Universitat Autònoma de Barcelona

ESCOLA TÈCNICA SUPERIOR D'ENGINYERIA

DEPARTAMENT D'ENGINYERIA QUÍMICA

EFFECT OF DIFFERENT CARBON SOURCES AND CONTINUOUS AEROBIC CONDITIONS ON THE EBPR PROCESS

Maite Pijuan Vilalta

PhD Thesis
October 2004

CARLES CASAS ALVERO, professor titular i JUAN ANTONIO BAEZA LABAT, professor lector, del Departament d'Enginyeria Química de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que la Llicenciada en Ciències Ambientals Maite Pijuan Vilalta ha realitzat sota la nostra direcció, el treball que amb títol "Effect of different carbon sources and continuous aerobic conditions on the EBPR process", es presenta en aquesta memòria, i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, presentem a l'Escola Superior d'Enginyeries de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a

Bellaterra, Octubre 2004

Dr. Carles Casas Alvero Dr.Juan Antonio Baeza Labat

"It is good to have an end to the journey towards;
but it is the journey that matters, in the end"

U.K. Le Guin

AGRAÏMENTS / ACKNOWLEDMENTS

No m'agradaria enquadernar aquesta tesi sense donar les gràcies a tots aquells que m'han ajudat en algun moment d'aquest treball tant a nivell científic com anímic. Gràcies especialment,

- Als meus dos directors, Juan i Carles; vam començar un tema nou amb tot el que això comporta. Espero que us ho hagueu passat tant bé com jo durant aquests anys.
- A en Javier, per apostar per mi tot i no conèixer-me i donar-me ànims en tot moment.
- A en Guisa, per totes les discussions científiques i les que no ho han estat. He après molt de tu i amb tu. Gràcies per la teva ajuda.
- A en Julián, David, Maria, Irene, Bartro i la resta de "Depuradors", per deixar-me aprendre una miqueta de cadascun de vosaltres.
- A la Rosi, por estar siempre dispuesta a ayudarme con los miles de tubitos. Gracias.
- A la gent que ha hagut d'escoltar algun que altre cop les meves cabòries: la Suau, la Paqui, la Neus, la Núria, en Resina, en Ramon, n'Ernest, la Luci. Gràcies per la vostra amistat.

I would like also to thank some people who I met when I was abroad:

- Linda, to give me the oportunity to work with you and to come to Aussie land twice.
- Aaron and Adrian, the two big guys in the P-team. I learnt a lot from both of you and I am not thinking just in science.
- Gildinya, una gran científica y amiga. Tu vitalidad contagia. Teia, Rikkie, Valeria, Chris... you help me to feel like home.

I per últim voldria donar les gràcies als meus pares i al Jaume. Jo tinc molt clar que sense vosaltres no estaria aquí. Un petó.

FINANCIAL SUPPORT

The author acknowledges ‘Generalitat de Catalunya’ for the grant FI0036. This work was supported by the “Comisión interministerial de ciencia y tecnología” (CICYT), project REN2000-0670/TECNO. The Department of Chemical Engineering of UAB is the Biochemical Engineering Unit of the Centre de Referència en Biotecnologia (CeRBa) of Generalitat de Catalunya.

TABLE OF CONTENTS

STATEMENT INDICATING THE PARTS OF THE THESIS THAT ARE NOT MY SOLE ORIGINAL WORK…….. I

LIST OF PUBLICATIONS AND AUTHOR'S CONTRIBUTION…….……... II

LIST OF ABBREVIATIONS……..……. IV

CHAPTER I: INTRODUCTION……. 1

CHAPTER II: LITERATURE REVIEW……...…….. 3

2.1. INTRODUCTION……... 3

2.2. ROLE OF PHOSPHORUS IN THE ENVIRONMENT……. 3

2.2.1. Natural conditions……... 3

2.2.2. Polices to control phosphate polution……. 4

2.3. ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL…….. 6

2.3.1. The mecanism underlying the EBPR…….. 6

2.3.2. Influence of the carbon source in the EBPR process…….. 10

2.3.3. PHA composition depending on substrate addition..…….. 14

2.3.4. Simultaneous acceptor and electron donor in the EBPR system…….…….. 16

2.4. REFERENCES…… 17

CHAPTER III: MATERIALS AND METHODS…….…… 23

3.1. CHEMICAL ANALYSES…….. 23

3.1.1. Total Suspended Solids and Volatile Suspended Solids……. 23

3.1.2. Volatile Fatty Acids…… 23

3.1.3. Phosphate, nitrite and nitrate…….. 24

3.1.4. Glycogen……. 24

3.1.5. Polyhydroxyalcanoates……... 24

3.1.6. Total phosphorus…… 25

3.2. MICROBIAL ANALYSES……. 25

3.2.1. Fluorescence in situ hybridization…….. 25

3.2.2. Chemical staining……... 29

3.3. RESPIROMETRY……... 30

3.4. MODELLING SOFTWARE……... 31

3.5. REFERENCES…… 31

CHAPTER IV: ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL IN A SEQUENCING BATCH REACTOR USING PROPIONATE AS THE SOLE CARBON SOURCE…… 33
4.1. INTRODUCTION……... 33
4.2. MATERIALS AND METHODS…… 33
4.2.1. Reactor…… 33
4.2.2. Synthetic wastewater…….. 34
4.3. RESULTS AND DISCUSSION…….. 35
4.3.1. Enriching an EBPR population……... 35
4.3.2. Cycle studies with propionate and acetate…….. 36
4.3.3. Propionate versus acetate: the main differences …… 41
4.3.4. Cycle studies at different pH…….. 42
4.3.5. Process modelling……... 45
4.4. CONCLUSIONS……. 51
4.5. REFERENCES…… 52

CHAPTER V: RESPONSE OF TWO DIFFERENT EBPR POPULATIONS ENRICHED WITH ACETATE AND PROPIONATE RESPECTIVELY TO DIFFERENT CARBON SOURCES……. 55
5.1. INTRODUCTION……... 55
5.2. MATERIALS AND METHODS…… 55
5.2.1. Pilot plant…… 55
5.2.2. Synthetic wastewater…….. 56
5.3. RESULTS…… 57
5.3.1. Comparison of the microbial community in the two SBRs… 57
5.3.2. Response of the Propionate-SBR biomass to different substrates……... 61
5.3.3. Response of the Acetate-SBR biomass to different substrates……... 66
5.4. CONCLUSIONS……. 71
5.5. REFERENCES…… 72

CHAPTER VI: RESPONSE OF FULL SCALE SLUDGE TO ACETATE AND PROPIONATE. COMPARISON WITH THE METABOLIC MODEL PREDICTIONS…… 73
6.1. INTRODUCTION……... 73
6.2. MATERIALS AND METHODS…… 74
6.2.1. Experimental setup: TOGA sensor……. 74
6.2.2. Synthetic wastewater…….. 76
6.3. RESULTS AND DISCUSSION…….. 77
6.3.1. Acetate experiments…… 77

6.3.2. Propionate experiments…… 80
6.4. CONCLUSIONS……. 84
6.5. REFERENCES…… 85

CHAPTER VII: AEROBIC PHOSPHORUS RELEASE LINKED TO ACETATE UPTAKE: PROCESS MODELLING USING OXYGEN UPTAKE RATE…… 89
7.1. INTRODUCTION……... 89
7.2. MATERIALS AND METHODS…… 90
7.2.1. Experimental design……... 90
7.2.2. Synthetic media…….. 92
7.3. RESULTS…… 92
7.3.1. Anaerobic/Aerobic experiments……. 92
7.3.2. Aerobic experiments……... 97
7.3.3. Validation experiments……...… 103
7.3.4. Discussion……... 104
7.4. CONCLUSIONS……. 106
7.5. REFERENCES…… 107

CHAPTER VIII: AEROBIC PHOSPHORUS RELEASE LINKED TO ACETATE UPTAKE: INFLUENCE OF PAO INTRACELLULAR STORAGE COMPOUNDS…….. 109
8.1. INTRODUCTION……... 109
8.2. MATERIALS AND METHODS…… 109
8.2.1. Experimental design……... 109
8.2.2. Synthetic wastewater…….. 110
8.3. RESULTS…… 110
8.3.1. Biomass characterisation…… 110
8.3.2. AnOx experiment…… 111
8.3.3. EAN and EOX experiments…… 111
8.3.4. Oxygen uptake rate measurements……. 114
8.3.5. Comparison between AnOx, EAN and EOX……. 115
8.4. CONCLUSIONS……. 117
8.5. REFERENCES…… 118

CHAPTER IX: NET P-REMOVAL DETERIORATION IN ENRICHED PAO SLUDGE SUBJECTED TO PERMANENT AEROBIC CONDITONS…… 119
9.1. INTRODUCTION……... 119
9.2. MATERIALS AND METHODS…… 120
9.2.1. Experimental design……... 120
9.2.2. Reactor…… 120
9.2.3. Synthetic wastewater…….. 121

9.3. RESULTS…… 121
9.3.1. OUR profiles……... 127
9.4. DISCUSSION…….. 128
9.5. CONCLUSIONS……. 131
9.6. REFERENCES…… 132

CHAPTER X: CONCLUSIONS AND FUTURE WORK…….. 133
10.1. CONCLUSIONS……... 133
10.2. FUTURE WORK…….. 135

STATEMENT INDICATING THE PARTS OF THE THESIS THAT ARE NOT MY SOLE ORIGINAL WORK

This thesis contains some important contributions that have been made by those with whom I have worked throughout the course of this PhD. These are acknowledged as follows:

- Albert Guisasola of the Department of Chemical Engineering of the Universitat Autònoma de Barcelona complemented part of the work presented with his engineering background. His main contribution was the calibration and the validation of the kinetic and stoichiometric model used in Chapter IV and Chapter VII. The interpretation and discussion of the results of this model were a joint effort. He also developed the LFS respirometer adapted in Chapters VII, VIII and IX and his help in the OUR interpretation is also acknowledged.

LIST OF PUBLICATIONS AND AUTHOR'S CONTRIBUTIONS

1. Pijuan M, Saunders AM, Guisasola A, Baeza JA, Casas C, Blackall LL. 2004. **Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source**. Biotechnology and bioengineering 85(1):56-67.

Author's contribution: Experimental design, experimental work and writing full paper. The model calibration and validation was performed by Guisasola A. Discussion and editing contributions from other authors.

2. Pijuan M, Baeza JA, Casas C, Lafuente J. (In press). **Response of an EBPR population developed in a SBR with propionate to different carbon sources**. Water Science and Technology.

Author's contribution: Experimental design, experimental work and writing full paper and preparation of the poster. Discussion and editing contributions from other authors. This paper was awarded for an outstanding poster presentation at the 3rd IWA Specialised Conference on Sequencing Batch Reactor Technology. 22-26 February 2004. Noosa, Australia.

3. Guisasola A, Pijuan M, Baeza JA, Carrera J, Casas C, Lafuente J. 2004. **Aerobic phosphorus release linked to acetate uptake in bio-P sludge: process modelling using oxygen uptake rate**. Biotechnology and bioengineering 85 (7): 722-733.

Author's contribution: All the experimental work in the AnaOx experiments and sharing the experimental work in the Aerobic experiments with Guisasola A. Equal contribution with Guisasola A. in the discussion of the results. Contribution in writing the paper.

4. Pijuan M, Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. (Submitted). **Aerobic phosphorus release linked to acetate uptake: influence of PAO intracellular storage compounds**. Biochemical Engineering Journal.

Author's contribution: Experimental design, experimental work and writing full paper and preparation of the poster. Editing contributions from other authors. This paper was presented in BIOTEC04, 19-23 July 2004. Oviedo, Spain.

5. Pijuan M, Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. (Submitted). **Net P-removal deterioration in enriched PAO sludge subjected to permanent aerobic conditions**. Biotechnology and bioengineering.

Author's contribution: Experimental design, experimental work and writing full paper. Discussion and editing contributions from other authors.

LIST OF ABBREVIATIONS

ASM	Activated Sludge Model
ATP	Adenosine triphosphate
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
CP	CO_2 Production
CT	CO_2 Transfer
DO	Dissolved Oxygen Concentration
DPAO	Denitrifying Polyphosphate Accumulating Organism
EBPR	Enhanced Biological Phosphorus Removal
EMP	Embden-Meyerhof-Parnas
FID	Flame Ionisation Detector
FISH	Fluorescence *In Situ* Hybridization
GAO	Glycogen Accumulating Organism
GC	Gas Chromatograph
HCl	Hydrogen Chloride
HP	Hydrogen ion Production
HRT	Hydraulic Retention Time
LPO	Lactic acid Producing Organism
OHO	Ordinary Heterotrophic Organism
OUR	Oxygen Uptake Rate
PAOs	Polyphosphate Accumulating Organisms
PHA	PolyHydroxyAlkanoates
PHB	Poly-β-HydroxyButyrate
PHV	Poly-β-HydroxyValerate
PH2MB	Poly-β-Hydroxy-2-MethylButyrate
PH2MV	Poly-β-Hydroxy-2-MethylValerate
PolyP	Polyphosphate
RNA	RiboNucleic Acid
SBR	Sequencing Batch Reactor
TOGA	Titration and Off-Gas Analysis
VFAs	Volatile Fatty Acids
SRT	Sludge Retention Time

TCA	Tricarboxylic Acid
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

CHAPTER I

INTRODUCTION

1.1. INTRODUCTION

Research in biological phosphorus removal has become one of the interest topics in the field of wastewater treatment, and after many years since the first study in EBPR, the metabolism of PAOs, responsible of the process, as well as their competitors, GAOs, is not fully established yet.

With the aim of contributing to the understanding of the process, a new research line based on biological phosphorus removal was started in 2000 in the Environmental Engineering Group of the Chemical Engineering Department at the Universitat Autònoma de Barcelona. The author started her PhD with the beginning of this new project. Since January of 2000 til December of 2001 some preliminar results were obtained and they were presented as "Eliminació biològica de fòsfor en un SBR" (in Catalan) that constituted the Master in Science of the author. Although no significant results were obtained, it was a useful work in terms of getting skilled in working with SBR and developing EBPR population.

The work presented in this thesis includes the experiments performed in the last 2,5 years, part of these performed in the Advanced Wastewater Management Centre (University of Queensland, Brisbane, Australia) during two stay periods from April to July of 2002 and November to March of 2003-04.

The research of the author has been focused in two different topics, both of them in the field of biological phosphorus removal. The effect of the carbon source in a PAO population has been studied in Chapters IV, V and VI. For the other hand, the EBPR activity of an enriched PAO population under strictly aerobic conditions has been studied and the results are presented in Chapter VII, VIII and IX

An EBPR system was developed using propionate as the only carbon source. Some cycle studies were performed with the PAO enriched population with different concentrations of propionate and acetate and a kinetic and stoichiometric model was developed comparing the acetate and the propionate responses. Moreover, the effect of the pH was studied with this population when propionate was used. It was the first study using an EBPR population developed with propionate as the sole carbon source. This work is presented in Chapter IV (*Enhanced biological phosphorus removal developed in a sequencing batch reactor using propionate as the sole carbon source*).

In the same line, two SBR-EBPR systems were enriched using acetate and propionate respectively as the sole carbon sources. Differences in the microbial community composition were observed and some cycle studies experiments were

performed with different substrates using biomass from both reactors. Results of this work are showed in Chapter V (*Response of two different EBPR populations enriched with acetate and propionate respectively to different carbon sources*).

In the last years, different studies have been carried out with acetate and propionate and some of them have been used to develop metabolic models to predict the behaviour of EBPR systems. Nevertheless, these experiments were carried out using enriched PAO cultures. Results from these studies found in the literature have been compared with the response of full scale sludge withdrawn from two different EBPR WWTP. The differences between the literature results and the response of full-scale EBPR sludge to acetate and propionate, where the percentage of PAOs is much lower than the enriched culture, has been studied. All these results and discussion are showed in Chapter VI (*Response of full scale EBPR sludge to acetate and propionate. Comparison with metabolic model predictions*).

The EBPR activity under strictly aerobic conditions of an enriched PAO population has been studied and the results are presented in Chapter VII, VIII and IX. PAO can take up acetate under aerobic conditions, linking this uptake with phosphorus release, glycogen degradation and PHA production as in anaerobic conditions although the rates observed are different from the ones obtained in anaerobic conditions. When substrate is depleted, PAO degraded its PHA to uptake phosphorus, to replenish the glycogen pools and to growth. All this phenomenon has been analysed and modelled and is presented in Chapter VII (*Aerobic phosphorus release linked to acetate uptake: process modelling using oxygen uptake rate).*

To complement Chapter VII, the effect of intracellular compounds in an enriched PAO population working under strictly aerobic conditions was studied. This work is presented in Chapter VIII (*Aerobic phosphorus release linked to acetate uptake: influence of PAO intracellular storage compounds*).

After observing that an enriched PAO sludge was able to behave in a similar way under strictly aerobic conditions than in anaerobic/aerobic conditions, achieving net phosphorus removal from the system in both cases, next step in this research topic was to study for how long this biomass could work under aerobic conditions without loosing its phosphorus removal activity. This study is presented in Chapter IX where an SBR reactor was seeded with an enriched EBPR biomass and was subjected to aerobic cycles for 11 days. Microbial population as well as the most significant compounds of the process were analysed throughout different cycle studies. Chapter IX summarise and discuss the results obtained (*Net P-removal deterioration in enriched PAO sludge subjected to permanent aerobic conditions*).

CHAPTER II

LITERATURE REVIEW

2.1. INTRODUCTION

This chapter is dedicated to an overview of the two aspects in the biological phosphorus removal process studied in this thesis. The effect of different substrates in the EBPR systems has been studied since the beginning of the nighties but this review will be focused in the latest studies although some comments on the early papers will be done. Moreover the articles that have studied the simultaneous presence of an electron donor and electron acceptor in EBPR systems will be commented although there is not much in the literature.

This chapter aims to be a tool to get easy access to the recent literature about these topics available until mid 2004.

2.2. ROLE OF PHOSPHORUS IN THE ENVIRONMENT

2.2.1. NATURAL CONDITIONS

Because phosphates are non-volatile, their occurrence is limited to soil and aquatic environments. In Figure 2.1 the water-based phosphate cycle is presented. Phosphorus is a macro-nutrient that is necessary to all living cells. It is a limiting nutrient with regard to growth of algae and plants in lakes. Bacteria and many other organisms use orthophosphates to create organic phosphates and build solid-phase structures. As such, it is an important component of adenosine triphosphate (ATP), nucleic acids (DNA and RNA) and phospholipids in cell membranes. In both prokaryotes and eukaryotes, phosphorus can be stored in intracellular granules as polyphosphates.

Anaerobic activity in the bottom of lakes accounts for release of orthophosphates to the standing water, with subsequent consumption by bacteria and algae (Reddy, 1998).

Pollution-related phosphorus compounds are orthophosphates, organic phosphorus and polyphosphates. The average concentration of total phosphorus (inorganic and organic forms) in wastewater is in the range of 5-20 mg P/L (Bitton, 1994) and usually below 25 mg P/L (SCOPE, 1998).

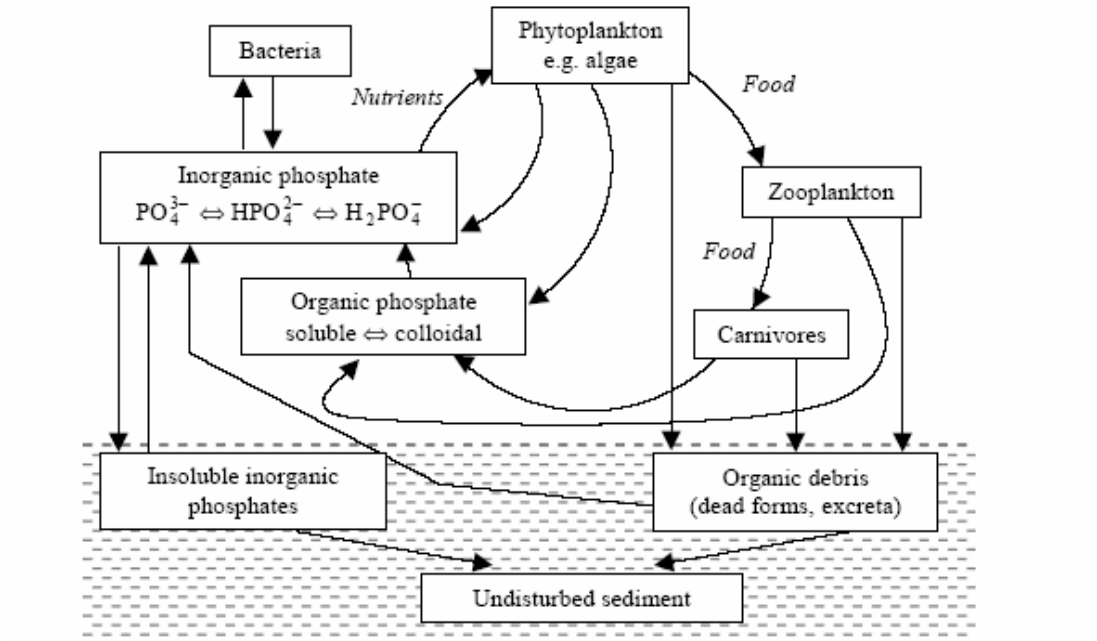

Figure 2.1. The water-based phosphate cycle (Reddy, 1998)

In natural conditions the phosphorus concentration in water is balanced, i.e. the accessible mass of this constituent is close to the requirements of the ecological system. However, if the input of phosphorus to waters is higher than the one that can be assimilated by a population of living organisms, the problem of excess phosphorus content occurs. This situation is commonly known as eutrophication. Eutrophication means high concentrations of plant nutrients, but nowadays the word is often used to refer to the adverse response of an ecosystem to an excess nutrients (Harper, 1991).

It has been scientifically established that the main causes of eutrophication are nitrogen, in the case of coastal waters, and phosphorus in the case of freshwaters. However, both nitrogen and phosphorus can be limiting factors, either together of in turn, depending on the algal species and the time of the year. It therefore is often necessary to reduce both nutrients.

2.2.2. POLICES TO CONTROL PHOSPHATE POLLUTION

Phosphorus constraints on industrial and sewage discharge to prevent eutrophication differ from country to country. Even within Europe different policies exist (i.e. legislation, conventions). With the publication of the urban wastewater Directive 91/271 (CEC, 1991), Member States were enforced to designate "sensitive" and "less sensitive" areas. An area is classified sensitive (SCOPE, 1998) when:

• the receiving water is eutrophic or which in the near future may become eutrophic if protective action is not taken.

• the water is used for drinking water.

• the water requires a more stringent than secondary treatment in order to accomplish other EU Directives.

Table 2.1. Technical compliance deadlines for collection and treatment requirements from Directive 91/271 (SCOPE, 1998).

	12/1998	**12/2000**	**12/2005**
Sewerage collection	>10000 p.e in sensitive areas	>10000 p.e in normal and less sensitive areas	>2000 p.e in all areas
Primary or secondary treatment		>15000 p.e in less sensitive areas	>10000 p.e in less sensitive areas
Secondary treatment		>15000 p.e in normal areas	>10000 p.e in normal and less sensitive areas
More advanced treatment	>10000 p.e in sensitive areas		

If the sewerage is collected in agglomerations<2000 p.e., appropriate treatment should be applied

In Table 2.1 the technical compliance deadlines for collection and treatment requirements enforced by the EC Directive 91/271 are presented. Although the two first dates have already past, they have been left in the table since not all Member States have fulfilled the requirements yet. By the beginning of 2002, Belgium, Denmark, Luxembourg, the Netherlands, Austria, Finland, Sweden and Germany were requiring stringent treatment levels for a very high percentage of the total wastewater load. Greece, Spain, Italy, Portugal and the UK foresaw more stringent wastewater treatment to protect sensitive areas for less than 10% of their wastewater load. France and Ireland foresaw about 24% and 86% respectively.

At the beginning of 2002, Germany and the Netherlands achieved above more than the requested 75% phosphorus reduction required by the Directive, but have not yet fully attained the 75% nitrogen reduction. Denmark and Austria were practically in full compliance with the Directive, whereas in Belgium, Greece, Portugal, Finland and the UK less than 50% of the wastewater load impacting on sensitive areas was receiving the appropriate level of treatment. Most Member States plan to achieve conformity with the Directive by 2005 or 2008 at the latest.

Table 2.2. Requirements for discharge from urban wastewater treatment plants to sensitive areas (CEC, 1991)

Parameters	Concentration	Minimum percentage of reduction[1]
Total phosphorus	10000-100000p.e. 2mgP/L	80%
	>100000p.e. 1mgP/L	
Total nitrogen	10000-100000 p.e.15 mgN/L	70-80%
	>100000 p.e. 10 mgP/L	

[1] Reduction in relation to the load of the influent.

When taking into account the options formulated in Table 2.2, it becomes clear that any biological process used to achieve the effluent standards and/or the removal ratio, should be manageable very well, i.e. the underlying processes should be understood thoroughly to avoid any malfunctioning to meet the standards. The process should also be operable under very stable conditions. As for the Enhanced Biological Phosphorus Removal (EBPR) process, stricter regulations have led a vast number of research institutes, researchers and plant operators to study the process. Since the development of the process by Barnard (1976), and even more since the introduction of activated sludge wastewater models, research papers increased enormously with positive effect on the application of EBPR to achieve the standards laid down in the Directive 91/271/EEC.

2.3. ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL

2.3.1. THE MECANISM UNDERLYING THE EBPR

The possibility to immobilise the nutrient phosphorus using a process without addition of chemicals has motivated many studies in order to understand and improve the performance of the processes involved in EBPR by activated sludge. EBPR is widely accepted as one of the most economical and sustainable processes to remove phosphorus from wastewater (Metcalf and Eddy, 1991), but at the same time, it is a complex process when compared to chemical oxygen demand (COD) or nitrogen removal. For this reason, EBPR has been left on the back burner although it has significant economical advantages and its implementation would make nitrogen removal more efficient. Currently nitrogen removal goals that need to be attained are getting much stricter and it is becoming more imperative that phosphorus removal be accomplished. This is because phosphorus is nearly always the limiting nutrient for

freshwater bodies, and is seasonally or regionally limiting for estuarine bodies of water. There is a need to increase the number of wastewater treatment plants that practice EBPR in addition to nitrogen removal, but a better understanding of its economic and process optimization advantages is needed by the wastewater treatment profession.

Since 1980s a great deal of research efforts has been dedicated to exploring the underlying reactions of EBPR. The EBPR process is based on the enrichment of activated sludge with polyphosphate accumulating organisms (PAOs) and details of their biochemical pathways are still only hypotheses without confirmation. The current knowledge puts the research community into two camps: the supporters of Comeau (Comeau *et al.* 1986; Comeau *et al.* 1987; Wentzel *et al.* 1991) and the supporters of Mino (Mino *et al.* 1987; Satoh *et al.* 1992; Smolders *et al.* 1994a,b; Liu *et al.* 1996a; Maurer *et al.* 1997; Brdjanovic *et al.* 1998). The main difference between both hypotheses was the origin of the reducing power required in the process. Under anaerobic conditions, PAOs take up organic substrates (preferably volatile fatty acids - VFAs) and store them as poly-hydroxyalkanoates (PHAs), while the reducing equivalents are provided by the TCA cycle (Comeau hypothesis, figure 2.2.Left) or by glycolysis of internally stored glycogen (Mino hypothesis, figure 2.2.Right).

Currently, there are other researchers who support hypotheses built on the ideas of both researchers, such as Pereira *et al.* (1996), Louie *et al.* (2000), Hesselman *et al.* (2000), Yagci *et al.* (2003) and others. The energy for this process is obtained partly from the glycogen utilisation but mostly from the hydrolysis of the intracellular stored polyphosphate (polyP), resulting in an orthophosphate release into solution. In the subsequent aerobic phase, PAOs take up excessive amounts of orthophosphate to recover the intracellular polyP levels by oxidising the stored PHA. Meanwhile they grow and replenish the glycogen pool using PHA as both carbon and energy sources (Figure 2.3) (Arun *et al.* 1988; Smolders *et al*., 1995). Net phosphorus removal is achieved by wasting sludge after the aerobic period when the biomass contains a high level of polyP (Figure 2.4).

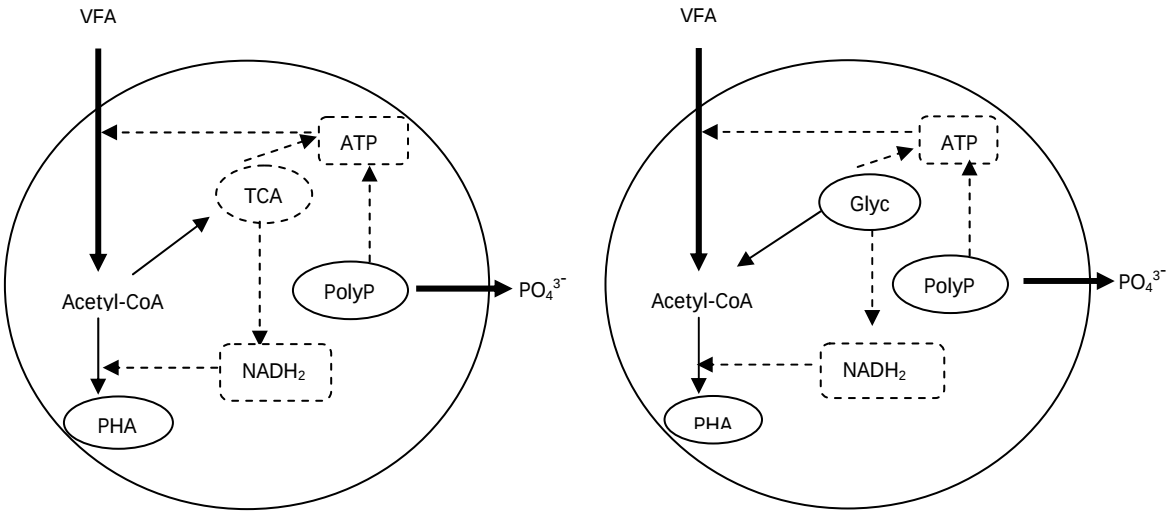

Figure 2.2. Schematic representation of the anaerobic PAO mechanism. Left- Comeau hypothesis. Right- Mino hypothesis.

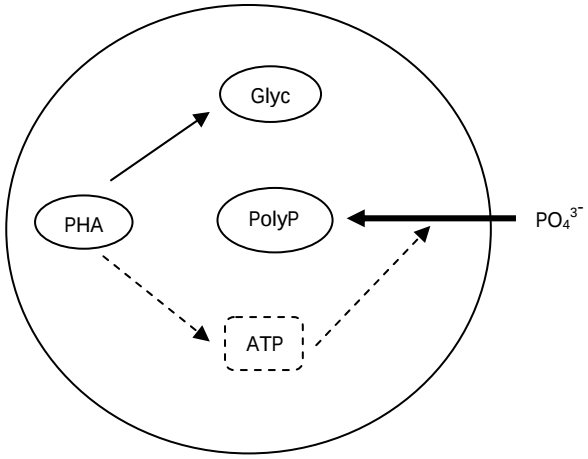

Figure 2.3. Schematic representation of the aerobic PAO mechanism (Smolders *et al.* 1995).

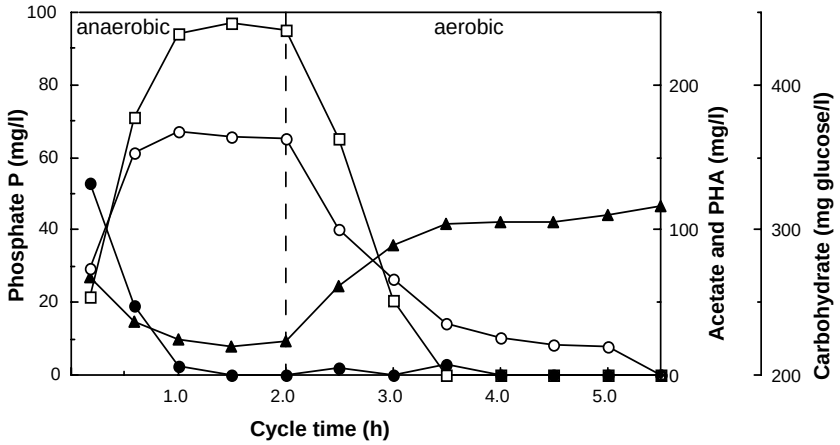

Figure 2.4. Profiles of extracellular phosphate-P (□), acetate (●), PHA (○), and glycogen (▲) during the anaerobic and aerobic reactor cycle stages of a typical PAO sludge (Bond *et al.* 1999).

The anaerobic phase was believed to provide a unique, positive environment for the PAOs, enabling them to reserve the necessary amount of carbon to themselves without having to compete with other microorganisms (Matsuo *et al.,* 1992). Later research provided evidence that other, so-called Glycogen Accumulating Organisms (GAOs) were able to store carbon sources as well and compete with PAOs in such systems (Figure 2.5 and 2.6) but without taking up phosphate.

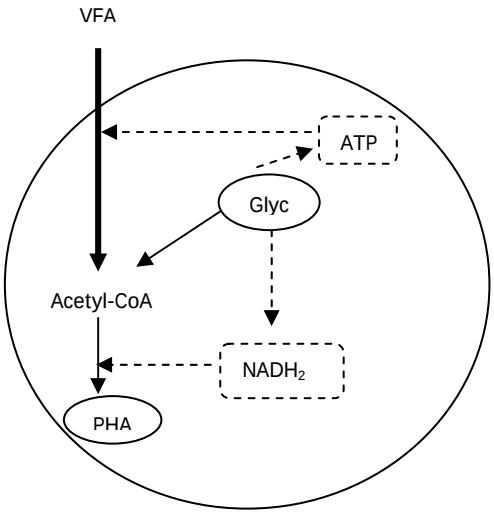

Figure 2.5. Schematic representation of the anaerobic GAO metabolism

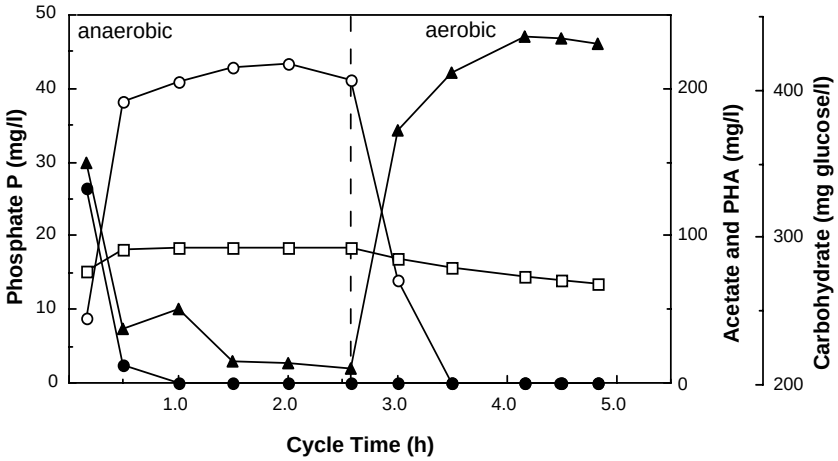

Figure 2.6 Profiles of extracellular phosphate-P (□), acetate (●), PHA (○), and glycogen (▲) during the anaerobic and aerobic reactor cycle stages of a typical GAO sludge (Bond *et al.* 1999).

It has been assumed that the availability of VFAs (also referred to as fermentation products, with as main constituent acetate) is a prerequisite for EBPR. In the absence of these components, fermentation of readily biodegradable carbon sources under

anaerobic conditions is necessary. Later research results also indicated good phosphorus removal with direct utilisation of readily biodegradable material.

2.3.2. INFLUENCE OF THE CARBON SOURCE IN THE EBPR PROCESS

Effect of VFAs and glucose

A substantial amount of research has been focused on EBPR response achieved with different organic compounds in the last years. Sometimes, deterioration of the EBPR in laboratory scale systems has been observed (Cech *et al.*, 1993) and it has been attributed to the carbon source supplied during the anaerobic phase of the EBPR process. More attention has to be paid to the type of carbon source in the wastewater and its effect on EBPR due to the heterogeneous organic matter in wastewater which often reaches the treatment plant without complete acid fermentation.

In initial studies concerning EBPR, authors continuously refer to good EBPR activity in connection with carbon sources belonging to the group of volatile fatty acids (VFAs). Fuhs and Chen (1975), Potgieter and Evans (1983), Malnou (1984), Ekama *et al.* (1984), Arvin and Kristensen (1985) and Comeau *et al.* (1987) all report a more important phosphorus release when acetate or propionate were used instead of other substrates. According to Reddy (1998) an accepted rule is that the readily available organic matter concentration, which corresponds to the group of VFAs, within the initial anaerobic phase, must be more than 25 mg COD/L to accomplish significant EBPR. Increases in the organic matter will increase the phosphorus release and the organic storage in the anaerobic zone, up to some optimum VFA *versus* phosphorus ratio. Conversely, the study by Randall and Chapin (1997) show that high concentrations of acetic acid (greater than 400 mg COD/L), also can cause failure of EBPR processes.

Since the discovery of EBPR, acetate has been the carbon source to which most attention has been attributed because of its positive influence on the process. Moreover, fermentation processes preceding or active in the anaerobic phase will provide large amounts of acetate although propionate is also abundant in real septic domestic wastewaters. Several metabolic models have been proposed for acetate as a substrate in EBPR, but among the alternative substrates, a metabolic model has been developed only for glucose (Wang *et al.* 2002). When acetate is used as the sole carbon source, the PHA produced is mostly poly-β-hydroxyButyrate (PHB). According to Comeau *et al*. (1987), poly-β-hydroxyvalerate (PHV) production was significantly higher than that of PHB when a substrate with an odd number of carbon

atoms was fed to activatd sludge. This observation was confirmed later for propionate consumption by Lemos *et al.* (1998). The composition of PHA derived from propionate was elucidated by Satoh *et al.* (1992) as a mixture of PHV, PHB, poly-β-hydroxy-2-methylvalerate (PH2MV) and poly-β-hydroxy-2-methylbutyrate (PH2MB) as can be observed in figure 2.7. Recently Lemos *et al.* (2003) described the metabolic pathway for propionate utilisation by PAOs using ^{13}C labelling and in vivo nuclear magnetic resonance and Oehmen *et al.* (submitted) developed a metabolic model for propionate utilisation by PAOs.

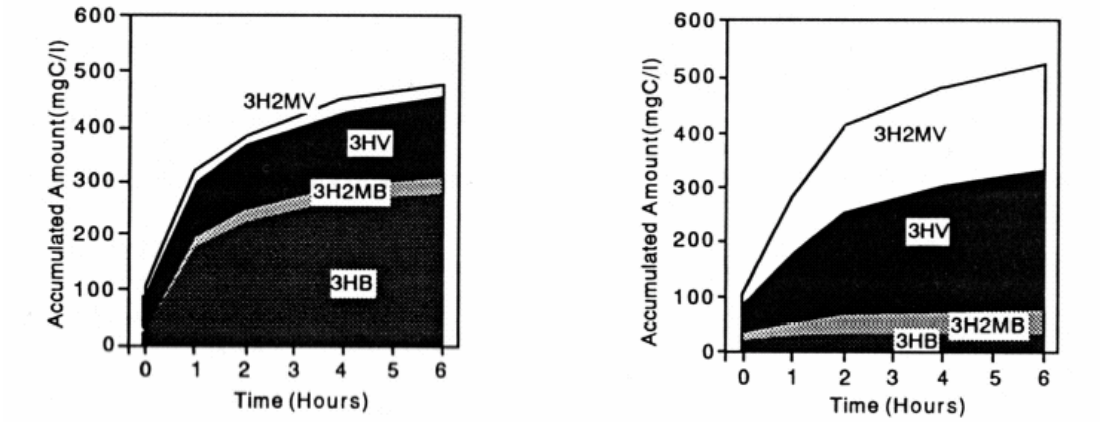

Figure 2.7. PHA composition for substrate uptake. Left.- Acetate. Right.- Propionate. (Satoh *et al.* 1992).

EBPR process can also occur successfully with organic substrates other than VFAs (such a mixture of peptone and glucose or even only glucose). The absence of both, their pre-conversion to VFAs and storage to PHAs show that different biochemical pathways have to be considered in which the role of acetate and PHAs may not be as essential as it has been assumed until now. Some discussion about the effect of glucose on the EBPR systems is described in the literature in the last years (Carucci *et al.* 1999; Sudiana *et al.* 1997; Jeon *et al.* 2000 and 2001; Wang *et al.* 2002).

With the discovery of the EBPR process (Levin and Shapiro, 1965), it was initially thought that glucose was the most important carbon source (Nicholls and Osborn, 1979). Although, in 1975 Fuhs and Chen postulated that an anaerobic phase was necessary for fermentation processes to produce VFA, they also postulated the fermentation products were consumed aerobically, i.e. no link was made between anaerobic and aerobic phosphorus related processes. With the basic background of the EBPR process becoming more and more clear from the 1980's on and the indication of a link between the anaerobic and aerobic processes, attention became more focussed on anaerobic usage of the VFA sources. However, since the studies by Fukase *et al.* (1985) and by Cech and Hartman (1993) indicating possible

deterioration of EBPR activity using glucose as a carbon source, more and more authors try to find experimental evidence for the fate of glucose in EBPR systems (Carucci *et al.* 1999; Sudiana *et al.* 1997; Jeon *et al.* 2000 and 2001). Regarding the relation between anaerobic glucose uptake and phosphorus release, only little information is encountered in literature. Using glucose feed Arvin and Kristensen (1985) and Fukase *et al*. (1985) observed a time delay effect, i.e. P-release is somewhat delayed relative to the glucose uptake.

Whether or not glucose uptake is coupled with fermentation is still unclear. Nakamura and Dazai (1989) and Carucci *et al.* (1999) reported on release of lactic acid in anaerobic batch tests on a glucose and peptone feed, indicating fermentation took place. Jeon and Park (2000) observed that EPBR with glucose supply was accomplished at least by two kinds of bacterial populations, a lactic acid producing organism (LPO) and a PAO. Jeon *et al.* (2000) described the morphological characteristics of the sludge present in these systems. Sudiana *et al.* (1997) observed small amounts of acetate and propionate in the bulk solution when using glucose as carbon source in batch experiments. Wentzel *et al.* (1991) stated that direct uptake of glucose by PAOs was not possible. Randall *et al.* (1994) on the contrary observed rapid transport and storage into the cells before fermentation.

Comparing these results with EBPR systems supplied with acetate, lower levels of phosphorus were released into the medium and PHA was enriched significantly with PHV. Wang *et al.* 2002 reasoned that the predominance of PHV was employed to balance the internal redox during the anaerobic condition. The anaerobic metabolism of absorbed glucose under the Embden-Meyerhof-Parnas (EMP) pathway could produce enough energy for all metabolic activities and prevent the energy derivation from polyphosphate hydrolysis. This could explain the easy deterioration of EBPR performance when glucose is utilized as the major substrate, probably due to the growth of GAOs in the systems.

