Ana Alexandra Barbosa Lanham

Licenciada

Full-scale biological phosphorus removal: quantification of storage polymers, microbial performance and metabolic modelling

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

Júri:

Presidente:	Prof. Doutor José Augusto Legatheaux Martins
Arguentes:	Doutora Maite Pijuan
	Doutor António Martins
Vogais:	Prof. Doutora Maria Ascensão Miranda Reis
	Prof. Doutor Per Halkær Nielsen
	Doutora Gilda de Sousa Carvalho Oehmen
	Doutor Adrian Michael Oehmen

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Para a minha Mãe
A pessoa que mais admiro e que me ensinou a ter coragem e a lutar pelos meus sonhos

Para os Tios Maria Elvira (1914-1999) e Fernando Vieira de Sá
A minha inspiração encontrada nos avós que nunca tive

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[...] Birds flyin' high, you know how I feel
Sun in the sky, you know how I feel
Breeze driftin' on by, you know how I feel
Its a new dawn, its a new day, its a new life for me
And this old world is a new world and a bold world for me [...]
Nina Simone

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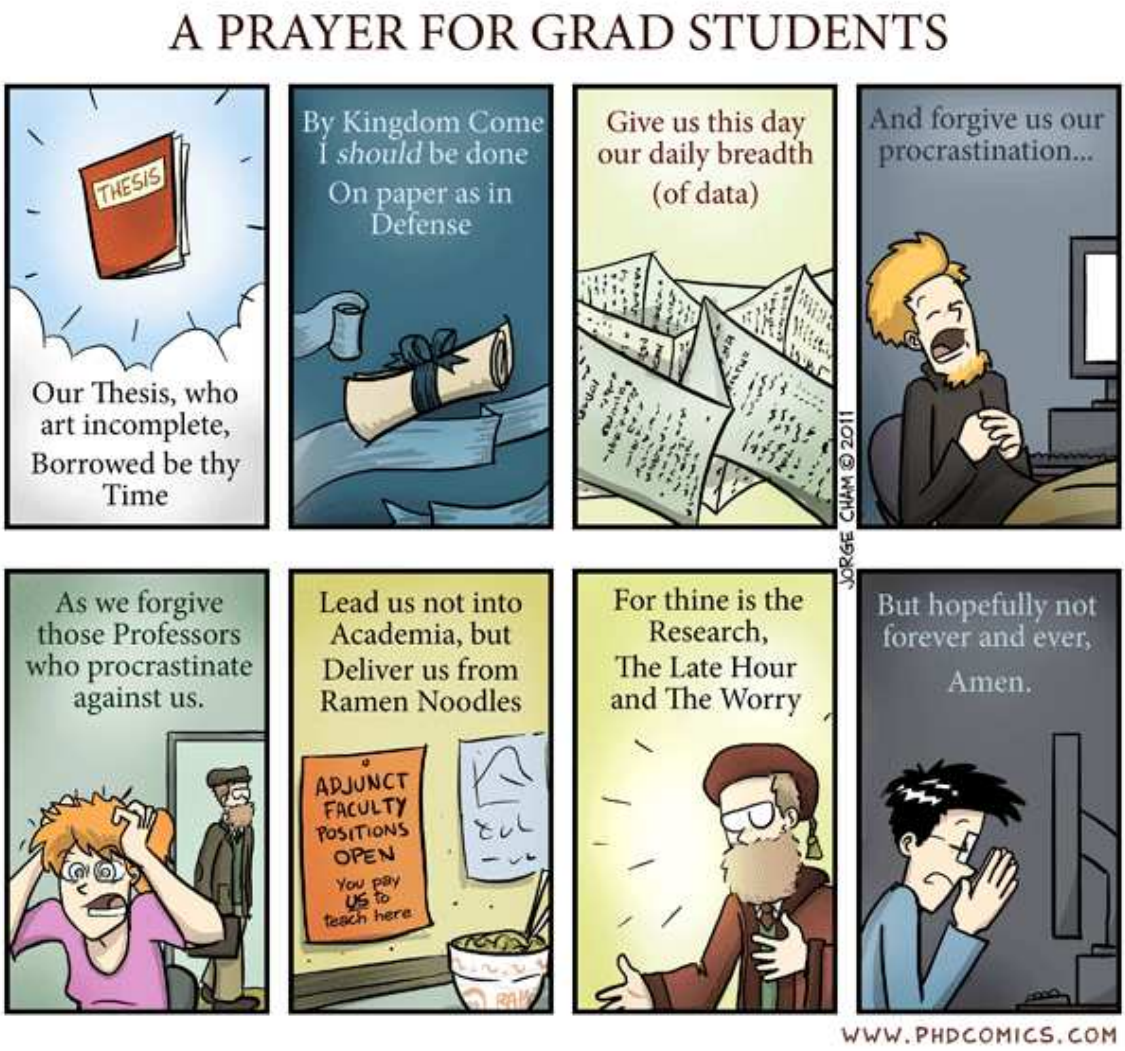

Faculty real legacy is people, not paper:
create environments
that develop professionals of whom you are proud.

David Patterson, Berkeley University
(2001)

Abstract

Enhanced biological phosphorus removal (EBPR) can be applied in wastewater treatment plants (WWTPs), as a sustainable and efficient way to remove phosphorus from wastewater and hence reduce its impact on eutrophication. This work characterises the performance, metabolism and identity of the microbial EBPR communities in full-scale WWTPs. The accurate quantification of the internal storage compounds, namely polyhydroxyalkanoate (PHA) and glycogen, is crucial to the characterisation of EBPR. The optimal glycogen and PHA quantification methods were sensitive to the heterogeneity of the sample, in terms of its microbial structure (floccular or granular) and, for PHA, in terms of the size and the number of substituents of the monomers forming the copolymer. Additionally, by characterising six full-scale EBPR WWTPs, in terms of their overall performance, microbial identity and metabolism, the composition of polyphosphate accumulating organisms (PAOs) was fairly similar in all plants. Also, a warmer climate was not sufficient to justify a higher presence of glycogen accumulating organisms (GAOs). Differing levels of denitrifying PAOs were obtained in different plants and the involvement of the tricarboxylic acid (TCA) cycle in the anaerobic metabolism of PAOs was observed. Furthermore, a metabolic model developed in this study, which incorporates the involvement of the anaerobic TCA cycle and a new description of the aerobic maintenance processes, was able to accurately describe the chemical cycling of soluble and intracellular compounds, while requiring a simple calibration procedure. A series of simulations demonstrated that lower acetate concentrations in the feed and higher aeration retention times would favour the TCA cycle metabolism over the glycolysis pathway, which would explain why the former has been more frequently encountered in WWTPs and the latter in lab-scale enriched cultures.

Keywords: Enhanced biological phosphorus removal; anaerobic TCA cycle; glycogen; PHA; metabolic modelling

Resumo

A remoção biológica de fósforo (EBPR) é um processo aplicado em estações de tratamento de águas residuais (ETARs), como uma forma sustentável de remover fósforo, atuando na prevenção do fenómeno de eutrofização. Este trabalho caracterizou o desempenho, o metabolismo e a identidade da comunidade microbiana de um sistema EBPR em ETARs. A quantificação dos polímeros internos de reserva, como o glicogénio e o polihidroxialcanoato (PHA), é crucial para a caracterização de um sistema EBPR. As condições ótimas foram sensíveis à heterogeneidade das amostras, tanto na estrutura microbiana (floculular ou granular) como, nos caso do PHA, do tamanho e do número de substituintes dos monómeros que formam o co-polímero. A caracterização de seis ETARs com EBPR, em termos do seu desempenho e da identidade e metabolismo microbiano, testado em experiências controladas com acetato, revelou que a composição dos organismos acumuladores de fosfato (PAOs) foi semelhante nas diferentes ETARs. Um clima mais quente não foi suficiente para justificar uma presença de organismos acumuladores de glicogénio (GAOs) mais elevada. Foram observados diferentes níveis de PAOs desnitrificantes, assim como o envolvimento do ciclo dos ácidos tricarboxílicos (TCA) em anaerobiose. Paralelamente, foi desenvolvido um modelo metabólico, que incorpora o ciclo do TCA, assim como uma nova descrição dos processos de manutenção aeróbios que descreveu corretamente as transformações químicas dos compostos extra- e intracelulares, necessitando apenas de alguns ajustes nos parâmetros cinéticos. Simulações de longo termo demonstraram que concentrações mais baixas de acetato, assim como períodos aeróbios mais longos, favoreciam o metabolismo do TCA em detrimento da glicólise, o que poderá explicar o facto de o TCA ter sido predominantemente observado em ETARs, ao invés da glicólise, que tem sido mais frequentemente observada em reatores à escala laboratorial.

Palavras-chave: Remoção biológica de fósforo; ciclo do TCA; glicogénio; PHA; modelação metabólica

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Notations and abbreviations

General terms

ΔP_{aer}	Amount of phosphate taken up in aerobic conditions
ΔP_{anox}	Amount of phosphate taken up in anoxic conditions
δ_{aer}	aerobic P/O ratio - the amount of ATP produced per oxidised $NADH_2$
δ_{anox}	anoxic P/O ratio - the amount of ATP produced per oxidised $NADH_2$
f_{DPAO}	Fraction of Denitrifying PAOs
f_{n-DPAO}	Fraction of non-Denitrifying PAOs
A/O	2-stage Phoredox
A2/O	3-stage Phoredox
AE	Aerobic conditions
AN	Anaerobic conditions
ASM	Activated Sludge Model
ATP	Adenosine Triphosphate
AX	Anoxic conditions
BNR	Biological Nutrient Removal
COD	Chemical Oxygen Demand
DK	Denmark
DNA	Deoxyribonucleic Acid
DOE	Design of Experiments

DPAO	Denitrifying PAO
EBPR	Enhanced Biological Phosphorus Removal
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhof-Parnas pathway
FISH	Fluorescence *in situ* Hybridisation
GAO	Glycogen Accumulating Organisms
GC	Gas Chromatography
GDP	Guanosine Triphosphate
HRT	Hydraulic Retention Time
MAR	Microautoradiography
MBR	Membrane Bio-Reactor
MC	Microbial Culture
MCL	Medium-Chain Length
MUCT	Modified University of Cape Town process
n-DPAO	non-Denitrifying PAO
NADH	Nicotinamide Adenine Dinucleotide
P	Phosphorus
PAO	Polyphosphate Accumulating Organisms
PH2MB	Poly-3-hydroxy-2-methylbutyrate
PH2MV	Poly-3-hydroxy-2-valerate
PHA	Polyhydroxyalkanoate
PHB	Poly-3-hydroxybutyrate
PHV	Poly-3-hydroxyvalerate
PPK	Polyphosphate Kinase
PT	Portugal
R^2	Coefficient of determination
RAS	Return Activated Sludge

rRNA	Ribosomal Ribonucleic Acid
RSS	Return sludge side-stream hydrolysis
SBR	Sequencing Batch Reactor
SRT	Solids Retention Time
SS	Suspended Solids
TCA	Tricarboxylic Acid
TS	Total Solids
UCT	University of Cape Town process
VFA	Volatile Fatty Acid
WWTP	Wastewater Treatment Plant
X	Active biomass

Specific statistic terms in Chapters 3 and 4

$\overline{y}$	Average of all experimental values
σ	Standard deviation
a_i	Model coefficient for variable x_i
N	Number of experiments
n	Number of replicates
P	Number of parameters
p	probability value
q	Predicted value for glucose or PHA concentration
x_i	Variable No. i
y	Experimental value for glucose or PHA concentration
z	Normalised predicted value for glucose or PHA concentration
A	GC Peak Area
A_{is}	GC Peak Area of Internal Standard
DF	Number of Degrees of Freedom
k	kinetic constant for the PHA or glycogen hydrolysis reaction

LOF	Lack Of Fit
MLR	Multiple Linear Regression
n	order of the hydrolysis reaction of PHA or glycogen hydrolysis
RSM	Response Surface Modelling

Specific terms in Chapter 6

$f_{GLY}^{\max}$	Maximum glycogen fraction
$\alpha_{j,HAc}$	Energy requirement for acetate transport across the cell membrane in organism j
$f_{ACCI,ACCII}$	Fraction of total *Accumulibacter* covered by Type I/Type II FISH probes
f_j	Fraction of organism j
$i_{BM,P}$	Phosphorus content in the biomass
ini	Initial
$K_{S,k}^{i,j}$	Half-saturation constant in conditions i, for organism j on compound k
k_j	Aerobic consumption or production rate for compound j
$m_{j,k}^{i}$	Maintenance coefficient in conditions i for organism j on compound k
n	Number of experimental values
$q_{HAc}^{\max}$	Maximum acetate uptake rate
S_j	Concentration of compound j in the bulk liquid
$x_{\max}^{exp}$	Maximum experimental value observed
$x_{\min}^{exp}$	Minimum experimental value observed
x_i^{exp}	Experimental value at time point i
X_j^i	Concentration of the internal compound j, for organism i
x_i^{model}	Modelled value at time point i
$Y_{k,l}^{i,j}$	Stoichiometric yield in conditions i, for organism j, of compound k on compound l
ACC	*Accumulibacter*
ACC_Gly	*Accumulibacter* metabolism with glycolysis
ACC_TCA	*Accumulibacter* metabolism with the anaerobic TCA cycle

AER	Aerobic
ANA	Anaerobic
DEF	*Defluviicoccus*
GB	*Competibacter*
GLY	Glycogen
HAc	Acetate
O_2	Oxygen
PO_4	Phosphate
PP	Polyphosphate
X	Biomass

1 Thesis outline

Summary *A summary of the motivation for this work is presented in this section, together with an outline of the contents of each chapter that constitute the thesis.*

Increasing levels of nutrients are being wasted, from domestic effluent to agricultural run-off, with extremely damaging results to receiving water bodies, which then suffer the consequences of eutrophication. As a new trend for urban management emerges, one where sustainability should be at the core of city life, wastewater treatment plants (WWTPs) will transit from rudimentary end-cycle facilities to complex technological industries where materials are separated, recovered, treated and recycled, hence providing a source of energy, water and resources such as nutrients. The process of enhanced biological phosphorus removal (EBPR) has been used for many years as a reliable and sustainable strategy to remove phosphorus from wastewater and thus reduce its impact on eutrophication. Additionally, good phosphorus removal is a promising key factor in order to achieve not only a satisfactory level of wastewater treatment, but also as one of the initial steps towards phosphorus recovery, which will be of vital importance since phosphorus is an essential element to life and its reserves are under severe depletion.

The main goals of the work presented in this thesis were to address different aspects pertinent to EBPR, focusing essentially on full-scale systems, so as to offer a step further in integrating and consolidating previous research work, mainly obtained in lab-scale systems. These range from the methods for quantification of storage polymers, to the identification and characterisation of the EBPR microbial communities and their metabolic pathways as well as developing metabolic models to describe the overall process. It is worthwhile to mention that the work was carried out in a collaboration with Portuguese and Danish WWTPs. Although EBPR facilities have been functioning successfully for many years in Denmark, this process is still relatively unexploited in Portuguese wastewater treatment plants and the potential of Portuguese EBPR WWTPs had never been characterised before. Hopefully, an ultimate goal, is that this work serves as an encouragement for further developing this strategy in Portugal.

The thesis includes the following chapters and content:

- In Chapter 2, the importance of the preservation of the phosphorus cycle is discussed, by explaining briefly the vital dependency of life on phosphorus and by reporting the abusive anthropogenic effect on phosphorus reserves. As the understanding of biological phosphorus removal is perfected, and as new technologies are arising to recover the phosphorus captured in the biomass, wastewater treatment plants will play a crucial role in converting this linear materials economy flow into a closed cycle. Since the main focus of this work is on EBPR, the major concepts defining EBPR, as well as the key findings on performance, eco-physiology, metabolism, microbiology and modelling are presented in Chapter 2, so as to set a base of the state of the art that will support the major findings uncovered in this work.

- One of the first aspects addressed in the thesis was the assessment of robust and

reliable methods for storage polymers quantification, namely glycogen (Chapter 3) and polyhydroxyalkanoate (PHA) (Chapter 4). These storage polymers are relevant parameters in the metabolism of organisms performing EBPR and many different methods are described in the literature for different systems and by different authors. Hence, when dealing with such a complex system such as WWTP sludge, it is important to unveil the factors that might have an impact on the quantification of these polymers, ranging from microbial composition and microbial structure to the levels of the stored polymer. Chapters 3 and 4 were based on the optimisation of the quantification methods in several microbial systems, including activated sludge systems, and hence were able to determine key factors that should be addressed in the quantification method and optimal analysis conditions, which could therefore be applied to quantifying glycogen and PHA in WWTP sludge.

- Then, six WWTPs performing EBPR were characterised in Chapter 5, by means of their microbial composition and the performance of the sludge in lab-scale batch tests with acetate as carbon source. The initial hypothesis was that when comparing WWTPs from a cold-climate country (Denmark) and a warm-climate country (Portugal), the microbial composition would differ, in particular with a higher presence of glycogen accumulating organisms (GAOs), which are not favourable towards the overall phosphorus removal process. GAOs have been suggested in the past to possess a competitive advantage over polyphosphate accumulating organisms (PAOs), the vital microorganisms that carry out the phosphorus removal process, at higher temperatures. In addition, in Chapter 5, important findings are reported on the metabolic pathways being used by PAOs and GAOs, which provides a further contribution to the understanding of the metabolic versatility of these organisms.

- Using the experimental results determined in Chapter 5, in Chapter 6 it was possible to modify the established metabolic models validated for lab-scale cultures to describe the data obtained with real sludge. This chapter addressed the necessary model calibration protocols required to describe the data and was performed with a view towards generating practically applicable strategies that can be implemented by the wastewater industry. Additionally, the metabolic model developed was also used as a tool capable of integrating a complex set of experimental data and of providing new insights into the metabolic capacities of the microbial communities involved in EBPR.

- Finally, in Chapter 7, a summary of the main findings is provided along with a description of some questions that have emerged from the present work and that should be addressed in future work.

2 State of the art

Summary *This chapter provides an introduction to the concepts presented and discussed in this thesis. It aims to put into perspective the environmental problem that gave rise to the biological nutrient removal process. Additionally, it serves the purpose of reviewing the pertinent literature, and therefore, the main advances put forward in the last years, so as to create the necessary base for a constructive and supported discussion of the results found from the current work.*

2.1 THE PHOSPHORUS CYCLE: CHALLENGES AND OPPORTUNITIES

"'With your intensive agriculture...you're simply draining the soil of phosphorus. More than half of 1% a year. Going clean out of circulation. And then the way you throw away hundreds of thousands of tons of phosphorus pentoxide in your sewage! Pouring it into the sea. And you call that progress. Your modern sewage systems!" His tone was witheringly scornful. "'You ought to be putting it back where it came from. On the land." Lord Edward shook an admonitory finger and frowned. "'On the land, I tell you."

Aldous Huxley, *in* Point Counter Point, 1928

Phosphorus (P) is a remarkable element and one of the few that is involved in the foundations of life itself, along with carbon, oxygen, hydrogen and nitrogen. It is part of the cell's energy carrier, the adenosine triphosphate molecule (ATP), the cell's genetic material, the desoxyribonucleic acid (DNA), the cell's barrier to the outside environment, the phospholipidic membrane, and finally, the skeleton of all vertebrates, as a major component of bones (Filippelli, 2008).

The natural biogeochemical cycle of P is unusual, in the fact that it is mostly restrained to the solid and aqueous phases, since under the pressure and temperature conditions existing on Earth, gaseous forms of phosphorus are not stable (Filippelli, 2008). P is rarely found in nature as a free element, but rather as a component of several minerals, mostly in the phosphate form. Historically, the first supplies of P were extracted from *guano*, the mineralised excrements of animals such as sea-birds or bats. However, the main resources of P are found in the form of phosphorite, or phosphate rock, a sedimentary rock composed essentially of apatites (calcium phosphate), resulting from the sedimentary deposition of bone, shell and P precipitates (Ashley et al., 2011).

The natural P cycling, as shown in Figure 2.1, involves the weathering of phosphate rocks, due to rain and wind erosion, releasing into the soil and aquatic environments a flow of inorganic P, mainly in the form of phosphates. These are absorbed, transformed into organic molecules and used firstly by plants, and then subsequently, by animals, as nutrients for growth. The produced biomass or excrements, as well as the remains of dead animals, return to the soil or water and are decomposed by microorganisms, which mineralise P back to its inorganic form (Filippelli, 2008). Surface run-off, resulting from rain, flooding, water infiltration and irrigation, carries some of the P contained in the soil to the discharging water body. The excess P in water, depending on environmental conditions, precipitates and sediments at the bottom of the water body, along with the remains of organic matter and solid phosphate-rich materials such as bone or shells. Over several million years, these deposits form a sedimentary layer of phosphate rock that is

progressively elevated to the surface by natural tectonic movements (Filippelli, 2008).

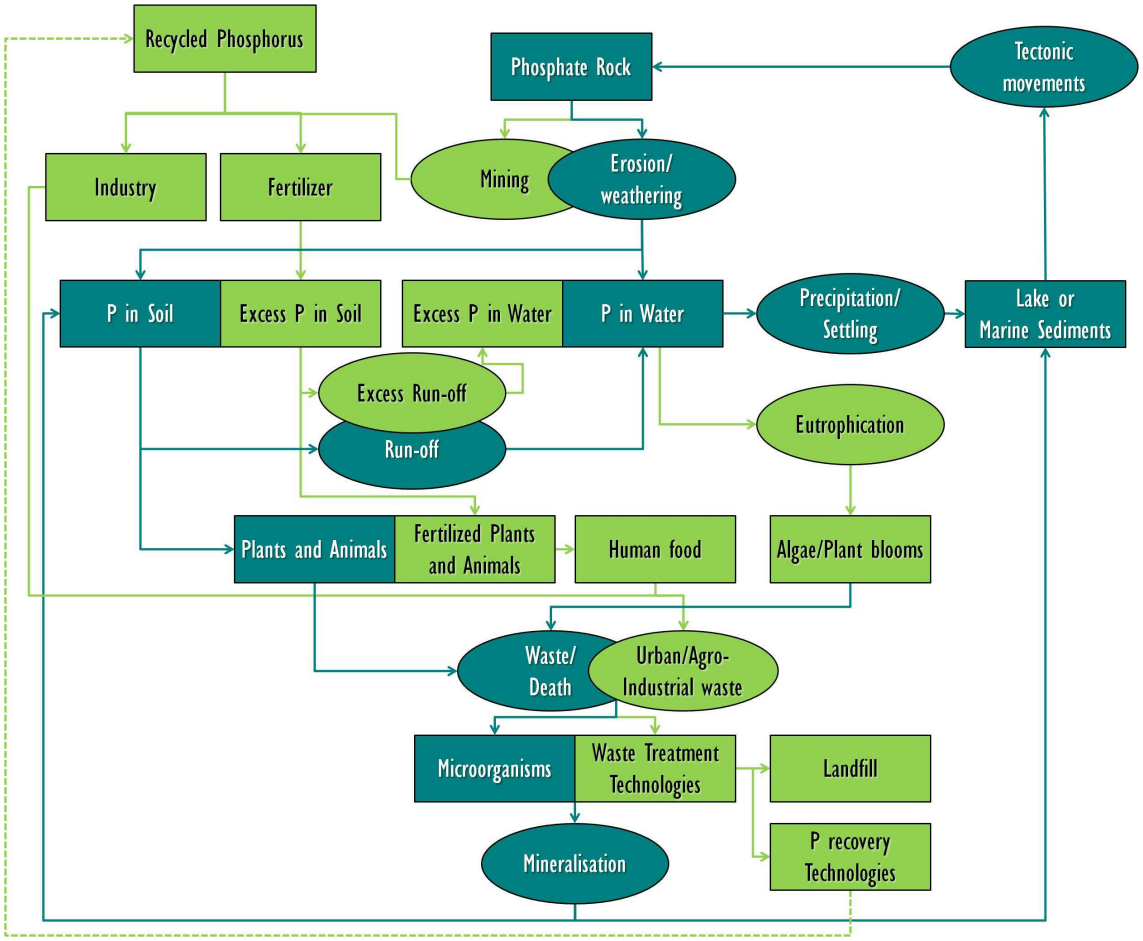

Figure 2.1: P cycle diagram illustrating the natural cycling of P (dark green) and also the interference of human activity on the natural cycle of P (light green) (Cordell et al., 2011; Cordell et al., 2009)

The anthropogenic effect on the P cycle has been exponential over the last decades (Ashley et al., 2011). Conventional agriculture relied on the natural concentration of P in the soil for crops and animal production. Food was produced locally and crop, animal, as well as human residues were recycled locally as fertiliser. With the industrial revolution there was a huge population migration, from rural areas to cities, resulting in a massive concentration of people in an area where nutrient recycling was no longer possible. Disease and hygiene were concepts that led to the so called Sanitation Revolution (ca. 1850, in the United Kingdom), where waste was no longer treated as a solid component returned to the soil, but as a liquid and diluted stream, centralised in the first sewer systems, whose fate ended in water streams such as rivers, lakes or the sea (Seviour et al., 2010b). This gradually led to an impoverishment of soils in nutrient concentrations, in particular P. However, soon after, as many advances were made in the agronomical and chemical sciences, both in the determination of soil composition, the optimisation

of crop yields and the discovery of chemical processes to produce fertilisers, the generalised use of phosphate rock as a P source began, in particular after World War II (Ashley et al., 2011). Although, the main application of P is for fertlisers (approximately 85% of the P mined, as indicated by Cordell et al. (2009)), other uses for P include sodium tripolyphosphate, used as a softener in detergents, organophosphorus compounds, used in the chemical industry as flame retardants or even herbicides, as well as its application in the steel and food industry.

Since the regeneration of phosphate rock from lake or marine sediments takes several million years, this resource is non-renewable in a human time scale and therefore our society is using phosphorus in a throughput direction, from a concentrated source, to the diffusion and dispersion of P in soil, landfills and water bodies. The implications of this use (or abuse) of phosphorus are social, economical, political and environmental. As an essential element to life and to sustain a balanced and productive environment, the depletion of P sources, which have been estimated to last for another 50-100 years (Cooper et al., 2011), will constitute a severe limitation that, contrary to other non-renewable compounds, such as fossil fuels, cannot be replaced. This will have an extremely negative impact on food production, especially in countries where food production already poses a problem. Additionally, the main P resources are located in only a handful of countries, in particular China, the USA and Morocco, where Morocco itself retains approximately 80% of the world's P supplies. In fact, most countries are expected to end their P reserves in the next decades, which would leave Morocco as the main P supplier in the world. The reliance on one country for such a vital resource will inevitably lead to an issue in terms of global phosphorus security (Cooper et al., 2011).

In environmental terms, as a cause of the excess P mining, instead of P recycling, there has been an anthropogenic enrichment of phosphate in water bodies, a phenomenon known as eutrophication (Figure 2.2). This enrichment overly stimulates the rapid growth of cyano-bacteria, algae and some aquatic plants to the point where the ecosystem is suffocated due to lack of oxygen and nutrients and due to a dense surface layer of organic matter that does not allow light to penetrate and promote photosynthesis (Anderson et al., 2002). Some cyano-bacteria also release toxins that can severely decrease the quality of water, especially in bathing areas or near potable water sources. Furthermore, the depletion of oxygen in the water body, as well as the progressive decomposition of the organic matter resultant from the algae-bloom, have profound negative effects on landscape, on the quality of air, on fisheries and on the diversity of the ecosystem (Anderson et al., 2002).

The increased perception that the human impact on the natural P cycle has brought, and will further bring, tremendous problems, has served as a driving force to develop

Figure 2.2: Photos of areas suffering from eutrophication in Portugal; a - Tâmega River (Magalhães, 2011), b - Flores Lagoon, Azores (Lusa, 2011), c - Oeiras stream (Viseu, 2011)

new solutions for a more sustainable use of P in the future (Ashley et al., 2011). Cordell et al. (2009) has mentioned that one of the main problems in the P cycle today are the huge losses in the chain of production, the losses of P due to run-off and also the low yield of P used for crops. From the P used as fertiliser, approximately 30% is lost due to run-off, another 35% stays in the soil in non-available forms to plants and only 35% is incorporated into plants and subsequently into animals. Also, from the total waste produced in the food chain, approximately 60% of the mined P is or can be recycled back to fertiliser (Cordell et al., 2009; Rittmann et al., 2011).

Therefore, many processes have been developed and are still being developed in order to increase the plant uptake of phosphorus and phosphorus availability in soil, to regulate the levels of application of fertilisers, to improve technologies and policies for animal and crop waste recycling, to contain the level of nutrients in run-off streams and finally to recover P from industrial and domestic wastes (Cordell et al., 2011; Rittmann et al., 2011; Vaccari et al., 2011). This may lead to a new nutrient revolution, where a multi-disciplinary approach must be considered - from the economical perspective, to the technical challenges and ending with the social perception issues. However, a new paradigm is already starting to come into place, one where nutrient recycling must be considered, perhaps even at a local level, where the dilution and dispersion of nutrients must be reversed to give rise to a concentration, valorisation and re-utilisation approach.

2.2 BIOLOGICAL PHOSPHORUS REMOVAL FROM WASTEWATER USING ACTIVATED SLUDGE PROCESSES

In the modern society materials economy, wastewater treatment plants (WWTPs) consist of one of the main points where liquid waste streams can be centralised and treated. Since many communities are facing scarcity of water and nutrients, and as new energy sources must be found to progressively replace the fossil fuel hegemony, it can no longer be overlooked the crucial potential of WWTPs as a worldwide industry transforming waste into

valuable materials, including nutrients, energy, water and other recyclable materials (Verstraete et al., 2009). In particular for phosphorus, approximately 15% of the total mined P flows through WWTPs and so far little is still recovered (Cordell et al., 2009). From the P available in WWTPs, only 6% is recycled to the soil in processes such as water reuse for irigation and composting of sludge, 53% is wasted into the receiving water bodies and 40% is disposed of in landfills, which leaves a huge margin for improved P recovery from WWTP (Cordell et al., 2009). Concentration and recovery of P in WWTPs is possible, making use of technologies that are already accessible such as, biological phosphorus removal coupled with chemical precipitation of P in the forms of struvite (magnesium ammonium phosphates) and apatite (calcium phosphates) (Rittmann et al., 2011). For an efficient and sustainable P removal and P recycle, it is then of the utmost importance to adequately control and understand the biological process of phosphorus removal, so as to develop a solid foundation at the base of a widespread P recovery in WWTPs. This subject is the focus of the present work.

The activated sludge process currently constitutes one of the most widespread means for wastewater treatment. It has been in place since the early 20th century, when Ardern and Lockett experimented with decoupling the hydraulic retention time (HRT) and the solids retention time (SRT) in a fully aerated treatment system similar to a sequencing batch reactor (SBR) (Ardern et al., 1914). Historically, the main goal of the first WWTPs was to eliminate the organic matter in order to reduce the anaerobic decomposition, the foul odours, the microbial pathogens and the heavily polluted charge to water bodies. This was achieved by running a fully aerated process that would allow both the chemical and the microbiological oxidation of the carbonaceous matter. With the discovery of the negative impact of nitrogen compounds, such as nitrate, on aquatic life, the activated sludge processes evolved to incorporate nitrification (conversion of ammonium to nitrate) and denitrification (conversion of nitrate to nitrogen gas). Hence, different plant configurations were developed to include both aerobic zones (nitrification) and zones without aeration, but where nitrate was present, or anoxic zones (denitrification). Finally, in the late 1970's, due to an increased perception of the effects of excess phosphorus being released on the quality of aquatic ecosystems, due to eutrophication, WWTPs began to include the removal of P.

The removal of inorganic P, or phosphate, can be done either by chemical precipitation, by adding most commonly iron, aluminium or calcium salts, or by biological activity. While the former seems like a more robust and reliable strategy, it also faces several limitations in the fact that reagents need to be added to perform the treatment, aggravating the costs and producing higher amounts of sludge (Oehmen et al., 2007). The latter was discovered in the late 1960's when several authors and in particular, James Barnard, began observing unexplained removal of phosphate in activated sludge plants.

After several experiments with different plant configurations, it was concluded that for phosphate removal to occur, an anaerobic phase should be provided, where a phosphate release occurred, followed by a dramatic drop in phosphate levels in the subsequent aerobic phase, as has been reviewed by Barnard, 1983.

Since then, the engineering has walked hand in hand with the chemistry and the microbiology and as the mechanisms underlying the process have become better understood, several different configurations have been developed to achieve reliable phosphorus removal from wastewater and, in most cases, coupled with nitrogen removal. Some examples include the conventional initial process, also called the A/O configuration or 2-stage Phoredox, and the A2/O process or 3-stage Phoredox (Figure 2.3). These two systems, include an anaerobic zone followed by an aerobic zone (A/O) or by an anoxic and then by an aerobic zone (A2/O) in order to remove phosphorus and nitrogen. Systems like the University of Cape Town process (UCT), the modified UCT (MUCT) and the Johannesburg configuration are all variations of the initial Phoredox process, which attempt to minimise the negative effects of nitrate on biological phosphorus removal as the return activated sludge (RAS) recycle stream is fed to the anaerobic zone. Therefore, these systems have altered the RAS such that it is recycled to a separate anoxic stage in order to fully remove nitrate. Configurations such as Biodenitro, and the improved version for phosphorus removal, that includes an anaerobic stage, Biodenipho (Figure 2.3), were designed as two systems in parallel that alternate between anoxic and aerobic conditions and which are known for a high level of nitrogen removal. Space is another crucial factor in plant design, since it is most often expensive and/or limited. Hence, other configurations, such as the SBR, the membrane bio-reactor (MBR) technology, as well as the activated sludge processes with granular sludge, are effective and more sophisticated systems that have increasingly been applied, however with some limitations due to difficulties in their operation or due to high costs, e.g, in the case of MBRs (Seviour et al., 2010b; Tchobanoglous et al., 1991). Finally, readily assimilable carbon substrates, such as volatile fatty acids, are often limiting and therefore strategies need to be found to provide the necessary substrates for adequate phosphorus removal and denitrification. Return sludge side-stream hydrolysis (RSS) was developed by replacing the conventional anaerobic stage with a long residence time stage, where approximately 20% of the return sludge is fermented and then fed to the head of the anoxic tank (Vollertsen et al., 2006).

Presently, the biological removal of phosphorus, also known as enhanced biological phosphorus removal (EBPR), is relatively well established from the operational point of view. However, difficulties still lie on achieving a reliable removal without the use of chemical precipitation as a polishing step in order to meet the regulatory limits, which can be quite stringent in areas sensitive to eutrophication. The dynamic conditions of a WWTP's operation, i.e., variations in climate, influent composition and flow and the

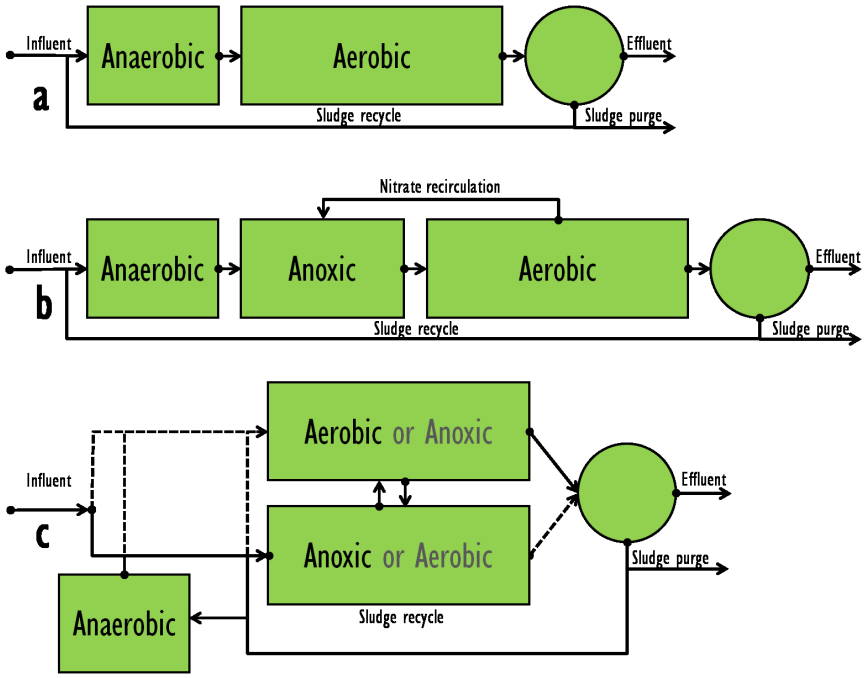

Figure 2.3: Examples of the EBPR configurations more relevant to this study: a - Conventional A/O process; b - A2/O process; c - Biodenitro process coupled with a return sludge side stream hydrolysis

presence of toxic chemicals, can have a negative impact on the EBPR process and originate episodes with poorer removal efficiency, or even failure (Oehmen et al., 2007). In general, the only added requirement for an EBPR plant, in comparison with a simple, fully aerated activated sludge process, is the inclusion of an anaerobic zone prior to the aerobic phase. EBPR also functions well with an anoxic stage, since phosphorus can be removed both in aerobic and anoxic conditions, although recirculation of nitrate to the anaerobic phase should be avoided (Barnard, 1982).

2.3 MICROBIOLOGY AND METABOLISM OF ORGANISMS INVOLVED IN EBPR

In WWTPs, the microbial community involved in EBPR only constitutes a minor fraction of approximately 5-30% of the ecosystem (Nielsen et al. (2011), in Denmark, Gu et al. (2008), in the USA, Wong et al. (2005), in Japan, Zhang et al. (2011), in China and Beer et al. (2006), in Australia). It is divided into two main groups of organisms defined by their phenotype: the polyphosphate accumulating organisms (PAOs), which contribute to the removal of phosphorus, and the glycogen accumulating organisms (GAOs), which do not contribute to the removal of phosphorus and therefore act as competitors to PAOs.

