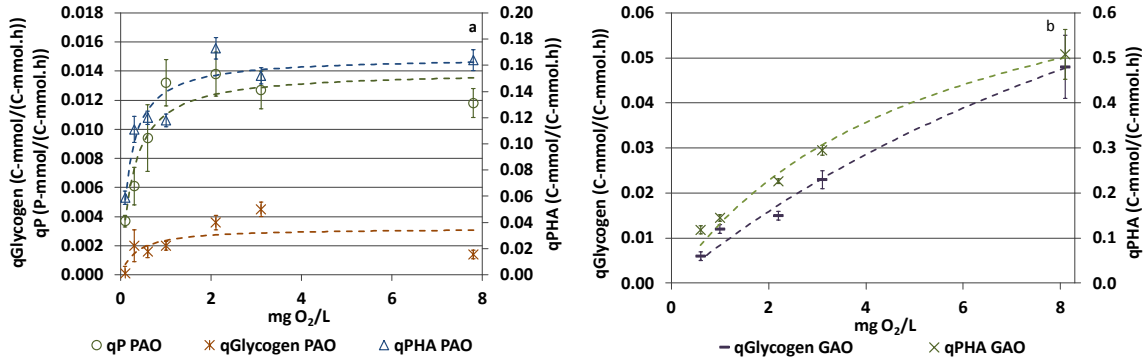


Figure 4.4 – Aerobic kinetic parameters for PAO (a) and GAO (b) culture as a function of the DO concentration.

The PAO and GAO oxygen affinity constant (K_{O2}) for each variable (PHA consumption and glycogen production for PAOs and GAOs and poly-P formation for PAOs) were determined by fitting the experimental data to the Michaelis-Menten equation (equation 4.9), using SigmaPlot 11 (Systat Software Inc.) (see Figure 4.4):

$$q = q_{max} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}} \quad (4.9)$$

The K_{O2} for PAOs was calculated for glycogen production, PHA consumption and P uptake. When comparing the K_{O2} obtained for P uptake (0.27 ± 0.10 mg O_2/L), PHA consumption (0.19 ± 0.06 mg O_2/L) and glycogen production (0.35 ± 0.44 mg O_2/L) for PAOs, the results were very similar and were in strong agreement with each other. For the case of GAOs, the K_{O2} for glycogen production (15.4 ± 7.2 mg O_2/L) was much higher than for PHA consumption (5.26 ± 1.28 mg O_2/L). These results show that PAOs have a much higher affinity for oxygen than GAOs. Furthermore, the high difference between the K_{O2} estimates for glycogen production and PHA consumption for

GAOs suggests that glycogen production is even more strongly influenced by the DO concentration, which may become a limiting factor for their survival in WWTPs. The high uncertainty associated with the K_{O2} estimates for GAOs, particularly that obtained for glycogen production, is due to their very low oxygen affinity. Since most of the data points are in the DO range of interest for WWTP operation, there is lower confidence when the K_{O2} is at such a high DO level. Indeed, the correlation shown in Figure 4.4b approaches that of a first-order relationship for GAO kinetics with respect to oxygen.

Microorganisms can often be referred to as either “r” or “K” strategists, depending on their affinity for a substrate. Through the results obtained in this study it is possible to conclude that GAOs are “r” strategists, due to their low affinity for oxygen and high K_{O2} coefficient, while PAOs are “K” strategists, due to their high affinity for oxygen, and low K_{O2} coefficient. The high affinity of PAOs for oxygen is an important factor that can be used to determine EBPR performance at different DO levels. The DO concentration is a factor that can promote the proliferation of PAOs over GAOs, as found in this study (see section 4.3.1) and can consequently be used as a means of improving the efficiency of P removal. The differences in oxygen affinity of PAOs and GAOs should also be incorporated into EBPR models in order to optimise biological nutrient removal efficiency from WWTPs at minimal energetic costs.

4.3.3 EFFECT OF AEROBIC HYDRAULIC RETENTION TIME (HRT)

The aerobic HRT of a WWTP also impacts the aeration energetic input. The effect of extending the aerobic period on the PAO culture was monitored over a period of 43 days to examine if the aerobic HRT also influences the PAO-GAO competition, in addition to the DO concentration. Prolonging the aeration period clearly resulted in a lower amount of P release and P uptake (Figure 4.5a and 4.5b), leading to incomplete P removal. Table 4.4 presents the evolution of polyphosphate content (%P), P release and uptake and VSS/TSS ratio during this study. As can be observed, the %P decreased and the VSS/TSS ratio increased correspondingly, reflecting the depletion of polyphosphate stored by PAOs. As shown in Figure 4.5a, even after the first day of operation with the extended aerobic period, a secondary release of phosphorus was observed at the end of the aerobic phase, likely used as an energy source for cell maintenance once the PHA degradation was complete. This not only led to a higher quantity of P in the SBR effluent, but reduced the amount of poly-P available for VFA uptake in the subsequent anaerobic phase. This secondary release could explain why

GAOs were favoured with an extended aerobic HRT. As poly-P content of PAOs decreased over time, the VFA uptake by PAOs would decrease, leaving more VFA available for GAOs, thereby promoting their proliferation.

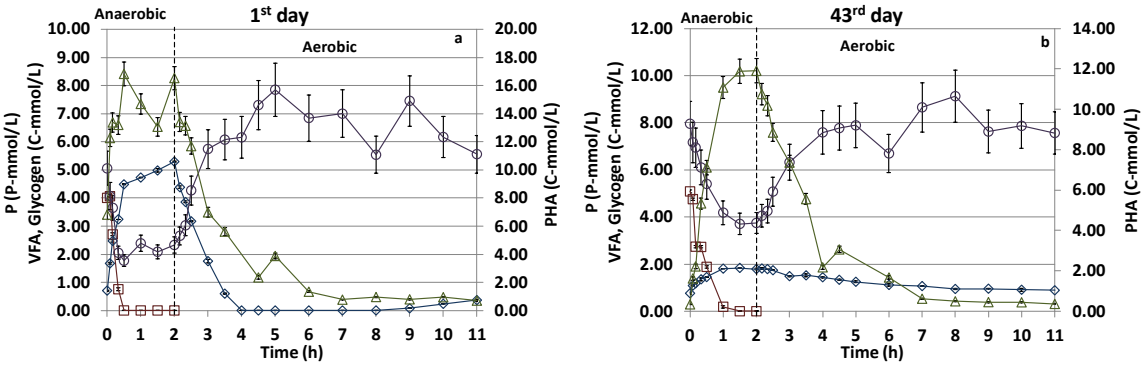


Figure 4.5 – Profile of carbon and P transformations in the PAO reactor on the 1st day (a) and 43rd day (b) (VFA (□), PHA (Δ), P (◊), glycogen (○).

Table 4.4 – Polyphosphate content and VSS/TSS ratio evolution for the reactor during the prolonged aeration period.

Day	%P	P release (P-mmol/L)	P uptake (P-mmol/L)	$\frac{P}{VFA}$	$\frac{VSS}{TSS}$
1st	13.3 ± 0.1	4.59 ± 0.04	4.93 ± 0.04	0.76 ± 0.01	0.56 ± 0.12
10th	10.5 ± 0.2	3.34 ± 0.01	3.48 ± 0.11	0.56 ± 0.02	0.66 ± 0.15
18th	11.1 ± 0.3	2.57 ± 0.09	2.61 ± 0.09	0.43 ± 0.02	0.77 ± 0.07
23rd	4.9 ± 0.1	1.68 ± 0.04	1.40 ± 0.06	0.28 ± 0.01	0.82 ± 0.10
33rd	3.3 ± 0.1	0.67 ± 0.08	0.91 ± 0.10	0.11 ± 0.01	0.93 ± 0.02
38th	1.0 ± 0.5	1.03 ± 0.10	0.80 ± 0.10	0.17 ± 0.02	0.89 ± 0.33
43rd	5.7 ± 0.3	1.03 ± 0.11	0.90 ± 0.11	0.17 ± 0.02	0.88 ± 0.08

Note: P/VFA is express in P-mol/C-mol and VSS/TSS in gVSS/gTSS.

Comparing the anaerobic carbon and P transformations for 1st and 43rd day (Table 4.5), it was observed that the decrease in P release per VFA uptake was accompanied by an increase in glycogen consumption and PHA production, suggesting increased GAO activity. FISH quantification showed that the population present in the reactor changed from a PAO-dominated biomass to a GAO-dominated biomass (Table 4.5). Overall, the results of this study showed that a longer aeration period affected the PAO-GAO competition, promoting the proliferation of GAOs over PAOs, and consequently leading to the deterioration of EBPR performance.

Table 4.5 – Anaerobic carbon and phosphorus transformations and evolution of the PAO and GAO population in the reactor during the prolonged aeration period.

Day	$\frac{\text{PHA}}{\text{VFA}}$	$\frac{\text{Glycogen}}{\text{VFA}}$	$\frac{\text{P}}{\text{VFA}}$	PAOs (%)	GAOs (%)
1st	1.62 ± 0.36	0.45 ± 0.05	0.76 ± 0.01	87 ± 2	12 ± 2
23rd				53 ± 4	45 ± 3
43rd	1.93 ± 0.09	0.70 ± 0.07	0.17 ± 0.02	12 ± 2	51 ± 4

Note: PHA/VFA and Glycogen/VFA are expressed in C-mol/C-mol and P/VFA is expressed in P-mol/C-mol.

4.3.4 IMPLICATIONS FOR FULL-SCALE EBPR SYSTEMS

The results obtained in this study show that more aeration (supplied either through maintaining a high DO level or an increased aerobic HRT) favours GAOs over PAOs. Therefore, it is advantageous for WWTPs to be operated at relatively low DO concentrations or with short aerobic zones, in order to minimise the growth of GAOs and improve P removal efficiency. Low aeration brings the added advantage of decreasing the energetic costs and improving the WWTP's energetic sustainability.