Effect of other carbon sources

Satoh *et al.* (1996) reported pyruvate (and succinate and lactate, see below) to disappear faster from the supernatant (about 5 minutes) than acetate, propionate and malate, although a lower net phosphorus release was observed compared to acetate or propionate. It was also observed that pyruvate was initially stored in a form other than PHA (not mentioned which), without phosphorus release. The accumulated material was then gradually converted to PHA with concomitant phosphorus release. Without really having conclusive experimental evidence, they assumed no

fermentation occurred and both internal storage and transformation to PHA was performed by PAOs. Possible adsorption of the carbon source to the sludge was not discussed.

Using lactate Comeau *et al.* (1987) reported immediate fast phosphorus release. Satoh *et al.* (1996) reported lactate to disappear faster from the supernatant than acetate, propionate and malate, although a lower net phosphorus release was observed compared to acetate or propionate. As for pyruvate, the authors observed a fast storage in a form other than PHA, followed by a phase with phosphorus release and concomitant PHA formation. Jeon and Park (2000) observed lactate production by fermentative bacteria with concurrent uptake of this produced lactate and phosphorus release by PAOs.

According to Satoh *et al.* (1996) the uptake of malate was the slowest with the lowest amount of phosphorus released (comparable to pyruvate) and of PHA formed. Malate also disappeared slower from the supernatant than succinate, pyruvate and lactate. Succinate disappeared faster from the supernatant than acetate, propionate and malate. As for pyruvate and lactate, the authors observed a fast storage in a form other than PHA, followed by a phase with phosphorus release and concomitant PHA formation.

Using starch, a glycogen like polymer, as feed for an SBR system, Randall *et al.* (1994) observed no EBPR activity. Marais *et al.* (1983) even reported the direct use of sewage substrate as carbon source.

To summarise the last paragraphs on non-VFAs, it can be concluded that whereas initially it was generally accepted that PAOs were unable to directly utilise carbon sources other than VFAs under anaerobic conditions, experimental evidence is such that PAOs appears to have mechanisms to directly use non-VFAs without prior fermentation by heterotrophic bacteria. Much controversy still exists concerning the impact of these carbon sources on the overall phosphorus removal capacity (Liu *et al.*, 1996b; Mino *et al.*, 1998).

2.3.3. PHA COMPOSITION DEPENDING ON SUBSTRATE ADDITION

Part of the EBPR research has been focused on understanding how different carbon substrates and their concentration can influence the production of PHA by PAOs. PHAs are the only linear polyesters biodegradable and biocompatible with numerous applications in medicine, pharmacy and packaging. These thermoplastic polymers are accumulated as intracellular granules by different bacteria as carbon and energy storage material under conditions of restricted growth (Anderson *et al.* 1990). More than 40 different hydroxyalkanoic acids (HA) have been detected as constituents of PHA, but only few homopolyesters beside PHB are available from bacteria (Steinbüchel *et al.* 1992). Depending on the type of microorganism and substrate fed, they may produce polymers with different composition.

Using acetate as a sole carbon source, Smolders *et al.* (1994a) observed mainly PHB production although some PHV (around 10%) was also detected. Nevertheless, in this study the PHV was considered jointly with PHB and referred to as PHB. Using the same carbon source, Murnleitner *et al.* (1997) considered 20% PHV and 80% PHB as an average value.

Lemos *et al.* (1998) tested different substrates. Acetate, propionate and butyrate were used as single substrates and mixed with increasing overall concentration. They reported on the production of a copolymer of HB and HV, with the HB units being dominant, when acetate was tested. When propionate was tested in the same study they obtained 28% PHB and 72% PHV. These percentages would be different if the PH2MV production had been measured. Using butyrate, they reported HB units to be produced to a higher extent, but the total amount of polymer formed was much less compared to acetate or propionate feed.

Louie *et al.* (2000) observed both PHB and PHV accumulation when acetate was used as carbon source. A PHV to PHB ratio of 0.6-0.75 of was observed. Feeding pyruvate combined with acetate resulted in accumulation of PHB and PHV in approximately a 1:1 ratio. Citrate addition did not result in PHA. Succinate or succinate combined with acetate resulted in a 2-fold higher accumulation of PHV than PHB. The yield of polymer produced per carbon consumed was found to diminish from acetate (0.97 C/C), to propionate (0.61 C/C), to butyrate (0.21 C/C). Using a mixture of acetate, propionate and butyrate and increasing the carbon concentration, but maintaining the relative concentration of each substrate, propionate was primarily consumed, thus PHA was enriched with HV units.

Hood *et al.* (2001) observed that acetic and isovaleric acids resulted in HB units while valeric and propionic acids resulted in 3HV or 3H2MV units. Later, Liu *et al.* (2002) correlated the different types of PHA with the P-uptake capacity, but only PHB and PHV were analysed. When acetic acid was used 75% of PHB and 25% of PHV were produced and when propionic acid was tested 90 % of PHV was quantified in front of 10% of PHB. Valeric acid was stored mainly as PHV (95%) and isovaleric acid produced 85% of PHB and 15% of PHV. The conclusions of this study were that PHB degradation resulted in greater aerobic phosphorus uptake than PHV degradation.

Levantesi *et al.* 2002 used a mixture of VFAs (propionate, acetate and butyrate) and they observed higher production of PHB than PHV. The molar ratios of HB:HV determined for the individual substrates were 3.04 for acetate, 0.39 for propionate and 1.48 for butyrate. Nevertheless, PH2MV was not quantified in this study and no reference to other PHA was described.

Lemos *et al.* 2003 studied the propionate metabolism by activated sludge in EBPR systems. During the anaerobic phase, the propionate was converted to PHA with the following monomer composition: 74.2% 3HV, 16.9% 3H2MV, 8.6% 3H2MB and 0.3% of 3HB.

Glucose has also been tested in numerous studies and different results in terms of PHA production were obtained. Sudiana *et al.* (1997) observed no difference in total PHA content between glucose and acetate fed systems showing good EBPR performance. However, no information is provided regarding the PHA fractionation. Liu (1996a) observed that glucose uptake was coupled to a decrease in the sludge's carbohydrate (glycogen) content with probable conversion to non-carbohydrates as PHA. No important PHB profile was recorded, but since PHB was the only component measured, the authors assumed that probably other PHAs were involved. Carucci *et al.* (1995), however, reported that PHAs were not detected during the anaerobic release of ortho-phosphate in an anaerobic/aerobic sequencing batch reactor when glucose and peptone were supplied as mixed carbon substrate. Glucose decrease in these systems was not connected to the release of ortho-phosphate, but overall good phosphorus removal was observed.

Jeon and Park (2000) observed that the amount of PHAs synthesised was less than the amount of glucose added since glucose was converted to other storage compounds by the LPO. Using glucose, Jeon *et al.* 2001 observed 36.5% 3HB, 51.0% 3HV, 5.7% 3H2MB and 6.7% of 3H2MV.

Wang *et al.* 2002 developed a biochemical model of glucose used by an enriched PAO population. They quantified 83% of PHV production versus 17% of PHB.

2.3.4. SIMULTANEOUS ACCEPTOR AND ELECTRON DONOR IN THE EBPR SYSTEM

The stability and efficiency of EBPR processes can be disturbed by several factors. It has been reported that, for example, deterioration of EBPR efficiency regularly occurred at some wastewater treatment plants after heavy rainfall or weekends. The phenomenon was attributed to low plant loading that took place during such events. In the case of heavy rainfall, the Wastewater Treatment Plant (WWTP) temporarily receives low concentrated sewage and high hydraulic loading. According to Henze (1995) prolonged exposure to storm water conditions will negatively affect EBPR processes. It is still not clear whether this is due to inhibition of PAOs or that the low COD concentrations in the influent are causing the problem.

There are two common causes of excessive aeration of activated sludge which occur in practice. Firstly, a combination of a heavy rain event and sewerage with large hydraulic gradient may result in a relatively high input of air into the sewer. The dissolved oxygen (DO) input to the sewage can further increase if screw pumps and aerated grit chambers are employed at the WWTP. This means that during rainfall events the anaerobic hydraulic retention time of EBPR systems may become temporarily shortened, making the aerobic phase longer than designed for. The second cause of over-aeration lies in the fact that some WWTPs have inadequate aeration control. In those plants, the control of aeration system can not be adjusted to the process requirements which made the activated sludge excessively aerated during weekends (Brdjanovic *et al.* 1998).

The EBPR process is based on the physical separation between the electron donor (COD) and the final electron acceptor (oxygen or nitrate). One step beyond in PAO knowledge is the analysis of the effect of simultaneous presence of both the electron donor and the final electron acceptor that can occur in the situations described above. Some research can be found in the literature (Brdjanovic *et al.* 1998; Serafim *et al.* 2002; Ahn *et al.* 2002) where P-release is linked to external substrate uptake.

In these studies it was confirmed that the presence of acetate under aerobic conditions provokes phosphate release. Brdjanovic *et al.* (1998) focused their study in the effect of the PHB content in the phosphate uptake and suggest that when COD is added to the system under aerobic conditions, P-release occurs, but the released phosphate can not be taken up fully again, because the PHB content limits the phosphorus uptake rate.

On the other hand, Ahn *et al.* 2002 focused their research in the transformations of relevant intracellular compounds in denitrifying phosphate accumulating organisms (DPAO) under different combinations of electron acceptor and electron donor. The difference between the metabolism of PAO and DPAO is that DPAO use nitrate instead of oxygen as an electron acceptor for phosphorus uptake (Bortone *et al.* 1999; Kuba *et al.* 1993; Zeng *et al.* 2003). Results of this study demonstrated that DPAOs have the ability to partly produce reduction equivalents through the TCA cycle in the presence of acetate under anoxic conditions. PHB production in the presence of acetate under aerobic conditions was related to the amounts of PHB and polyphosphate accumulated inside the cell.

Serafim *et al.* (2002) showed that the change in operational conditions (from anaerobic/aerobic systems to strictly aerobic conditions) allowed an enriched PAO population to modify the metabolism of phosphorus and PHA accumulation. The change in metabolism seems not to be caused by a modification in the composition of the population because of the immediate response observed when conditions were modified. Under aerobic dynamic feeding, phosphorus accumulation was almost hindered while the amount of PHA accumulation increased significantly.

2.4. REFERENCES

Ahn J, Daidou T, Tsuneda S, Hirata A. 2002. Transformation of phosphorus and relevant intracellular compounds by a phosphorus accumulating enrichment culture in the presence of both the electron acceptor and electron donor. Biotechnol Bioeng 79:83-93.

Anderson AJ, Dawes EA. 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalcanoates. Microbial Rev 54:450-472.

Arun V, Mino T, Matsuo T. 1998. Biological mechanisms of acetate uptake mediated by carbohydrate consumption in excess phopshorus removal systems. Water Res 22:565-570.

Arvin E, Kristensen GH. 1985. Exchange of organics, phosphate and cations between sludge and water in biological phosphorus and nitrogen removal processes. Water Sci Technol 17:147-162.

Barnard JL. 1976. A review of biological phosphorus removal in the activated sludge process. Water SA 2:126-144.

Bitton G. Wastewater Microbiology, 1st Ed, (ed. Mitchel), Wiley-Lys, New York, US. (1994).

Bond PL, Keller J, Blackall LL. 1999. Bio-P and non-bio-P bacteria identification by a novel microbial approach. Water Sci Technol 39(6):13-20.

Bortone G, Libelli SM, Tilche A, Wanner J. 1999. Anoxic phosphate uptake in the DEPHANOX process. Water Sci Technol 40:177-185.

Brdjanovic D, Slamet A, van Loosdrecht MCM, Hooijmans CM, Alaerts GJ, Heijnen JJ. 1998. Impact of excessive aeration on biological phosphorus removal from wastewater. Water Res 32:200-208.

Carucci A, Lindrea K, Majone M, Ramadori R. 1995. Dynamics of the anaerobic utilization of organic substrates in an anaerobic/aerobic sequencing batch reactor. Water Sci Technol 31**:**35-43.

Carucci A, Lindrea K, Majone M, Ramadori R. 1999. Different mechanisms for the anaerobic storage of organic substrates and their effect on EBPR. Water Sci Technol 39:21-28.

CEC, Council Directive concerning urban waste water treatment. (91/271/EC) Official Journal L135/40 (1991)

CEC, Implementation of Council Directive 91/271/EEC of 21 May 1991 concerning urban waste water treatment, as amended by Commission Directive 98/15/EC of 27 February 1998: Summary of the measures implemented by the Member States and assessment of the information received pursuant toarticles 17 and 13 of the Directive. COM(98)775 (1998)

Cech J S, Hartman P. 1993. Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. Water Res 27:1219-1225.

ComeauY, Hall KJ, Hancock REW, OldhamWK. 1986. Biochemical model for enhanced biological phosphorus removal. Water Res 20:1511-1521.

Comeau Y, Oldham WK, Hall KJ. 1987. Dynamics of carbon reserves in biological dephosphation of wastewater. Proc. IAWPRC Int. Conf. on Biological Phosphate Removal from Wastewaters, Rome, Italy Adv. Water Pollut. Cont. (ed. Ramadori), 39-55.

Ekama GA, Marais GvR, Siebritz IP. 1984. Biological excess phosphorus removal. In: Theory, design and operation of nutrient removal activated sludge processes. 7:1-32 (Water Research Commission) Pretoria, South Africa.

Fuhs GW, Chen M. 1975. Microbiological basis of phosphate removal in the activated sludge process for treatment of wastewater. Microbiol Ecol 2:119-138.

Fukase T, Shibata M, Miyaji Y. 1985. Factors affecting biological removal of phosphorus. Water Sci Technol 17:187-198.

Harper, D. 1991. Eutrophication of freshwaters. Chapman and Hall, London.

Henze M, Gujer W, Mino T, Matsuo T, Wentzel MC, Marais GvR. 1995. Activated Sludge Model No. 2. IAWQ Scientific and Technical Report No.3. London, UK.

Hesselmann RPX, Von Rummell R, Resnick SM, Hany R, Zehnder JB. 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res 34:3487-3494.

Hood CR, Randall AA. 2001. A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. Water Res 35:2758-2766.

Jeon CO, Park JM. 2000. Enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose as a sole carbon source. Water Res 34:2160-2170.

Jeon CO, Lee DS, Park JM. 2001. Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: characteristics of carbon metabolism. Water Environm Res 73:295-300.

Kuba T, Smolders G, van Loosdrecht MCM, Heijnen JJ. 1993. Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. Water Sci Technol 27:241-252.

Lemos PC, Viana C, Salgueiro EN, Ramos AM, Crespo JPSG, Reis MAM. 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate accumulating mixed culture. Enzyme Microbial Technol 22:662-671.

Lemos PC, Serafim LS, Santos M, Reis MAM, Santos H. 2003. Metabolic pathway for propionate utilization by phosphorus accumulating organisms in activated sludge: ^{13}C labelling and in vivo nuclear magnetic resonance. Applied and environmental microbiology 69:241-251.

Levantesi C, Serafim LS, Crocetti GR, Lemos PC, Rossetti S, Blackall LL, Reis MAM, Tandoi V. 2002. Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. Enviornm Microb 4:559-569.

Levin GV, Shapiro J. 1965. Metabolic uptake of phosphorus by waste water organisms. J Water Pollut Control Fed 37:800-821.

Liu WT, Mino T, Matsuo T, Nakamura K. 1996a. Biological phosphorus removal processes - Effect of pH on anaerobic substrate metabolism. Water Sci Technol 34: 25-32.

Liu WT, Mino T, Nakamura K, Matsuo T. 1996b. Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. Water Res 30:75-82.

Liu Y, Geiger C, Randall AA. 2002. The role of poly-hydroxy-alkanoate form in determining the response of enhanced biological phosphorus removal biomass to volatile fatty acids. Water Environ Res 74:57-67.

Louie TM, Mah TJ, Oldham W, Ramey WD. 2000. Use of metabolic inhibitors and gas chromatography/mass spectroscopy to study poly-β-hydroxyalkanoates metabolism involving cryptic nutrients in enhanced biological phosphorus removal systems. Water Res 34:1507-1514.

Malnou D, Meganck M, Faup GM, Rostu M. 1984. Biological phosphorus removal: study of the main parameters. Water Sci Technol 16.

Marais GvR, Loewenthal RE, Siebritz IP. 1983. Observations supporting phosphate removal by biological excess uptake. A review. Water Sci Technol 15:15-41.

Matsuo T, Mino T, Satoh H. 1992. Metabolism of organic substances in anaerobic phase of biological phosphate uptake process. Water Sci Technol 25:83-92.

Maurer M, Gujer W, Hany R, Bachmann S. 1997. Intracellular carbon flow in phosphorus accumulating organisms from activated sludge systems. Water Res 31:907-917.

Metcalf and Eddy. Wastewater Engineering: Treatment, Disposal and Reuse. McGraw-Hill, New York (1991).

Mino T, Arun V, Tsuzuki Y, Matsuo T. 1987. Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal. In Ramadori R., editor. Biological phosphate removal from wastewaters (Advances in Water Pollution Control 4). Oxford: Pergamon Press. pp. 27-38.

Mino T, van Loosdrecht MCM, Heijnen JJ. 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Review paper. Water Res 32:3193-3207.

Murnleitner E, Kuba T, van Loosdrecht MCM, Heijnen JJ. 1997. An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnol Bioeng 54:434-450.

Nakamura K, Dazai M. 1989. Growth characteristics of batch-cultured activated sludge adits phosphate elimination capacity. J Ferment Bioeng 64:433-439.

Nicholls HA, Osborn DW. 1979. Bacterial stress: prerequisite for biological removal of phosphorus. J Water Pollut Control Fed 51:557-569.

Oehmen A, Zeng RJ, Yuan Z, Keller J. (submitted). A metabolic model of anaerobic propionate utilisation by polyphosphate accumulating organisms (PAO) in enhanced biological phosphorus removal systems. Biotechnol Bioeng.

Pereira H, Lemos PC, Reis MA, Crespo JPSG, Carrondo MJT, Santos H. 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo 13C-NMR labelling experiments. Water Res 30:2128-2138.

Potgieter DJJ, Evans BW. 1983. Biochemical changes associated with luxury phosphate uptake in a modified phoredox activated sludge system. Water Sci Technol 15:105-116.

Randall AA, Benefield LD, Hill WE. 1994. The effect of fermentation products on enhanced biological phosphorus removal, polyphosphate storage and microbial population dynamics. Water Sci Technol 30:213-219.

Randall CW, Chapin RW. 1997. Acetic acid inhibition of biological phosphorus removal. Water Environ Res 69:955-960.

Reddy M. Biological and chemical systems for nutrient removal. WEF, special publication (1998).

Satoh H, Mino T, Matsuo T. 1992. Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal process. Water Sci Technol 26:933-942.

Satoh H, Ramey WD, Koch FA, Oldham WK, Mino T, Matsuo T. 1996. Anaerobic substrate uptake by the enhanced biological phosphorus removal activated sludge treating real sewage. Water Sci Technol 34:9-16.

SCOPE, Newsletter, Special edition on "Implementation of the 1991 EU Urban Waste Water Directive and its role in reducing phosphate discharges", 34 (1998).

Serafim LS, Lemos PC, Reis MAM. 2002. Change in the metabolism of PHA accumulation by activated sludge modifying operating conditions. Water Sci Technol 46(1-2):353-356.

Smolders GJF, van der Meij J, van Loosdrecht MCM Heijnen JJ.1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. Biotechnol Bioeng 43:461-470.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-848.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1995. A structured metabolic model for the anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol Bioeng 47: 277-287.

Steinbüchel A, Wiese SA. 1992. Pseudomonas strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain length 3-hydroxyalkanoic acids. Appl Microbiol Biotechnol 37:691-697.

Sudiana IM, Mino T, Satoh H, Nakamura K, Matsuo T. 1997. Metabolism of enhanced biological phosphorus removal and non enhanced biological phosphorus removal sludge with acetate and glucose as carbon source. BRN3 Conference, Brisbane, 41-47.

Wang N, Peng J, Hill G. 2002. Biochemical model of glucose induced enhanced biological phosphorus removal under anaerobic condition. Water Res 36:49-58.

Wentzel MC, Ekama GA, Marais GvR. 1991. Kinetics of nitrification denitrification biological excess phosphorus removal systems - a review. Water Sci Technol 23:555-565.

Yagci N, Artan N, Ubay Çokgör E, Randall CW, Orhon D. 2003. Metabolic model for acetate uptake by a mixed cultura of phosphate and glycogen accumulating organisms under anaerobic conditions. Biotechnol Bioeng 84:360-373.

Zeng R, Saunders AM, Yuan Z, Blackall LL, Keller J. 2003. Identification and comparison of aerobic and denitrifying polyphosphate accumulating organisms. Biotechnol Bioeng 83:140-148.

CHAPTER III

MATERIALS AND METHODS

The work presented in this thesis has been performed in two different laboratories and some analysis techniques for the same compound differs between them because of the techniques available in each place.

Different experimental set ups have been used along this thesis. These are described in each chapter to avoid any misunderstanding.

3.1. CHEMICAL ANALYSES

3.1.1. TOTAL SUSPENDED SOLIDS (TSS) AND VOLATILE SUSPENDED SOLIDS (VSS)

Mixed liquor suspended solids (TSS) and mixed volatile suspended solids (VSS) were analysed according to the methods 2540 D and 2540 E respectively of the standard methods for the examination of water and wastewater (APHA, 1995).

3.1.2. VOLATILE FATTY ACIDS (VFAs)

Acetate, propionate and butyrate were measured by Gas Chromatography (GC). A volume of 0.1 mL of 10% phosphoric acid was added to 0.9 mL of filtered sample (Millipore 0.2 μm pore size) and stored at 4ºC. Two different chromatographs were used depending in the laboratory where the experiments were performed.

A Hewlet Packart 5890 GC equipped with a HP-7673 column (30 m × 0.53 mm × 1.0 μm; length × internal diameter × film thickness) and a flame ionisation detector (FID) was used. The injector temperature was 260ºC, and a sample volume of 1 μL was injected. The carrier gas was high purity helium at a flow rate of 49 mL/min with precolumn and the column temperature was 240ºC. The run time was 22 min. The software used was Millenium 3.20 Waters.

A Perkin Elmer Autosystem GC equipped with a DB-FFAP column (15 m × 0.53 mm × 1.0 μm; length × internal diameter × film thickness) and a flame ionisation detector (FID) was also used. The injector temperature was 220ºC, and a sample volume of 1 μL was injected. The carrier gas was high purity helium at a flow rate of 30 mL/min and the column temperature was 140ºC. The run time was 16 min and the detector temperature was 250ºC.

3.1.3. PHOSPHATE, NITRITE, NITRATE AND AMONIA

Analyses of orthophosphate-phosphorus (PO_4^{3-}-P), nitrite, nitrate and amonia in 0.45 μm filtered samples were performed using two different systems depending on the laboratory where the experiments were performed.

An Electrophoresis Capillar System (*Quanta 4000E CE - WATERS*) was used. The electrolyte used was a commercial solution (*Ionselect High Mobility Anion Electrolite*)

Orthophosphate-phosphorus (PO_4^{3-}-P), nitrite, nitrate and ammonia were also analysed using a Lachat QuikChem8000 Flow Injection Analyser (FIA) (ProTech Group Pty Ltd, Coolum, Australia). A 0.45 μm filtered sample was appropriately diluted to bring the sample into the test range and analysed by FIA.

3.1.4. GLYCOGEN

Glycogen was determined by the modified method of Smolders *et al.* (1994a). A volume of 5 mL of 0.6 M HCl was added to weighed freeze-dried biomass in screw topped glass tubes, and then heated at 100°C for 6 h. After cooling and centrifugation, 1 mL of the supernatant was transferred to a HPLC vial for glucose analysis. A volume of 50 μL was injected into a Hewlett-Packard X-87H 300 mm x 7.8 mm, BioRad Aminex ion exclusion HPLC column at 65°C. 0.008 N H_2SO_4 sparged with helium was used as carrying solution at a flow rate of 0.6 mL/min. A Perkin Elmer Refractive Index Detector was used and operated at 35°C.

A Yellow Spring Instrument (2700 Select) was also used for glucose determination.

3.1.5. POLYHIDROXYALCANOATES (PHA)

PHB, PHV and PH2MV were measured according to a modification of the method of Comeau *et al.* (1988). Weighed freeze-dried biomass was put into screw topped glass tubes and methylated with 2 mL of acidified methanol (3% H_2SO_4) and 2 mL of chloroform during 20 hours at 100 ºC. After cooling, 1 mL of Milli-Q water was added and the contents were shaken vigorously for 10 min. One hour of settling was allowed for phase separation. The chloroform phase was then extracted from the samples, mixed with 1g of sodium sulphate, and then separated from the solid phase and transferred to GC vials. Benzoic acid was used as an internal standard. The calibration of the method was performed using a 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer (7:3) as standard for PHB and PHV (Fluka, Buchs SG, Switzerland) and 2-hydroxycaproic acid as standard for PH2MV (Aldrich). The

analyses were performed in a GC system, and a volume of 3 μL of the chloroform phase was injected in 1:25 split mode with column DB-5 (30 m × 0.25 mm × 0.25 mm) and a FID temperature of 240 ºC. The following temperature program was used: hold at 100ºC for 1 min, increase temperature by 10ºC/min to 150ºC, then by 45ºC/min to 270ºC.

3.1.6. TOTAL PHOSPHORUS

For the biomass P content determination, an amount of 20 mg of lyophilised sludge samples were digested with 2 ml of HNO_3 in a microwave oven. P content was assessed through ICP-OES spectrometry.

3.2. MICROBIAL ANALYSES

3.2.1. FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Oligonucleotides (short strands of nucleic acids) complementary to 16S rRNA sequence regions with an intermediate degree of conservation and characteristic for phylogenetic entities like genera, families, subclasses, have been used successfully for rapid identification of bacteria. The oligonucleotides are able to enter fixed bacterial cells and once inside the cells, they may form stable associations (hybrids via hydrogen bonding between complementary nucleotides) with the 16S rRNA in the ribosomes. If the complementary sequence for the oligonucleotide is not present in the 16S rRNA in the ribosome, stable hybridisation does not occur and the oligonucleotide is washed from the bacterial cell. In order to observe when hybridisation occurs, the oligonucleotides also contain a "reporter" molecule which usually is a fluorochrome. Cells in which the fluorescently-labelled oligonucleotide has hybridized with the 16S rRNA in the ribosome can be directly visualised by epifluorescent microscope or a confocal laser scanning microscope. The technique is called fluorescent *in situ* hybridization or whole cell probing and at least in the activated sludge area, this field has been dominated by the research group of R. Amann in Germany.

Fluorescence *in situ* hybridization (FISH) was performed as in Amann (1995) with Cy5-labelled EUBMIX probes (for most bacteria; Daims *et al*., 1999) and either Cy3-labelled PAOMIX probes (for *Accumulibacter*, comprising equal amounts of probes PAO462, PAO651 and PAO846, Crocetti *et al*., 2000) or Cy3-labelled GAOMIX (for *Competibacter*, comprising equal amounts of probes GAOQ431 and GAOQ989, Crocetti *et al*., 2002). The probes used are summarised in Table 3.1.

Table 3.1. Oligonucleotide probes used in this thesis

Probe name	Probe sequence (5'-3')	rRNA target site*	Specificity	% Formamide	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338-355	Many but not all *Bacteria*	0-70	Amann et al. (1990)
EUB338-II	GCAGCCACCCGTAGGTGT	16S, 338-355	*Planctomycetales*	0-50	Daims et al. (1999)
EUB338-III	GCTGCCACCCGTAGGTGT	16S, 338-355	*Verrucomicrobiales*	0-50	Daims et al. (1999)
PAO462	CCGTCATCTACWCAGGGTATTAAC*	16S, 462-485	"*Candidatus* Accumulibacter phosphatis"	35	Crocetti et al. (2000)
PAO651	CCCTCTGCCAAACTCCAG	16S, 651-668	"*Candidatus* Accumulibacter phosphatis"	35	Crocetti et al. (2000)
PAO846	GTTAGCTACGGCACTAAAAGG	16S, 846-866	"*Candidatus* Accumulibacter phosphatis"	35	Crocetti et al. (2000)
GAOQ431	TCCCCGCCTAAAGGGCTT	16S, 431-448	"*Candidatus* Competibacter phosphatis"	35	Crocetti et al. (2002)
GAOQ989	CACCTCCCGACCACATTT	16S, 989-1006	"*Candidatus* Competibacter phosphatis"	35	Crocetti et al. (2002)

*rRNA, *Escherichia coli* numbering (Brosius et al. 1981).
EUB338, EUB338-II, EUB338-III were used in a mixture called EUBMIX.
PAO462, PAO651, PAO846 were used in a mixture called PAOMIX.
GAOQ431 and GAOQ989 were used in a mixture called GAOMIX.
W=A+T

Description of the FISH technique

A) SAMPLE FIXATION

Paraformaldehyde (see below) is used to fixate the samples. 3 volumes of paraformaldehyde fixative is added to 1 volume of sample and hold at 4ºC for 1 to 3 hr. Pellet the cells by centrifugation (5000 × g) and remove fixative. Wash the cells with 0.01M phosphate buffered saline (PBS) described in Table 3.1 and resuspend in one volume of 0.01M PBS per one volume of ice cold ethanol and mix. Fixed cells can be spotted onto glass slides or stored at -20ºC for several months.

Paraformaldehyde: heat 65 ml of high purity water to 60ºC. Add 4 g paraformaldehyde. Add a drop of 2 M NaOH solution and stir rapidly until the solution has nearly clarified (1-2 min). Remove from the heat source and add 33 mL of 0.03M PBS. Adjust pH to 7.2 with HCl. Remove any remaining crystals by sterile filtration (0.2 µm). Quickly cool to 4ºC and store at this temperature.

Table 3.1. Phosphate Buffered Saline or PBS (for pH 7.2, the ratios of disodium:sodium phosphates must be 7.2:2.8)

	0.3 M P-PO_4^{3-}	**g for 1000 mL**
$Na_2HPO_4 \cdot 12H_2O$	0.216 M	77.37
$NaH_2PO_4 \cdot 2H_2O$	0.084 M	13.1
NaCl	3.9 M	226.2

To prepare 0.01 M PBS dilute 1 0.3M in 30.

B) APPLICATION OF SAMPLES TO SLIDES

Apply 10 µL of fixed sample to the glass slide, let dry, and dehydrate in ethanol series (3 min in each): 50%, 80% and 98% ethanol. Slides can be stored indefinitely.

C) PROBE HYBRIDIZATION

The hybridization buffer is prepared in 2 mL microcentrifugue tubes at the time of use. Two mL prepared allows adding 8 µL to each well on the slide and the remainder in the hybridization tube for keeping the chamber in the tube moist.

The hybridization buffer composition is:

- 360 µL of 5 M NaCl (autoclaved)
- 40 µL of 1 M Tris /HCl (autoclaved)
- 2 µL of 10% SDS- not autoclaved- placed in the lid of the centrifuge tube

- 700 µL of formamide
- 898 µL of milliQ water

The working formamide concentration is 35%.

A volume of 8 µL is added to each well on the slide and the remainder is used to moisten a tissue paper in the 50 mL tube. Then add 1 µL of probe at 25 ng/ µL and mix carefully. Place the slice in the 50 mL tube containing the moistened tissue. Close and put in the hybridization oven at 46ºC for 1.0 to 2.0 hr.

D) WASHING

After hybridization, the slides are carefully removed from the tube and rinsed immediately in water or in wash buffer.

Preparation of the wash buffer (total volume = 50 mL):

- 80 µL of NaCl 5M autoclaved
- 500 µL EDTA 0.5M
- 1 mL Tris/HCl 1 M autoclaved
- 43.8 mL milli-Q water autoclaved
- 50 µL 10% SDS not autoclaved (added last)

Warm the wash buffer in the bath at 48ºC during the hybridization. Washing has to be done at 48ºC.

After hybridization, slides are carefully removed from their tube and splashed with warm wash buffer into a beaker. Slide is then placed into the washing buffer tube and into the water bath at 48ºC for 10-15 min. Rapid transfer of slides prevents cooling which leads to non specific probe binding.

After wash, the slide is gently rinsed in milliQ water. Water is directed above wells and allowed to flood over them. Gloves used must not have too much powder on them as this is highly autofluorescent. Both sides of the slide are washed to remove any salt which is highly autofluorescent. After the washing step, all droplets of water have to be removed from the wells. Applying compressed air direct at the side of the slide ensures that all water droplets are removed.

E) MOUNTING SLIDES

A few drops of Citifluor AF1 are applied on the slide where are the FISH samples. Then, a coverslip is applied and the Citifluor covers the wells.

Visualisation and quantification

FISH preparations were visualised with a BioRad Radiance confocal laser scanning microscope (CLSM) or Leica DMIRB confocal laser scanning microscope (CLSM) and quantification of *Accumulibacter or Competibacter* as a proportion of all bacteria was done using methods previously reported (Zeng *et al*., 2003). Briefly, 40 randomly chosen CLSM fields from different x, y, and z coordinates were imaged (600× magnification) from each sample and were exported to Image Pro Plus (Media Cybernetics) software package for image analysis. The area containing Cy3-labelled specific probe (PAOMIX) cells was quantified as a percentage of the area of Cy5-labelled bacterial probe (EUBMIX) cells. The area of specific cell signal was expressed as a mean percentage of the area of bacterial cell signal for the 40 images. This method is a slight adaptation of those previously reported by Bouchez et al. 2000 and Crocetti et al., 2002.

3.2.2. CHEMICAL STAINING

Sudan Black stain (Murray et al. 1994)

The ability of Sudan Black to dissolve in fatty material because of its lipophilic properties allows this stain to be used to detect polyhydroxyalcanoates (PHA), reserves stored as granules within the cells of some bacterial species, which would otherwise not be visible under light microscopy. However, Sudan Black is not specific for PHA and stains any lipophilic material.

A) PREPARATION OF REAGENTS

Solution 1: Sudan Black B (IV) (0.3g) plus 60% ethanol (100mL).

Solution 2: Safranin O (0.5g) dissolved in distilled water (100 mL).

B) PROCEDURE

Using Solution 1, stain air-dried smear for 10 min adding extra stain if slide begins to dry, and follow with a 1-s rinse with water. Shake off excess water and stain with Solution 2 for 10s, rinse well with water and allow drying prior to examination. The preparation is examined through a bright-field objective lens (400-

1000×magnification). Blue/black granules in a clear or lightly coloured back-ground indicate de presence of lipophilic material, usually PHA.

Loeffer's methylene blue stain (Murray et al. 1994)

This stain is used for polyanions including polyphosphate. A red/pink coloration against a dark blue background is positive for the presence of poly P.

A) PREPARATION OF REAGENTS

Solution 1: Methylene blue (0.3g) dissolved in 60% ethanol (100mL).
Solution 2: Potassium hydroxide (10mg) dissolved in distilled water (100mL).
Mix solutions 1 and 2; mixed solutions are stable.

B) PROCEDURE

Stain Air-dried smear for 10-30s, rinse slide well with tap water. Shake off excess water and allow to dry prior to examination. Examine the prepared specimen through a bright field objective lens (400-1000× magnification). Bright pink to violet inclusions against a blue background indicate the presence of poly P. Typical staining reactions are that often the entire cell will stain pink-violet, and this can be interpreted as indicating the storage of a large amount of polyphosphate.

3.3. RESPIROMETRY

The OUR profile was obtained by measuring the DO profile (respirogram) in the reactor. The DO was measured in the liquid phase of the reactor, which was working on static conditions (there were neither inputs nor outputs). In addition, the reactor was continuously aerated with a steady flow. Hence, the reactor worked as a respirometer known as LFS respirometer (Spanjers et al., 1997). The DO balance in the liquid phase of the reactor must be solved in order to obtain the OUR profile. Assuming constant liquid volume and distinguishing between the OUR due to the endogenous process (OUR_{END}) from the OUR due the exogenous process (OUR_{EX}), equation 5.1 can be obtained (Vanrolleghem et al., 1994).

$$OUR_{EX}(t) = k_L a \cdot \left[S_{OE} - S_O(t)\right] - \frac{dS_O}{dt} \quad (5.1)$$

where S_{OE} is the constant equilibrium value of DO reached when the biomass is aerated under endogenous conditions. The k_La of the reactor was calculated following the procedure originally described by Bandyopadhyay et al. (1967). The

k_La was estimated through a least square optimisation process on the reaeration profile obtained with the action of turning the aeration off and subsequently turning it on.

3.4. MODELLING SOFTWARE

The modelling software used was MATLAB 6.1 (TheMathWorks, Natick, MA). The differential equations were solved using the function *ode45*. This solver is based on an explicit Runge-Kutta (4,5) formula, the Dormand-Prince pair. Parameter optimisation to fit with experimental data was carried out by using the *fminsearch* function, which uses the Nelder-Mead-Simplex search method.

3.5. REFERENCES

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. App Environ Microbiol 56:1919-1925.

Amann RI. 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular Microbial Ecology Manual*, pp. MMEM-3.3.6/1-MMEM-3.3.6/15. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. London: Kluwer Academic Publications.

APHA.1995. Standard methods for the examination of water and wastewater, 19th ed. American Publishers Health Association.

Bandyopadhyay B, Humprey AE, Taguchi H. 1967 Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. Biotechnol Bioeng 9:533-544.

Bouchez T, Patureau D, Dabert P, Juretschko S, Dore J, Delgenes P, Moletta R, Wagner M. 2000. Ecological study of a bioaugmentation failure. Envioron Microbiol 2:179-190.

Comeau Y, Hall KJ, Oldham WK. 1988. Determination of poly-hidroxybutyrate and poly-hydroxyvalerate in activated sludge by gas-liquid chromatography. Appl Environ Microbiol 54:2325-2327.

Crocetti GR, Hugenholtz P, Bond PL, Schuler A, Keller J, Jenkins D, Blackall LL. 2000. Identification of polyphosphate accumulating organisms and design of 16S

rRNA-directed probes for their detection and quantitation. App Environ Microbiol 66:1175-1182.

Crocetti GR, Banfield JF, Keller J, Bond PL, Blackall LL. 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology 148:3353-3364.

Daims H, Bruhl A, Amann R, Schleifer KH, Wagner M. 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 22:434-444.

Murray RGE, Robinow CF. 1994. Light microscopy in methods for general and molecular bacteriology. Eds. P Gerhardt, RGE Murray, WA Wood and NR Krieg, American Society for Microbiology, Washington DC, pp. 7-20.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994a. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-849.

Spanjers H, Vanrolleghem PA, Olsson G, Dold PL. 1997. Respirometry in control of the activated sludge process: principles. Scientific and technical report, no7 IWA Publishing. London.

Vanrolleghem PA, Kong Z, Rombouts G, Verstraete W. 1994. An on-line respirographic biosensor for the characterization of load and toxicity of wastewaters. J Chem Technol Biotechnol 59:321-333.

Zeng RJ, Saunders AM, Yuan Z, Blackall LL, Keller J. 2003. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnol Bioeng 83:140-148.

CHAPTER IV

ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL IN A SEQUENCING BATCH REACTOR USING PROPIONATE AS THE SOLE CARBON SOURCE

This chapter has been published as:

Pijuan M, Saunders AM, Guisasola A, Baeza JA, Casas C, Blackall LL. 2004. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnology and bioengineering 85(1):56-67.

4.1. INTRODUCTION

Acetate and propionate are the two most common VFAs present in septic domestic wastewaters. However, most mechanistic models for EBPR are developed by treating all VFAs as acetate. When acetate is used as the sole carbon source, the PHA produced is mostly PHB, while when propionate becomes the predominant carbon source, most PHA produced appears as PHV (Lemos *et al.* 1998; Randall and Liu, 2002). If the type of VFA used by PAOs in EBPR has an effect on their metabolism, the acetate:propionate ratio should be taken into account for mechanistic modelling (Hood and Randall, 2001). In this work the behaviour of an EBPR population developed using propionate as the sole carbon source was studied. The majority of studies on EBPR have been carried out using acetate as the sole carbon source or acetate in combination with other substrates. (Smolders *et al.* 1994a,b; Mino *et al.* 1994; Hood and Randall, 2001; Levantesi *et al*. 2002). Propionate as a carbon source has not been commonly reported upon since in the literature although some recent works can be found (Lemos *et al*. 2003).

In this study, the abundance of "*Candidatus* Accumulibacter phosphatis" and "*Candidatus* Competibacter phosphatis" (from now on called *Accumulibacter* and *Competibacter*, respectively) in a propionate fed SBR was followed using FISH probing. Population abundances were compared with the EBPR process performance data. Moreover, cycle studies were performed with the propionate-enriched biomass using propionate and acetate separately as the sole carbon source. The effect of pH on the process performance during cycle studies was also evaluated in order to determine the optimum working pH range. A modification of the ASM2 model based on glycogen economy was used to describe the experimental data obtained in the cycle study experiments performed with propionate and acetate.

4.2. MATERIALS AND METHODS

4.2.1. REACTOR

Experiments were conducted in an SBR with a working volume of 1.8 litres, inoculated with activated sludge from an EBPR plant (Thornside WWTP, Queensland, Australia). It was operated in 4 cycles of 6 h per day. Each cycle consisted of 2.5 h anaerobic react, 3 h aerobic react, 30 min settling and, in the last 5 min of settling, extraction of 900 mL supernatant. A volume of 900 mL of synthetic wastewater (composition below) was added during the first 7 min of the subsequent

cycle, producing a HRT of 12 h. The SRT was kept at 9 d by periodic sludge wastage during the react phase. Since the pH was not controlled, it increased from 7.2 to 7.6 during the anaerobic react and it increased further to 8.5 during the aerobic react, due to the stripping of CO_2. The SBR operating temperature was 23-24ºC during all stages. To achieve anaerobic conditions, nitrogen gas was bubbled through the liquid and to achieve aerobic conditions, air was bubbled. Figure 4.1 shows the experimental set up used in this study.

Figure 4.1. Experimental set up used in this study

4.2.2. SYNTHETIC WASTEWATER

Two separate solutions called "concentrated feed" (constituting 45 mL per 900 mL synthetic wastewater) and "P-water" (constituting 855 mL per 900 mL synthetic wastewater), were mixed prior to addition to the SBR and collectively they formed the synthetic wastewater used in this study. The concentrated feed (adapted from Smolders *et al.*, 1994a) consisted of (g/L RO water): 5.6 propionic acid (to achieve 140 mg/L in the reactor), 2.44 peptone, 0.84 NH_4Cl, 1.8 $MgSO_4*7H_2O$, 3.2 $MgCl_2*6H_2O$, 0.84 $CaCl_2*2H_2O$, 0.4 yeast extract, 10 allylthiourea (ATU) to inhibit nitrification and 12 mL of nutrient solution (Zeng *et al.* 2003). The nutrient solution consisted of (g/L RO water): 1.5 $FeCl_3*6H_2O$, 0.15 H_3BO_3, $CuSO_4*5H_2O$, 0.18 KI, 0.12 $MnCl_2*4H_2O$, 0.06 $Na_2MoO_4*4H_2O$, 0.12 $ZnSO_4*7H_2O$, 0.15 $CoCl_2*6H_2O$ and 68.5 mL EDTA 0.5M. Propionate was the sole carbon source and the initial anaerobic concentration in the SBR was increased progressively over a 20 d period from 20 mg to 140 mg propionate/L. The pH of the concentrated feed was adjusted to 5.5 with 2 M NaOH and autoclaved to prevent contamination.