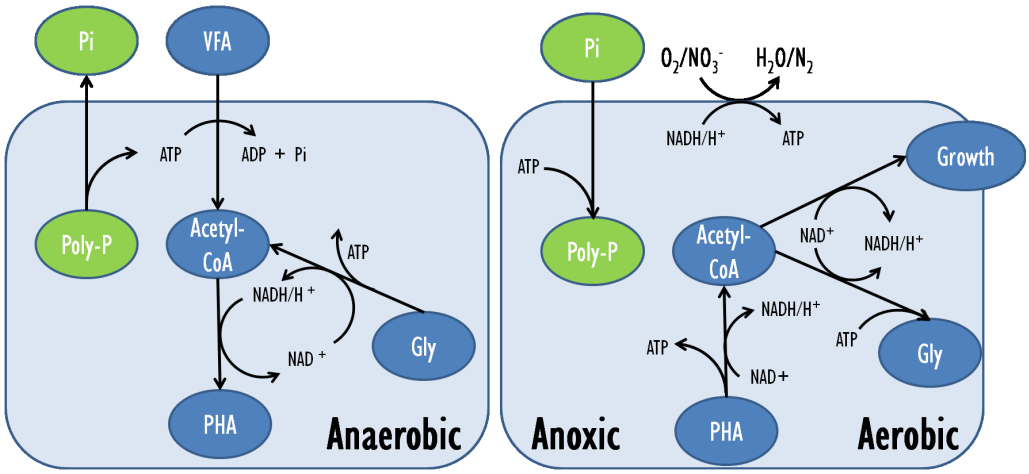

Figure 2.4: Schematic representation of the accepted PAO metabolism in anaerobic and aerobic/anoxic conditions; For simplification purposes, reducing power is only presented as NAD+,NADH/H+ but also includes NADP+,NADPH/H+ and FAD,$FADH_2$

PAOs consist of a group of aerobic organisms, capable of taking up, under anaerobic conditions, carbon substrates, such as acetate and propionate and store them as intracellular carbon polymers, such as polyhydroxyalkanoate (PHA). The energy and the reducing equivalents required to perform this biochemical reaction are obtained from the hydrolysis of internal polyphosphate, producing ATP, and therefore releasing ortophosphate into the extracelullar medium, and from the hydrolysis of internal pools of glycogen, using the glycolysis pathway. In the presence of an electron acceptor, such as oxygen or nitrate, PAOs are able to use their internal stocks of PHA to produce energy and building blocks for the regeneration of their glycogen and polyphosphate pools, as well as for growth (Oehmen et al., 2007). A schematic representation of the accepted metabolism of PAOs is depicted in Figure 2.4. The general metabolism accepted for GAOs is equivalent to the one of PAOs, however, without the ability to produce or consume polyphosphate reserves.

Although the PAO and GAO phenotype has been quite well established, it is important to note that these acronyms refer to groups of organisms, whose identity and full diversity is yet unknown. The most relevant microorganisms involved in the EBPR process have so far never been isolated in pure cultures, and thus, the knowledge obtained has been the result of lab-scale cultures containing enriched communities of organisms with a PAO or a GAO phenotype. While these enriched systems continue to provide invaluable insight into the metabolic capacities of PAOs and GAOs, it is important to stress that in real WWTPs, these communities would be flanked by many others, hence forming a network of interactions that is yet difficult to infer from only lab-scale studies. Those findings need to be continuously supported and confirmed with information obtained from real systems, in an iterative way, in order to progress into a more complete characterisation of such a complex dynamic system.

With the advances in molecular biology, some very interesting methods have proven extremely useful in deciphering the identity and the metabolism of organisms involved in EBPR without the need to resort to classical isolation techniques. One of the first important contributions was the design of clone libraries from 16S ribosomal ribonucleic acid (rRNA) from lab-scale enriched cultures (e.g., Crocetti et al. (2000); Crocetti et al. (2002); Kong et al. (2002)) and its application to designing oligonucleotide probes for fluorescence *in situ* hybridisation (FISH) for environmental samples (Bottari et al., 2006; Nielsen, 2009; Zwirglmaier, 2005). This technique targets rRNA with oligonucleotide probes to visualise and quantify certain microorganisms or groups of microorganisms in mixed microbial cultures. Coupling this technique with methods such as chemical staining for PHA or polyphosphate visualisation (Crocetti et al., 2002; Kawaharasaki et al., 1999; Levantesi et al., 2002; Serafim et al., 2002) or even to microautoradiography (MAR) (Wagner et al., 2006) provides a powerful insight into the identity of the microorganisms as well as their activity, or metabolism, *in situ*. Microbiological and eco-physiological studies have taken advantage of advanced molecular biology methods to provide detailed knowledge on the phylogenetic diversity within PAOs and GAOs, not only by targeting the 16S rRNA regions (Kong et al., 2005; Kong et al., 2007; Slater et al., 2010; Wong et al., 2007) but also by targeting the genes for the polyphosphate kinase enzymes (PPK1 and PPK2 as discussed by He et al. (2007)), which catalyse the conversions of polyphosphate into energy (ATP and guanosine triphosphate or GDP) (Flowers et al., 2009; He et al., 2007; Kim et al., 2010; Peterson et al., 2008). This approach has largely contributed to improve the resolution in the phylogeny of PAOs and to distinguish between different types within the same organism, with potentially different metabolic capacities, for instance concerning denitrification (Carvalho et al., 2007; Flowers et al., 2009). Further progress has been made with the support of more sophisticated techniques, namely metagenomics, which came to resolve some controversial aspects of the abilities of PAOs, by providing evidence of the available genomic pathways (Albertsen et al., 2011; García-Martín et al., 2006).

The main PAO identified until now in full-scale systems and obtained in highly enriched cultures is *Candidatus* Accumulibacter phosphatis, hereafter referred to as *Accumulibacter*, for which several FISH probes are available to detect and quantify its presence in mixed microbial systems (Crocetti et al., 2000; Flowers et al., 2009). *Accumulibacter* has been extensively studied, showing its ability to take up different carbon sources, preferably VFAs such as acetate or propionate, but also butyrate and valerate (Hood et al., 2001; Pijuan et al., 2004b). *Accumulibacter* can withstand lower temperatures, down to 5°C (Brdjanovic et al., 1997) and is favoured by neutral to higher pH (7-7.5) (Filipe et al., 2001b). Phylogenetic studies using the PPK enzyme have been able to differentiate between five different Clades (I, IIA-D) that appear unevenly distributed in different EBPR systems, suggesting they have different ecological niches or functions (He et al., 2007). Based on these results, FISH probes were designed to distinguish between Type I (Clade IA and

other Type I Clades) and Type II (Clades IIA, IIC and IID) *Accumulibacter* (Flowers et al., 2009).

Some organisms belonging to the *Tetrasphaera* genus have also been suggested as putative PAOs (Kong et al., 2005; Nguyen et al., 2011; Seviour et al., 2008a). However, an enriched culture has never been successfully obtained and therefore most of the information known on their metabolism was obtained by MAR-FISH and staining studies (Kong et al., 2005; Nguyen et al., 2011). Within *Tetrasphaera*, three Clades were identified (Nguyen et al., 2011) and were shown to actively cycle phosphate when shifting from anaerobic to aerobic conditions (Kong et al., 2005; Nguyen et al., 2011). *Tetrasphaera* seem to occupy a slightly different ecological niche than *Accumulibacter*, since the carbon sources assimilated are mostly casamino acids, glucose and, to a lesser extent, acetate (Kong et al., 2005; Nguyen et al., 2011). Additionally, it is still unclear what type of storage compounds, besides polyphosphate, it is able to store (Seviour et al., 2008b).

Concerning GAOs, two main groups have been identified with corresponding studies in lab-scale reactors leading to the development of clone libraries: *Candidatus* Competibacter phosphatis (Crocetti et al., 2002; Kong et al., 2002), hereafter referred to as *Competibacter* and *Defluviicoccus vanus* related GAOs, hereafter referred to as *Defluviicoccus*, including the more well-studied Clusters I and II (Meyer et al., 2006; Wong et al., 2004) and the recently discovered Clusters III and IV (McIlroy et al., 2009; Nittami et al., 2009) although only Cluster III has been suggested as having the expected GAO phenotype (McIlroy et al., 2010). *Competibacter* has been the most studied GAO thus far and its similarities with *Accumulibacter*'s metabolism are many. However, they do bear some differences, notably in that *Competibacter* is unable to take up propionate efficiently (Oehmen et al., 2005c), seems to be stimulated by higher temperatures (25-30°C) (Panswad et al., 2003) and lower pH values (6.5-7) (Filipe et al., 2001a). *Defluviicoccus* seem more versatile in terms of assimilable carbon sources, taking up acetate and propionate (Burow et al., 2007; Wong et al., 2004).

Accumulibacter has been detected in many full-scale EBPR systems with numerous configurations in countries such as Denmark, Japan, Australia, USA and the Netherlands, in numbers that vary from barely detectable limits up to 20% of the biovolume of all bacteria. However, most *Accumulibacter* levels are quite stable from plant to plant, averaging approximately 9% (Beer et al., 2006; Gu et al., 2008; Kong et al., 2007; Lopez-Vazquez et al., 2008a; Nguyen et al., 2011; Saunders et al., 2003; Silva et al., 2012; Thomsen et al., 2007; Wong et al., 2005). *Competibacter* levels are far more dynamic, from non-detectable up to 30%, in samples from Japanese WWTPs (Wong et al., 2005). Most of the time, the abundance of *Competibacter* is lower than the abundance of *Accumulibacter*. However, when the reverse is observed, the GAO fraction often led to a deteriorated EBPR performance

(Gu et al., 2008; Kong et al., 2007; Lopez-Vazquez et al., 2008a; Saunders et al., 2003; Wong et al., 2005; Zhang et al., 2011). It is worthwhile to note that even in the case where GAOs do not upset the EBPR function, they are still competing with PAOs for carbon, which results in increased carbon requirements leaving a lower fraction for other functions such as denitrification (Saunders et al., 2003). The fraction of *Defluviicoccus*-related GAOs has been less studied, although the abundance is generally lower. Until now, only in Australia have significant amounts of *Defluviicoccus* related GAOs been observed, from none detectable up to values higher than 25% (only Clusters II and III) (McIlroy et al., 2009). Additionally, significant values of Cluster II have only been found in two Danish plants out of eleven (Burow et al., 2007), suggesting that the presence of these organisms is less widespread. The abundance of *Tetrasphaera*-PAOs is generally higher than the fraction of *Accumulibacter* or *Competibacter*, usually ranging on average from 5% to 30% (Beer et al., 2006; Kong et al., 2005; Nguyen et al., 2011).

During the past 10-20 years, some aspects of the PAOs and GAOs metabolic characteristics and capacities have been quite controversial. One of these aspects has been the complete description of the anaerobic metabolism, and in particular, the source of reducing equivalents. While the first biochemical model, proposed by Comeau et al. (1986) and by Wentzel et al. (1986), suggested the involvement of the tricarboxylic acid (TCA) cycle in anaerobic conditions to generate reducing equivalents, a second hypothesis, proposed by Mino et al. (1987) and later by Arun et al. (1989), based on the use of glycolysis, has gained a more general acceptance due to the support of experimental stoichiometric results obtained mainly from lab-scale enriched cultures (cf. Figure 2.5 for the representation of the different metabolic pathways possible). However, many experimental results have shown that the TCA cycle could be active (Burow et al., 2008; Hesselmann et al., 2000; Maurer et al., 1997; Pereira et al., 1996; Pijuan et al., 2008; Zhou et al., 2009), either to its full extent or partially (e.g., the glyoxylate cycle or the split TCA cycle), and the metagenomic analysis conducted in 2006 by García-Martín et al. revealed that *Accumulibacter* does possess the necessary genes that encode for the enzymes for the functioning of either the full or the partial TCA cycle. As has been reviewed by Zhou et al. (2010), this controversy could result from a diversity in metabolic pathways within the *Accumulibacter* population or simply from the fact that all of these options are present and can be activated under different conditions.

A second controversial aspect has been related to the denitrification capacities of PAOs. It has long been shown that anoxic phosphorus removal is twice as advantageous since it allows to remove phosphorus as well as nitrate with the same carbon requirements (Kuba et al., 1996b). Furthermore, since the anoxic metabolism is approximately 40% less efficient than with oxygen, this process has an overall lower sludge production which entails less costs for sludge disposal, not to mention the cost-reductions with less

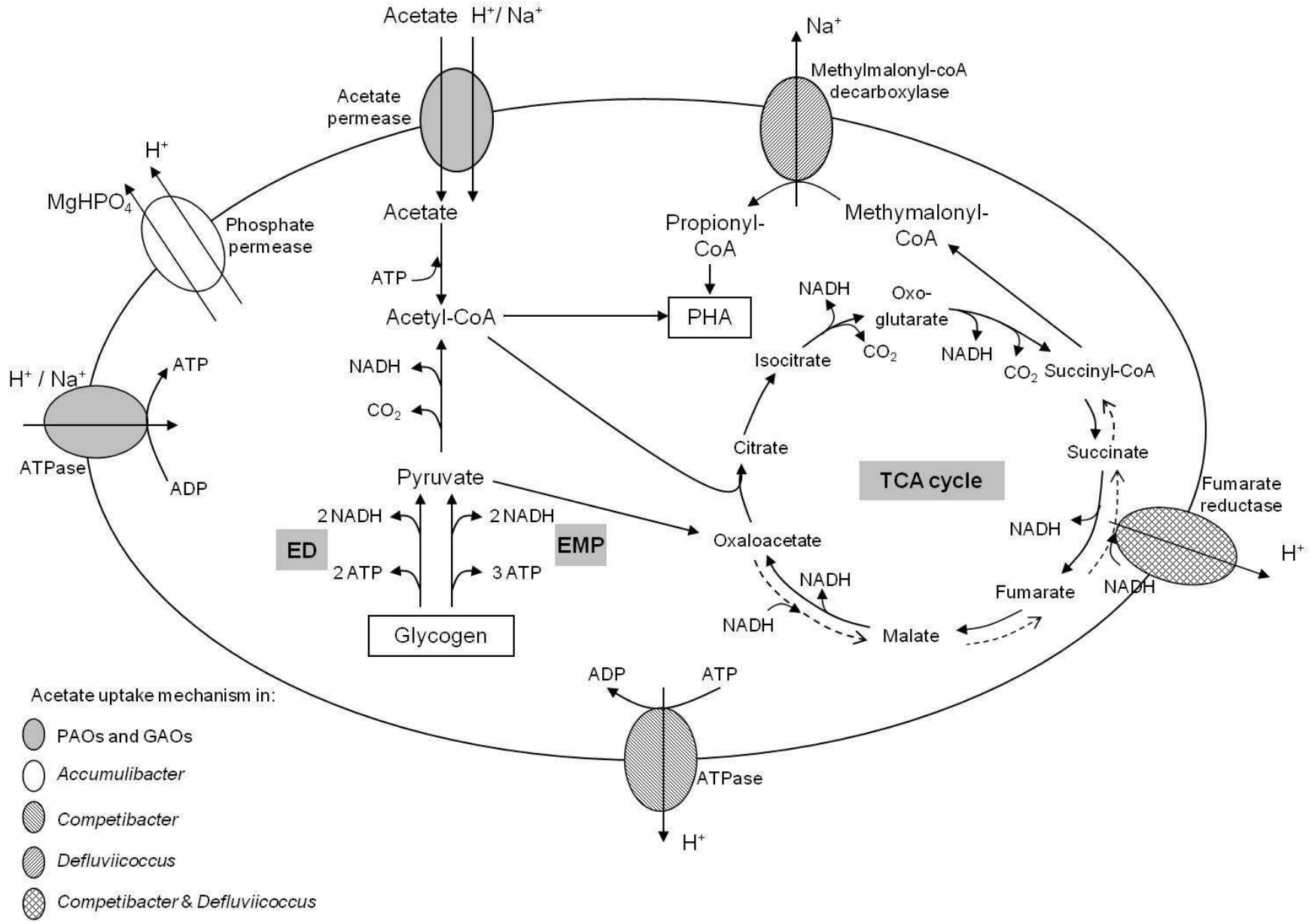

Figure 2.5: Biochemical anaerobic pathways proposed for *Accumulibacter*, *Competibacter* and *Defluviicoccus*, illustrating the glycolysis (either via the Entner-Doudoroff (ED) or the Embden-Meyerhof-Parnas (EMP) pathway) as well as the anaerobic full- or split-TCA cycle utilisation. Representation of the the acetate transport mechanisms, as well as the proton and sodium efflux pumps that maintain the proton motive force. Figure according to Oehmen et al. (2010a)

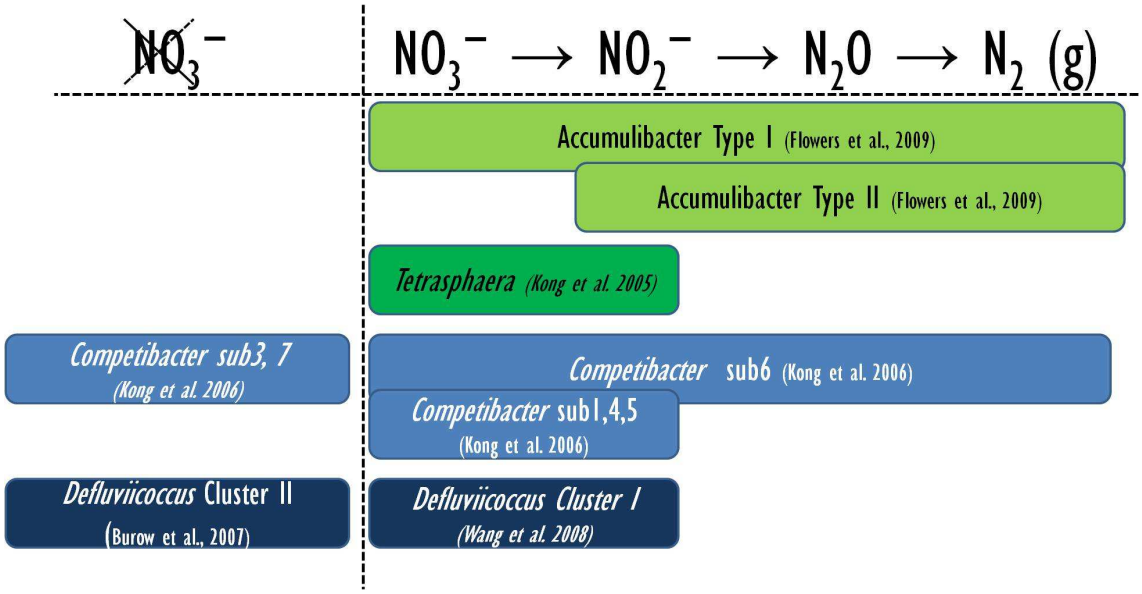

Figure 2.6: Different denitrification abilities of PAOs and GAOs known to date (Burow et al., 2007; Flowers et al., 2009; Kong et al., 2005; Kong et al., 2006; Wang et al., 2008)

aeration required (Kuba et al., 1996a). However, differing denitrification capacities were obtained in different studies which would suggest contradictory results in terms of *Accumulibacter*'s ability to use nitrate as an electron acceptor, as reviewed in Oehmen et al. (2007). Finally, a finer characterisation of the diversity within *Accumulibacter* was able to clear that different Types would have different affinities towards nitrate (Carvalho et al., 2007; Flowers et al., 2009): whereas Type I is proposed to denitrify from nitrate to nitrogen gas, Type II would only denitrify from nitrite to nitrogen gas (Carvalho et al., 2007; Flowers et al., 2009) (Figure 2.6). This is yet another example of the variability of functions being carried out by different sub-groups within the main organisms relevant to EBPR and the selective pressures on each Type has not been identified yet. In the same way, contrasting denitrification capacities have been shown for *Tetrasphaera*-PAOs and for both GAOs, often with differences between Types or Clades. Some organisms within *Tetrasphaera* were able to use nitrate, but not nitrite, for phosphorus cycling, most likely indicating that they were unable to use nitrate all the way to nitrogen gas (Kong et al., 2005) (Figure 2.6). As shown in Figure 2.6, subgroups of *Competibacter* revealed different extents of denitrification (Kong et al., 2006; Zeng et al., 2003b) and the same applied for *Defluviicoccus*, where Cluster I was shown to denitrify from nitrate to nitrite (Wang et al., 2008), whereas Cluster II was not (Burow et al., 2007).

Finally, a main aspect to be considered is the factors impacting on the competition between PAOs and GAOs, so as to control and reduce the presence of GAOs in full-scale systems. The main factors suggested to negatively affect the performance of EBPR due to competition with GAOs include higher temperatures (>20°C), the composition of the

VFAs available (e.g., acetate over propionate), low carbon to P ratio, lower pH (<7) and longer SRT, as reviewed in Oehmen et al. (2007). Many of these factors have been first suggested in lab-scale cultures, although a much smaller number have been confirmed in full-scale WWTPs (Gu et al., 2008; Lopez-Vazquez et al., 2008a; Zhang et al., 2011). Thus, EBPR control strategies that are applicable to full-scale systems require further study, especially since the ecological complexity and dynamics of EBPR makes it difficult to point out isolated factors.

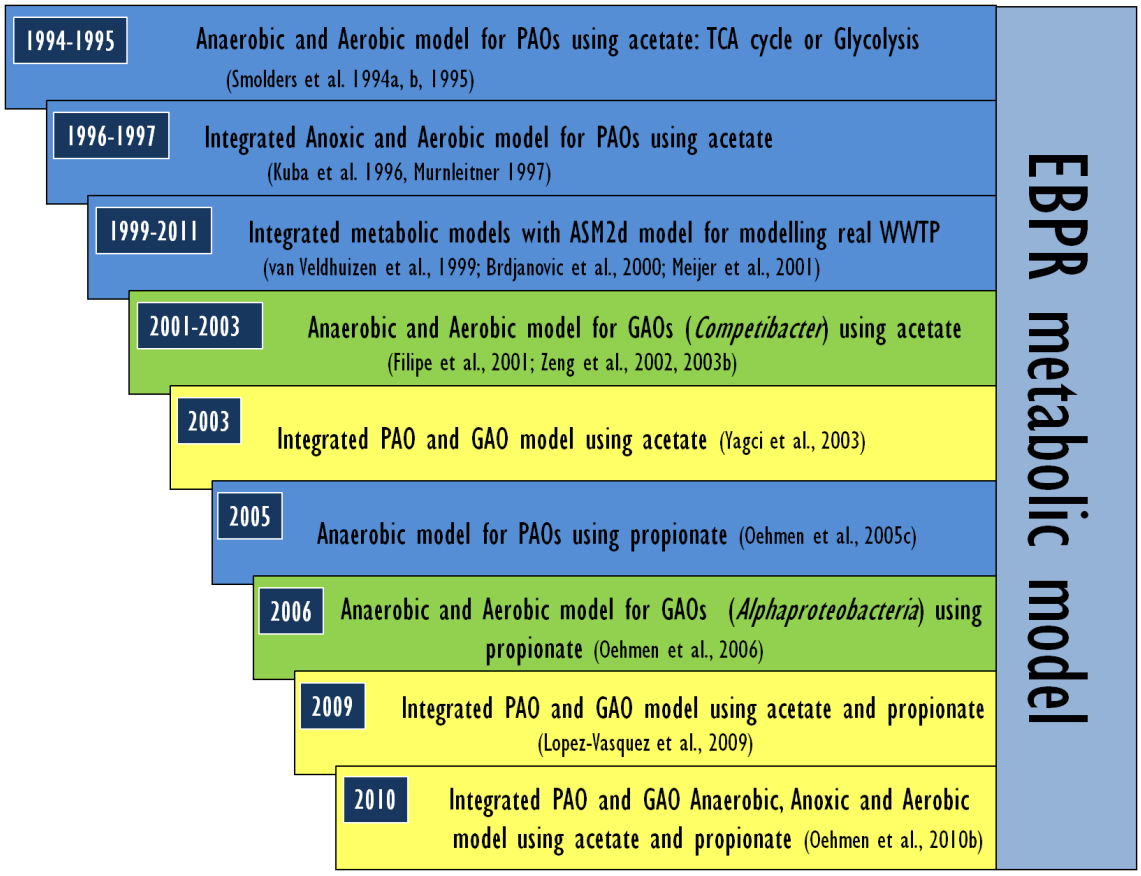

Figure 2.7: Improvement of EBPR metabolic models based on the contributions of Brdjanovic et al. (2000); Filipe et al. (2001a); Kuba et al. (1996a); Lopez-Vazquez et al. (2009a); Meijer et al. (2001); Murnleitner et al. (1997); Oehmen et al. (2010b); Oehmen et al. (2005b); Oehmen et al. (2006); Smolders et al. (1995); Smolders et al. (1994a); Smolders et al. (1994b); Veldhuizen et al. (1999); Yagci et al. (2003); Zeng et al. (2002); Zeng et al. (2003a)

Dealing with such complex and dynamic microbial systems requires capturing the inter-correlation between all the different relevant parameters: the microbial composition, the levels of internal reserve compounds and the environmental and operational conditions. Each of these parameters, either individually or in association with other parameters, has an impact on the kinetics, the stoichiometry and hence on the overall efficiency of these organisms. Metabolic models, based on the biochemical knowledge

and on the experimental results obtained in lab-scale cultures, have for many years attempted to describe the functioning of complex EBPR systems, mostly, so far, at lab-scale. They provide a very useful tool in describing and predicting the behaviour of complex microbial systems due to the built-in mechanistic correlations between the different parameters. These provide an advantage over conventional activated sludge models (ASM), since as they rely on mass, energy and redox balances, they would purportedly require a simpler calibration procedure (Seviour et al., 2010b). Nevertheless, they require a more detailed knowledge of the system, namely the type of organisms present and their metabolic characteristics. Smolders et al. (1994b) developed one of the first metabolic models describing the anaerobic and the aerobic metabolism of PAOs, using either the anaerobic TCA cycle or glycolysis, with acetate as the carbon source. Since then, a number of improvements and modifications have been added to the initial model, as illustrated in Figure 2.7, to include the metabolism of PAOs and GAOs in anaerobic, anoxic and aerobic conditions, with different carbon sources such as acetate and propionate. Hence, metabolic modelling serves a dual purpose as a bridge between the knowledge on the biochemical and molecular mechanisms regulating PAOs and GAOs and its impact on describing and predicting the functioning of real systems (Oehmen et al., 2010a). On one hand, the models offer a comprehensive tool able to condensate and interconnect many fundamental findings, and in that sense they are able to validate hypotheses or identify missing correlations (Oehmen et al., 2010a). On the other hand, they can transpose as much of this acquired information as necessary to accurately describe the performance of EBPR systems in lab-scale (Lopez-Vazquez et al., 2009a; Oehmen et al., 2010b) as well as in full-scale (Meijer et al., 2001; Veldhuizen et al., 1999).

2.4 CHALLENGES IN UNDERSTANDING MICROBIAL EBPR COMMUNITIES AND THEIR METABOLISM IN FULL SCALE SYSTEMS

The driving force behind research on EBPR systems has always been the need to improve the final process and its performance, reliability and predictability. The assumption is that by better understanding the microbiology, eco-physiology and biochemistry of the organisms involved, the external factors can be thus controlled to stimulate a better performance and to reduce competition with other organisms, such as GAOs. However, major difficulties arise when studying systems whose microbial communities are so diverse and still largely unknown, and whose conditions on site are exceedingly dynamic, ranging from variations in carbon source and nutrient availability, electron acceptors, the presence of toxic compounds and environmental conditions such as temperature and pH. The scientific approach to characterise such a complex system inevitably implies the study of the effect of isolated parameters in controlled lab-scale systems and then the testing in full-scale systems of the hypotheses produced. Currently, a lot of information

has been produced by lab-scale systems and it is needed to apply the existing hypotheses to real systems and to try to integrate the lab-scale knowledge with its application in reality. This should constitute an on-going cycling process, from molecular and biochemical studies of simple enriched cultures in lab-scale, to its validation in real systems, preferably using metabolic modelling as a bridge, as discussed previously, only to formulate hypotheses with a broader scope and involving more complex systems that then will, in turn be tested in controlled experiments in lab-scale. With the development of more sophisticated tools, in particular the ones involving *meta* approaches such as *meta*-genomics, i.e., recovering the genome of multiple communities present in an environmental sample, as opposed to the traditional concept of relying only on clonal cultures, it has been possible to obtain a wider picture of the genetic potential of a community, rather than of one individual microorganism.

One of the challenges presented in this work was to determine whether the methods for the quantification of the carbon storage compounds relevant in EBPR, glycogen and PHA, presently available were sufficiently robust and accurate, especially when used in such complex matrices as activated sludge. The quantification methods for both these compounds rely on cell lysis mechanisms and hydrolysis reactions and they both have suffered variations over time. Hence, it was unclear which conditions would be most favourable for the analysis of the mixed microbial communities of activated sludge, which contain not only a highly diverse population, congregated in various cellular structures, from single-cell to heterogeneous-sized flocs, containing different levels of internal polymers. Moreover, a reliable method is needed in order to accurately describe the small changes often observed.

Concerning the identity and the metabolism of EBPR organisms, the main challenge comes with asserting whether the observations put forward in lab-scale conditions are transferable to full-scale systems. As a more detailed knowledge on these microbial communities is attained, in particular now with the availability of increasingly complex molecular tools, it is crucial to interpret this information in WWTPs and to correlate the eco-physiology with selective pressures or environmental factors. For instance, what is the distribution of the different microorganisms known to date as related to EBPR in function of climate or operational conditions such as carbon availability? In permanently fluctuating conditions, are the metabolisms expressed the same or contrasting to those observed in lab-scale enriched cultures, and if there are differing results, what are the hypotheses for the differences observed?

Finally, since metabolic models already provide the compilation of a significant amount of knowledge on the main organisms involved in EBPR systems, an attempt has to be made to apply these models to complex real systems. Although a combination of metabolic

models and ASM models has previously been successfully applied to WWTP systems (Meijer et al., 2001; Veldhuizen et al., 1999), it is important to assess how well the present metabolic models, which have evolved to a new degree of complexity by incorporating the activity of GAOs and its competition with PAOs, describe the behaviour of the sludge and what should be the necessary calibration procedure. These answers would provide an important step forward to the application of metabolic modelling, so that it becomes a powerful predicting tool for real WWTPs.

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3 Optimisation of glycogen quantification in mixed microbial cultures*

Summary *This study addressed the key factors affecting the extraction and quantification of glycogen from floccular and granular mixed microbial cultures collected from activated sludge, nutrient removal systems and photosynthetic consortiums: acid concentration, hydrolysis time and concentration of biomass in the hydrolysis. Response surface modelling indicated that 0.9 M HCl and a biomass concentration of 1 mg.mL^{-1} were optimal conditions for performing acid hydrolysis. Floccular samples only needed a 2-h hydrolysis time whereas granular samples required as much as 5 h. An intermediate 3 h yielded an error of 10% compared to the results obtained with the hydrolysis times specifically tailored to the type of biomass and can thus be recommended as a practical compromise.*

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3.1 INTRODUCTION

Glycogen, a polysaccharide formed of glucose units, is used as a storage compound by different organisms (Preiss, 1984). The reversible interconversion of glycogen to glucose and then to pyruvate through the glycolysis and the gluconeogenesis pathways, provide cells with a useful source of carbon, energy (ATP) and reducing equivalents (NADH). Therefore, the utilisation of storage compounds such as glycogen, but also polyphosphate and polyhydroxyalkanoate (PHA), is often used as a survival strategy for bacteria present in dynamic systems subjected to variable environmental conditions (Loosdrecht et al., 1997).

The analysis and quantification of bacterial glycogen has been essential to the understanding of the complex microbial communities in various environments and glycogen has been included as a major component in metabolic models for activated sludge (Kuba et al., 1996a; Oehmen et al., 2010a; Oehmen et al., 2006; Smolders et al., 1994b).

Consequently, there is often a necessity to accurately quantify the glycogen content of cells and to estimate the kinetics and stoichiometry of the process. Nevertheless, metabolic modelling predictions for glycogen have often exhibited error rates as high as 20-25% exceeding those for PHA, phosphorus or acetate (Brdjanovic et al., 2000; Lopez-Vazquez et al., 2009b). Therefore, an optimisation of the analytical method for glycogen is needed.

Glycogen analysis can be carried out in several ways, depending on the type of cells that are being investigated and also on whether the purpose of the analysis lies only on its quantification or on its simultaneous recovery (Ernst et al., 1984; Good et al., 1933; Maurer et al., 1997; Palmstierna, 1956; Parrou et al., 1997). Most methods include extraction, precipitation and hydrolysis of the glycogen and measurements of the glucose produced. The extraction can be performed under alkaline or acidic conditions and precipitation is done with ethanol (Ernst et al., 1984; Good et al., 1933). Hydrolysis is achieved with acids or enzymes (Ernst et al., 1984; Maurer et al., 1997). The analysis methods of the resulting glucose include chromatography (Smolders et al., 1994b), colorimetric methods (phenol-sulphuric method, anthrone method) (Fang et al., 2000) or enzymatic kits (Coats et al., 2011).

A simple and high-throughput quantification method was developed by Smolders et al. (1994b) and Maurer et al. (1997) to process several samples simultaneously. The method most frequently used at present includes acidic hydrolysis of the biomass pellet or suspension with dilute hydrochloric acid to simultaneously extract and hydrolyse glycogen.

The main limitation of this method is that it quantifies not only the glucose derived from glycogen but the total glucose. For this reason, some studies refer to glycogen values

as total carbohydrates (Bond et al., 1999; Fang et al., 2000; Maurer et al., 1997).

Since the established glycogen determination methods were developed for pure cultures, cells from muscle and liver tissues or yeast (Ernst et al., 1984; Good et al., 1933; Palmstierna, 1956; Parrou et al., 1997), they might not be useful for environmental samples containing mixed populations assembled in heterogeneous structures from single cells to flocs or granules, usually bound together by extracellular polymers.

Acid concentration, hydrolysis time and biomass concentration play an important role in the cell lysis and glycogen hydrolysis, as shown by their differing values used by different authors, but these parameters have not been systematically optimised. Therefore, in the present study, a design of experiments (DOE) strategy was used to assess the influence of acid concentration, duration of the hydrolysis and amount of biomass on glycogen determination.

3.2 MATERIALS AND METHODS

3.2.1 Design of experiments and response surface modelling

3.2.1.1 Factor selection and design of experiments

The effect of acid concentration was assessed with 0.3 to 0.9 M HCl with 0.6 M as the central point, which is also the concentration most widely reported in the literature (Bond et al., 1999; Carvalho et al., 2007; Guisasola et al., 2007; Maurer et al., 1997; Zeng et al., 2003c). Since the reported hydrolysis times ranged from 2 to 6 h (Carvalho et al., 2007; Guisasola et al., 2007; Serafim et al., 2002; Zeng et al., 2003c) hydrolysis times from 2 to 10 h with a central point of 6 h were applied. Biomass concentrations were chosen in the range from 1 to 4 $mg.mL^{-1}$ with a central point of 2.5 $mg.mL^{-1}$.

The goal was to assess the impact of these parameters, for different microbial cultures, on one response factor: the glucose concentration per gram of biomass or total solids (TS). For each microbial culture, the combination of factors and the number of experiments were determined using a central composite face-centred design with three levels, one central point and one replicate for each experiment (Eriksson et al., 2008).

3.2.1.2 Model building

The model for estimating the optimal working conditions was conceived using a response surface modelling approach (RSM) (Montgomery, 2000) which fits a quadratic equation (q) to the experimental values of glucose concentration (Eq. 3.1), considering not only linear and quadratic coefficients (a) of the variables (x) but also all possible

interactions between them. Coefficients were determined by multiple linear regression (MLR).

$$q(x_1, x_2, x_3) = a_0 + \sum_{k=1}^{3} a_k x_k + \sum_{(i,j=1)}^{3} a_{ij} x_i x_j + a_{123} x_1 x_2 x_3 \tag{3.1}$$

In order to estimate each model's coefficients, the values of glucose concentration were normalised (z_i) using the standard score method given by Eq. 3.2 (Cela, 2000). This method, known as auto-scaling, transforms each value into the number of standard deviations (σ) above or below the average of all the values obtained ($\overline{y}$). This normalisation was especially relevant when modelling combined results of two or more bacterial cultures (cf. Sections 3.3.2 and 3.3.3) since each set of results displayed different ranges of glucose concentration.

$$z_i = \frac{y_i - \bar{y}}{\sigma} \tag{3.2}$$

The model coefficients were computed with Matlab 2006b (Mathworks Inc, USA) using the regstats function. The validation of the models was based on: the coefficient of determination (R^2), the regression's goodness of fit and the lack of fit (LOF). The R^2 test measures how well the regression model fits the experimental data. It compares the sum of squares of the residuals (difference between the predicted value, q_i, and the experimental value, y_i) to the total sum of squares (difference between each experimental value and the average of all experimental values, $\bar{y}$) as indicated in Eq.3.3. $R^2 = 1$ indicates a perfect fit with no deviation between the model prediction and the raw data.

$$R^2 = 1 - \frac{\sum_i (y_i - q_i)^2}{\sum_i (y_i - \bar{y})^2} \tag{3.3}$$

The goodness of fit test compares the error of the fit of the model to the variance of the predicted results. It uses an F-test to determine whether the sum of squares of regression (difference between each predicted value and the average of all experimental values) is significantly lower than the residual sum of squares as displayed in Eq. 3.4, where each component is divided by their respective number of degrees of freedom (DF). For the sum of squares of regression, DF is the number of parameters (P) of the model equation, whereas, for the residual sum of squares, DF is the difference between the total number of experiments performed (N) and the number of model parameters minus 1. This criterion is satisfied in a 95% confidence interval when the probability value (p), determined through the F-test, is lower than 0.05 (Montgomery, 2000).

$$F = \frac{\sum_{ni} (q_i - \bar{y})^2}{P} \times \frac{N - P - 1}{\sum_{ni} (y_i - q_i)^2} \tag{3.4}$$

The LOF test, as given in Eq. 3.5, uses an F-test to assess whether the model's error (difference between the average of replicates for each experiment, $\bar{y}_i$, and the predicted value) is comparable to the replicate error (difference between the experimental value and the average of replicates for each experiment). If a model has a good fitting, then its error (also called lack of fit) should be comparable to the error between replicates (also called pure error), for a data set where n replicates are available for each experimental condition i. The DF of each term is $n-2$ and $N-n$, respectively. Using an F-test, this criterion is satisfied within a 95% confidence interval when the resulting p-value is higher than 0.05 Eriksson et al., 2008.

$$F = \frac{\sum_{n,i} n(\bar{y}_i - q_i)^2}{n-2} \times \frac{N-n}{\sum_{n,i} (y_{n,i} - \bar{y}_i)^2} \tag{3.5}$$

3.2.2 Characteristics of the bacterial cultures

Four systems were chosen containing mixed microbial cultures with different characteristics such as feed source, scale of operation, type of bacterial aggregates (flocs or granules), operation cycle and concentration of glycogen.