Nevertheless, it is necessary to consider all relevant factors that low DO concentrations can cause to full-scale systems. It is clear that sufficient aeration is needed to achieve not only complete P removal, but the aeration level supplied to WWTPs is also dependent on other biological processes, such as nitrification. Nitrification is affected by the DO concentration, where an inhibition of this process is often found at low DO levels. Ginige et al. (2013) observed that a decrease in DO

concentration from 2 to 0.8 mg O_2/L promoted an increase in P removal efficiency, but a decrease in nitrification rates, likely due to oxygen limitation. Besides the decrease in nitrification rates, low DO concentrations have also been found to increase N_2O emissions during nitrification (Kampschreur et al., 2009). Increased N_2O emissions negatively impact the greenhouse gas emissions of the WWTP, since N_2O is a very strong greenhouse gas (>300 times stronger than CO_2) and has been found to have a substantial impact on the total greenhouse gas budget of WWTPs (Flores-Alsina et al., 2014). Due to the impact of DO concentration on N_2O emissions and nitrification rates, it is necessary to maintain sufficient DO during nitrification in order to minimise its inhibition and the resulting N_2O emissions. Furthermore, low DO levels may lead to anaerobic/anoxic conditions in secondary clarifiers, promoting secondary P release (Mikola et al., 2009) or rising sludge caused by denitrification (Flores-Alsina et al., 2010). The DO concentration has also been found to affect the sludge settleability. Martins et al. (2003) showed that, at low DO concentrations (≤ 1.1 mg O_2/L), the sludge settleability was negatively affected, where the proliferation of filamentous bacteria was observed.

Thus, it is important to determine the DO concentration that will improve EBRP and enable lower energetic costs without provoking operational problems. The knowledge gained from this work could be incorporated into WWTP models in order to optimise biological nutrient removal at minimal energetic costs. The application of such a model would enable the study of the effect of DO concentration on the competition between all relevant organisms, improving our capacity to optimise WWTPs.

4.4 CONCLUSIONS

The DO concentration is an important operational factor that affects the PAO-GAO competition, where PAOs are favoured at low DO levels. PAOs possess a higher affinity for oxygen and showed a clear kinetic advantage over GAOs at low DO concentrations, thus EBPR systems should be operated at low DO levels to minimise the proliferation of GAOs. Operation at high DO levels was shown to lead to higher growth and activity of GAOs. A high aerobic HRT was shown to promote the proliferation of GAOs over PAOs, decreasing the P removal efficiency. Low aeration can not only improve EBPR performance, but decrease the energetic costs and consequently improve the cost-effectiveness of the WWTP. Incorporation of the oxygen

affinity of PAOs and GAOs into WWTP models could be highly useful to optimise EBPR processes.

REFERENCES

Ahn, C., Park, J., 2008. Critical factors affecting biological phosphorus removal in dairy wastewater treatment plants. KSCE J. Civ. Eng. 12 (2), 99–107.

Brdjanovic, D., Slamet, A., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998. Impact of excessive aeration on biological phosphorus removal from wastewater. Water Res. 32 (1), 200–208.

Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2014. The effect of substrate competition on the metabolism of polyphosphate accumulating organisms (PAOs). Water Res. 64, 149–159.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001. pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. Water Environ. Res. 73 (2), 223–232.

Flores-Alsina, X., Arnell, M., Amerlinck, Y., Corominas, L., Gernaey, K. V, Guo, L., Lindblom, E., Nopens, I., Porro, J., Shaw, A., Snip, L., Vanrolleghem, P.A., Jeppsson, U., 2014. Balancing effluent quality, economic cost and greenhouse gas emissions during the evaluation of (plant-wide) control/operational strategies in WWTPs. Sci. Total Environ. 466–467 (0), 616–624.

Flores-Alsina, X., Comas, J., Rodriguez-Roda, I., 2010. Analysis of rising sludge risk in Activated Sludge Systems: from operational strategies to clarifier design, in: Swayne, D.A., Yang, W., Voinov, A.A., Rizzoli, A., Filatova, T. (Eds.), International Congress on Environmental Modelling and Software, Ottawa, Canada.

Ginige, M.P., Kayaalp, A.S., Cheng, K.Y., Wylie, J., Kaksonen, A.H., 2013. Biological phosphorus and nitrogen removal in sequencing batch reactors: effects of cycle length, dissolved oxygen concentration and influent particulate matter. Water Sci. Technol. 68 (5), 982–90.

Griffiths, P.C., Stratton, H.M., Seviour, R.J., 2002. Environmental factors contributing to the “G bacteria” population in full-scale EBPR plants. Water Sci Technol 46 (4-5), 185–192.

Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S.M., van Loosdrecht, M.C.M., 2009. Nitrous oxide emission during wastewater treatment. Water Res. 43, 4093–4103.

Lanham, A., Oehmen, A., Saunders, A., Carvalho, G., Nielsen, P., Reis, M.,2014. Metabolic modelling of full-scale enhanced biological phosphorus removal sludge. Water Res. 66(1), 283-295.

Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. Process Biochem. 48 (11), 1626–1634.

Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2012. Optimisation of glycogen quantification in mixed microbial cultures. Bioresour. Technol. 118 (0), 518–525.

Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z., 2006. Identifying causes for N2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. J. Biotechnol. 122 (1), 62–72.

Li, H., Chen, Y., 2011. Research on polyhydroxyalkanoates and glycogen transformations: Key aspects to biologic nitrogen and phosphorus removal in low dissolved oxygen systems. Front. Environ. Sci. Eng. China 5 (2), 283–290.

Li, H., Chen, Y., Gu, G., 2008. The effect of propionic to acetic acid ratio on anaerobic–aerobic (low dissolved oxygen) biological phosphorus and nitrogen removal. Bioresour. Technol. 99 (10), 4400–4407.

Liu, W.-T., Nakamura, K., Matsuo, T., Mino, T., 1997. Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors—Effect of PC feeding ratio. Water Res. 31 (6), 1430–1438.

Lopez, C., Pons, M.N., Morgenroth, E., 2006. Endogenous processes during long-term starvation in activated sludge performing enhanced biological phosphorus removal. Water Res. 40 (8), 1519–1530.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Res. 43 (2), 450–462.

Lu, H., Keller, J., Yuan, Z., 2007. Endogenous metabolism of Candidatus Accumulibacter phosphatis under various starvation conditions. Water Res. 41 (20), 4646–4656.

Martins, A.M.P., Heijnen, J.J., Van Loosdrecht, M.C.M., 2003. Effect of dissolved oxygen concentration on sludge settleability. Appl. Microbiol. Biotechnol. 62, 586–593.

Mikola, A., Rautiainen, J., Vahala, R., 2009. Secondary clarifier conditions conducting to secondary phosphorus release in a BNR plant. Water Sci. Technol. 60 (9), 2413–2418.

Molinos-Senante, M., Hernández-sancho, F., Sala-garrido, R., Hernández-Sancho, F., Sala-Garrido, R., 2012. Economic feasibility study for new technological alternatives in wastewater treatment processes: a review. Water Sci. Technol. 65 (5), 898.

Muga, H.E., Mihelcic, J.R., 2008. Sustainability of wastewater treatment technologies. J. Environ. Manage. 88 (3), 437–447.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Res. 41 (11), 2271–2300.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res. 44 (15), 4473–4486.

Oehmen, A., Saunders, A.M., Vives, M.T., Yuan, Z., Keller, J., 2006. Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. J. Biotechnol. 123 (1), 22–32.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2005. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol. Bioeng. 91 (2), 162–168.

Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnol. Bioeng. 85 (1), 56–67.

Reichert, P., 1994. AQUASIM - a tool for simulation and data analysis of aquatic systems. Water Sci. Technol. 30 (2), 21–30.

Rosso, D., Stenstrom, M.K., Larson, L.E., 2008. Aeration of large-scale municipal wastewater treatment plants: state of the art. Water Sci. Technol. 57 (7), 973–978.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.

Vargas, M., Yuan, Z., Pijuan, M., 2013. Effect of long-term starvation conditions on polyphosphate- and glycogen-accumulating organisms. Bioresour. Technol. 127 (0), 126–131.

Weissbrodt, D.G., Schneiter, G.S., Fürbringer, J.-M., Holliger, C., 2013. Identification of trigger factors selecting for polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge sequencing batch reactors. Water Res. 47 (19), 7006–7018.

Whang, L.-M., Park, J.K., 2006. Competition between Polyphosphate- and Glycogen-Accumulating Organisms in Enhanced-Biological-Phosphorus-Removal Systems: Effect of Temperature and Sludge Age. Water Environ. Res. 78 (1), 4–11.

Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z., Keller, J., 2003. Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnol. Bioeng. 81 (1), 92–105.

Zhang, C., Chen, Y., Liu, Y., 2007. The long-term effect of initial pH control on the enrichment culture of phosphorus- and glycogen-accumulating organisms with a mixture of propionic and acetic acids as carbon sources. Chemosphere 69 (11), 1713–1721.

Zheng, X., Tong, J., Li, H., Chen, Y., 2009. The investigation of effect of organic carbon sources addition in anaerobic–aerobic (low dissolved oxygen) sequencing batch reactor for nutrients removal from wastewaters. Bioresour. Technol. 100 (9), 2515–2520.

5

THE SURVIVAL STRATEGIES OF POLYPHOSPHATE ACCUMULATING ORGANISMS AND GLYCOGEN ACCUMULATING ORGANISMS UNDER CONDITIONS OF LOW ORGANIC LOADING

SUMMARY: Enhanced biological phosphorus removal (EBPR) is usually limited by organic carbon availability in wastewater treatment plants (WWTPs). Polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) were operated under extended periods with low organic carbon loading in order to examine its impact on their activity and survival. The decrease in organic carbon load affected PAOs and GAOs in different ways, where the biomass decay rate of GAOs was approximately 4 times higher than PAOs. PAOs tended to conserve a relatively high residual concentration of polyhydroxyalkanoates (PHAs) under aerobic conditions, while GAOs tended to deplete their available PHA more rapidly. This slower oxidation rate of PHA by PAOs at residual concentration levels enabled them to maintain an energy source for aerobic maintenance processes for longer than GAOs. This may provide PAOs with an advantage over GAOs in surviving the low organic loading conditions commonly found in full-scale wastewater treatment plants.