The P-water consisted of (mg/L RO water): 81.6 KH_2PO_4 and 62.0 K_2HPO_4 and the pH was adjusted to 10.0 with 2 M NaOH. The beginning COD:P ratio was 4 and it increased to a final value of 14, when the SBR was working at 140 mg propionate/L.

4.3. RESULTS AND DISCUSSION

4.3.1. ENRICHING AN EBPR POPULATION

An EBPR biomass was enriched using propionate as the sole carbon source. After three weeks, the amount of propionate was 140 mg/litre at the beginning of the anaerobic phase, and this concentration was maintained until steady state was achieved. After three SRTs (approximately 27 days), the reactor showed substantial P removal. During this period, the amount of PAOMIX-binding cells (*Accumulibacter*) rapidly increased from 7% of all bacteria on the 2nd day of operation to 54% of all bacteria on the 17th day of operation. The micrographs in Figure 4.2 visually show this shift in microbial population. During steady state operation of the SBR, when all cycle study experiments were carried out, 50-55% of all bacteria were *Accumulibacter* and they were presumed to be the dominant PAO.

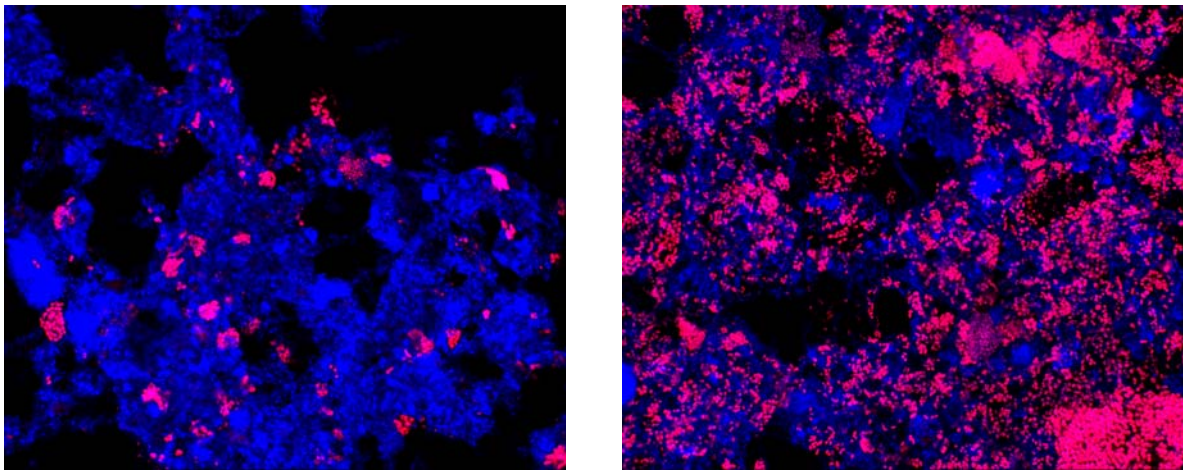

Figure 4.2. CLSM micrographs of FISH of the EBPR sludge hybridized with probe specific for PAO (In pink, Cy3-labelled PAOMIX probes) and probe for bacteria (In blue, Cy5-labelled EUBMIX probes). a) After 2 days of SBR operation. b) After 15 days of operation.

The inoculum for the SBR contained GAOMIX-binding cells (*Competibacter)*, and although their abundance could not be accurately quantified due to such low amounts, they probably comprised 1-2% of all bacteria. On the 17th day of operation, the abundance of *Competibacter* cells had dramatically declined to just a few cells every few fields observed microscopically, which would be substantially less than

even 1% of all bacteria. After 6 weeks of operation, *Competibacter* cells were rarely observed in the biomass.

It has been suggested that propionate could selectively enrich PAOs over other organisms like glycogen-accumulating organism (GAOs). Hood and Randall (2001) hypothesised that propionate may be relatively easily sequestered and metabolised by PAOs, but that it might not be readily utilizable by other microorganisms also requiring VFA in the anaerobic zone. Thomas *et al.* (2002) also provide evidence supporting the GAO competition for acetate and PAO competition for other substrates including propionate. The population shifts in *Accumulibacter* (a PAO) and *Competibacter* (hypothesised to be the abundant GAO) in the SBR with propionate was dramatic and rapid and it could be explained by the carbon source, as suggested by others (Mino *et al.*, 1994; Hood and Randall, 2001). Therefore, the use of propionate, instead of acetate, seems to be beneficial in obtaining a culture with as many PAOs as possible. This is a priority in current EBPR research, since it will provide important information for understanding the physiology and biochemistry of PAOs and GAOs (Blackall *et al.* 2002; Seviour *et al.*, 2003).

4.3.2. CYCLE STUDIES WITH PROPIONATE AND ACETATE

After obtaining steady state in the SBR, two sets of cycle study experiments were carried out in order to determine the behaviour of the EBPR microbial community developed with propionate. The experiments were performed in the SBR with the same operational conditions and also without pH control. Since cycle studies were generally carried out once per day, at least three 6-h SBR cycles with normal VFA concentration elapsed between each study. The first set of experiments was done with different propionate concentrations at the beginning of the cycle.

Propionate as substrate

Results of the cycle studies performed with different initial concentrations of propionate are presented in Figure 4.3.A (P profiles), 4.3.C (propionate profiles) and 4.3.E (ammonia profiles). The rates for different transformations are presented in Table 4.1. During these cycle studies, the anaerobic P-release (mmol P/g VSS) increased with increasing propionate concentration in the feed, but the concomitant aerobic P-uptake for the two highest propionate concentrations employed was not complete. The aerobic P-uptake presented for the normal SBR operating concentration (1.35 mmol propionate/g VSS) was complete after 2 h of aerobiosis (Figure 4.3.A).

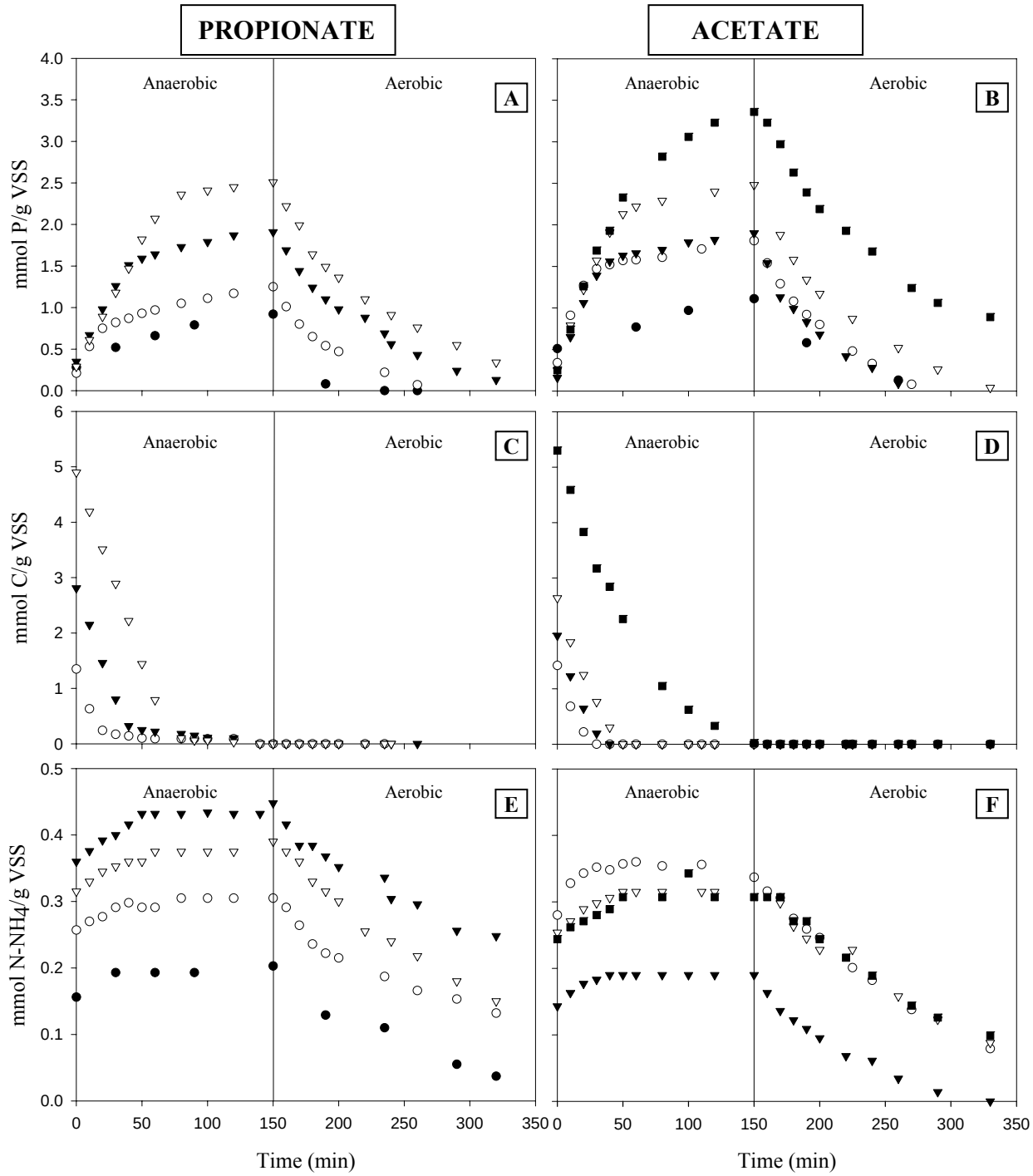

Figure 4.3. Experimental profiles of different compounds during the anaerobic/aerobic cycles of the SBR. **A** (phosphorus profile)**, C** (propionate profile) **and E** (ammonia profile)**:** Profiles obtained with different amounts of propionate: ● 0 mmol C/g VSS, ○ 1.35 mmol C/g VSS, ▼ 2,81 mmol C/g VSS, ▽ 4.90 mmol C/g VSS . **B** (phosphorus profile)**, D** (acetate profile) **and F** (ammonia profile)**:** Profiles obtained with different amounts of acetate: ● 0 mmol C/g VSS, ○ 1.42 mmol C/g VSS, ▼ 1.96 mmol C/g VSS, ▽ 2.64 mmol C/g VSS, ■ 5.30 mmol C/g VSS.

During the anaerobic period, the phosphorus was released at two different rates. The first rate took place when propionate was present in the mixed liquor. The second P-release, slower than the first one, was detected when propionate was completely sequestered. The rates of the first P-release when propionate was provided are very similar (Table 4.1) and thus, it seems that this rate does not depend on the concentration of propionate. The second P-release rates when propionate was provided are also quite similar. However, the P-release rate in the study employing the highest concentration of propionate was a bit lower than the other two (Table 4.1). This difference could be due to the fact that the second P-release rate was determined in a shorter period of time (Figure 4.3.A). The cycle study with no added propionate demonstrated P-release could occur without any VFA consumption (Figure 4.3.A). The P-release rate for this latter study, 0.005 mmol P/g VSS min, is higher than those of the second release from the other experiments (Table 4.1). Nevertheless, all the second P-release rates, including that measured with no added propionate, are in the same order of magnitude (Table 4.1) and they are about one order of magnitude lower than P-release rates when propionate was still present (Table 4.1). One hypothesis explaining this P-release, which seems not to be linked with the uptake of substrate, could be that the enriched PAOs use their excess intracellular polyP to generate energy that could be required for maintenance. An additional hypothesis is that the microorganisms are using substrates other than propionate such as peptone or yeast extract for their P-release. The SBR medium contained peptone at levels slightly higher than those typically used by other researchers (Bond *et al.* 1999; Smolders *et al.* 1994a; Zeng *et al.* 2003).

Table 4.1. Phosphorus release rates, propionate and phosphorus uptake rates, and phosphorus release *vs* substrate uptake ratios calculated with the experimental data of cycle studies performed with different amounts of propionate in the SBR.

	Propionate concentrations during cycle studies (mmol C/g VSS)			
	0	**1.35**	**2.81**	**4.90**
P-release rate * (mmol P/g VSS min)	-----	0.027	0.029	0.026
Second release rate (mmol P/g VSS min)	0.005	0.003	0.003	0.002
Propionate uptake rate (mmol C/g VSS min)	-----	0.056	0.062	0.069
Propionate upt. rate (mmol prop/g VSS min)	-----	0.018	0.021	0.023
P-release/ C-uptake* (mmol P/mmol C)	-----	0.49	0.46	0.38
P-release/propionic upt.* (mmol P/mmol prop)	-----	1.48	1.39	1.13
P-uptake rate (mmol P/g VSS min)	0.016	0.012	0.012	0.012

* Values calculated using the period when there is substrate in the mixed liquor.

Barnard (1984) first described this second P-release in the anaerobic zone after VFA removal. Smolders *et al.* (1995) introduced a maintenance parameter in their anaerobic metabolic model of 0.001 mmol P/g VSS min, which is a lower value than that obtained in this study with propionate (Table 4.1; 0.003-0.005 mmol P/g VSS min). However, no further comment is made about this in their work and, moreover, it is mostly absent in other EBPR literature. In our study, the secondary P-release measured could not be neglected because it is equivalent to 18% of the total amount of P-released under anaerobic conditions.

Acetate as substrate

After the propionate cycle studies, a second set of cycle studies with acetate was performed in the reactor. The acetate experiments spanned 5 days, during which the carbon source fed to the SBR was changed from 1.89 mmol propionate/L (196 mg COD/L) to 2.83 mmol acetate/L (187 mg COD/L). During this 5 day period, one cycle per day was used to study different acetate additions. All other SBR cycles were operated with 2.83 mmol acetate/L. The main reason for changing the SBR operating carbon source was to explore the acclimation of the biomass to acetate. After these 5 days, acetate was changed back to propionate in order to return to the original operating conditions of the SBR. In the first SBR cycle with acetate (1.42 mmol C/g VSS), the propionate-enriched biomass was able to use acetate immediately for EBPR. The observed acetate uptake rate did not change markedly during the 5 days of acetate operation, meaning that no acclimation period was required.

Results of the cycle studies performed with different initial concentrations of acetate are presented in Figure 4.3.B (P profiles), 4.3.D (acetate profiles) and 4.3.F (ammonia profiles). The rates for different transformations are presented in Table 4.2.

Table 4.2. Phosphorus release rates, acetate and phosphorus uptake rates, and phosphorus release *vs* substrate uptake ratios calculated with the experimental data of cycle studies performed with different amounts of acetate in the SBR.

	Acetate concentrations during cycle studies (mmol C/g VSS)				
	0	**1.42**	**1.96**	**2.64**	**5.30**
P-release rate* (mmol P/g VSS min)	-----	0.047	0.041	0.041	0.042
Second release rate (mmol P/g VSS min)	0.004	0.003	0.003	0.004	**
Acetate uptake rate (mmol C/g VSS min)	-----	0.060	0.059	0.062	0.061
Acetate uptake rate (mmol acetate/g VSS min)	-----	0.030	0.029	0.031	0.030
P-release/ C uptake* (mmol P/mmol C)	-----	0.78	0.70	0.66	0.68
P-release/acetic uptake* (mmol P/mmol acet.)	-----	1.56	1.39	1.33	1.37
Phosphorus uptake rate (mmol P/g VSS min)	0.009	0.016	0.016	0.016	0.013

* Values calculated using the period when there is substrate in the mixed liquor.

** Substrate present during all the anaerobic phase.

During these cycle studies, the anaerobic P-release (mmol P/g VSS) increased with increasing acetate concentration in the feed. The following aerobic P-uptake was complete, with the only exception of the highest acetate concentration. When the SBR operation employed 1.42-1.96 mmol C/g VSS, the aerobic P-uptake was complete after approximately 2 h of aerobiosis (Figure 4.3B).

As with propionate, two different P-release rates were observed. The first one was higher than the second (Table 4.2), which occurred when acetate was depleted (Figure 4.3.B and 4.3.D). Values of this second P-release obtained using either acetate or propionate were very similar as were the results performed without added VFA. The type of VFA seems not to affect the second P-release. This finding could provide support for the hypothesis that the organisms are using peptone in the medium for P-release, since this component is common to both experiments.

Ammonia results

Ammonia was measured during all the experiments performed with propionate (Figure 4.3.E) and acetate (Figure 4.3.F). An increase of ammonia was detected in all the experiments during the anaerobic period likely due to hydrolysis of the organic nitrogen from peptone to ammonia by the microorganisms. The diminution of ammonia in aerobic conditions can not be assigned to nitrification, because nitrate was not detected in the reactor. Therefore, the biomass consumed the ammonia for

growth, supporting the metabolic models showing that PAOs grow under aerobic conditions.

4.3.3. PROPIONATE VERSUS ACETATE: THE MAIN DIFFERENCES

The results of the experiments performed using the two different VFAs show that there were some differences in the behaviour of the selected population when they used propionate or acetate. The main results are summarised in Table 4.3. One main difference observed was the rate of P-release when VFA is still present. The results of this study contrast with some of those reported in the literature (Hood and Randall, 2001) where the experiments performed with propionate showed a higher P-release than with acetate. In the experiments, the mmol P-release per mmol of VFA uptaken for propionate (1.33) was very similar to the results obtained in the case of acetate (1.40). When these data were expressed as mmol P-release/mmol C uptake, a difference is clear – 0.46 for propionate and 0.72 for acetate. High P-release was detected when acetate (in terms of C-mmol) was uptaken, which agrees with Liu *et al.* (2002). A possible explanation could be that the same amount of ATP is required to convert propionate to propionyl-CoA (3 carbons) as is required to convert acetate to acetyl-CoA (2 carbons). So, more energy could be required per C-mol to convert acetate to acetyl-CoA than to convert propionate to propionyl-CoA. This energy requirement is also related to the fractions of PHB, PHV or PH2MV in the PHA.

Table 4.3. Mean rates of phosphorus release, substrate and phosphorus uptake, and mean ratios of phosphorus release *vs* substrate uptake calculated with the experimental data of cycle studies performed with different amounts of propionate and acetate in the SBR.

Parameters average	Propionate experiments	Acetate experiments
P-release rate * (mmol P/g VSS min)	0.027 ± 0.002	0.043 ± 0.003
VFA uptake rate (mmol C/g VSS min)	0.062 ± 0.006	0.060 ± 0.001
VFA uptake rate (mmol VFA/g VSS min)	0.020 ± 0.001	0.029 ± 0.001
P-release/VFA-uptake(mmol P/mmol VFA)	1.33 ± 0.18	1.40 ± 0.11
P release/ C uptake* (mmol P/mmol C)	0.46 ± 0.03	0.72 ± 0.04
P-uptake rate ** (mmol P/g VSS min)	0.012 ± 0.001	0.015 ± 0.002

* Values calculated using the period when there is substrate in the mixed liquor.

** Values calculated without using the value obtained in the cycle study performed without substrate.

The rate of the VFA uptake, in terms of mmol C uptaken/g VSS min, was very similar for propionate and acetate (Table 4.3) but when converted to mmol VFA uptaken/g VSS min, acetate uptake by PAOs was more rapid than was propionate uptake. Consequently, the P-release when acetate was the carbon source was faster

than when propionate was the carbon source, since PAOs expend the same energy to uptake both VFAs, as was hypothesised in the previous paragraph.

During aerobic conditions the uptake of phosphorus took place. The values of the P-uptake rate in the experiments with acetate were higher than in the experiments performed with propionate (Table 4.3). As suggested Randall and Liu (2002), this P-uptake can be influenced by the PHA composition at the beginning of the aerobic phase. The hypothesis that higher PHB percentages imply higher P-uptake agrees with the experimental results of this work.

Analyses of the intracellular polymers were carried out during the different cycle study experiments. Acetate was mainly stored as PHB while when propionate was used, most of it was stored as PHV. The results are shown in Table 4.4. The types of polymers produced by the propionate-fed biomass agree with those reported by Randall and Liu (2002), but they found 10% PHB and 90% PHV, while in this work 36% PHB and 64% PHV was found.

Table 4.4. Polyhydroxyalkanoate composition of the SBR biomass analysed at the end of the anaerobic phase in the cycle studies performed with propionate or acetate as carbon source.

Substrate	**PHB (%)**		**PHV (%)**	
	Average	Standard Deviation	Average	Standard Deviation
Propionate	36	2	64	2
Acetate	86	3	14	3

4.3.4. CYCLE STUDIES AT DIFFERENT pH

A set of cycle studies was performed in the SBR at steady state to assess the effect of pH from 6.5 to 8.0 on EBPR performance, when propionate was the sole carbon source. A pH controller was installed in the reactor to perform one SBR cycle study on each of four consecutive days at pH values of 6.5, 7.0, 7.5 and 8.0 during the whole cycle. A study without pH control was also carried out in order to see the P and propionate transformations in a typical cycle (Figure 4.4). One anaerobic-aerobic cycle of the reactor had the pH controlled and there were three cycles at uncontrolled pH between the experimental cycles. The VFA in all the experiments was propionate at 196 mg COD/L (1.89 mmol propionate/L) at the beginning of the anaerobic phase.

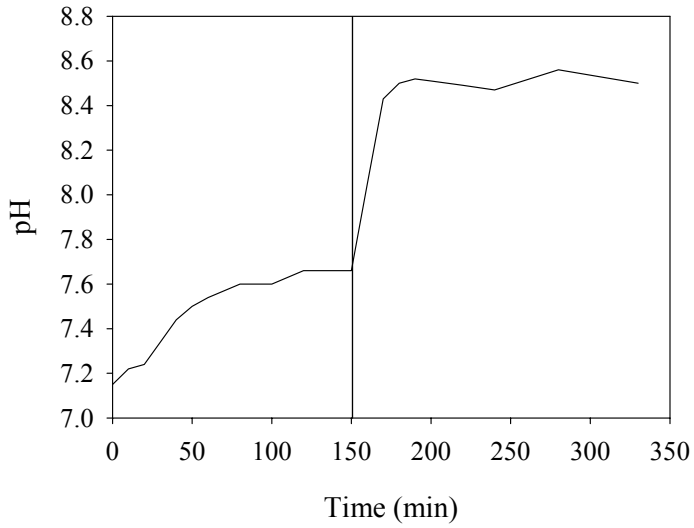

Figure 4.4. pH profile obtained in a common cycle of the SBR with propionate as carbon source and without pH control.

Increasing the pH from 6.5 to 8.0 or operation at uncontrolled pH (Figure 4.4) led to increased propionate uptake rates (Figure 4.5 and Table 4.5). The best result obtained in anaerobic conditions was working at pH of 8.0 or in an uncontrolled pH operation (Table 4.5). These results differ from those reported by Liu *et al.* (1996) for EBPR with acetate, where an optimum anaerobic pH of 6.8 was found. However, other studies on EBPR with acetate found best results with no pH control in the anaerobic period because the system pH tended to increase anaerobically (Schuler and Jenkins, 2002).

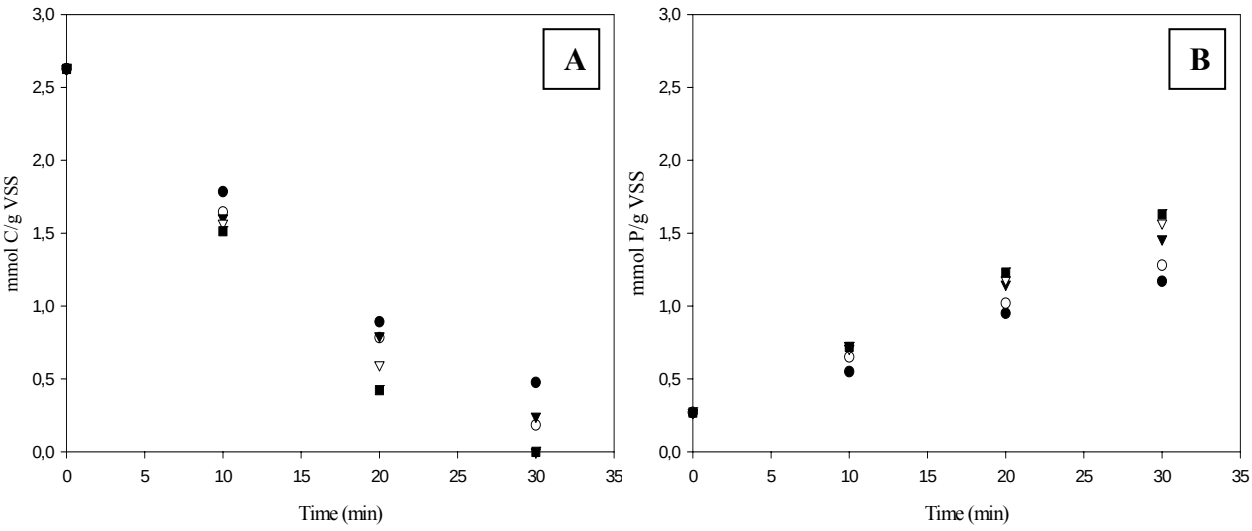

Figure 4.5. Experimental data for propionate (A) and phosphate (B) in the mixed liquor during the anaerobic phase of a cycle study performed in the SBR at different controlled pH values (● pH 6.5; ○ pH 7.0; ▼ pH 7.5; ∇ pH 8.0; ■ pH without control).

A significant effect of the pH on the P-uptake rate was found in the aerobic period (Figure 4.5 and Table 4.5). A low pH value like 6.5 or no pH control led to the lowest P-uptake rates, while the highest P-uptake rates were recorded at pH 7.5 and 8.0. Consequently, this pH range was recommended as the optimal working pH under aerobic conditions. Nevertheless, when the anaerobic and aerobic data are considered jointly (Figure 4.5 and 4.6 and Table 4.5), the overall optimum operating pH for EBPR with propionate was around 7.5.

Table 4.5. Phosphorus release rate and propionate and phosphorus uptake rates calculated with the experimental data of cycle studies performed at different controlled pH.

	pH				
	6.5	**7.0**	**7.5**	**8.0**	**No control****
P-release rate* (mmol P/g VSS min)	0.030	0.034	0.039	0.041	0.036
Propionic uptake rate (mmol C/g VSS min)	0.067	0.081	0.079	0.082	0.100
P-uptake rate (mmol P/g VSS min)	0.012	0.021	0.025	0.025	0.020

* Values calculated using the period when there is substrate in the mixed liquor.

**See Fig.4.4 for profile during anaerobic and aerobic periods.

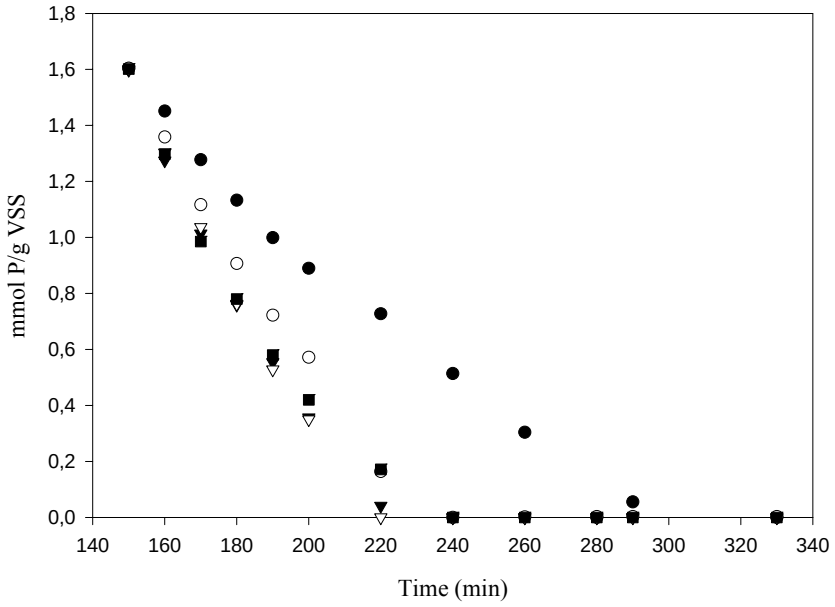

Figure 4.6. Experimental data for phosphate in the mixed liquor during the aerobic phase of a cycle study performed in the SBR at different controlled pH values (● pH 6.5; ○ pH 7.0; ▼ pH 7.5; ∇ pH 8.0; ■ pH without control).

4.3.5. PROCESS MODELLING

A kinetic and stoichiometric model developed as a modification of ASM2 (Henze *et al.* 2000) was fitted to the experimental profiles. Only PAO microorganisms are considered in the model because OHOs and nitrifying bacteria were not favoured in the operational conditions of our system. The main modification introduced consisted in the inclusion of glycogen economy. This modification is based on other models found in the literature (Smolders *et al.* 1994 a,b.; Mino *et al.* 1995; van Veldhuizen *et al*. 1999, Filipe and Daigger 1999, Manga *et al.* 2001).

This model considered nine reactions involved in PAO mechanisms including glycogen economy and the second P-release. Storage polymers (PHA, glycogen and polyP) are treated separately from active biomass to describe reliably the process. This consideration implies the appearance of the lysis reactions to ensure that polymeric storage products decay together with the biomass. Nitrogen is not considered as a state variable because ammonia concentration was high enough to avoid limitations during the experiments. In addition, anoxic processes related to PAO have been omitted because nitrates were not present in the reactor.

A description of this model is shown in Figure 4.7 with a graphical representation, along with the kinetics and stoichiometry (Table 4.6) and a brief description of the parameters (Table 4.7). The maintenance process of the model was used to describe the second P-release observed in all the experiments performed with both VFAs when these were completely depleted. In fact, this release would take place during all the anaerobic period, but it is only observed when the substrates are depleted or in the experiments performed without adding VFA. When the VFA is still in the medium, this second release is hidden by the normal release of phosphorus due to the substrate uptake, because this one is much higher than the maintenance P-release.

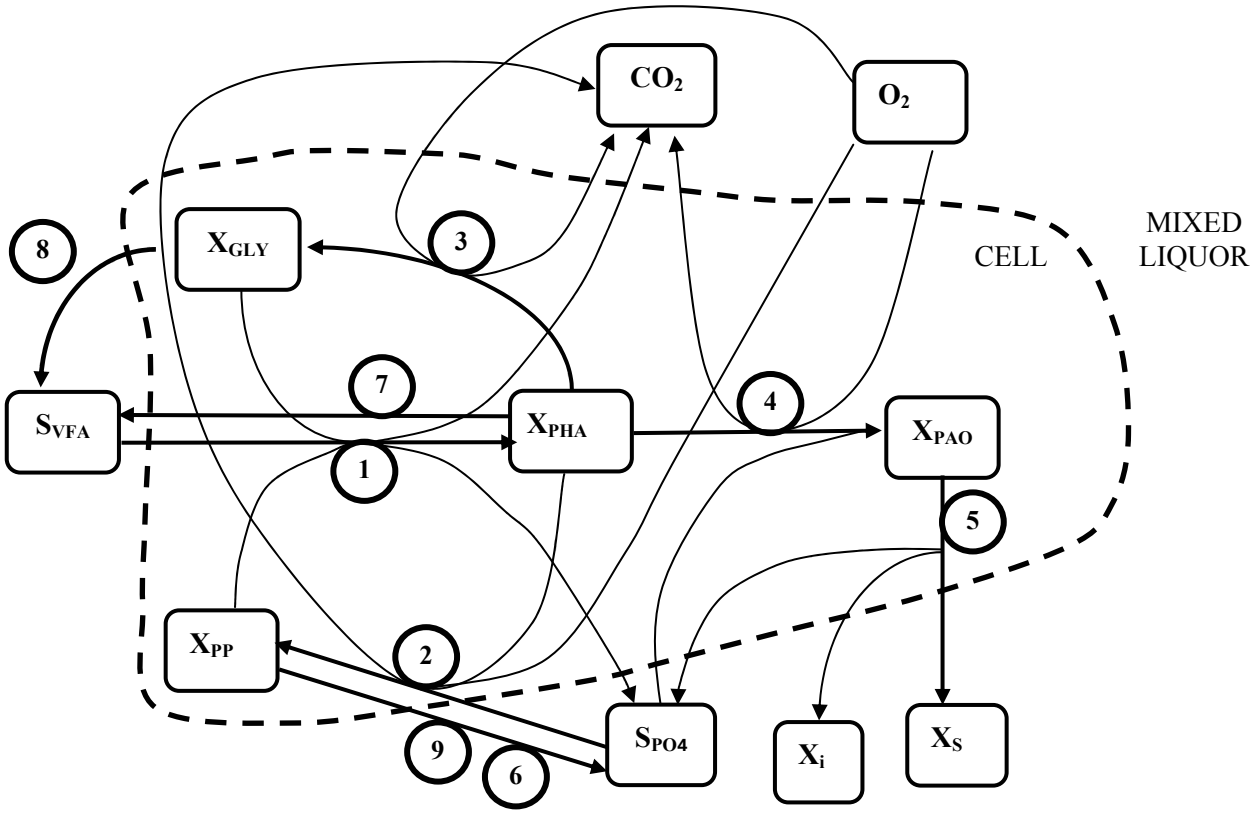

Figure 4.7. Schematic diagram of the model used in this study (1-PHA storage, 2-Poly-P storage , 3-Glycogen storage, 4-PAO growth, 5- PAO lysis, 6- Poly-P lysis, 7- PHA lysis, 8-Glycogen lysis, 9-Anaerobic poly-P lysis for maintenance).

The aerobic phase is described by four conversion rates: PHA, biomass, glycogen and poly-P. With three of these rates known, the remaining one can be calculated. In this model, the biomass formation rate was calculated using the other ones because the biomass increase was not measured in the experiments performed.

Table 4.6 – Stoichiometry and kinetics of the model used in this study.

Process	Stoichiometry								
	S_{VFA}	S_{O2}	S_{PO4}	X_{PHA}	X_{GLY}	X_{PP}	X_{PAO}	X_S	X_I
1 X_{PHA} storage	$-Y_{VFA1}$		Y_{PO}	1	$-Y_{GLY1}$	$-Y_{PO}$			
2 X_{PP} storage		$-Y_{PHA}$	-1	$-Y_{PHA}$		1			
3 X_{GLY} storage		$-(1-Y_{GLY3})$		-1	Y_{GLY3}				
4 X_{PAO} growth		$-(1-Y_{PAO})/Y_{PAO}$	$-i_{BPM}$	$-1/Y_{PAO}$			1		
5 X_{PAO} lysis			$\nu 5_P$				-1	$(1-f_{XI})$	f_{XI}
6 X_{PP} lysis			1			-1			
7 X_{PHA} lysis	1			-1					
8 X_{GLY} lysis	1				-1				
9 Maintenance			1			-1			

Process	kinetics
1 X_{PHA} storage	$q_{PHA}\frac{S_{VFA}}{K_S+S_{VFA}}\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{X_{GLY}/X_{PAO}}{K_{GLY}+X_{GLY}/X_{PAO}}X_{PAO}$
2 X_{PP} storage	$q_{PHA}\frac{S_{VFA}}{K_S+S_{VFA}}\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{X_{GLY}/X_{PAO}}{K_{GLY}+X_{GLY}/X_{PAO}}X_{PAO}$
3 X_{GLY} storage	$q_{PHA}\frac{S_{VFA}}{K_S+S_{VFA}}\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{X_{GLY}/X_{PAO}}{K_{GLY}+X_{GLY}/X_{PAO}}X_{PAO}$
4 X_{PAO} growth	$q_{PHA}\frac{S_{VFA}}{K_S+S_{VFA}}\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{X_{GLY}/X_{PAO}}{K_{GLY}+X_{GLY}/X_{PAO}}X_{PAO}$
5 X_{PAO} lysis	$b_{PAO}X_{PAO}$
6 X_{PP} lysis	$b_{PP}X_{PP}$
7 X_{PHA} lysis	$b_{PHA}X_{PHA}$
8 X_{GLY} lysis	$b_{GLY}X_{GLY}$
9 Maintenance	$q_{MANT}\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{K_O}{K_O+S_O}X_{PAO}$

Calibration and validation of the model

This model was calibrated using one cycle study experiment of the set performed with each VFA. The calibrations performed for both substrates are shown in Figure 4.8 (A and B). PHA comprises PHV and PHB compounds. In table 4.7, the model parameters obtained are shown and compared to ASM2 values. Only four parameters of the model are different when propionate or acetate is used as substrate.

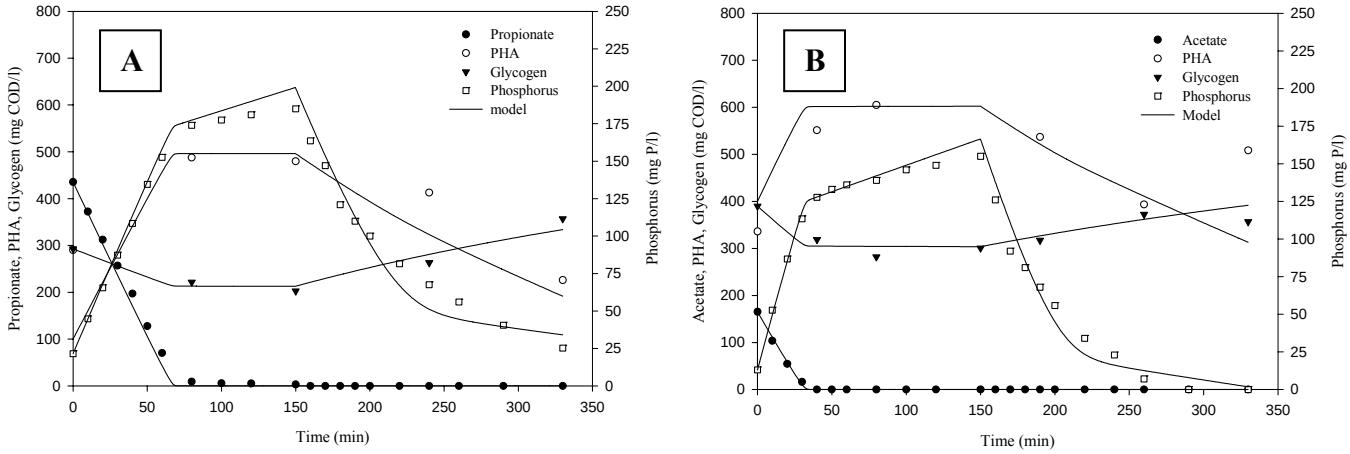

Figure 4.8. Cycle studies used for model calibrations. **A** - Experimental measures and simulated values for propionate cycle study (4.9 mmol C / g VSS). **B** - Experimental measures and simulated values for acetate cycle study (1.96 mmol C / g VSS). (□ Phosphorus, ● VFA, ▼Glycogen, ○ PHA, — fitted model).

Table 4.7–Value and description of the parameters of the model and comparison with the ASM2 parameters (COD_X = biomass COD, COD_{VFA} = external substrate COD (acetate or propionate), COD_{PHA} = PHA COD, COD_G = Glycogen COD, COD_I = Inert COD).

Proc	Param	Description	ASM2	Prop.	Acet.	Units
STOICHIOMETRIC PARAMETERS						
1	Y_{VFA1}	VFA uptake per PHA stored	1	1.1	0.82	g COD_{VFA}· g^{-1} COD_{PHA}
1	Y_{PO}	P released per PHA stored	0.4	0.33	0.55	g P · g^{-1} COD_{PHA}
1	Y_{GLY1}	Glycogen degraded per PHA stored	-	0.2	0.42	g COD_G · g^{-1} COD_{PHA}
2	Y_{PHA}	PHA degraded per poly-P stored	0.2	0.2	0.2	g COD_{PHA} · g^{-1} P
3	Y_{GLY3}	Glycogen stored per PHA converted	-	1.0 **	1.0 **	g COD_G · g^{-1} COD_{PHA}
4	i_{BPM}	P content of biomass	0.02	0.02	0.02	g P · g^{-1} COD_X
4	Y_{PAO}	PAO Yield biomass/PHA	0.625	0.625	0.625	g COD_X · g^{-1} COD_{PHA}
5	ν_{5P}	P released in PAO lysis	0.01	0.01	0.01	g P · g^{-1} COD_X
5	f_{XI}	X_I produced in PAO lysis	0.1	0.1	0.1	g COD_I · g^{-1} COD_X
KINETIC PARAMETERS						
1	q_{PHA}	PHA storage rate constant	*	5.76	5.76	g COD_{PHA} · g^{-1} COD_X· d^{-1}
1	K_S	Saturation coefficient for VFA	4	4	4	mg COD_{VFA} · L^{-1}
1,9	K_{PP}	Saturation coefficient for poly-P	0.0075	0.0075	0.0075	g P · g^{-1} COD_X
1	K_{GLY}	Saturation coefficient for glycogen	-	1e-3 **	1e-3 **	g COD_G · g^{-1} COD_X
2	q_{PP}	Poly-P storage rate constant	1.5	3.64	4.07	g P · g^{-1} COD_X· d^{-1}
2	K_{PS}	Saturat. coeff. for P in poly-P storage	0.2	0.2	0.2	mg P·L^{-1}
2	K_{PHA_P}	Saturation coefficient for PHA in poly-P storage	*	0.133	0.133	g COD_{PHA} · g^{-1} COD_X
2	K_{MAX}	Maximum ratio of X_{PP}/X_{PAO}	0.255	0.255	0.255	g P · g^{-1} COD_X
2	K_{IPP}	Inhibition coefficient for X_{PP} storage	0.015	0.015	0.015	g P · g^{-1} COD_X
2,3,4,9	K_O	Saturation coefficient for DO	0.20	0.20	0.20	mg OD·L^{-1}
3	q_{GLY}	Glycogen storage rate constant	-	5	5	g COD_G · g^{-1} COD_X· d^{-1}
3	X_{GLYMAX}	Maximum ratio of X_{GLY}/X_{PAO}	-	0.28 ***	0.28 ***	g COD_G · g^{-1} COD_X
3,4	K_{PHA}	Saturation coefficient for PHA	0.0125	0.0125	0.0125	g COD_{PHA} · g^{-1} COD_X
4	μ_{PAO}	Maximum PAO growth rate	1.0	0.5	0.5	d^{-1}
4	K_P	Saturat. coeff. for P in PAO growth	0.01	0.01	0.01	mg P·L^{-1}
5	b_{PAO}	PAO lysis rate constant	0.2	0.2	0.2	d^{-1}
6	b_{PP}	Poly-P lysis rate constant	0.2	0.2	0.2	d^{-1}
7	b_{PHA}	PHA lysis rate constant	0.2	0.2	0.2	d^{-1}
8	b_{GLY}	Glycogen lysis rate constant	0.2	0.2	0.2	d^{-1}
9	q_{MANT}	Maintenance rate constant	0.29	0.29	0.29	g P· g^{-1} COD_X· d^{-1}

* These values of ASM2 parameters are not comparable to the ones obtained in this work, since the kinetics of the process in which these parameters are involved are based on different approaches.