The first bacterial culture (1-FH) came from a 4.4L lab-scale sequencing batch reactor (SBR), operated under photosynthetic non-aerated conditions, fed with a synthetic wastewater containing acetate as the carbon source. It was enriched in photosynthetic polyhydroxyalkanoate (PHA) and glycogen accumulating organisms, composed essentially by bacteria and algae, aggregated in flocs, and containing approximately 8% (w/w) of glycogen.

The second microbial culture (2-FL) came from a wastewater treatment plant (WWTP) (Beirolas, SIMTEJO, Lisbon, Portugal), operated under sequential anaerobic, anoxic and aerobic conditions and fed with wastewater containing a mixture of domestic and industrial fractions. Its microbial population was composed by flocs containing a high diversity of organisms, including a high fraction of filaments and an overall low amount of intracellular glycogen of approximately 3% (w/w).

The third culture (3-GL) was obtained from a 30-L lab-scale SBR performing biological nutrient removal (BNR), operating under alternating anaerobic-anoxic-aerobic conditions, fed with domestic wastewater obtained from Quart WWTP (Girona, Spain). The microbial population was aggregated in flocs and granules and contained an enrichment in polyphosphate- and glycogen-accumulating organisms, with a total glycogen content of approximately 3% (w/w).

The fourth culture (4-GH) was collected from a 2-L enhanced biological phosphorus removal (EBPR) SBR, operated under anaerobic-aerobic conditions, fed with synthetic

wastewater containing 75% acetate and 25% propionate. Its microbial population was aggregated into small-sized granules and was enriched in polyphosphate-accumulating organisms containing approximately 9% (w/w) intracellular glycogen.

3.2.3 Glycogen quantification

Each biomass was collected by centrifugation (10 000 x g; 5 min) and freeze-dried overnight. The pellets were weighed into air-tight Pyrex tubes, to which 2 mL of a dilute solution of HCl was added. The tubes were incubated in a heating-block at 100°C for the selected hydrolysis time. The samples were cooled in an ice bath, the supernatant was extracted, filtered (0.2 μm pore size), and glucose was analysed using an ion-exchange Aminex HPX-87H HPLC column (BIORAD, USA), coupled to a refractive index detector (Merck-Hitachi, Germany) and operated at 30°C and 0.5 mL.min^{-1}, with 0.005 M H_2SO_4 as the eluent. Samples were analysed in duplicate.

3.2.4 Stereomicroscopic imaging

Images of the microbial cultures were taken using a stereomicroscope (SteREO Discovery V12, Zeiss, USA) with transmitted light (Schott KL2500) and coupled to a digital camera (Coolpix-4500, Nikon, Japan).

3.3 RESULTS AND DISCUSSION

3.3.1 Individual models for each microbial culture

3.3.1.1 Model compilation and validation

The analytical conditions for a set of 15 different experiments were determined through the DOE and were applied in duplicate, for each microbial culture.

These conditions and the resulting glucose concentrations are outlined in Table 3.1. The results cover a range where the maximum value is approximately three times higher than the minimum value and significantly different considering the replicate error. For example for culture 1-FH, the minimum value was 47 $\pm$ 4 mg.gTS^{-1} and the maximum value was 120 $\pm$ 21 mg.gTS^{-1}. This implied that the factors chosen had a significant impact on the amount of glucose extracted.

Using each set of results, a quadratic model was compiled and its coefficients determined through MLR (cf. Table 3.2). It should be noted that the given model coefficients were estimated using normalised y-values, according to Eq. 3.2. The validity of the models is presented in Table 3.3.

Table 3.1: Average glucose concentrations, in mg of glucose per total solids (TS), obtained in each set of experiments

Experimental conditions				**Glucose concentration (mg.gTS^{-1})**			
Exp. n°	**Acid conc.** (M)	**Hyd. time** (h)	**Bio. conc.** (mg.mL^{-1})	**1-FH**	**2-FL**	**3-GL**	**4-GH**
3	0.3	2	1	61 ± 2	29 ± 8	22.10 ± 0.08	61 ± 10
1	0.3	2	4	47 ± 4	14 ± 1	16 ± 1	42 ± 4
5	0.3	6	2.5	81 ± 1	25.9 ± 0.9	26 ± 4	101.02 ± 0.04
4	0.3	10	1	75 ± 12	30 ± 1	25 ± 7	105 ± 15
9	0.3	10	4	76 ± 2	25.3 ± 0.4	29 ± 1	102 ± 2
2	0.6	2	2.5	82 ± 1	19 ± 1	22 ± 6	74 ± 8
13	0.6	6	1	101 ± 3	29 ± 5	32 ± 1	122 ± 32
7	0.6	6	2.5	86 ± 4	25.8 ± 0.2	27.6 ± 0.7	98 ± 17
12	0.6	6	4	79 ± 7	25.1 ± 0.7	29 ± 3	96 ± 4
6	0.6	10	2.5	86 ± 1	20.6 ± 0.2	28 ± 10	104 ± 1
8	0.9	2	4	69 ± 7	16 ± 4	28 ± 5	63 ± 38
10	0.9	6	2.5	73 ± 5	28 ± 6	29 ± 7	100 ± 2
11	0.9	10	1	73 ± 25	N.D.	29 ± 9	101 ± 11
14	0.9	10	4	53 ± 2	25 ± 4	34 ± 13	102 ± 16
15	0.9	2	1	120 ± 21	42 ± 5	41.6 ± 0.2	109 ± 4

Table 3.2: Estimated model coefficients (1: acid concentration; 2: hydrolysis time; 3: cell concentration).

	1-FH	**2-FL**	**3-GL**	**4-GH**	**Flocular model**	**Granular model**	**Global model**
a_0	-5.19	-1.08	-1.6	-3.88	-3.06	-3.31	-2.89
a_1	16.55	3.38	5.27	7.35	10.09	6.6	7.93
a_2	0.6	0.82	0.38	0.89	0.73	0.73	0.68
a_3	-0.05	-0.57	-0.65	-0.31	-0.36	-0.4	-0.47
a_{11}	-7.46	1.83	0.12	-2.31	-2.84	-0.77	-1.69
a_{12}	-1.03	-0.97	-0.27	-0.53	-1.02	-0.47	-0.65
a_{13}	-0.73	-0.68	0.3	-0.4	-0.69	-0.36	-0.48
a_{22}	-0.02	-0.04	-0.02	-0.04	-0.03	-0.04	-0.03
a_{23}	-0.01	-0.01	0.03	-0.001	-0.008	0.01	0.008
a_{33}	0.01	-0.05	0.05	0.03	0.03	0.03	0.04
a_{123}	0.1	0.13	-0.006	0.05	0.11	0.02	0.05

The value of R^2 for the correlation between experimental and prediction values was higher than 0.8 for cultures 1-FH and 2-FL and higher than 0.7 for culture 4-GH. Although typical R^2 values for adequate fits are usually higher than 0.9, a value of R^2 higher than 0.7 was considered acceptable (Lundstedt, 1998). Additionally, it is important to state that the method for glycogen analysis is prone to a significant error between replicates, whose causes have not yet been identified. The high error between replicates contributed to a dispersion in the values, affecting the quality of the fit. In fact, for the 3-GL model, a low R^2 was obtained (0.61), mainly due to the quality of the results produced for this particular microbial culture, since the replicate values indicated the highest error of all. Sample heterogeneity may be one of the main sources for error between replicates since, in fact, it is observed that the cultures with a higher heterogeneity have a higher error: 1-FH culture being the most homogenous system and 3-GL being a more heterogeneous system composed of a mixture of flocs and granules. Other error sources, affecting all analyses, may derive from the sensitivity of glucose molecules to oxidation and rapid degradation (Slimestad et al., 2006).

Table 3.3: ANOVA coefficients for floccular (Floc.), granular (Gran.), and global (Glob.) models and the corresponding optimum predicted values

	1-FH	**2-FL**	**3-GL**	**4-GH**	**Floc.**	**Gran.**	**Glob.**
R^2	0.81	0.89	0.61	0.77	0.85	0.79	0.73
p-value (<0.05)	0.00	0.00	0.02	0.00	0.00	0.00	0.00
LOF (>0.05)	0.24	0.55	0.77	0.75	0.57	0.30	0.35
Average replicate error (%)	8	10	17	12	-	-	-
Hydrolysis time (h)	2	2	3	6	2	5	3
Acid concentration (M)	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Biomass concentration ($mg.mL^{-1}$)	1	1	1	1	1	1	1

Nevertheless, all the model predictions, including the model for biomass 3-GL, lie within a 95% confidence interval, since the p-values were below 5%. Simultaneously, all the models have a good fitting of the data since the LOF values were higher than 5%, indicating that the error of the model was lower than the error between replicates within a 95% confidence interval. Therefore, despite the low coefficient of determination, in particular for the 3-GL model, its predictions were considered and discussed. Furthermore, its outcome agreed well with the main trends observed in the other models, i.e. that a higher acid concentration and a lower biomass content leads to improved results. Also, the conditions predicted for the maximum glucose concentration match the conditions tested experimentally (experiment number 15 of Table 3.1).

3.3.1.2 *Determination of optimal conditions*

The conditions leading to maximum glucose recovery, predicted from the equations obtained for each model, are shown in Table 3.3. For all the cultures, the optimum method conditions imply the use of a 0.9 M HCl and a biomass concentration in the reaction volume of 1 mg.mL^{-1}. The optimum hydrolysis time, on the other hand, varies from 2 h for cultures 1-FH and 2-FL to 6 h for culture 4-GH.

According to the models, the biomass concentration has a strong impact on the quantity of glycogen extracted, at least in the range of acid and hydrolysis times tested (Fig. 3.1). Fixing the acid concentration at the optimum value, i.e. 0.9 M, the highest concentrations of glucose were obtained for lower biomass concentrations and for a particular hydrolysis time interval, which varied according to the microbial culture. For lower hydrolysis times, an increase in the biomass concentration from 1 to 4 mg.mL^{-1} led to a decrease in the glucose obtained by approximately 30-40%. This effect may be caused by an incomplete extraction or hydrolysis of glycogen when the concentration of cells is increased. Also, biomass concentrations lower than 1 mg.mL^{-1} might still further improve the analysis. However, due to the experimental conditions used in this study (type of tube, acid volume, heating-block model, scale precision), 1 mg.mL^{-1} of biomass was considered to be the minimum value possible within an acceptable error range for the weighed value of 5%. In order to reduce this biomass concentration even further either less biomass or a higher volume should be employed. The former will increase the weighing error and will also reduce the portion of the sample analysed which might then affect the reproducibility of the analysis as discussed in section 3.3.4. The latter could be employed to a certain extent, as long as the volume is fully encased inside the thermoblock. However, as will also be shown in section 3.3.4, an increase in volume reduced the glucose concentration obtained.

The effect of acid concentration and hydrolysis time can be visualised in the surface response graph represented in Fig. 3.2, where the third variable tested, the biomass concentration, was fixed at the optimum predicted for all models (i.e. 1 mg.mL^{-1}).

The acid concentration had a positive effect on the extraction of glycogen for all cultures, especially for lower hydrolysis periods. In fact, an interaction effect between acid concentration and duration of hydrolysis was observed (Fig. 3.2): the higher the acid concentration, the lower the hydrolysis time needed. Actually, increasing the hydrolysis time beyond its optimum value might even result in a decrease in the glycogen concentration. The inverse is not necessarily true, meaning that lowering the acid concentration cannot be compensated by increasing the hydrolysis time. Therefore, a higher acid concentration is essential to obtain maximum glucose extraction.

Since the optimal acid concentration indicated by the models (0.9 M) was also the maximum concentration tested, an additional experiment was carried out to test higher

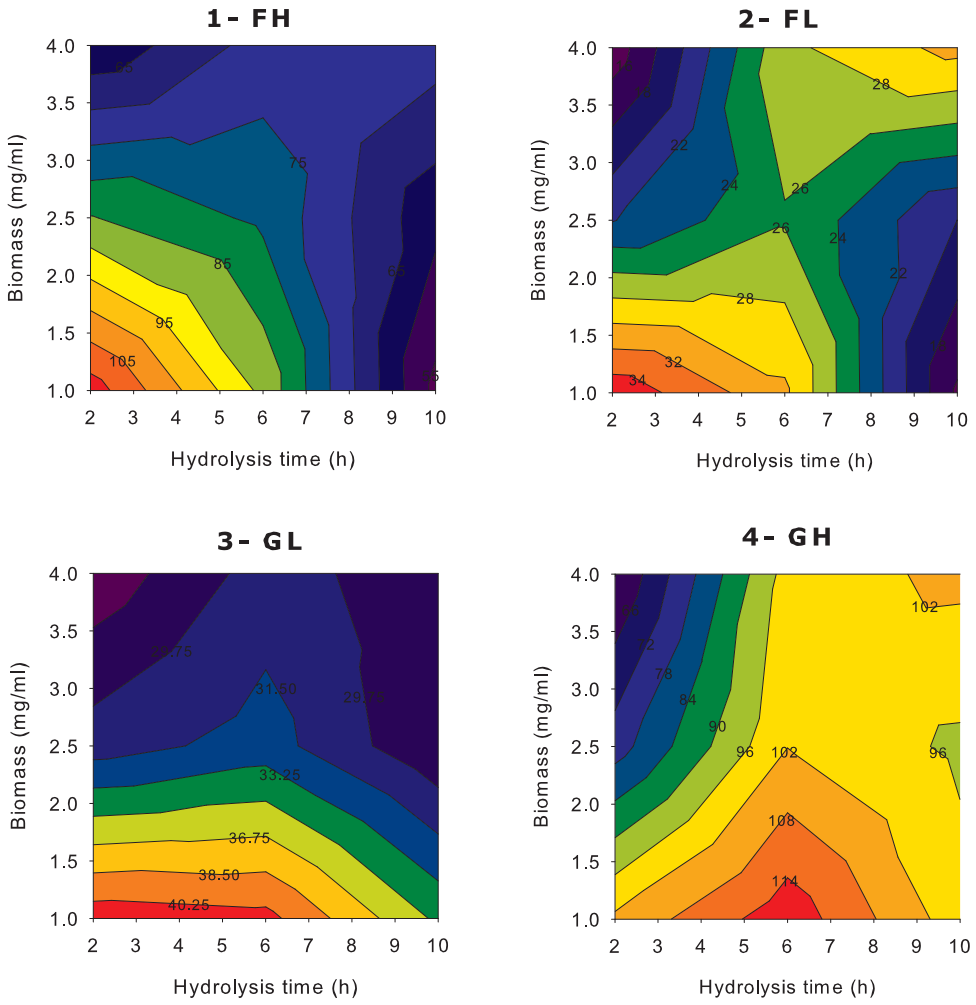

Figure 3.1: Surface contour plots indicating the models' prediction of the glucose concentration per biomass (mg.gTS^{-1}; shown in the colour gradients and values in boxes) using different biomass concentrations and different hydrolysis times with the acid concentration fixed at 0.9 M HCl

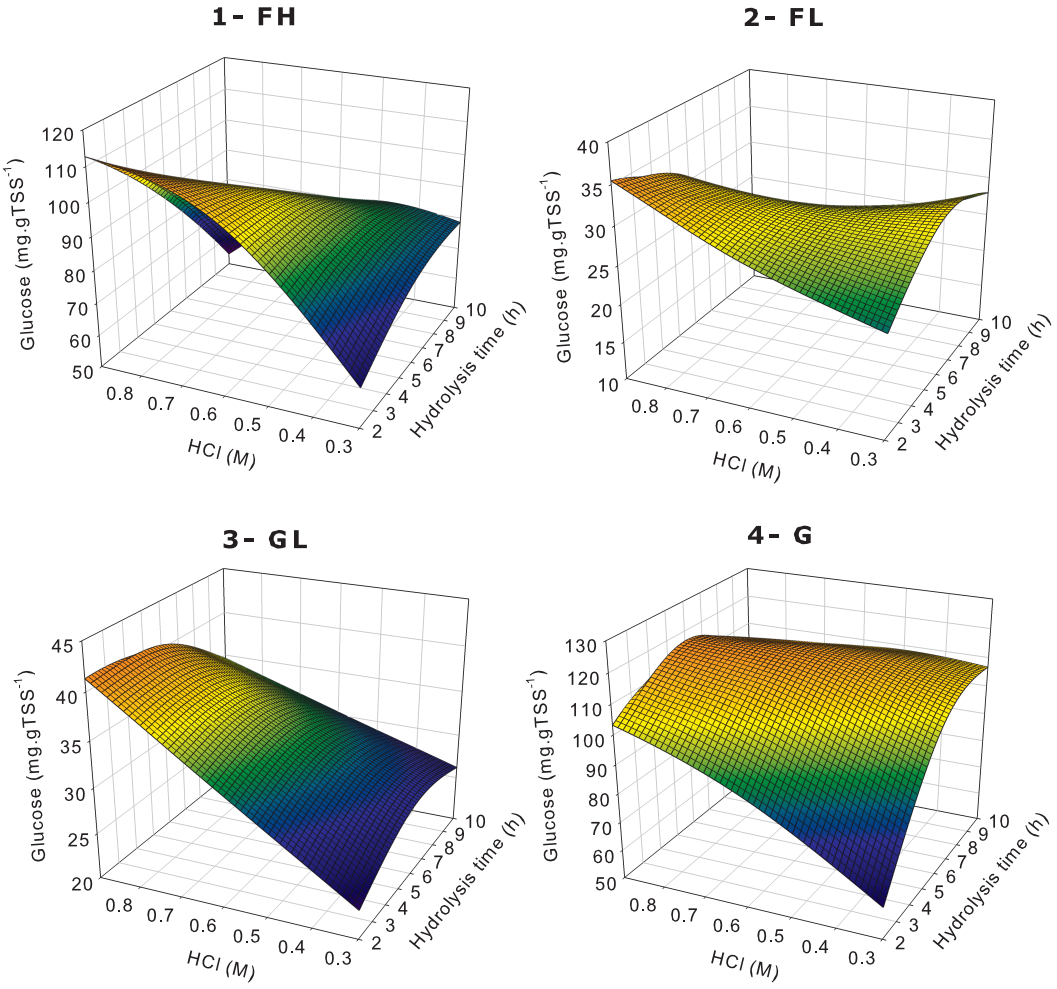

Figure 3.2: Surface response graphs of the glucose extracted for each microbial culture at different acid concentrations and hydrolysis times; the biomass parameter was fixed at the optimum predicted by the model (1 mg.mL^{-1})

acidic concentrations of 1.2 M and 1.5 M (results not shown). Both of these concentrations led to a poor resolution in the HPLC analysis. Therefore, 0.9 M was chosen as the optimal acid concentration when using the HPLC analytical method chosen in this study.

The duration of the hydrolysis was the only factor whose optimum differed among the microbial cultures. The models for floccular cultures indicated that the minimum hydrolysis time tested (i.e., 2 h) already leads to the maximum recovery of glycogen, whereas the models for cultures containing granules indicated the need for a longer hydrolysis time (>3 h). However, the optimum method for the 3-GL culture required considerably less hydrolysis time than the 4-GH culture (3 h and 6 h, respectively). The longer hydrolysis time for 4-GH could have been due to the higher glycogen concentration or to differences in the granule's structure resulting in different mass-transfer rates.

3.3.1.3 Glycogen extraction and hydrolysis kinetics

In order to confirm whether the glycogen content affected the optimum digestion time, the glycogen hydrolysis was followed over time using microbial cultures with high and low glycogen content (1-FH and 2-FL), as well as glycogen from bovine liver (type IX, Sigma, USA) as control (1 $g.L^{-1}$). The experiment was performed using the optimum conditions predicted by the models, namely 0.9 M HCl and 1 $mg.mL^{-1}$ for the biomass concentration. In order to determine the kinetic equation of each reaction, the experimental results were handled using the Euler method. Each set of results fitted Eq. 3.6 with an R^2 of 0.97, 0.95 and 0.91 for 1-FH and 2-FL cultures and glycogen from bovine liver respectively, thus, indicating that the hydrolysis rate followed a similar first order kinetic profile for the microbial cultures and the pure glycogen. Therefore, the hydrolysis rate is linearly dependent on the concentration of glycogen: the higher the glycogen concentration, the faster the hydrolysis (c.f. Fig. 3.3).

$$\frac{dq}{dt} = -0.022q \tag{3.6}$$

Even though the rate of hydrolysis is directly proportional to the concentration of glycogen, a full hydrolysis can be defined as the time when a certain threshold (i.e., extraction of 90% of total glycogen) is extracted, hence, this time is independent of the initial glycogen concentration. According to this model, 104 min was the minimum time needed for the hydrolysis of 90% of the initial glycogen for all three examples tested. This result also validated the optimum hydrolysis time obtained by the design of experiments where, according to this kinetic model, 2 h would be sufficient for a 93% recovery of the total glycogen content.

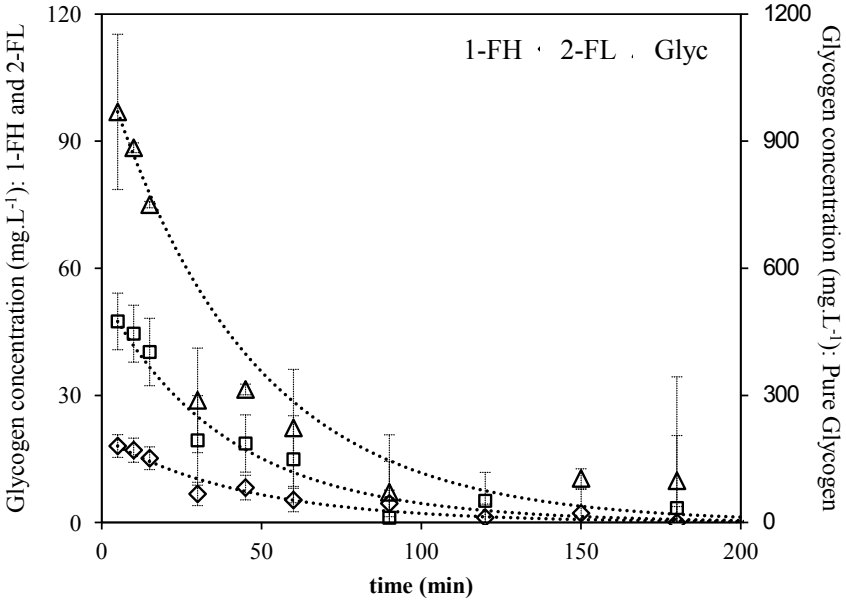

Figure 3.3: Glycogen hydrolysis profile for a high (1-FH) and low (2-FL) glycogen content microbial culture compared with the hydrolysis curve of pure glycogen from bovine liver at 0.9 M HCl

3.3.1.4 Cell aggregation differences

Since the concentration of glycogen does not affect the hydrolysis time, the other viable hypothesis to explain differences in the optimum hydrolysis period for floccular and granular biomasses is related to cell aggregation which would hinder cell lysis or permeation of acid inside the cell. In order to assess this hypothesis, samples of each microbial culture were observed under a stereomicroscope to evaluate differences in cell agglomeration structures. Whereas cultures 1-FH and 2-FL present relatively homogeneous floccular structures, culture 3-GL reveals a mixture of heterogeneous flocs and granules in variable sizes and culture 4-GH is mainly composed of small sized granules (Fig.3.4). This suggested that the complexity and the compaction in cell aggregation are directly related with the need for a longer hydrolysis time.

3.3.2 Models for floccular and granular biomass

The main goal of this study was to contribute towards a glycogen analysis method that could be used universally. However, since the cell aggregation state of the microbial cells was identified as affecting the method's efficiency, the possibility of developing separate models for floccular and granular cultures was investigated. Results for both of these models are shown in Table 3.3.

The values of R^2 for both models were 0.85 and 0.79, which are acceptable when

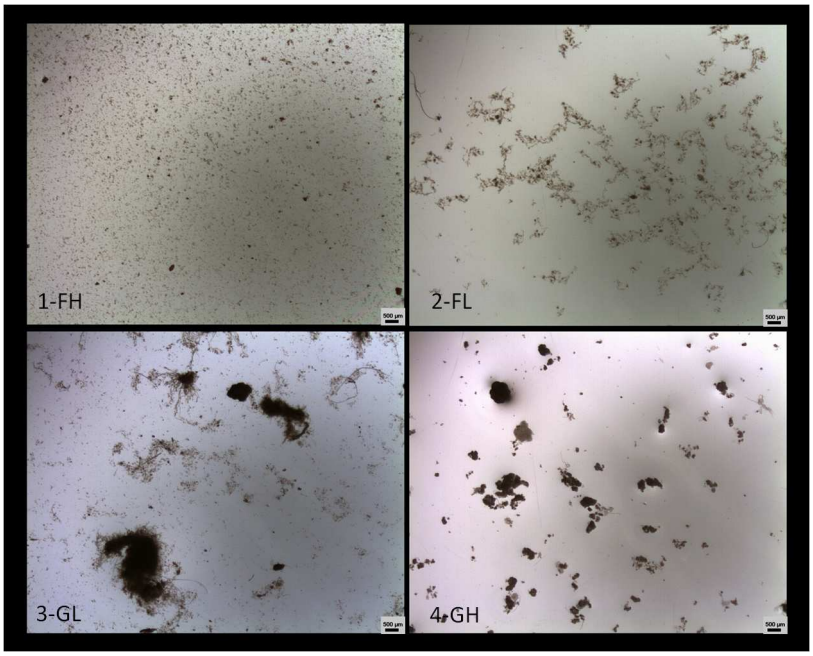

Figure 3.4: Stereomicroscopic images of each microbial culture tested (bar corresponds to 500 μm)

applied to biological cultures. Both models are statistically acceptable according to the F-test (p-value) and by showing no lack of fit (LOF).

As expected, these models indicate an optimum hydrolysis time of 2 h for the floccular cultures and a hydrolysis time of 4.7 h for granular cultures. If both granular microbial cultures were to use this method instead of their optimised methods, the values obtained would differ by less than 2% (Table 3.4). This indicated that, according to the type of cell aggregation of the microbial culture to be analysed, it is reliable to use shorter hydrolysis periods for mostly floccular cultures, i.e. 2 h and longer digestion times for granular cultures, i.e. 5 h.

Table 3.4: Sensitivity analysis of the glucose concentration obtained for each microbial culture using the methods developed from the floccular, granular and global models, as well as methods from the literature

Glucose predicted (mg.gTS^{-1})	**1-FH**	**2-FL**	**3-GL**	**4-GH**
Individual model condts.	113.2	35.6	41	118.1
Floccular model condts. (0.9 M/2 h/1 mg.mL^{-1})	113.2	35.6	40.8	103.3
Granular model condts. (0.9 M/5 h/1 mg.mL^{-1})	98.8	33.5	40.5	116.9
Global model condts. (0.9 M/3 h/1 mg.mL^{-1})	108.4	35.4	41	110.1
Lit. method n^{o}1 - 0.6 M/1 h/5 mg.mL^{-1} (Smolders et al., 1994b)	79.4	14.6	20.6	51.8
Lit. method n^{o}2 - 0.6 M/2 h/2.5 mg.mL^{-1} (Carvalho et al., 2007)	97.6	26.3	29.4	82.8
Lit. method n^{o}3 - 0.6 M/6 h/4 mg.mL^{-1} (Guisasola et al., 2007)	94	26.8	24.7	104.4

3.3.3 Global Model

Although it was possible to identify cell aggregation as the main factor behind variable hydrolysis times needed for maximal glycogen extraction, it is not always possible to know before-hand what is the type of aggregation of the samples to analyse. Therefore, a global model was developed, using all of the experiments done for the four different types of microbial cultures (Table 3.3). This global model described the set of results within a 95% confidence interval since the p-value and LOF were lower and higher than 0.05 respectively and the R^2 value was higher than 0.7. As a result of the inclusion of two floccular, one semi-granular and one granular culture, the model predicts an optimum hydrolysis time of 3 h.

When comparing the models for each individual culture with the floccular, granular and global models, it was possible to analyse what would be the difference when using the optimised method for flocs or granules versus the global method (Table 3.4).

For all the cases, the glucose concentration obtained with the floccular or granular model was similar to the one obtained with the individual model i.e., within the replicate error. For cultures 1-FH, 2-FL and 4-GH, using the non-adequate model e.g., using the floccular model for the granular culture, resulted in losses equal or higher to the maximum replicate error in the range of 6 to 12%. So, when in doubt of the aggregation type, it is recommended to use the global method. However, for culture 3-GL, maximum glucose concentration results were very similar using either the individual, or any of the other models.

The concentration of glucose obtained using the global model method was within the error range for all cultures, showing that this method would work appropriately regardless of the type of biomass. Finally, according to the models, it was possible to determine the glucose concentrations for each of the cultures if the methods available in the literature had been used. Methods using 1 h hydrolysis (Literature method No.1) showed 30-60% lower results than the methods obtained in this study. Methods using 2 and 6 h (Literature method No.2 and No.3) also showed lower values, although with less of a difference (15-30%), since the hydrolysis time was equal or higher than the minimum value of 2 h obtained in this study. In particular, Literature method No.3 showed a low deviation for the 4-GH culture, since its conditions approximated the optimum ones determined for granular cultures.

3.3.4 Other relevant factors for practical implementation

Besides the effect of acid concentration, hydrolysis time and biomass concentration, as well as the type of aggregates in the system, other details might also be of interest. The

volume of the acid solution could be important to control, but these considerations depend on which experimental setup is available in the lab, namely the type of heating-block used and the maximum volume allowed in each tube. The level of the liquid must be fully encased inside the heating-block in order to assure complete thermal homogeneity. As an example, three experiments were conducted using 16 replicate samples in each experiment where, while maintaining a constant biomass concentration, the reaction volume was increased from 2 to 5 to 8 mL. Even though both 2 mL and 5 mL volumes were still encased inside the heating-block, the glycogen content decreased 12% with each of the increases in volume. Therefore, it is recommended that hydrolysis volumes should be kept low to minimise temperature gradients inside the tubes.

An important reference should be made to the high replicate errors obtained for the glycogen analysis (8-17%). The heterogeneity of samples is a factor that may influence greatly the error of glycogen analysis in environmental samples (Apostolides et al., 1981). This was observed in this study, since the replicate error increased in accordance with sample heterogeneity, with the highest error obtained for biomass 3-GL, which was composed of a mixture of flocs and granules. If the composition is heterogeneous, in terms of its aggregation mechanisms, the sample composition captured in a 1-2mg portion will have some variability, therefore reducing its reproducibility, especially since, from the results presented here, there is a great difference in the parameters needed for floccular and granular biomasses. Further investigation of these factors should still be undertaken to fully understand their impact on glycogen quantification and provide solutions to minimise their occurence.

3.4 CONCLUSIONS

Glycogen analysis is sensitive to acid concentration, hydrolysis time and biomass concentration. It is proposed that a new method should include using 0.9 M acid and 1 $mg.mL^{-1}$ of biomass. The time of the hydrolysis should be adapted according to the granulation type of the culture: 2 h for floccular cultures, 5 h for granular cultures and an intermediate value of 3 h when the aggregation state is unknown or dynamic.

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4 Factors impacting on polyhydroxyalkanoate (PHA) quantification in mixed microbial cultures

Summary *Polyhydroxyalkanoate (PHA) is a polymer that has gained much interest due to its biodegradability, biocompatibility and its role in environmental microbial cultures. Through the years, many studies have referenced different conditions for the acidic methanolysis quantification method. However, although some studies have addressed the optimisation of this method, there has not been a systematic approach towards understanding the mechanisms leading to an optimal PHA quantification in different microbial cultures and for different PHA monomers. This study used independent experiments as well as a design of experiments approach to determine the effect on PHA quantification of acid concentration, hydrolysis time and biomass concentration during the hydrolysis. Experiments were carried out in six different mixed microbial cultures from different systems: from full-scale activated sludge systems to specific PHA accumulating cultures, containing high and low PHA fractions and from floccular to granular sludge. Results indicated that the overall hydrolysis kinetics was limited by the kinetics of the PHA hydrolysis in floccular cultures, whereas in granular cultures, it was limited by the cell lysis step. The monomeric composition of the polymer also has an impact on the hydrolysis rate, therefore a higher acid concentration and a longer hydrolysis should be employed when quantifying monomers with more substituents. The biomass concentration used should be between 3 and 10 mg/mL in order to obtain the maximum monomer recovery.*

The contents of this chapter were adapted from the publication: Lanham, AB; Ricardo, AR; Albuquerque, MGE; Pardelha, F; Carvalheira, M; Coma, M; Fradinho, J; Carvalho, G; Oehmen, A; Reis, MAM. 2012. Analysis of the factors impacting on polyhydroxyalkanoate (PHA) quantification in mixed microbial cultures. Appl Microbiol Biotechnol. submitted

4.1 INTRODUCTION

Polyhydroxyalkanoate (PHA) are polymers composed by a family of polyester monomers which include over 110 different molecules with varying backbone length from 4 to 16 carbons (Rijk et al., 2005). PHA synthesis from an external carbon source has been identified in a great number of different bacteria, who utilise this polymer as a carbon, energy and reducing equivalents storage product when facing limiting conditions (e.g. nutrients or oxygen) (Lee, 1996; Loosdrecht et al., 1997). PHA is accumulated inside the bacterial cells as discrete granules that are often composed of different monomers, forming a co-polymer, e.g., poly-(3-hydroxybutyrate-co-3-hydroxyvalerate).

PHA can largely contribute as a biodegradable and renewable alternative to replace the fossil-produced polyesters that currently hold the majority of the plastics market. Using pure or mixed cultures fed on waste material or industrial by-products, several authors were able to show the feasibility of producing a new generation of more sustainable and biocompatible plastics (Reis et al., 2003).

Additionally, PHA also plays a significant role as a storage polymer in environmentally engineered processes (Loosdrecht et al., 1997). Due to the dynamic conditions of these processes, either purposely imposed or due to variations in climate, flows or influent composition, they often favour the selection of organisms that are able to take advantage of storage molecules in order to survive. Therefore, PHA has for a long time been included in biological wastewater treatment models (Smolders et al., 1994b) and is often a key compound when addressing the metabolism of bacteria present in such systems (Mino et al., 1998; Oehmen et al., 2007).

The method that is most widely used for PHA quantification in bacterial systems was developed by Braunegg et al. (1978) for a polyhydroxybutyrate (PHB) producing pure culture, *Alcaligenes eutrophus*. He proposed using an acidic methanolysis reaction (3% v/v sulphuric acid) at 100°C for 3.5 h. The combination of acid and temperature will induce cell lysis, so as to allow access to the polymer, and the combination of acid with the methanol allows for the polymer's hydrolysis and conversion into methyl-ester monomers. The resulting monomers are then analysed by gas chromatography (GC).

Since the design of the initial method, several modifications and improvements have optimised the length of the hydrolysis, the acid concentration needed, as well as the type of sample preparation (Apostolides et al., 1981; Baetens et al., 2002; Comeau et al., 1988; Huijberts et al., 1994; Jan et al., 1995). In particular, one important modification has been the adaptation of the initial method, designed for pure cultures, to complex systems, such as mixed microbial cultures (Apostolides et al., 1981; Jan et al., 1995). In this case, microorganisms are not in suspension but form complex and heterogeneous structures, such as flocs or granules, aggregated due to polymers, such as exopolysaccharides. This situation could lead to a loss of reproducibility in the PHA analysis, as well as a more

difficult access of the acid and methanol to the PHA polymer. Comeau et al. (1988) further validated the method for polyhydroxyvalerate (PHV) and several authors followed in adapting the method for medium-chain length (MCL) monomers (Brandl et al., 1988; Gross et al., 1989; Lageveen et al., 1988; Oehmen et al., 2005a). A summary of the methods used by different authors can be found in Table 4.1.

The need to quantify PHA in different systems containing different PHA compositions, as summarised in Table 4.1, from pure cultures producing only PHB as stated in Braunegg et al. (1978), to complex mixed cultures producing PHB, polyhydroxy-2-methylbutyrate (PH2MB), PHV, polyhydroxy-2-methylvalerate PH2MV and polyhydroxy-2-hexanoate PHHX as reported by e.g., Liu et al. (1996), has resulted in multiple variations of the initial PHA quantification method.

This work intended to resolve what are the impacting factors on PHA quantification, their specific effect on the different monomers and on different microbial cultures, in order to obtain a systematic and universal understanding of what is the best approach to quantify PHA in different systems. Although the main focus was on the acidic methanolysis method, major findings on the factors affecting biomass and polymer hydrolysis could also be transferable to other methods using an acidic alcoholysis method (e.g., Werker et al. (2008)). The effect of acid concentration, hydrolysis time and biomass concentration was first determined in detail for 2-4 cultures as well as in standards. Thereafter, to confirm the universality of the first results a systematic approach using design of experiments was conducted for six microbial cultures from different processes, with different microbial characteristics and producing a variety of PHA monomers from PHB and PHV to PH2MB and PH2MV. These cultures included PHA accumulating organisms (higher PHA content), activated sludge (lower PHA content) as well as floccular and granular systems. The impact of these factors on different microbial systems accumulating co-polymers with different compositions was determined, with the aim to help researchers choose the most suitable PHA quantification method.