PUBLISHED AS: Mónica Carvalheira, Adrian Oehmen, Gilda Carvalho, Maria A.M. Reis. "Survival strategies of polyphosphate accumulating organisms and glycogen accumulating organisms under conditions of low organic loading", Bioresource Technology, 172 (2014), 290–296.

5.1 INTRODUCTION

In enhanced biological phosphorus removal (EBPR) wastewater treatment processes, polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) compete for the limited supply of organic carbon sources such as volatile fatty acids (VFAs) (Oehmen et al., 2007). Mixed culture enrichments containing PAOs and/or GAOs have been often used to study operational and environmental factors that impact the competition between these two groups of organims, such as carbon source composition, pH, temperature, sludge retention time, influent acetate to phosphorus (P) ratio and substrate feeding rate (Lopez-Vazquez et al., 2009; Tu and Schuler, 2013; Whang and Park, 2006). Nevertheless, the VFA load used in lab-scale systems is substantially higher than the typical load received at most full-scale wastewater treatment systems. The reason for this is that it is often desirable to achieve highly enriched cultures at the expense of the elimination of other organisms, such as autotrophs and ordinary heterotrophs, thus the VFA load under anaerobic conditions is increased to maintain high selectivity of the culture for PAOs and/or GAOs.

The efficiency of the EBPR process is usually limited by the content of VFAs present in domestic wastewater (Barnard, 1984). Although the VFA content is dependent on the wastewater characteristics, their concentration in domestic wastewater typically lies between 10 and 74 mg COD/L (Siedlecka et al., 2008; Thomas et al., 2003; Zeng et al., 2006). Some studies have focussed on the effect of short-term (i.e. 1-3 d) decreases in organic load in an effort to simulate the decrease in organic load that occurs during weekends in many wastewater treatment plants (Ahn et al., 2006; Carucci et al., 1999; Miyake and Morgenroth, 2005; Temmink et al., 1996). Results have shown that after a rapid decrease in the initial organic carbon concentration (from 200-400 mg COD/L to levels between 10-100 mg COD/L), the EBPR performance deteriorated, most notably through a decrease in aerobic P uptake. Moreover, Temmink et al. (1996) observed a depletion in the polyhydroxyalkanoates (PHAs) pool, which affected the P uptake, since the aerobic P uptake rate is highly dependent on the level of PHA. Ahn et al. (2006) showed that an abrupt decrease in the organic concentration from 150 mg/L to 50 mg/L, PAOs could not adapt themselves to these sudden changes and the EBPR system became unstable. However, these studies did not evaluate the impact of prolonged low carbon loading periods on the metabolism of PAOs and GAOs, and consequently, the effect of this factor on the competition between both organisms. Knowledge about microbial competition under

stress conditions (such as low organic loading) is very important for EBPR systems, since it will contribute towards the development of strategies that will promote the proliferation of PAOs and, consequently, improve EBPR performance.

Tu and Schuler (2013) studied the effect of lowering the acetate concentration in an EBPR system through decreasing the feeding rate, while maintaining the total acetate load to the bioreactor (i.e. 200 mg COD/L per cycle). With this approach, it was found that PAOs were able to outcompete GAOs. Nevertheless, the impact of long periods of low total organic carbon loading, such as those normally present in continuous full-scale WWTPs (e.g. with influent in the 10-74 mg COD/L range), has not been previously assessed. Under conditions of low total organic loading, the maintenance processes of PAOs and GAOs become increasingly crucial, since each group of organisms will experience an increased quantity of time in the absence of external carbon sources. This will lead to the consumption of their internal carbon and energy sources: polyhydroxyalkanoates (PHAs) and glycogen, as well as polyphosphate in the case of PAOs.

Previous studies have investigated the effect of long-term starvation on PAOs (21 days) and GAOs (26 days) (Vargas et al., 2013), and have found that PAOs display a faster decay rate as compared to GAOs, while the specific acetate uptake capacity of PAOs is also decreased at a higher rate as compared to GAOs. While these tests focused on the response of each organism to extended periods without external carbon source, it is unclear if a similar response may be expectable under a situation of low organic loading, at a level close to that typically found in full-scale wastewater treatment plants (WWTPs).

In the present study, the effect of prolonged low carbon loading on PAO and GAO metabolism was investigated for the first time in order to understand the impact of this factor on their activity and survival.

5.2 MATERIALS AND METHODS

In this study, two sequencing batch reactors (SBRs - see Appendix H) were operated initially under conditions of high organic concentration (VFA_{in} = 200 mg COD/L) in order to enrich for PAOs in one SBR, and GAOs in the other. Then, each system was subjected to a decrease in the initial VFA concentration, to about 33 mg COD/L. This concentration was chosen since it is within the typical range observed in full-scale WWTPs.

5.2.1 PAO AND GAO PARENT REACTORS

Two sequencing batch reactors (SBR) with 2L of working volume were operated to select for PAO and GAO, respectively, and were seeded from the lab-scale PAO and GAO reactors described in section 4.2.1 – Chapter 4. The SBRs operation and feed solution were the same described in section 4.2.1 – Chapter 4. In the PAO reactor, a mixture of acetate (HAc) and propionate (HPr) (75-25% HAc-HPr) was used and in the GAO reactor only HAc was fed. This was performed according to previous studies showing that mixed VFA feeds favour the growth of PAO over GAO, as compared to single substrates (Chapter 3; Lopez-Vazquez et al., 2009).

In both SBRs, the hydraulic retention time (HRT) was 12 hours and the solid retention time (SRT) was 8 days. To maintain anaerobic conditions, argon was bubbled into the reactors at a flow rate of approximately 15 mL/min. During the aerobic phase, the dissolved oxygen (DO) concentration was controlled at 2.2 ± 0.4 mg O_2/L for the PAO reactor and 1.9 ± 0.3 mg O_2/L for the GAO reactor, using an on-off valve. The temperature was controlled at 20 ± 1ºC and pH was controlled at 7.3 ± 0.1 for the PAO reactor and 7.0 ± 0.0 for the GAO reactor, by automatic addition of 0.1M HCl. These conditions were maintained for >3 SRT in each system, 36 and 27 days for PAOs and GAOs, respectively.

Monitoring of the SBRs involved samples taken at various time points for volatile fatty acids (VFAs), phosphorus (P), polyhydroxyalkanoates (PHAs) and glycogen. The total suspended solids (TSS) and volatile suspended solids (VSS) were sampled at the end of the aerobic phase, while fluorescence *in situ* hybridisation (FISH) samples were taken at the end of the anaerobic and aerobic phases.

5.2.2 PAO AND GAO REACTORS WITH LOW ORGANIC LOAD

The carbon source concentration was decreased to 32.7 ± 0.9 mg COD/L to study the effect of low organic load on PAO and GAO cultures, maintaining an identical HAc:HPr ratio in each system as indicated in Section 5.2.1. Due to the low organic load, the SRT was increased to 50 days to prevent biomass washout. Except for the VFA concentrations, the composition of solutions A and B as well as all other operational conditions were maintained identical to the parent reactors. Each system was operated for 29 days under these conditions. Samples taken for monitoring both SBRs were identical to that outlined in section 5.2.1.

5.2.3 CHEMICAL AND MICROBIAL ANALYSIS

The VFAs, TSS, VSS, PHA, glycogen, phosphate and total phosphate were determined as described in section 3.2.3 – Chapter 3. Fluorescence *in situ* hybridisation (FISH) was performed as described in section 3.2.3 – Chapter 3. The probes used were: EUBMIX (EUB338, EUB338-II and EUB338-III), PAOMIX (PAO 651, 462 and 846); GAOMIX (GAOQ989 and GB_G2); *Defluviicoccus vanus-related* GAOs cluster I (TFO_DF218 and TFO_DF618), cluster II (DF988 and DF1020), cluster III (DF1013 and DF1004) and cluster IV (DF181A and DF181B).

5.2.4 DECAY RATES

The active biomass concentration of PAOs and GAOs was determined as the VSS minus both the PHA and glycogen content. Biomass decay rates were determined using the active biomass concentration in g/L of PAOs or GAOs. The PAO and GAO anaerobic activity decay rates were determined using the anaerobic phosphorus release rate (PAOs) or glycogen consumption rate (GAOs). The aerobic activity decay rates for PAOs and GAOs were determined using the aerobic maintenance coefficient (m_{ATP}). Both the biomass decay and activity decay rates were calculated via the following equation, developed by Lesouef et al. (1992):

$$b = -\ln\left(\frac{R_t}{R_0}\right) \times \frac{1}{t_d} \qquad (5.1)$$

where, b corresponds to the biomass/activity decay rate for PAOs or GAOs, R_0 and R_t are the active biomass concentrations, phosphorus release rate (PAO), glycogen consumption rate (GAO) or aerobic maintenance coefficient (m_{ATP}) at time 0 and time t, respectively, and t_d is the duration of the experiment (29d). Previous studies investigating the effect of starvation conditions on PAOs and GAOs have also determined the biomass and activity decay rates through a similar methodology (Hao et al., 2010; Vargas et al., 2013).

5.2.5 AEROBIC MAINTENANCE COEFFICIENT

Aerobic maintenance processes of PAOs and GAOs have been found to be generated by PHA oxidation, or glycogen consumption following PHA depletion

(Vargas et al., 2013). Thus, the aerobic maintenance coefficient (m_{ATP}) for PAOs and GAOs was calculated either using the aerobic PHA degradation (m_{PHA}) (equation 5.2 – see Lopez-Vazquez et al. (2009)) or aerobic glycogen consumption ($m_{Glycogen}$) (equation 5.3 – see Lanham et al., (2014)) rates, as shown below (see Table 5.1 for definition of parameters):

$$m_{ATP} = \frac{m_{PHA} \times (6\lambda + 27\lambda\delta + 8\beta + 30\beta\delta)}{12} \qquad (5.2)$$

$$m_{ATP} = m_{Glycogen} \times \left(\frac{1}{2} + \frac{\delta}{2}\right) \qquad (5.3)$$

The PHA degradation rates were determined from the experimental data obtained in each sampling day after the soluble P had been depleted (PAOs) or after the glycogen concentration was stable (GAOs). The glycogen consumption rates were determined after PHA consumption ceased.