** Manga *et al.* 2001

*** Filipe and Daigger 1999

One of the most controversial stoichiometric parameters is Y_{VFA1}, which describes the VFA uptake per PHA stored. The values obtained in this study (1.1 g COD_{VFA}/g COD_{PHA} when propionate is used and 0.82 g COD_{VFA}/g COD_{PHA} when acetate is used) show a smaller value in the case of acetate than in the case of propionate. These results mean that the amount of PHA produced per acetate uptake is higher than the PHA produced when propionate is used as substrate. This result agrees with the values presented by Liu *et al.* (2002). The main reason can be the production of other PHA polymers not considered in this work, as PH2MV or PH2MB when propionate is used as carbon source (Satoh *et al*., 1992). Another possible explanation could be that there was less glycogen consumption when propionate was used and therefore, more propionate has to be consumed to produce the same amount of PHA (Chen *et al*. 2002).

The results obtained for glycogen degraded per PHA stored (Y_{GLY1}) are quite different when propionate or acetate was used. A value of 0.20 g COD_{GLY}/g COD_{PHA} was obtained for propionate, while 0.42 was obtained for acetate. These values indicate that a smaller amount of reducing power is required to store propionate than acetate. Hood and Randall (2001) summarised the stoichiometry and $NADH_2$ production of probable pathways for PHA biosynthesis and their finding, that less reducing power is required to store propionate, agrees with the results of this work. There is a greater need for reducing equivalents to convert acetate to PHB than to convert propionate to PHV and/or PH2MV, which are respectively PAOs normal PHA storage compounds.

The phosphate released per PHA stored (Y_{PO}) also differs when propionate or acetate were used as substrates (0.33 g P/g COD_{PHA} with propionate and 0.50 g P/g COD_{PHA} with acetate).These values indicate that more phosphate is released to produce the same amount of PHA in terms of COD when acetate is used.

Finally, the only kinetic parameter different for both VFA is the Poly-P storage rate constant (q_{PP}). The value obtained for acetate (4.07 g P / g COD_X d) is higher than the one obtained for propionate (3.64 g P / g COD_X d), in agreement with the observed experimental results.

Once the model was calibrated, the validation was carried out with the other cycle studies. Figure 4.9 (A and B) shows one example of this validation for each VFA. The validations show that this model gives an accurate description of the behaviour of the different compounds involved in the process for each VFA used.

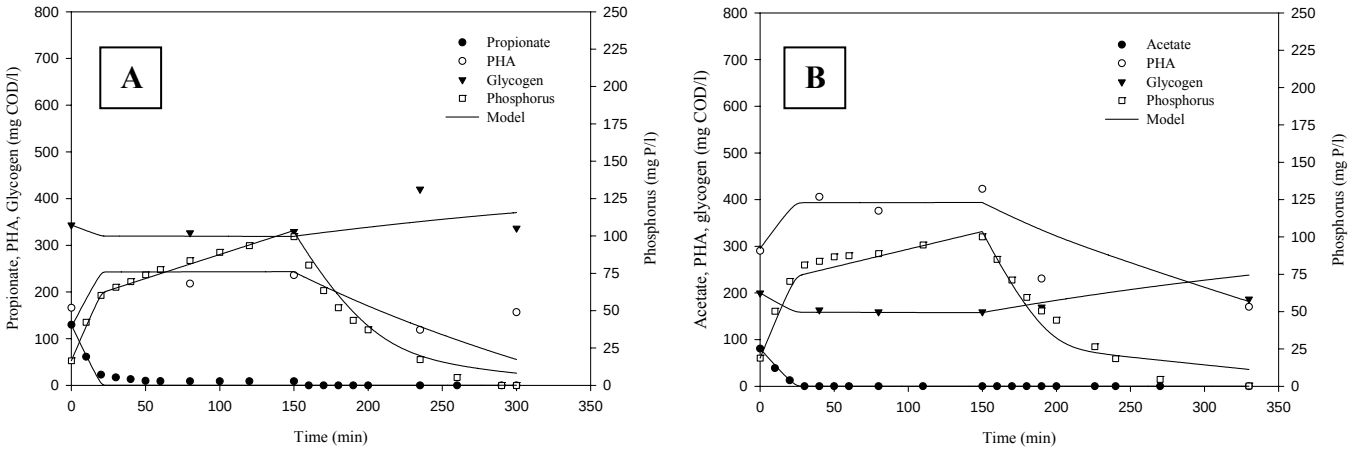

Figure 4.9. Cycle studies used for model validations. **A** - Experimental measures and simulated values for propionate cycle study (1.35 mmol C / g VSS). **B** - Experimental measures and simulated values for acetate cycle study (1.42 mmol C / g VSS). (□ Phosphorus, ● VFA, ▼Glycogen, ○ PHA, — model prediction)

4.4. CONCLUSIONS

The main conclusions from this study are:

1. *Accumulibacter* (55% of all bacteria) were the PAOs in a propionate operating anaerobic-aerobic SBR and GAOs (*Competibacter*) appeared to be unable to compete for propionate.

2. When propionate and acetate were used as carbon sources for EBPR, PAOs demonstrated similar anaerobic VFA-uptake rates (mmol C/g VSS min), although there was less anaerobic P-release with propionate compared to acetate. In addition, a higher aerobic P-uptake rate for acetate was observed.

3. A remarkable second P-release possibly linked to PAO maintenance or to use of peptone as a carbon source is reported. It was only detectable after the VFA substrate was exhausted but it probably occurred throughout the anaerobic period.

4. The optimal working pH range for EBPR with propionate as the sole carbon source was around 7.5. According to the literature, this value is similar to that obtained with acetate as the carbon source.

5. The model presented in this study gives a good prediction of the behaviour of the EBPR system when propionate or acetate is used as the carbon sources. A maintenance reaction is added in order to explain the second P-release detected in the experiments.

4.5. REFERENCES

Barnard JL. 1984. Activated primary tanks for phosphate removal. Water SA 10:121-126

Blackall LL, Crocetti GR, Saunders AM, Bond PL. 2002. A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. Antonie van Leeuwenhoek 81:681-691.

Bond PL, Keller J, Blackall LL. 1999. Bio-P and non-bio-P bacteria identification by a novel microbial approach. Water Sci Technol 39(6):13-20.

Chen Y, Trujillo M, Biggerstaff J, Ahmed G, Lamb B, Eremekdar FG, McCue T, Randall AA. 2002. The Effects Of Propionic Versus Acetic Acid Content Of Domestic Sewage On Enhanced Biological Phosphorus Removal, Proceedings Water Environment Federation 75th Annual Conference and Exposition (WEFTEC) 2002, Research Symposium oral presentation, Sept 28 –Oct 2, 2002, Chicago, Illinois, USA (CD-Rom).

Filipe CDM, Daigger GT. 1999. Evaluation of the capacity of phosphorus-accumulating organisms to use nitrate and oxygen as final electron acceptors: A theoretical study on population dynamics. Water Environ Res 71:1140-1150.

Henze M, Gujer W, Mino T, van Loosdrecht MCM. 2000. Activated Sludge Models ASM1, ASM2, ASM2d and ASM3: Scientific and Technical Report nº9. IWA Task Group on Mathematical Modelling for Design and Operation of Biological Wastewater Treatment.

Hood CR, Randall AA. 2001. A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. Water Res 35:2758-2766.

Lemos PC, Viana C, Salgueiro EN, Ramos AM, Crespo JPSG, Reis MAM. 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. Enz Microb Technol 22:662-671.

Lemos PC, Serafim, LS, Santos MM, Reis MAM, Santos H. 2003. Metabolic pathway for propionate utlization by phosphorus accumulating organisms in activated sludge: ^{13}C labelling and in vivo nuclear magnetic resonance. App Environ Microbiol 69:241-251.

Levantesi C, Serafim LS, Crocetti GR, Lemos PC, Rossetti S, Blackall LL, Reis MAM, Tandoi V. 2002. Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. Environ Microbiol 4:559-569.

Liu W, Mino T, Matsuo T, Nakamura K. 1996. Biological phosphorus removal processes. Effect of pH on anaerobic substrate metabolism. Water Sci Technol 34 (1-2):25-32.

Liu Y, Geiger C, Randall AA. 2002. The role of poly-hydroxy-alkanoate form in determining the response of enhanced biological phosphorus removal biomass to volatile fatty acids. Water Environ Res 74:57-67.

Manga J, Ferrer J, Garcia-Usach F, Seco A. 2001. A modification to the Activated Sludge Model No. 2 based on the competition between phosphorus-accumulating organisms and glycogen-accumulating organisms. Water Sci Technol 43(11):161-171.

Mino T, Satoh H, Matsuo T. 1994. Metabolisms of different bacterial populations in enhanced biological phosphate removal processes. Water Sci Technol 29 (7):67-100.

Mino T, Liu W, Kurisu F, Matsuo T. 1995. Modelling glycogen storage and denitrification capability of microorganisms in EBPR processes. Water Sci Technol 31(2):25-34.

Randall AA, Liu Y. 2002. Polyhydroxyalkanoates form potentially a key aspect of aerobic phosphorus uptake in enhanced biological phosphorus removal. Water Res 36:3473-3478.

Satoh H, Mino T, Matsuo T. 1992. Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. Water Sci Technol 26(5-6):933-942.

Schuler AJ, Jenkins D. 2002. Effects of pH on enhanced biological phosphorus removal metabolisms. Water Sci Technol 46(4-5):171-178.

Seviour R.J, Mino T, Onuki M. 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol Rev 27:99-127.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 42:461-70.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-848.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1995. A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorous removal process. Biotechnol Bioeng 47:277-87.

Thomas M, Wright P, Blackall LL, Urbain V, Keller J. 2002. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. Third IWA World Water Congress, Melbourne, Australia, 7-12 April, 2002.

van Veldhuizen HM, van Loosdrecht MCM, Heijnen JJ. 1999. Modelling biological phosphorus and nitrogen removal in a full scale activated sludge process. Water Res 33:3459-3468.

Zeng RJ, Saunders AM, Yuan Z, Blackall LL, Keller J. 2003. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnol Bioeng 83:140-148.

CHAPTER V

RESPONSE OF TWO DIFFERENT EBPR POPULATIONS ENRICHED WITH ACETATE AND PROPIONATE RESPECTIVELY TO DIFFERENT CARBON SOURCES

Part of this chapter has been accepted as:

Pijuan M, Baeza JA, Casas C, Lafuente J. (In press). Response of an EBPR population developed in a SBR with propionate to different carbon sources. Water Science and Technology.

5.1. INTRODUCTION

Sometimes, EBPR deterioration in laboratory scale systems has been attributed to the carbon source supplied during the anaerobic phase (Cech *et al.*, 1990). More attention has to be paid to the type of carbon source in the wastewater and its effect on EBPR due to the heterogeneous organic matter in wastewater which often reaches the treatment plant without complete acid fermentation. At this point, research in the response of EBPR systems to different substrates has been increased in the last years (Hood and Randall, 2001; Levantesi *et al.*, 2002; Randall and Liu, 2002).

In the study presented in this chapter, two SBRs with the same operational conditions were inoculated with the same sludge. The only difference between them was the carbon source used. Acetate was employed as the carbon source in one SBR and propionate in the other. Moreover and aiming to understand the different responses of these EBPR biomasses, several substrates were tested in different cycle studies in both reactors. Several cycles studies with different organic substrates (such as butyrate and glucose) were conducted for a better understanding of the carbon source effect. The synthesis of different PHA depending on the substrate used as well as the reducing power required to store these substrates were analysed in each cycle study. Moreover, the responses to different substrates in both reactors was compared and related to the different microbial community present.

5.2. MATERIALS AND METHODS

5.2.1. PILOT PLANT

A pilot plant consisting in two SBRs completely monitored and controlled was built to enrich the two EBPR microbial populations (Figure 5.1). Both reactors were inoculated with activated sludge from a non-EBPR plant (Granollers WWTP, Catalonia, Spain) and the only difference between them was the different carbon source used: propionate or acetate. They were operated in 4 cycles of 6 h per day. Each cycle consisted of 2 h anaerobic react, 3 h aerobic react, 55 min of settling and in the last 5 min, extraction of 5 L supernatant. A volume of 5 L of synthetic wastewater was added during the first 5 min of the subsequent cycle, producing a HRT of 12 h. The SRT was kept at 9 d in both reactors by periodic sludge wastage during the react phase. The pH was controlled during the aerobic period at 7.0 ± 0.1 with 1M HCl. Temperature was also controlled at 25ºC. To achieve anaerobic and aerobic conditions, nitrogen gas and air were bubbled through the liquid respectively.

The cycle studies were conducted after 90 days of SBRs operation, when the reactors were working with a microbial community in steady state. The biomass concentration was around 4300 mg VSS·L^{-1} in the Acetate-SBR, with a VSS/TSS ratio of 0.87 at the end of the aerobic period, and 4000 mg VSS·L^{-1} in the Propionate-SBR, with a VSS/TSS ratio of 0.65 at the end of the aerobic period.

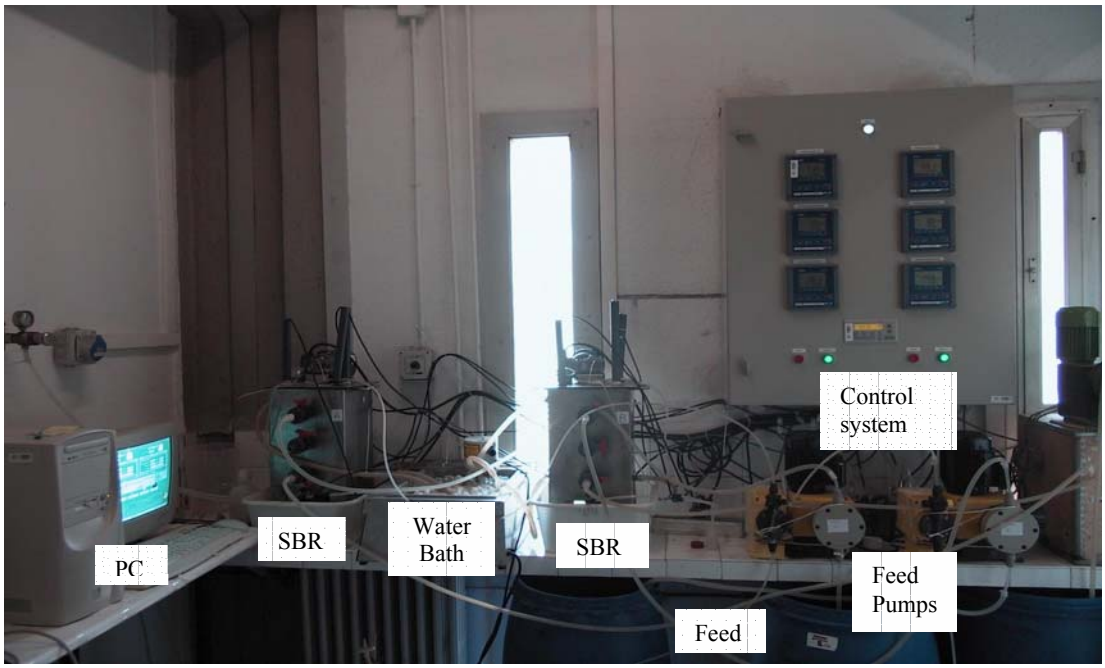

Figure 5.1. Pilot plant.

5.2.2. SYNTHETIC MEDIA

Two separate solutions called "concentrated feed" (constituting 0.25 L per 5 L synthetic wastewater) and "P-water" (constituting 4.75 L per 5 L synthetic wastewater) collectively formed the synthetic wastewater used in this study. The composition of the synthetic wastewater used in this study was the same as the one used in Chapter IV. The only difference between the synthetic media used in both reactors was the carbon source and the fact that no peptone was added. One SBR was working using acetate as the only carbon source (Acetate-SBR) and the initial concentration in the SBR (after the feeding phase) was increased progressively over a 26 d period from 30 mg to 200 mg/L. The other SBR was developed using propionate (Propionate-SBR) as the sole carbon source and the initial concentration in the SBR (after the feeding phase) was increased progressively over a 26 d period from 20 mg to 160 mg/L. The pH of the concentrated feed was adjusted to 5.5 with 2 M NaOH and autoclaved to prevent contamination.

The P-water consisted initially of (mg/L RO water): 81.6 KH_2PO_4 and 62.0 K_2HPO_4 and the pH was adjusted to 10.0 with 2 M NaOH. When the acetate and the

propionate were increased to 200 and 160 mg/L respectively the phosphorus concentration was doubled in the media to avoid any limitation.

The synthetic media described above was used in the cycle studies, except for the carbon source which was substituted by the one used in each study.

5.3. RESULTS

5.3.1. COMPARISON OF THE MICROBIAL COMUNITY IN THE TWO SBRs

Both SBRs were inoculated with the same sludge coming from a non-EBPR WWTP with a low PAOs percentage. FISH was performed in this initial sludge to detect the presence of PAOs and GAOs in it. The results are showed in figure 5.2.

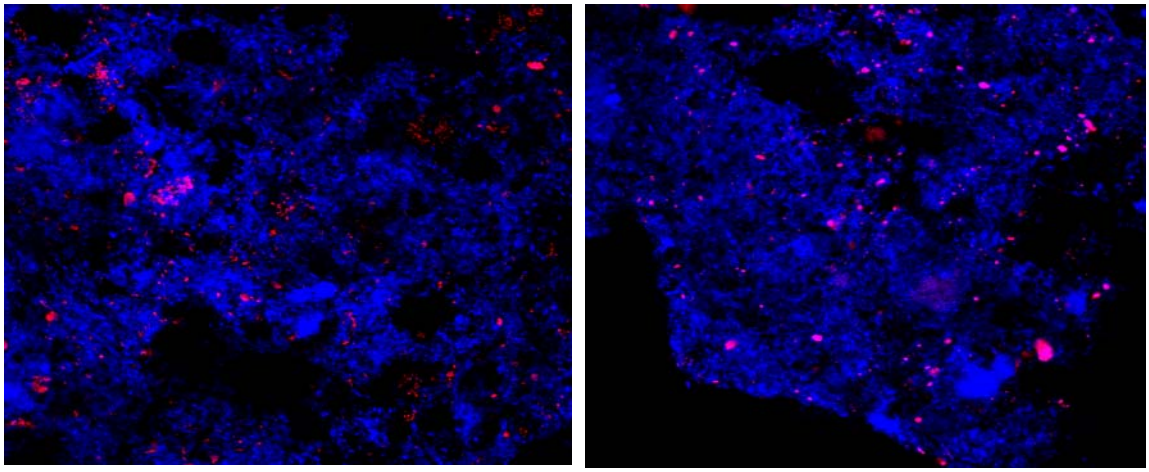

Figure 5.2. CLSM micrographs of FISH of the inoculum sludge coming from a non-EBPR wastewater treatment plant. A) Sludge hybridized with probe specific for PAO (In pink, Cy3-labelled PAOMIX probes) and probe for bacteria (In blue, Cy5-labelled EUBMIX probes). B) Sludge hybridized with probe specific for GAO (In pink, Cy3-labelled GAOMIX probes) and probe for bacteria (In blue).

<u>Propionate-SBR</u>

Propionate-SBR showed a good net P-removal after 15 days of the start up and increase progressively until the steady state was achieved around 30 days after the start up. The FISH analyses presented in figure 5.3 performed when the reactor was in steady state conditions show the abundant presence of *Accumulibacter* in detrimental of the presence of *Competibacter*.

During the steady state, the amount of PAOMIX-binding cells (*Accumulibacter*) was around 50% of all bacteria. On the contrary, the abudance of *Competibacter* cells declined to just a few cells. This supports the hypothesis that GAOs are less competitive than PAOs when propionate is used as substrate (Hood and Randall 2001).

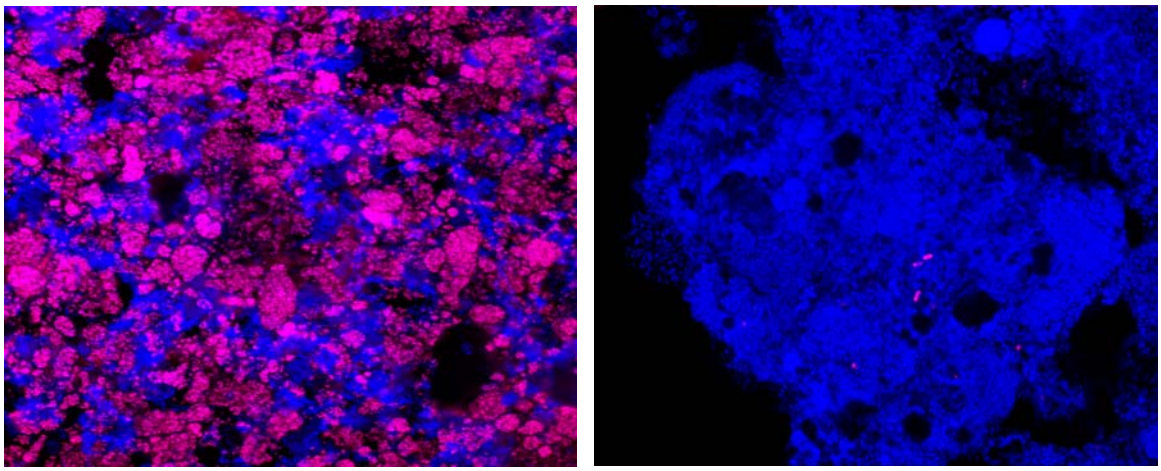

Figure 5.3. CLSM micrographs of FISH of the sludge present in the Propionate-SBR after 90 days of operation. A) Sludge hybridized with probe specific for PAO (In pink, Cy3-labelled PAOMIX probes) and probe for bacteria (In blue, Cy5-labelled EUBMIX probes). B) Sludge hybridized with probe specific for GAO (In pink, Cy3-labelled GAOMIX probes) and probe for bacteria (In blue).

Some chemical stains were performed to complement the FISH information. Figure 5.4 shows the results of Sudan Black and the Methylene Blue stains performed at the beginning and at the end of the anaerobic period of one cycle. A difference is clear in the Sudan Black stain. A qualitative increase in the PHA amount was observed between figure 5.4A and 5.4B when propionate was depleted.

The Methylene Blue stains (Figure 5.4C and 5.4D) show the polyP granules in violet against a blue background. Results of this stain show the difference between the beginning (Figure 5.4C) and the end (Figure 5.4D) of the anaerobic period is not notorious. The variation in the polyP amount along the cycle is not very important and can not be qualitatively detected with this stain.

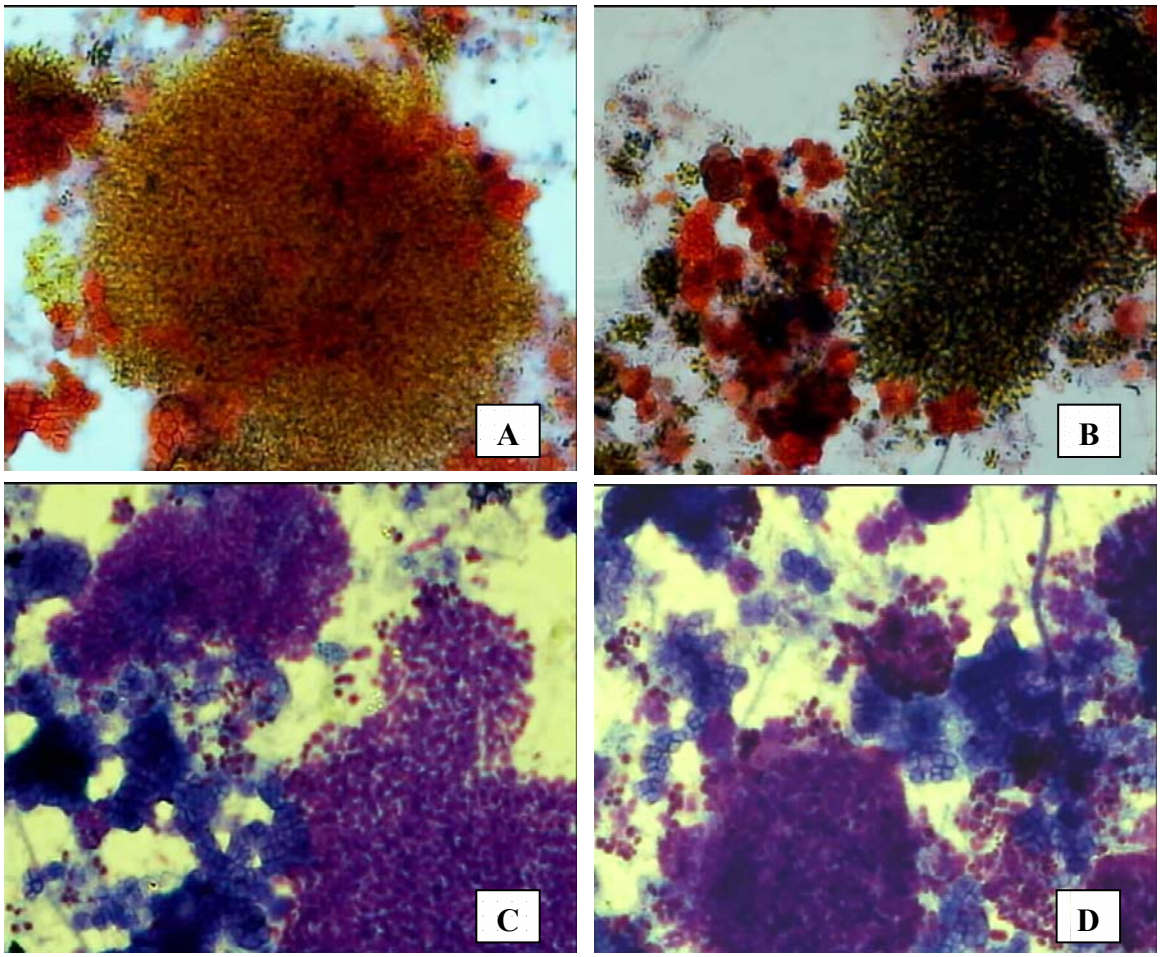

Figure 5.4. Sludge from Propionate-SBR. A) Sudan Black stain at the beginning of the anaerobic period. B) Sudan Black stain at the end of the aerobic period. (Black granules in a clear or lightly coloured back-ground indicate de presence PHA). C) Methylene Blue stain at the beginning of the anaerobic period. D) Methylene Blue stain at the end of the anaerobic period (A violet coloration against a dark blue background is positive for the presence of poly P).

Acetate-SBR

The EBPR efficiency was lower in the Acetate-SBR (when compared to the Propionate-SBR) eventhough both reactors were inoculated with the same sludge. Moreover, more sludge disgregation was observed in this reactor and the settling was not as good as in the Propionate-SBR. FISH was performed in the sludge and a few cells of *Accumulibacter* were detected. The same technique indicates than *Competibacter* was also present in the sludge approximately in the same amount than *Accumulibacter*. Figure 5.5 shows the FISH micrographs from this reactor, after 90 days of operation.

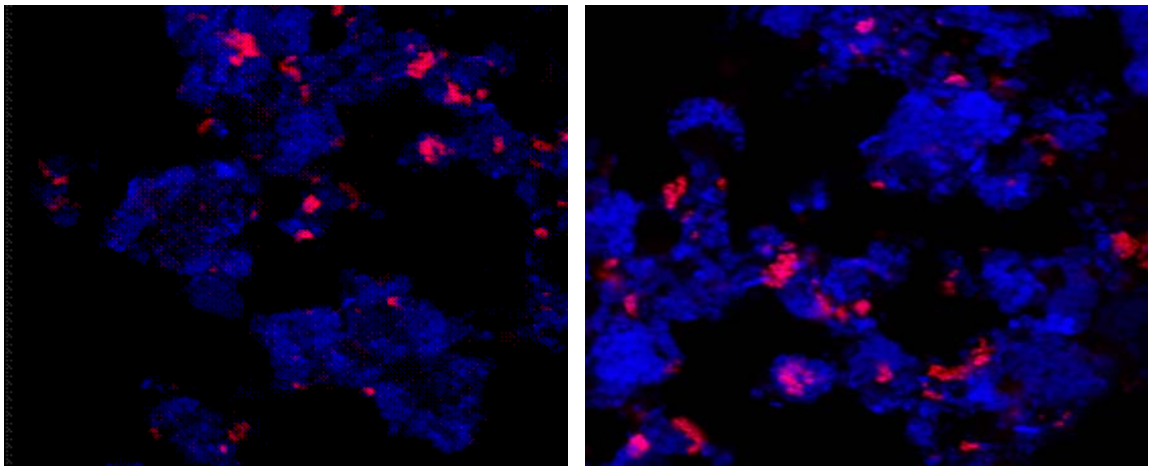

Figure 5.5. CLSM micrographs of FISH of the sludge present in the Acetate SBR after 90 days of operation. A) Sludge hybridized with probe specific for PAO (In pink, Cy3-labelled PAOMIX probes) and probe for bacteria (In blue, Cy5-labelled EUBMIX probes). B) Sludge hybridized with probe specific for GAO (In pink, Cy3-labelled GAOMIX probes) and probe for bacteria (In blue).

Chemical stains were performed in the sludge. Filament bacteria were quite abundant in the sludge which contributed to reduce the settling efficiency in the system.

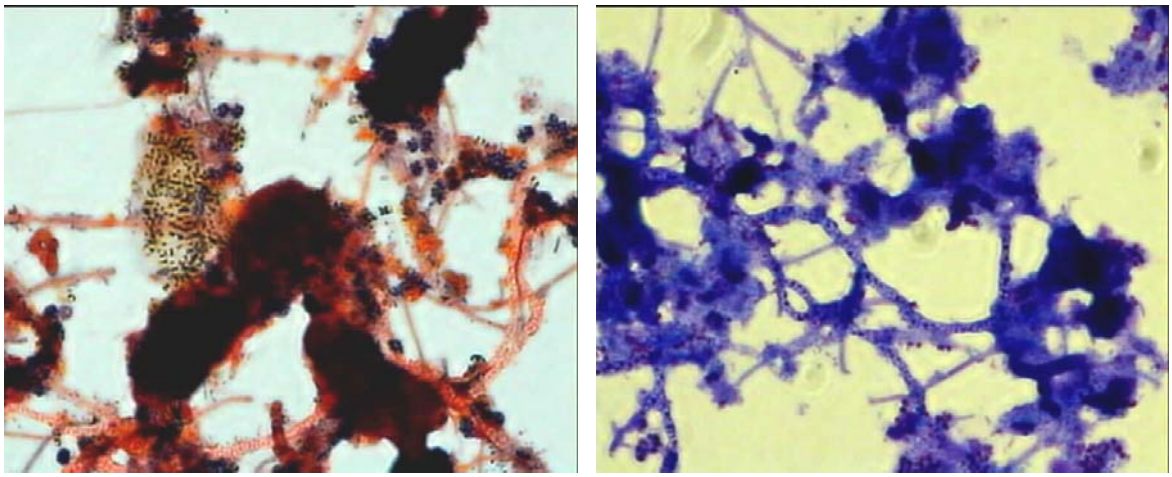

Figure 5.6. A) Sudan Black stain of the Acetate-SBR sludge (Black granules in a clear or lightly coloured back-ground indicate de presence PHA). B) Methylene Blue stain of the Acetate-SBR sludge (A violet coloration against a dark blue background is positive for the presence of poly P).

Sudan Black stain (Figure 5.6.A) shows the PHA presence in the sludge at the end of the anaerobic period of the cycle. Nevertheless, the Methylene Blue stain shows that a few polyP granules are present in this sludge. This fact complements the FISH analyses (Figure 5.5.A) where few *Accumulibacter* cells can be observed.

Different microbial population was observed between the SBRs although both were inoculated with the same sludge. The Acetate-SBR did not achieve the EBPR microbial population as it did the Propionate-SBR where the stability of the system was higher.

5.3.2. RESPONSE OF THE PROPIONATE-SBR BIOMASS TO DIFFERENT SUBSTRATES

The responses of the Propionate-SBR population to four different individual substrates (propionate, acetate, butyrate and glucose) were analysed. An amount of COD (approximately 280 mg COD·L^{-1}) was added in the cycle studies performed with VFA, while the cycle study performed with glucose, less concentration was added (approximately 200 mg COD·L^{-1}) to avoid the presence of this substrate under aerobic conditions. These concentrations were slightly higher than the normal initial propionate working concentration in the reactor (approximately 240 mg COD ·L^{-1}) and for this reason the levels of glycogen at the end of the different cycle studies used to be slightly higher than the initials.

The experimental profiles obtained in each of the cycle studies are shown in figure 5.7. Although all the carbon sources were depleted during the anaerobic period, propionate and acetate were consumed during the first 50 min of the cycle and butyrate and glucose needed more time to be depleted. Specific substrate uptake rates (mmol substrate/g VSS min) for propionate and acetate were similar and higher than those obtained for butyrate and glucose (Table 5.1). It is difficult to determine if the biomass was acclimated to these substrates, the consumption rate may had been higher. Similar results were obtained by Lemos *et al*. (1998) who found the highest consumption rate for acetate followed by propionate and butyrate, using a biomass adapted to consume simultaneously these different fatty acids.

Two ratios for P-release were calculated (Table 5.2): one versus substrate uptake and another one versus PHA accumulated. A higher P-release *vs* substrate uptake was observed for acetate (0.319 mmol P/mmol C), followed by propionate, butyrate and glucose. These results are comparable with values found in the literature (Liu *et al.* 2002;) and in Chapter IV. Regarding P-release *vs* PHA accumulated, a higher ratio was observed when butyrate was used (0.466 mmol P/mmol C) followed by propionate, acetate and glucose. This different trend was observed because the ratio PHA produced *vs* substrate uptake was very different depending on the carbon source (Table 5.3), and in the case of butyrate one PHA monomer produced could not be quantified.

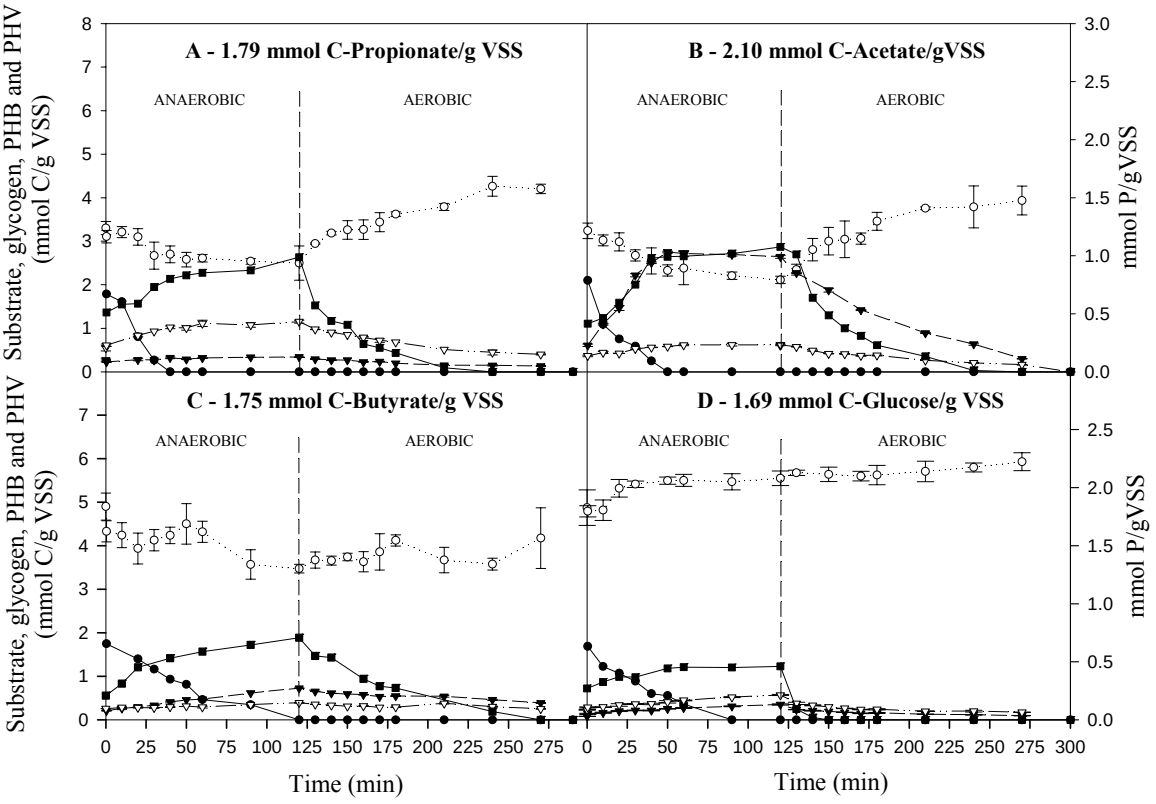

Figure 5.7. Experimental profiles of different compounds during the anaerobic/aerobic cycles of the Propionate-SBR, acetate, butyrate and glucose as carbon sources.
(● substrate; ■ phosphorus; ○ glycogen; ▼ PHB; ∇ PHV).

Table 5.1. Rates obtained in the cycle studies performed with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate in the Propionate-SBR

Rates	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
P-release* (mmol P/gVSS min)	0.0073	0.0142	0.0035	0.0017	0.0117
Substrate-uptake (mmol C/gVSS min)	0.051	0.046	0.016	0.022	0.057 (Prop) 0.020 (Buty) 0.015 (Acet) 0.018 (Gluc)
Substrate-uptake (mmol subs./gVSSmin)	0.017	0.023	0.004	0.004	0.019 (Prop) 0.005 (Buty) 0.007 (Acet) 0.003 (Gluc)
P-uptake (mmol P/gVSS min)	0.010	0.010	0.007	0.039	0.006

* This rate was calculated when there was still substrate in the mixed liquor.

Table 5.2. Ratios obtained in the cycle studies performed with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate in the Propionate-SBR. These ratios were calculated using the initial and final concentration of each compound in the corresponding phase.

Ratios	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
P-release/Subst-upt. (mmol P/mmol C)	0.268	0.319	0.217	0.053	0.199
P-release/PHA-prod. (mmol P/mmol C)	0.425	0.248	0.466	0.147	0.438
Gly-degra./Subst-upt. (mmol C/mmol C)	0.447	0.628	0.491	0.278*	0.295
P-upt./PHAoxidized (mmol P/mmol C)	0.721	0.384	1.291	0.552	0.356

*When glucose was used as substrate, glycogen was synthesized not degraded.

Glycogen degradation *vs* substrate uptake is shown in Table 5.2. When propionate was consumed, less reducing power was needed to uptake VFA and, therefore, less glycogen was degraded. On the contrary, when acetate was used, the highest glycogen degradation was detected per VFA uptake. When glucose was used as carbon source, glycogen was produced instead of being degraded. This carbon source was not comparable with the VFA because it followed a different pathway to be stored and part of it was stored as glycogen.

With respect to the aerobic phase, PAO sequestered phosphorus at the same rate when they used propionate or acetate as carbon source (Table 5.1). These values are similar to previous results shown in Chapter IV. When butyrate was used, this rate was smaller and more time was required for total P-uptake (Figure 5.1).

The effect of the different carbon sources on PHA production was also assessed. Tables 5.3 and 5.4 show the different PHA monomers quantified for each substrate and its molar percentages. Acetate uptake resulted in the highest accumulation and PHB was the polymer mainly formed. Similar results were obtained by Satoh *et al*. (1992) and Lemos *et al.* (1998). When propionate was used as the sole carbon source, three monomer units were detected: 3HV, 3HB and 3H2MV. When butyrate was used as substrate, a different unidentified monomer was detected with the chromatographic analysis (data not shown). This monomer only appeared when butyrate was used as carbon source and it seemed the main monomer synthesized using butyrate because of the high area detected in the analysis. Glucose utilisation produced a relative lower amount of stored PHA, because part of it was stored as glycogen instead of PHA.

The lowest carbon recovery ratio was observed when butyrate was used, while acetate presented the highest one. The low recovery ratios detected with some substrates imply a low efficiency of PHA storage. Nevertheless, it is important to

remark that the butyrate carbon recovery ratio would be higher if the contribution of the PHA monomer detected could be taken into account.

Table 5.3. Accumulated PHA (PHB+PHV+PH2MV) during the anaerobic phase in the different cycle studies performed with propionate, acetate, butyrate and glucose as single substrates or when they were used as mixed substrate in the Propionate-SBR.

Ratios	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
PHB-prod./Subst-upt. (mmol C/mmol C)	0.04	1.15	0.36	0.16	0.29
PHV-prod./Subst-upt. (mmol C/mmol C)	0.34	0.13	0.08	0.20	0.16
PH2MV-prod/Subst-upt (mmol C/mmol C)	0.26	0	0.02	0.0	0.0
PHA total-prod./Subst-upt. (mmol C/mmol C)	0.64	1.28	0.46	0.36	0.45
Carbon recovery ratio (mmol C/mmol C)	0.44*	0.81*	0.31*	0.64**	0.35

*(PHA produced)/(substrate taken up + glycogen degraded)

**(PHA produced + Glycogen produced)/(substrate taken up)

Table 5.4. Molar percentages of PHB, PHV and PH2MV production during the anaerobic period, obtained in the cycle studies using propionate, acetate, butyrate and glucose as single substrates and when they were used as mixed substrate in the Propionate-SBR.

	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
% PHB	6.2	89.6	78.5	44.1	64
% PHV	53.4	10.4	16.4	55.9	36
% PH2MV	40.4	0	5.1	0	0

Cycle study with mixed substrate

A cycle study with a mixture of propionate, acetate, butyrate and glucose was carried out to study PAO behaviour in front of different substrates, knowing its response to these substrates individually (cycles studies described before). The initial substrates concentrations for this cycle study were (mmol C/g VSS): 0.68 propionate, 0.93 acetate, 1.27 butyrate, 0.63 glucose. Figure 5.8A shows the experimental profiles obtained for the different substrates and phosphate. All substrates were completely exhausted during the first 60 min of the anaerobic period, but phosphate was not totally sequestered under aerobic conditions. Glycogen, PHB and PHV experimental data are presented in figure 5.8B.