4.2 MATERIALS AND METHODS

4.2.1 PHA quantification method

The PHA content in biomass was hydrolysed into methyl-ester monomers through a methanolysis reaction, followed by their analysis by gas chromatography. All microbial samples were centrifuged at 10 000 g, the supernatant was discarded and the pellet was freeze-dried over night. A precise amount of lyophilised biomass (error of 0.01 mg) was weighed into a Pyrex tube, where 1 mL of acidic methanol (3-20% sulphuric acid v/v) and 1 mL of chloroform were added. The chloroform solution contained 1 mg/mL

Table 4.1: Summary of the conditions for methanolysis used by several authors for PHA analysis[a]

PHA composition	System type	Biomass concentration (mg/mL $CHCl_3$)	Acid concentration (v/v %)	Hydrolysis time (h)	Reference
PHB	PC	n.s.	1-3	4	Braunegg et al. (1978)
PHB	MMC	25	3	3	Apostolides et al. (1981)
MCL PHA	PC	4	15	2.3	Brandl et al. (1988)
PHB, PHV	MMC	10	3	3.5	Comeau et al. (1988)
MCL PHA	PC	n.s.	15	2.3	Lageveen et al. (1988)
PHB and total PHA	PC	10	15	2-4	Huijberts et al. (1994)
PHB	PC	5.5	3	3	Jan et al. (1995)
PHB, PH2MB, PHV, PH2MV, PHHX	MMC	n.s.	15	2.3	Liu et al. (1996)
PHB, PHV	MMC	n.s.	3	20	Bond et al. (1999)
PHB, PHV	MMC	10	3	6	Zeng et al. (2003c)
PHB, PHV	PC	n.s.	15	2.3	Hai et al. (2004)
PHB, PHV, PH2MV	MMC	7.5	3	6	Pijuan et al. (2004a)
PHB	MMC	n.s.	20	3.5	Serafim et al. (2004)
PHB, PHV, PH2MV	MMC	10	3-10	2-20	Oehmen et al. (2005d)
PHB, PHV	MMC	10	3	3.5	Whang et al. (2006))
PHB, PHV, PH2MV	MMC	10	3	20	Zhou et al. (2008)
PHB, PHV	MMC	n.s.	20	3.5	Albuquerque et al. (2010)
PHB, PHV, PHHX	PC	n.s.	5	4	Li et al. (2011)
PHB, PHV, PHHX	PC	10	15	2.3	Ng et al. (2011)

[a]MCL - Medium-chain length; PC - pure culture; MMC - mixed microbial culture; PHHX - poly-3-hydroxyhexanoate; n.s. - not specified

heptadecane (Fluka) as internal standard. The tubes were sealed with an air tight Teflon-lined screw cap and incubated at 100°C in a dry-heat thermo-block for the necessary time (1-20 h). The tubes were then cooled on ice for 30 min. Water (0.5 mL) was added to aid the two phase separation and the phases were mixed using a vortex for 1 min. The lower phase, containing the chloroform, was extracted into a GC vial and dried using molecular sieves (4 Å, Prolabo) to remove traces of water.

2 μL of sample were injected in a Varian CP-3800 gas chromatograph (Varian, CA, USA) equipped with a FID detector and a ZB-WAX plus column (60 m, 0.53 mm internal diameter, 1 μm film thickness, Phenomenex, USA) coupled with a guard-column (0.32 mm internal diameter). Helium was used as a carrier gas, at constant pressure (14.5 psi). The temperature of injection was 280°C, the temperature of the detector was 230°C and the temperature ramp started at 40°C, increased at a rate of 20°C/min until 100°C, further increased at a rate of 3°C/min until 175°C and finally increased again at 20°C/min until 220°C, to ensure a cleaning step of the column after each injection.

A co-polymer of PHB-PHV (88:12 wt, Aldrich) was used as a standard for PHB, PHV and PH2MB, while 2-hydroxy-caproic acid (Aldrich) was used as a standard for PH2MV. Standards were processed in the same way as the samples, after being dissolved into a chloroform solution.

Results were presented as the response factor for each compound in terms of area of the selected peak (A), divided by the area of the internal standard peak (A_{is}) - A/A_{is}. This procedure corrected for error in the volume measurement in the reaction process as well as errors in the volume of injection in the gas chromatograph. The results were then normalised with the amount of biomass weighed. To find the concentration of polymer, the area of each peak, divided by the area of the internal standard, was calibrated using a 6 point calibration curve.

4.2.2 Microbial cultures tested

Six microbial cultures from different microbial systems and displaying different PHA accumulation capacities and compositions were selected for this study. These cultures result from ongoing work at the authors' laboratories.

The first and the second microbial cultures tested (MC1 and MC2) were from the same PHA producing culture, maintained in a 800 mL sequencing batch reactor (SBR), run in aerobic dynamic feeding conditions and fed with fermented sugar cane molasses. MC1 was obtained through a PHA accumulation batch test, in order to achieve a higher content of internal PHA (30% w/w). MC2 was withdrawn from the selection SBR and contained only 1% (w/w) of PHA. MC1 and MC2 contained a co-polymer composed of mainly PHB (72-78%) and PHV (22-26%). The third microbial culture (MC3), consisted of

a mixture of both bacteria and algae, obtained from a 4-L lab-scale SBR, operated under anaerobic and photosynthetic conditions. This PHA accumulating culture was fed with a synthetic wastewater, containing acetate as the carbon source. MC3 contained approximately 5% (w/w) PHA, mainly composed of PHB (97%) and a fraction of PHV (3%). The fourth microbial culture (MC4) was collected from a 2-L enhanced biological phosphorus removal (EBPR) SBR, operated under anaerobic-aerobic conditions, fed with synthetic wastewater containing 75% acetate and 25% propionate. Its microbial population was aggregated into a dynamic mixture of flocs and small-sized granules and consisted of a high enrichment in polyphosphate accumulating organisms. MC4 contained approximately 6% (w/w) PHA, mainly composed of PHB (57%) and PHV (32%) but also containing 3% PH2MB and 8% PH2MV. The fifth microbial culture (MC5) was obtained from a 30-L lab-scale SBR performing biological nutrient removal (BNR), operating under alternating anaerobic-anoxic-aerobic conditions, fed with domestic wastewater (Quart WWTP, Girona, Spain). The microbial population was aggregated in both flocs and granules. It contained a low amount of PHA (less than 1% w/w) composed of mainly PHB (66%) and PHV (29%), but also of small fractions of PH2MB (3%) and PH2MV (2%). The sixth microbial culture was sludge from a wastewater treatment plant (WWTP), sampled either from Aalborg West WWTP (Aalborg, Denmark), operated under a Biodenipho^{TM}system coupled with side-stream hydrolysis (MC6a), or from Beirolas WWTP (Lisbon, Portugal) and operated under sequential anaerobic, anoxic and aerobic conditions (MC6b). Both these cultures contained approximately 2% (w/w) PHA composed mainly of PHB (80%) and PHV (20%). MC6b also contained a small fraction of less than 3% of PH2MB and PH2MV.

4.2.3 Design of experiments and response surface modelling

4.2.3.1 Factor choice and design of experiments

This study used the Design of Experiments (DOE) tool to optimise the methanolysis reaction of different PHA monomers for 6 different microbial cultures in order to assess the conditions where full hydrolysis was achieved. The 3 main impacting factors chosen were the concentration of sulphuric acid in the methanol solution, the length of the hydrolysis time and the concentration of biomass in the chloroform phase used in the analysis.

According to the literature, and also shown in Table 4.1, the sulphuric acid concentrations used in most available methods ranged from 3% to 20% acid and therefore that was the range chosen for DOE. Concerning the length of the hydrolysis, most authors had proposed hydrolysis durations from 2 to 20 h (Table 4.1), however from preliminary tests, 3 h had already been established as the minimum for PHB analysis and therefore the interval chosen was between 3 and 20 h. Finally, the quantity of biomass used for

each assay is for most authors around 10 mg/mL (Table 4.1) and its optimum has not been discussed. Due to the author's experience, the range chosen was from 2 mg/mL up to 8 mg/mL $CHCl_3$.

In short, 6 DOE were conducted, one for each of the microbial cultures tested. Each DOE consisted of 15 experiments performed in duplicate, whose conditions were determined by varying the 3 input factors (acid, hydrolysis time and biomass quantity) to assess their impact on 2-4 output factors depending on the polymer composition in each culture (PHB, PH2MB, PHV, PH2MV). The output factors were introduced into the DOE as the area produced by each peak obtained in the chromatogram, divided by the area of the internal standard - A/A_{is} and normalised by the weight of the biomass pellet used. A full list of the 15 experiments is shown in Table 4.2.

Table 4.2: Description of the conditions of the experiments conducted for each microbial culture; each experiment was performed in duplicate

Exp. No.	Acid concentration (%)	Hydrolysis duration (h)	Biomass concentration (mg/mL $CHCl_3$)
1	3	3	2
2	11.5	11.5	2
3	3	3	8
4	3	20	2
5	20	3	2
6	11.5	11.5	8
7	11.5	3	5
8	20	3	8
9	3	20	8
10	20	20	2
11	3	11.5	5
12	20	20	8
13	20	11.5	5
14	11.5	20	5
15	11.5	11.5	5

For each microbial culture tested, the combination of factors and the number of experiments were determined using a central composite face-centred design with 3 levels, 1 central point and 1 replicate for each experiment Eriksson et al., 2008.

4.2.3.2 Model building

A similar approach to what was described in Section 3.2.1 was applied to the area of PHB, PHV, PH2MB and PH2MV and the best fitted model was determined.

4.3 RESULTS

4.3.1 The effect of the acid concentration

The impact of the acid concentration on the co-polymer hydrolysis kinetics was assessed by quantifying the different PHA monomers at increasing hydrolysis times. Two parallel hydrolysis curves were obtained using acidic methanol with 3 and 20% sulphuric acid (Figure 4.1) and approximately 3 mg/mL of biomass. The GC peak areas were normalised with the internal standard and the biomass weighed for each sample.

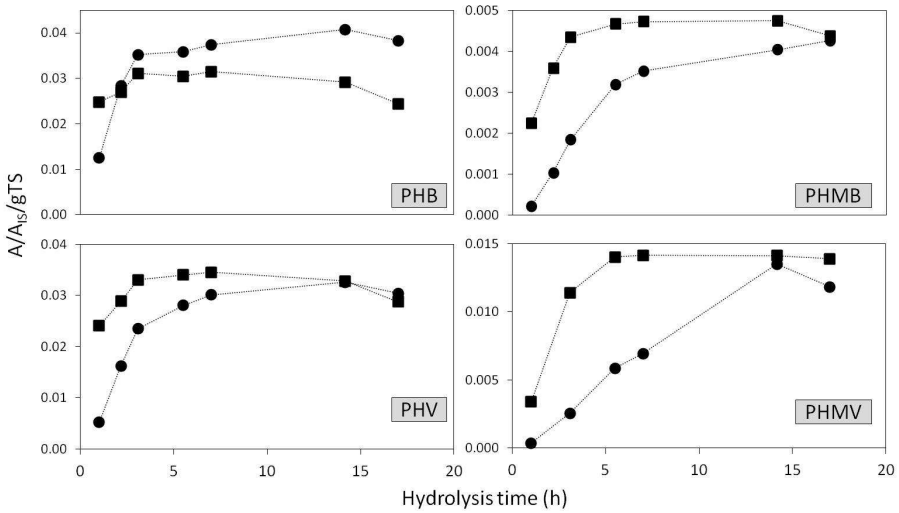

Figure 4.1: Quantification of each PHA monomer throughout the hydrolysis of MC4 samples using 3% (circles) and 20% (squares) acidic methanol. Peak areas presented were normalised with the internal standard and the biomass weighed for each sample (approx. 3 mg)

The hydrolysis rates increased from 2 to 4 fold when using 20% acidic methanol instead of 3%. For example, the PHB hydrolysis rate with 3% (43.2 A/(A_{is}.gTS.s)) doubled to 90 A/(A_{is}.gTS.s) at 20% acid and the rate of PH2MV increased 4-fold when using 20% (14.4 A/(A_{is}.gTS.s)) instead of 3% (3.6 A/(A_{is}.gTS.s)).

Also, for the same acid concentration, the hydrolysis rate varied depending on the type of monomer. Hence, at 3% acid, PHB and PHV had the lowest hydrolysis times, of approximately 3-4 h, while PH2MB and PH2MV required a much longer hydrolysis time of approximately 8 to 15 hours.

For PHB analysis, the peak areas obtained with 20% acid were approximately 20% lower than the ones obtained with 3% acid. A decrease in PHB peak areas, when increasing the acid concentration, was also observed for culture MC2, only containing PHB and PHV, in a similar set of experiments (results not shown). This effect was not observed for any of the other monomers, suggesting it was specific or more enhanced for PHB in comparison with other monomers.

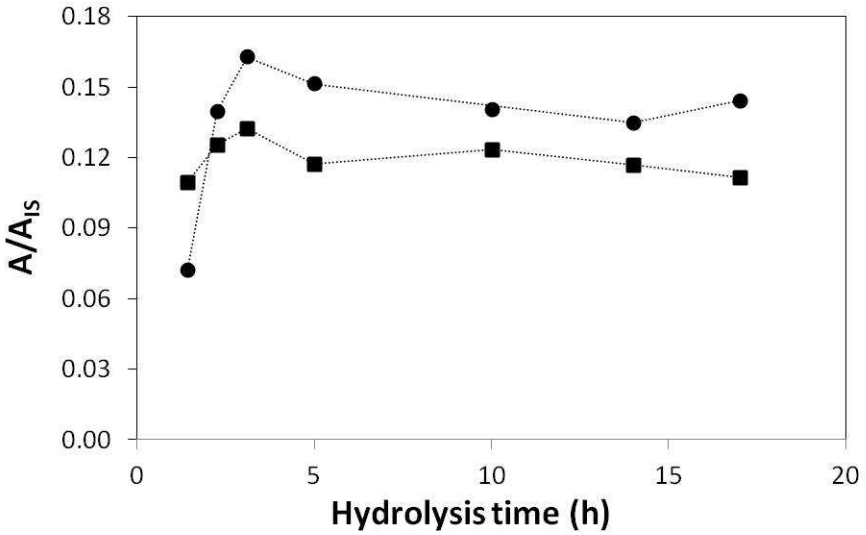

Figure 4.2: Quantification of PHB monomers in a PHB-PHV copolymer standard using 3% (circles) and 20% (squares) acidic methanol

When hydrolysing the co-polymer standards with acid concentrations of 3 and 20%, PHB, but not PHV, displayed the same effects observed in the sludge samples: a decrease in peak areas and an increase in the hydrolysis rate for higher acid concentrations, as shown in Figure 4.2. However, when calculating the ratio of the areas of PHB in the samples and in the standard ($A_{sample}/A_{standard}$) using 3 and 20% acid, the average of the values obtained for each time point was equal within the standard deviation (0.113 ± 0.008 and 0.102 ± 0.007 for MC2 and 0.25 ± 0.04 and 0.24 ± 0.03 for MC4 at 3 and 20% acid respectively). Concerning the hydrolysis rates, the difference observed for PHB standards hydrolysed using 3 or 20% acid was only approximately 30%, which was lower than what was observed for the sludge samples.

4.3.2 The effect of monomer composition and sludge structure on the hydrolysis rate of PHA

The profile of PHB, PHV, PH2MB and PH2MV hydrolysis rates were determined using 3% acid concentration in a PHA producing culture (MC2) and full-scale activated sludge (MC6a), as well as in standards. These results were compared with the ones already obtained for MC4 (Figure 4.3a-b). However, while MC2 and MC6a only contained a co-polymer of PHB:PHV, MC4 contained a tetra-polymer of PHB:PHV:PH2MB:PH2MV and therefore results are also shown at 20% acid concentration in comparison with the hydrolysis rate of the standard for PH2MV, caproic acid (Figure 4.3c).

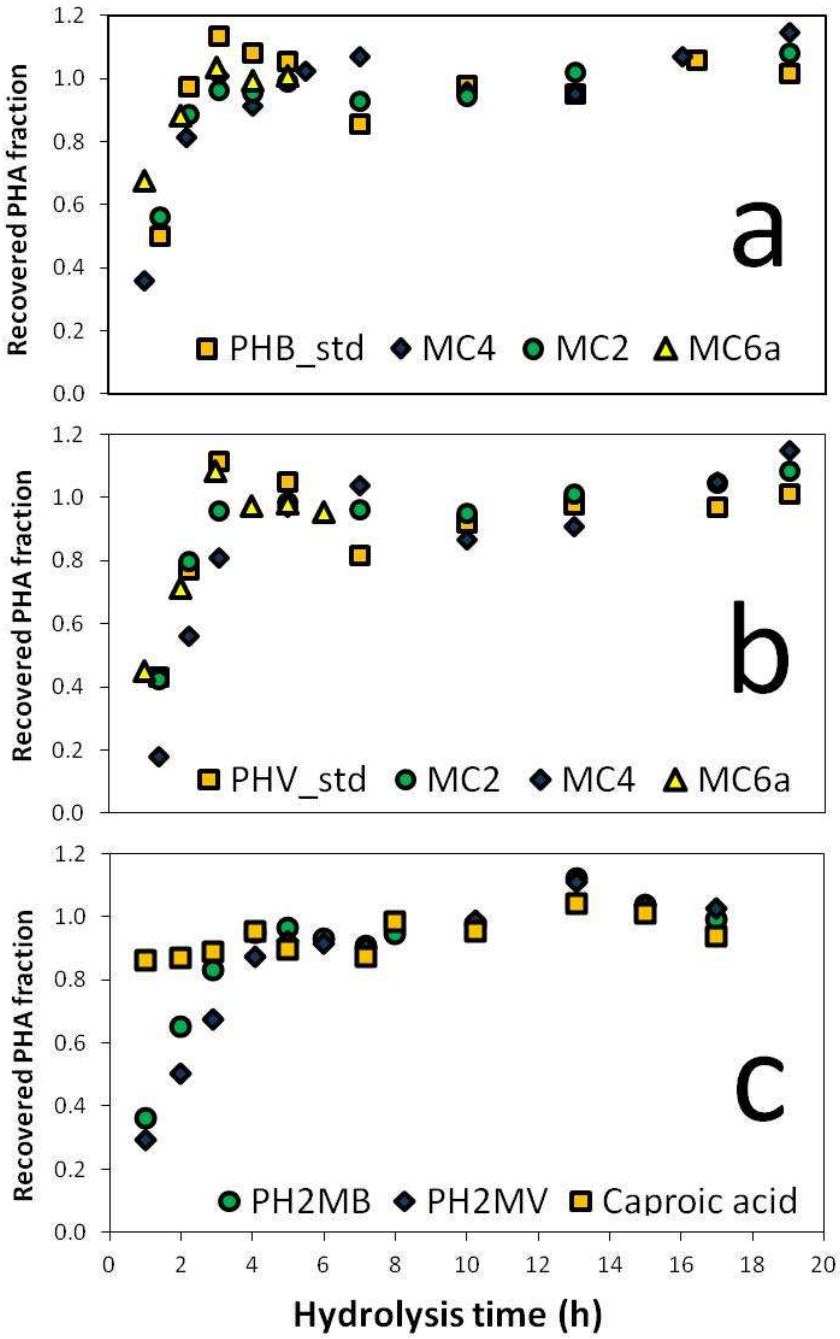

Figure 4.3: Hydrolysis kinetic profile for PHB, PHV, PH2MB and PH2MV monomers; a- PHB hydrolysis profile with 3% acidic methanol; b- PHV hydrolysis profile with 3% acidic methanol; c- PH2MB and PH2MV hydrolysis profile in MC4 with 20% acidic methanol; standards (std) - squares; for (a) and (b): MC2 - circles, MC4 - diamonds, MC6a - triangles; for (c): PH2MB - circles, PH2MV - squares

Table 4.3: Kinetic constants (k) and optimum hydrolysis times for PHA monomers in cultures MC2, MC4 and MC6a and for standards using 3% acid and 20% acid methanolysis

3% acid												
	PHB			PHV			PH2MB			PH2MV		
	k	R^2	t (h)	k	R^2	t (h)	k	R^2	t (h)	k	R^2	t (h)
Standard	0.88	0.89	2.6	0.71	0.88	3.2	-	-	-	-	-	-
MC2	0.88	0.98	2.6	0.77	0.95	3	-	-	-	-	-	-
MC4_1	0.64	0.93	3.6	0.41	0.95	5.7	0.22	0.81	10.3	0.15	0.83	15.7
MC6a	0.92	0.98	2.5	0.77	0.94	3	-	-	-	-	-	-
20% acid												
	PHB			PHV			PH2MB			PH2MV		
	k	R^2	t (h)	k	R^2	t (h)	k	R^2	t (h)	k	R^2	t (h)
MC4_1	2	0.95	1.1	1.5	0.96	1.6	0.95	0.95	2.4	0.52	0.99	4.4
MC4_2	1.2	0.92	1.9	1	0.98	2.3	0.58	0.93	4	0.41	0.97	5.6

The monomer concentration increased during the hydrolysis until reaching a maximum value. The profile of PHA recovered fraction for each microbial culture and standards tend to agree, indicating a common optimal hydrolysis time, except in the case of caproic acid which hydrolyses much faster at 20% acid concentration than PH2MB or PH2MV.

In order to further clarify the mechanism of the reaction, the kinetic constant, k, and the order of the reaction, n, were determined using the iterative Euler method programmed into Excel (Office 2007, Microsoft) and using the solver function. This method determined that the reactions were of first order (n=1). The kinetic constants for each culture, indicated in Table 4.3, decreased from PHB to PH2MV, i.e., $k_{PHB} > k_{PHV} > k_{PH2MB} > k_{PH2MV}$. This was verified for hydrolyses done in both acidic conditions, 3 and 20%. Furthermore, the effect of acid on the kinetics of the hydrolysis, already observed in the previous section, was confirmed since $k_{PHA}^{3\%} < k_{PHA}^{20\%}$ for all monomers in culture MC4.

The kinetic constant for PHB and PHV standards was similar to the kinetic constant of cultures MC2 and MC6a, but higher than the one of culture MC4 (Table 4.3). The lower kinetic constant for culture MC4, composed of granules and flocs, was confirmed in another experiment undertaken with a different MC4 sample (MC4_2). In fact, the MC4_2 sample was homogenised by mechanical shear to disrupt granules and the kinetic constant was even lower.

Using the kinetic constants, it was possible to predict the optimum time needed, at these conditions, to achieve at least 90% hydrolysis. The hydrolysis of PHB and PHV in standards and cultures MC2 and MC6a should be completed in between 2-3 h, when using a 3% acidic methanol solution. For MC4 a higher hydrolysis time, between 3-6 h, was needed. This hydrolysis time could be decreased to approximately 2 h for both MC4

samples if the acid concentration was increased to 20%. Results for PH2MB and PH2MV were only available for culture MC4 which determined that at 20% acid concentration a hydrolysis time between 3 and 6 h could be employed.

4.3.3 The effect of biomass concentration

The effect of the biomass concentration on the hydrolysis reaction was assessed using the optimum conditions for PHB analysis in cultures MC2 and MC6a (3 hours hydrolysis and 3% acidic methanol). Four different microbial cultures were tested: PHA accumulating organisms with higher and lower PHA concentrations (MC1 and MC2), a floccular lab-scale culture (MC3), and WWTP sludge (MC6a). Results, shown in Figure 4.4, reveal a similar trend for all the microbial cultures, where a 95% recovery of PHB was obtained for analyses using between approximately 3 to 10 mg/mL of biomass.

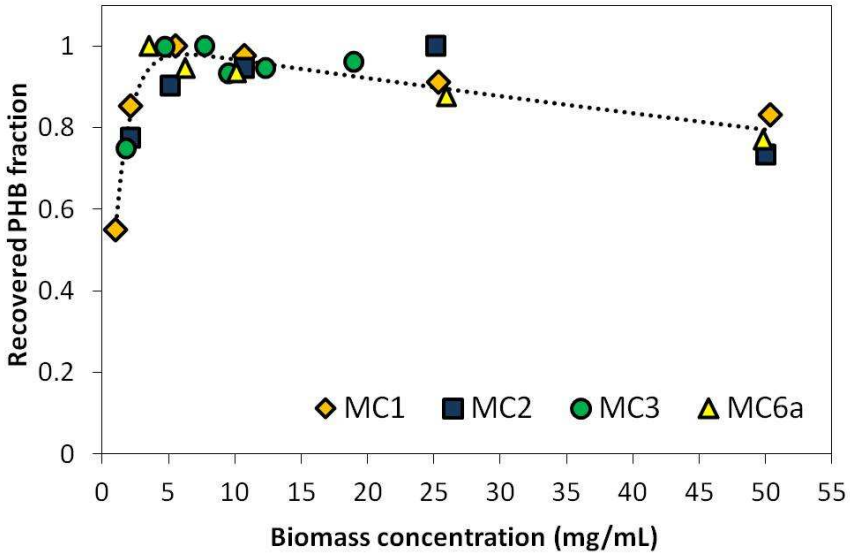

Figure 4.4: Effect of the biomass concentration on the PHB extraction for 4 different cultures (3%, 3 h): MC1 (diamonds), MC2 (squares), MC3 (circles), MC6a (triangles)

For biomass concentrations lower than 3 mg/mL, the PHB recovered fraction was only in the range of 60-80% of the maximum value. This was verified both for higher (MC1) and lower (MC2 and MC3) PHB concentrations. For biomass concentrations equal or higher than 10 mg/mL, the recovered fraction slowly decreased until approximately 80% at 50 mg/mL. The same effect was observed on the quantification of PHV in cultures MC1, MC2 and MC6a, suggesting that this effect was independent of the PHA monomer (results not shown).

4.3.4 Design of experiments (DOE)

In addition to the experimental results carried out for some microbial cultures, an experimental design strategy was conducted in six different microbial cultures, some of them already tested previously but using different sample batches: MC1-MC5 and MC6b. This allowed for a comprehensive confirmation of the trends observed previously.

A quadratic model was built for each microbial culture and for each individual monomer (PHB, PHV, PH2MB and PH2MV) which described well the results as indicated by the R^2 higher than 0.8, the p-value lower than 0.05 and the LOF value higher than 0.05 (cf. Table 4.4).

Table 4.4: Statistical parameters for the models determined using the DOE approach

	R^2	p-value	LOF value		R^2	p-value	LOF value
		PHB				**PHV**	
MC1	0.89	0	0.13	MC1	0.78	0.02	0.07
MC2	0.82	0	0.19	MC2	0.77	0.01	0
MC3	0.98	0	0.43	MC3	0.96	0	0.4
MC4	0.89	0	0.85	MC4	0.93	0	0.91
MC5	0.91	0	0.79	MC5	0.92	0	0.23
MC6b	0.85	0	0.82	MC6b	0.88	0	0.08
		PH2MB				**PH2MV**	
MC4	0.95	0	0.87	MC4	0.93	0	0.13
MC5	0.92	0	0.96	MC5	0.92	0	0.19
MC6b	0.89	0	0.89	MC6b	0.89	0.01	0.08

2 DOE conditions were not fulfilled: condition No. 10 and No. 12 (cf. Table 4.2), due to extraction difficulties for samples subjected to high hydrolysis periods and high acid concentration. Under these conditions, a greater instability between the two phases was observed, which reduced significantly the feasibility of the extraction step and the concentration of monomeric ester quantified. For this reason, although the models satisfied the statistical criteria established, they lacked sensitivity to accurately predict the optimum hydrolysis time for each microbial culture. The accuracy of the prediction for the optimum hydrolysis time decreased even further since a wide time interval was chosen from 3 to 20 h, in order to verify both PHB and PH2MV optimum conditions.

The model's coefficients indicated the effect and the weight of each parameter on the output result, in this case, the PHA monomer concentration. An average of the model coefficients for all cultures for each monomer is shown in Figure 4.5 with the corresponding error bars indicating their significance. Coefficients were only considered significant if the error bar was lower than the coefficient's value. The coefficients were normalised between -1 and 1 and therefore their values may be directly compared to each other. Positive coefficients imply a positive effect and negative coefficients, a negative contribution

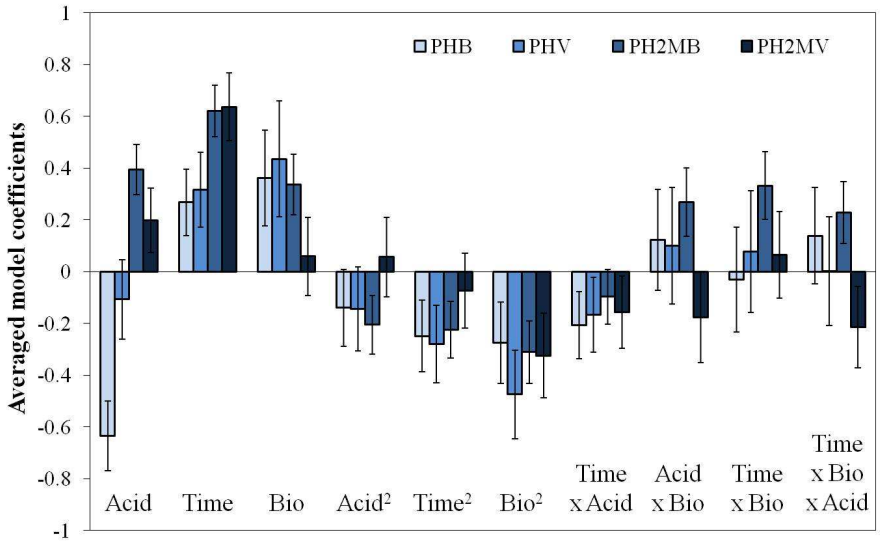

Figure 4.5: Averaged model coefficients for all the microbial cultures and for each PHA monomer. Error bars represent the significance of each coefficient therefore, coefficients with an error bar higher than the value itself were not considered significant to the model. PHB, PHV, PH2MB and PH2MV model coefficients are presented in increasingly darker shades of blue

to the PHA monomer concentration.

The most obvious aspects can be derived from the linear coefficients associated to acid concentration, hydrolysis time and biomass concentration, as separate parameters. Acid has a strong negative effect for PHB whereas it brings a moderate positive effect to PH2MB and PH2MV. The hydrolysis time brings a positive effect to all PHA monomers, but with higher values for PH2MB and PH2MV, as expected. The biomass coefficient was also positive and significant for all monomers except for PH2MV, meaning that higher biomass concentrations, in the tested range of 2-8 mg/mL, improved the recovery of PHA monomers.

The quadratic coefficients dictate the form of the quadratic curve obtained from the model. When considering normalised coefficients between -1 and 1, and considering a positive linear coefficient, a positive quadratic coefficient implies a curve with a positive growth, whereas a negative quadratic coefficient implies a curve with a negative growth. When the linear and the quadratic coefficients have the same sign, they reinforce either a positive or a negative effect. When they have opposite signs, the effect of the linear coefficient is cancelled and inversed for a certain value of the parameter equal to $-a_i/2a_{ii}$, where a_i and a_{ii} are the values of the linear and the quadratic coefficients for each parameter (cf. Equation 3.1).

The quadratic coefficient for acid was not significant for most monomers, except in the case of PH2MB, where it was slightly negative. Since the linear coefficient for acid

was moderately positive, the result was that the positive effect of acid was progressively diminished for higher concentrations of acid. The quadratic coefficient for time was moderately negative for all monomers except in the case of PH2MV. For PHB and PHV, since the linear coefficient was positive and in the same range as the quadratic one, this means that the overall positive effect of time was very slight and that a change in trend can be anticipated for long hydrolysis periods, as expected. For PH2MB, the linear coefficient was approximately 3 times higher than the quadratic one, therefore stressing the positive effect of this parameter and indicating that a turning point was not observed in the time interval chosen. The fact that PH2MV does not have a significant negative quadratic coefficient indicated that the positive linear effect was not reduced for the time interval chosen. The quadratic coefficient for biomass concentration was moderately negative for all monomers and in the same range as the linear coefficient. Hence, the biomass concentration had an overall slight positive effect, in the interval of 2 to 8 mg/mL, and this effect was diminished for higher biomass values.

The interaction coefficients, implicating 2 different variables at once, describe the synergistic effect that each variable has on the other. A positive interaction coefficient will further enhance an already positive variable. On the other hand, a negative interaction coefficient will moderate and eventually reduce the positive trend. Considering the acid x time coefficient, it was slightly negative for all monomers. Therefore, considering that the hydrolysis time has an overall positive effect for all monomers, this tendency will be moderated with the increase of the acid concentration. The other significant interaction coefficients only implicate PH2MB and apply to the time x biomass coefficient and to the acid x biomass coefficient, which are both moderately positive interactions. Considering the overall positive effect of time and acid on the hydrolysis of PH2MB, this positive effect was even more noticeable for higher biomass concentrations. This may be explained by the fact that the low concentrations of PH2MB observed (3% w/w, in cultures MC4, MC5 and MC6) will be more easily extracted not only at conditions using higher acid and time but also at conditions where the biomass content was higher in order to yield a detectable GC peak. However, the same effect was not noticeable for PH2MV.

During the design of experiments procedure, a total of approximately 160 samples were processed. All of these samples were performed in duplicate by a total of 7 different operators over a 2-week period. The average of the replicate error obtained for all samples was 5%. When comparing the replicate error for each biomass, there were no significant differences i.e., all errors are the same within the standard deviation (results not shown). When comparing the error from operators, also no significant differences were noted. However, when comparing the conditions of the samples, the ones where the biomass amount was 2 mg/mL had a higher error (7$\pm$ 2%) than samples with 5 or 8 mg/mL (4 $\pm$ 2%), likely due to a higher weighing error, or due to the small portion of sample used being not representative of the entire sample.

Table 4.5: Coefficients of the models obtained for each culture and for each monomer. Coefficients in light grey are not significant within a 95% confidence interval

			PHB			
	MC1	MC2	MC3	MC4	MC5	MC6b
Acid (a_1)	-1.1	-0.71	-0.34	-0.51	-0.51	-0.63
Hyd. Time (a_2)	0.31	0.16	0.14	0.46	0.38	0.17
Bio. (a_3)	-0.41	0.57	0.93	-0.28	0.71	0.65
Acid2 (a_{11})	-0.19	-0.22	-0.16	-0.1	-0.07	-0.11
Hyd Time2 (a_{22})	-0.23	-0.43	-0.15	-0.44	-0.06	-0.19
Bio2 (a_{33})	0.04	-0.12	-0.31	-0.52	-0.24	-0.5
Acid x Hyd. Time (a_{12})	0.11	-0.37	-0.1	-0.11	-0.31	-0.47
Acid x Bio (a_{13})	-0.12	0.22	0.03	-0.15	0.63	0.13
Hyd Time x Bio (a_{23})	-0.65	0.02	0.19	-0.31	0.34	0.22
Acid x Hyd Time x Bio (a_{123})	-0.76	0.42	0.2	-0.1	0.66	0.41
			PHV			
Acid (a_1)	-0.7	-0.02	0.08	0.06	-0.18	0.11
Hyd. Time (a_2)	0.08	0.35	0.34	0.55	0.43	0.17
Bio. (a_3)	-1.3	0.97	0.99	-0.06	0.99	1.02
Acid2 (a_{11})	-0.280	-0.33	0.06	-0.16	-0.1	-0.05
Hyd Time2 (a_{22})	-0.26	-0.25	-0.26	-0.64	-0.1	-0.17
Bio2 (a_{33})	-0.61	-0.27	-0.35	-0.59	-0.45	-0.57
Acid x Hyd. Time (a_{12})	-0.32	0	0.01	-0.16	-0.29	-0.25
Acid x Bio (a_{13})	-1.3	0.39	0.21	0.05	0.76	0.48
Hyd Time x Bio (a_{23})	-1.52	0.26	0.47	-0.02	0.62	0.66
Acid x Hyd Time x Bio (a_{123})	-1.64	0.4	0.19	-0.06	0.63	0.49
		PH2MB			**PH2MV**	
	MC4	MC5	MC6b	MC4	MC5	MC6b
Acid (a_1)	0.28	0.45	0.46	0.37	0.03	0.19
Hyd. Time (a_2)	0.7	0.77	0.4	0.77	0.63	0.5
Bio. (a_3)	-0.23	0.57	0.67	-0.37	0.31	0.23
Acid2 (a_{11})	-0.09	-0.23	-0.29	-0.19	0.04	0.31
Hyd Time2 (a_{22})	-0.49	0.04	-0.22	-0.4	0.2	-0.03
Bio2 (a_{33})	-0.49	-0.12	-0.33	0.12	-0.29	-0.81
Acid x Hyd. Time (a_{12})	-0.03	-0.09	-0.17	-0.2	-0.22	-0.05
Acid x Bio (a_{13})	-0.12	0.65	0.28	-0.48	-0.14	0.09
Hyd Time x Bio (a_{23})	-0.15	0.58	0.56	0.37	0.03	0.19
Acid x Hyd Time x Bio (a_{123})	-0.11	0.53	0.27	0.77	0.63	0.5

4.4 DISCUSSION

4.4.1 The overall effect of each parameter on the quantification of PHA

Acid concentration, time of hydrolysis and biomass concentration had an effect on the PHA hydrolysis rate and quantification. The results from the hydrolysis profiles, performed on only some cultures, were consistent with the results from the DOE.

The biomass concentration limited the recovered fraction of PHB and PHV in values lower than 3 mg/mL, which was supported by the DOE results since they indicated that in the 2-8 mg/mL tested range, biomass concentration had a positive effect, moderated towards higher values. For biomass concentrations higher than 10 mg/mL, the recovered PHA fraction starts to decrease suggesting that there was an incomplete hydrolysis. Since saturation was not observed for calibration curves, the incomplete hydrolysis could be related to excess of biomass. In fact, the resulting chromatograms had an increase in the number of peaks, intensifying the noise in the analysis and therefore hindering the peak's resolution.

Although an increase in acid concentration had a negative effect on PHB recovery, it increased the hydrolysis rates of all monomers, which could be particularly useful in the determination of PH2MB and PH2MV, since they possess a considerably slower kinetics than PHB and PHV. The DOE results showed a positive impact of time on all monomers, however, the combined effect of time and acid was slightly negative, suggesting that finding the optimum conditions for each monomer implies finding a compromise between the acid concentration and the hydrolysis time: the more acid, the less time needed or the reverse. The hydrolysis time should be sufficiently high to achieve a maximal conversion of the polymer into the monomers, but increasing the hydrolysis time further than the optimum point does not lead to a decrease in the monomer concentration, as was also shown in Huijberts et al. (1994).

Apart from the acid concentration and the hydrolysis time interaction, the different parameters, in general, do not have a synergistic effect, i.e., the interaction coefficients were not significant within a 95% confidence interval. This indicated that, if needed, each parameter can be further optimised for a particular system in an independent way.