Table 5.1 – Parameter description used for estimation of the aerobic maintenance coefficient (Oehmen et al., 2010).

Parameter	Description	Value	Units
δ	ATP produced per NADH oxidized	1.85	ATP-mol/NADH-mol
λ	Percentage of Acetyl-CoA* in PHA	$d + \frac{2e}{5}$	C-mol/C-mol
β	Percentage of Propionyl-CoA* in PHA	$f + \frac{3e}{5}$	C-mol/C-mol
d	Polyhydroxybutyrate (PHB) fraction in PHA	$\frac{X_{PHB}}{X_{PHA}}$	C-mol/C-mol
e	Polyhydroxyvalerate (PHV) fraction in PHA	$\frac{X_{PHV}}{X_{PHA}}$	C-mol/C-mol
f	Polyhydroxy-2-methylvalerate (PH_2MV) fraction in PHA	$\frac{X_{PH_2MV}}{X_{PHA}}$	C-mol/C-mol

5.3 RESULTS AND DISCUSSION

5.3.1 PAO AND GAO ENRICHMENT UNDER HIGH ORGANIC LOAD

Firstly, each reactor was operated at a high initial organic concentration (200 mg COD/L) in order to obtain enrichments of PAOs and GAOs, respectively, prior to the decrease in VFA concentration. Figure 5.1 presents the profile obtained for each reactor, showing a typical phenotype for PAO (Figure 5.1a) and GAO (Figure 5.1b). In both reactors, VFAs were completely consumed during the anaerobic phase and converted to PHA, through the energy and reducing power generated by glycogen consumption and P release (PAO reactor only). In the aerobic phase, PHAs were consumed to replenish glycogen and for biomass growth, in both cultures, and also for P uptake in the PAO reactor.

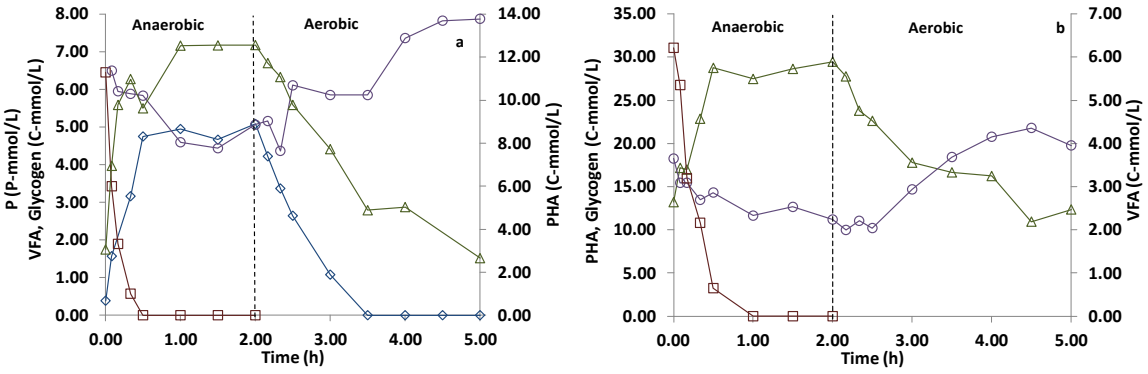


Figure 5.1 – Profile of carbon and phosphorus transformations for the PAO reactor (a) and carbon transformations for the GAO reactor (b) obtained under high organic loading (VFA (□), P (◊), PHA (Δ), glycogen (○), ammonium (+)).

Over the experimental period, the net P release and P uptake for the PAO reactor were 3.3 ± 1.5 P-mmol/L and 4.3 ± 0.9 P-mmol/L, respectively, with an average $P_{release}/VFA_{uptake}$ ratio of 0.5 ± 0.2 P-mol/C-mol. This behaviour is typical for enriched PAO cultures (Chen et al., 2004; Smolders et al., 1994). The average TSS and VSS, for the PAO reactor, was 3.52 ± 0.94 g/L and 2.28 ± 0.44 g/L, respectively, obtaining an average VSS/TSS ratio of 0.66 ± 0.08 gVSS/gTSS. For the GAO reactor, the TSS and VSS were 2.88 ± 0.17 g/L and 2.79 ± 0.18 g/L, respectively, obtaining a VSS/TSS ratio of 0.97 ± 0.01 gVSS/gTSS. This difference between the VSS/TSS ratios for PAOs and GAOs was expected due to the storage of polyphosphate in the case of PAOs only, and supports the high abundance of polyphosphate in the PAO reactor (12.3 ± 4.9%)

and low abundance in the GAO reactor (1.1 ± 0.4%). This agrees well with the FISH results, where *Accumulibacter* PAOs were the dominant organisms in the PAO reactor, representing 74 ± 4% of all bacteria, where *Competibacter* GAOs only represented 21 ± 1%. In the GAO reactor, the main organisms present were *Competibacter* GAOs, representing 73 ± 2% of all bacteria. *Defluviicoccus vanus*-related GAOs were not observed in either reactor.

5.3.2 EFFECT OF LOW ORGANIC LOADING ON PAOS AND GAOS

With the objective of studying the effect of low initial organic carbon concentrations on PAO and GAO metabolism, the initial VFA concentration was decreased directly from 200 mg COD/L to 32.7 ± 0.9 mg COD/L. As can be observed in Figure 5.2, the VFA consumption in the PAO reactor slowed down over time, while for the GAO reactor, the consumption of VFA remained similar along the study.

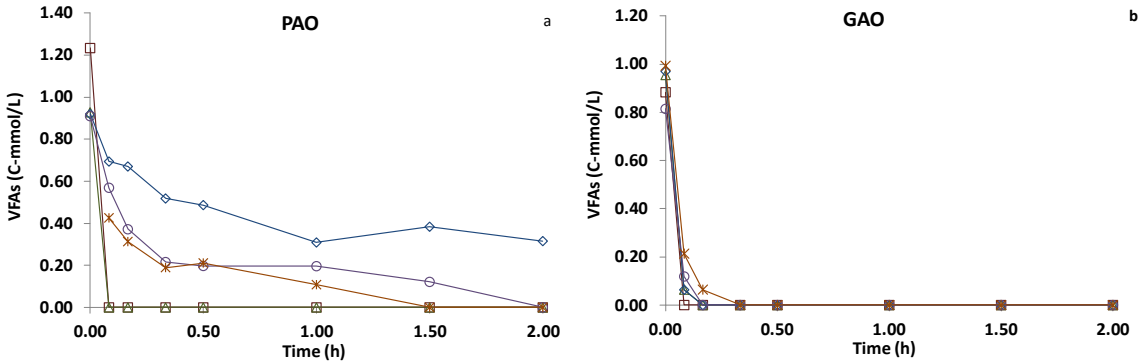


Figure 5.2 – Profile of VFA consumption for the PAO (a) and GAO (b) reactors obtained with low organic load (1st day (□); 8th day (Δ); 15th day (◊); 22nd day (○); 29th day (*)).

The reason for the decrease in VFA uptake rate in the PAO reactor along the time may have been due to the depletion of intracellular polyphosphate. Figure 5.3a shows that a decrease in the quantity of phosphorus release and uptake were observed along the study, which was accompanied by a decrease in the polyphosphate content of the cells (Figure 5.3b), from about 27% to 1.5%, and an increase in the VSS/TSS ratio (0.56 gVSS/gTSS to 0.90 gVSS/gTSS). The depletion of polyphosphate content led to a lower quantity of P available for release, which would also decrease the capacity of PAOs to take up VFA anaerobically.

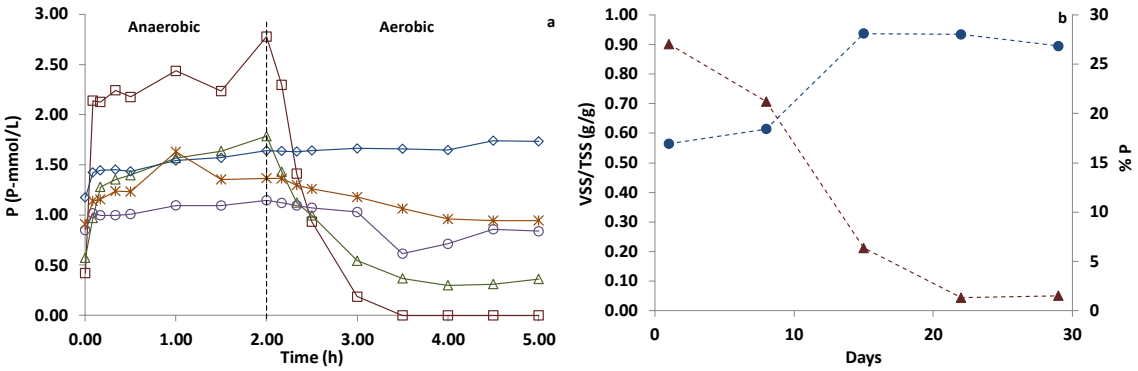


Figure 5.3 – Phosphorus profile for the PAO culture with low organic load (a) (1st day (□); 8th day (Δ); 15th day (◊); 22nd day (○); 29th day (*)) and the corresponding VSS/TSS ratio and total phosphorus concentration (b) (VSS/TSS (●); %P (▲)).