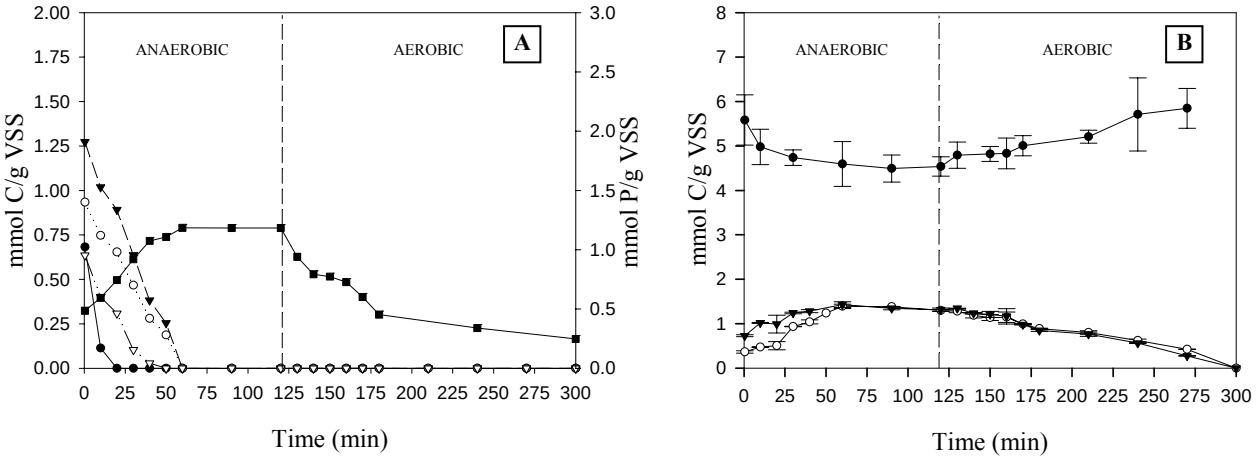

Figure 5.8. Experimental profiles of different compounds during the cycle study of the SBR performed with a mixture of 4 substrates. A- Substrates and phosphorus: ○ acetate, ● propionate, ▼ butyrate; ∇ glucose; ■ phosphorus. B- Intracellular compounds: ● glycogen; ○ PHB; ▼ PHV.

Tables 5.1 shows also the different rates obtained for this cycle study. The substrate uptake rates obtained for propionate, butyrate and glucose were very similar to the ones obtained in the cycle studies using single substrates, meanwhile, acetate uptake rate decreased significantly. The cycles studies performed with different substrates added individually, showed that PAOs could uptake propionate and acetate at a very similar rate (mmol substrate/g VSS min). Nevertheless, when this population had the mixed substrate, substrate uptake rate for propionate was almost maintained, while for acetate it was significantly reduced.

When the mixture of substrates was used, the ratio of P-uptake in the aerobic period *vs* PHA oxidised was lower than the ones obtained in the cycle studies with single substrates. One possible explanation to this ratio could be the composition of the intracellular PHA. Randall and Liu (2002) observed a correlation between aerobic P-uptake and PHA composition, but taking into account only the PHB and PHV. Nevertheless, when butyrate was used as the only carbon source or even when it was used in combination with other substrates, a different PHA was detected. The fraction of this non-identified monomer unit seemed to be important and might determine the P-uptake rate which presented a similar value when butyrate was used individually (0.007 mmol P/g VSS min) and when it was used in a mixed substrate (0.006 mmol P/g VSS min).

Some differences were observed in the comparison of the ratios of glycogen utilised *vs* substrate uptake obtained in the cycle studies performed with individual substrates and that obtained with the mixed substrate. The cycle study carried out with mixed

substrate presented the lowest glycogen degradation (0.297 mmol C/mmol C). This could be due to the fact that the glucose present in the mixed substrate was acting as a source of reducing power as suggested Jeon *et al.* (2001) and therefore, less glycogen had to be degraded to store the different VFA as PHA.

5.3.3. RESPONSE OF THE ACETATE-SBR BIOMASS TO DIFFERENT SUBSTRATES

Two sets of experiments were performed in the Acetate-SBR when it was working with a microbial community in steady state conditions. The experiments were the same as the ones performed in the Propionate-SBR. Acetate, propionate, butyrate and glucose were tested as sole substrates in different cycle studies performed in the reactor and also they were tested together as a mixed substrate. Approximately the same amount of COD (280 mg COD·L^{-1}) was added in each cycle study performed with different substrates, but in the case of the cycle study performed with butyrate, the substrate *vs* biomass ratio was higher.

The experimental profiles of the different compounds analysed in each cycle study are represented in figure 5.9. Only acetate and glucose were consumed in the anaerobic period, meanwhile in the cycle studies performed with propionate and butyrate, some COD remained at the beginning of the aerobic phase. Net P-removal was only observed in the acetate cycle study. However, phosphate was not completely depleted and the P-removal value was very low. Glucose and acetate presented the highest substrate uptake rates (Table 5.5) and propionate and butyrate the lowest rates respectively. P-release rate also shows the same trend as C-uptake rate. The highest P-release rate was in the cycle study performed with glucose followed by acetate, butyrate and propionate.

The P-uptake rate presented the highest value in the cycle study performed with butyrate, followed by acetate, glucose and propionate.

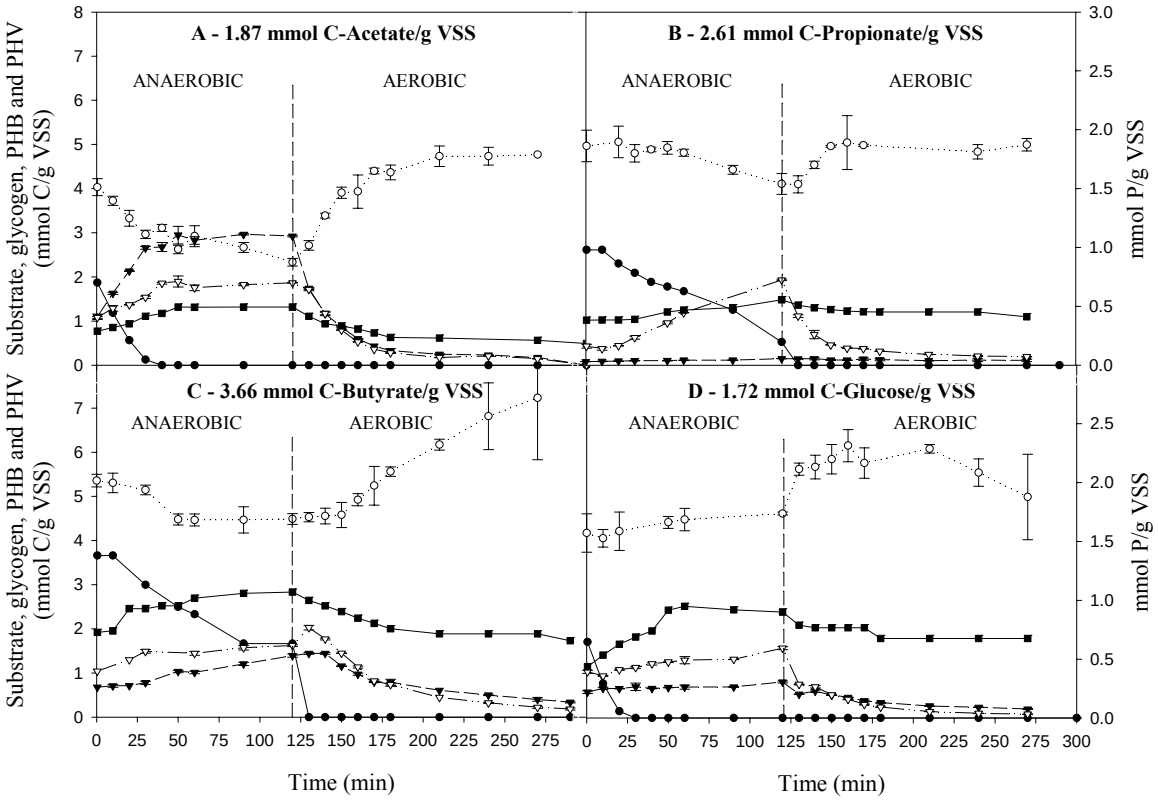

Figure 5.9. Experimental profiles of different compounds during the anaerobic/aerobic cycles of the Acetate-SBR with acetate, propionate, butyrate and glucose as carbon sources. (● substrate; ■ phosphorus; ○ glycogen; ▼ PHB; ∇ PHV).

Table 5.5. Rates obtained in the cycle studies performed in Acetate-SBR with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate.

Rates	Single Substrate				Mixed Substrate	
	Propionate	Acetate	Butyrate	Glucose		
P-release* (mmol P/gVSS min)	0.0015	0.0043	0.0028	0.0095	0.005	
Substrate-uptake (mmol C/gVSS min)	0.0174	0.0583	0.0167	0.0785	0.019(Prop) 0.010 (Acet)	0.009(Buty) 0.019(Gluc)
Substrate-uptake (mmol subs./gVSSmin)	0.0058	0.0292	0.0042	0.0131	0.006(Prop) 0.005 (Acet)	0.002 (Buty) 0.003 (Gluc)
P-uptake (mmol P/gVSS min)	0.0010	0.0017	0.0028	0.0013	0.0019	

* This rate was calculated when there was still substrate in the mixed liquor.

Table 5.6 summarises the ratios calculated for the different measured compounds in this study. The highest P-release *vs* C-uptake ratio was detected with butyrate as substrate and the lowest was observed when propionate was used. Nevertheless, the P/C ratios observed in all the cycle studies presented a very low value, which is indicative of the presence of GAOs in the reactor.

The highest glycogen degradation *vs* substrate uptake ratio was observed when acetate was used as substrate (0.909 mmol C/mmol C). This value indicates the presence of GAOs in the reactor, which was corroborated by the FISH analyses. Nevertheless, this ratio decreased to similar values as the Propionate-SBR results, when other substrates were tested. This fact could suggest that this microbial community enriched with acetate, might not be able to consume propionate or butyrate as fast as they consume acetate. More glycogen would be degraded when acetate was consumed because probably more GAOs were able to uptake it, but with the other substrates, less GAOs would uptake them, involving less glycogen degradation. As in the cycle studies performed with the Propionate-SBR, when glucose was used as substrate, glycogen production was observed in the anaerobic period due to the direct storage of glucose as glycogen.

P-uptake *vs* PHA-oxidised ratio shows the highest value for butyrate, and the lowest was observed when acetate was used. This could be linked to the glycogen degradation ratio. When acetate was tested, the glycogen degradation ratio showed a high value, indication of the activity of the GAOs in the system. On the contrary, less glycogen was degraded when butyrate was used; this fact was linked to GAO activity, then in the cycle study performed with butyrate less GAOs would take part in the system. This would increase the P-uptake ratio. Nevertheless it has to take into account that with butyrate as substrate, another PHA was synthetised and this was not considered in the P-uptake *vs* PHA-oxidised ratio. This ratio should decrease if this PHA monomer could be considered.

Table 5.6. Ratios obtained in the cycle studies performed in Acetate-SBR with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate. These ratios were calculated using the initial and final concentration of each compound in the corresponding phase.

Ratios	Single Substrate				Mixed
	Propionate	Acetate	Butyrate	Glucose	Substrate
P-release/Subs-upt. (mmol P/mmol C)	0.086	0.107	0.170	0.121	0.120
P-release/PHA-prod. (mmol P/mmol C)	0.117	0.077	0.130	0.600	0.149
Gly-degra./Subs-upt. (mmol C/mmol C)	0.416	0.909	0.435	0.121*	0.257
P-upt./PHAoxidized (mmol P/mmol C)	0.084	0.069	0.146	0.105	0.115

*When glucose was used as substrate, glycogen was synthesized, not degraded.

Four different PHA monomers were detected in the chromatographic analyses in the different cycle studies performed although just three of them could be quantified. Table 5.7 summarises the ratios of the different PHA analyses *vs* substrate uptake and table 5.8 shows the percentages of the different monomers synthetised depending on the substrates tested.

Table 5.7. Accumulated PHA (PHB+PHV+PH2MV) during the anaerobic phase in the different cycle studies performed in Acetate-SBR with propionate, acetate, butyrate and glucose as single substrates or when they were used as mixed substrate.

Ratios	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
PHB-prod./Subst-upt. (mmol C/mmol C)	0.02	0.97	0.43	0.13	0.14
PHV-prod./Subst-upt. (mmol C/mmol C)	0.72	0.42	0.45	0.31	0.53
PH2MV-prod/Subst-upt (mmol C/mmol C)	0.0	0.0	0.04	0.0	0.0
PHA total-prod./Subst-upt. (mmol C/mmol C)	0.74	1.39	0.92	0.44	0.67
Carbon recovery ratio (mmol C/mmol C)	0.52*	0.73*	0.64*	0.77**	0.53

*(PHA produced)/(substrate taken up + glycogen degraded)

**(PHA produced + Glycogen produced)/(substrate taken up)

Table 5.8. Molar percentages of PHB, PHV and PH2MV production during the anaerobic period, obtained in the cycle studies in the Acetate-SBR using propionate, acetate, butyrate and glucose as single substrates and when they were used as mixed substrate.

	Single Substrate				Mixed Substrate
	Propionate	Acetate	Butyrate	Glucose	
% PHB	2.7	69.9	46.7	29.9	20.9
% PHV	97.3	30.1	48.9	70.1	79.1
% PH2MV	0.0	0.0	4.4	0.0	0.0

Propionate was mainly stored as PHV and no PH2MV was detected differing from the cycle study performed with the same substrate in the Propionate-SBR. In the cycle study performed with acetate, PHB was the main monomer synthetised although a significant amount of PHV was also produced. PHV production needs propionyl-CoA and acetyl-CoA as precursors and when acetate is used as substrate, the only metabolic pathway to obtain propyonil-CoA is through the degradation of glycogen. It seems logic then, that the more glycogen degraded, the more PHV synthetised. Some PHV production is attributed to PAO metabolism when acetate is used as the sole carbon source (Pereira *et al*. 1996, Hesselmann *et al*. 2000) although some authors considered only PHB production when acetate is used (Smolders *et al*. 1994a,b) and PHV is linked to the GAOs activity.

Cycle study with mixed substrate

A cycle study with a mixture of propionate, acetate, butyrate and glucose was carried out. The objective of this study was to assess the response of this biomass to a mixture of the substrates tested independently in the cycle studies described before. The initial substrate concentrations for this cycle study were (mmol C/g VSS): 1.33 propionate, 1.5 acetate, 2.04 butyrate, 1.17 glucose. Figure 5.10A shows the experimental profiles obtained for the substrates and phosphate analysed. PHB, PHV and glycogen profiles are presented in Figure 5.10B.

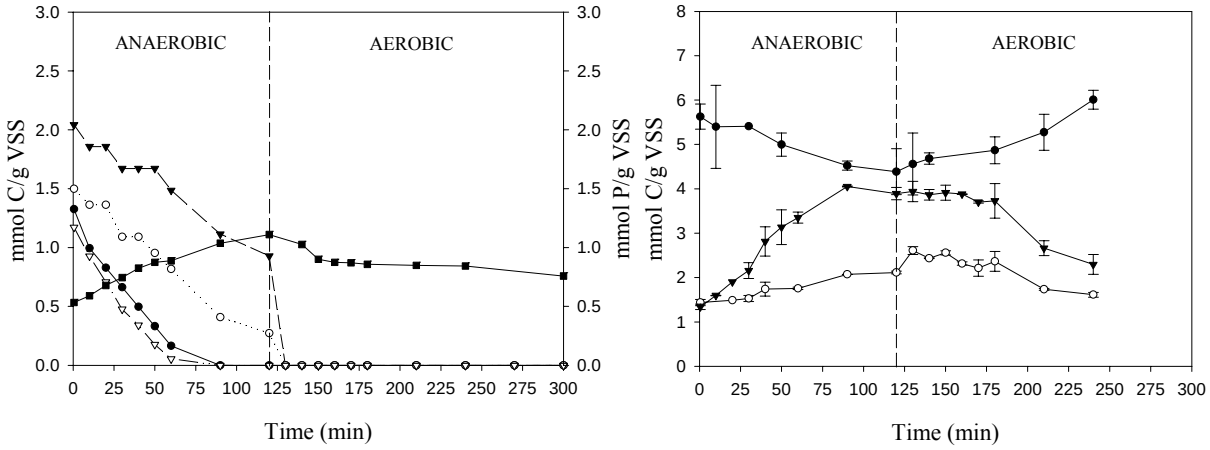

Figure 5.10. Experimental profiles of different compounds during the cycle study of the Acetate-SBR performed with a mixture of 4 substrates. A- Substrates and phosphorus: ○ acetate, ● propionate, ▼ butyrate; ∇ glucose; ■ phosphorus. B- Intracellular compounds: ● glycogen; ○ PHB; ▼ PHV.

Table 5.5 show the different rates obtained in this cycle study. All the substrates consumption rates, except in the case of propionate, were reduced because all the substrates were simultaneously consumed. The value obtained in P-release *vs* C-uptake ratio (0.120 mmol P/mmol C, table 5.6) was very similar to the one obtaining when this ratio was calculated considering the ratios obtained for each substrate individually and multiplying it by the amount of substrate taken up (0.119 mmol P/mmol C, calculated value). Therefore, it seems that this ratio is a sum of the individual ratios contribution obtained when the substrates were used independently and no synergic effect exist.

For the other hand, glycogen degradation *vs* C-uptake ratio (0.257 mmol C/mmol C) presented a lower value than the one that should have if the substrates were added individually (0.416 mmol C/mmol C, calculated value). Although glucose was acting as a source of reducing power in the mixed substrate experiment, it seems than when the mixed substrate was used, less reducing power was required to store the different substrates in PHA. This could be due to the different sorts of PHA synthetised.

Tables 5.7 and 5.8 summarise the PHA monomers synthetised in the mixed substrate cycle study as well as the percentages of the different monomers obtained. Only PHB and PHV were quantified despite of detecting another peak as in the case of the cycle study performed using butyrate. PH2MV was not detected in the mixed substrate experiment.

5.4. CONCLUSIONS

Two different SBRs were used to perform this study. The only difference between them was the feeding carbon source; one was working using propionate as the sole carbon source and the other using acetate. The evolution of the microbial communities from each reactor was studied. Moreover, two sets of experiments were performed in both reactors to study the effect of different carbon sources in each biomass. The main conclusions obtained from this study are:

- The SBR working with propionate as carbon source presented a better net P-removal capacity in the system. The microbial analyses demonstrated that a higher amount of PAOs were present in the Propionate-SBR sludge and almost no GAOs were present in the sludge. On the contrary, the SBR working with acetate, presented a very few amount of PAOs and also presented some GAOs which negatively affected the net phosphorus removal of the system.

- PHA composition was influenced strongly by the carbon source. Acetate was mainly stored as PHB, meanwhile propionate was mainly stored as PHV. PH2MV was also produce when propionate was feed but only in the Propionate-SBR.

- When butyrate was used as substrate, another monomer unit was detected in the chromatographic analysis. This monomer only appeared when butyrate was used as carbon source and it seemed to be the main monomer synthesized.

- Some PHB and PHV storage was observed when glucose was used.

- The ratio of glycogen degraded *vs* substrate uptake in the cycle studies performed with individual substrates was higher than the obtained with the mixed substrate. This could be due to the fact that glucose present in the mixed substrate was acting as a source of reducing power and therefore, less glycogen had to be degraded to store the different VFA as PHA. The different types of PHA synthetised could also contribute in the reduction of the reducing power requirements.

5.5. REFERENCES

Cech J.S. and Hartman P. (1990). Glucose induced breakdown of enhanced biological phosphate removal. Environ Technol 11:651-658.

Hood C.R. and Randall A.A. (2001). A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. Water Res 35:2758-2766.

Jeon C.O., Lee D.S. and Park J.M. (2001). Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: characteristics of carbon metabolism. Water Environ Res 73:295-300.

Lemos P.C., Viana C., Salgueiro E.N., Ramos A.M., Crespo J.P.S.G. and Reis M.A.M.(1998). Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. Enz Microb Technol 22:662-671.

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

Liu Y., Geiger C. and Randall A.A. (2002). The role of poly-hydroxy-alkanoate form in determining the response of enhanced biological phosphorus removal biomass to volatile fatty acids. Water Environ Res 74:57-67.

Pereira H, Lemos PC, Reis MAM, Crespo JPSG, Carrondo MJT, Santos H. 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo 13 C-NMR. Water Res 30:2128-2138.

Randall A.A. and Liu Y. (2002). Polyhydroxyalkanoates form potentially a key aspect of aerobic phosphorus uptake in enhanced biological phosphorus removal. Water Res 36:3473-3478.

Satoh H., Mino T. and Matsuo T. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. Water Sci Technol 26:933-942.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 42:461-70.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-848.

CHAPTER VI

RESPONSE OF FULL SCALE EBPR SLUDGE TO ACETATE AND PROPIONATE. COMPARISON WITH METABOLIC MODEL PREDICTIONS

6.1. INTRODUCTION

In the last years, EBPR has become a well-established activated sludge process and major research efforts have been focused on the understanding of the metabolic pathways of the microorganisms implicated. Interpretation of the stoichiometry involved on the process has become one of the main goals to achieve in this field, and in this line some metabolic models have been developed using enriched cultures of PAOs and GAOs.

The first metabolic models developed in this field were focused on PAOs. It was suggested that the necessary reducing power for PHB synthesis was supplied from the oxidation of acetate to CO_2 through the TCA cycle (Comeau *et al*. 1986; Wentzel *et al*. 1986). Later on, Mino *et al*. (1987) suggested that the degradation of intracellular glycogen was the only source of reducing power, rejecting the two previous hypotheses. Pereira *et al*. (1996) demonstrated that the reducing power was mainly produced by the degradation of the glycogen but some reducing power was obtained simultaneously through the oxidation of some part of the acetate in the TCA cycle.

PHB was presented as the only organic storage polymer in the anaerobic metabolism of PAO in the early models (Smolders *et al*. 1994a.b), but some other authors considered also PHV production when acetate was used as the only substrate (Pereira *et al*. 1996; Hesselmann *et al*. 2000; Yagci *et al*. 2003).

Moreover, some metabolic models for GAO have been developed in the last years (Filipe *et al*. 2001; Zeng *et al*. 2002; Zeng et *al.* 2003).

All these models have been formulated using enriched laboratory cultures using acetate as the main substrate. Recently, some studies have been focused on propionate as a carbon source and the metabolic pathways hypothetically used by PAO and GAO with this substrate (Oehmen *et al*. submitted). Nevertheless, there is an increasing need to contrast the predictions of the models with full scale sludge in EBPR wastewater treatment plants, where the presence of PAO and GAO is much lower than in the communities used to develop these models. In order to do this comparison, four sets of experiments were performed using sludge withdrawn from two different full scale EBPR plants. Acetate and propionate were used independently to analyse the different responses of this sludge to these two substrates.

6.2. MATERIALS AND METHODS

6.2.1. EXPERIMENTAL SETUP: TOGA SENSOR

Four sets of batch experiments were performed using the TOGA sensor (Pratt *et al*. 2003) in order to obtain the anaerobic CO_2 production.

The TOGA consists of a bioreactor working as SBR, a quadrupole mass spectrometer (Omnistar, Balzers AG, Liechtenstein) used to measure the off-gas concentration from the reactor, as well as gas flow controllers (Bronkhorst Hi-Tech, El-Flow, Netherlands) to measure and control the inlet gas flowrates. There is also a pH control system (WP91 unit, TPS, Brisbane, Australia), which is used maintain the pH in the reactor at a pre-set constant level (in this study 7.00 ± 0.01) through titrating acid or base and. The hydrogen ion consumption or production rate caused by the biological as well as physical and chemical processes is then determined as the rate of acid or base addition. In this work a 3 L reactor was used. Liquid temperature and dissolved oxygen were both measured using a dissolved oxygen electrode (YSI model 5739, Yellow Springs USA). The pH was measured using a pH electrode (Ionode IJ44, TPS, Brisbane Australia). The TOGA sensor is schematically shown in Figure 6.1 and the real sensor set-up is shown in Figure 6.2.

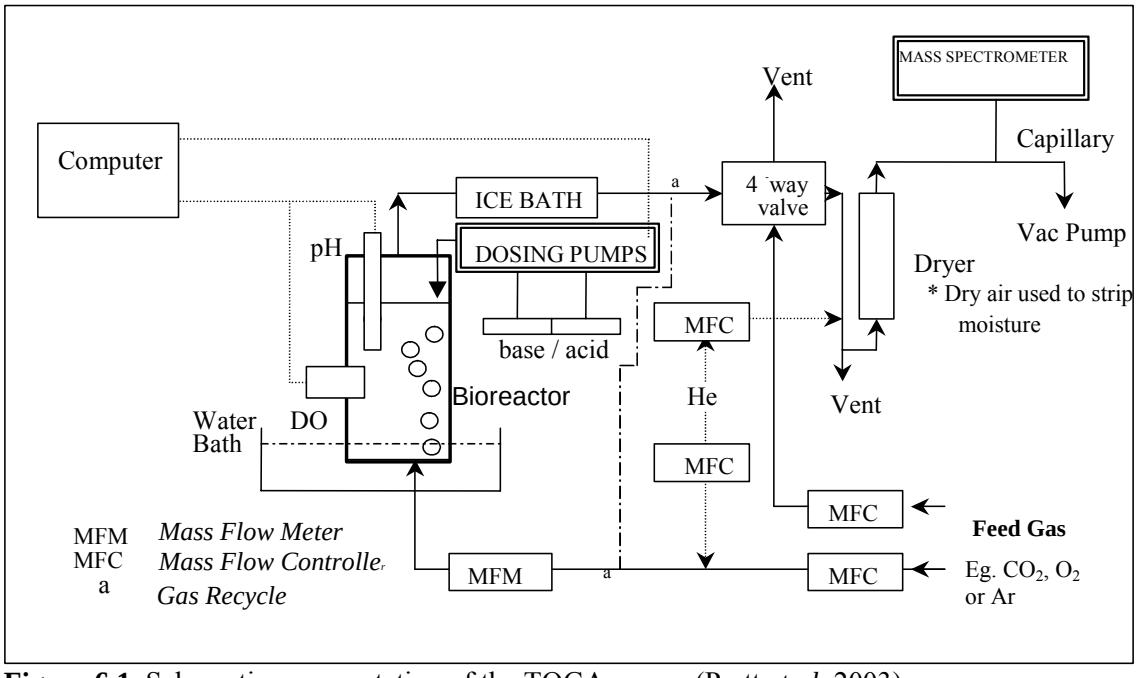

Figure 6.1. Schematic representation of the TOGA sensor (Pratt *et al.* 2003).

The CO_2 produced rate (CPR) in the anaerobic period is either stripped from the liquid into the gas phase, or is dissolved in the liquid phase in the form of bicarbonate (HCO_3^-) depending on pH and on HCO_3^- concentration .The CO_2 transfer rate (CTR) from the liquid to the gas phase is determined by the TOGA

sensor using the method of Gapes *et al*. (2001). The dissolved CO_2 concentration was determined through the on–line measurements of hydrogen ion production rate (HPR) from the TOGA sensor, in conjunction with off-line measurements of P release and VFA uptake. The overall relationship describing CO_2 production is shown in equation 6.1 for acetate as substrate and 6.2 for propionate as substrate (adapted from Pratt *et al*. 2003).

$$CPR = (1+10^{pKa1-pHop})(HPR + \frac{CTR}{1+10^{pKa1-pHop}} - \frac{r_{acetate}}{2} - \frac{r_{H3PO4}}{1+10^{pka2-pHop}}) \qquad \text{eq. 6.1}$$

$$CPR = (1+10^{pKa1-pHop})(HPR + \frac{CTR}{1+10^{pKa1-pHop}} - \frac{r_{propionate}}{3} - \frac{r_{H3PO4}}{1+10^{pka2-pHop}}) \qquad \text{eq. 6.2}$$

Where pKa1= 6.35 $\quad CO_2 + H_2O \leftrightarrow H_2CO_3 \xleftrightarrow{pka1} H^+ + HCO_3^-$

pKa2= 7.20 $\quad PO_3^- + H_2O \leftrightarrow H_2PO_4^- \xleftrightarrow{pka2} H^+ + HPO_4^{2-}$

pHop= 7.00

and $r_{acetate}$ = rate of acetate removal (mmol C/h)

$r_{propionate}$= rate of propionate removal (mmol C/h)

r_{H3PO4} = rate of phosphate release (mmol P/h)

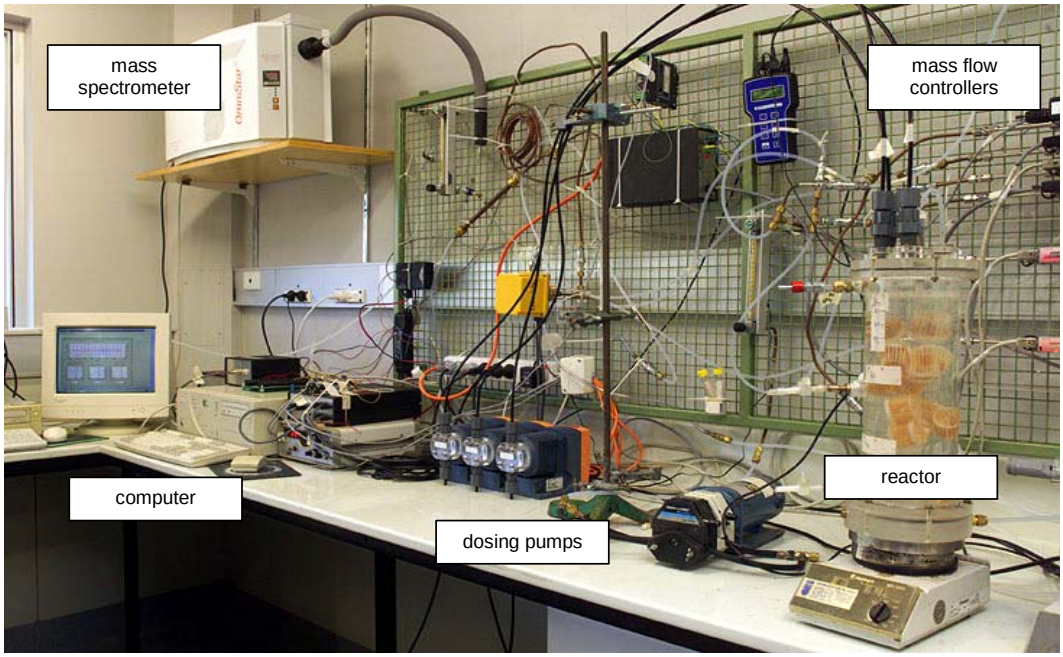

Figure 6.2. The TOGA sensor (Pratt *et al*. 2003).

Batch experiments were performed using full scale sludge from two WWTP performing EBPR in Queensland, Australia, namely the Thorneside WWTP and the Noosa WWTP. The sludge was taken from the aerobic part of the plant and was then aerated for 4 hours. Afterwards, the mixed liquor was let to settle, the supernatant was removed. The sludge was mixed with a synthetic feed solution to avoid any nutrient limitation in the experiments, which was then added to the TOGA sensor. The working volume was 3 L. The mixed liquor was buffered with 1 g/L Hepes (Sigma Aldrich chemicals), and the pH was controlled at 7.00 ± 0.01 through dosage of 0.04 M HCl and 0.04 M NaOH. In the 2 hours of the anaerobic period, helium was bubbled through the sludge to maintain strict anaerobic conditions. Measurements of the VFA, orthophosphate, ammonia, nitrite and nitrate were carried out throughout the batch experiments. PHB, PHV, PH2MV and glycogen were also measured at the beginning and at the end of each batch experiment and triplicates were done of each sample.

The endogenous processes in biological wastewater treatment systems are poorly understood at present. They likely cover a range of mechanisms, including cell maintenance, predation by eukaryotic microorganisms, cell inactivation (death or dormancy), cell lysis, and regeneration. In each of the operating phases there is some degree of endogenous activity. The effect of this activity on the measured signals has to be accounted for. This is achieved by monitoring the sensor signals prior to substrate addition. It is then assumed that the level of endogenous activity remains unchanged during substrate removal. The endogenous CPR is calculated from the measured background HPR and CTR. In order to consider the endogenous CPR in the final CTR result, 6 hours anaerobic experiment without adding any substrate was performed for both sludges.

The TSS concentration along the experiments was around 3200 mg $\cdot L^{-1}$ with a VSS/TSS ratio of 0.70.

6.2.2. SYNTHETIC MEDIA

The synthetic media used in this study was a concentrated feed (constituting 100 mL per 1.5 L) and P-water (constituting 30 mL per 1.5 L) and adapted from Chapter IV. Both were mixed and diluted with RO water to 1.5 L prior to addition to the TOGA sensor. The concentrated feed consisted of (g/L RO water): 0.84 NH_4Cl, 1.8 $MgSO_4 \times 7H_2O$, 3.2 $MgCl_2 \times 6H_2O$, 0.84 $CaCl_2 \times 2H_2O$, 0.4 yeast extract, 1 allylthiourea (ATU) to inhibit nitrification and 12 mL of nutrient solution (Zeng *et al*. 2003).

P-water consisted of (mg/L RO water): 81.6 KH_2PO_4 and 62.0 K_2HPO_4. The working concentration of acetate and propionate in the beginning of each batch experiment was 50 mg/L and 40 mg/L respectively.

6.3. RESULTS AND DISCUSSION

6.3.1. ACETATE EXPERIMENTS

Three different batch experiments using acetate as the only carbon source were carried out using each full scale sludge. Table 6.1 shows the stoichiometric ratios obtained in the three acetate experiments performed using the Thorneside WWTP sludge. Table 6.2 summarises these ratios obtained with the Noosa WWTP sludge.

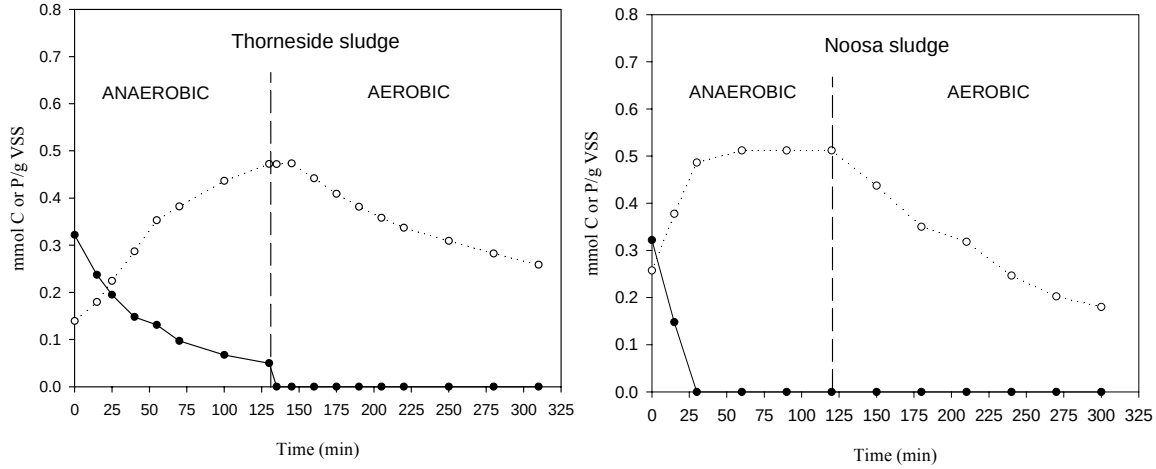

Figure 6.3. Typical time profiles of phosphate (○) and acetate (●) obtained in the batch experiments performed with both sludges.

Table 6.1. Ratios obtained in Thorneside sludge experiments using acetate.

Ratios (mmol Por C/mmol C)	Thorneside WWTP sludge			
	T.Acetate-1	T.Acetate-2	T.Acetate-3	Average
P-release/C-uptake	0.59	0.61	0.57	0.59
Glyc. degraded/C-uptake	0.35	0.37	0.31	0.34
PHB synthetised/C-uptake	1.11	1.15	0.89	1.05
PHV synthetised/ C-uptake	0.21	0.07	0.16	0.15
PH2MV synthetised/ C-uptake	0	0	0	0
PHA total/C-uptake	1.32	1.22	1.05	1.20
CO_2 production/C-uptake	0.22	0.39	0.45	0.35
Carbon recovery ratio*	114%	118%	115%	116%

*Carbon recovery ratio= (PHA+CO_2)/(Acetate+Glyc) *100

Table 6.2. Ratios obtained with Noosa sludge using acetate.

Ratios	Noosa WWTP sludge			
	N. Acetate-1	**N. Acetate-2**	**N. Acetate-3**	**Average**
P-release/C-uptake	0.51	0.39	0.36	0.42
Glyc. degraded/C-uptake	0.42	0.24	0.53	0.40
PHB synthetised/C-uptake	1.21	0.90	1.10	1.07
PHV synthetised/C-uptake	0.20	0.35	0.27	0.27
PH2MV synthetised/ C-uptake	0	0	0	0
PHA total/C-uptake	1.42	1.25	1.37	1.35
CO_2 production/C-uptake	0.39	0.39	0.21	0.33
Carbon recovery ratio	127%	133%	103%	120%

*Carbon recovery ratio= (PHA+CO_2)/(Acetate+Glyc) *100

Figure 6.3 shows typical acetate and phosphate profiles measured during batch tests. Noosa sludge displayed a higher acetate consumption rate compared to Thorneside sludge. Although the batch experiments consisted of 2 hours anaerobic period and 3 hours aerobic period, in this study just the anaerobic period was used to compare with the data presented in the literature.

FISH was performed in the two sludges and *Accumulibacter* could not be quantified because its presence was less than 10% of the total amount of bacteria. No *Competibacter* cells were detected, therefore if these organisms were in the sludge they were present in very low percentage.

All the values obtained in this study have been compared with some data presented in the literature for enriched PAO population. The literature values are summarised in Table 6.3.

Table 6.3. Literature results presented in experiments performed with enriched PAO cultures.

Ratios (mmol P or C/mmol C)	**Reference**			
	Smolders *et al.* 1994	**Pereira *et al.* 1996**	**Hesselmann *et al.* 2000**	**Yagci *et al.* 2003**
P-release/C-uptake	0.44	0.16	0.37	0.48
Glyc. degraded/C-uptake	0.50	0.69	0.63	0.48
PHB synthetised/C-uptake	1.33	1.02	1.11	1.08
PHV synthetised/C-uptake	NM	0.45	0.29	0.12
PH2MV synthetised/C-uptake	NM	NM	NM	0.03
PHA total/C-uptake	1.33	1.47	1.40	1.23
CO_2 production/C-uptake	0.17*	0.22*	0.23*	0.18*

*Not measured. Calculated from C or O/R balance.

NM= No-measured

The values obtained with full scale sludge using acetate were quite similar to the literature results. Nevertheless, some differences in the ratios could be observed between full scale sludge containing les than 10% of the total bacteria as PAOs, and enriched cultures where the percentage of this group is considerably higher.

The results of the experiments performed with Thorneside sludge showed a higher P/C ratio than the literature results although the working pH was 7.00 as in the literature. The average of the three experiments performed (T-Acetate1, T-Acetate2 and T-Acetate3) was 0.59. This value could indicate that more phosphorus was released to take up acetate, so more energy for acetate uptake and conversion would have come from the poly-P breakdown.

Another source of energy in the PAO metabolism comes from the glycogen degradation. Glycogen degradation versus acetate uptake ratio in the experiments performed with the Thorneside sludge had a mean value of 0.34. This value was lower than the range of values presented in the literature. This correlates well with the higher P/C ratio obtained for the same experiments.

The experiments performed with Noosa sludge using acetate showed similar results in terms of P/C and glycogen degradation/C-uptake ratios to the literature results. But even in this sludge, slightly lower value in the glycogen ratio was observed.

These results could indicate that more reducing power would have come from the TCA cycle than the amount predicted in the bibliography (Pereira et al. 1996; Yagci et al. 2003). Another hypothesis is that some COD present but not quantified could have been degraded through the TCA cycle obtaining some reducing power, and for this reason less glycogen would be degraded.

Before Pereira et al. 1996, only a few studies reported on the PHV content of the polyhydroxyalkanoate polymer in activated sludge, and many assumed that PHB was the only PHA formed when acetate was used as substrate (Smolders et al. 1994). Nevertheless, the latest metabolic models developed have considered PHV formation from acetate (Hesselmann et al. 2000; Yagci et al. 2003). The experiments performed with acetate using Thorneside and Noosa sludge showed that PHB and PHV were formed from acetate. The percentages of the different PHA formed in both sludges are presented in Table 6.4.

Table 6.4. Molar percentages of PHB, PHV and PH2MV production during the anaerobic period, obtained in the batch experiments performed with acetate as substrate with full scale sludge from Thorneside WWTP and Noosa WWTP.

	Thorneside sludge			**Noosa sludge**		
	T.Acet-1	**T.Acet-2**	**T.Acet-3**	**N.Acet-1**	**N.Acet-2**	**N.Acet-3**
%PHB	84	95	84	86	72	81
%PHV	16	5	16	14	28	19
%PH2MV	0	0	0	0	0	0
	Literature results					
	Smolders 1994		**Pereira 1996**	**Hesselmann 2000**		**Yagci 2003**
%PHB	100		74	79		87
%PHV	0		26	21		10
%PH2MV	0		0	0		3

The experimental values obtained for Thorneside and Noosa WWTPs were similar to the literature values presented in the metabolic models that consider PHB-PHV formation (Pereira *et al*. 1996; Hesselmann *et al*. 2000; Yagci *et al*. 2003). The experiments performed with Noosa sludge produced in general higher PHV percentages than those with Thorneside sludge. This could be because more glycogen was degraded in the experiments with Noosa sludge (Table 6.1 and 6.2) and therefore, more propionyl-CoA could be produced, which in conjunction with acetyl-CoA, forms PHV.

The CO_2 values obtained showed a wide variation between the experiments. In general, more CO_2 was produced than the theoretical values described in the literature. The carbon recovery ratios shown in Tables 6.1 and 6.2 for Thorneside and Noosa sludges respectively indicates an overestimation in the carbon balance. This could be due to some COD present in the mixed liquor but not quantified, was consumed during the experiments, increasing the CO_2 production. Moreover, the degradation of this COD could contribute to the production of the reducing power necessary to store PHA and that will explain the lower experimental glycogen degradation values compared to the literature values.

6.3.2. PROPIONATE EXPERIMENTS

Three different batch experiments were performed with each sludge using propionate as the only carbon source. Tables 6.5 and 6.6 summarise the main ratios obtained in the experiments performed with Thorneside and Noosa sludge respectively.

Figure 6.4 shows the typical experimental profiles obtained when propionate was tested using both sludges. As in the case of acetate, the highest propionate uptake rate was found in the batch experiments performed with Noosa sludge.