4.4.2 Different cultures, different methods?

In the previous experimental sections, slower kinetics were found for MC4, in comparison with the kinetics of standards and even of other microbial cultures such as MC2 and MC6a. Culture MC4 has shown a dynamic behaviour in terms of the aggregation state of the biomass, alternating between floccular and granular phases. For instance, MC4_1 and the sample used in the DOE corresponded to a semi-granulation state, whereas MC4_2

corresponded to a complete granulated state.

While floccular cultures MC2 and MC6a showed a similar kinetic constant as the standards, indicating that the limiting reaction was the hydrolysis of the polymer, the granular culture MC4 displayed slower kinetics, indicating that the limiting mechanism could be cell disaggregation and lysis, likely indicating internal mass transfer limitations of acid within the granules. Additionally, the more granulated the sludge, the slower the kinetics, as shown by the slower kinetics of MC4_2 compared with MC4_1. This effect was also noticeable in the DOE results for cultures MC4 and MC5 (also semi-granular), which revealed the highest value for the hydrolysis time coefficients in the models of each monomer (results shown in Table 4.4). The effect of slower kinetics could be compensated by either increasing the hydrolysis time or by increasing the acid concentration. In fact, results shown in Figures 4.1 and 4.2 indicated that, when increasing the acid concentration from 3 to 20%, the hydrolysis rate increased more in culture MC4 than in the standards, confirming that the acid impacts not only the PHA hydrolysis kinetic constant, as shown in Table 4.3, but also the cell lysis/disaggregation mechanism. A difference in conditions for hydrolysing granular and floccular biomasses for glycogen quantification was also found in Chapter 3.

4.4.3 The combined analysis of different monomers

Results from the kinetic profiles indicated that the longer and the more substituents contained in the chain, the slower the polymer hydrolysis Table 4.3. Also, the DOE experiments revealed that acid concentration and hydrolysis time have a greater positive effect on PH2MB and PH2MV than on PHB and PHV, which suggested that these monomers require longer digestions at higher acid concentrations in order to achieve a complete hydrolysis. The acidic methanolysis reaction implies that the acid attacks the most hydrophilic part of the poly-ester molecule, which lies in the carboxylic group -COOH. With the increase in size and number of the substituents, e.g. from a methyl group in PHB (carbon 3), to an ethyl group in PHV (carbon 3) and to a methyl and an ethyl group in PH2MV (carbons 2 and 3, respectively), the hydrophobicity around the main carbon chain increases, which leads to a higher steric hindrance to the acid attack. The steric hindrance was even more accentuated in the case of PH2MB and PH2MV because of the added substituents in carbon 2, which are closer to the carboxyl group. Although scarce, literature results for higher chain-length monomers also indicated that a higher acid concentration or a higher hydrolysis time should be used (Brandl et al., 1988; Huijberts et al., 1994; Oehmen et al., 2005a). However, the reason for this had not been identified.

In terms of PHB, the negative impact of an increased acid concentration, observed in the hydrolysis profiles and in the DOE results, has been often discussed in the literature (Braunegg et al., 1978; Jan et al., 1995; Oehmen et al., 2005a). The acid effect on PHB has

been suggested to derive from its degradation into crotonic acid or other degradation products (Braunegg et al., 1978; Huijberts et al., 1994; Jan et al., 1995; Lageveen et al., 1988). However, both Jan et al. (1995) and Huijberts et al. (1994) have shown that no secondary hydrolysis products were formed in the reaction and Jan et al. (1995) suggested that this decrease in PHB recovery was due to a shift in the partition coefficient of HB between the chloroform and the methanol phase during the extraction procedure, most likely due to pH. Therefore, the quantification of this monomer should preferably use lower acid concentrations. However, this effect was equivalent in samples and in standards, as shown by the same values of the A_$sample$/$A_{standard}$ ratios at 3 and 20% acid concentrations. Hence, it can be corrected at higher acid concentrations, as long as the standards for the calibration curves have been subjected to the same hydrolysis conditions as the samples.

The time needed for achieving maximal PHB hydrolysis was in agreement with results found in literature. For PHB and PHV, most authors used a common method (Comeau et al., 1988; Oehmen et al., 2005a) of 3-4 h and 3% acid concentration (cf. Table 4.1). According to Table 4.3, these conditions would also be sufficient for the analysis of PHB and PHV in all standards and floccular cultures. Granular cultures might need a longer hydrolysis period, up to 6 h. Most of the results in the literature available for PHB analysis using 3% acid concentration are displayed in Figure 4.6, together with all the results obtained in this study, including the DOE results. It is interesting to note that the lower recovered fractions (<0.8), obtained in this study, for hydrolysis times of 3 h and using an acid concentration of 3%, were all either from granular cultures, from samples with low biomass concentrations (approx. 2mg/mL) or from both, as pointed out in Figure 4.6.

4.4.4 Choosing an optimised PHA quantification method - what to conclude

An optimised method should use the least resources (lowest acid concentrations) and be the least time consuming (shortest hydrolysis times), while achieving the highest polymer recovery. When developing a PHA quantification method, the type of biomass and the type of monomers analysed should be considered: the higher the complexity of the monomers and the number and size of granules, the longer the hydrolysis time and the higher the acid concentration. The kinetic perspective carried out in detail for only some cultures, combined with a statistical confirmation of the trends observed in 6 different microbial cultures pointed out the importance of choosing the appropriate conditions for PHA quantification in different systems. Floccular cultures containing PHB and PHV, which constitute the majority of the cases in mixed microbial cultures, can be analysed using a 3-h hydrolysis with 3% acid concentration. If the culture also contains PH2MB and PH2MV, the acid concentration should be increased to 20% and the samples should be hydrolysed at least 4 h. When dealing with granular biomass, the best option could

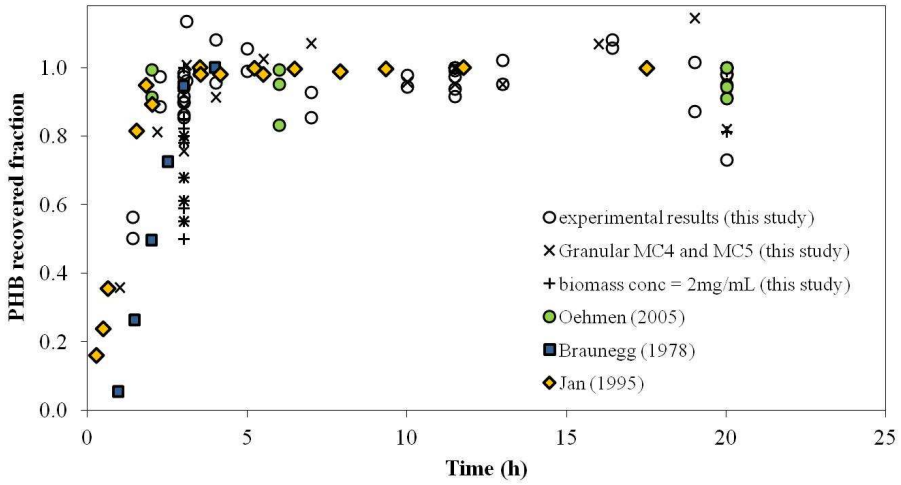

Figure 4.6: Hydrolysis kinetic profile for PHB using all the results collected during this study (empty circles crosses and plus signs), complemented with results from Braunegg et al. (1978) - pure culture (squares), Jan et al. (1995) - pure culture (diamonds) and Oehmen et al. (2005a) - EBPR floccular culture (circles), during a hydrolysis with 3% acid concentration. Experimental results from granular cultures (MC4 and MC5) (crosses) and using low biomass concentrations (approx. 2 mg/mL) (plus signs) were singled out with different markers.

be either to still use 3% acid and longer digestion periods, e.g., 6 h, or to increase the acid concentration. Using an acid concentration of 20%, the hydrolysis time could be increased to 4-6 h, particularly if the granular biomass also contains higher chain monomers such as PH2MB and PH2MV. The major mechanisms uncovered in this work impacting on the type of biomass and the PHA composition should be applicable for other acidic alcoholysis methods, however further work should provide this confirmation.

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5 Microbial and metabolic analysis of full-scale EBPR wastewater treatment plants

Summary *Enhanced biological phosphorus removal (EBPR) has been a widely used nutrient removal process in Denmark and is being introduced in Portuguese wastewater treatment plants. This study compared the microbial community and its performance in terms of EBPR of 3 Danish and 3 Portuguese WWTPs by using fluorescence in situ hybridisation (FISH) and by performing laboratory batch tests with acetate as the carbon source in anaerobic-anoxic-aerobic and anaerobic-aerobic conditions. This enabled distinguishing between the activity of polyphosphate accumulating organisms (PAOs) and denitrifying -PAOs (DPAOs). The microbial communities comprised Accumulibacter fractions of 3-6%. Tetrasphaera putative polyphosphate accumulating organisms (PAOs) were found in higher numbers ranging from 15 to 25%. Even though Portugal has a warmer climate, glycogen accumulating organisms (GAOs) were found only in one plant from each country, in numbers ranging from 3% in the Danish plant to 5-8% in the Portuguese plant, most likely due to selection factors other than temperature alone. The performance of the sludge with acetate indicated stoichiometric anaerobic yields concordant with different levels of utilisation of the TCA cycle and glycolysis, in different plants and at different sampling dates. All plants displayed anoxic PAO activity, although with different levels, which seemed to be inversely correlated with the amount of total nitrogen loaded to the plant.*

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5.1 INTRODUCTION

Enhanced biological phosphorus removal (EBPR) has been widely used in many wastewater treatment plants (WWTPs) as an efficient, economical and sustainable way to remove phosphorus from wastewater (Oehmen et al., 2007). The process relies on alternating anaerobic with aerobic and/or anoxic conditions. This strategy imposes a selection factor promoting the survival of organisms that are able to internally stock carbon- and energy-providing polymers, namely polyphosphate, glycogen and polyhydroxyalkanoate (PHA), through the uptake of carbon substrates, mainly volatile fatty acids (VFA), in anaerobic conditions (Oehmen et al., 2007). Two main groups of organisms have been identified: polyphosphate accumulating organisms (PAOs), characterised by their capacity of internally storing phosphate as polyphosphate, and glycogen accumulating organisms (GAOs), with a similar metabolism as PAOs but without the capacity to accumulate polyphosphate and hence negatively contributing to the overall phosphorus removal process. GAOs have been studied due to their competition with PAOs for carbon substrates leading to a deteriorated phosphorus removal capacity in EBPR plants (Oehmen et al., 2007; Seviour et al., 2000). The presence of GAOs has been correlated with several operational factors, such as pH, carbon source and temperature, as reviewed in Oehmen et al. (2007). In particular, it has been demonstrated that PAOs have a more advantageous metabolism at lower temperatures (10-20°C) and that GAOs become stronger competitors at higher temperatures (20-30°C) (Lopez-Vazquez et al., 2009b) and thus that they would be more likely to proliferate in warmer climates or seasons (Gu et al., 2008; Oehmen et al., 2007).

The most well known and studied PAO has been "*Candidatus* Accumulibacter phosphatis", a non-isolated organism (Crocetti et al., 2000). Other putative PAOs have also been reported, including organisms belonging to the *Tetrasphaera* genus, which were also shown to accumulate polyphosphate although it is still unknown which specific storage polymers are being used for phosphorus uptake (Nguyen et al., 2011).The main GAOs identified include "*Candidatus* Competibacter phosphatis" (Crocetti et al., 2002; Kong et al., 2002) and organisms related to *Defluviicoccus vanus* including clusters I, II and III (McIlroy et al., 2010; Meyer et al., 2006; Wong et al., 2004). The anaerobic metabolism of *Accumulibacter*-PAOs, in particular with acetate, has been well established. Acetate is taken up and transformed into PHA by using ATP generated by the hydrolysis of polyphosphate. However, the source of reducing equivalents necessary for the conversion of acetate to PHA has been the subject of several controversial findings, possibly indicating that the PAO anaerobic metabolism could vary according to external or internal factors such as glycogen storage levels or temperature (Zhou et al., 2010). Initial results suggested the anaerobic involvement of the tricarboxylic acid (TCA) cycle (Comeau et al., 1986; Wentzel et al., 1986) while others implicated the utilisation of glycogen as a source for reducing equivalents and energy (Mino et al., 1987). However, several findings have

indicated the co- existence of both, in lab-scale cultures (e.g., Hesselmann et al. (2000); Pereira et al. (1996) as well as in full-scale (Pijuan et al., 2008). Zhou et al. (2009) recently determined that when under glycogen limited conditions, a laboratory enrichment of PAOs utilised the TCA cycle instead of the glycolytic pathway.

Phosphorus uptake occurs both in aerobic conditions but also in anoxic conditions (Kerrn-Jespersen et al., 1993). A subset of PAOs or GAOs are also able to use nitrate and nitrite as an electron acceptor for growth and P uptake, usually referred to as denitrifying PAOs (DPAOs) or DGAOs. *Accumulibacter* type I (Carvalho et al., 2007; Flowers et al., 2009), *Competibacter* (Kong et al., 2006; Zeng et al., 2003b) and *D. vanus* cluster I related GAOs (Wang et al., 2008) have all been shown to denitrify. This fact has led to the optimisation of phosphorus removal also in anoxic conditions, which would be beneficial since it would lead to the simultaneous removal of phosphorus and nitrate using the same carbon requirements (Kuba et al., 1996a). However, the activity of DPAOs in full-scale systems, especially in light of the new findings for *Accumulibacter* type I, has not been well characterised yet.

While the identity and characteristics of the main organisms responsible for phosphorus removal activity have been characterised in laboratory experiments, investigations at the full-scale level are still few and present new challenges on how to deal with a greater number of parameters and a more complex and dynamic system. Some studies have addressed the microbial diversity in WWTPs (e.g., He et al. (2008); Nielsen et al. (2010); Wong et al. (2005)), while others have focused on the metabolic performance of these microbial communities (e.g., Gu et al. (2008); Lopez-Vazquez et al. (2008b); Pijuan et al. (2008); Tykesson et al. (2006)). However, a clearer understanding is still needed on how the microbial population, the operational parameters and the environmental conditions can be accurately linked to the performance of the EBPR process and further integrated in the rest of a WWTP's microbial community and operation.

This study intended to investigate and compare the microbial community and its EBPR activity in WWTPs in Portugal, a warm climate country, where the performance of EBPR plants has never been assessed before, and in Denmark, a cold climate country, where EBPR is well established. In each country, 3 EBPR plants with different operational modes were chosen and the main organisms known to date to be involved in the EBPR process were assessed using quantitative fluorescence *in situ* hybridisation (qFISH). The activity of the sludge from each plant was tested in anaerobic, aerobic and anoxic conditions in controlled batch tests. Based on the known metabolic capacities of PAOs, GAOs and DPAOs, their function and their preferential selective conditions, determined mostly in lab-scale studies, it was possible to investigate their occurrence in full-scale systems, verify their activity and correlate it to the operation of the plants. This work takes one step further towards the missing link between the knowledge obtained for simplified systems (well defined, laboratory scale) with the more complex and dynamic processes

that are occurring in real systems.

5.2 MATERIALS AND METHODS

5.2.1 Sampling campaign and WWTP characteristics

In order to assess and compare the EBPR activity in WWTPs in Portugal and in Denmark, 6 plants performing bio-P removal were chosen, 3 in Portugal and 3 in Denmark. In Portugal, the 3 WWTPs chosen included Beirolas WWTP (SIMTEJO, Lisbon), Setúbal WWTP (Águas do Sado, Setúbal), in the Lisbon region, and Carvoeiro (Águas do Algarve, Faro), in the south of Portugal. All 3 used a conventional bio-P configuration, including a sequence of anaerobic, anoxic and aerobic tanks (A2O). Beirolas was chosen as the main WWTP investigated, with 7 sampling dates, 5 in summer and 2 in winter, and Setúbal as the second WWTP with 4 sampling dates, 2 in summer and 2 in winter. The survey took place from June 2010 to March 2011 with two main sampling periods: one from June to September 2010 (summer sampling), where the average air temperature was 25°C (19°C minimum and 31°C maximum) and one in March 2011 (winter sampling) , where the average air temperature was 15°C (10°C minimum and 17°C maximum). The third Portuguese plant (PT_3) was sampled only for FISH since, although it operated under a biological phosphate removal configuration, it dealt with very stringent phosphate limits and therefore there was also a high addition of chemical precipitants which impaired the normal biological phosphate removal process, as observed in one lab experiment. For this reason this plant was not included further in the study. FISH results shown include only one sampling date in summer conditions. All WWTPs treat mainly domestic wastewater and Setúbal receives an important industrial contribution from a bread yeast factory which increased the COD load.

In Denmark, the 3 WWTPs chosen included Aalborg West and Aalborg East WWTP (Aalborg City) and Hjørring WWTP (Hjørring). The WWTPs in Aalborg used an adapted Biodenitro^{TM} configuration, which alternates in the same tank anoxic and aerobic conditions, coupled with a return-sludge side-stream hydrolysis process (RSS) that digests anaerobically approximately 20% of the return sludge with a residence time of 20-30 h and recirculates this fermented stream along with the influent to the head of the anoxic tank. Hjørring operated a conventional bio-P system, equivalent to the ones used in Portugal. Aalborg West was chosen as the main WWTP investigated, with 5 sampling dates. Aalborg East and Hjørring were sampled 3 and 2 times respectively. The Danish sampling campaign took place between October and November 2010 under Danish winter conditions with an average ambient temperature of 7°C (5°C minimum and 8°C maximum) and some precipitation. All Danish plants treated mainly domestic wastewater. A description of the main characteristics for each plant is listed in Table 5.1. From here on, for text simplification, the plants shall be called by their code name: PT for Portuguese

plants and DK for Danish plants. The values provided were obtained from the plant operators and correspond to averages of the available values during the year the campaign was conducted, i.e. May 2010 to May 2011. For each sampling date, sludge samples were collected from the end of the aerobic phase for the microbial characterisation and for the laboratorial batch tests. In Portuguese WWTPs, grab samples from all influent and effluent streams, as well as from each biological compartment were also collected.

5.2.2 Quantitative fluorescence *in situ* hybridisation

Quantitative fluorescence *in situ* hybridisation (FISH) was carried out according to Nielsen (2009) and Mielczarek et al. (2012). Sludge samples were collected for each sampling date from the end of the aerobic tank, fixed in 4% paraformaldehyde (PFA) for gram-negative bacteria and for ethanol gram-positive bacteria and stored at -18°C. Target organisms were quantified by their biovolume against the total bacterial biovolume as determined using a generic probe targeting all bacteria (EUBmix, containing a mixture of EUB338, EUB338II and EUB338III) (Amann et al., 1990; Daims et al., 1999). Specific oligonucleotide probes included PAO mix (PAO651, PAO462 and PAO846) targeting *Accumulibacter*-PAOs (Crocetti et al., 2000); Acc-I-444 and Acc-II-444 targeting type-I and type-II *Accumulibacter*-PAOs (Flowers et al., 2009); Tet2-174 (clade II), Tet1-266 (clade I), Tet3-654 (clade III) and Tet2-892 (clade II) targeting Tetraesphaera putative P-accumulating organisms (Nguyen et al., 2011); GAOmix (GAOQ431, GAOQ989 and GB_G2) targeting *Competibacter*-GAOs (Crocetti et al., 2002; Kong et al., 2002); DEF1mix (TFO_DF218 and TFO_DF618) targeting *Defluviicoccus vanus* related GAOs cluster I (Wong et al., 2004); DEF2mix (DF988 and DF1020) targeting *D. vanus* related GAOs cluster II (Meyer et al., 2006); DF1013 and DF1004 targeting phylotypes within cluster III *Defluviicoccus* (Nittami et al., 2009), indicated as putative GAOs (McIlroy et al., 2010). The results are presented as a percentage of all bacteria and given as an average of duplicate samples. A standard deviation of 20% is not shown in figures.

5.2.3 Batch tests

A 5-L sludge sample was taken from the end of the aerobic phase and stored at 4°C overnight. The sludge was diluted at a final concentration of approximately 3 g/L volatile suspended solids (VSS). The sludge was washed (3 x, mineral medium) and sparged with argon (Portugal) or nitrogen (Denmark) for at least 15 min to attain anaerobic conditions. The sludge was subjected to a sequence of anaerobic-aerobic or anaerobic-anoxic conditions (Figure 5.1) at pH 7.0 $\pm$ 0.2, 20 $\pm$ 1°C and with oxygen levels close to saturation. A subset of tests were also exposed to an aerobic period following the anoxic conditions (batch tests PT_1 n° 4, 5 and 7, PT_2 n° 1-4, DK_1 n° 2-4, DK_2 n° 1-2 and DK_3 n° 1). For batch tests performed in Denmark and for winter-Portuguese batch tests, a blank

Table 5.1: Summary of some of the main characteristics of the WWTPs investigated, the incoming influent and their treatment efficiency. All results presented constitute averaged values based on the information provided by the WWTPs for one year of operation at the time the sampling was conducted

			Wastewater treatment plants[a]				
	Main characteristics and parameters		**Beirolas (PT_1)**	**Setúbal (PT_2)**	**Aalborg West (DK_1)**	**Aalborg East (DK_2)**	**Hjørring (DK_3)**
Operational condts.	Layout		A_2O	A_2O	Biodenitro + RSS	Biodenitro + RSS	A_2O
	Average flow	10^3 m^3/day	48 ± 7	$12 \pm$ n/a	49 ± 8	17 ± 5	13 ± 6
	Chemical precipitation[b]		no	no	yes ($FeCl_3$)	yes ($FeCl_3$)	yes ($FeCl_3$)
	Typical pH		7.5 ± 0.2	7.5 ± 0.2	$7.0 \pm$ n/a	7.3 ± 0.1	6.96 ± 0.05
	Temperature range	°C	15-23	12-25	8-20	8-18	6-18
	SRT	d	12	5	19	30	43
Influent	Average COD	mg/L	253 ± 75	1032 ± 196	198 ± 36	626 ± 206	402 ± 272
	Suspended solids	mg/L	81 ± 23	292 ± 59	79 ± 19	182 ± 111	537 ± 467
	Average N	mg-N/L	43 ± 13	98 ± 15	30 ± 5	52 ± 14	37 ± 16
	Average NH3	mg-N/L	33 ± 10	68 ± 16	n/a	38 ± 8	18 ± 10
	Average Total P	mg-P/L	5 ± 0.9	5 ± 3	4 ± 1	10 ± 5	10 ± 6
	C:N:P	mg	55:10:01	204:19:01	50:07:01	64:05:01	40:04:01
Efficiency	COD removal	%	85	91	88	94	92
	N removal	%	64	75	77	91	86
	P removal	%	43	85	94	96	92
	NH3 removal	%	60	92	n/a	98	96
	Total P effluent	mg-P/L	2 ± 1	0.7 ± 0.9	0.2 ± 0.1	0.3 ± 0.2	0.8 ± 0.5
Sludge[c]	SS	g/L	2.9 ± 0.7	3.7 ± 0.8	4.3 ± 0.8	4.8 ± 0.5	5.0 ± 1.4
	PHA	mg-C/gTS	2.5 ± 1	4.8 ± 1.3	3.7 ± 0.3	3.1 ± 0.4	1.2 ± 0.8
	Glycogen	mg-C/g TS	4.3 ± 0.8	14.6 ± 1.6	6.1 ± 0.5	5.0 ± 0.3	6.3 ± 0.9

[a] SRT- Sludge retention time; COD - Chemical oxygen demand; SS - Suspended solids; TS - Total solids; RSS - Return-sludge side-stream hydrolysis; n/a - not available
[b] chemical precipitants were only added in Danish WWTPs as a polishing step for P removal
[c] averaged values for aerobic sludge grab samples collected and analysed in the laboratory

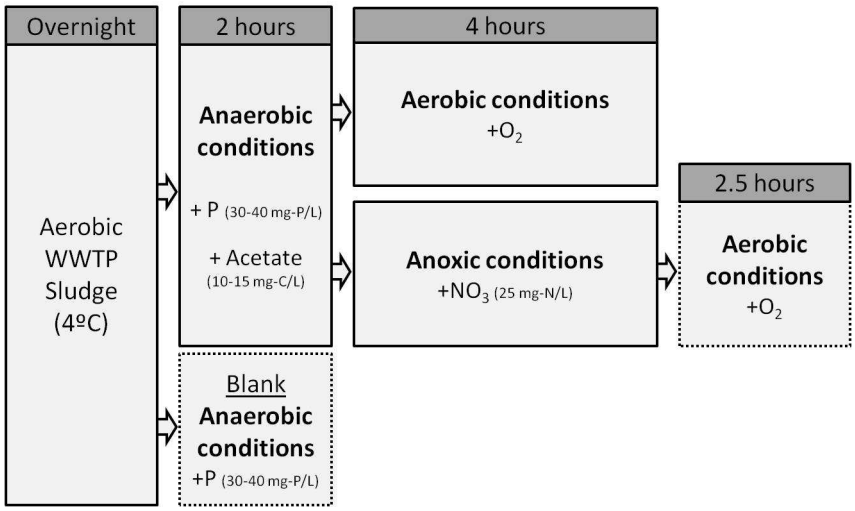

Figure 5.1: Sequence of batch tests performed on WWTP sludge; the blank anaerobic batch tests and the aerobic batch tests following the anoxic batch tests, marked in a dotted contour line, were not executed for all the sampling dates

test in anaerobic conditions was also carried out, where no acetate was provided.

Samples were periodically taken for analysis of acetate, phosphate, nitrate and ammonia concentration in the supernatant and to determine PHA, glycogen and total phosphorus in the biomass. Samples were also taken at the beginning and end of each phase for the determination of the mixed liquor suspended solids concentration and the volatile suspended solids (VSS). The mineral medium used was similar to the one used in Carvalho et al. (2007) without acetate nor phosphate. Each test commenced with the addition of an acetate (10-15 mg-C/L) and phosphate (30-40 mg-P/L) pulse. A pulse of approximately 25 mg-N/L of nitrate was added at the beginning of the anoxic phase. The concentration of the nutrients was optimised so that it would be possible to achieve in a 4-6 hour period the maximal utilisation of the PHA internal reserves of PAOs (or GAOs) as recommended in Oehmen et al. (2010c), obtained when a stabilisation of phosphorus levels was observed.

5.2.4 Chemical analysis

Samples for the analysis of acetate, phosphate, ammonia and nitrate or nitrite were filtered (0.2 μ m) and acidified (40 mM H_2SO_4). Samples for phosphate were stored at 4°C prior to analysis and samples for acetate, ammonia and nitrate or nitrite were stored at -18°C. Acetate and phosphate were analysed as described in Carvalho et al. (2007). Ammonia, nitrate and nitrite were analysed as described in Lanham et al. (2011). Samples for PHA and glycogen analysis were fixed for at least 1 h (8% formaldehyde), washed in 0.9% NaCl, then freeze-dried before analysis as described in Chapters 3 and 4. Total and volatile suspended solids (TSS and VSS) were analysed according to standard methods

(APHA et al., 1995).

5.2.5 Calculations

The chemical concentrations given in this study were calculated per C-mol of active biomass (X). The concentration of active biomass was calculated by subtracting the amount of glycogen and PHA from the VSS. The general biomass formula used was $CH_{1.84}O_{0.5}N_{0.19}$ as reported by Zeng et al. (2003d).

5.3 RESULTS AND DISCUSSION

5.3.1 Microbial composition of EBPR relevant bacteria

One of the goals of this study was to investigate the differences in microbial composition in Portuguese and Danish EBPR WWTPs. The microbial composition of Portuguese EBPR plants, as well as their efficiency in terms of P-removal, had never been assessed before.

5.3.1.1 PAO activity and the diversity within Accumulibacter

Accumulibacter was present in all plants (Figure 5.2). Some variation was seen within the range of 3.5 to 6%, with *Accumulibacter* marginally more abundant in DK_2. Greater variation was observed in the relative abundance of *Accumulibacter* Type I and II (Figure 5.3), where the abundance of Type I and Type II doubled or tripled in both PT_1 and PT_2 in winter time as compared to summer. A considerable fraction of the *Accumulibacter* was not targeted by either of the two sub-group probes, suggesting that there is an undescribed diversity of *Accumulibacter* that was widespread among the sampled plants or, more likely, that these probes did not target all Type I and II *Accumulibacter* in the plants. This had already been put forward by Flowers et al. (2009), since Acc-I-444 seemed to hybridise with some but not all of the Type I organisms and Acc-II-444 also did not hybridise with all the Type II organisms.

The total abundance of *Tetrasphaera*-PAOs was 3 to 5 times higher than the abundance of *Accumulibacter*, ranging from 15% in PT_2 to almost 30% in DK_2 (Figure 5.2). The abundance of clade I and II organisms within *Tetrasphaera* was moderately correlated (R^2 of 0.45-0.55), suggesting that the selection pressures existent in each plant have a similar effect on them. There was no correlation between the *Accumulibacter* abundance and the *Tetrasphaera* abundance, suggesting that the selection pressures for both groups of organisms were independent.

The different phosphate removal activities observed for the different plants, in terms

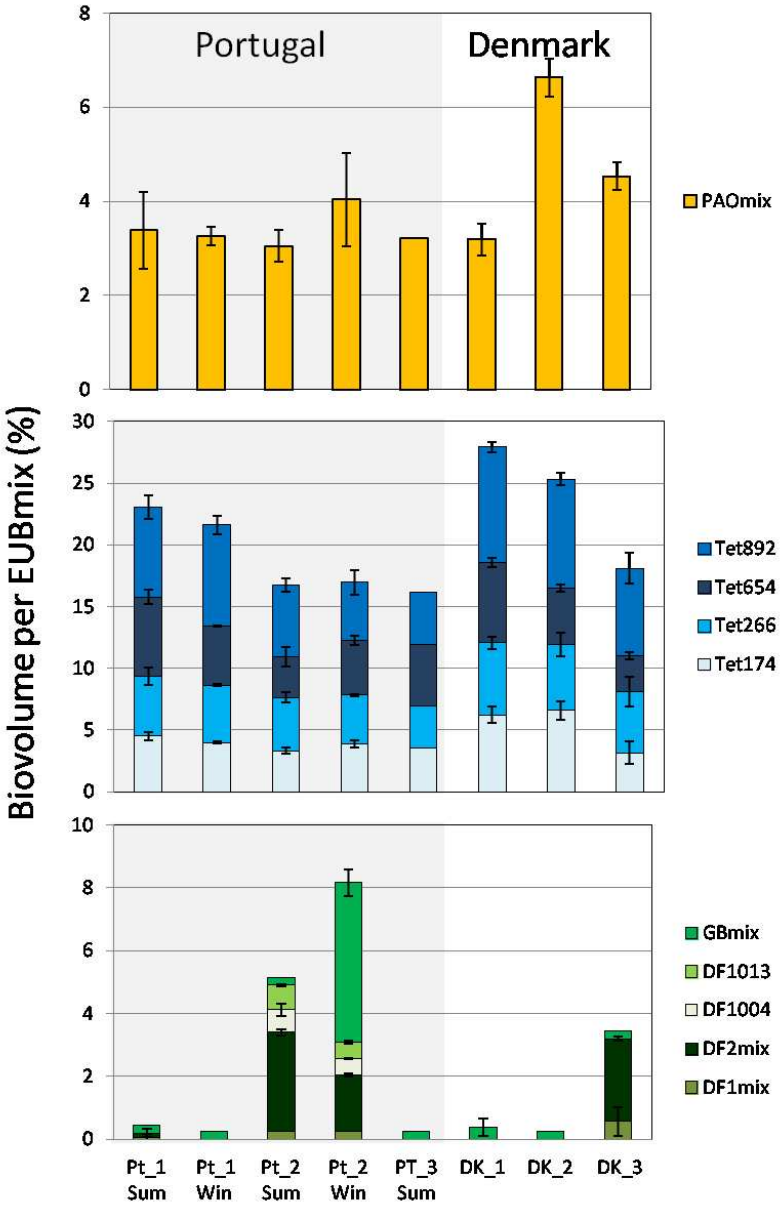

Figure 5.2: Averaged microbial composition of the EBPR-related organisms in the sampled WWTPs in Portugal and in Denmark; Win- Winter; Sum - Summer

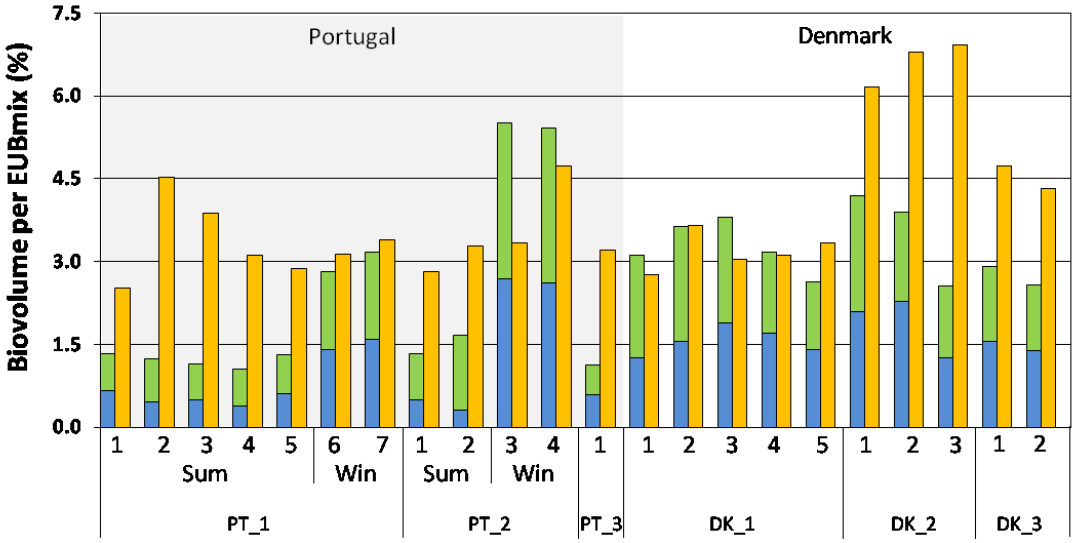

Figure 5.3: Cumulative abundance of *Accumulibacter* Type I and Type II sub-groups (targeted by probes Acc-I-444 and Acc-II-444 probes, respectively) (left-side bar, bottom (blue) and top (green) compartments, respectively) compared to the total abundance of *Accumulibacter* (targeted by the probe set PAOmix) (right-side bar - yellow). Win- winter; Sum - summer

of their P release per acetate uptake yield (P/HAc) and their aerobic or anoxic P-uptake performance, did not correlate with the quantity of *Accumulibacter* observed in the different WWTPs surveyed, nor with the quantity of *Accumulibacter*+*Tetrasphaera*. It had been previously reported, that when quantifying the total P-accumulating organisms by staining microscopical techniques using DAPI, *Accumulibacter* only accounted for 49-60% (He et al., 2008). Also, although some *Tetrasphaera* were shown to store polyphosphate and to take up acetate (Nguyen et al., 2011), the extent of their contribution to the P-removal process remains unknown.

DK_1 and PT_1 presented similar *Accumulibacter* abundances and results from the batch tests indicated similar P removal capacities (Table 5.3), however, DK_1 showed a better yearly P-removal performance with an efficiency of 94% compared to an efficiency of only 43% in PT_1 (Table 5.1). PT_1's influent COD concentration was equivalent to the COD concentration of DK_1, which were lower than the other plants surveyed. However, DK_1 is operated with a process of RSS, where a fraction of the sludge produced is hydrolysed anaerobically to increase the readily assimilable carbon fraction fed to the biological process. Also, on average, in the aerobic tank, PT_1 presented the lowest glycogen content (Table 5.1). In fact, a correlation was observed in PT_1 between the P release observed *in situ* with the amount of carbon in the influent (R^2 of 0.86), suggesting that the main problem affecting the biological P removal process overall in this plant was a shortage of carbon. Finally, PT_3, the plant where a heavy chemical dosage for P precipitation was being carried out, showed the same abundance of *Accumulibacter* and *Tetrasphaera* than PT_1 or DK_1, although the activity of the sludge was very low with no P release or

P uptake observed (results not shown). Therefore, the quantity of PAOs detected by FISH were not the sole indication of the activity occurring in the process, which depended as well on the conditions provided for these organisms to take P up, namely on process configuration and on the addition of chemical precipitants.

5.3.1.2 The presence and activity of GAOs

Significant GAO abundances (higher than 10% of the PAO population) were detected in only two of the 6 plants tested: one Portuguese plant (PT_2) and one Danish plant (DK_3) (Figure 5.2). Despite the presence of GAOs in PT_2 and DK_3, the overall efficiency of these plants in terms of phosphate removal did not seem hindered, showing, respectively, 85 and 92% P-removal efficiency based on yearly data (Table 5.1), though in DK_3 a polishing chemical precipitation step is carried out. In the lab-scale batch tests, both sludges demonstrated a higher aerobic P uptake than anaerobic P release, thus showing the capacity to perform net P-removal (see Table 5.3). PT_2, presenting the highest GAO fraction of all the plants, did show the lowest P removal performance in the batch tests.

DK_3 presented approximately 3% of *D. vanus*-related GAOs, mainly belonging to cluster II. PT_2 had a population change from summer to winter samples: a substantial increase in *Competibacter* from insignificant levels to approximately 5% was observed and the *D.vanus* related GAOs, also mainly cluster II, decreased slightly from 4 to 3%. Although *Defluviicoccus* have been shown to take up acetate (Burow et al., 2007), the P/HAc yield in the batch tests (see Table 5.2) was only considerably lower than expected in plant PT_2 but not in DK_3, where most of the GAO fraction belonged to *D. vanus* cluster II, suggesting that this population might not have contributed to the overall acetate cycling observed. So far, there is not much information available on the metabolism of cluster II or cluster III *D. vanus* with acetate as carbon source. MAR-FISH experiments have shown that they are both able to take up acetate as well as propionate (Burow et al., 2007; McIlroy et al., 2010). However, no significant enrichments in cluster II or III have been reported yet where their kinetics with acetate could be determined and all lab-scale studies where cluster II was present used propionate as carbon source (Lanham et al., 2008; Meyer et al., 2006; Oehmen et al., 2005b; Oehmen et al., 2005c), therefore indicating that they may be more competitive with propionate than with acetate.