Figure 5.4 presents the PHA profiles for the PAO (a) and GAO (b) reactors. In the GAO reactor, a substantial decrease in the PHA storage accompanied the decrease in organic load, where the PHA was almost completely consumed during the aerobic phase, reaching an average final concentration of 0.2 ± 0.2 C-mmol/L between the 8th and 29th day. This complete aerobic consumption of PHA was unsurprising since PHA is widely recognised to serve as the primary carbon and energy source of PAOs and GAOs under aerobic conditions (Oehmen et al., 2007; Vargas et al., 2013). Nevertheless, a similar trend was not observed in the PAO reactor. The PHA profile remained similar throughout the study, where the aerobic PHA consumption was comparable to the PHA produced under anaerobic conditions. The average final PHA content of PAOs was observed to be 5.8 ± 1.3 C-mmol/L between the 8th and 29th day, which was significantly higher than the GAO SBR (0.2 ± 0.2 C-mmol/L). It should also be noted that all PHA fractions (i.e. PHB, PHV and PH_2MV) remained in the same proportion at the end of the aerobic phase throughout the study, showing that there was not a preference to oxidise a specific PHA fraction. The reason for the difference in PHA depletion between PAOs and GAOs is unclear and requires further investigation regarding PAO and GAO metabolism (using e.g. advanced microbial ecology tools), but suggests that PAOs tend to avoid the complete exhaustion of their internal PHA, unlike GAOs. The PHA oxidation rate of PAOs is known to be dependent upon the PHA storage fraction of the organisms (Murnleitner et al., 1997), however, the results from this study suggest that the PHA oxidation rate is also dependent upon the quantity of PHA produced in the preceding anaerobic phase. Moreover, previous tests using full-scale EBPR sludge have also shown that PHA is not fully consumed under

aerobic conditions (Lanham et al., 2013), supporting the findings of this study. As discussed below, this difference in PHA oxidation profile under low organic loading may influence the capacity of PAOs and GAOs to survive under such conditions, which are commonly found in full-scale plants.

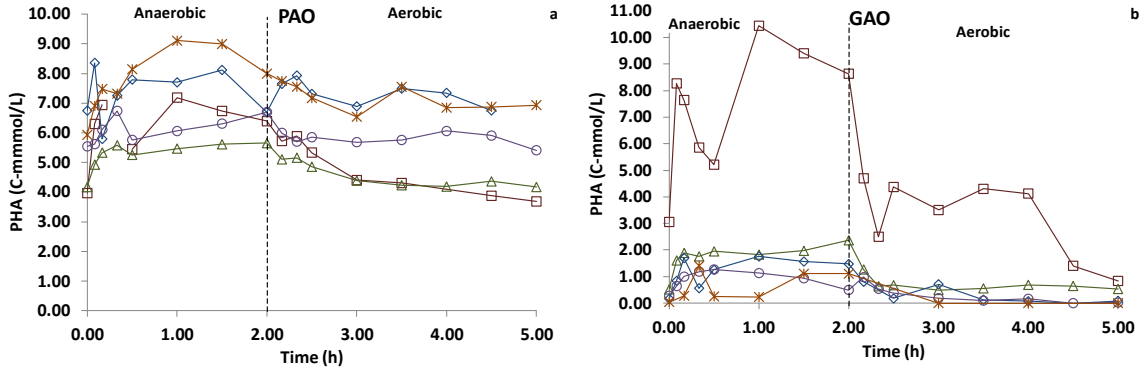


Figure 5.4 – PHA profile for the PAO (a) and GAO (b) reactors under low organic load (1st day (□); 8th day (Δ); 15th day (◊); 22nd day (○); 29th day (*)).

Glycogen profiles obtained for the PAO and GAO cultures are presented in Figure 5.5. In both cultures, a decrease in glycogen concentration between the 1st and 8th day was observed, which remained relatively constant after the 8th day. After this day, the anaerobic consumption and aerobic production of glycogen was very low in the PAO culture, and only residual glycogen levels remained in the cells. In the GAO culture, the anaerobic consumption and aerobic production of glycogen was also very low after day 8, although the residual glycogen level was substantially higher in this system. Therefore, GAOs appeared to prioritise the preservation of their glycogen pools under low organic loading, while PAOs prioritised the preservation of their PHA pools.

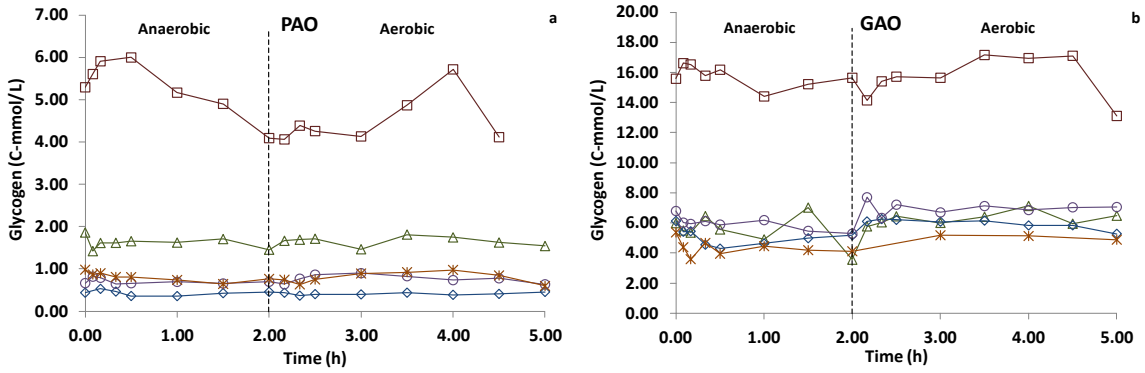


Figure 5.5 – Glycogen profile for the PAO (a) and GAO (b) reactors under low organic load (1st day (□); 8th day (Δ); 15th day (◊); 22nd day (○); 29th day (*)).

5.3.3 BIOMASS AND ACTIVITY DECAY OF PAO AND GAO

The concentration of active biomass decreased along the study in both the PAO and GAO reactors (Figure 5.6), although only a ~20% reduction was observed in the PAO biomass (from 72.57 to 57.43 C-mmol/L), while the GAOs decreased by ~50% (from 102.46 to 52.36 C-mmol/L).

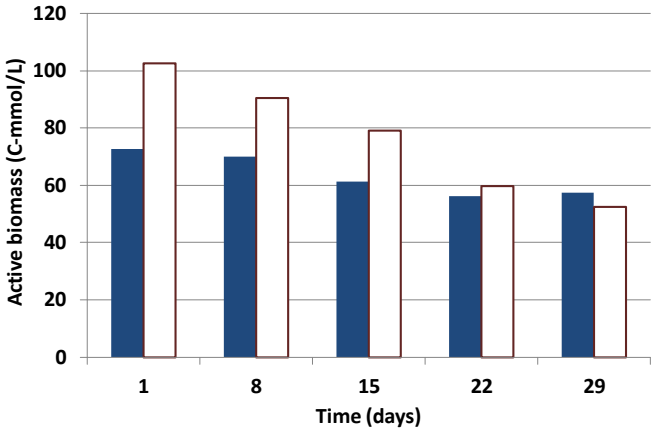


Figure 5.6 – Evolution of the active biomass concentration for PAOs and GAOs under low organic load (PAOs (■); GAOs (□)).

Indeed, the biomass decay rate was approximately 4 times higher for GAOs then for PAOs (see Table 5.2). Vargas et al. (2013) also determined the biomass decay rates for PAOs and GAOs, however their results contrasted with those of this

study, where PAOs were observed to display a substantially higher decay rate as compared to GAOs. The differences in operational conditions between the two studies can likely explain this difference, since Vargas et al. (2013) performed their experiments under starvation conditions (i.e. no organic carbon addition), while in this study the reactor was maintained under low organic loading. The results from this work show that PAOs are more able to survive at low organic loading conditions than GAOs. The difference observed in biomass decay may be related with the fact that PAOs did not consume their entire PHA reserves, unlike GAOs. Thus, under aerobic conditions, GAOs would not have PHA available as an energy source for maintenance processes, although some glycogen was still present and likely partially used for aerobic maintenance. The aerobic maintenance coefficient for PAOs and GAOs decreased with the decrease of carbon concentration along the 29 days of the experiment. Under high organic load, the aerobic maintenance coefficient for PAOs and GAOs were 0.120 mol ATP/(C-mol X.h) and 0.199 mol ATP/(C-mol X.h), respectively, decreasing to 0.026 mol ATP/(C-mol X.h) (PAOs) and 0.079 mol ATP/(C-mol X.h) (GAOs) in the first day under low organic conditions. Between 8^{th} and 29^{th} day, the aerobic maintenance coefficient was 0.007 ± 0.003 mol ATP/(C-mol X.h) for PAOs and 0.010 ± 0.006 mol ATP/(C-mol X.h) for GAOs. As can be observed, the GAOs maintenance coefficient was higher than PAOs, showing that GAOs need more energy for aerobic maintenance. Since GAOs depleted their PHA and only contained glycogen for maintenance energy generation, their high biomass decay rate suggested that PHA was a more efficient substrate for aerobic maintenance processes. The lower biomass decay rates observed for PAOs suggests that preserving some PHA reserves in favour of e.g. glycogen may provide a competitive advantage to PAOs over GAOs under situations of low organic loading, such as those encountered in continuous full-scale WWTPs.

Although PAOs were able to survive low organic loading conditions, their specific anaerobic activity decreased, and the aerobic phosphorus uptake achieved by the culture was very low. The anaerobic activity decay was determined in this study through the phosphorus release rate in the PAO reactor, and glycogen consumption rate in the GAO reactor. As can be observed by the results presented in Table 5.2, the PAO anaerobic activity decay rate was equal to that obtained by Lopez et al. (2006) and comparable with the study of Hao et al. (2010), although somewhat lower than in the study of Vargas et al. (2013). For the GAO culture, the anaerobic activity decay rate is within the same range that obtained by Vargas et al. (2013), although lower than

in the study of Hao et al. (2010) (Table 5.2). As previously mentioned, it is possible that the low organic loading applied in this study instead of the starvation conditions that were applied in these previous studies might had also affected the activity decay rates. As also discussed by Hao et al. (2010), the decay rate for GAOs is more difficult to determine than PAOs because it is determined through e.g. the glycogen consumption rate, and the presence of other microorganisms in the sludge that can consume glycogen or other carbohydrates may influence the result.

Table 5.2 – Comparison of biomass and activity decay rates between PAOs and GAOs calculated using equation 5.1.