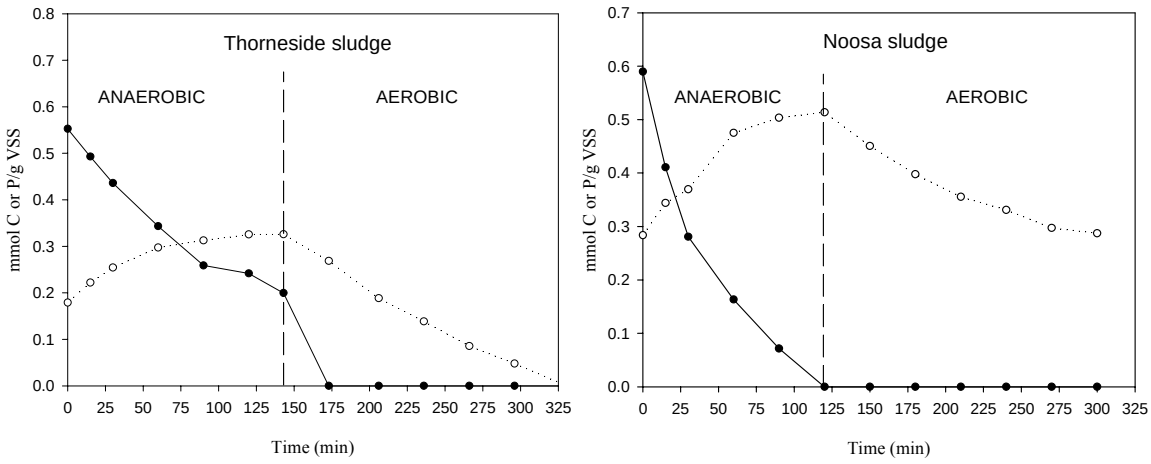

Figure 6.4. Typical time profiles of phosphate (○) and propionate (●) obtained in the batch experiments performed with both sludges.

Table 6.5. Ratios obtained with Thorneside sludge experiments using propionate.

Ratios	**Thorneside WWTP sludge**			
(mmol P or C/mmol C)	**T.Propionate-1**	**T.Propionate-2**	**T.Propionate-3**	**Average**
P-release/C-uptake	0.38	0.30	0.30	0.33
Glyc. degraded/ C-uptake	0.29	0.13	0.30	0.24
PHB synthetised/ C-uptake	0.12	0.0	0.0	0.04
PHV synthetised/ C-uptake	0.34	0.37	0.70	0.47
PH2MV synthetised/ C-uptake	0.71	0.69	0.63	0.68
PHA total/C-uptake	1.17	1.07	1.34	1.19
CO_2 production/ C-uptake	0.142	0.283	0.289	0.238
Carbon recovery ratio	102%	119%	124%	115%

*Carbon recovery ratio= (PHA+CO_2)/(Acetate+Glyc) *100

Table 6.6. Ratios obtained in Noosa sludge experiments using propionate.

Ratios	Noosa WWTP sludge			
(mmol P or C/mmol C)	N. Propionate-1	N. Propionate-2	N. Propionate-3	Average
P-release/C-uptake	0.44	0.43	0.31	0.39
Glyc. degraded/ C-uptake	0.22	0.23	0.32	0.26
PHB synthetised/ C-uptake	0.05	0.05	0.06	0.05
PHV synthetised/ C-uptake	0.63	0.62	0.59	0.61
PH2MV synthetised/ C-uptake	0.52	0.52	0.48	0.51
PHA total/C-uptake	1.20	1.18	1.14	1.17
CO_2 production/ C-uptake	0.101	0.275	0.266	0.214
Carbon recovery ratio	107%	119%	106%	110%

*Carbon recovery ratio= (PHA+CO_2)/(Acetate+Glyc) *100

Propionate has been shown to be present in EBPR plant influents and is also generated in high abundance by many plant prefermentors (Thomas *et al*. 2003; von Munch 1998). Detailed metabolic models describing the metabolism of PAOs and GAOs have been developed using acetate as the sole carbon source (Smolders *et al*. 1994; Filipe *et al*. 2001; Zeng *et al*. 2003). The first metabolic model developed with propionate as the sole carbon source has been submitted recently by Oehmen *et al*. (submitted), and details the PAO anaerobic pathways when propionate is the only substrate. With propionate, the PHA composition of PAOs consists of PHB, PHV, PH2MB and PH2MV. Two acetyl-CoA molecules form one 3HB molecule, one acetyl-CoA molecule and one propionyl-CoA molecule form either one 3HV molecule or one 3H2MB molecule, and two propionyl-CoA molecules form one 3H2MV molecule. Since PHV and PH2MB are isomers of each other, they will henceforth be grouped together and referred to simply as PHV, which indicates the sum of these two polymers. Since it has not yet been determined if acetyl-CoA preferentially binds to propionyl-CoA, a comparison between completely random and completely selective binding of these molecules was performed in the model presented by Oehmen *et al*. (submitted). This model presents two hypotheses in the metabolism of PAO using propionate. The first one is called “random” hypothesis and predicts some PHB synthesis from propionate. The “non-random” hypothesis predicts no PHB production when propionate is used. When propionate is taken up, they suggested that PAO behave according to the “non-random” hypothesis and no PHB is produced with propionate as the sole substrate.

The ratios presented in the experiments carried out to develop this model have been summarised in Table 6.7.

Table 6.7. Values obtained in the two hypothesis proposed by Oehmen *et al*. (submitted) in the metabolic model developed with propionate as the only carbon source.

mmol P or C/mmol C	**PAO-PROPIONATE MODEL (Oehmen *et al*. submitted)**	
	Random	**Non-random**
P-release/C-uptake	0.40	0.40
Glyc. degraded/C-uptake	0.33	0.33
PHB synthetised/C-uptake	0.06	0
PHV synthetised/C-uptake	0.42	0.56
PH2MV synthetised/C-uptake	0.75	0.67
PHA total/C-uptake	1.22	1.22
CO_2 production/C-uptake	0.11	0.11

The only difference between the two hypotheses is the PHA percentage production. The other ratios are the same in the random and non-random hypotheses.

The results obtained in this study using propionate for both sludges show that the P-release/C-uptake ratio was slightly lower than the prediction of the model in the Thorneside sludge, meanwhile Noosa sludge results were very close to the model prediction. The glycogen degradation was also lower than the model prediction although the difference was not very large. The main discrepancy between the "random" and "non-random" model predicted values and the full scale sludge results was the percentages of the different monomers in the composition of PHA. Table 6.8 shows these percentages for the experiments performed with propionate as well as the percentages predicted by the model.

Table 6.8. Molar percentages of PHB, PHV and PH2MV production during the anaerobic period, obtained in the batch experiments performed with propionate as substrate with full scale sludge from Thorneside WWTP and Noosa WWTP.

	Thorneside sludge				**Noosa sludge**			
	T.Prop1	**T.Prop2**	**T.Prop3**	**Average**	**N.Prop1**	**N.Prop2**	**N.Prop3**	**Average**
%PHB	10	0	0	3	4	5	6	5
%PHV	29	35	53	39	52	59	52	54
%PH2MV	61	65	47	58	43	36	42	40
	PAO-PROPIONATE MODEL (Oehmen *et al*. submitted)							
	Random model				**Non-random model**			
%PHB	5				0			
%PHV	34				45			
%PH2MV	61				55			

Some PHB production was found in most of the experiments performed meanwhile the non-random model does not predict any PHB formation. Conversely, PHV and PH2MV percentages were quite similar among the experiments performed with the same sludge, but they were different comparing the average of the results obtained for each sludge and comparing to the model predictions too. Therefore, it seems difficult to predict the fraction of different polymers that will be synthetised in sludge.

The metabolic model predicts different PHA production percentages as is shown in Table 6.8. However, the model was developed with a biomass enriched on propionate and this biomass was working under the same operational conditions and with the same synthetic media concentrations for some time. Biomass from a full scale process usually receive different sorts and concentrations of substrates and might produce a different response when propionate is used as a sole substrate. Nevertheless, in terms of total PHA produced, the results with full scale sludge and the predictions of the model were very similar.

When propionate was used as the sole carbon source, CO_2 production was the most variable parameter measured as in the case of the acetate experiments. The carbon recovery ratio exceeded 100% in all the cases. As in the acetate experiments, the overestimation in the carbon production could be due to some COD that has not been considered in the feed because it was coming from the mixed liquor of the treatment plant.

6.4. CONCLUSIONS

The study presented in this chapter is the first that compares experimental data obtained with enriched cultures (mostly developed with acetate and cultivated in lab-scale sequencing batch reactors) with full scale EBPR sludges. The only metabolic model developed with propionate (Oehmen et al. submitted) has also been compared with some full-scale sludge experiments carried out with propionate. The main conclusions obtained from this study are:

- The literature values agree reasonably well with the results obtained with experiments performed with full-scale EBPR sludge using acetate or propionate. Nevertheless, slight differences were observed.

- Less glycogen degradation was observed in both sludges with both substrates. It could be that full-scale EBPR biomass requires less reducing power to store

the added substrate as PHA or perhaps in these experiments, some COD not quantified was oxidised producing some reducing power. That would also explain the overestimation in the CO_2 production values in all the experiments.

- The amount of total PHA produced for each substrate was close to the literature values. Less PHA production was observed when propionate was used and less glycogen was degraded, which contributed to the PHA production. Nevertheless, some differences in the PHA composition were observed. When acetate was used as substrate, mainly PHB was produced but also some PHV was synthetised. The percentage found in the experiments agrees with the values of recent literature which also predict PHV formation from acetate.

  When propionate was the sole carbon source added, PHV and PH2MV were mainly produced but PHB was also detected. These results differ from the non-random PAO metabolic model developed recently for PAO using propionate. Additionally, different percentages of PHV and PH2MV were found between the two full-scale sludges analysed and the metabolic model predictions. This could be because enriched sludges with propionate produce different responses of full-scale sludges receiving different types of substrates in different concentrations and varying along the time. Therefore, the percentages of the different PHA monomers generated by full-scale sludges could be poorly predictable compared to enriched sludges.

- CO_2 production obtained in all the sets of experiments performed was the most variable parameter determined. The carbon recovery ratio obtained in all the experiments was overestimated. This fact could be due to some COD present in the mixed liquor which was not quantified as carbon uptake in the final results.

6.5. REFERENCES

Comeau Y, Hall KJ, Hancock REW, Oldham WK. 1986. Biochemical model for enhanced biological phosphorus removal. Water Res 20: 1511-1521.

Filipe CDM, Daigger GT, Grady CPL. 2001. A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH. Biotechnol Bioeng 76:17-31.

Gapes D, Keller J. 2001. Analysis of biological wastewater treatment processes using multicomponent gas phase mass balancing. Biotechnol Bioeng. 76: 361-375.

Hesselmann RPX, Von Rummell R, Resnick SM, Hany R, Zehnder JB. 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res 34:3487-3494.

Mino T, Arun V, Tsuzuki Y, Matsuo T. 1987. Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal. In Ramadori R., editor. Biological phosphate removal from wastewaters (Advances in Water Pollution Control 4). Oxford: Pergamon Press. pp. 27-38.

Oehmen A, Zeng RJ, Yuan Z, Keller J. (submitted). A metabolic model of anaerobic propionate utilisation by polyphosphate accumulating organisms (PAO) in enhanced biological phosphorus removal systems. Biotechnol Bioeng.

Pereira H, Lemos PC, Reis MAM, Crespo JPSG, Carrondo MJT, Santos H. 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo 13 C-NMR. Water Res 30:2128-2138.

Pratt S, Yuan Z, Gapes D, Dorigo M, Zeng R, Keller J. 2003. Development of a novel titration and off-gas analysis (TOGA) sensor for study of biological processes in wastewater treatment systems. Biotechnol Bioeng 81: 482-495.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 42:461-70.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-848.

Thomas M, Wright P, Blackall LL, Urbain V, Keller J. 2002. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. Third IWA World Water Congress, Melbourne, Australia, 7-12 April, 2002.

Von Munch E. 1998. DSP prefermentor technology book. Brisbane, Qld, Australia: Science Traveller International, CRC WMPC Ltd.

Wentzel MC, Lötter LH, Lowenthal RE, Marais GvR. 1986. Metabolic behaviour of Acinetobacter spp. in enhanced biological phosphorus removal. A biochemical model. Water SA 12: 209-224.

Yagci N, Artan N, Ubay Çokgör E, Randall CW, Orhon D. 2003. Metabolic model for acetate uptake by a mixed cultura of phosphate and glycogen accumulating organisms under anaerobic conditions. Biotechnol Bioeng 84:360-373.

Zeng RJ, Yuan Z, van Loosdrecht MCM, Keller J. 2002. Proposed modifications to metabolic model for glycogen accumulating organisms under anaerobic conditions. Biotechnol Bioeng 80:277-279.

Zeng RJ, van Loosdrecht MCM, Yuan Z, Keller J. 2003. Metabolic model for glycogen accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnol Bioeng 81:92-105.

Zeng RJ, Yuan Z, Keller J. 2003. Model-based analysis of anaerobic acetate uptake by a mixed culture of polyphosphate-accumulating and glycogen-accumulating organisms. Biotechnol Bioeng 83:293-302.

CHAPTER VII

AEROBIC PHOSPHORUS RELEASE LINKED TO ACETATE UPTAKE: PROCESS MODELLING USING OXYGEN UPTAKE RATE

This chapter has been published as:

Guisasola A, Pijuan M, Baeza JA, Carrera J, Casas C, Lafuente J. 2004. Aerobic phosphorus release linked to acetate uptake in bio-P sludge: process modelling using oxygen uptake rate. Biotechnology and bioengineering 85 (7): 722-733

7.1. INTRODUCTION

The EBPR process is based on the physical separation between the electron donor (COD) and the final electron acceptor (oxygen or nitrate). One step beyond in PAO knowledge is the analysis of the effect of the simultaneous presence of both the electron donor and the final electron acceptor. Some research can be found in the literature about the effects of the coexistence of COD as electron donor and nitrate as electron acceptor (Kuba *et al*., 1996; Pereira *et al*., 1996; Filipe and Daigger, 1999). These works were focused in studying the importance of the DPAOs in the bio-P sludge. On the other hand, P-release under strictly aerobic conditions linked to external substrate uptake has already been described in the literature (Henze *et al*., 2000; Ahn *et al*., 2002). This release is related to PAOs, which are also capable to take up organic substrates under aerobic conditions.

However, it is not clear whether, under aerobic conditions, PAOs store organic substrates as PHA linked to oxygen consumption or linked to glycogen degradation and P-release, as under anaerobic conditions. It is not clear either the response of an EBPR biomass in front of an organic substrate (acetate) under strictly aerobic conditions, when this biomass contains different levels of PHA, polyphosphate and glycogen.

Hence, this chapter is focused in studying the bio-P sludge behaviour when acetate and oxygen were present simultaneously. A comparison was carried out between the behaviour of a bio-P sludge using acetate under anaerobic/aerobic conditions and its behaviour under aerobic conditions. This comparison was carried out by measuring acetate, phosphorus, PHB and glycogen along the experiments, measuring in-situ the OUR and modelling the experimental data.

The analysis of PAO activity under these conditions will contribute to a deeper knowledge of these organisms. For example, knowing PAO behaviour under aerobic conditions will contribute to a better understanding of the OUR profiles obtained in batch experiments with acetate addition to a biomass coming from a WWTP with EBPR.

7.2. MATERIALS AND METHODS

7.2.1. EXPERIMENTAL DESIGN

Two different sets of experiments were performed. On the one hand, experiments conducted under classical anaerobic/aerobic conditions in an EBPR SBR. On the other hand, experiments conducted under aerobic conditions in a different aerated batch reactor with biomass coming from the mentioned SBR.

Anaerobic/aerobic (AnOx) experiments

The first AnOx set of experiments was conducted in a SBR with a working volume of 10 L, seeded with activated sludge from a non-EBPR plant (Granollers WWTP, Catalonia, Spain). It was operated with 4 cycles per day, with a controlled temperature of 25ºC. Each cycle consisted of 2 h anaerobic react, 3.5 h aerobic react, 25 min of settling and, in the last 5 min, withdrawing 5 L of the supernatant. A volume of 5 L of synthetic wastewater was added during the first 15 min of the subsequent cycle, resulting in a HRT of 12 h. The SRT was kept at 9 d by sludge wastage at the end of the aerobic period, but before the mixing was stopped. The biomass concentration during the SBR operation was around 4900 mgVSS·L^{-1}, with a VSS/TSS ratio of 0.80. The pH was controlled during the aerobic period at 7.0 ± 0.1 with 1M HCl.

Two different tests were performed after 150 days of SBR operation, when the reactor was working with an EBPR population in steady state. These experiments were conducted with an initial concentration in the SBR of 500 and 250 mg COD·L^{-1} as acetate, using the same cycle configuration as the normal operation.

A detail of the pilot plant used to enrich the EBPR population and to perform these experiments is showed in figure 7.1.

Figure 7.1. Pilot plant used to enrich the EBPR population and to perform the AnOx experiments.

Aerobic experiments

A 10 L batch reactor with a controlled temperature of 25ºC was used in this set of experiments. This reactor was inoculated with biomass withdrawn from the SBR at the end of the aerobic phase, and left only with aeration during 10 hours to ensure the absence of any external organic substrate. Afterwards, and once the DO level reached a constant value, a pulse of substrate was added. Two different tests were performed with initial concentrations in the SBR of 300 and 250 mg COD L^{-1} as acetate. In each test, the biomass was coming from the SBR using the procedure described above. The biomass concentration during these experiments was around 2200 mgVSS·L^{-1}, with a VSS/TSS ratio of 0.80. The pH was controlled during all the experiments at 7.0 ± 0.1 with 1M HCl. The DO was higher than 2 mg L^{-1} throughout the experiments. DO and pH were measured with an InoLab pH/Oxi Level 3 (WTW, Weilheim, Germany).

In figure 7.2 is showed the experimental set up for the aerobic batch experiments presented in this chapter.

Figure 7.2. Experimental set up used in the aerobic set of experiments

7.2.2. SYNTHETIC MEDIA

The feed for normal SBR operation was prepared daily from a stock solution and distilled water. It had the following composition ($mg \cdot L^{-1}$): 594 $NaCH_3COO$, 270 KH_2PO_4, 101 NH_4Cl, 8.5 $CO(NH_2)_2$, 9 yeast extract, 38 NaCl, 126 $MgCl \cdot 6H_2O$, 45 $CaCl_2$, 3.1 $FeSO_4 \cdot 7\ H_2O$, 1.8 $MnSO_4 \cdot H_2O$, 3.1 $ZnSO_4 \cdot 7H_2O$, 1.5 $CuSO_4 \cdot 5H_2O$, 0.015 H_3BO_3, 0.050 KI, 0.046 $CoCl_2 \cdot 7H_2O$, 0.015 $Na_2MoO_4 \cdot 2H_2O$ and 3.1 EDTA. The initial COD concentration in the normal SBR operation was 250 mg $COD \cdot L^{-1}$ as acetate. In the batch experiments, a different amount of acetate was added according to each experiment.

7.3. RESULTS

7.3.1. ANAEROBIC/AEROBIC (AnOx)

In this set of experiments, acetate, phosphate, PHB and glycogen were analysed under AnOx conditions (Figure 7.3). The experimental profiles obtained exhibited a trend similar to typical EBPR system. PHA are polymers of different hydroxyalkanoic acids, but when acetate is used as the only carbon source, PHA appears mainly as PHB (Satoh *et al*., 1992). For simplification, in this study PHA was considered as PHB.

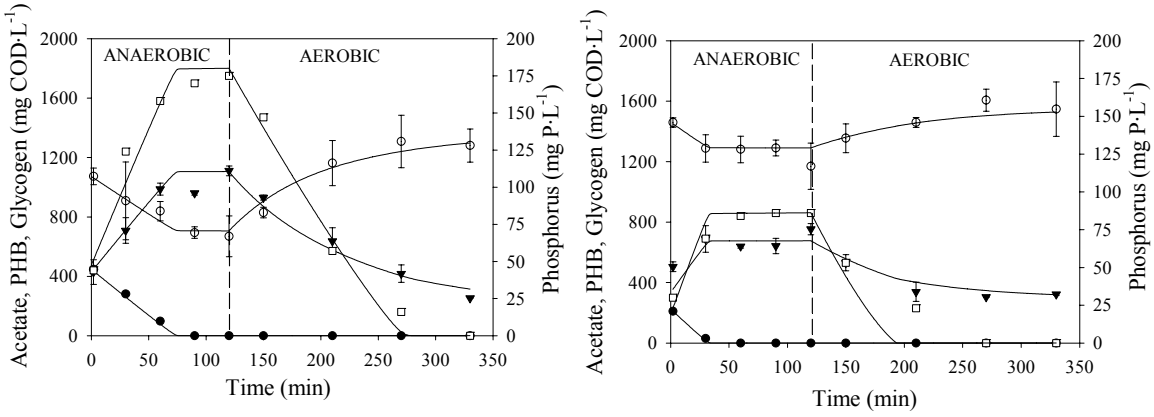

Figure 7.3. Cycle studies used for AnOx model calibration and validation. Left - Experimental measurements and simulated values for calibration cycle study, with an initial concentration of acetate of 500 mg $COD \cdot L^{-1}$. Right - Experimental measurements and simulated values for validation cycle study, with an initial concentration of acetate of 250 mg $COD \cdot L^{-1}$. **(**●-Acetate, □-Phosphorus, ○-Glycogen, ▼-PHB, —— model)

A kinetic and stoichiometric model (Chapter IV), developed as a modification of the ASM2 (Henze *et al*., 2000), was used to describe the experimental profiles. The main modification introduced consists in the inclusion of glycogen economy, as many other models found in the literature (Smolders *et al*., 1994a,b; Mino *et al*., 1995; Filipe and Daigger, 1999; Van Veldhuizen *et al*., 1999; Manga *et al*., 2001; Hao *et al*.; 2001). The stoichiometry and kinetics of this model are shown in Table 7.1.

The model was calibrated with the experimental data obtained with an initial concentration in the SBR of 500 mg $COD \cdot L^{-1}$ as acetate (Figure 7.3. left). Then, it was validated in another experiment with an initial concentration of 250 mg $COD \cdot L^{-1}$ (Figure 7.3. right). As can be seen in both figures, the model describes reasonably well the experimental profiles obtained. The parameters of this model are presented in table 7.2. Most of the values derive from the literature, and only eight of them were modified through an optimisation process.

Table 7.1. Stoichiometry and kinetics of the AnOx model.

STOICHIOMETRY

PROCESS	S_S	S_{O2}	S_{PO4}	S_{CO2}	X_{PHA}	X_{GLY}	X_{PP}	X_{PAO}	X_I
	mg COD·L^{-1}		mg P·L^{-1}	mol·L^{-1}	mg COD·L^{-1}	mg COD·L^{-1}	mg P·L^{-1}	mg COD·L^{-1}	mg COD·L^{-1}
1 X_{PHA} storage	$-Y_{AC1}$		$Y_{PO}\ \frac{1}{8}\cdot\left(\frac{Y_{AC1}}{\gamma_A}+\frac{Y_{GLY1}}{\gamma_G}-\frac{1}{\gamma_P}\right)$		1	$-Y_{GLY1}$	$-Y_{PO}$		
2 X_{PP} storage		$-Y_{PHA}$	-1	$\frac{-Y_{PHA}}{8\gamma_P}$	$-Y_{PHA}$		1		
3 X_{GLY} storage		$-(1-Y_{GLY3})$		$\frac{(1-Y_{GLY3})}{8\gamma_P}$	-1	Y_{GLY3}			
4 X_{PAO} growth		$\frac{-(1-Y_{PAO})}{Y_{PAO}}$	$-i_{BPM}\ \frac{1}{8}\left(\frac{1}{Y_{PAO}\cdot\gamma_P}-\frac{1}{\gamma_X}\right)$	$\frac{-1}{Y_{PAO}}$				1	
5 X_{PAO} lysis			ν_{5P}				-1	$(1-f_{XI})$	f_{XI}
6 X_{PP} lysis			1				-1		
7 X_{PHA} lysis	1				-1				
8 X_{GLY} lysis	1					-1			

KINETICS

$$q_{PHA}\cdot M_{AC}\cdot\frac{X_{PP}/X_{PAO}}{K_{PP}+X_{PP}/X_{PAO}}\frac{X_{GLY}/X_{PAO}}{K_{GLY}+X_{GLY}/X_{PAO}}\cdot X_{PAO}$$

$$q_{PP}\cdot M_{PO4}\cdot M_O\cdot\frac{X_{PHA}/X_{PAO}}{K_{PHA\_P}+X_{PHA}/X_{PAO}}\frac{K_{MAX}-X_{PP}/X_{PAO}}{K_{IPP}+K_{MAX}\ X_{PP}/X_{PAO}}X_{PAO}$$

$$q_{GLY}\cdot M_O\cdot(X_{GLYMAX}-\frac{X_{GLY}}{X_{PAO}})\cdot\frac{X_{PHA}/X_{PAO}}{K_{PHA}+X_{PHA}/X_{PAO}}X_{PAO}$$

$$\mu_{PAO}\cdot M_{PO4}\cdot M_O\cdot\frac{X_{PHA}/X_{PAO}}{K_{PHA}+X_{PHA}/X_{PAO}}X_{PAO}$$

$$b_{PAO}X_{PAO}$$

$$b_{PP}X_{PP}$$

$$b_{PHA}X_{PHA}$$

$$b_{GLY}X_{GLY}$$

Nomenclature:

Degree of reduction (Roels, 1983): γ_A (acetate)= 4, γ_P (PHB)= 4.5, γ_G (glycogen)= 4 and γ_X (biomass)= 4.2 considering elemental composition of biomass as $CH_{1.8}O_{0.5}N_{0.2}$ (Smolders *et al.* 1994)

$$M_O=\frac{S_O}{K_O+S_O}\qquad M_{PO4}=\frac{S_{PO4}}{K_{PS}+S_{PO4}}\qquad M_{AC}=\frac{S_S}{K_S+S_S}$$

An analysis of some parameters obtained in table 7.2 may indicate the presence of GAO in the bio-P sludge used in this work. The value obtained for Y_{PO} (0.195) could indicate that a fraction of GAO were present in the SBR even though this parameter has a wide variability in the literature. Actually, it is shown to be quite difficult to avoid the presence of GAO in a typical bio-P sludge coming from a standard AnOx process.

A lot of research about GAO and PAO competition is being conducted, as for example in Liu *et al*. (1996), Filipe *et al*. (2001a,b), Manga *et al*. (2001) or Zeng *et al*. (2003). However, distinguishing between the two populations was beyond the scope of this study, which was analysing the behaviour of a bio-P sludge (which may contain GAOs) when organic carbon source and oxygen were present.

The kinetics of glycogen storage process consisted in product saturation kinetics as described in Filipe and Daigger (1999). In the PHB storage process, the CO_2 production under anaerobic conditions was considered as described in Smolders *et al*. (1994a) and Zeng *et al*. (2003). The ratio of CO_2 production per acetate uptaken predicted in this work (0.16 C-mol / C-mol) agrees with the ratio obtained in these other studies.

Besides, some rates are higher than those found in the literature, as q_{PHA} (Henze *et al*., 2000) and q_{GLY} (Manga *et al*., 2001). The reason could be that the system of this study (SBR) is much more dynamic in the COD variations compared to the cited systems of the literature. Hence, SBR will probably be more effective in the selection for biomass that take up and store substrate very fast (Dircks *et al*., 2001).

Table 7.2. Value and description of the parameters of the AnOx model (COD_X = biomass COD, COD_S = external substrate (acetate) COD, COD_{PHA} = PHA COD, COD_G = Glycogen COD, COD_I = Inert COD)

Symbol	Description		Units	From
Y_{AC1}	Ac consumed per PHA stored	**0.663 ± 0.016**	g $COD_S \cdot g^{-1}$ COD_{PHA}	This study
Y_{PO}	P released per PHA stored	**0.195 ± 0.007**	g $P \cdot g^{-1}$ COD_{PHA}	This study
Y_{GLY1}	Glyc. degraded per PHA stored	**0.54 ± 0.05**	g $COD_G \cdot g^{-1}$ COD_{PHA}	This study
Y_{PHA}	PHA degraded per poly-P stored	0.20	g $COD_{PHA} \cdot g^{-1}$ P	Henze *et al*. 2000
Y_{GLY3}	Glyc. stored per PHA converted	1	g $COD_G \cdot g^{-1}$ COD_{PHA}	Manga *et al.* 2001
Y_{PAO}	PAO Yield biomass/PHA	0.3	g $COD_X \cdot g^{-1}$ COD_{PHA}	Henze *et al*. 2000
q_{PHA}	PHA storage rate constant (PAO)	**8.0 ± 0.3**	g $COD_{PHA} \cdot g^{-1}$ $COD_X \cdot d^{-1}$	This study
q_{PP}	Poly-P storage rate constant	**1.350 ± 0.001**	g $P \cdot g^{-1}$ $COD_X \cdot d^{-1}$	This study
q_{GLY}	Glyc. storage rate constant	**22 ± 9**	g $COD_G \cdot g^{-1}$ $COD_X \cdot d^{-1}$	This study
μ_{PAO}	Maximum PAO growth rate	**0.463± 0.021**	d^{-1}	This study
b_{PAO}	PAO lysis rate constant	0.2	d^{-1}	Henze *et al*. 2000
b_{PP}	Poly-P lysis rate constant	0.2	d^{-1}	Henze *et al*. 2000
b_{PHA}	PHA lysis rate constant	0.2	d^{-1}	Henze *et al*. 2000
b_{GLY}	Glyc. lysis rate constant	0.2	d^{-1}	This study
K_O	Saturation coefficient for DO	0.20	mg $OD \cdot L^{-1}$	Henze *et al*. 2000
K_S	Saturation coefficient for acetate	4	mg $COD_S \cdot L^{-1}$	Henze *et al*. 2000
K_{PP}	Saturation coefficient for poly-P	0.01	g $P \cdot g^{-1}$ PAO	Henze *et al*. 2000
K_{GLY}	Saturation coefficient for glycogen	0.001	mg $COD_G \cdot L^{-1}$	Manga *et al.* 2001
K_{PHA-P}	Saturation coeff. for PHA in poly-P storage	0.07	g $COD_{PHA} \cdot g^{-1}$ COD_X	Manga *et al.* 2001
K_{PS}	Saturation coeff. for P in poly-P storage	0.2	mg $P \cdot L^{-1}$	Henze *et al*. 2000
K_{MAX}	Maximum ratio of X_{PP}/X_{PAO}	0.34	g $P \cdot g^{-1}$ PAO	Henze *et al*. 2000
K_{IPP}	Inhibition coefficient for X_{PP} storage	0.02	g $P \cdot g^{-1}$ PAO	Henze *et al*. 2000
X_{GLYMAX}	Maximum ratio of X_{GLY}/X_{PAO}	**0.71 ± 0.06**	g $COD_G \cdot g^{-1}$ COD_X	This study
K_{PHA-G}	Saturation coefficient for PHA	0.01	g PHA $\cdot g^{-1}$ PAO	Henze *et al*. 2000
K_P	Saturation coeff. For P in PAO storage	0.01	mg $P \cdot L^{-1}$	Henze *et al*. 2000
i_{BPM}	P content of biomass	0.02	g $P \cdot g^{-1}$ COD_X	Henze *et al*. 2000
ν_{5P}	P released in PAO lysis	0.01	g $P \cdot g^{-1}$ COD_X	Henze *et al*. 2000
f_{XI}	X_I produced in PAO lysis	0	g $COD_I \cdot g^{-1}$ COD_X	Henze *et al*. 2000

Finally, it can be observed that the confidence intervals of the parameters obtained were low, with the exception of the glycogen storage rate constant (q_{GLY}). The reason was that the experimental glycogen measures showed an important variability.

7.3.2 AEROBIC EXPERIMENTS

In this set of experiments, a pulse of acetate was added under aerobic conditions to a bio-P sludge. This biomass was withdrawn from the reactor where the AnOx set of experiments was previously performed. In figure 7.4, two different phases can be distinguished, despite the aeration conditions were not changed along the experiment.

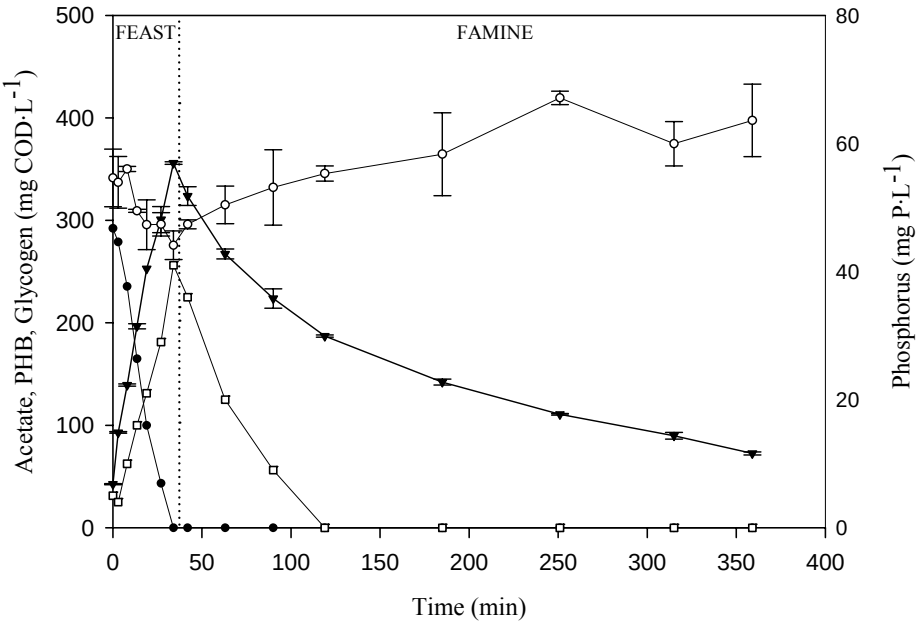

Figure 7.4. Experimental profiles of different compounds during the aerobic experiment with an initial concentration of acetate of 300 mg COD·L^{-1}. ●-Acetate, □-Phosphorus, ○-Glycogen, ▼-PHB

The first phase coincided with the presence of acetate, which was stored as PHB. Afterwards, in the second phase, PHB was consumed and biomass grew on the accumulated polymer. These two phases of accumulation and subsequent growth are known in the literature as feast / famine phases when referring to heterotrophic consumption of an external substrate (e.g. Majone *et al*., 1999; Dircks *et al*., 2001). In this work, these phases were also analogous to the anaerobic / aerobic phases of the EBPR process according to the trends of acetate, phosphate, PHB and glycogen.

Accordingly, while acetate was consumed in the feast phase, PHB was stored. In this storage process, glycogen degradation and phosphate release were experimentally observed. These events are uniquely characteristic of PAO behaviour, and they are carried out in order to obtain the necessary reducing power and energy to store PHB. Hence, PAOs seem to behave the same way than in anaerobic conditions in presence of acetate, although the PHB storage was performed under aerobic conditions.

In addition, OUR was measured (Figure 7.5) in order to obtain complementary information. The OUR value obtained was considerably high during acetate consumption (from 0 to 35 min). When acetate was depleted, the OUR value decreased, but some oxygen consumption was observed until minute 370. The high OUR in the feast phase could be assigned to acetate storage as PHB, linked to oxygen consumption.

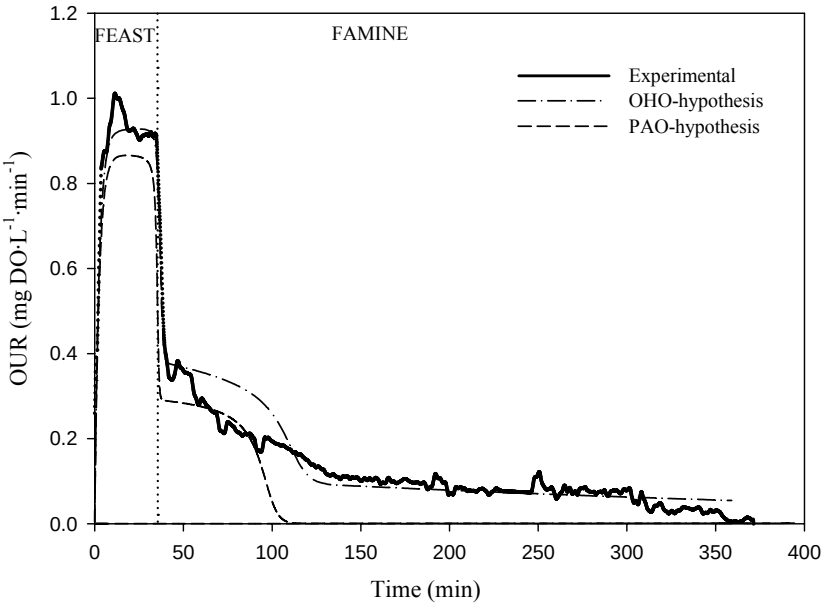

Figure 7.5. Experimental and simulated OUR profiles during the aerobic experiment with an initial concentration of acetate of 300 mg COD·L^{-1}.

In the famine phase, once the acetate was exhausted, the profiles obtained coincided with the typical EBPR behaviour in the aerobic phase: PHB was degraded (allegedly for PAO growth and to restore the glycogen internal pool), glycogen was accumulated and phosphate was taken up.

At this point, the AnOx model described previously was simulated with the initial conditions of the relevant components of the aerobic experiment (Figure 7.6 solid line) and compared with the experimental data. The kinetic and stoichiometric parameters of the processes related to PAO were not modified with respect to the

parameters obtained in the experiment under EBPR conditions, except for the X_{GLYMAX} parameter of the glycogen storage process. This parameter was changed from 0.71 to 0.40 g $COD_G \cdot g^{-1}$ COD_X because the initial glycogen levels were very different between both sets of experiments. Moreover, in the kinetics of poly-P storage, glycogen storage and PAO growth (processes 2, 3 and 4 of table 7.1) a new term was required in order to prevent these processes from occurring simultaneously with PHB storage when acetate was present, and hence obtaining an analogous behaviour to PAO under anaerobic conditions. This term was a switch on acetate: $K_{AC}/(S_{AC}+K_{AC})$, with K_{AC} estimated as 10 mg $COD \cdot L^{-1}$. In the AnOx model (conventional EBPR), these processes cannot occur simultaneously because they include an inhibition term on DO, so that the processes cannot "be active" in the anaerobic phase.

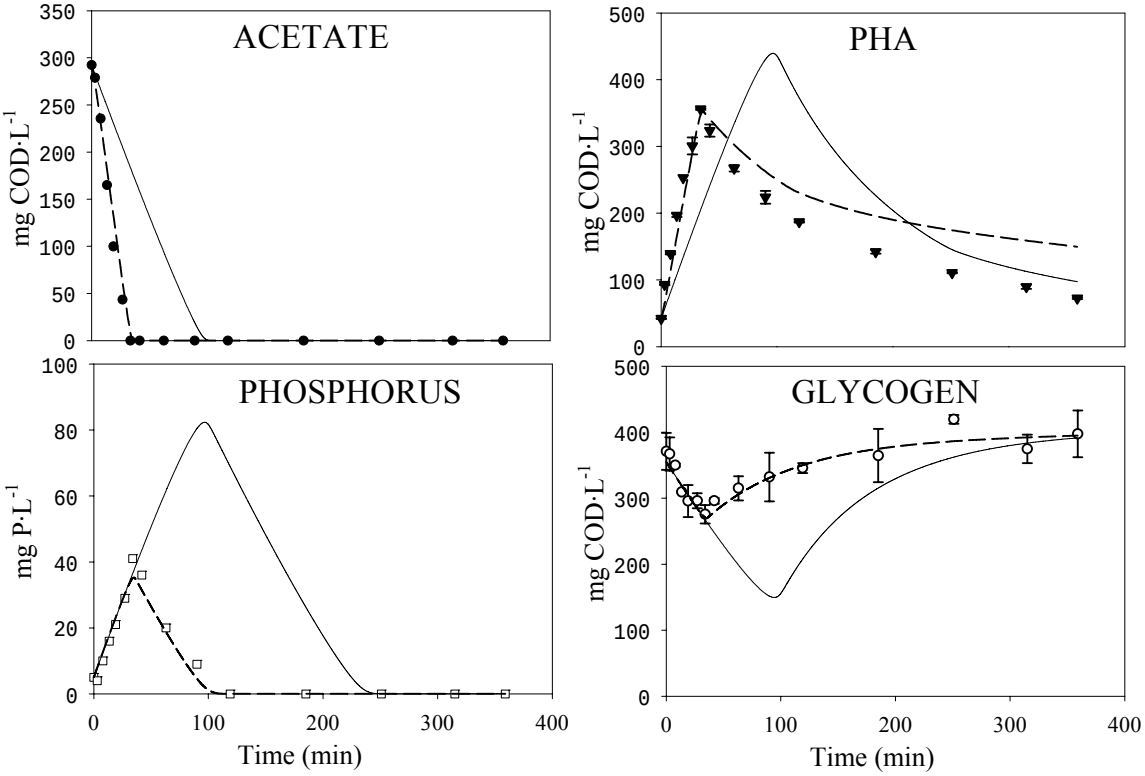

Figure 7.6. Experimental measurements of different compounds during the aerobic experiment with an initial concentration of acetate of 300 mg $COD \cdot L^{-1}$. Simulated values with the AnOx model (——). Simulated values with the PAO-hypothesis model (-------).

In figure 7.6, the simulation of acetate, phosphate, glycogen and PHB are shown together with the experimental data. P-release and glycogen degradation in the feast phase were well predicted by the AnOx model. These profiles were characteristic of PAO behaviour. However, the model prediction did not agree with the evolution of

these compounds along the famine phase, because the point where the acetate was totally consumed (end of the feast phase) was not well predicted.

On the other hand, the AnOx model underestimated the rates of acetate consumption and PHB storage as observed by Kuba *et al*. (1996) in their experiments with nitrate as final electron acceptor. This fact indicated that existed an additional consumption of acetate for PHB storage, with neither P-release nor glycogen degradation. Kuba *et al*. (1996) also observed that part of acetate was oxidised through the TCA, to obtain energy and reduction equivalents for PHB storage, when nitrate was present. In addition, the high OUR observed in the feast phase (Figure 7.5) showed that the consumption of this "excess" of acetate for PHB storage was linked to oxygen consumption.

At this point, two different hypotheses were proposed. Both hypotheses considered that PAOs, under aerobic conditions, uptake acetate coupled to PHB storage, glycogen degradation and phosphorus release as in anaerobic conditions. Moreover, the first hypothesis (PAO-hypothesis) considered that PAOs were able to store additional acetate as PHB linked to oxygen consumption. The second one (OHO-hypothesis) considered that a different biomass fraction: OHOs were the only responsible for this additional consumption of acetate. Both hypotheses were evaluated by simulation, extending the AnOx model with additional equations, and using the parameters of table 7.2 without modifications.

PAO-hypothesis implies that PAOs can oxidise part of the acetate using oxygen as electron acceptor in order to obtain energy to store PHB. Then, PAOs should take advantage of the most efficient aerobic metabolism. In addition, P-release was observed under aerobic conditions, so PAOs also use the classical polyP hydrolysis mechanism for energy supply. This hypothesis was considered and modelled by introducing a new process in the AnOx model (Table 7.3, process 9a), so that the PAOs can uptake aerobically acetate and store it as PHB as described in the ASM3 model (Henze *et al*., 2000). A factor was added to this PHB storage process in order to describe the time delay in reaching the maximum OUR value experimentally observed after adding the acetate. This phenomenon, known as "start-up", is probably caused by many factors such as mixing, substrate diffusion or metabolic pathways activation, and can be mathematically described by a first order delay (Vanrolleghem *et al*., 1998; Guisasola *et al*., 2003).