Temperature, as well as pH and carbon source, has been indicated as a preponderant factor on the PAO-GAO competition. Therefore, by comparing the microbial population of EBPR plants in a cold climate country (Denmark, where the average winter temperature was 7°C) and in a warm climate country (Portugal, where the average summer and winter temperatures were 15 and 25°C), a higher presence of GAOs in the Portuguese plants was expected than in the Danish ones. Although in a small number of plants sampled (3 Portuguese and 3 Danish), significant GAO numbers (>1% of bacteria) were found

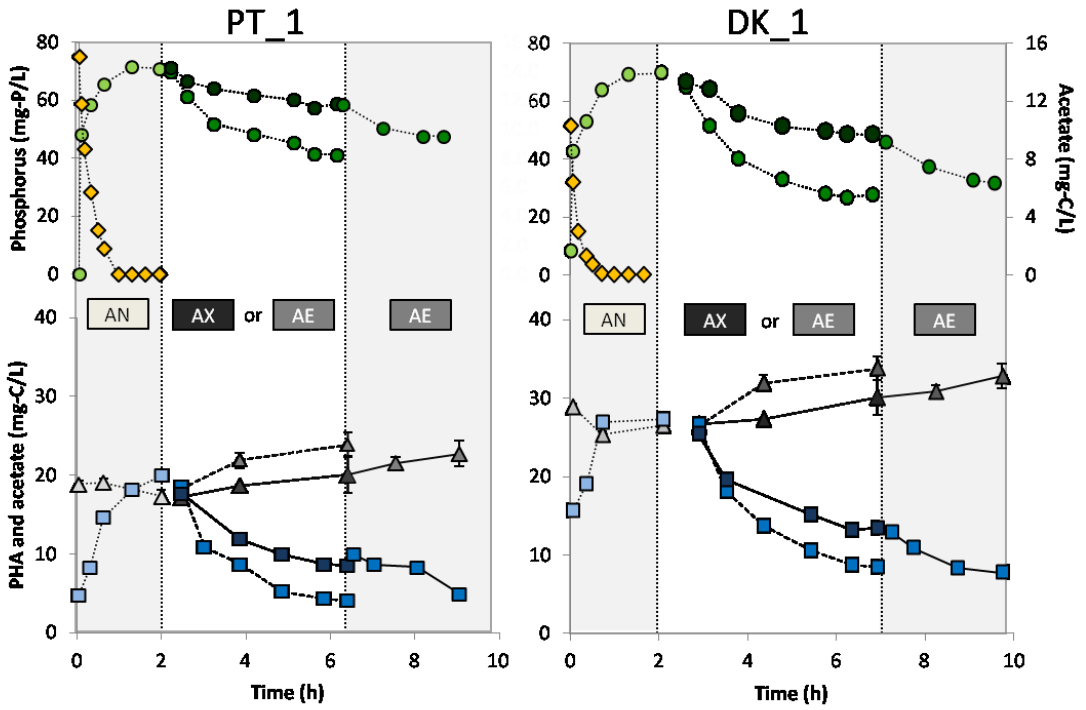

Figure 5.4: Chemical transformations occurring in the batch tests conducted, illustrating the cycling of phosphorus (circles), PHA (squares), glycogen (triangles) and the uptake of acetate (diamonds) in sludge from a Portuguese WWTP (PT_1) and from a Danish WWTP (DK_1). AN - anaerobic (light shade); AX - anoxic (dark shade); AE - aerobic (medium shade).

only in one Portuguese plant and in one Danish plant, indicating that the warm climate alone is perhaps not the sole condition to determine a higher incidence of GAOs. For instance, PT_2 had a very high C:P ratio (204:1), which is a factor that likely stimulated the proliferation of GAOs as well as PAOs (Mino et al., 1998).

5.3.2 Anoxic vs. aerobic performance

Parallel aerobic and anoxic-aerobic batch tests were carried out with two objectives: to determine the denitrifying PAO activity in each WWTP, and to compare the anoxic vs. aerobic P-removal potential of full-scale plants. An example of the phosphate and carbon transformations occurring during a Portuguese and a Danish batch test, for two of the main WWTPs studied, is represented in Figure 5.4.

The amount of phosphate taken up in aerobic (ΔP_{aer}) or anoxic (ΔP_{anox}) tests was determined for each test. The fraction of DPAOs (f_{DPAO}) and n-DPAOs (f_{n-DPAO}) over total PAO was calculated by modifying the method described in Oehmen et al. (2010c), resulting in Equation 5.1 and Equation 5.2. This modification takes into account the lower energetic efficiency of nitrate as an electron acceptor due to a reduced ATP production

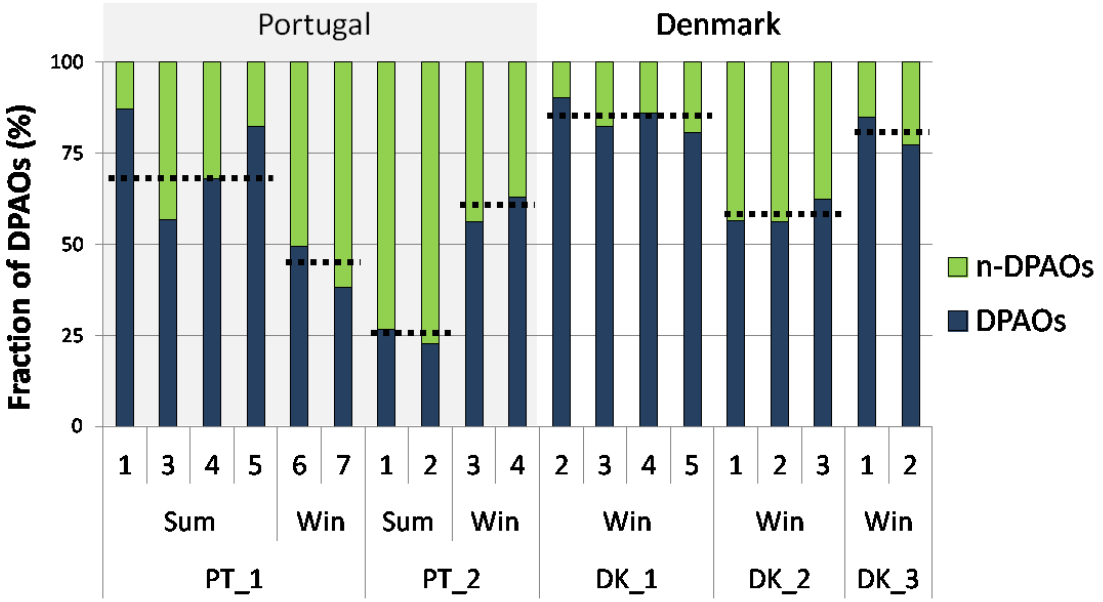

Figure 5.5: Fraction of denitrifying PAOs and non-denitrifying PAOs in total PAOs, estimated based on the uptake of phosphorus in parallel batch tests run in aerobic conditions and anoxic conditions; the dotted lines correspond to the average value for each plant.

during the oxidative phosphorylation mechanism (Kuba et al., 1996a; Murnleitner et al., 1997). Therefore, the phosphorus uptake in anoxic conditions was corrected with the P/O ratios, i.e. the amount of ATP produced per oxidised $NADH_2$, for oxygen (δ_{aer}) and nitrate (δ_{anox}) as electron acceptors, with values of 1.85 (Smolders et al., 1994b) and 1 (Kuba et al., 1996a), respectively (Figure 5.5). This modification was confirmed by using the same approach but considering the PHA consumed instead of the phosphorus. When GAOs were not present, the PHA pools used in anoxic conditions approximately corresponded to the fraction of DPAOs in the total PAO community (Equation 5.3). The fraction of DPAOs obtained with one method was compared to the fraction obtained with the other, so as to determine the ratio of $\delta_{aer}/\delta_{anox}$ (Equation 5.3). The experimental value obtained was 1.5 ± 0.6, which was close to the theoretical value of 1.85.

$$f_{DPAO} + f_{n-DPAO} = 1 \tag{5.1}$$

$$f_{DPAO} = \frac{\Delta P_{anox}}{\Delta P_{aer}} \times \frac{\delta_{aer}}{\delta_{ax}} \tag{5.2}$$

$$f_{DPAO} = \frac{\Delta PHA_{anox}}{\Delta PHA_{aer}} = \frac{\Delta P_{anox}}{\Delta P_{aer}} \times \frac{\delta_{aer}}{\delta_{ax}} \tag{5.3}$$

The highest denitrification performance was found in DK_1, with approximately 85%

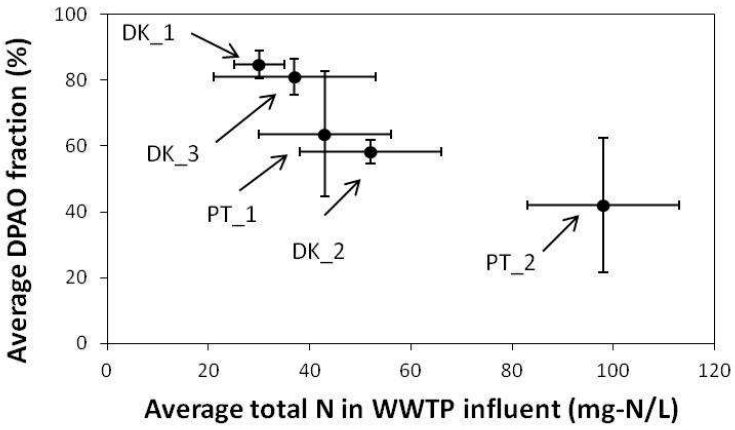

Figure 5.6: Correlation between the average DPAO fraction and the total N measured in the influent of each WWTP

of the PAO population being DPAOs, and the lowest (25%) was found in PT_2 during the summer sampling. It is interesting to note that for these 5 WWTPs, the higher the concentration of total N in the influent, the lower the DPAO fraction seems to be (Figure 5.6). While the reasons for this are unclear, many studies have referred the negative impact that the presence of nitrate in the anaerobic zone produces on EBPR (Barnard, 1982; Kuba et al., 1994), because of the purpose of the anaerobic stage for phosphorus release and VFA uptake being lost when an electron acceptor is present. In this case, it is reasonable to assume that in the plants with higher nitrogen loading, the concentration of nitrate at the end of the aerobic phase will also be higher and therefore, some nitrate might be carried over, in the sludge recirculation stream, to the anaerobic tank. Despite the already overall negative impact that the presence of nitrate could have on the selection of PAOs, its effect could even be more negative on the selection of DPAOs vs. non-DPAOs, since, while the former could potentially denitrify with external carbon, therefore accumulating less PHA, the latter could not and hence they would perform their anaerobic metabolism, even in the presence of nitrate, as long as carbon is not limiting.

No correlation was found between the fraction of Type I *Accumulibacter*, suggested to be able to denitrify from nitrate (Flowers et al., 2009; Lanham et al., 2011) and the fraction of DPAOs. This suggested that denitrification is carried out by only some strains within this clade (which were absent or represent variable fractions of Type I in the tested sludges), by other clades within *Accumulibacter* or that other PAOs besides *Accumulibacter*, such as members of *Tetrasphaera*, were also able to denitrify.

The second goal of these tests was to compare the outcome of the process using only aerobic conditions (both non-DPAOs and DPAOs active) to when using anoxic conditions (only DPAOs active) followed by aerobic conditions (only non-DPAOs active, as DPAOs would have depleted their PHA pools). Despite the lower efficiency that characterises the anoxic activity (40% lower than with oxygen as determined by Kuba et al.

Table 5.2: Summary of the aerobic and anoxic yields obtained for the batch tests; standard deviations represent the variation observed between different sampling dates and not analytical error

			Wastewater treatment plants							PAO models[a]
	Yield (C or P-mol)		PT_1 summer	PT_1 winter	PT_2 summer	PT_2 winter	DK_1 winter	DK_2 winter	DK_3 winter	
Aerobic	$Y_{P/PHA}$		1.1	0.9	0.2	0.3	0.8	0.7	1.6	0.41
		±	0.3	0.1	0.1	0	0.1	0.1	0.3	
	$Y_{Glyc/PHA}$		0.7	0.4	0.2	0.3	0.3	0.3	0.8	0.42
		±	0.3	0.3	0	0.2	0.1	0.1	0.6	
	$Y_{P_{AE}/P_{AN}}$		1.8	1.2	1.3	1.2	1.4	1.8	1.1	
		±	0.8	0.3	0.7	0.2	0.2	0.1	0.4	
Anoxic	$Y_{P/PHA}$		0.7	0.7	0	0.2	0.7	0.7	1	0.30
		±	0.1	0.6	0	0	0.2	0.3	0.1	
	$Y_{Glyc/PHA}$		0.7	0.3	0.1	0.1	0.5	0.3	0.5	0.41
		±	0.5	0.1	N.D	0	0.6	0.1	0.6	
	$Y_{P_{AX}/P_{AN}}$		0.8	0.3	0.2	0.4	0.7	0.6	0.5	
		±	0.3	0	0.1	0	0.1	0.1	0.2	
Aerobic (after anoxic)	$Y_{P/PHA}$		1.7	0.9	0.3	0.2	1.3	0.9	1.4	0.41
		±	0.7	N.D	0	0	0.2	0.1	N.D	
	$Y_{Glyc/PHA}$		0.9	0.3	0.1	0.5	0.7	0.5	1.1	0.42
		±	0.2	N.D	N.D	0.1	0.2	0.2	N.D	
	$Y_{P_{AE}/P_{AN}}$		0.8	0.7	1.1	0.6	0.7	1.1	0.5	
		±	0.4	N.D	0	0.7	0.5	N.D	N.D	

[a] According to the PAO model with acetate developed in Smolders et al. (1995); 2 According to the DPAO model with acetate developed in Kuba et al. (1996a)

(1996a)), the anoxic/aerobic strategy was able to remove approximately 90% of what was removed in sole aerobic conditions (Figure 5.7), where the corresponding PHA and glycogen consumed and produced were approximately 10% higher. This suggests that, although the anoxic metabolism should be less efficient, overall the outcome was similar with a slightly lower phosphorus removal at the expense of a higher PHA consumption and therefore the anoxic/aerobic strategy could be successfully employed in WWTPs to remove phosphorus, without a significant decrease in efficiency.

Concerning the anoxic and aerobic yields, in particular those of P/PHA (Table 5.2, they presented much higher values than expected, when compared to the metabolic models. Nevertheless, the aerobic metabolic models have always been developed assuming the utilisation of the glycolysis pathway in anaerobic conditions. However, Pijuan et al. (2008) observed the partial use of the anaerobic TCA cycle in full-scale WWTPs. If this were the case here, it could be that when the TCA cycle is used, there is less PHA available and therefore, there might be a shift in the carbon fluxes within the cell, sacrificing growth in favour of P uptake or glycogen storage, as has been suggested by Murnleitner et al. (1997). In fact, Zhou et al. (2009), who studied the involvement of the TCA cycle in glycogen starved cultures, also observed a higher P/PHA yield than what was predicted in the models, of approximately 0.8 P-mol/C-mol. In view of these results, the involvement of the TCA cycle in the activated sludge from the plants studied was further investigated.

5.3.3 The importance of the TCA cycle in anaerobic conditions

The anaerobic stoichiometric data obtained for each plant (Table 5.3) was compared with the metabolic models available, which condense the information known so far on the metabolism of PAOs and GAOs. A range of different anaerobic Glyc/HAc yields was observed between plants and even between different samples within the same plant, which was correspondingly reflected in the PHA/HAc yields. In some cases, such as in most PT_1 samples, the glycogen yields were close to 0, in accordance with the utilisation of the TCA cycle (Smolders et al., 1994b), while in some other cases, such as in most samples of DK_1, they were closer to what is expected when glycolysis is used (i.e. 0.5 C-mol/C-mol HAc - (Smolders et al., 1994b)). PHA yields were in accordance with the glycogen results, i.e. presenting lower values (close to 0.9 C-mol/C-mol VFA - (Smolders et al., 1994b)) when glycogen yields were low, and presenting higher values (closer to 1.3 C-mol/C-mol VFA - (Smolders et al., 1994b)) when the glycogen yields were higher. Since no GAOs were observed in these plants, this suggested that in some cases the TCA cycle was being used, while in other cases the glycolysis pathway was used, which is in accordance with Pijuan et al. (2008).

Zhou et al. (2009) reported that a shift from using the glycolysis pathway to the

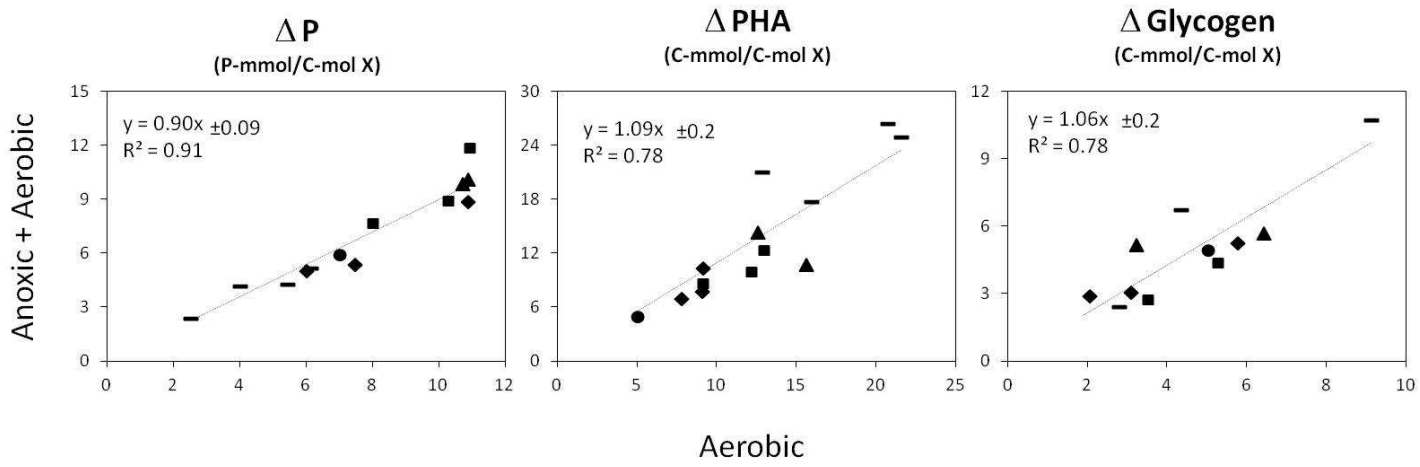

Figure 5.7: Comparison of the normalised consumption of phosphorus and PHA and the production of glycogen for the two different strategies tested for P-removal: only aerobic conditions and anoxic+aerobic conditions. - PT_1 (diamonds), PT_2 (dashes), DK_1 (squares), DK_2 (triangles), DK_3 (circles)

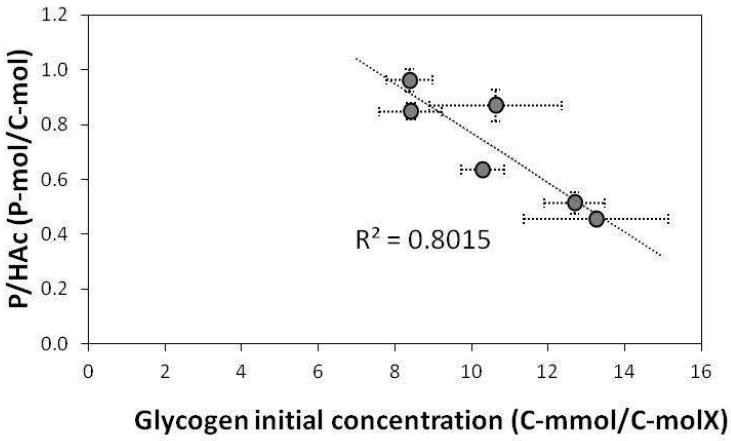

Figure 5.8: Correlation between the initial glycogen concentration and the resulting P/HAc yield obtained for the Portuguese WWTP PT_1

TCA cycle was due to glycogen starvation conditions. The data obtained in this study showed the same trends as the data obtained by Zhou et al. (2009), when correlating the PHA/HAc yields with the Glyc/HAc yields (Figure 5.9). The initial concentration of glycogen was also correlated with the P/HAc yields in PT_1 (Figure 5.8), where the higher the glycogen concentration at the beginning of the anaerobic phase, the lower the P/HAc yield, indicating a higher utilisation of glycolysis. Additionally, the amount of glycogen consumed in anaerobic conditions constitutes on average approximately only 30-60% of the glycogen produced during the aerobic phase of the batch tests (for an example see Figure 5.4), which further supports the hypothesis that glycogen was limiting in the sludge when it was collected from the WWTP.

It should be noted that the phosphate yields resulted in higher values than expected for the Danish plants, which was not concordant with the glycogen and PHA yields (Table 5.3). In the blank tests conducted in this study, the P release rate in all the Danish plants was approximately the double of the value obtained in the Portuguese blank tests (1.3 ± 0.2 and 0.6 ± 0.1 P-mmol/C-molX.h, respectively). Regarding that the PHA and glycogen variations were equivalent in the Portuguese and Danish tests, another mechanism, other than maintenance, might have taken place in the Danish blank tests that influenced the P release observed. A possibility is the chemical release of phosphate from iron-phosphate precipitates due to the activity of iron reducing bacteria (Nielsen, 1996). Interestingly, from the plants analysed in Table 5.1, only the Danish plants use chemical precipitation and therefore it is plausible that iron-phosphate precipitates present in these sludges could be re-dissolved in anaerobic conditions, resulting in over-estimates of the P/HAc yields for these plants as shown in Figure 5.9.

The stoichiometric results obtained in this study seem to indicate that in the different WWTPs observed there is a "'metabolic gradient"' that is determined by the glycogen's

Table 5.3: Anaerobic yields for chemical transformations involving P, PHA and Glycogen (Glyc) cycling (Average ± Standard deviation)

			Portugal				Denmark			Models[a]		
			PT_1 summer	**PT_1 winter**	**PT_2 summer**	**PT_2 winter**	**DK_1 winter**	**DK_2 winter**	**DK_3 winter**	**TCA PAO**	**Glyc. PAO**	**GAO**
P/HAc	P or C-mol		0.6	0.9	0.3	0.5	1.1	1	1	0.8	0.5	0
		±	0.2	0.1	0.3	0	0.2	0.4	0			
PHA/HAc			0.8	0.9	1.6	1.8	1.3	1.4	0.9	0.9	1.3	1.9
		±	0.2	0	0.6	0.4	0.2	0.4	0.2			
Glyc/HAc			0.2	0.1	0.2	0.7	0.3	0.4	0.2	0	0.5	1.1
		±	0.1	0	0	0.2	0.1	0	0.1			
PHB	%		79	84	67	67	83	77	75	100	100	73
		±	3	7	1	0	8	0	9			
PHV			18	14	29	32	17	20	25	N.D.	N.D.	24
		±	5	7	3	0	8	0	9			
PH2MB			1	1	1	0	0	2	0	N.D.	N.D.	N.D.
		±	0	0	0	0	0	0	0			
PH2MV			2	1	3	0	0	1	0	N.D.	N.D.	2
		±	2	0	2	0	0	1	0			
GAO[b]/PAO[c]			0.1	0.1	1.7	2.1	0.1	0	0.8			
		±	0	0	0.3	0.7	0.1	0	0.1			
Competi.[d]/PAO			0.1	0.1	0.1	1.3	0.1	0	0.1			
		±	0	0	0	0.4	0.1	0	0			

[a] Acetate PAO models introduced by Smolders et al. (1994b) and Acetate GAO model introduced by Zeng et al. (2002)
[b] GAO refers to total GAO as measured by qFISH (sum of GAOmix, DEF1mix, DEF2mix and DF1013 and DF1004);
[c] PAO refers to total *Accumulibacter* as measured by qFISH and the PAOmix probe
[d] Competi. refers to *Competibacter* as measured by qFISH with GAOmix

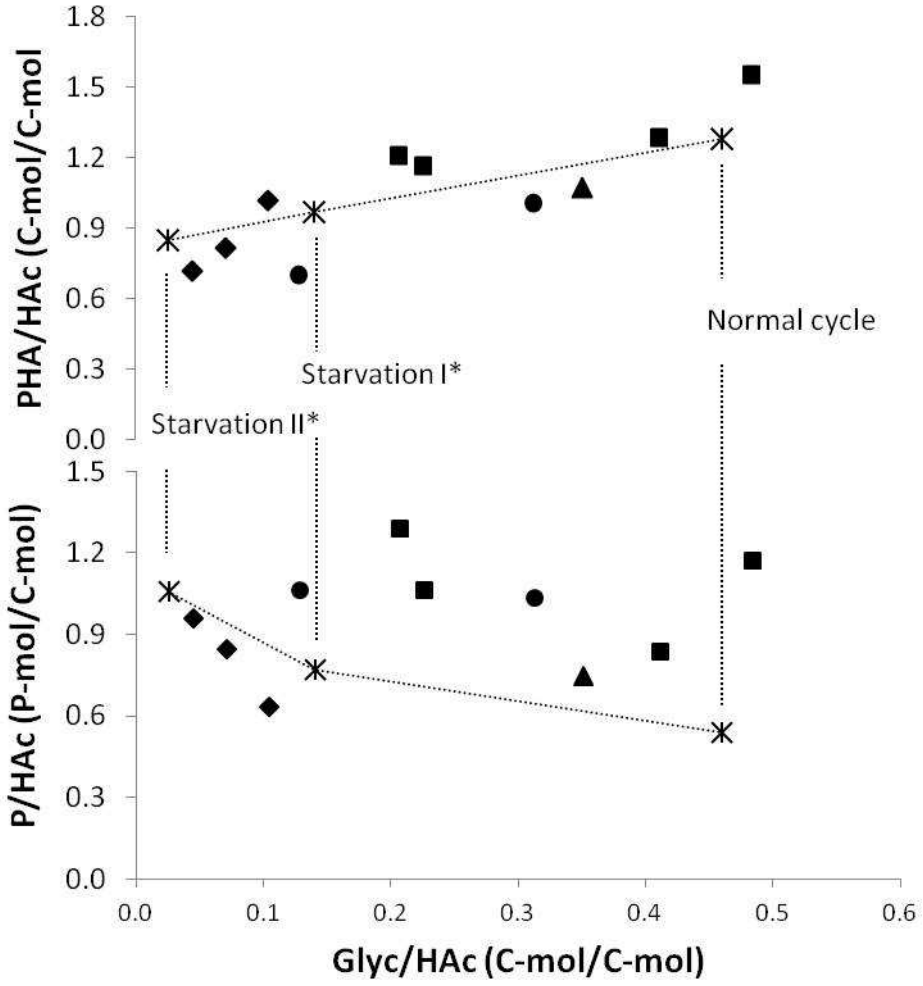

Figure 5.9: Correlation between the glycogen, the PHA and the phosphate yield per acetate consumed in anaerobic conditions for the different WWTPs tested - PT_1 (diamonds), DK_1 (squares), DK_2 (triangles), DK_3 (circles) in comparison with the values obtained by Zhou et al. (2009) (stars) for a typical cycle operation and for 2 different starvation conditions (I and II). (*) A detailed description of the conditions used to produce these starvation phases are described in Zhou et al. (2009)

availability: if microorganisms are starved or have low glycogen available they will resort to the TCA cycle to generate their reducing equivalents anaerobically. However, as glycogen becomes more available, they progressively use the TCA cycle less and glycolysis more, which is in fact energetically more favourable (as discussed below).

Therefore the TCA cycle is suggested to play a more important role than initially predicted by laboratory-enriched cultures, as was also determined by Pijuan et al. (2008). This could be due to the fact that in WWTPs the aerobic phases have longer durations (ranging in this case from 5 to 15 h) than in laboratory reactors (ranging from 2 to 4 h) (e.g., Carvalho et al. (2007); Flowers et al. (2009); Lanham et al. (2011); Smolders et al. (1994b); Zhou et al. (2009)). This longer aerobic phase may lead to glycogen depletion, rather than polyphosphate depletion or cell decay, once the PHA reserves are exhausted, as shown by Lopez et al. (2006). Additionally, sludge in WWTPs still has to endure another starvation period during the settling and recycling process, where glycogen pools are most likely to be further depleted.

The hypothesis that the TCA cycle is active to different extents in WWTP sludge due to glycogen limitation leads to the question of what is the effect that this shift in metabolism might bring to the EBPR performance. Theoretically, the glycolysis metabolism should be more efficient and beneficial for EBPR, since at the end of the anaerobic period cells will contain more PHA (1.33 C-mol PHA/C-mol acetate with glycolysis vs. 0.89 C-mol PHA/C-mol acetate with the TCA cycle) and will have released less phosphate anaerobically. This was verified experimentally (Figure 5.10), where the net P removed per net P released ratio increased from approximately 1.0 (P-mol) to 1.5 (P-mol), while the glycogen per acetate yield increased from 0.05 to 0.5 C-mol/C-mol. This increase in efficiency was even greater if taking into account the P released by other processes such as by chemical P release as discussed above.

Therefore, by using the TCA cycle, PAOs lose a fraction of their potential in the EBPR process and that carbon limitation might affect negatively the efficiency of the process. Interestingly, DK_1 and DK_2, where the strategy of RSS was used, precisely as a way to increase readily biodegradable carbon loading to the WWTP, presented the best results with a lower utilisation of the TCA cycle as when compared to PT_1 or DK_3.

In order to obtain a better understanding of the EBPR process, further research should be carried out to determine in more detail the factors that trigger the use of the TCA cycle over the glycolysis (as also pointed out by Zhou et al. (2010), as well as the impact of the TCA cycle on the aerobic and anoxic yields of P removal. If this information were incorporated into the metabolic models, it would be possible to describe a higher level of metabolic versatility for the EBPR microorganisms, and also to determine what would be the role of this metabolic versatility in the competition between PAOs and GAOs. Further work is needed to relate PAO/GAO metabolic models to the results generated from full-scale sludge. Additionally, it is important to stress that the role and metabolism

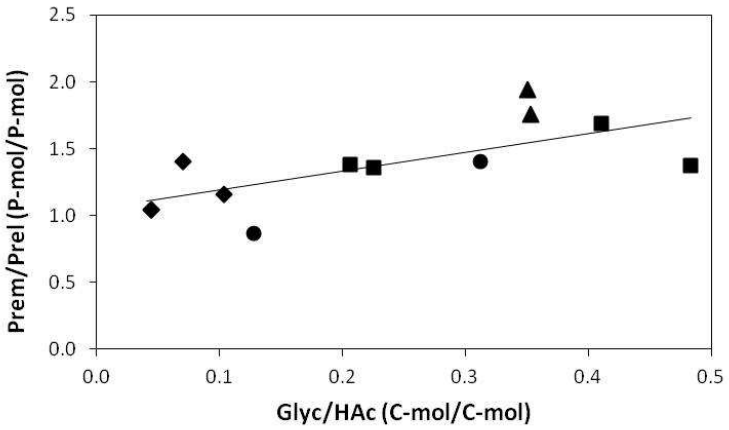

Figure 5.10: Correlation between the Glyc/HAc yield, as an indication for the use of the TCA cycle vs. glycolysis, and the efficiency of the EBPR process expressed in terms of the net P removed per net P released anaerobically - PT_1 (diamonds), DK_1 (squares), DK_2 (triangles), DK_3 (circles).

of other unknown PAOs and GAOs is still unclear, e.g., little could be inferred on the role of *Tetrasphaera*, which could potentially affect the stoichiometry and the kinetics of the process. Therefore, future work should provide a deeper insight into the metabolism and physiology of lesser known PAOs and GAOs, which could then be tested in experiments with activated sludge fed with complex substrates in order to better understand their contribution to the process.

5.4 CONCLUSIONS

The following main conclusions can be taken from this work:

- All plants had a significant PAO population, composed of *Accumulibacter* and *Tetrasphaera* PAOs. Only 2 of the 6 plants surveyed, one Portuguese and one Danish, contained an abundant GAO population (4-8%). Though it was hypothesised that GAOs might be more abundant in the Portuguese plants due to the higher operating temperatures, this did not seem to be the case in this study.

- The anaerobic metabolism in acetate-fed batch tests showed differences between plants in the relative importance of the TCA cycle and glycolysis as a source of reducing equivalents. This was apparently due to differences in the initial glycogen pools that may cause glycogen limitation in some of the sludges. This demonstrated that both mechanisms are important to various extents in different WWTPs, or even in the same WWTP in different periods of time, and that these differences probably resulted from the operating conditions or the characteristics of the influent.

- Overall, it was determined that many of the factors expected from lab-scale experience to impact on EBPR were not found to fully explain the results obtained with full-scale sludge, most likely due to the impact of other less well characterised factors and also due to the higher dynamism and complexity observed in full-scale plants. Therefore, in order to obtain a comprehensive understanding of the system, further research should focus on capturing the metabolic versatility of the involved microorganisms when facing non-steady-state conditions and under limitation of substrates and/or nutrients.

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6 Metabolic modelling of full-scale enhanced biological phosphorus removal sludge: anaerobic TCA cycle vs. glycolysis

Summary *This study investigates, for the first time, the application of metabolic models incorporating polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) towards describing the biochemical transformations of full-scale enhanced biological phosphorus removal activated sludge from four wastewater treatment plants (WWTPs). For this purpose, previous metabolic models applied to lab-scale systems were modified by incorporating the anaerobic utilisation of the TCA cycle and the aerobic maintenance processes based on sequential utilisation of PHA, followed by glycogen and polyphosphate. The abundance of the PAO and GAO populations quantified by fluorescence in situ hybridisation (FISH) served as the initial conditions of each biomass fraction, whereby the models were able to describe accurately the experimental data. The kinetic rates were found to change among the different plants, or even in the same plant during different seasons, either suggesting the presence of additional PAO or GAO organisms, or varying microbial activities for the same organisms. Nevertheless, these variations in kinetic rates were found to be proportional to the difference in acetate uptake rate, suggesting a viable means of calibrating the model. The application of the metabolic model to full-scale sludge also revealed that different Accumulibacter clades likely possess different acetate uptake mechanisms, as a correlation was observed between the energetic requirement for acetate transport across the cell membrane with the diversity of Accumulibacter present. Using the model as a predictive tool, it was shown that lower acetate concentrations in the feed as well as longer aerobic retention times, favour the dominance of the TCA metabolism over glycolysis, which could explain why the anaerobic TCA pathway seems to be more relevant in full-scale WWTPs than in lab-scale systems.*

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6.1 INTRODUCTION

The biological removal of phosphate (also known as enhanced biological phosphorus removal or EBPR) has been incorporated into several wastewater treatment plant (WWTP) configurations and provides a more economical and sustainable alternative to chemical precipitation methods of P removal (Mino et al., 1998; Oehmen et al., 2007).

For more than 20 years, an effort has been made to develop and apply activated sludge models (ASM) to describe and predict the activated sludge processes, which are a useful tool for plant design and optimisation (Henze et al., 2000). While ASM models use a grey-box approach and focus on macroscopic processes, a different modelling approach, relying on metabolic and biochemical pathways, describes the energy, redox and mass balances of the cell processes (Smolders et al., 1994b). When comparing the two strategies, ASM models require a plant-tailored calibration procedure that can affect a higher number of variables, whereas metabolic models have been reported to require a simpler calibration procedure, since all of the equations for the microbial processes are inter-dependant (Seviour et al., 2010a). Both approaches have been combined in the Technical University of Delft model (TUDP) and successfully applied for full-scale WWTPs, describing anaerobic, anoxic and aerobic processes of polyphosphate accumulating organisms (PAOs) (Brdjanovic et al., 2000; Meijer et al., 2001; Veldhuizen et al., 1999).

EBPR is a complex process to model, since PAOs function in anaerobic, anoxic and aerobic conditions and use three different internal storage compounds, i.e., polyphosphate, glycogen and polyhydroxyalkanoate (PHA), as a resource for energy, reducing power and carbon. Additionally, PAOs have to withstand competition from glycogen accumulating organisms (GAOs), for which external parameters, such as temperature, pH, COD:P ratio and carbon source, play a significant role (Oehmen et al., 2007). Hence, the initial metabolic model developed in Delft for PAOs (Kuba et al., 1996a; Murnleitner et al., 1997; Smolders et al., 1995; Smolders et al., 1994b), was expanded to include the metabolic pathways of two main GAO-like organisms, i.e., *Competibacter* and *Defluviicoccus* vanus-related organisms, as well as the effects of temperature, carbon source and pH on their metabolism (Lopez-Vazquez et al., 2009b). Additionally, the denitrification capacities of *Accumulibacter*, the main PAO known, and *Competibacter*- and *Defluviicoccus*-GAOs were further added by Oehmen et al. (2010b).

However, these new additions have only been validated in lab-scale systems containing enrichments of PAOs and GAOs and have not previously been tested on full-scale sludge. While simplified metabolic model calibration strategies have been proposed based on lab-scale results (Oehmen et al., 2010b), it is necessary to test these theories using full-scale sludge in order to evaluate their applicability to more complex situations. It is noteworthy to mention that when modelling the performance of full-scale systems there is an added complexity, since, not only could there be unknown PAOs and/or GAOs

whose contribution to the phosphorus removal process is still unknown, but known PAOs such as Tetrasphaera could be active, whose metabolism related to EBPR is still largely unclear. Adding to this complexity is the fact that PAOs and GAOs make up a much smaller fraction of the total microbial community in full-scale sludge as compared to lab-scale systems, which can potentially have an impact on their metabolic behaviour.

Although PAOs have been typically modelled as using the glycolysis pathway as their sole source of anaerobic reducing power generation, it has been suggested that the role of the anaerobic TCA cycle in real WWTPs might be greater than expected as compared to lab-scale results (Pijuan et al., 2008 and Chapter 5). In fact, Zhou et al. (2009) have shown that the TCA might have a particularly prominent role when PAOs face conditions of glycogen shortage. Since WWTPs deal with variable influent compositions and often with limited carbon substrate availability, this might be the reason for a greater reliance on the TCA cycle in WWTPs as opposed to lab-scale reactors (cf.,Chapter 5). Therefore, in order to improve the applicability of metabolic models, particularly with respect to full-scale situations, the relevance of incorporating the TCA cycle activity into the model should be assessed.