		Anaerobic		Aerobic	
	Reference	**PAOs**	**GAOs**	**PAOs**	**GAOs**
Biomass decay rate (d^{-1})	This study	-	-	0.006	0.025
Biomass decay rate (d^{-1})	Vargas et al. (2013)	-	-	0.029	negligible
Activity decay (d^{-1})	This study	0.15	0.08	0.051	0.100
	Lopez et al. (2006)	0.15	-	-	-
	Hao et al. (2010)	0.181	0.132	-	-
	Vargas et al. (2013)	0.25	0.047	-	-

As in other studies, the anaerobic activity decay of PAOs was higher than that of GAOs, showing that low carbon loading can negatively affect PAOs activity, although PAOs appear better equipped to survive under this condition. Indeed, the difference in anaerobic activity decay rates for PAOs and GAOs may explain the difference in VFA uptake rate between PAOs and GAOs (Figure 5.2), since PAOs capacity to take up VFA decreased more than GAOs. Despite the capacity of PAOs to better survive over GAOs under periods of low organic loading, it is clear that sufficient organic carbon must be provided in order to ensure effective EBPR activity and P removal performance by PAOs. Furthermore, the higher aerobic maintenance requirements (and aerobic activity decay rate) of GAOs than PAOs may be a contributing factor to their higher biomass decay rate. It has been found that most heterotrophic and autotrophic organisms present in wastewater, including PAOs, decay much faster under aerobic conditions than under anaerobic or anoxic conditions (Hauduc et al., 2013; Lu et al., 2007; Siegrist et al., 1999). Thus, the higher aerobic

maintenance requirements of GAOs than PAOs may contribute more significantly than their inferior anaerobic maintenance requirements to their survival in wastewater treatment plants.

Tu and Schuler (2013) demonstrated that PAOs proliferated over GAOs under continuous feeding as opposed to dump feeding, suggesting that PAOs possess a higher substrate affinity. This work further showed that PAOs are also less prone to decay under conditions of low total organic load, perhaps due to the preference of PAOs for maintaining PHA reserves instead of glycogen for maintenance energy purposes and their inferior aerobic activity decay rate. These results indicate that PAOs are more suited for long term survival under the typical low organic loads found in full-scale WWTPs.

5.4 CONCLUSIONS

Extended periods of low organic loading affects the metabolism of PAO and GAO in different ways. The biomass decay rate of GAOs was about 4 times higher than PAOs, showing that PAOs are better adapted to survive in low carbon loading conditions. The aerobic consumption of PHA was notably different for each culture, where PAOs tended to maintain a PHA reserve, while GAOs exhausted all of their PHA, which may have contributed to their higher biomass decay rate. PAOs appear better equipped to survive the conditions of low organic carbon load typically encountered in WWTPs as compared to GAOs.

REFERENCES

Ahn, C.H., Park, J.K., Kim, K.S., 2006. Microbial adaptability to organic loading changes in an enhanced biological phosphorus removal process. JOURNAL OF ENVIRONMENTAL ENGINEERING-ASCE 132, 909–917.

Amann, R.I., 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes, in: Akkermans van Elsas, J.D., de Bruijn, F.J., A.D.L. (Ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publications, Dordrecht, Holland, pp. 1–15.

APHA/AWWA, 1995. Standard Methods for the Examination of Water and Wastewater. Port City Press, Baltimore.

Barnard, J.L., 1984. Activated primary tanks for phosphate removal. Water SA 10, 121–126.

Carucci, A., Kühni, M., Brun, R., Carucci, G., Koch, G., Majone, M., Siegrist, H., 1999. Microbial competition for the organic substrates and its impact on EBPR systems under conditions of changing carbon feed. Water Science and Technology 39, 75–85.

Chen, Y., Randall, A.A., McCue, T., 2004. The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. Water Research 38, 27–36.

Hao, X., Wang, Q., Cao, Y., van Loosdrecht, M.C.M., 2010. Experimental evaluation of decrease in the activities of polyphosphate/glycogen-accumulating organisms due to cell death and activity decay in activated sludge. Biotechnology and Bioengineering 106, 399–407.

Hauduc, H., Rieger, L., Oehmen, A., van Loosdrecht, M.C.M., Comeau, Y., Héduit, A., Vanrolleghem, P.A., Gillot, S., 2013. Critical review of activated sludge modeling: State of process knowledge, modeling concepts, and limitations. Biotechnology and Bioengineering 110, 24–46.

Lanham, A., Oehmen, A., Saunders, A., Carvalho, G., Nielsen, P., Reis, M., 2014. Metabolic modelling of full-scale enhanced biological phosphorus removal sludge. Water Research 66, 283-295.

Lanham, A.B., Oehmen, A., Saunders, A.M., Carvalho, G., Nielsen, P.H., Reis, M.A.M., 2013. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. Water Research 47, 7032–7041.

Lesouef, A., Payraudeau, M., Rogalla, F., Kleiber, B., 1992. Optimizing Nitrogen Removal Reactor Configurations by On-Site Calibration of the IAWPRC activated sludge model. Water Science & Technology 25, 105–123.

Lopez, C., Pons, M.N., Morgenroth, E., 2006. Endogenous processes during long-term starvation in activated sludge performing enhanced biological phosphorus removal. Water Research 40, 1519–1530.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Research 43, 450–462.

Loy, A., Maixner, F., Wagner, M., Horn, M., 2007. probeBase--an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic acids research 35, D800–D804.

Lu, H., Keller, J., Yuan, Z., 2007. Endogenous metabolism of Candidatus Accumulibacter phosphatis under various starvation conditions. Water Research 41, 4646–4656.

Miyake, H., Morgenroth, E., 2005. Optimization of enhanced biological phosphorus removal after periods of low loading. Water Environment Research 77, 117–127.

Murnleitner, E., Kuba, T., van Loosdrecht, M.C.M., Heijnen, J.J., 1997. An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnology and Bioengineering 54, 434–450.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Research 41, 2271–2300.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Research 44, 4473–4486.

Siedlecka, E.M., Kumirska, J., Ossowski, T., Glamowski, P., Gołebiowski, M., Gajdus, J., Kaczyński, Z., Stepnowski, P., 2008. Determination of volatile fatty acids in environmental aqueous samples. Polish Journal of Environmental Studies 17, 351–356.

Siegrist, H., Brunner, I., Koch, G., Phan, L.C., Le, V.C., 1999. Reduction of biomass decay rate under anoxic and anaerobic conditions. Water Science and Technology 39, 129–137.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnology and Bioengineering 43, 461–470.

Temmink, H., Petersen, B., Isaacs, S., Henze, M., 1996. Recovery of biological phosphorus removal after periods of low organic loading. Water Science and Technology 34, 1–8.

Thomas, M., Wright, P., Blackall, L., Urbain, V., Keller, J., 2003. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. Water science and technology : a journal of the International Association on Water Pollution Research 47, 141–148.

Tu, Y., Schuler, A.J., 2013. Low Acetate Concentrations Favor Polyphosphate-Accumulating Organisms over Glycogen-Accumulating Organisms in Enhanced Biological Phosphorus Removal from Wastewater. Environmental Science & Technology 47, 3816–3824.

Vargas, M., Yuan, Z., Pijuan, M., 2013. Effect of long-term starvation conditions on polyphosphate- and glycogen-accumulating organisms. Bioresource Technology 127, 126–131.

Whang, L.-M., Park, J.K., 2006. Competition between Polyphosphate- and Glycogen-Accumulating Organisms in Enhanced-Biological-Phosphorus-Removal Systems: Effect of Temperature and Sludge Age. Water Environment Research 78, 4–11.

Zeng, R.J., Yuan, Z., Keller, J., 2006. Effects of solids concentration, pH and carbon addition on the production rate and composition of volatile fatty acids in prefermenters using primary sewage sludge. Water science and technology: a journal of the International Association on Water Pollution Research 53, 263–269.

6

GENERAL CONCLUSIONS AND FUTURE WORK

6.1 GENERAL CONCLUSIONS

In this thesis, the effect of operational parameters relevant to full-scale EBPR systems was studied with respect to their impact on the PAO-GAO competition, their metabolism and modelling. The results will contribute towards the improvement of EBPR processes by increasing our knowledge regarding the response of key microorganisms to these operational conditions, and our ability to describe and predict EBPR systems for process optimisation purposes.

PAOs were observed to outcompete GAOs using a combined VFA feed with a ratio of 75-25% acetate to propionate, which is a VFA composition that commonly occurs in EBPR plants. Tests were performed at varying acetate:propionate ratios, in order to study the impact of substrate competition on the VFA uptake kinetics. A PAO metabolic model was modified in order to incorporate this substrate competition, and described well the experimental data observed. The aerobic kinetic parameters (phosphorus uptake, PHA degradation and glycogen production) were relatively independent of the concentration of acetate or propionate, showing that it is not necessary to change the metabolic model parameters as a function of the VFA source, which was proposed in earlier studies. This simplifies the metabolic model calibration procedure substantially, improving their applicability for full-scale EBPR process optimisation. The long-term simulation showed that the model developed in this work is robust, resulting in a good description of the process.

PAOs presented an advantage over GAOs at low DO concentrations. This was determined through reactor operation at different DO levels and through the determination of the maximum specific aerobic metabolic rates as a function of the DO concentration for each group of organisms. PAOs were observed to have a substantially higher affinity for oxygen as compared to GAOs. Furthermore, an increase in the aerobic HRT promoted the proliferation of GAOs over PAOs, which also shows that low aeration favours PAOs. Low aeration can be achieved in EBPR processes either through operation at low DO levels, or through designing the system with smaller aerobic zones. This work showed that either of these strategies would be effective in decreasing GAO activity.

Under low organic carbon loading conditions, the PAO and GAO metabolism was affected in different ways. The aerobic PHA oxidation of PAOs and GAOs proceeded differently, where PAOs tended to avoid complete exhaustion of their internal PHA reserves, while GAOs tended to consume all of their internal PHA

reserves, suggesting that these conditions may influence PAO and GAO survival. The higher aerobic maintenance requirements for GAOs promoted a higher biomass decay rate, suggesting that PHA is a more advantageous substrate for aerobic maintenance processes than glycogen. This faster decay rate of GAOs may provide a competitive advantage to PAOs in EBPR systems. This work showed that PAOs seem more able to survive under conditions of low organic carbon loading; however, it is necessary to ensure sufficient organic carbon in order to achieve good EBPR efficiency.