Table 7.3. Stoichiometry and kinetics of the PAO-hypothesis model (9a) and the OHO-hypothesis model (9b and 10).

PROCESS		STOICHIOMETRY					KINETICS
		S_S	S_{O2}	S_{CO2}	$X_{PHA,HET}$	X_H	
		mg COD·L^{-1}		mol·L^{-1}	mg COD·L^{-1}	mg COD·L^{-1}	
	PAO-hypothesis						
9a	Aerobic X_{PHA} storage by X_{PAO}	$\frac{-1}{Y_{STO}}$	$\frac{-(1-Y_{STO})}{Y_{STO}}$	$\frac{1}{8\gamma_{STO}}\cdot(\frac{\gamma_{STO}}{Y_{STO}\cdot\gamma_S}-1)$	1		$k_{STO}\cdot M_{AC}\cdot M_O X_{PAO}\cdot(1-e^{-t/\tau})$
	OHO-hypothesis						
9b	Aerobic X_{PHA} storage by X_H	$\frac{-1}{Y_{STO}}$	$\frac{-(1-Y_{STO})}{Y_{STO}}$	$\frac{1}{8\gamma_{STO}}\cdot(\frac{\gamma_{STO}}{Y_{STO}\cdot\gamma_S}-1)$	1		$k_{STO}\cdot M_{AC}\cdot M_O X_H\cdot(1-e^{-t/\tau})$
10	X_H growth		$\frac{-(1-Y_H)}{Y_H}$	$\frac{1}{8\gamma_X}\cdot(\frac{\gamma_X}{Y_H\cdot\gamma_{STO}}-1)$	$\frac{-1}{Y_H}$	1	$\mu_H\cdot M_O\cdot\frac{X_{PHA}/X_H}{K_{STO}+X_{PHA}/X_H}X_H$

Nomenclature:

Degree of reduction (Roels, 1983): γ_{STO} (cell internal storage product) = 4.5, γ_S (external substrate (acetate)) = 4

$$M_O=\frac{S_O}{K_O+S_O} \qquad M_{AC}=\frac{S_S}{K_S+S_S}$$

The model was fitted maintaining the previous values for the parameters of processes 1-8. For the process 9a, the value for the yield Y_{STO} was assumed to be the same of ASM3, and the storage rate constant (k_{STO}) was modified in order to fit the experimental profiles for acetate.

As can be observed in figure 7.6 (dashed line), the feast phase was well described using this new process but, in the famine phase, PHB degradation was underestimated. On the other hand, this hypothesis underestimated the OUR of both feast and famine phases (dashed line in figure 7.5).

In order to verify the OHO-hypothesis, two new processes related to the OHO were added to the AnOx model. These processes (9b and 10 of table 7.3) were adapted from the ASM3 model. Two different contributions of PHB were considered: PHB from PAO and PHB from OHO. The sum of both contributions should be equal to the PHB measured.

The model was fitted maintaining the previous values for the parameters of the 8 first processes. In the processes 9b and 10, the value for the yield Y_{STO} was assumed to be the same of ASM3. The parameters used in this model are presented in table 7.4. The confidence intervals for the estimated parameters are much lower than the previous case. This is because of the extra amount of information provided by the OUR measurement every 5 seconds.

Moreover, the number of parameters in this case is only 5 compared to the 8 of the previous case. The obtained k_{STO} (8.9 g $COD_{PHA}\cdot g^{-1}$ $COD_X \cdot d^{-1}$) was higher than the value recommended in the ASM3 model (5 g $COD_{PHA}\cdot g^{-1}$ $COD_X \cdot d^{-1}$). The reason could be the same as the previously commented for q_{PHA} and q_{GLY}. In addition, the obtained Y_H (0.7 g $COD_X \cdot g^{-1}$ COD_{PHA}) was higher than the value recommended in the ASM3 model (0.63 g $COD_X \cdot g^{-1}$ COD_{PHA}). Nevertheless, others authors found values of Y_H very close to the obtained in this study (Dircks *et al*., 2001).

Table 7.4. Value and description of the parameters of the PAO and OHO hypotheses models (COD_X = biomass COD, COD_S = external substrate (acetate) COD, COD_{PHA} = PHA COD, COD_G = Glycogen COD)

Symbol	Description		Units	From
Y_{STO}	PHA produced per acetate uptaken	0.85	g $COD_{PHA} \cdot g^{-1}$ COD_S	Henze *et al*. 2000
Y_H	Heterotrophic growth yield	**0.70 ± 0.06**	g $COD_X \cdot g^{-1}$ COD_{PHA}	This study
k_{STO}	Storage rate constant	**8.93 ± 0.04**	g $COD_{PHA} \cdot g^{-1}$ $COD_X \cdot d^{-1}$	This study
μ_H	Heterotrophic growth rate	**2.88 ± 0.03**	d^{-1}	This study
K_{STO}	Storage process constant	1	g $COD_{PHA} \cdot g^{-1}$ COD_X	Henze *et al*. 2000
K_{S_HET}	Heterotrophic sat. coeff. for Acetate	**10.00 ± 0.06**	mg $COD_S \cdot L^{-1}$	This study
τ	Time constant	**3.0 ± 0.1**	min	This study

As shown in figure 7.7, the model described accurately the evolution of all the components in both feast and famine phases. In addition, it made a good prediction of the OUR profile (dashed-dotted line in figure 7.5) during both phases. This prediction was better than the obtained with the PAO-hypothesis.

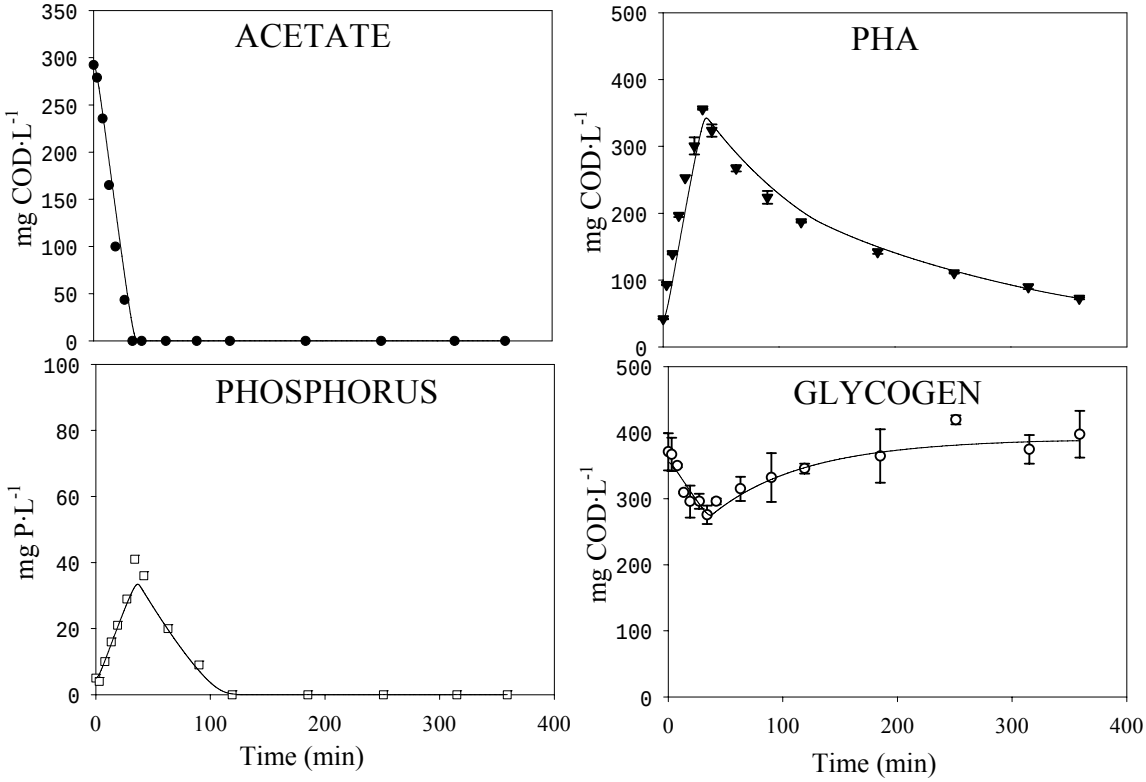

Figure 7.7. Experimental measurements of different compounds during the aerobic experiment with an initial concentration of acetate of 300 mg $COD \cdot L^{-1}$. Simulated values with the OHO-hypothesis model (——).

7.3.3 VALIDATION EXPERIMENT

Another experiment was carried out in order to test whether the experimental results agree with the observed behaviour in the first aerobic experiment. In addition, it was tested whether the calibrated model for each hypothesis was able to describe the new experimental data.

The experimental results shown in figure 7.8, confirm that, under aerobic conditions, P-release occurred simultaneously to acetate uptake, glycogen degradation and PHB storage. Also, figure 7.8 shows the predicted profiles for both hypotheses.

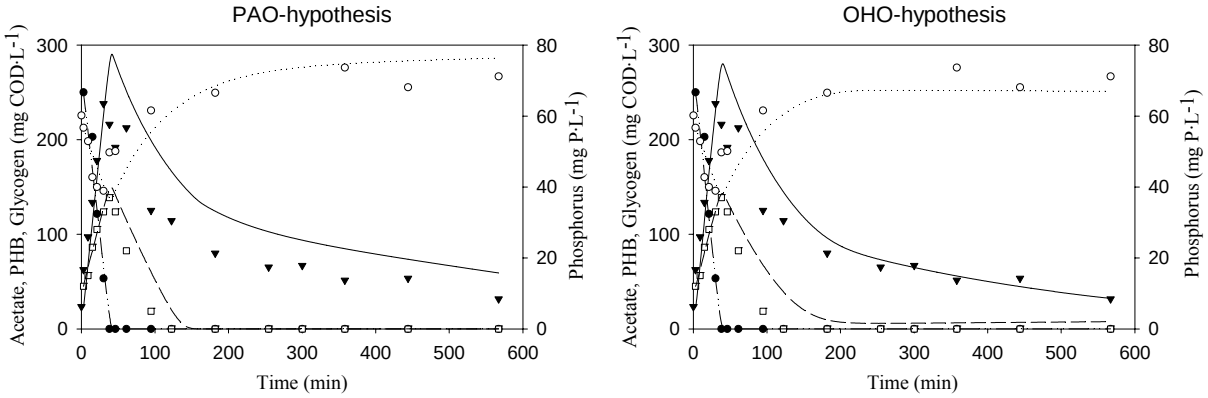

Figure 7.8. Experimental measurements of different compounds during the validation aerobic experiment, with an initial concentration of acetate of 250 mg $COD \cdot L^{-1}$. Left - Simulated values with the PAO-hypothesis model. Rigth - Simulated values with the OHO-hypothesis model. ●-Acetate, □-Phosphorus, ○-Glycogen, ▼-PHB, -···- Acetate model, -- Phosphorus model, ··· Glycogen model, — PHB model.

The PAO-hypothesis model described adequately the feast phase, although the PHB degradation and the polyP storage in the famine phase were underestimated. The observed behaviour for PHB was quite similar to the observed in the calibration experiment of this hypothesis (Figure 7.6). However, the polyP storage process was better described in the calibration experiment.

The OHO-hypothesis model also described adequately the feast phase and underestimated the polyP storage in the famine phase. However, the OHO-hypothesis provided a better description of the PHB degradation in the famine phase. A good description of PHB degradation in this phase was also obtained in the calibration experiment of this hypothesis (figure 7.7).

7.3.4. DISCUSSION

A conventional bio-P sludge obtained from a SBR operated with normal EBPR conditions (anaerobic/aerobic cycles) was used to test its behaviour under aerobic conditions. The results obtained showed that this bio-P sludge stored acetate as PHB, linked to glycogen degradation and P-release in aerobic conditions. These results could not be reliably described using a modified ASM2 model, including glycogen economy, calibrated for the same bio-P sludge in anaerobic/aerobic conditions (AnOx model, figure 7.3). This model underestimated the acetate uptake and the PHB storage in the feast phase (solid line in figure 7.6). Moreover, it did not describe the high experimental OUR observed during the feast phase (figure 7.5).

Two hypotheses were proposed to describe the experimental measures. Both hypotheses considered that PAO, under aerobic conditions, uptake acetate coupled to

PHB storage, glycogen degradation and phosphorus release as in anaerobic conditions. However, the additional consumption of acetate was attributed to different biomass fractions depending on the hypothesis.

Both hypotheses were evaluated by simulation, extending the AnOx model with additional equations. The OHO-hypothesis described the experimental profiles more accurately than the PAO-hypothesis (Figures 7.5, 7.6 and 7.7). The main differences were the better predictions for PHB degradation during the famine phase and the OUR profile during both feast and famine phases.

The different PHB degradation predicted by both hypotheses was due to the growth of OHO (process 10 of Table 7.3). This ASM3 process predicts heterotrophic biomass growth using PHB. This additional consumption provided a better description of the PHB profile for OHO-hypothesis.

The most important difference between both hypotheses was observed in the OUR profiles (Figure 7.5). During the feast phase, the oxygen consumption predicted by the PAO-hypothesis was lower than the predicted by the OHO-hypothesis. This last OUR was very similar to the experimental. The difference between both models during the feast phase was that the OUR of the OHO-hypothesis was due to the simultaneous PHB storage and OHO growth while, in the PAO-hypothesis, OUR was only due to aerobic PHB storage. Both hypotheses did not consider PAO-growth when acetate was present. However, if PAO growth was considered when acetate was present, the OUR of the feast phase predicted by the PAO-hypothesis was also lower than the experimental. Moreover, this possibility underestimated the P-release in the feast phase and the PHB degradation in the famine phase (data not shown).

During the famine phase, the oxygen consumption predicted by the PAO-hypothesis model finished around minute 100, whereas the predicted by the OHO-hypothesis model described all the experimental oxygen consumption until minute 360. The reason for this discrepancy was that the kinetics of poly-P storage and PAO growth (processes 2 and 4 of table 7.1) depend on P concentration, according to ASM2 model (Henze *et al*., 2000). However, the kinetics of OHO growth (process 10 of table 7.3) were predicted in spite of phosphorus absence, according to ASM3 model (Henze *et al*., 2000).

In accordance with these results, the best description seemed to be provided by the OHO-hypothesis model. Nevertheless, this model gave these predictions only when considering a high fraction in OHO similar to the fraction of PAO. One possible reason to this high fraction in OHO could be that the SBR during normal operation

was not bubbled with nitrogen during the anaerobic phase. Then, some oxygen could be transferred through the surface, favouring somehow the OHO presence in the bio-P sludge. In addition, some organic matter could be provided by means of the lysis processes, giving some additional substrate for OHO growth during aerobic phases. However, a rough estimation of the amount of oxygen transferred through the surface indicates that only about 3% of COD could be consumed aerobically. This contradicts somehow the OHO-hypothesis.

Assuming that PAO and OHO populations are well described by the modified ASM2 model and the ASM3 model respectively, the OHO-hypothesis describes better the experimental profiles. Nevertheless, none of both hypotheses can be totally rejected and it is also possible that its combination could describe better the experimental results. More research is needed in this field in order to clarify the experimental observations.

7.4. CONCLUSIONS

In this study, a comparison between the behaviour of an EBPR sludge working under anaerobic/aerobic conditions and the behaviour of the same sludge with simultaneous presence of an electron donor (acetate) and a final electron acceptor (oxygen) was performed.

The main conclusions obtained are:

- PHB storage from acetate by PAO observed in anaerobic conditions seems not to be affected by the oxygen presence. Under strictly aerobic conditions, PAO can uptake acetate obtaining energy from poly-P hydrolysis and reducing power from glycogen degradation.

- Part of the acetate consumed in aerobic conditions is stored as PHB with oxygen consumption.

- Two different hypotheses were formulated and simulated to describe this aerobic acetate consumption and PHB storage. The PAO-hypothesis considered that PAO store acetate as PHB linked to oxygen consumption, in addition to store acetate as PHB linked to P-release and glycogen degradation. The OHO-hypothesis considered that PAO store acetate as PHB linked to P-release and glycogen degradation, and that OHO are the only responsible for the additional acetate consumption and PHB storage.

- Assuming that PAO and OHO populations are well described by the utilized models, the OHO-hypothesis described better the experimental profiles.

7.5. REFERENCES

Ahn J, Daidou T, Tsuneda S, Hirata A. 2002. Transformation of phosphorus and relevant intracellular compounds by a phosphorus accumulating enrichment culture in the presence of both the electron acceptor and electron donor. Biotechnol Bioeng 79:83-93.

Dircks K, Henze M, van Loosdrecht MCM, Mosbaek H, Aspegren H. 2001. Storage and degradation of poly-β-hydroxybutyrate in activated sludge under aerobic conditions. Water Res 35:2277-2285.

Filipe CDM, Daigger GT. 1999. Evaluation of the capacity of Phosphorous-Accumulating Organisms to use nitrate and oxygen as final electron acceptors: a theoretical study on population dynamics. Water Environ Res 71:1140-1150.

Filipe CDM, Daigger GT, Grady CPL Jr. 2001a. Stoichiometry and kinetics of acetate uptake under conditions by an enriched culture of phosphorus-accumulating organisms at different pHs. Biotechnol Bioeng 76:32-43.

Filipe CDM, Daigger GT, Grady CPL Jr. 2001b. A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: Stoichiometry, kinetics and the effects of pH. Biotechnol Bioeng 76:17-31.

Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. 2003. An off-line respirometric procedure to determine inhibition and toxicity of biodegradable compounds in biomass from an industrial WWTP. Water Sci Technol. In press.

Hao X, Van Loosdrecht MCM, Meijer SCF, Qian Y. 2001. Model-based evaluation of two BNR processes – UCT and A_2N. Water Res 35:2851-2860.

Henze M, Gujer W, Mino T, Van Loosdrecht MCM. 2000. Activated Sludge Models ASM1, ASM2, ASM2d and ASM3: Scientific and Technical Report nº9. IWA Task Group on Mathematical Modelling for Design and Operation of Biological Wastewater Treatment.

Kuba T, Wachtmeister A, van Loosdrecht MCM, Heijnen JJ. 1994. Effect of nitrate on phosphorous release in biological phosphorous removal systems. Water Sci Technol 30(6):263-269.

Liu WT, Mino T, Nakamura K, Matsuo T. 1996. Biological removal processes: Effect of pH on anaerobic substrate metabolism. Water Sci Technol 30(1-2):25-32.

Majone M, Dircks K, Beun JJ. 1999 Aerobic storage under dynamic conditions in activated sludge processes. The state of the art. Water Sci Technol 39 (1):61-73.

Manga J, Ferrer J, Garcia-Usach F, Seco A. 2001. A modification to the Activated Sludge Process No.2 based on the competition between phosphorus-accumulating organisms and glycogen-accumulating organisms. Water Sci Technol 43(11):161-171.

MATLAB. Optimization Toolbox. 1999. User's Guide. Version 2 (Release 11). The MathWorks. Natick, MA, USA.

Mino T, Liu W, Kurisu F, Matsuo T. 1995. Modelling glycogen storage and denitrification capability of microorganisms in EBPR processes. Water Sci Technol 31(2):25-34.

Pereira H, Lemos P, Reis M, Crespo J, Carrondo M, Santos H. 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo C-NMR labeling experiments. Water Res 30:2128-2138.

Satoh H, Mino T, Matsuo T. 1992. Uptake of organic substrates and accumulation of polyhydroxyalcanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. Water Sci Technol 26(5-6):933-942.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 42:461-70.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng 44:837-848.

van Veldhuizen HM, van Loosdrecht MCM, Heijnen JJ.1999. Modelling biological phosphorus and nitrogen removal in a full scale activated sludge process. Water Res 33:3459-3468.

Vanrolleghem PA, Gernaey K, Coen F, Petersen B, De Clerq B, & Ottoy JP. 1998. Limitations of short term experiments designed for identification of activated sludge biodegradation models by fast dynamic phenomena. In: Proceedings 7th IFAC Conference on Computer Applications in Biotechnology CAB7. Osaka, Japan, May 31-June 4 1998.

Zeng RJ, Yuan Z, Keller J. 2003. Model-based analysis of anaerobic acetate uptake by a mixed culture of polyphosphate-accumulating and glycogen-accumulating organisms. Biotechnol Bioeng 83:293-302.

CHAPTER VIII

AEROBIC PHOSPHORUS RELEASE LINKED TO ACETATE UPTAKE: INFLUENCE OF PAO INTRACELLULAR STORAGE COMPOUNDS

This chapter has been submitted as:

Pijuan M, Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. Aerobic phosphorus release linked to acetate uptake: influence of PAO intracellular storage compounds. Biochemical Engineering Journal.

8.1. INTRODUCTION

The aim of this study was to assess the response of an EBPR biomass in front of an organic substrate (acetate) under aerobic conditions, when this biomass contained different levels of PHA, polyphosphate and glycogen. This study was conducted using activated EBPR sludge cultivated in a SBR. Three different experiments were performed in order to compare the standard reactor operation with the results obtained in batch experiments performed under aerobic conditions with sludge withdrawn at the end of the anaerobic period of the SBR and at the end of the aerobic period.

8.2. MATERIALS AND METHODS

8.2.1. EXPERIMENTAL DESIGN

To carry out this study three different experiments were performed. The SBR was normally fed with propionate but acetate was chosen as the substrate in the batch experiments, to use the predominant substrate in the literature. In addition, it has been shown that PAO normally fed with propionate can move to acetate consumption with any time delay (Chapter IV).

Anaerobic/aerobic experiment

The first experiment (AnOx) consisted in a monitorisation of a steady state anaerobic-aerobic cycle of the SBR where the conventional behaviour of PAO can be observed and using acetate as the sole carbon source, with an initial concentration of 260 mg/L. A detail of the pilot plant used to perform this experiment is showed in figure 5.1 from Chapter V.

Aerobic batch experiments

The second set of experiments consisted in two aerobic batch experiments performed in a different 10 L batch reactor with a controlled temperature of 25ºC. In the first batch experiment (EAN), this reactor was inoculated with biomass withdrawn from the SBR at the end of the anaerobic phase and in the second batch experiment (EOX) the reactor was inoculated with biomass withdrawn at the end of the aerobic period. These tests were performed with an initial concentration of 200 mg/L of acetate. The pH was controlled during all the experiments at 7.0 ± 0.1 with 1M HCl. The DO was higher than 2 mg L^{-1} throughout the experiments to avoid oxygen limitations. DO and pH were measured with an InoLab pH/Oxi Level 3 (WTW, Weilheim, Germany). A detail of the experimental set up used for these experiments is shown in figure 7.2 of chapter VII.

8.2.2. SYNTHETIC MEDIA

Two separate solutions called "concentrated feed" (constituting 0.25 L per 5 L synthetic wastewater) and "P-water" (constituting 4.75 L per 5 L synthetic wastewater) collectively formed the synthetic wastewater used in this study. The synthetic wastewater had the same composition as described in Chapter IV but no peptone was added. The EBPR population of the reactor was developed using propionate as the sole carbon source and the normal working concentration in the SBR (after the feeding phase) was 160 mg/L of propionate.

8.3. RESULTS

8.3.1 BIOMASS CHARACTERISATION

An initial characterisation of the biomass used in both sets of experiments was performed to assess the amount of PAO and GAO present. FISH was carried out in the sludge. The micrographs in Figure 8.1 visually show the abundance of PAOMIX-binding cells (*Accumulibacter*) and how GAOMIX-binding cells (*Competibacter*) were rarely observed. PAO were quantified as around 45 % of total microorganisms via image analysis.

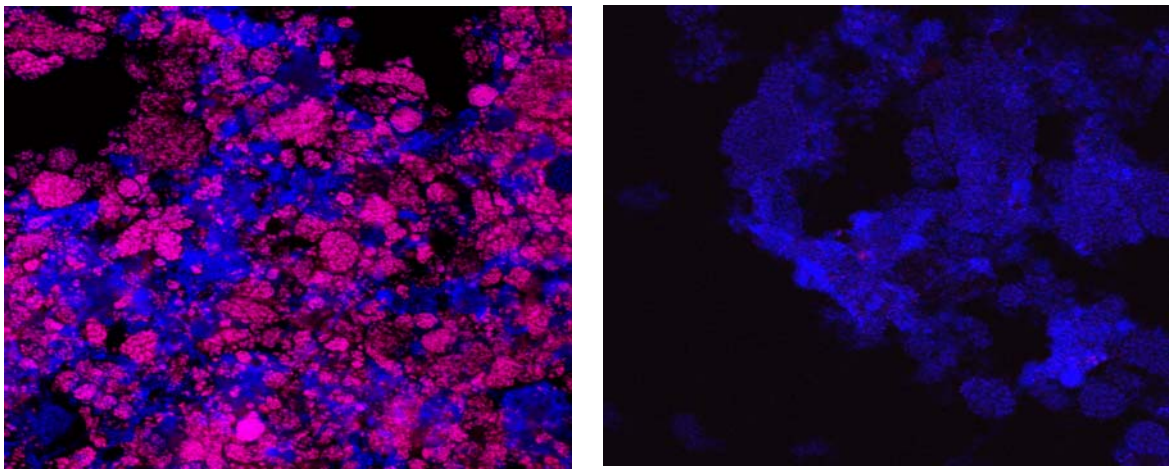

Figure 8.1. CLSM micrographs of FISH of the EBPR sludge used in the batch experiments. A.- Sludge hybridized with probe specific for PAO (Cy3-labelled PAOMIX probes in pink) and probe for bacteria (Cy5-labelled EUBMIX probes in blue). B) Sludge hybridized with probe specific for GAO (Cy3-labelled GAOMIX probes not detected) and probe for bacteria (Cy5-labelled EUBMIX probes in blue).

8.3.2. AnOx EXPERIMENT

A standard cycle study performed in the SBR using acetate as the only carbon source (AnOx experiment) was carried out in order to compare the EBPR performance of the system with the response obtained in the aerobic batch experiments performed in the second set of experiments. Figure 8.2 shows the typical EBPR profiles obtained in this cycle study. During the anaerobic phase, acetate was consumed and PHA was produced meanwhile glycogen was degraded and phosphate was released to the medium. After acetate depletion under anaerobic conditions, very small variations in the measured components were observed. In the aerobic phase, PHA was consumed and glycogen was formed, while phosphate was taken up from the medium.

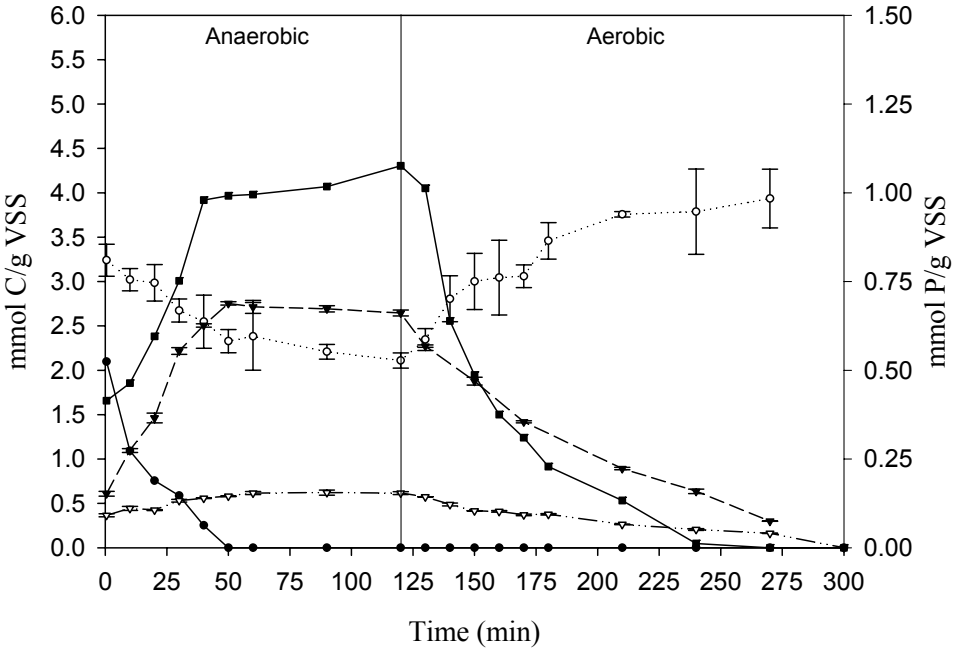

Figure 8.2. Experimental profiles of the different compounds during the anaerobic/aerobic cycle study performed in the SBR with acetate (AnOx experiment): (●) acetate, (■) phosphorus, (○) glycogen, (▼) PHB, (▽) PHV.

8.3.3. EAN AND EOX EXPERIMENTS

The second set of experiments was performed with biomass withdrawn at the end of the anaerobic period of the SBR (EAN) and from the end of the aerobic period (EOX). Table 8.1 shows the values obtained for the different storage products present in the biomass at the beginning of each batch experiment. The differences observed between the initial values from EAN and EOX were due to the SBR cycle period when the biomass was withdrawn at. The cycle previous to both experiments

was fed with propionate as carbon source and, then, a considerable amount of PHV and PH2MV was observed in the PHA composition. This effect was less significant in the EOX experiment, where PHA was consumed during the previous aerobic phase. EAN was carried out with biomass withdrawn at the end of the anaerobic period of the SBR, where PAO had used its polyP to uptake the substrate and to store it as PHA, degrading part of its glycogen pools to get the reducing power required for this PHA storage. The biomass used for EOX was withdrawn at the end of the aerobic period of the SBR, where PAO had degraded its PHA to recover its glycogen levels and to uptake the phosphorus from the media to replenish its polyP pools.

Table 8.1. Initial levels of the different storage compounds of the biomass used in both experiments.

Initial conditions	EAN	EOX
PHB (mmol C/g VSS)	0.35	0.46
PHV (mmol C/g VSS)	0.41	0.06
PH2MV (mmol C/g VSS)	0.56	0.21
Total PHA (mmol C/g VSS)	1.32	0.73
Glycogen (mmol C/g VSS)	3.73	4.53
polyP (mg P/ mg TSS)	0.089	0.091
Biomass (mg VSS/ L)	1500	1350

The profiles obtained in EAN and EOX are depicted in figure 8.3 and 8.4 respectively. The same trends were observed in both experiments, although the initial phosphate level was higher in the EAN experiment because phosphorus was previously released in the anaerobic period. As observed in Chapter VII, two different phases could be distinguished, despite the aeration conditions did not change along the experiment. The first phase coincided with the presence of acetate and it is known as feast phase.

Afterwards, in the second phase, PHA was consumed and biomass grew on the accumulated polymer and it is known as famine phase. During the feast phase, acetate was consumed and PHA was produced meanwhile glycogen was degraded and phosphate was released to the media. These processes are uniquely characteristic of PAO behaviour and, hence, it seems that PAO behave in a similar way as under anaerobic/aerobic conditions. In the famine phase, PHA was consumed and glycogen was formed, while phosphate was taken up from the media as in the conventional EBPR process where the second phase is also aerobic.

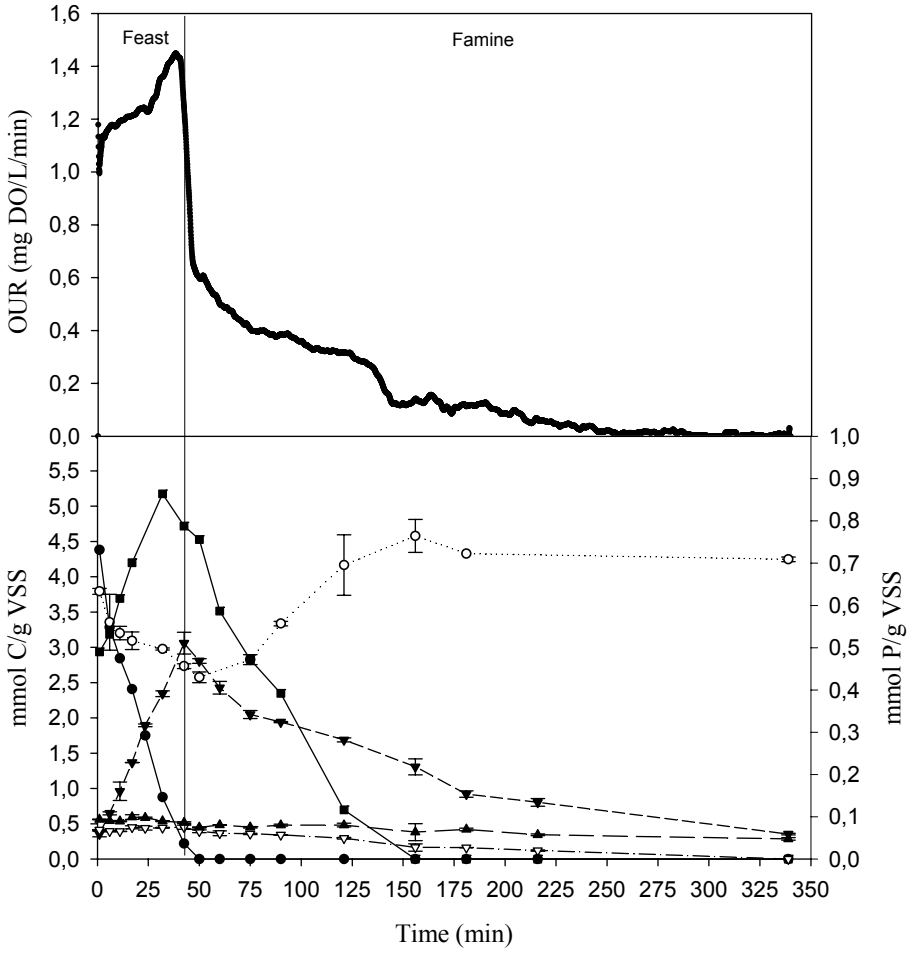

Figure 8.3. Batch experiment performed with sludge withdrawn at the end of the anaerobic period (EAN). A.- Experimental OUR profile. B.- Experimental profiles of different compounds: (●) Acetate, (■) Phosphorus, (○) Glycogen, (▼) PHB, (▽) PHV, (▲) PH2MV.

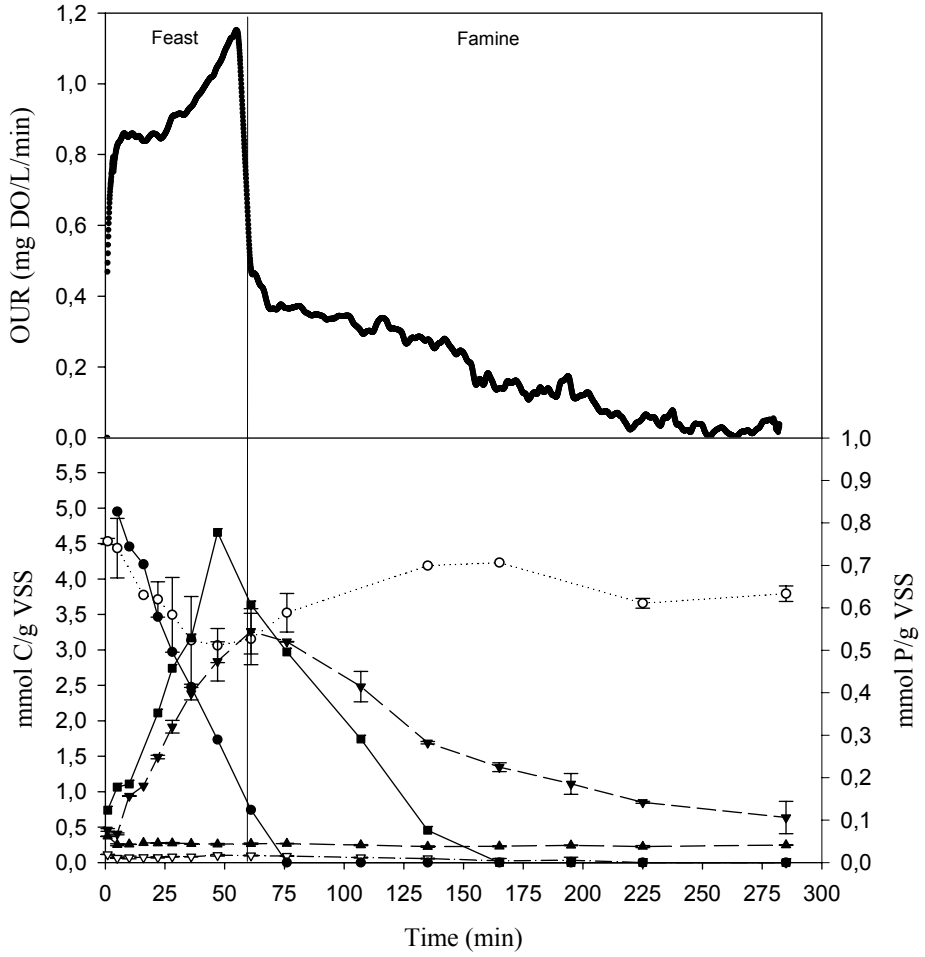

Figure 8.4. Batch experiment performed with sludge withdrawn at the end of the aerobic period (EOX). A.- Experimental OUR profile. B.- Experimental profiles of different compounds: (●) Acetate, (■) Phosphorus, (○) Glycogen, (▼) PHB, (∇) PHV, (▲) PH2MV.

8.3.4. OXYGEN UPTAKE RATE MEASUREMENTS

In addition, OUR was measured in both aerobic batch experiments for a better insight of the process. The major OUR value was detected during the feast period when acetate was still present. This fact is quite outstanding since oxygen consumption has obviously never been linked to acetate consumption in the EBPR process. During the famine phase, OUR decreased, but two different rates could be found in this period. The first one took place when there was still phosphate in the medium (end of feast phase until 150 min in both experiments). During this period a higher PHA degradation was observed as well as glycogen production. When phosphate was completely depleted, in both experiments, glycogen production also stoped and PHA degradation rate as well as OUR decreased, but there were still some consumption of PHA and oxygen until the end of the experiments. This oxygen consumption when phosphate was depleted and the notably high initial oxygen

consumption was discussed in Chapter VII whether these observations were related to PAO metabolism in aerobic conditions or to a heterotrophic presence in the biomass population. Both options were tested via modelling in that work and none of them could strongly be rejected.

8.3.5. COMPARISON BETWEEN AnOx, EAN AND EOX

Table 8.2 summarises the ratios obtained in the three experiments performed. In the aerobic experiments, the swap between the feast and famine processes was not as clearly observed as in the AnOx experiment. When acetate was close to depletion, there was an intermediate phase when the trends of the measured compounds began to change.

Table 8.2. Ratios obtained in the experiments performed in this study.

	ANOX	**Aerobic batch experiments**	
		EAN	**EOX**
P/C (mmol P/mmol C)	0.31	0.14	0.18
PHA_F/Ac_{UPT} (mmol C/mmol C)	1.28	0.77	0.78
Gly_{DEG}/Ac_{UPT} (mmol C/mmol C)	0.63	0.24	0.48
Gly_{DEG}/PHA_F (mmol C/mmol C)	0.49	0.32	0.45
Gly_F/PHA_D (mmol C/mmol C)	0.65	0.46	0.23

In the AnOx experiment, this intermediate phase between feast and famine was not observed because the switch from anaerobic to aerobic was forced to be long after the acetate was depleted. Hence, in the aerobic experiments, the ratios were calculated through the initial rates, when acetate was in excess.

A difference is clear between the ratios obtained in the AnOx experiment and the aerobic batch experiments. The ratios obtained in the AnOx experiment agree with the typical literature values for an EBPR system. In the experiments under aerobic conditions less P-release, PHA production and glycogen degradation were observed. Not all of the acetate taken up is metabolised as described in the conventional EBPR process. Part of it is oxidised for energy obtention, which seems a more efficient process than polyP hydrolysis. Hence, only part of the acetate is taken up linked to phosphorus release and glycogen degradation and then the respective ratios (P/C and Gly_{DEG}/Ac_{UPT}) decrease in the aerobic experiments. Some of these facts could be also observed in similar experiments (Brdjanovic *et al*. 1998, Ahn *et al*. 2002, Serafim *et al*. 2002) and also in Chapter VII.

Moreover, some differences can be also detected between EAN and EOX. A higher P/C ratio in aerobic conditions was observed in EOX. Moreover, the glycogen degraded versus acetate taken up or PHA produced was also higher in EOX (Table 8.2). These differences could be attributed to the fact that in EOX more polyP was present in the sludge because it was withdrawn at the end of the aerobic period of the SBR. These results (under aerobic conditions) are in agreement with Hesselmann *et al*. (2000), who described a positive correlation between polyP content and P release under anaerobic conditions. The values obtained in these ratios suggested a higher PAO activity in the EOX experiment, although the ratio of PHA formed per acetate consumed did not change between both aerobic experiments.

In the famine phase of the experiment EAN, more glycogen was produced per PHA degraded. This ratio would seem inconsistent because the amount of glycogen degraded versus PHA formed in this experiment was lower than the value obtained from the same ratio in EOX. Nevertheless, it has to be taken into account that the biomass used in EAN experiment was withdrawn at the end of the anaerobic period of the SBR, where some glycogen had already been degraded to uptake the substrate under anaerobic conditions. Therefore, in the famine phase of the EAN experiment, PAO had to recover the glycogen pools used in the anaerobic period of the SBR plus the glycogen used in the feast phase of the batch experiment. For the other hand, in EOX experiment less glycogen was produced versus PHA taken up, because PAO had just to recover the glycogen pools that they used in the feast phase. The P-uptake ratios are not comparable between the experiments because the phosphorus was totally taken up during the aerobic period of the AnOx experiment or during the famine period in EAN or EOX experiments. Therefore, it is not possible to know the maximum P-uptake of the sludge in these different conditions.

In table 8.3, the values related to the oxygen uptake rate profiles are compared between the two aerobic experiments.

Table 8. 3. Oxygen consumption values obtained in the two batch experiments performed.

	EAN	**EOX**
Initial substrate (mg COD/L)	213.4	213.4
Total oxygen consumed (mg O_2/L)	108	94
Feast oxygen consumed (mg O_2/L)	64	56
Total yield (g $COD_X \cdot g^{-1}COD_S$)	0.49	0.56
Feast yield (g $COD_X \cdot g^{-1}COD_S$)	0.79	0.82

As can be seen, the global yield obtained in both experiments was very close to the ones predicted by ASM3 (Henze *et al*. 2000) for storage of readily biodegradable substrates for heterotrophic biomass [(Y_H=0.63) · (Y_{STO}=0.85) = (Y_{GLOBAL}=0.535)]. The values of the yield obtained considering only the oxygen consumption in the feast phase are lower than the value proposed by ASM3 (0.85). This is not a strange fact and many authors have proposed storage yields lower than 0.85 since it is considered that not all the acetate is uniquely used for storage but also for growth (Guisasola *et al*. submitted).