Furthermore, in previous metabolic models the aerobic maintenance processes predict cell decay at low PHA levels, which is not consistent with literature findings. Experiments on the endogenous metabolism of PAOs (Lopez et al., 2006; Lu et al., 2007) observed that the aerobic maintenance processes were dependant on glycogen and polyphosphate degradation following PHA depletion, with minimal cell decay. This is a particularly relevant factor to include when applying the model to full-scale systems, whereby the level of polymers stored by the sludge is much lower as compared to lab-scale systems.

In this study, a simplified version of the metabolic models previously developed by Lopez-Vazquez et al. (2009b) and Oehmen et al. (2010b) were adapted in order to incorporate the anaerobic TCA utilisation of PAOs, in addition to the previously implemented glycolytic pathway. The resulting model was tested by describing the anaerobic/aerobic chemical transformations observed in activated sludge batch tests fed with acetate as carbon source, from four different EBPR WWTPs with differing microbial compositions (*Accumulibacter*, *Competibacter* and *Defluviicoccus*) and metabolisms, as shown in Chapter 5. Special attention was paid to the required calibration procedure necessary in order to describe the activity of each biomass, and where possible, simplified calibration procedures that could be applicable to the modelling industry were evaluated. In addition, theoretical simulation studies were conducted between PAOs using solely the TCA cycle (ACC_TCA) and PAOs using glycolysis (ACC_Glyc) in order to better understand the conditions which may lead to the use of one metabolic pathway over the other. Thus, this study is also relevant to improve our knowledge about factors that influence the microbial metabolism in EBPR systems, which is necessary in order to better understand

and optimise the performance of the process.

6.2 MATERIALS AND METHODS

6.2.1 Experimental results

Four WWTPs were modelled in order to describe the anaerobic and aerobic chemical transformations observed in activated sludge tested in lab-scale batch tests, fed with acetate, at neutral pH (7) and at 20°C. The WWTPs studied had either an A2/O configuration (Portuguese WWTPs: PT_1 and PT_2) or a Biodenitro configuration coupled to a return sludge side-stream hydrolysis process (RSS) (Danish WWTPs: DK_1 and DK_2). Experiments were carried out in winter and summer for the Portuguese WWTPs and only in winter for the Danish WWTPs and for most cases (PT_1 winter, PT_2 winter and summer and DK_2) the experimental results were averaged from two replicate batch tests. Two of the WWTPs (PT_1 and DK_2) only had *Accumulibacter*-PAOs (approximately 4% as determined by quantitative fluorescence *in situ* hybridisation (qFISH)), whereas PT_2 had significant amounts of *Defluviicoccus*- and *Competibacter*-GAOs (4-8%). DK_1 presented one time point with almost 1% of *Competibacter*. Also, Type I and Type II *Accumulibacter* were quantified (Flowers et al., 2009) and while in the Portuguese winter tests and in the tests from DK_1 an equivalence between the sum of Type I and Type II and total *Accumulibacter* was observed, in the Portuguese summer tests and in the tests from DK_2, the total *Accumulibacter* population was not described by the sum of Type I and Type II, suggesting an unknown diversity of other *Accumulibacter* Types, as high as 50% of the total *Accumulibacter* population. *Tetrasphaera*-PAOs were also present in all plants (15-25%), however since their metabolism is yet unclear, this group was not considered in the model. A complete account of the WWTPs characterisation, the batch test results and the microbial population quantification can be seen in Chapter 5.

6.2.2 Model description

The model developed in this study was based on a simplified version of previous metabolic models defined by Lopez-Vazquez et al. (2009b) and Oehmen et al. (2010a) and was compiled using AQUASIM software (v. 2.1, Reichert (1994)). The present model focused only on acetate as the sole external carbon source, converted into polyhydroxyalkanoate (PHA). It does not address pH nor temperature dependencies (since these were controlled at 7 and 20°C in all batch tests) and aims at describing the anaerobic and aerobic transformations of *Accumulibacter*-PAOs (abbreviated to ACC) and *Competibacter* and *Defluviicoccus*-GAOs (abbreviated to GB and DEF).

The model describes the acetate (S_{HAc}) and the phosphate (S_{PO4}) concentrations observed in the medium, the fraction of PHA (X_{PHA}), glycogen (X_{GLY}) and polyphosphate (X_{PP}) inside the bacterial cells, as well as the concentration of PAOs and GAOs (X_{ACC}, X_{GB} and X_{DEF}). The initial values for these parameters in the model were based on the experimental results. The initial concentrations of the PAO and GAO biomass fractions were based on the active biomass concentrations (as given by the volatile suspended solids (VSS) minus the organic storage polymers, PHA and glycogen, cf., Smolders et al. (1994a) multiplied by the fraction of *Accumulibacter*, *Competibacter* and *Defluviicoccus* detected by qFISH (Oehmen et al., 2010b). In systems containing PAOs and GAOs simultaneously, their initial fraction of glycogen and PHA was estimated based on the specific anaerobic yields for each compound (cf., Table 6.1) in each type of organism as exemplified in Eq. 6.1 for the initial PHA fraction of *Competibacter* ($X^{GB}_{PHA,i}$).

$$X^{GB}_{PHA,ini} = X_{PHA,ini} \times \frac{f_{GB} \times Y^{GB}_{PHA,HAc}}{f_{GB} \times Y^{GB}_{PHA,HAc} + f_{ACC} \times Y^{ACC}_{PHA,HAc} + f_{DEF} \times Y^{DEF}_{PHA,HAc}} \quad (6.1)$$

where f_{GB}, f_{ACC} and f_{DEF} are the fraction of each of these organisms as exemplified in Eq. 6.2 for *Competibacter*.

$$f_{GB} = \frac{X_{GB}}{X_{GB} + X_{ACC} + X_{DEF}} \quad (6.2)$$

The anaerobic stoichiometry of PAOs was based on acetate uptake coupled with polyphosphate hydrolysis, phosphate release, glycogen degradation and PHA production. Anaerobic maintenance processes were modelled as polyphosphate hydrolysis and subsequent phosphate release, which was followed by glycogen degradation, if low polyphosphate levels were attained. A complete account of the anaerobic reactions and kinetics in the model is given in Appendix A-I. The anaerobic yields were defined based on the utilisation of the anaerobic TCA cycle or the glycolysis pathway, as determined experimentally by the anaerobic glycogen per acetate yield determined in the activated sludge batch tests (Chapter 5). The overall reactions for the TCA cycle or the glycolysis stoichiometry were based on Smolders et al. (1994b) and are presented in Eqs. 6.3 and 6.4, respectively. Greater explanation of the incorporation of the TCA cycle stoichiometry is detailed in Section 6.3.1.

The overall acetate uptake reaction where the TCA cycle is incorporated is shown below (C-mol basis):

$$\begin{aligned} &Acetate + (0.5 + \alpha_{ACC})HPO_3 + (\frac{1}{3} - 0.5 + \alpha_{ACC})H_2O \\ &\rightarrow 0.89PHA + 0.11CO_2 + (0.5 + \alpha_{ACC})H_3PO_4 \end{aligned} \quad (6.3)$$

The overall acetate uptake reaction where glycolysis is incorporated is shown below (C-mol basis):

$$Acetate + 0.5Glycogen + (0.25 + \alpha_{ACC})HPO_3 \rightarrow 1.33PHA + 0.17CO_2 + (0.25 + \alpha_{ACC})H_3PO_4 + \left(\frac{5}{12} - (0.25 + \alpha_{ACC})\right) H_2O \quad (6.4)$$

where α_{ACC} is the energy of transport of one C-mol of acetate across the cell membrane.

GAOs were modelled in the same way as PAOs, except with a different stoichiometry (see Table 6.1) and excluding the processes dependant on polyphosphate or phosphate. The overall anaerobic acetate uptake of GAOs is shown in Eq. 6.5.

$$Acetate + (1 + 2\alpha_{GAO})Glycogen \rightarrow (1.75 + \frac{5}{3}\alpha_{GAO})PHA + (\frac{1}{4} + \frac{1}{3}\alpha_{GAO})CO_2 \quad (6.5)$$

Table 6.1: Anaerobic stoichiometric parameters for *Accumulibacter* (ACC), *Competibacter* (GB) and *Defluviicoccus* (DEF) at pH 7, 20°C and with acetate as the carbon source. The yields for the two possible metabolisms for *Accumulibacter*, with glycolysis (ACC_Gly) and with the anaerobic TCA cycle (ACC_TCA) are indicated.

C-mol or P-mol[a]		ACC_Gly	ACC_TCA	GB	DEF
Phosphate released per acetate yield	$Y_{PO4,HAc}$	0.5	0.75	0	0
Glycogen consumed per acetate yield	$Y_{GLY,HAc}$	0.5	0	1.12	1.12
PHA produced per acetate yield	$Y_{PHA,HAc}$	1.33	0.89	1.86	1.86

[a] *Accumulibacter* yields given by Smolders et al. (1994b) and GAO yields given by Filipe et al. (2001a); Zeng et al. (2002)

The aerobic stoichiometry and kinetics of the PAO model are based on the method proposed by Murnleitner et al. (1997), where PHA is degraded to contribute to phosphate uptake, polyphosphate formation and glycogen replenishment. Growth is determined in the model from the difference between the degraded PHA and the glycogen and polyphosphate produced (Figure 6.1). The aerobic stoichiometry depends on the ATP production yield per NADH oxidised with oxygen, through the oxidative phosphorylation mechanism, known as the P/O ratio or δ. The same processes are applied to the GAO models with the exception of the phosphate uptake and the polyphosphate formation processes. As explained in more detail in Section 6.3.1, the aerobic maintenance processes rely sequentially on PHA, glycogen and polyphosphate as energy sources. The kinetic processes and parameters utilised in this model, summarised in Appendixes A-I,

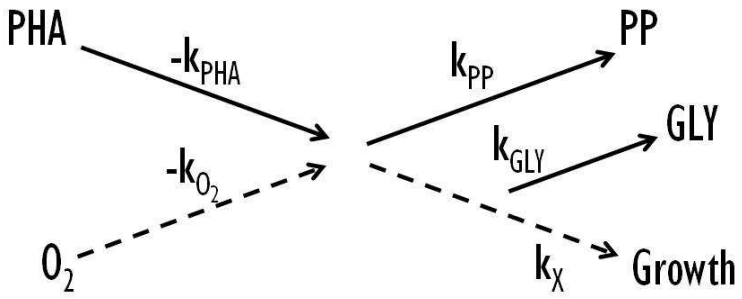

Figure 6.1: Kinetic structure of the aerobic model illustrating that the growth rate (k_X) and the oxygen consumption rate (k_{O2}) are modelled indirectly from the PHA degradation rate (k_{PHA}) and the polyphosphate and glycogen formation rates (k_{PP} and k_{GLY} respectively) (Adapted from Murnleitner et al. (1997))

were mostly consistent with those specified in Lopez-Vazquez et al. (2009b); Oehmen et al. (2010b), except for the differences specified in Section 6.3.1.

6.2.3 Model calibration

The PAO model was first calibrated using the results from two batch tests of plant PT_1, in winter time, at standard conditions (acetate as the carbon source, pH=7 and 20°C). The calibration procedure was mainly performed on the kinetic parameters, namely $q_{HAc}^{\max}$, k_{PP}, k_{PHA} and k_{GLY}. A full account of the altered parameters is given in Table 6.5. The resulting calibrated model was adjusted to the experimental values obtained in PT_1, but in summer, and to the experimental values obtained in the other WWTPs. The adjustments were based on recalibrating $q_{HAc}^{\max}$, which then largely affected the aerobic rates proportionally, as suggested by Oehmen et al. (2010b). During this procedure, the initial concentrations of acetate, phosphate, PHA, glycogen and polyphosphate were set to the initial experimental values.

The *Competibacter* model and the *Defluviicoccus* model were first calibrated in one batch test of plant DK_1 and two batch tests of plant PT_2 (summer), respectively. The same procedure as for PAOs was applied to adapt the models to the experimental results of plant PT_2 (winter).

6.2.4 Sensitivity and error analysis and simulation studies

The deviation for n data points of the values predicted by the model (x_i^{model} at time point i) and the experimental values ($x_i^{\exp}$ at time point i) was determined by calculating the normalised root mean squared deviation (NRMSD) as stated in Eq. 6.6:

$$NRMSD = \frac{\sqrt{\frac{\sum_{i=1}^{n}(x_i^{\exp} - x_i^{model})^2}{n}}}{x_{\max}^{\exp} - x_{\min}^{\exp}} \tag{6.6}$$

where $x_{\max}^{\exp}$ and $x_{\min}^{\exp}$ are the maximal and minimal experimental values measured for that parameter.

For long-term simulations, which were performed either for sensitivity analysis purposes or for microbial competition estimates, a sequencing batch reactor (SBR) was defined in AQUASIM as a mixed 8-L reactor compartment with variable volume, with a sludge retention time (SRT) of 10 d, a hydraulic retention time of approximately 0.6 d, and a 7-h cycle. The simulations were executed for 40 d until steady state conditions were achieved (4 x SRT) (similar to what is described in Oehmen et al. (2010b).

Sensitivity analyses were performed to assess the impact of the aerobic PHA degradation rate and of the initial polyphosphate concentration by varying the value of these parameters by $\pm$ 50% and then determining the effect on growth, levels of storage compounds (PHA, glycogen and polyphosphate) and on phosphorus removal.

Simulations were performed to determine the theoretical competition between the two anaerobic metabolisms that are at the focus of this work. In this set of simulations, either the anaerobic TCA metabolism or the glycolysis metabolism was modelled in a distinct PAO population, with all the other parameters being equivalent between the different "organisms". While it is recognised that PAOs can potentially perform both processes simultaneously, this approach allowed examination of the factors that influence the competition between different metabolic pathways. Due to hypotheses suggested in Chapter 5, the competition between the two metabolisms was assessed at different acetate concentrations in the feed, from 0.25 to 5 C-mM, and at different aerobic phase durations, from 3 to 12 h.

6.3 RESULTS AND DISCUSSION

6.3.1 Model development

The goal of this study was primarily to adapt metabolic models developed for lab-scale systems to describe the processes occurring in full-scale sludge. Although it is undeniable that lab-scale systems are essential to achieve a deeper understanding about key metabolic transformations, the higher complexity of full-scale WWTP might require adjustments to the models developed upon lab-scale data, regarding the diversity of influent characteristics and operating conditions existing in WWTPs. In particular for EBPR systems, the diversity of PAOs and GAOs, and of their metabolic activities, in real plants might be much higher than that obtained in lab-scale reactors. Moreover, the enrichments

obtained in the latter can contain a greatly amplified abundance of these specific populations with respect to full-scale systems, thus diluting possible competition and synergetic interactions of flanking populations. In this study, some parameters and concepts had to be adjusted from the models developed by Lopez-Vazquez et al. (2009b) and Oehmen et al. (2010a), as detailed below.

6.3.1.1 Incorporating the TCA cycle stoichiometry

Pijuan et al. (2008) and also in Chapter 5, two EBPR studies concerning full-scale activated sludge, have observed the partial utilisation of the anaerobic TCA cycle to different extents, as shown by the stoichiometric yields, which, as reviewed by Oehmen et al. (2010a), can indicate that different biochemical pathways are being employed. Smolders et al. (1994b) proposed two metabolic models for the anaerobic metabolism of PAOs, one using the TCA cycle and the other employing glycolysis. The anaerobic TCA cycle utilisation is associated with a higher phosphorus release to acetate uptake yield ($Y^{ACC}_{PO4,HAc}$) and a lower glycogen consumption and PHA production per acetate uptake ($Y^{ACC}_{GLY,HAc}$and $Y^{ACC}_{PHA,HAc}$, respectively) than what is observed when using the glycolytic pathway (Smolders et al. (1994b) cf., Table 6.1). In Chapter 5 different degrees of TCA cycle utilisation were observed in different plants, and even in the same plant at different periods in time, which were proportional to the glycogen consumption to acetate uptake yield. Therefore, the incorporation of the TCA cycle metabolism was performed by adjusting the $Y^{ACC}_{PO4,HAc}$and the $Y^{ACC}_{PHA,HAc}$yields such that they are dependent on the $Y^{ACC}_{GLY,HAc}$yields observed in the batch tests.

Another important aspect is that, within the cases where the anaerobic TCA cycle was relevant, a discrepancy was observed between some of the $Y^{ACC}_{PO_4,HAc}$values obtained experimentally and those predicted by the TCA model. The reason for this likely stems from the fact that $Y^{ACC}_{PO_4,HAc}$is dependent on the energetic requirement for acetate uptake across the cell membrane (α_{PAO}), which may vary depending on whether PAOs employ the TCA cycle or glycolysis. Thus, the α_{ACC} value that Smolders et al. (1994b) found using the glycolysis pathway (via a lab-scale culture) is not necessarily transferable to the TCA cycle model. This energetic requirement (α_{ACC}), is known to be dependent on pH (Filipe et al., 2001a; Smolders et al., 1994b) but also on the VFA uptake mechanism of the cell (Oehmen et al., 2010a). Since pH was controlled at 7 in all experiments, we hypothesised that the reason for this occasional discrepancy was due to the α_{ACC} parameter changing as a function of the PAO population, with different *Accumulibacter* sub-groups possessing different VFA uptake mechanisms. Supporting this hypothesis is the fact that a correlation was observed between the α_{ACC} parameter and the abundance of *Accumulibacter* Type I and Type II vs. total *Accumulibacter*. In cases where total *Accumulibacter* correlates closely (between 75 and 100%) with the sum of Type I plus Type II, a higher

α_{ACC}, and consequently a higher $Y^{ACC}_{PO_4,HAc}$was observed. When Type I plus Type II accounted for between 25 and 75% of the total *Accumulibacter* population, the α_{ACC} agrees well with that predicted by the glycolysis model. Interestingly, Zhou et al. (2009) also found a higher $Y^{ACC}_{PO_4,HAc}$value than the one predicted by Smolders et al. (1994b) when the TCA cycle was active, corroborating the results of this study. Therefore, the value of $Y^{ACC}_{PO4,HAc}$ was incorporated in the model (Eqs. 6.7 and 6.8) by introducing a first term that accounts for a higher $Y^{ACC}_{PO4,HAc}$value when the TCA cycle is active instead of glycolysis, due to the necessity to increase the hydrolysis of polyphosphate to accommodate for all the ATP production that is no longer being generated by glycolysis.

The second term defines a new value for α_{ACC}, according to the diversity observed in the *Accumulibacter* population. Furthermore, it is important to note that this increase in ATP requirement for acetate transport was not observed when the glycolysis pathway was active, but only when the TCA cycle was used and the total *Accumulibacter* population was well described by the sum of Type I and Type II. This suggests that different *Accumulibacter* sub-populations possess different acetate uptake mechanisms. Additionally, when comparing the VFA uptake energetic requirements of PAOs and GAOs (cf., Table 6.2), it can be observed that this parameter appears to be dependent on the level of glycolysis activity of the cell, in addition to the VFA uptake mechanism. For instance, PAOs and GAOs are capable of using additional VFA uptake mechanisms besides the direct use of ATP (see Oehmen et al. (2010a) for a review). These mechanisms become more prevalent when higher quantities of glycogen are used by the cell, and thus, they are more significant in GAOs than PAOs. Furthermore, *Defluviicoccus* GAOs have been found to have two simultaneous extra proton motive force (pmf) generation capacities (i.e. through fumarate reductase and methylmalonyl-CoA decarboxylase), while *Competibacter* GAOs only have one (through fumarate reductase) (Burow et al., 2008; Saunders et al., 2007). This correlates very well with the ATP requirement for transport found in these organisms through metabolic models (Filipe et al., 2001a; Oehmen et al., 2006).

$$Y^{ACC}_{PO4,HAc} = \frac{1}{4} + (Y^{ACC\_Gly}_{GLY,HAc} - Y^{ACC\_TCA}_{GLY,HAc}) \times (1 - \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}}) + \alpha^{ACC}_{HAc} \tag{6.7}$$

$$\alpha^{ACC}_{HAc} = -1.1 + 0.19 \times pH + \frac{1}{4}(1 - \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}}) \times f_{ACCI,ACCII} \tag{6.8}$$

where $f_{ACCI,ACCII}$is 1 for a higher coverage of total *Accumulibacter* by Types I and II (75-100% of total *Accumulibacter*) and 0 for a lower coverage (25-75% of total *Accumulibacter*). As previously reported by Smolders et al. (1994b), the range of α is constrained between 0 and 0.5 mol ATP / C-mol acetate uptake. The values of the yields for the two *Accumulibacter* metabolisms (ACC_TCA and ACC_Gly) are stated in Table 6.1.

$Y^{ACC}_{PHA,HAc}$ was defined using a similar strategy as for $Y^{ACC}_{PO_4,HAc}$ (Eq. 6.9), where more PHA is produced via the glycolysis pathway as compared to the TCA cycle per mole of acetate uptake.

$$Y^{ACC}_{PHA,HAc} = Y^{ACC\_TCA}_{PHA,HAc} + (Y^{ACC\_Gly}_{PHA,HAc} - Y^{ACC\_TCA}_{PHA,HAc}) \times \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}} \tag{6.9}$$

6.3.1.2 *Anaerobic maintenance processes*

The batch tests conducted in WWTPs that carried out a chemical phosphate precipitation polishing step, namely DK_1 and DK_2, presented a relatively high anaerobic P release, which could be partially due to anaerobic dissolution of iron-phosphate precipitates caused by iron-reducing bacteria (Nielsen, 1996). For simplicity reasons, it was opted to model this additional P-release as an increased anaerobic maintenance coefficient, instead of adding a new chemical P release process. The anaerobic maintenance coefficient (m^{ANA}_{ACC}) was determined in a blank test without the addition of acetate, and its value was determined by calculating the rate of ATP production from the rate of P release observed during this test (Table 6.5).

6.3.1.3 *Aerobic maintenance processes*

In previous models, the aerobic maintenance was modelled as a function of cell growth, which is indirectly related to PHA degradation (Murnleitner et al., 1997). For this reason, at low PHA concentrations, the model predicted negative growth or cell decay, which contrasted with studies where the endogenous mechanisms of PAOs were characterised (Lopez et al., 2006; Lu et al., 2007). Based on the results of Brdjanovic et al. (1998), Lopez et al. (2006) and Lu et al. (2007), the aerobic maintenance process was expanded to include a sequential dependence on PHA, glycogen and then polyphosphate. These studies have shown that PAOs tend to rely on energy generation from their storage polymers prior to cell decay, an effect that is of high importance particularly in the substrate-limited conditions that normally exist in full-scale WWTPs, unlike lab-scale systems. The three sequential aerobic maintenance processes are defined in Eqs. 6.10 to 6.12.

$$m^{AER}_{ACC,PHA} = m^{AER}_{PHA} \times X_{ACC} \times \frac{X^{ACC}_{PHA}}{X^{ACC}_{PHA} + K^{ACC}_{PHA}} \tag{6.10}$$

$$m^{AER}_{ACC,GLY} = m^{AER}_{GLY} \times X_{ACC} \times \left(1 - \frac{X^{ACC}_{PHA}}{X^{ACC}_{PHA} + K^{ACC}_{PHA}}\right) \times \frac{X^{ACC}_{GLY}}{X^{ACC}_{GLY} + K^{ACC}_{GLY}} \tag{6.11}$$

Table 6.2: Summary of the energy requirement for acetate transport (α) in PAOs and GAOs

	$f_{ACCI,ACCII}$	α	Additional active pmf generating mechanisms	References
ACC_TCA	1	0.5	none	This study, (Zhou et al., 2009)
ACC_TCA	0	0.25	Fumarate reductase?	This study, (García-Martín et al., 2006; Smolders et al., 1994b)
ACC_Gly	-	0.25	Fumarate reductase?	(García-Martín et al., 2006; Smolders et al., 1994b)
Competi	-	0.06	Fumarate reductase	(Filipe et al., 2001a; Saunders et al., 2007)
Defluvi	-	0	Fumarate reductase and Methylmalonyl-CoA decarboxylase	(Burow et al., 2008; Oehmen et al., 2006)

$$m_{ACC,PP}^{AER} = m_{PP}^{AER} \times X_{ACC} \times \left(1 - \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{PHA}^{ACC}}\right) \times \left(1 - \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{GLY}^{ACC}}\right) \times \frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{PP}^{ACC}} \tag{6.12}$$

where, m_{PHA}^{AER}, m_{GLY}^{AER} and m_{PP}^{AER} are the aerobic maintenance coefficients based on PHA, glycogen and polyphosphate respectively. m_{PHA}^{AER} was defined according to Lopez-Vazquez et al. (2009b) (Eq.6.15), while m_{GLY}^{AER} and m_{PP}^{AER} are defined in this study. From Eqs. 6.13 and 6.14, the degradation of one C-mol of glycogen or of one P-mol of polyphosphate produces $\frac{1+\delta}{2}$ or 1 ATP-mol, respectively. These coefficients are proportional to how much glycogen or polyphosphate would be needed to produce the ATP requirements for aerobic maintenance, defined as m_{ATP}^{AER} (Smolders et al., 1994b), hence obtaining the expressions presented in Eqs. 6.16 and 6.17.

Glycogen degradation to produce ATP (C-mol basis):

$$Glycogen + \frac{1}{4}O_2 \rightarrow \frac{2}{3}PHA + \frac{1}{3}CO_2 + \frac{1}{3}H_2O + \frac{1+\delta}{2}ATP \tag{6.13}$$

Polyphosphate degradation to produce ATP:

$$HPO_3 + H_20 \rightarrow ATP + H_3PO_4 \tag{6.14}$$

$$m_{PHA}^{AER} = \frac{12 \times m_{ATP}^{AER}}{6 + 27\delta} \tag{6.15}$$

(cf., Lopez-Vazquez et al., 2009)

$$m_{GLY}^{AER} = \frac{2 \times m_{ATP}^{AER}}{1 + \delta} \tag{6.16}$$

$$m_{PP}^{AER} = m_{ATP}^{AER} \tag{6.17}$$

The stoichiometric matrix for these maintenance coefficients is given in Table 6.3.

It was assumed that the aerobic maintenance processes would be similar in GAOs, as in PAOs, i.e. with the sequential utilisation of PHA and glycogen for aerobic maintenance, excluding of course the process dependant on polyphosphate. However, GAOs aerobic maintenance processes have been far less studied than PAOs, therefore further research is needed to confirm this assumption.

Table 6.3: Stoichiometric matrix for the aerobic maintenance coefficients. For a definition of the different yield coefficients, cf., Appendix A-I

	Processes		
	$m^{AER}_{ACC,PHA}$	$m^{AER}_{ACC,GLY}$	$m^{AER}_{ACC,PP}$
Components	(Lopez-Vazquez et al., 2009b)	this study	this study
S_{O2}	$\frac{Y^{ACC}_{X,HAc}}{Y^{ACC}_{O_2,X}} - 1$	$-\frac{1}{4}$	
S_{PO4}	$i_{BM,P} \times Y^{ACC}_{X,HAc}$		1
X_{ACC}	$-Y^{ACC}_{X,HAc}$		
X^{ACC}_{PHA}		$\frac{2}{3}$	
X^{ACC}_{GLY}		-1	
X^{ACC}_{PP}			-1

6.3.2 Model calibration and application in the different WWTPs

One of the main differences in transposing a model that was developed and validated in lab-scale cultures to WWTP sludge is that the proportion of PAOs and GAOs shifts from high quantities in enriched cultures (normally >60% of the total population), to only a minor fraction of the total community (in this study, usually approximately 5% of the total population, and never surpassing 12%). The strategy used in this study was to input the abundance of each type of organism (*Accumulibacter*, *Competibacter* and *Defluviicoccus*), as determined by quantitative FISH, multiplied by the active biomass concentration (i.e. VSS minus PHA and glycogen, converted to CmM via the biomass formula of $CH_{1.84}O_{0.5}N_{0.19}$ (Zeng et al., 2003d) for the initial biomass fractions of each group (Oehmen et al., 2010b). This step alone yielded a fairly correct description of the chemical transformations, with some adjustment required with respect to the kinetic parameters. In general the rates were slightly lower than the values obtained by (Lopez-Vazquez et al., 2009b), except for glycogen, which had a slightly faster rate than found in lab-scale systems (Table 6.5). After the calibration procedure, the model fitted remarkably well with the experimental results, with error values below 5%, as determined by the NRMSD (Table -6.7).

Similarly to lab-scale studies, the calibration procedure involved the adjustment of the $q^{\max}_{HAc}$, the k_{PHA}, the $k_{PP}$and the k_{GLY} parameters (Table 6.5). Other minor modifications from the lab-scale models are shown in Table 6.4, notably of which was the need to increase the maximal glycogen fraction ($f^{\max}_{GLY}$), which was found to exceed the previously estimated threshold (0.27 C-mol/C-mol as used in Lopez-Vazquez et al. (2009))

when measured in the activated sludge. This parameter was introduced in the model to prevent the prediction of an unrealistically high level of glycogen accumulation (Meijer et al., 2002). However, considering the low quantity of PAOs and GAOs in full-scale sludge, the measured glycogen value might be more highly influenced by e.g. the presence of other hydrolysable sugar-polymers resulting from exopolymeric substances, for example, from the presence of other EBPR bacteria (e.g. *Tetrasphaera* or unidentified populations) or other populations present in the sludge. It is noticeable that despite the fact that glycogen levels are approximately 1 C-mmol/L in sludge from PT_1, the microorganisms hardly consume glycogen anaerobically (Figure 6.2), which could suggest a depletion of their internal storage reserves and that the remaining glycogen quantified resulted from other glucose sources.

A similar effect was observed for PHA levels, which seem to not decrease beneath a value of approximately 0.5 C-mmol/L (Figure 6.2 and Figure 6.3). This suggests there could be other PHA sources from PHA accumulating organisms other than PAOs and GAOs (also suggested by Meijer et al. (2002)). Further, aerobic phosphate uptake seemed to be influenced by a factor other than PHA limitation, since a deceleration was observed even when PHA was still available. This was likely due to the level of poly-P accumulation within PAOs, which is known to affect the P uptake rate (Smolders et al., 1995) and was taken into account by adjusting the initial poly-P concentration in the modelling of some batch tests. Nevertheless, a good correlation was not found between the quantifiable total phosphorus and the adjusted initial polyphosphate concentration of the model. This was likely due to the fact that other organisms besides *Accumulibacter* can also store poly-P, and the impact from chemical P precipitates. The total phosphorus measured was 2 to 3 times higher (30.6 to 61.5 mg-P/g VSS for a 5% enrichment in *Accumulibacter*) than what is expected from other literature results (e.g., Acevedo et al. (2012) obtained 300 mg-P/g VSS for an 80%-enrichment in PAOs). However, a sensitivity analysis in long term simulations (40 d) showed that even when varying the initial concentrations of polyphosphate by 50%, the models converged to the same steady-state conditions (less than 1% error) and therefore this effect is only relevant to accurately describe the start-up conditions.

The calibration of the GAO models necessitated the inclusion of PAOs as well, since in the set of WWTPs modelled, there was no situation where GAOs, either *Competibacter* or *Defluviicoccus*, were present without PAOs. Despite this challenge, the phosphate profile was effectively described by introducing, as initial concentrations, the GAO abundance in the proportions determined by qFISH, confirming once more the success of this methodology. However, although the stoichiometry of glycogen and PHA was generally well described, some discrepancies arose for these two parameters, especially for glycogen, when calibrating the experimental results from PT_2, which could result from the fact that the redox balances did not close in plant PT_2 (Chapter 5)

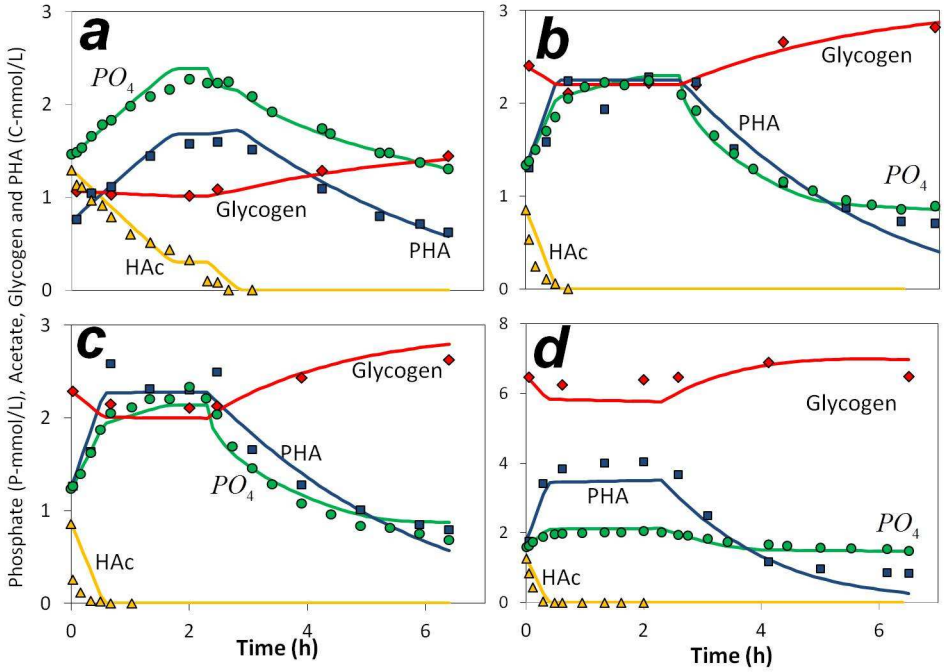

Figure 6.2: Calibration for a) PAOs in PT_1 (winter), b) PAO model to DK_1, c) *Competibacter* and PAOs in DK_1, d) *Defluviicoccus* and PAOs in PT_2 (summer). Experimental data shown for phosphate (circles), PHA (squares), acetate (triangles) and glycogen (diamonds)

Table 6.4: Adjusted parameters during the calibration

		PAO model (Portugal)	PAO model (Denmark)
m_{ACC}^{ANA}	mol-ATP/C-mol/h	2.35[a]	40
$K_{S,PP}^{ANA}$	C-mol/C-mol/h	0.01	0.01
$f_{GLY}^{\max}$	C-mol/C-mol	0.8	0.8

[a] According to Smolders et al. (1994b)

The next step was to apply the calibrated models to other experiments for the same plant and also for different plants. Once more, the strategy of inputting the PAO and GAO abundance as determined by FISH proved very effective in determining the overall cycling of the target parameters (Figure 6.3 and Table 6.7).

When transposing the model to the Danish plants, the anaerobic maintenance coefficient had to be adjusted in order to account for a higher phosphorus release. This was resolved by determining the experimental value of the maintenance coefficient from the phosphorus release in a batch test performed without the feeding of acetate (Table 6.4).

What differed from plant to plant, as well as in experiments within the same plant, were the kinetic parameters presented in Table 6.5. Interestingly, different kinetics were observed in Portuguese plants when comparing summertime (faster) and wintertime (slower) experiments. In Denmark, where experiments were all performed in winter-like conditions, while DK_1 revealed the same kinetics as the summertime Portuguese experiments, DK_2 agreed with the wintertime kinetics (Table 6.5 and Table 6.6), suggesting this effect might not entirely be related to seasonal effects, but could translate either different levels of activity of PAO and GAO cells, or the presence of other PAOs and GAOs besides *Accumulibacter*, *Competibacter* and *Defluviicoccus*. It is noteworthy to mention that the factor by which the anaerobic kinetics changed also generally correlated well with the changes in aerobic kinetics, which is in agreement with the findings from lab-scale studies (Oehmen et al., 2010b).

One exception, however, was the aerobic PHA degradation rate, which remained constant in most experiments (except in DK_2, cf., Table 6.5) and therefore was independent of the overall activity factor discussed above. Since PHA is not an exclusive polymer of PAOs and GAOs, it could be produced or consumed by other organisms present in the sludge, thus influencing the rates observed. Nevertheless, Meijer et al. (2002) already discussed that the model was more sensitive to stoichiometry than to kinetics, in steady state conditions. In fact, when varying the aerobic PHA degradation rate by $\pm$ 50%, a difference was observed in the PHA and in the glycogen levels by 55 and 27% respectively, whereas a difference of less than 10% was observed for growth and for the concentration of phosphate after 40 d, which in fact are the most vital parameters for modelling EBPR. Considering the finding that the k_{PHA}rarely changed, and when it did change an appreciable effect on the prediction of P removal or growth was not observed, it is suggested that this kinetic parameter can be assumed to be constant while $q_{HAc}^{\max}$, k_{PP} and k_{GLY} can be assumed to vary by an identical factor. This could be a useful strategy during future full-scale metabolic model calibration endeavours, thereby avoiding the direct measurement of PHA and glycogen, which are usually not feasible to quantify at full-scale facilities.