6.2 FUTURE WORK

Based on the work developed and on the results obtained, the following suggestions for future work can be proposed:

Firstly, it is important to understand if the metabolic model developed in this work can be successfully applied to full-scale WWTPs. For this, a bioreactor using real wastewater should be operated in order to validate the metabolic model developed for substrate competition and also verify if the aerobic processes can be described by the aerobic parameters determined in this work. The PAOs advantage at low DO levels over GAOs should also be verified through bioreactor operation at low and high DO concentrations using real wastewater and similar initial quantities of PAOs and GAOs.

As low DO concentrations can affect other WWTP processes besides EBPR (e.g. nitrification, N_2O production, sludge settleability), the DO concentration of WWTPs should be optimised considering all relevant processes together to improve the cost-effectiveness and overall sustainability of the system. For this purpose, a model should be developed and experimentally validated in order to better predict the impact of DO concentration on WWTP performance. This could involve the combination of metabolic and ASM models, incorporating the effect of DO on the PAO-GAO competition studied in this work.

Since *Tetrasphaera* are PAO-related organisms and they are present in WWTPs often in higher abundances than PAOs, it would be interesting to understand their role in full-scale EBPR plants and study the competition between these organisms and other key organisms in activated sludge, such as other PAOs and GAOs. In order to improve phosphorus removal in EBPR processes, the optimal conditions (e.g. pH, temperature, dissolved oxygen concentration) for *Tetrasphaera* should be established,

as well as their relative contribution towards phosphorus removal and their ecological niche in WWTPs.

Occasionally, the phosphorus concentration occurring in the treated effluent can be higher than that allowed by legislation, sometimes due to the secondary release of P in the clarifier, where it is necessary to reduce the phosphorus concentration further. Some types of nanoparticles, such as iron nanoparticles, are able to remove phosphorus from wastewater. It would be interesting to study their utilisation in WWTPs as a tertiary treatment or, eventually, their utilisation coupled to the biological treatment. Firstly, a cost-effectiveness study should be performed in order to determine the viability of this method as compared to standard chemical precipitation. The operational conditions (e.g. nanoparticle concentration and retention time) should be optimised, as well as the conditions necessary for nanoparticle recycling. Related studies could include the possibility of simultaneously removing other contaminants (e.g. metals or organic micropollutants) through these nanoparticles.

APPENDICES – CHAPTER 3 AND 4

APPENDIX A. Stoichiometric matrix for *Accumulibacter* PAO

	Process	Components							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
		S_{O2}	S_{HAc}	S_{HPr}	S_{PO4}	X_{PAO}	$X_{PAO,PHA}$	$X_{PAO,Gly}$	$X_{PAO,PP}$
	Accumulibacter								
1	Anaerobic acetate uptake		-1		$Y_{PO4\_Ac,PAO}$		$Y_{Ac\_PHA,PAO}$	$-Y_{Gly\_Ac,PAO}$	$-Y_{PO4\_Ac,PAO}$
2	Anaerobic propionate uptake			-1	$Y_{PO4\_Prop,PAO}$		$Y_{Prop\_PHA,PAO}$	$-Y_{Gly\_Prop,PAO}$	$-Y_{PO4\_Prop,PAO}$
3	Anaerobic maintenance				1				-1
4	Aerobic PHA degradation	$-Y_{O2\_PHA\_PAO}$			$-Y_{PO4\_X,PAO}$	$Y_{PHA\_X,PAO}$	-1		
5	Aerobic glycogen production	$Y_{O2\_Gly\_PAO}$			$Y_{PO4\_Gly,PAO}$	$-Y_{X\_Gly,PAO}$		1	
6	Aerobic Poly-P formation	$Y_{O2\_PP\_PAO}$			$-Y_{PO4\_PP,PAO}$	$-Y_{X\_PP,PAO}$			1
7	Aerobic maintenance	$m_{PAO,OX}$			$m_{PAO,PO4}$	$-Y_{PHA\_X,PAO}$			

$Y_{O2\_PHA,PAO} = Y_{PHA\_X}/Y_{O2\_X}$

$Y_{O2\_Gly,PAO} = Y_{PHA\_X}/(Y_{O2\_X} . Y_{PHA\_Gly})-(1/Y_{O2\_Gly})$

$Y_{O2\_PP,PAO} = Y_{PHA\_X}/(Y_{O2\_X} . Y_{PHA\_PP})-(1/Y_{O2\_PP})$

$m_{PAO,OX} = (Y_{PHA\_X}/Y_{O2\_X})-1$

$Y_{PO4\_X,PAO} = i_{BM,P} . Y_{PHA\_X}$

$Y_{PO4\_Gly,PAO} = i_{BM,P} . Y_{PHA\_X}/ Y_{PHA\_Gly}$

$Y_{PO4\_PP,PAO} = i_{BM,P} .(Y_{PHA\_X}/ Y_{PHA\_PP})-1$

$m_{PAO,PO4} = i_{BM,P} . Y_{PHA\_X}$

$Y_{X\_Gly,PAO} = Y_{PHA\_X}/ Y_{PHA\_Gly}$

$Y_{X\_PP,PAO} = Y_{PHA\_X}/ Y_{PHA\_PP}$

where:

Units

$$Y_{PHA\_X} = \frac{250(106\lambda + 127\beta)(6\lambda + 27\lambda\delta + 8\beta + 30\beta\delta)}{201930\lambda + 318000K1_2 + 678771\lambda\delta + 813435\beta\delta + 269240\beta + 381000K2\beta}$$

C-mol/C-mol

$$Y_{PHA\_PP} = \frac{\varepsilon(6\lambda + 27\lambda\delta + 8\beta + 30\beta\delta)}{12(\varepsilon + \delta)}$$

P-mol/C-mol

$$Y_{PHA\_Gly} = \frac{(3\lambda + 4\beta)(6\lambda + 27\lambda\delta + 8\beta + 30\beta\delta)}{24(2\lambda + 3\lambda\delta + 2\beta + 4\beta\delta)}$$

C-mol/C-mol

$$Y_{O2\_X} = \left(\frac{RedoxPHA}{4} \cdot \frac{1}{Y_{PHA\_X}} - \frac{RedoxBM}{4}\right)^{-1}$$

C-mol/O_2-mol

$$Y_{O2\_PP} = \left(\frac{RedoxPHA}{4} \cdot \frac{1}{Y_{PHA\_PP}}\right)^{-1}$$

P-mol/O_2-mol

$$Y_{O2\_PP} = \left(\frac{RedoxPHA}{4} \cdot \frac{1}{Y_{PHA\_Gly}} - 1\right)^{-1}$$

C-mol/O_2-mol

APPENDIX B. Anaerobic stoichiometric parameters for *Accumulibater* PAO

Parameter	Value	Units	Description	Source
$Y_{Ac\_PHA,PAO}$	4/3 $X_{PAO,PHB}$	C-mmol	PHA stored per 1 C-mmol acetate taken up	Smolders et al. (1994a)
$Y_{Gly\_Ac,PAO}$	1/2	C-mmol	Glycogen consumed per 1 C-mmol acetate taken up	Smolders et al. (1994a)
$Y_{PO4\_Ac,PAO}$	1/4 + $\alpha_{PAO,Ac}$	P-mmol	Poly-P consumed per 1 C-mmol acetate taken up	Smolders et al. (1994a)
$\alpha_{PAO,Ac}$	$0.19 \cdot pH - 1.1$	ATP-mmol	ATP necessary to transport 1 C-mmol acetate through cell membrane	Smolders et al. (1994a)
$Y_{Prop\_PHA,PAO}$	0.56 $X_{PAO,PHV}$ + 0.67 $X_{PAO,PH2MV}$ = 1.22 $X_{PAO,PHA}$	C-mmol	PHA produced per 1 C-mmol propionate taken up	Oehmen et al. (2005a), Oehmen et al. (2005b)
$Y_{Gly\_Prop,PAO}$	1/3	C-mmol	Glycogen consumed per 1 C-mmol propionate stored	Oehmen et al. (2005a), Oehmen et al. (2005b)
$Y_{PO4\_Prop,PAO}$	0.4	P-mmol	Poly-P consumed per 1 C-mmol propionate stored	Oehmen et al. (2005a), Oehmen et al. (2005b)

APPENDIX C. Aerobic parameters for *Accumulibacter* PAO.

Parameter	PAO or GAO	Units	Description	Reference
m_{PHA}	$\frac{12m_{ATP}}{6\lambda+27\lambda\delta+8\beta+30\beta\delta}$ *where* $m_{ATP,OX}$ = 0.019 ATPmol/(C-mol · h)	C-mol/(C-mol · h)	Aerobic maintenance coefficient on PHA	Smolders et al. (1994b), Zeng et al. (2003)
δ (Y_{NADH_ATP})	1.85	ATP-mol/NADH-mol	ATP produced per NADH oxidized (Aerobic P/O ratio)	Smolders et al. (1994b), Lopez-Vazquez et al. (2009)
K_1	1.7	ATP-mol/C-mol	ATP needed for biomass synthesis from Acetyl-CoA*	Smolders et al. (1994b), Zeng et al. (2003)
K_2	1.38	ATP-mol/C-mol	ATP needed for biomass synthesis from Propionyl-CoA*	Zeng et al. (2003)
ε	7	P-mol/NADH-mol	Aerobic phosphate transport coefficient (PAO only)	Smolders et al. (1994b)
RedoxPHA	$4.5a+4.8b+5c$	Number of electrons per C-mol	PHA degree of reduction	Zeng et al. (2003)
λ	$a+(2b/5)$	C-mol/C-mol	Percentage of Acetyl-CoA* in PHA	Zeng et al. (2003)
β	$c+(3b/5)$	C-mol/C-mol	Percentage of Propionyl-CoA* in PHA	Zeng et al. (2003)

a	X_{PHB}/X_{PHA}	C-mol/C-mol	PHB fraction in PHA	Zeng et al. (2003)
b	X_{PHV}/X_{PHA}	C-mol/C-mol	PHV fraction in PHA	Zeng et al. (2003)
c	X_{PH2MV}/X_{PHA}	C-mol/C-mol	PH2MV fraction in PHA	Zeng et al. (2003)
RedoxBM	$4+i_{BM,H}-2\cdot i_{BM,O}-3\cdot i_{BM,N}+5\cdot i_{BM,P}$	Number of electrons per C-mol	Biomass degree of reduction	Lopez-Vazquez et al. (2009)
$i_{BM,H}$	1.84	H-mol/C-mol	Hydrogen content in biomass	Lopez-Vazquez et al. (2009)
$i_{BM,O}$	0.50	O-mol/C-mol	Oxygen content in biomass	Lopez-Vazquez et al. (2009)
$i_{BM,N}$	0.19	N-mol/C-mol	Nitrogen content in biomass	Lopez-Vazquez et al. (2009)
$i_{BM,P}$	0.015	P-mol/C-mol	Phosphorus content in biomass	Lopez-Vazquez et al. (2009)