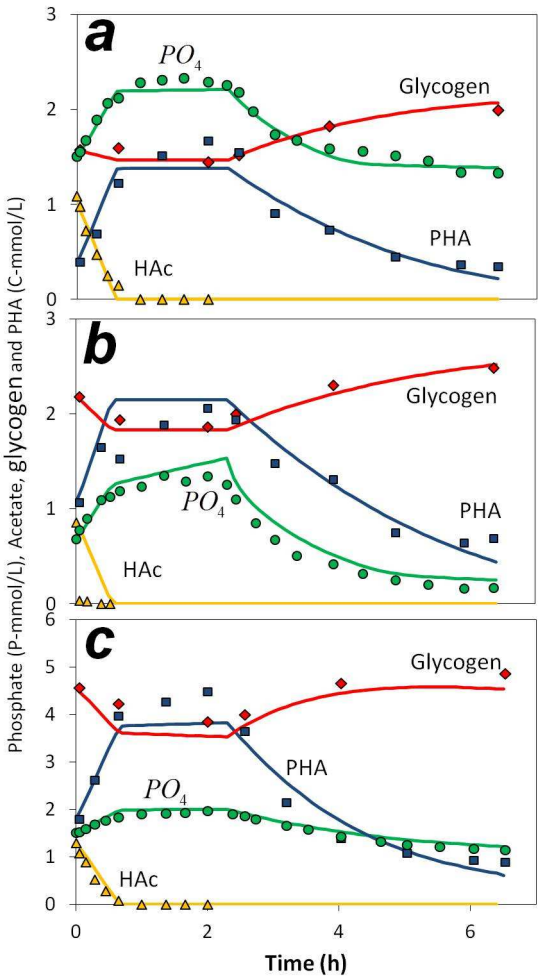

Figure 6.3: Model description of a) and b) PAO model in PT_1 and DK_1, c) PAO and GAO model in PT_2 (winter), with experimental data for phosphate (circles), PHA (squares), acetate (triangles) and glycogen (diamonds)

Table 6.5: Kinetic constants for the PAO model applied to different tests, given as a function of the initially calibrated k_{GLY}, k_{PHA}, k_{PP} and q^{max}_{HAc} for PT_1 (winter)

C-mol/C-mol/h or P-mol/C-mol/h	PT_1 (winter)	PT_1 (summer)	DK_1 (winter)	PT_2 (summer)	PT_2 (winter)	DK_2 (winter)	PAO [a] model
q^{max}_{HAc}	0.15	3 q^{max}_{HAc}	3 q^{max}_{HAc}	3 q^{max}_{HAc}	1 q^{max}_{HAc}	1 q^{max}_{HAc}	0.2
k_{PP}	0.01	3 k_{PP}	3 k_{PP}	3 k_{PP}	1 k_{PP}	1 k_{PP}	0.02
k_{PHA}	0.2	1 k_{PHA}	1 k_{PHA}	1 k_{PHA}	2 k_{PHA}	1 k_{PHA}	0.8
k_{GLY}	0.02	3 k_{GLY}	3 k_{GLY}	3 k_{GLY}	1 k_{GLY}	1 k_{GLY}	0.015

[a] (Lopez-Vazquez et al., 2009b)

Table 6.6: Kinetic constants for the GAO models in applied to different experiments, given as a function of the initially calibrated rates in DK_1 (*Competibacter*) and PT_2_summer (*Defluviicoccus*)

	Calibration		Kinetic adjustment	
	GB	DEF	GB	DEF
C-mol/C-mol/h	DK_1	PT_2 (summer)	PT_2 (winter)	
q^{max}_{HAc}	0.3	0.2	0.5 $q^{max}_{HAc,GB}$	0.4 $q^{max}_{HAc,DEF}$
k_{PHA}	0.3	0.2	1 k^{GB}_{PHA}	0.8 k^{DEF}_{PHA}
k_{GLY}	0.2	0.13	0.5 k^{GB}_{GLY}	0.4 k^{DEF}_{GLY}

Table 6.7: The normalised mean root squared deviation (NRMSD) between the experimental results and model predictions in the different experiments

	NRMSD (%)		
	Glycogen	PHA	PO_4
PAO model			
PT_1 (winter)	2.4	2.4	1.7
PT_1 (summer)	4.5	3.8	1.7
DK_1, exp. 1	3.7	4.1	1.4
DK_1, exp. 2	5.8	5.4	2.4
DK_2	7	3.2	0.6
PAO and GAO models			
DK_1, exp. 3	8.5	3.2	1.5
PT_2 (summer)	28.3	3.8	3.6
PT_2 (winter)	13.3	2.9	2

6.3.3 Competition between PAOs using glycolysis vs. TCA

The additional elements incorporated in this study to the metabolic models developed previously (Lopez-Vazquez et al., 2009b; Oehmen et al., 2010b) were successful in describing the overall transformations observed in activated sludge performing enhanced biological phosphorus removal with acetate as the carbon source. Thus, the model was used as a tool to understand the mechanisms that could lead to an advantage of the TCA metabolism (ACC_TCA) over the glycolysis metabolism (ACC_Gly). It is desirable to understand the factors that lead to the observation of the TCA metabolism in WWTPs, and explain why it is observed at a lesser extent in lab-scale SBRs.

In Chapter 5, it was hypothesised that low carbon substrate levels in the influent, as well as long aeration periods, could lead to a lower availability of glycogen and hence to the use of the TCA cycle over the glycolysis pathway. Simulations were carried out at different acetate concentrations in the influent and also in SBR cycles with different aeration periods (Figure 6.4). Although higher acetate concentrations clearly led to higher overall growth as compared to lower acetate concentrations, the relative proportion of ACC_TCA and ACC_Gly was investigated, and indeed found to vary. The results reveal that at higher substrate concentrations, e.g., at 5 C-mmol/L of acetate in the feed, a situation similar to the conditions used in most lab-scale reactors (e.g., 3.4 C-mmol/L in Acevedo et al. (2012), 4.7 C-mmol/L in Zhou et al. (2009) and 6.25 C-mmol/L in Lopez et al. (2006)), a predominance of the glycolytic metabolism is observed, whereas for lower substrate concentrations, i.e., lower than 2 C-mmol/L, which is closer to the acetate or total volatile fatty acid (VFA) concentration available in WWTPs (Zeng et al., 2006), the TCA metabolism gains an advantage. As seen by the simulations, this advantage likely derives from the fact that the aerobic replenishment of storage polymer is prioritised as compared to growth (Murnleitner et al. 1997), therefore the ACC_Gly will spend more PHA on restoring the consumed glycogen in aerobic conditions than the ACC_TCA. Thus, at lower acetate concentrations, the ACC_TCA metabolism gains an advantage, since less PHA will be used for glycogen production, and therefore, more PHA will be available for growth in comparison with ACC_Gly. Additionally, at lower acetate concentrations, PAOs become limited in glycogen resources and hence, while the ACC_TCA will continue to be able to take up acetate anaerobically, since this process is not dependant on glycogen, the glycolytic metabolism will no longer be able to support acetate uptake. The reason why the glycolysis pathway likely dominates in lab-scale systems, which are commonly operated with higher carbon concentrations, is because ACC_Gly are able to produce more PHA anaerobically than ACC_TCA. At non-limiting acetate concentrations, this higher PHA content will lead to more biomass growth by ACC_Gly than ACC_TCA.

The effect of the aeration phase duration is less pronounced on the selection of one

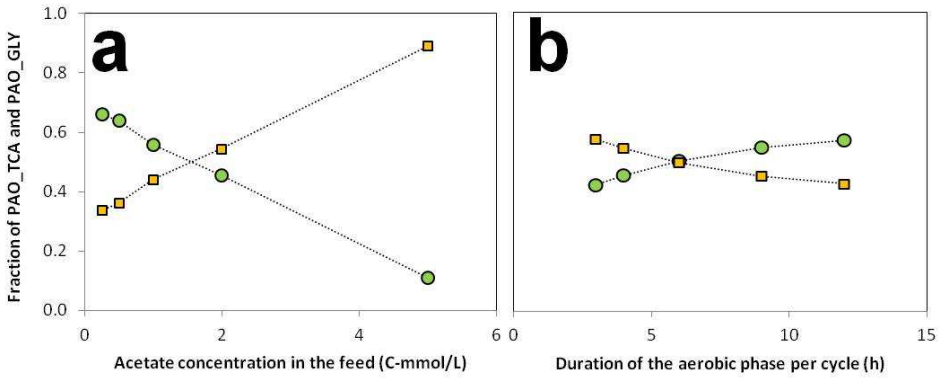

Figure 6.4: Effect of acetate feed concentration (a) and duration of the aerobic phase (b) on the competition between the two metabolisms: TCA cycle (circles) vs. glycolysis (squares). Simulations were run for 40 d using the PT_1 (summer) calibrated model. Simulations in (a) were conducted with an aeration period of 4 h and simulations in (b) were performed at an initial acetate concentration of 2 C-mM

metabolism over the other. However, for aeration periods lasting 3-4 h, which constitute the most common conditions in lab-scale reactors (e.g., 2.7 h to 3.5 h in Lopez et al. (2006), Zhou et al. (2009) and Acevedo et al. (2012)) the glycolytic metabolism is prevalent, whereas for longer aeration periods, such as higher than 6-7 h, which are typical aeration retention times in WWTPs (e.g. the four WWTPs modelled in this study) the TCA metabolism appears more favourable. This effect likely results from glycogen limitation as well, but due instead to the aerobic maintenance processes, which in this model rely on glycogen and then on polyphosphate after PHA depletion, as suggested by Lopez et al. (2006) and Lu et al. (2007). The previous models, which based aerobic maintenance indirectly on PHA through modelling a decrease in the biomass concentration (Murnleitner et al., 1997; Lopez-Vazquez et al., 2009; Oehmen et al., 2010b), predicts aerobic cell decay when PHA levels are depleted, observable even in cycles of 6 h (typical of lab-scale SBRs). This does not describe what is known for PAO's aerobic endogenous metabolism, where cell decay was minimal as compared to glycogen and polyphosphate degradation (Lopez et al., 2006; Lu et al., 2007). Therefore, the model proposed in this study predicts a consumption of glycogen, and eventually, if glycogen levels are depleted, a release of phosphate, which agrees better with what was observed in previous studies (Lopez et al., 2006; Lu et al., 2007).

6.4 CONCLUSIONS

In this study, a metabolic model was applied to describe the activity of PAOs and GAOs in sludge collected from four WWTPs. The model incorporates the anaerobic TCA cycle activity of PAOs in order to describe the observed biochemical transformations, as well as

the modification of previously proposed anaerobic and aerobic maintenance processes. The model was able to predict accurately the experimental results, by defining the initial abundance of PAOs and GAOs with the fraction quantified by FISH. The changes in anaerobic acetate uptake rate amongst the different sludges correlated very well with changes in aerobic P uptake rate and glycogen production rate, while the aerobic PHA kinetics remained largely stable in different plants and for different seasons. This suggests a simplified model calibration procedure that avoids the need for PHA and glycogen measurements. Additionally, it was suggested that the acetate uptake mechanism of *Accumulibacter* varies according to the different clades, with differences observed in the energetic requirements for acetate uptake depending on the *Accumulibacter* diversity. Long-term simulations using the model showed that low carbon substrate concentrations in the feed and long aerobic phases lead to an advantage of the anaerobic TCA metabolism over the glycolysis metabolism, which would explain the higher importance of this metabolism in WWTPs as opposed to what is most commonly observed in lab-scale reactors. This study illustrated how metabolic modelling could be used as a research tool to test hypotheses and to determine new metabolic properties of *Accumulibacter*, particularly with respect to its acetate uptake mechanism. It was found to be a robust and simplified tool to describe and predict with relative ease the EBPR metabolism observed in full-scale WWTPs. Further research should be conducted to integrate this improved version of the metabolic model with ASM models in order to describe the operation of biological nutrient removal plants in steady-state or dynamic conditions.

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7 Conclusions and future work

Summary *This chapter presents some of the main overall conclusions that resulted from this thesis. In addition, some aspects that could be addressed in the future are discussed.*

This work provided several contributions that constitute another step forward towards an improved understanding of microbial EBPR communities and mechanisms in full-scale WWTPs.

First of all, special attention was paid to the analysis and quantification of internal carbon storage polymers (glycogen and PHA), since they constitute a vital resource for EBPR bacteria and also since they are related to the overall phosphorus and carbon removal performance. It was made clear that these methods depend on the structure of the biomass, whether it is aggregated in flocs or in granules, and for PHA, on the type of monomers composing the co-polymer. Therefore, the application of these methods should consider the type of system, the composition of the polymer and the type of biomass. Once a reliable method for PHA and glycogen quantification was established, it was possible to characterise the identity and the performance of the EBPR microbial communities in activated sludge collected from six different WWTPs. PAOs were present in all plants with similar abundances, whereas GAOs were only systematically observed in two plants, one in Portugal and one in Denmark, which suggested that a warmer climate was not the sole factor for GAO selection. Parallel aerobic and anoxic batch tests showed different levels of activity of denitrifying PAOs in the WWTPs tested. Anaerobic batch tests with acetate revealed that the anaerobic metabolism relied, to different extents, on the anaerobic utilisation of the TCA cycle and glycolysis, hence demonstrating a metabolic diversity from plant to plant and even within the same plant but at different periods.

The incorporation of the TCA cycle in metabolic models was successfully used to describe the experimental data of the anaerobic-aerobic batch tests conducted with sludge from the different WWTPs. Interestingly, through describing the anaerobic metabolism of PAOs and correlating it with the abundance of organisms as quantified by FISH, different acetate uptake mechanisms in different Types of PAOs were revealed. By simulating the TCA metabolism in PAOs, vs. the glycolysis mechanism, in addition to the adjustment of the aerobic maintenance processes, the model described well the experimental data obtained with full-scale sludge. In long-term simulations, the anaerobic TCA metabolism was shown to be favoured by lower acetate concentrations and longer aeration phases. This explains why the TCA metabolism was observed to a greater extent in full-scale systems as opposed to glycolysis, more frequently observed in lab-scale enriched cultures.

After more than 30 years of research into the operational, microbiological and metabolic aspects of EBPR, there is an undisputable solid foundation of knowledge on this process, which has allowed for the development of new configurations and control strategies to improve its operation. So far, engineers and microbiologists have been assembling information that forms mainly a two-dimensional plot, where one axis is the identity of the organisms and the other their metabolism. Recent work, and this study is no exception, has been expanding the limits of these axes by exploring the diversity within

the PAO and GAO groups, by studying new organisms, or by finding a higher diversity within the ones already known. Recent findings suggest that different organisms possess different metabolic capacities, therefore future work should continue this line of investigation and use advanced microbiological or molecular biology techniques to clarify the phylogeny and activity of PAOs and GAOs. On the other hand, there is evidence that the EBPR-related microorganisms might have more complex and adaptable metabolisms than initially thought and therefore, further research should address the behaviour of EBPR in limiting conditions, stress conditions and continuously dynamic conditions, such as the ones encountered in real systems. The so called "omic" studies could provide useful insight on the metabolic versatility of PAOs and GAOs by identifying their genetic potential, as well as different levels of gene expression in response to operational conditions.

While these two axes should continue to expand, a more complicated task will be to incorporate a third dimension related to the ecophysiology of the microorganisms. This activity should be indirectly related to interactions between the EBPR microbial community with the other communities and with system's conditions, in regard to the operational parameters and to processes such as predation, competition and symbiotic interactions. To do this, experiments will have to incorporate higher levels of complexity, for instance by using more complex substrates such as real wastewater, and will need more powerful analytical tools that can measure in real time the cycling of components in different organisms and their reaction to dynamic conditions.

As the complexity of the information grows, it will be necessary to employ sophisticated mathematical and statistical tools to interpret and integrate the results. Metabolic modelling, alone or in conjunction with ASM models, will most likely play a strong role in this task, since it will be able to compile high amounts of interactions, while still requiring simple and easily obtainable inputs. However, as the models become more complete, the challenge is to keep them easy to use. Therefore, a very important aspect will be to critically judge when will the resolution be enough. This question may have two answers, according to the specific goals in mind: a lower resolution could be enough for describing real systems and a higher resolution could be extremely useful to use the model as a tool to understand particular aspects of the process.

A tool that is emerging in many sciences is augmented reality, a virtual tool that allows to modify by computer software the perception of reality. Rosling (2010), a Swedish medical doctor and statistician, in his work "200 Countries, 200 Years, 4 Minutes" clearly demonstrated the power of integrating in a virtual environment large quantities of data, in both number and complexity, and extracting very visual and clear interactions. It could be extremely interesting to compile the information available so far, on ecology, metabolic diversity, microbial identity and interactions in activated sludge, in such a virtual environment, to simulate the microbial interactions in a WWTP. This could potentially be a

multidisciplinary project, involving experts from the different areas relevant to biotechnological processes and wastewater treatment, entitled "2 million bugs, 200 days, 4 tanks (AN/AX/AE/settler)" where the goal would be to define the new gaps in knowledge and new possibilities in concepts such as symbiosis, competition, adaptability and resilience. This could provide interesting new concepts that could potentially be used to find new treatment systems or even be transferable to other areas such as health or even to human sociology.

Furthermore, future work should definitely address public perception and that is not only the role of the science promoters or communicators, but it is the role of each scientist. Due to the emergent phosphorus crisis, with potentially dramatic economical, social and political consequences, and to the even more drastic water crisis, it is important to debate what contributions could be made to revolutionise the role of WWTPs, in order to further promote the paradigm "from waste to value" and transform WWTPs in a centre for value and for strategic resources.

REFERENCES

Rosling, H (2010). *200 countries, 200 years, 4 minutes*. URL: http://www.youtube.com/watch?v=jbkSRLYSojo.

Appendices - Structure of the metabolic model in Chapter 6

Appendix A: Stoichiometric matrix for *Accumulibacter*-PAOs

		Process	Components 1 S_{O2}	2 S_{HAc}	3 S_{PO4}	4 X_{ACC}	5 X_{PHA}^{ACC}	6 X_{GLY}^{ACC}	7 X_{PP}^{ACC}
		Accumulibacter							
Anaerobic Processes PAO	**1**	Anaerobic acetate uptake Gycolysis		-1	$Y_{PO4,HAc}^{ACC\_Gly}$		$Y_{PHA,HAc}^{ACC\_Gly}$	$-Y_{GLY,HAc}^{ACC\_Gly}$	$-Y_{PP,HAc}^{ACC\_Gly}$
	2	Anaerobic acetate uptake TCA			$Y_{PO4,HAc}^{ACC\_TCA}$		$Y_{PHA,HAc}^{ACC\_TCA}$	$-Y_{GLY,HAc}^{ACC\_TCA}$	$-Y_{PP,HAc}^{ACC\_TCA}$
	3	Anaerobic maintenance on polyphosphate			1				-1
	4	Anaerobic maintenance on glycogen					$\frac{5}{6}$	-1	
Aerobic Processes PAO	**5**	Aerobic PHA degradation	$-Y_{O_2,PHA}^{ACC}$		$-Y_{PO_4,X}^{ACC}$	$Y_{X,PHA}^{ACC}$	-1		
	6	Aerobic glycogen production	$Y_{O_2,GLY}^{ACC}$		$Y_{PO_4,GLY}^{ACC}$	$-Y_{GLY,X}^{ACC}$		1	
	7	Aerobic Poly-P formation	$Y_{O_2,PP}^{ACC}$		$-Y_{PO_4,PP}^{ACC}$	$-Y_{PP,X}^{ACC}$			1

		Process	Components 1 S_{O2}	2 S_{HAc}	3 S_{PO4}	4 X_{ACC}	5 X_{PHA}^{ACC}	6 X_{GLY}^{ACC}	7 X_{PP}^{ACC}
	Accumulibacter (cont.)								
Aerobic Processes PAO (cont.)	**8**	Aerobic maintenance on PHA	$\frac{Y_{X,HAc}^{ACC}}{Y_{O_2,X}^{ACC}} - 1$		$i_{BM,P} \times Y_{X,HAc}^{ACC}$	$-Y_{X,HAc}^{ACC}$			
	9	Aerobic maintenance on glycogen	$-\frac{1}{4}$				$\frac{2}{3}$	-1	
	10	Aerobic maintenance on polyphosphate			1				-1

Appendix B: Stoichiometric matrix for GAOs: *Competibacter and Defluvicoccus*

		Process	Components 1 S_{O2}	2 S_{HAc}	3 S_{PO4}	8 X_{GB}	9 X_{PHA}^{GB}	10 X_{GLY}^{GB}
		Competibacter (GB)						
Anaerobic Processes GB	**11**	Anaerobic acetate uptake		-1			$Y_{PHA,HAc}^{GB}$	$-Y_{GLY,HAc}^{GB}$
	12	Anaerobic maintenance					$\frac{5}{6}$	-1
Aerobic Processes GB	**13**	Aerobic PHA degradation	$-Y_{O_2,PHA}^{GB}$		$-Y_{PO_4,X}^{GB}$	$Y_{X,PHA}^{GB}$	-1	
	14	Aerobic glycogen production	$Y_{O_2,GLY}^{GB}$		$Y_{PO_4,GLY}^{GB}$	$-Y_{GLY,X}^{GB}$		1
	15	Aerobic maintenance on PHA	$\frac{Y_{HAc,X}^{GB}}{Y_{O_2,X}^{GB}}-1$		$i_{BM,P}\times Y_{X,HAc}^{GB}$	$-Y_{X,HAc}^{GB}$		
	16	Aerobic maintenance on glycogen	$-\frac{1}{4}$				$\frac{2}{3}$	-1

Appendix B (cont): Stoichiometric matrix for GAOs: *Competibacter and Defluvicoccus*

			Components					
			1	**2**	**3**	**11**	**12**	**13**
		Process	S_{O2}	S_{HAc}	S_{PO4}	X_{DEF}	X_{PHA}^{DEF}	X_{GLY}^{DEF}
		Defluviicoccus (DEF)						
Anaerobic Processes DEF	**17**	Anaerobic acetate uptake		-1			$Y_{PHA,HAc}^{DEF}$	$-Y_{GLY,HAc}^{DEF}$
	18	Anaerobic maintenance					$\frac{5}{6}$	-1
Aerobic Processes DEF	**19**	Aerobic PHA degradation	$-Y_{O_2,PHA}^{DEF}$		$-Y_{PO_4,X}^{DEF}$	$Y_{X,PHA}^{DEF}$	-1	
	20	Aerobic glycogen production	$Y_{O_2,GLY}^{DEF}$		$Y_{PO_4,GLY}^{DEF}$	$-Y_{X,GLY}^{DEF}$		1
	21	Aerobic maintenance on PHA	$\frac{Y_{HAc,X}^{DEF}}{Y_{O_2,X}^{DEF}}-1$		$i_{BM,P}\times Y_{X,HAc}^{DEF}$	$-Y_{X,HAc}^{DEF}$		
	22	Aerobic maintenance on glycogen	$-\frac{1}{4}$				$\frac{2}{3}$	-1

Appendix C: Anaerobic and aerobic yields for *Accumulibacter, Competibacter* and *Defluviicoccus*

Accumulibacter

$$Y_{O_2,PHA}^{ACC} = \frac{Y_{X,PHA}}{Y_{O_2,X}}$$

$$Y_{O_2,GLY}^{ACC} = \frac{Y_{X,PHA}}{Y_{O_2,X} \times Y_{GLY,PHA}} - \frac{1}{Y_{O_2,GLY}}$$

$$Y_{O_2,PP}^{ACC} = \frac{Y_{X,PHA}}{Y_{O_2,X} \times Y_{PP,PHA}} - \frac{1}{Y_{O_2,PP}}$$

$$Y_{PO_4,X}^{ACC} = i_{BM,P} \times Y_{X,PHA}$$

$$Y_{PO_4,GLY}^{ACC} = \frac{i_{BM,P} \times Y_{X,PHA}}{Y_{GLY,PHA}}$$

$$Y_{PO_4,PP}^{ACC} = \frac{i_{BM,P} \times Y_{X,PHA}}{Y_{PP,PHA}} - 1$$

$$Y_{GLY,X}^{ACC} = \frac{Y_{X,PHA}}{Y_{GLY,PHA}}$$

$$Y_{PP,X}^{ACC} = \frac{Y_{X,PHA}}{Y_{PP,PHA}}$$

Competibacter

$$Y_{O_2,PHA}^{GB} = \frac{Y_{X,PHA}}{Y_{O_2,X}}$$

$$Y_{O_2,GLY}^{GB} = \frac{Y_{X,PHA}}{Y_{O_2,X} \times Y_{GLY,PHA}} - \frac{1}{Y_{O_2,GLY}}$$

$$Y_{PO_4,X}^{GB} = i_{BM,P} \times Y_{X,PHA}$$

$$Y_{PO_4,GLY}^{GB} = \frac{i_{BM,P} \times Y_{X,PHA}}{Y_{GLY,PHA}}$$

$$Y_{GLY,X}^{GB} = \frac{Y_{X,PHA}}{Y_{GLY,PHA}}$$

D. vanus related GAOs

$$Y_{O_2,PHA}^{DEF} = \frac{Y_{X,PHA}}{Y_{O_2,X}}$$

$$Y_{O_2,GLY}^{DEF} = \frac{Y_{X,PHA}}{Y_{O_2,X} \times Y_{GLY,PHA}} - \frac{1}{Y_{O_2,GLY}}$$

$$Y_{PO_4,X}^{DEF} = i_{BM,P} \times Y_{X,PHA}$$

$$Y_{PO_4,GLY}^{DEF} = \frac{i_{BM,P} \times Y_{X,PHA}}{Y_{GLY,PHA}}$$

$$Y_{GLY,X}^{DEF} = \frac{Y_{X,PHA}}{Y_{GLY,PHA}}$$

Appendix C (cont.): Anaerobic and aerobic yields for *Accumulibacter, Competibacter* and *Defluviicoccus*

Where,

$$Y_{X,PHA} = \frac{250(106\lambda + 127\beta) \times (6\lambda + 27\lambda\delta + 8\beta + 30\beta\delta)}{201930\lambda + 318000K_1\lambda + 678771\lambda\delta + 813435\beta\delta + 269240\beta + 381000K_2\beta}$$

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

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

$$Y_{O_2,X} = \left(\frac{\mathrm{Re}\, doxPHA}{4 \times Y_{X,PHA}} - \frac{\mathrm{Re}\, doxBM}{4} \right)^{-1}$$

$$Y_{O_2,PP} = \left(\frac{\mathrm{Re}\, doxPHA}{4 \times Y_{PP,PHA}} \right)^{-1}$$

$$Y_{O_2,GLY} = \left(\frac{\mathrm{Re}\, doxPHA}{4 \times Y_{GLY,PHA}} - 1 \right)^{-1}$$

Appendix D. Anaerobic stoichiometric parameters of *Accumulibacter*, *Competibacter* and *Defluviicoccus*.

Parameter	Value	Units	Description	Source
$Y_{PHA,HAc}^{ACC\_Gly}$	1.33	C-mol	PHA stored per C-mol acetate taken up (Glycolysis)	Smolders et al. (1994a)
$Y_{PHA,HAc}^{ACC\_TCA}$	0.89	C-mol	PHA stored per C-mol acetate taken up (TCA)	Smolders et al. (1994a)
$Y_{GLY,HAc}^{ACC\_Gly}$	0.5	C-mol	Glycogen consumed per C-mol acetate taken up (Glycolysis)	Smolders et al. (1994a)
$Y_{GLY,HAc}^{ACC\_TCA}$	0	C-mol	Glycogen consumed per C-mol acetate taken up (TCA)	Smolders et al. (1994a)
$Y_{PP,HAc}^{ACC\_Gly}$	$0.25 + \alpha_{ACC}$	P-mol	Poly-P consumed per C-mol acetate taken up (Glycolysis)	Smolders et al. (1994a)
$Y_{PP,HAc}^{ACC\_TCA}$	$0.5 + \alpha_{ACC}$	P-mol	Poly-P consumed per C-mol acetate taken up (TCA)	Smolders et al. (1994a)
$\alpha_{ACC,HAc}$	$0.19 \cdot pH - 1.1$	ATP-mol	ATP necessary to transport C-mol acetate through cell membrane	Smolders et al. (1994a)
$Y_{PHA,HAc}^{GB}$	$\left(1.75 + \frac{5}{3}\alpha\right) X_{GB,PHA}$	C-mol	PHA stored per C-mol acetate taken up	Zeng et al. (2003b)
$Y_{GLY,HAc}^{GB}$	$1 + 2 \cdot \alpha_{GB,Ac}$	C-mol	Glycogen consumed per C-mol acetate taken up	Filipe et al. (2001)
$\alpha_{GB,HAc}$	$0.057 \cdot pH - 0.34$	ATP-mol	ATP necessary to transport C-mol acetate through cell membrane	Filipe et al. (2001)
$Y_{PHA,HAc}^{DEF}$	$\left(1.75 + \frac{5}{3}\alpha\right) X_{DEF,PHA}$	C-mol	PHA stored per C-mol acetate taken up	Zeng et al. (2003b)
$Y_{GLY,HAc}^{DEF}$	$1 + 2 \cdot \alpha_{DEF,Ac}$	C-mol	Glycogen consumed per C-mol acetate taken up	Filipe et al. (2001)
$\alpha_{DEF,HAc}$	$0.057 \cdot pH - 0.34$	ATP-mol	ATP necessary to transport C-mol acetate through cell membrane	Filipe et al. (2001)

Appendix E. Aerobic parameters for *Accumulibacter,* GB, and DEF.

Parameter	PAO or GAO	Units	Description	Reference
m_{PHA}^{AER}	$\frac{12 m_{ATP}^{AER}}{6+27\delta}$ *where* $m_{ATP}^{AER} = 0.019$ ATP-mol/(C-mol.h)	C-mol/(C-mol · h)	Aerobic maintenance coefficient on PHA	Smolders et al. (1994b);
m_{GLY}^{AER}	$\frac{2 \times m_{ATP}^{AER}}{1+\delta}$	C-mol/(C-mol · h)	Aerobic maintenance coefficient on glycogen	this study
m_{PP}^{AER}	m_{ATP}^{AER}	C-mol/(C-mol · h)	Aerobic maintenance coefficient on polyphosphate	this study
δ (Y_{NADH_ATP})	1.85	ATP-mol/NADH-mol	ATP produced per NADH oxidized (Aerobic P/O ratio)	Smolders et al. (1994b)
K_1	1.7	ATP-mol/C-mol	ATP needed for biomass synthesis from Acetyl-CoA*	Zeng et al. (2003a)
K_2	1.38	ATP-mol/C-mol	ATP needed for biomass synthesis from Propionyl-CoA*	Zeng et al. (2003a)
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. (2003a);
λ	$a+(2b/5)$	C-mol/C-mol	Percentage of Acetyl-CoA* in PHA	Zeng et al. (2003a);
β	$c+(3b/5)$	C-mol/C-mol	Percentage of Propionyl-CoA* in PHA	Zeng et al. (2003a);
a	X_{PHB}/X_{PHA}	C-mol/C-mol	PHB fraction in PHA	Zeng et al. (2003a);
b	X_{PHV}/X_{PHA}	C-mol/C-mol	PHV fraction in PHA	Zeng et al. (2003a);
c	X_{PH2MV}/X_{PHA}	C-mol/C-mol	PH2MV fraction in PHA	Zeng et al. (2003a);
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 F. Kinetic expressions for *Accumulibacter* (Meijer et al., 2002; Oehmen et al., 2010; Lopez-Vazquez et al., 2009).

	Process	Expression	Switching functions
1	Anaerobic acetate uptake	$q_{HAc}^{ACC} \cdot \frac{S_{HAc}}{S_{HAc} + K_{S,HAc}} \cdot X_{ACC}$	$\frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{S,PP}} \cdot \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{S,GLY}} \cdot \frac{f_{PHA,\max} - f_{PHA}^{ACC}}{f_{PHA,\max} - f_{PHA}^{ACC} + K_{s,fPHA}}$
2	Anaerobic maintenance on polyphosphate	$m_{ACC}^{ANA} \cdot X_{ACC}$	$\frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{S,PP}}$
3	Anaerobic maintenance on glycogen	$m_{ACC}^{ANA} \cdot X_{ACC}$	$\frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{S,GLY}} \times \left(1 - \frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{S,PP}}\right)$
4	Aerobic PHA degradation	$q_{PHA}^{AER,ACC} \cdot f_{PHA}^{ACC2/3} \cdot X_{ACC}$	$\frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{S,fPHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
5	Aerobic glycogen production	$q_{GLY}^{AER,ACC} \cdot f_{PHA}^{ACC2/3} \cdot \frac{1}{f_{GLY}^{ACC}} \cdot X_{ACC}$	$\frac{f_{GLY,\max}^{ACC} - f_{GLY}^{ACC}}{f_{GLY,\max}^{ACC} - f_{GLY}^{ACC} + K_{S,GLY}} \cdot \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
6	Aerobic Poly-P formation	$q_{PP}^{AER,ACC} \cdot \frac{1}{f_{PP}^{ACC}} \cdot X_{ACC}$	$\frac{f_{PP,\max}^{ACC} - f_{PP}^{ACC}}{f_{PP,\max}^{ACC} - f_{PP}^{ACC} + K_{S,PP}} \cdot \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{S,PHA}} \cdot \frac{S_{S,PO4}}{S_{S,PO4} + K_{S,PO_4}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
7	Aerobic maintenance on PHA	$m_{ACC,PHA}^{AER} \cdot X_{ACC}$	$\frac{S_{O2}}{S_{O2} + K_{S,O2}}$
8	Aerobic maintenance on Glycogen	$m_{ACC,GLY}^{AER} \times X_{ACC}$	$\left(1 - \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{PHA}^{ACC}}\right) \times \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{GLY}^{ACC}}$
9	Aerobic maintenance on polyphosphate	$m_{ACC,PP}^{AER} \times X_{ACC}$	$\left(1 - \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{S,PHA}^{ACC}}\right) \times \left(1 - \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{S,GLY}^{ACC}}\right) \times \frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{S,PP}^{ACC}}$

Appendix G. Kinetic expressions for *Competibacter* (GB) (Lopez-Vazquez et al., 2009; Oehmen et al., 2010).

	Process	Expression	Switching functions
1	Anaerobic acetate uptake	$q_{HAc}^{GB} \cdot \frac{S_{HAc}}{S_{HAc} + K_{S,HAc}} \cdot X_{GB}$	$\frac{X_{GLY}^{GB}}{X_{GLY}^{GB} + K_{S,GLY}} \cdot \frac{f_{PHA,\max} - f_{PHA}^{GB}}{f_{PHA,\max} - f_{PHA}^{GB} + K_{s,fPHA}}$
2	Anaerobic maintenance on glycogen	$m_{GB}^{ANA} \cdot X_{GB}$	$\frac{X_{GLY}^{GB}}{X_{GLY}^{GB} + K_{S,GLY}}$
3	Aerobic PHA degradation	$q_{PHA,OX}^{GB} \cdot f_{PHA}^{GB\ 2/3} \cdot X_{GB}$	$\frac{X_{PHA}^{GB}}{X_{PHA}^{GB} + K_{S,fPHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
4	Aerobic glycogen production	$q_{GLY,OX}^{GB} \cdot f_{PHA}^{GB\ 2/3} \cdot \frac{1}{f_{GLY}^{GB}} \cdot X_{GB}$	$\frac{f_{GLY,\max}^{GB} - f_{GLY}^{GB}}{f_{GLY,\max}^{GB} - f_{GLY}^{GB} + K_{S,GLY}} \cdot \frac{X_{PHA}^{GB}}{X_{PHA}^{GB} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
5	Aerobic maintenance on PHA	$m_{GB,PHA}^{AER} \cdot X_{GB}$	$\frac{S_{O2}}{S_{O2} + K_{S,O2}}$
6	Aerobic maintenance on Glycogen	$m_{GB,GLY}^{AER} \times X_{GB}$	$\left(1 - \frac{X_{PHA}^{GB}}{X_{PHA}^{GB} + K_{S,PHA}^{GB}}\right) \times \frac{X_{GLY}^{GB}}{X_{GLY}^{GB} + K_{S,GLY}^{GB}}$

Appendix H. Kinetic expressions for *Defluviicoccus*-GAO (DEF) (Lopez-Vazquez et al., 2009; Oehmen et al., 2010).

	Process	Expression	Switching functions
1	Anaerobic acetate uptake	$q_{HAc}^{DEF} \cdot \frac{S_{HAc}}{S_{HAc} + K_{S,HAc}} \cdot X_{DEF}$	$\frac{X_{GLY}^{DEF}}{X_{GLY}^{DEF} + K_{S,GLY}} \cdot \frac{f_{PHA,\max} - f_{PHA}^{DEF}}{f_{PHA,\max} - f_{PHA}^{DEF} + K_{s,fPHA}}$
2	Anaerobic maintenance on glycogen	$m_{DEF}^{ANA} \cdot X_{DEF}$	$\frac{X_{GLY}^{DEF}}{X_{GLY}^{DEF} + K_{S,GLY}}$
3	Aerobic PHA degradation	$q_{PHA,OX}^{DEF} \cdot f_{PHA}^{DEF\,2/3} \cdot X_{DEF}$	$\frac{X_{PHA}^{DEF}}{X_{PHA}^{DEF} + K_{S,fPHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
4	Aerobic glycogen production	$q_{GLY,OX}^{DEF} \cdot f_{PHA}^{DEF\,2/3} \cdot \frac{1}{f_{GLY}^{DEF}} \cdot X_{DEF}$	$\frac{f_{GLY,\max}^{DEF} - f_{GLY}^{DEF}}{f_{GLY,\max}^{DEF} - f_{GLY}^{DEF} + K_{S,GLY}} \cdot \frac{X_{PHA}^{DEF}}{X_{PHA}^{DEF} + K_{S,PHA}} \cdot \frac{S_{O2}}{S_{O2} + K_{S,O2}}$
5	Aerobic maintenance on PHA	$m_{DEF,PHA}^{AER} \cdot X_{DEF}$	$\frac{S_{O2}}{S_{O2} + K_{S,O2}}$
6	Aerobic maintenance on Glycogen	$m_{DEF,GLY}^{AER} \times X_{DEF}$	$\left(1 - \frac{X_{PHA}^{DEF}}{X_{PHA}^{DEF} + K_{S,PHA}^{DEF}}\right) \times \frac{X_{GLY}^{DEF}}{X_{GLY}^{DEF} + K_{S,GLY}^{DEF}}$

Appendix I. Kinetic coefficients for PAOs and GAOs.

Kinetic coefficient	Description	Value	Units	Source
$K_{S,Ac}$	Half-saturation coefficient for acetate	0.001	C-mmol/L	Oehmen et al. (2010)
$K_{S,PHA}$	Half-saturation coefficient for PHA	0.01	C-mmol/L	Oehmen et al. (2010)
$K_{S,GLY}$	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 or GAO biomass concentration	1.00	C-mol/C-mol	Oehmen et al. (2010)
$f_{PAO,GLY,\max}$	Maximum glycogen content per PAO biomass concentration	0.80	C-mol/C-mol	Smolders et al. (1995)
$f_{GB,GLY,\max}$	Maximum glycogen content per *Competibacter* biomass concentration	0.35	C-mol/C-mol	Lopez-Vazquez et al. (2008)
$f_{DEF,GLY,\max}$	Maximum glycogen content per *Defluviicoccus* biomass concentration	0.35	C-mol/C-mol	Lopez-Vazquez et al. (2008)
$m_{ACC,ATP}^{ANA}$	ATP necessary for anaerobic maintenance purposes of PAOs	2.35	P-mmol / (C-mol · h)	Smolders et al. (1994a), Brdjanovic et al. (1998)
$m_{GB,ATP}^{ANA}$ & $m_{DEF,ATP}^{ANA}$	ATP necessary for anaerobic maintenance purposes of GAOs	4.70	C-mmol / (C-mol · h)	Zeng et al. (2003a) Lopez-Vazquez et al. (2007)

Appendix J. References for the Supplementary Material.

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