APPENDIX D. Kinetic expressions for *Accumulibacter* PAO (Meijer et al., 2002; Murnleitner et al., 1997; Oehmen et al., 2010)

Process	Expression	Switching functions
Anaerobic acetate uptake	$q_{PAO,Ac\_PHA} \cdot \frac{S_{Ac}}{S_{Ac} + K_{S,Ac}} \cdot X_{PAO}$	$\frac{X_{PAO,PP}}{X_{PAO,PP} + K_{S,PP}} \cdot \frac{X_{PAO,GLY}}{X_{PAO,GLY} + K_{S,GLY}} \cdot \frac{f_{PHA,max} - f_{PAO,PHA}}{f_{PHA,max} - f_{PAO,PHA} + K_{s,fPHA}}$
Anaerobic propionate uptake	$q_{PAO,Prop\_PHA} \cdot \frac{S_{Prop}}{S_{Prop} + K_{S,Prop}} \cdot X_{PAO}$	$\frac{X_{PAO,PP}}{X_{PAO,PP} + K_{S,PP}} \cdot \frac{X_{PAO,GLY}}{X_{PAO,GLY} + K_{S,GLY}} \cdot \frac{f_{PHA,max} - f_{PAO,PHA}}{f_{PHA,max} - f_{PAO,PHA} + K_{s,fPHA}}$
Anaerobic maintenance	$m_{PAO,AN} \cdot X_{PAO}$	$\frac{X_{PAO,PP}}{X_{PAO,PP} + K_{S,PP}} \cdot \left[1 - \frac{S_{O2}}{S_{O2} + K_{S,O2}}\right]$
Aerobic PHA degradation	$q_{PAO,PHA,OX} \cdot f_{PAO,PHA}^{2/3} \cdot X_{PAO}$	$\frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,fPHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
Aerobic glycogen production	$q_{PAO,GLY,OX} \cdot f_{PAO,PHA}^{2/3} \cdot \frac{1}{f_{PAO,GLY}} \cdot X_{PAO}$	$\frac{f_{PAO,GLY,max} - f_{PAO,GLY}}{f_{PAO,GLY,max} - f_{PAO,GLY} + K_{S,GLY}} \cdot \frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
Aerobic Poly-P formation	$q_{PAO,PO4\_PP,OX} \cdot \frac{1}{f_{PAOI,PP}} \cdot X_{PAO}$	$\frac{f_{PAO,PP,max} - f_{PAO,PP}}{f_{PAO,PP,max} - f_{PAO,PP} + K_{S,PP}} \cdot \frac{X_{PAO,PHA}}{X_{PAO,PHA} + K_{S,PHA}} \cdot \frac{S_{S,PO4}}{S_{S,PO4} + K_{S,SPO4}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
Aerobic maintenance	$m_{PAOI,PHA,OX} \cdot X_{PAO}$	$\frac{S_{O2}}{S_{O2} + K_{S,O2}}$

APPENDIX E. Kinetic coefficients for *Accumulibacter* PAO.

Kinetic coefficient	Description	Value	Units	Source
$K_{S,Ac}$	Half-saturation coefficient for acetate	0.001	C-mmol/L	Oehmen et al. (2010)
$K_{S,Prop}$	Half-saturation coefficient for propionate	0.001	C-mmol/L	Oehmen et al. (2010)
$K_{S,PHA}$	Half-saturation coefficient for PHA	0.02	C-mmol/L	This study
$K_{S,GLY}$	Half-saturation coefficient for glycogen	0.01	C-mmol/L	Oehmen et al. (2010)
$K_{S,PP}$	Half-saturation coefficient for poly-phosphate (poly-P)	0.01	P-mmol/L	Oehmen et al. (2010)
$K_{S,PO4}$	Half-saturation coefficient for orthophosphate	0.01	P-mmol/L	Oehmen et al. (2010)
$K_{S,O2}$	Half-saturation coefficient for oxygen	0.01	O_2-mmol/L	Oehmen et al. (2010)
$K_{S,fPHA}$	Half-saturation coefficient for the fraction of PHA in biomass	0.01	C-mol/C-mol	Oehmen et al. (2010)
$f_{PAO,PP,max}$	Maximum poly-P content per PAO biomass concentration	0.30	P-mol/C-mol	Wentzel et al. (1989)
$f_{PHA,max}$	Maximum PHA content per PAO biomass concentration	1.00	C-mol/C-mol	Oehmen et al. (2010)
$f_{PAO,GLY,max}$	Maximum glycogen content per PAO biomass concentration	0.8	C-mol/C-mol	Lanham et al. (2014)
$m_{PAO,AN}$	ATP necessary for anaerobic maintenance purposes of PAOs	2.35	P-mmol /(C-mol · h)	Smolders et al. (1994a), Brdjanovic et al. (1998)

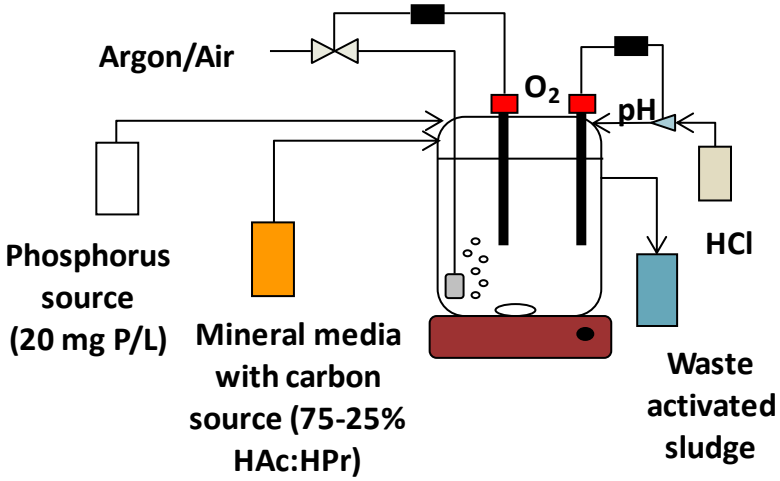
APPENDIX F. Maximum specific aerobic metabolic rates for PAOs.

	DO concentration (mg O_2/L)						
	7.8	**3.1**	**2.1**	**1.0**	**0.6**	**0.3**	**0.1**
q_{PHA} (C-mmol/(C-mmol X.h))	0.164 ± 0.008	0.152 ± 0.006	0.173 ± 0.008	0.118 ± 0.005	0.120 ± 0.005	0.111 ± 0.010	0.059 ± 0.005
$q_{Glycogen}$ (C-mmol/(C-mmol X.h))	0.0014 ± 0.0003	0.0045 ± 0.0005	0.0036 ± 0.0005	0.0020 ± 0.0003	0.0016 ± 0.0004	0.0020 ± 0.0011	<0.001 ± 0.0005
q_P (P-mmol/(C-mmol X.h))	0.0118 ± 0.0010	0.0127 ± 0.0013	0.0138 ± 0.0015	0.0132 ± 0.0016	0.0094 ± 0.0023	0.0061 ± 0.0013	0.0037 ± 0.0004

APPENDIX G. Maximum specific aerobic metabolic rates for GAOs.

	DO concentration (mg O_2/L)				
	8.1	**3.1**	**2.2**	**1.0**	**0.6**
q_{PHA} (C-mmol/(C-mmol X.h))	0.508 ± 0.055	0.295 ± 0.011	0.226 ± 0.005	0.145 ± 0.007	0.118 ± 0.009
$q_{Glycogen}$ (C-mmol/(C-mmol X.h))	0.048 ± 0.007	0.023 ± 0.002	0.015 ± 0.001	0.012 ± 0.001	0.006 ± 0.001

APPENDIX H. SBR experimental apparatus



SBR cycle

	Filling (5 min)
	Anaerobic period (2 hour)
	Aerobic period (3 hour)
	Settle/decant period (1 hour)

REFERENCES

Brdjanovic, D., Logemann, S., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998. Influence of temperature on biological phosphorus removal: Process and molecular ecological studies. WATER RESEARCH 32 (4), 1035–1048.

Lanham, A., Oehmen, A., Saunders, A., Carvalho, G., Nielsen, P., Reis, M., 2014. Metabolic modelling of full-scale enhanced biological phosphorus removal sludge. Water Research 66 (1), 283-295.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Research 43 (2), 450–462.

Meijer, S.C.F., van Loosdrecht, M.C.M., Heijnen, J.J., 2002. Modelling the start-up of a full-scale biological phosphorous and nitrogen removing WWTP. Water Research 36 (19), 4667–4682.

Murnleitner, E., Kuba, T., van Loosdrecht, M.C.M., Heijnen, J.J., 1997. An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnology and Bioengineering 54 (5), 434–450.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Research 44 (15), 4473–4486.

Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J., 2005a. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Research 39 (15), 3727–3737.

Oehmen, A., Zeng, R.J., Yuan, Z., Keller, J., 2005b. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnology and Bioengineering 91 (1), 43–53.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnology and Bioengineering 43 (6), 461–470.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnology and Bioengineering 44 (7), 837–848.

Wentzel, M.C., Ekama, G.A., Loewenthal, R.E., Dold, P.L., Marais, G.R., 1989. Enhanced Polyphosphate Organism Cultures in Activated-Sludge Systems .2. Experimental Behavior. Water Sa 15 (2), 71–88.

Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z., Keller, J., 2003. Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnology and Bioengineering 81 (1), 92–105.