8.4. CONCLUSIONS

In this work, the response of the same biomass with different levels of intracellular storage compounds to acetate under aerobic conditions was studied. Three different experiments were performed with biomass from an SBR. The first one consisted in a cycle study of the SBR keeping the operational conditions; meanwhile the other two experiments were performed under aerobic conditions and with sludge withdrawn at the end of the anaerobic period of the SBR and at the end of the aerobic period of the same SBR. The main conclusions obtained are:

- When substrate was fed under aerobic conditions, PAO could uptake it in a similar way as under anaerobic conditions: linking this uptake to phosphate release and glycogen degradation. This behaviour was observed despite the different levels of intracellular polymers in EAN or EOX experiments.

- Afterwards, when the substrate was depleted, PAO took up the phosphate from the medium, achieving a net phosphorus removal.

- A higher P/C, Gly_{DEG}/Ac_{UPT} and Gly_{DEG}/PHA_F ratios were observed in the EOX experiment. These differences could be attributed to the fact that more polyP was present in the EOX experiment. These ratios suggest a higher PAO activity in this experiment, although the ratio of PHA formed per acetate consumed does not change between both aerobic experiments.

- The values of the yield obtained considering the oxygen consumption linked to the acetate taken up are in agreement with the typical storage values described in the literature.

8.5. REFERENCES

Ahn J, Daidou T, Tsuneda S, Hirata A. 2002. Transformation of phosphorus and relevant intracellular compounds by a phosphorus accumulating enrichment culture in the presence of both the electron acceptor and electron donor. Biotechnol Bioeng 79:83-93.

Brdjanovic D, Slamet A, van Loosdrecht MCM, Hooijmans CM, Alaerts GJ, Heijnen JJ. 1998. Impact of excessive aeration on biological phosphorous removal from wastewater. Water Res 32:200-208.

Dircks K, Henze M, van Loosdrecht MCM, Mosbaek H, Aspegren H. 2001. Storage and degradation of poly-β-hydroxybutyrate in activated sludge under aerobic conditions. Water Res 35:2277-2285.

Guisasola A, Sin G, Baeza JA, Carrera J, Vanrolleghem PA. 2004. Limitations of ASM1 and ASM3: a comparison based on batch OUR profiles from different full-scale WWTP. Oral presentation. IWA World Water Congress Marrakech 2004.

Hesselmann RPX, VonRummell R, Resnick SM, Hany R, Zehnder AJB. 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res. 34:3487-3494.

Majone M, Dircks K, Beun JJ. 1999 Aerobic storage under dynamic conditions in activated sludge processes. The state of the art. Water Sci Technol 39 (1):61-73.

Serafim LS, Lemos PC, Reis MAM. 2002. Change in the metabolism of PHA accumulation by activated sludge modifying opearating conditions. Water Sci Technol 46(1-2):353-356.

Van Loosdrecht MCM, Pot MA, Heijnen JJ. 1997. Importance of bacterial storage polymers in bioprocesses. Water Sci Technol 35(1):41-47.

CHAPTER IX

NET P-REMOVAL DETERIORATION IN ENRICHED PAO SLUDGE SUBJECTED TO PERMANENT AEROBIC CONDITIONS

This chapter has been submitted as:

Pijuan M, Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. Net P-removal deterioration in enriched PAO sludge subjected to permanent aerobic conditions. Biotechnology and bioengineering.

9.1. INTRODUCTION

It has been suggested that excessive aeration of activated sludge in EBPR systems deteriorates the EBPR efficiency (Brdjanovic *et al*. 1998). This fact can occur in a WWTP in a heavy rain period, and sewerage with large hydraulic gradient may result in a relatively high input of air into the sewer. The DO input to the wastewater can further increase if screw pumps and aerated grit chambers are employed at the WWTP. This means that during rainfall events the anaerobic hydraulic retention time may become temporarily shortened, making the aerobic phase longer than designed for.

The EBPR process is based on the physical separation between the electron donor (organic substrates) and the electron acceptor (oxygen or nitrate). Recent studies have been focused in studying the response of an enriched PAO population when both, the donor and the acceptor coexist (Brdjanovic *et al*. 1998; Ahn *et al*. 2002) and how the feast/famine periods described in the literature (van Loosdrecht *et al*. 1997; Majone *et al*. 1999; Dircks *et al*. 2001) are analogous to the anaerobic/aerobic phases present in a common EBPR system (Chapter VII). PAOs have the ability of uptake acetate under aerobic conditions, linking this consumption to phosphorus release, PHA formation and glycogen degradation. Afterwards, when substrate is depleted, phosphorus is taken up linked with PHA degradation and glycogen synthesis.

The feast phase is described as the period where organic external substrate is still available in the medium and the famine period is the phase where the storage products are consumed.

To study this phenomenon, batch experiments have been performed with EBPR enriched cultures coming from anaerobic/aerobic systems and in these cases EBPR was also observed under strictly aerobic conditions. But it seems clear that PAO will lose its EBPR capacity if the aerobic conditions were maintained for a period of time. Otherwise, EBPR process would be observed in all the WWTP. This study aims to assess for how long a PAO community can keep an EBPR system, when subjected under permanent aerobic conditions. Moreover, microbial community composition as well as changes produced to the compounds analysed (phosphorus, glycogen, PHA) and in the OUR profile along the time were studied.

Hence, there is still much to be learned about EBPR and PAO and the knowledge acquired should be useful in helping to design and run these activated sludge systems better, and in responding to EBPR failure in a rational and scientific manner (Seviour *et al*. 2003). The study presented will contribute to clarify the net biological phosphorus removal phenomenon carried out under permanent aerobic conditions.

9.2. MATERIALS AND METHODS

9.2.1. EXPERIMENTAL DESIGN

A set of 7 cycle studies (named 1-OX to 7-OX) were performed in a SBR working with aerobic conditions with an enriched PAO biomass during 11 days. Acetate, phosphorus, PHA and glycogen were analysed throughout all the experiments and the OUR was measured. FISH analyses were performed at the beginning (1-OX) and at the end of the study (7-OX).

9.2.2. REACTOR

Experiments were conducted in a SBR with a working volume of 10 L, inoculated with activated sludge from the end of the aerobic period of an EBPR- SBR which had been working during 150 days with anaerobic/aerobic alternating conditions. Each cycle in the aerobic SBR consisted of 5.5 h of aerobic react, 25 min of settling and, in the last 5 min, extraction of 5 L of supernatant. It was operated in 4 cycles of 6 h per day. A volume of 5 L of synthetic wastewater was added during the first 5 minutes of the subsequent cycle, producing a HRT of 12 hours. The SRT was kept at 9 d by periodic sludge wastage at the end of the react phase. The TSS concentration along the experiments was around 2200 mg $\cdot L^{-1}$ with a VSS/TSS ratio of 0.64 at the end of the aerobic phase. The pH was controlled at 7.0 ± 0.1 with 1M HCl and the temperature was kept at 25ºC.

The DO was higher than 2 mg L^{-1} throughout the experiments to avoid oxygen limitations. DO and pH were measured in the cycle studies with and InoLab pH/Oxi Level 3 (WTW, Weilheim, Germany). A detail of the experimental set-up is showed in figure 9.1.

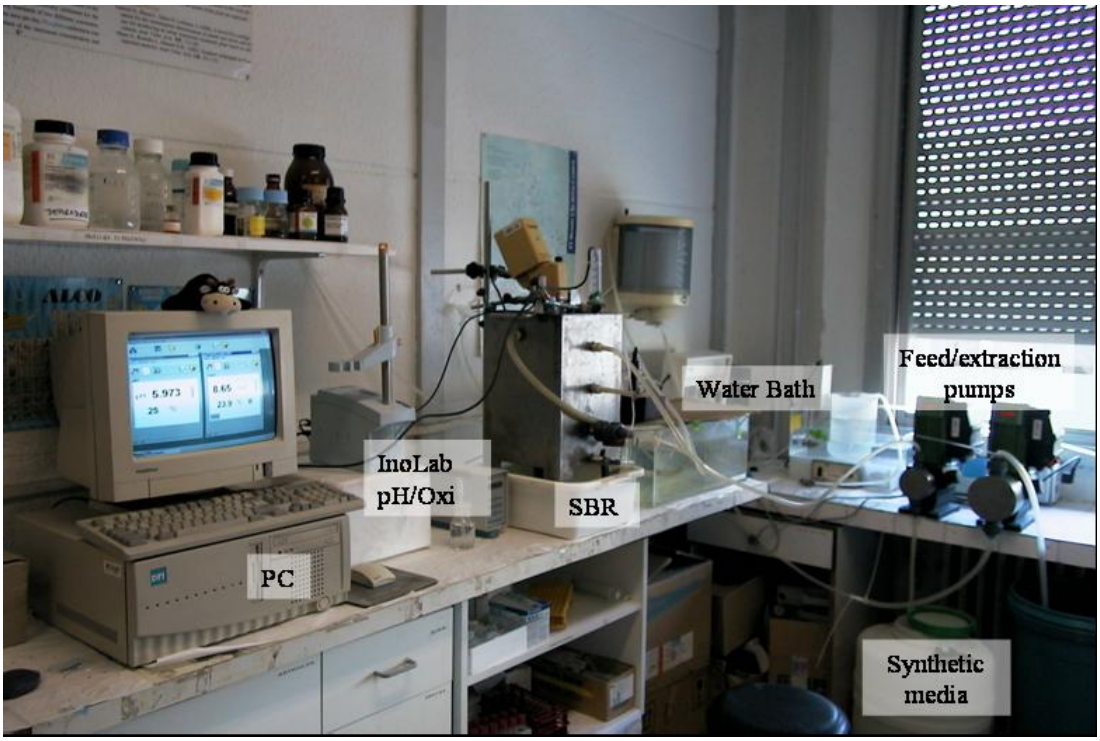

Figure 9.1. Experimental SBR setup where this study was performed.

9.2.3. SYNTHETIC MEDIA

Two separate solutions called "concentrated feed" (constituting 0.25 L per 5 L synthetic wastewater) and "P-water" (constituting 4.75 L per 5 L synthetic wastewater) collectively formed the synthetic wastewater used in this study. The composition of this synthetic wastewater can be found in Chapter IV, but no peptone was added. Acetate was used as carbon source and the working concentration at the beginning of the feast phase in the reactor was 250 mg/L acetate.

9.3. RESULTS

Different cycle studies were carried out and most significant compounds of the system (phosphorus, acetate, PHA, glycogen) as well as OUR were analysed. Although the profiles of most of these compounds changed, these changes could not be attributed to a modification in the composition of the biomass because FISH performed at the beginning and at the end of the period studied demonstrated that the microbial community was approximately the same. The percentage of PAO-MIX binding cells (*Accumulibacter*, presumed to be the predominant PAO) was found in

the first day and after 11 days in aerobic conditions (around 50 %), although more than one SRT passed. This fact clearly indicates that PAO could survive under strictly aerobic conditions and moreover, they were able to compete with the other bacteria present in this sludge during more than one SRT under aerobic conditions. The micrographs in figure 9.2 visually show this similarity in microbial population.

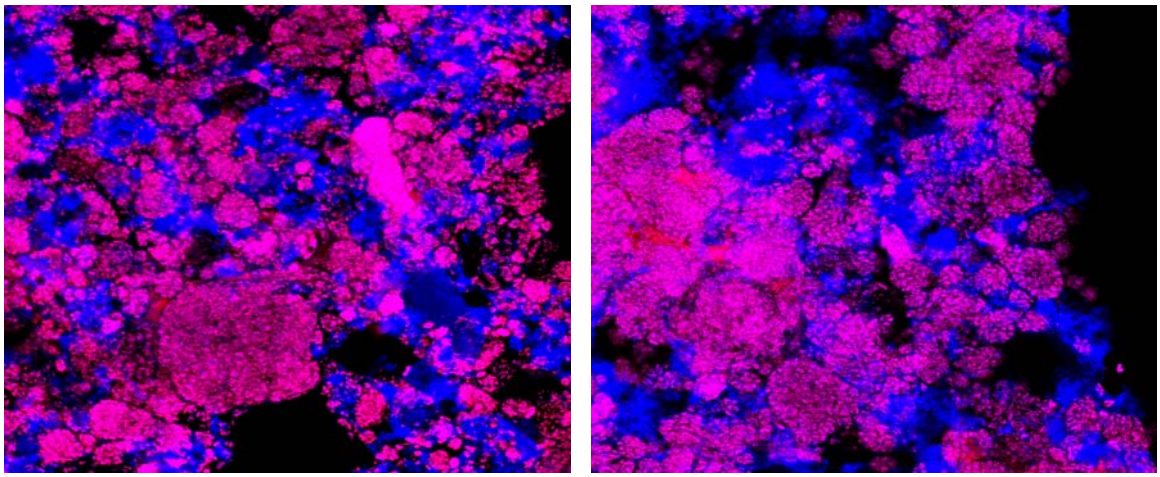

Figure 9.2. CLSM micrographs of FISH of the EBPR sludge hybridized with probe specific for PAO (In pink, Cy3-labelled PAOMIX probes) and probe for bacteria (In blue, Cy5-labelled EUBMIX probes). a) First day of reactor operation under aerobic conditions. b) After 11 days of reactor operation under aerobic conditions.

Although the microbial community kept the same composition along this study, the profiles of the compounds measured in the cycle studies during these 11 days suffered some changes. Nevertheless, the first cycle study performed (experiment 1-OX) had the same response as described before in Chapter VII. Two phases could be distinguished that are commonly known as feast/famine phases. In the feast phase, acetate was consumed linked to phosphorus release and glycogen degradation (characteristic of PAO behaviour) and also linked to PHA storage and oxygen consumption. When acetate was depleted, famine phase started. In this phase PHA was degraded related to glycogen production and phosphorus uptake (characteristic of PAO) and oxygen consumption. These two phases are analogous to the anaerobic/aerobic periods in a common EBPR system where the oxygen is the switch factor whereas the presence of external substrate is the switch factor in the feast/famine systems.

Figure 9.3 shows the trends of the different compounds analysed in the cycle study 1-OX. The highest oxygen consumption was detected in the feast phase, when acetate was still present in the media. When phosphorus was completely depleted from the system, there was a slightly decrease in the OUR (around minute 140). It occurred

because the phosphorus uptake process is linked to oxygen consumption. When phosphorus was depleted from the media, no more polyphosphate could be produced; therefore the oxygen consumption was due to PHA degradation to produce glycogen and biomass growth. Net phosphorus removal was achieved in the system.

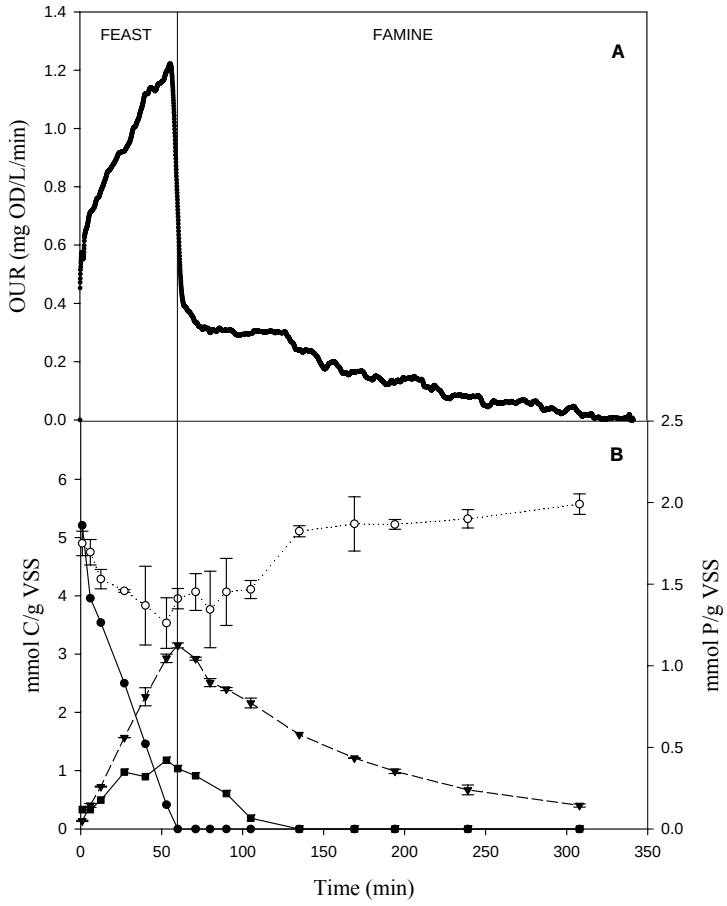

Figure 9.3. First cycle study (OX-1) performed in the SBR working with aerobic conditions with a biomass enriched in PAO withdrawn from an ANA/OX SBR reactor. A.-Experimental OUR profile. B.-Experimental profiles of different compounds: (●) Acetate, (■) Phosphorus, (○) Glycogen, (▼) PHB.

The last cycle study carried out in the reactor after 11 days of operation (7-OX) showed some differences respect to the first one (Figure 9.4). Acetate was completely consumed resulting in a similar consumption rate and close PHB amount was produced. Nevertheless, the level of glycogen present in biomass was significantly lower than in cycle 1-OX and the glycogen degraded in the feast phase and the glycogen produced in the famine phase was reduced respect to cycle 1-OX. The amount of phosphorus released in the medium in the feast phase was higher and less was taken up in the famine phase, resulting in a higher amount of phosphorus present in the medium at the end of the cycle than at the beginning. The OUR profile was similar in both cycle studies. In 7-OX cycle study no different trends can be

observed in the famine phase probably because there was phosphorus in the medium during all the cycle and its uptake was slower than in OX-1.

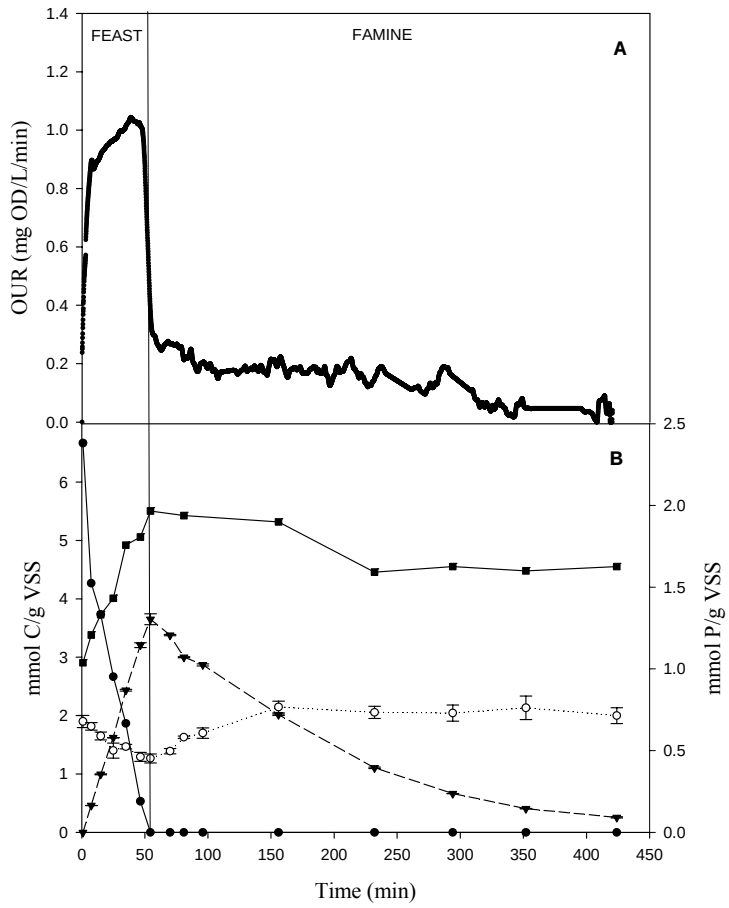

Figure 9.4. Cycle study in the SBR performed after 11 days (OX-7) of operation under permanent aerobic conditions. A.- Experimental OUR profile. B.-Experimental profiles of different compounds: (●) Acetate, (■) Phosphorus, (○) Glycogen, (▼) PHB.

Table 9.1 summarise the values obtained as a difference between beginning and end of feast/famine phases in all the cycle studies performed. In table 9.2 the ratios between the values are presented.

Table 9.1. Values obtained in the cycle studies performed in this study.

mmol C or P/g VSS	**1-OX (day 1)**	**2-OX (day 2)**	**3-OX (day 3)**	**4-OX (day 4)**	**5-OX (day 7)**	**6-OX (day 8)**	**7-OX (day 11)**
Acetate uptake	5.21	5.41	5.95	6.20	6.05	6.38	6.67
P-Release	0.30	0.39	0.31	0.51	0.41	0.68	0.93
P-Uptake	0.42	0.89	0.57	0.58	0.27	0.42	0.34
Glyc-degradation	1.57	1.40	0.99	0.87	0.76	0.75	0.63
Glyc-formation	2.04	1.24	0.87	0.79	0.71	0.65	0.73
PHA-formation	3.01	2.68	3.19	3.12	2.95	3.26	3.65
PHA-degradation	2.75	2.10	3.10	2.81	2.75	3.18	3.39

Table 9.2. Ratios obtained in the experiments performed in this study.

mmol C or P/ mmol C or P	1-OX (day 1)	2-OX (day 2)	3-OX (day 3)	4-OX (day 4)	5-OX (day 7)	6-OX (day 8)	7-OX (day 11)
P-rel / C-upt	0.06	0.07	0.05	0.08	0.07	0.11	0.14
Glyc-d / C-upt	0.30	0.26	0.17	0.14	0.12	0.12	0.09
PHA-f / C-upt	0.58	0.49	0.54	0.50	0.49	0.51	0.55
P-upt / P-rel	1.40	2.28	1.84	1.13	0.66	0.62	0.36
P-upt / PHA-d	0.15	0.42	0.18	0.21	0.10	0.13	0.10
Glyc-f / PHB-d	0.74	0.59	0.28	0.28	0.26	0.20	0.21

Glycogen was the component that seemed to be more affected because of the oxygen presence. Comparing the first cycle (1-OX) study with the last one (7-OX), a decrease in the glycogen degradation as well as a decrease in the glycogen formation can be observed (Table 9.1). This diminution in the glycogen is reflected also in the glycogen ratios (Table 9.2): a progressive reduction in the glycogen degradation versus acetate uptake ratio is observed along the study. This decrease suggests that the enriched PAO biomass could synthetise PHA from acetate without using as much glycogen as they did in classical anaerobic conditions. Similar amount of PHA was produced for C-uptake along the study. Consequently, glycogen formation from PHA was decreasing throughout the study resulting in a diminution in the absolute value of glycogen present in the biomass (Figures 9.3 and 9.4). Figure 9.5 reflects the progressive reduction of the glycogen content in the biomass at the end of the famine phase, as well as the decrease in the glycogen degradation and formation values.

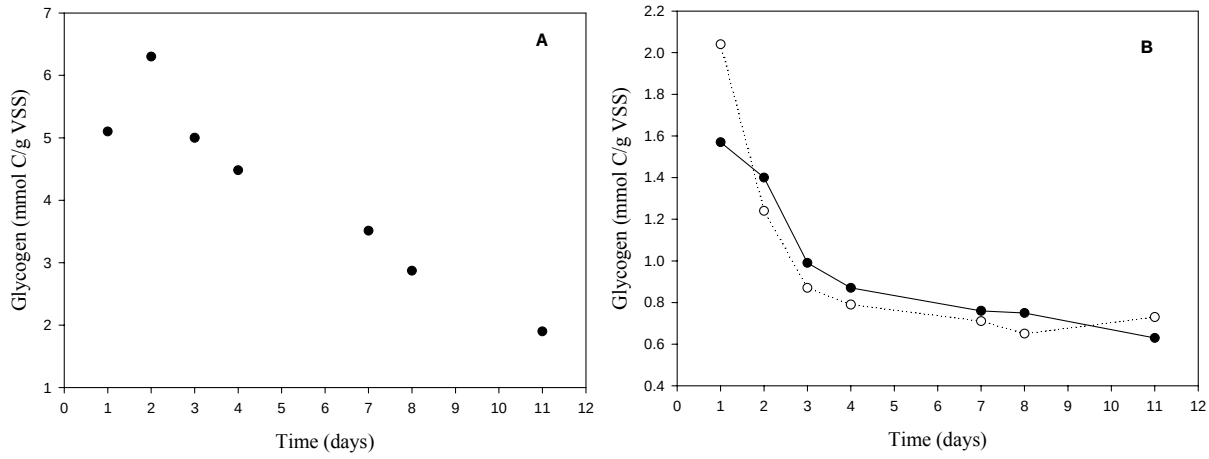

Figure 9.5. A.- Total amount of glycogen present in the biomass at the end of the famine phase in the SBR working with permanent aerobic conditions. B.- Glycogen degradation (●) in the feast period and glycogen formation (○) in the famine period.

Although the role of glycogen under aerobic conditions is less important than under anaerobic conditions, the values obtained in the experiments show that a slightly higher value of glycogen was degraded than synthetised and hence, the glycogen pools in the biomass were decreasing along the study. This fact may be attributed to a minium requirement of glycogen in PAO.

The other compound more linked to PAO behaviour is phosphorus (Table 9.2). A progressive decrease was observed in the P-uptake versus P-release ratio (Table 9.2 and figure 9.6A). The increase in the P-release value affected to net phosphorus removal. Net phosphorus removal was achieved in the system until day 4, while this ratio was higher than 1. In day 7 this ratio became lower and decreased progressively until day 11, resulting in an increase of phosphorus in the effluent of the reactor. In figure 9.6B are represented total P-release and total P-uptake in the cycles studies performed throughout all this study. A progressive increase in the absolute value of P-release was observed in parallel to a decrease in the total phosphorus uptake.

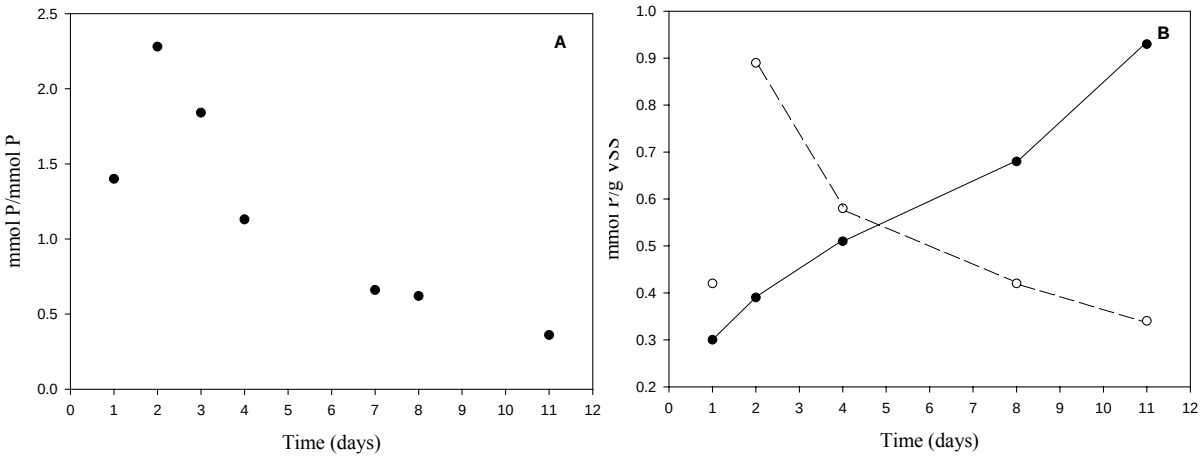

Figure 9.6. A- P-uptake versus P-release ratio along the study. B.- P-release (●) and uptake (○) along the study.

The first value of P-uptake versus P-release ratio (Figure 9.6A) and the first P-uptake value (Figure 9.6B) were lower than the others because the phosphorus was completely depleted from the system and probably if it had been more in the media, the uptake would have been higher.

PHA production remains constant along the study as can be seen in the ratio of PHA production versus acetate consumption ratio (Table 9.2). This fact agrees with Serafim *et al*. (2000) who observed similar production of PHA in an EBPR system submitted to aerobic conditions for a few days. Under classical anaerobic conditions, glycogen degradation contributes to the PHA formation. However in this work the amount of PHA produced seems not to be affected by the amount of glycogen

degraded in the aerobic system. Although less glycogen was degraded in the last experiment (7-OX) than in the first one (1-OX), a very similar amount of PHA was synthetised.

9.3.1. OUR PROFILES

OUR was followed in some cycles along the study. Differences in the OUR shapes were observed although the total oxygen consumption was almost the same (Figure 9.7 and Table 9.3). The highest oxygen consumption rate was observed in the feast period, when acetate was still present in the media. In this phase, the oxygen was consumed because of the oxidation of part of the acetate to CO_2 and the storage of part of it as PHB. Nevertheless, the OUR profile in the feast phase differed from a typical OUR feast profile (i.e. a rectangular peak). In this study, the OUR in the feast phase increased progressively, resulting in a sharp pointed peak in the OUR profile.

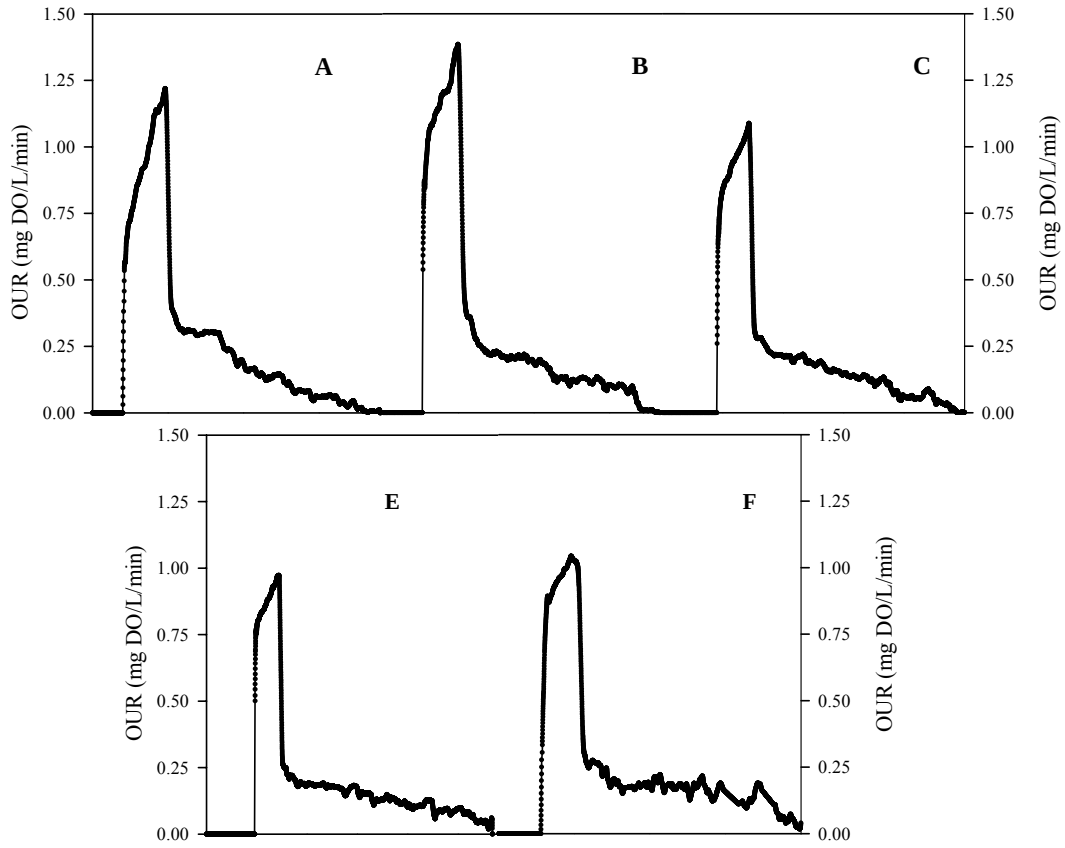

Figure 9.7. OUR profiles obtained in five cycle studies performed along this study.

This sharp pointed OUR peak in presence of external substrate is usually explained when biomass growth or substrate inhibition occurs. However, none of these phenomena happened. Ammonia was measured in the experiments and no noticeable consumption was detected in the feast period, therefore, no important growth was

expected. Moreover, the acetate consumption rate remains constant along the study and no inhibition for acetate has been described in the literature at this working concentration (Randall *et al*. 1997). Also, a decrease in the slope of the OUR peak along the experiments was observed (Figure 9.7).

In the famine phase of the cycle study OX-1, two different shoulders in the OUR profile can be observed (figure 9.3). Lower OUR was detected after the depletion of phosphate in minute 140. This change in the OUR was due to the PHA degradation needed for the P-uptake process. This process requires oxygen and when phosphorus is completely exhausted, the oxygen profile diminishes (Brdjanovic *et al*. 1998; also described in Chapter VII). This can be only observed in the first experiment, where the phosphorus was completely taken up in the famine phase. In all the other cycle studies, phosphorus was not exhausted, so this change in the OUR profile can not be observed.

Total oxygen consumption, corresponding to the area under the OUR profile, was similar for all the cycle studies performed. Table 9.3 summarises the values obtained for the OUR in the different experiments as well as the oxygen consumption in the feast and in the famine periods.

Table 9.3. Oxygen consumption values obtained in different cycles studies performed along the study.

	OXYGEN CONSUMPTION (mg O_2/L)				
	1-OX (day 1)	**2-OX (day 3)**	**3-OX (day 3)**	**4-OX (day 4)**	**7-OX (day 11)**
Feast period	59	62	56	47	49
Famine period	40	38	45	54	49
Entire cycle	99	100	101	101	98

9.4. DISCUSSSION

This study shows that PAOs remained in the sludge with a similar percentage respect the total amount of bacteria after 11 days of working under aerobic conditions. This fact suggests that these microorganisms were competitive with the other bacteria present in this sludge under unusual conditions for them. However, the system progressively lost the net phosphorus removal capacity. The oxygen presence throughout all the cycle involved an important change in the profiles of compounds unequivocally linked to PAO metabolism (glycogen and phosphorus), meanwhile other parameters remained almost constant (acetate consumption, PHA production and degradation). The significant decrease in the total glycogen amount in the sludge suggested that PAO under aerobic conditions did not require as much glycogen as in

anaerobic conditions. Pereira *et al*. (1996) demonstrated that under anaerobic conditions PAO degraded part of the acetate through the TCA cycle to obtain also some reducing power. Nevertheless, under anaerobic conditions just a small part of the substrate is derived to this cycle because it seems not to be favoured. But with acetate under aerobic conditions, PAO's tendency is to activate the most efficient (in terms of energy) metabolic pathway, the TCA cycle. Only in that way, PAOs could compete with the other bacteria present in the sludge, probably OHOs. Therefore, PAOs tend to reduce the amount of glycogen stored because in presence of oxygen seems not to be as useful as before.

A progressive increase in the P-release versus C-uptake ratio is observed along the study. Under anaerobic conditions, PAOs use its polyphosphate pools to obtain energy resulting in a release of phosphorus in the media. With oxygen, the energy would be supplied by the oxidation of acetate through the TCA cycle in OHOs. If it supposed that PAOs tended to behave as OHOs, then a logic PAO behaviour would be to reduce the P-release along the study.

However, PAOs are different than OHOs and even accepting that they can get energy in the TCA cycle, the P-release versus C-uptake ratio increased along the study. This increase can be explained jointly with the OUR profile.

The slope in the sharp pointed peak in the OUR profile diminished along the study. This phenomenon could be explained if some processes attributed only to the famine phase (P-uptake, glycogen production, PHA degradation) started in the feast phase. The oxygen consumption due to famine processes can be observed when the acetate is depleted. If some of them started although acetate was not exhausted yet, the slope of the sharp pointed peak in the OUR profile would increase as was observed in figure 9.7. Nevertheless, this hypothesis can not be confirmed by the experimental measurements of the different compounds because only the sum of the processes is observed. Figure 9.8A shows a schematic representation of a common OUR profile obtained with OHO when acetate is added (a classical rectangular peak). When this substrate is added to an EBPR biomass under aerobic conditions, the OUR profile obtained is shown in Figure 9.8B. As it was suggested before, the sharp pointed peak in the OUR could be due to the famine processes which would start in the feast phase.

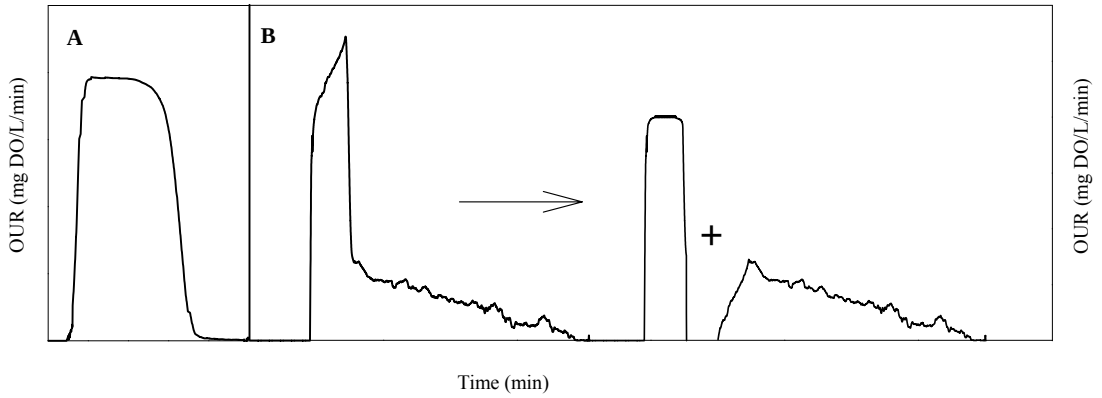

Figure 9.8. Schematic representation of the explanation of the OUR profile obtained along the study. A- Common OHO OUR profile. B- OUR profile obtained with an EBPR biomass feed with acetate under aerobic conditions.

This slope in the sharp pointed peak in the OUR decreased progressively along the study, suggesting that less oxygen consumption related to the famine processes was occurring simultaneously in the feast period. This could explain the increase in the P-release versus C-uptake ratio. In the experiment 1-OX, phosphorus release process was probably occurring simultaneously to P-uptake but it could not be detected in the phosphorus analysis because P-release was faster than P-uptake and hided this last process. Nevertheless, the ability of uptake phosphorus in parallel with P-release was diminishing along the study, so in the last experiment (7-OX) the effect of the P-uptake was not as important as in the first experiment, resulting in an increase in the P-release versus C-uptake ratio.

Moreover, this increase in the P-release versus C-uptake ratio can also be attributed to energy requirements. Under anaerobic conditions, glycogen degradation supplies part of the energy necessary to uptake acetate and store it as PHA, so it is probable that glycogen degradation under aerobic conditions imply a production of energy. But this energy would be reduced because less glycogen was degraded throughout the study. To compensate the lost of energy in this process, PAO could tend to release more phosphorus obtaining more energy in this process.

9.5. CONCLUSIONS

An enriched PAO population was submitted under aerobic conditions during more than one SRT (11 days) and the main conclusions obtained after this study are:

- Net phosphorus removal was achieved in the system until experiment 4-OX, after 4 days of operation under strictly aerobic conditions.

- The feast/famine periods observed in the first cycles studies (1-OX to 4-OX, from day 1 until day 4) performed in strictly aerobic conditions presented an analogous behaviour than a common anaerobic/aerobic EBPR system. Phosphorus release linked to glycogen degradation and PHA formation was detected when acetate was consumed in the feast period. In the famine phase, phosphate was taken up from the media and glycogen formation as well as PHA degradation could be observed. A progressive decrease in the amount of glycogen in the biomass as well as in the ratios of glycogen degradation and formation was observed along the study. This circumstance suggests that PAO tend to deplete its internal glycogen which in aerobic conditions is less useful than in anaerobic conditions.

- Similar percentage of PAOs respect the total amount of bacteria was present in the sludge at the beginning of the study and at the end, after working 11 days in aerobic cycles.

- The maximum OUR profile suggested that some famine process would occur simultaneously to the feast process. This would affect to the phosphorus release ratio which was lower in 1-OX than in 7-OX where the simultaneous P-uptake would be less important.

- PHA production ratio was maintained constant throughout the study. It suggests that the glycogen contribution in the PHA production was less significant under aerobic conditions than in a common EBPR system, with alternating anaerobic/aerobic conditions.

9.6. REFERENCES

Ahn J, Daidou T, Tsuneda S, Hirata A. 2002. Transformation of phosphorus and relevant intracellular compounds by a phosphorus accumulating enrichment culture in the presence of both the electron acceptor and electron donor. Biotechnol Bioeng 79:83-93.

Brdjanovic D, Slamet A, van Loosdrecht MCM, Hooijmans CM, Alaerts GJ, Heijnen JJ. 1998. Impact of excessive aeration on biological phosphorus removal from wastewater. Water Res 32:200-208.

Dircks K, Henze M, van Loosdrecht MCM, Mosbaek H, Aspegren H. 2001. Storage and degradation of poly-β-hydroxybutyrate in activated sludge under aerobic conditions. Water Res 35:2277-2285.

Majone M, Dircks K, Beun JJ. 1999 Aerobic storage under dynamic conditions in activated sludge processes. The state of the art. Water Sci Technol 39 (1):61-73.

Randall CW, Chapin RW. 1997. Acetic acid inhibition of biological phosphorus removal. Water Environm Res 69(5):955-960.

Serafim LS, Lemos PC, Reis MAM. 2002. Change in the metabolism of PHA accumulation by activated sludge modifying opearating conditions. Water Sci Technol 46(1-2):353-356.

Seviour R.J, Mino T, Onuki M. 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol Rev 27:99-127.

Van Loosdrecht MCM, Pot MA, Heijnen JJ. 1997. Importance of bacterial storage polymers in bioprocesses. Water Sci Technol 35(1):41-47.

CHAPTER X

CONCLUSIONS AND FUTURE WORK

10.1. CONCLUSIONS

This PhD thesis has been focused in two aspects of the EBPR process.

The effect of different substrates in the EBPR process was studied and the results were presented in Chapters IV, V and VI. The main conclusions obtained in this topic are summarised next.

- Propionate was a good substrate to develop an EBPR system. With this substrate seemed that GAOs (*Competibacter*) were unable to compete with PAOs. This was observed in the experiments presented in Chapter IV. To study in depth the effect of the carbon source in the evolution of the EBPR systems, some experiments were carried out. The results are showed in Chapter V. Two SBRs were inoculated with sludge withdrawn from the same WWTP. One was working with acetate and the other with propionate. The Propionate-SBR presented a better net P-removal capacity in the system than the other reactor. The microbial analyses (FISH) demonstrated that a higher amount of PAOs were present in the Propionate-SBR sludge and almost no GAOs were detected. On the contrary, the Acetate-SBR presented a few amount of PAOs and also presented a similar amount of GAOs which negatively affected the net phosphorus removal of the system.
- PHA composition was strongly influenced by the carbon source. Acetate was mainly stored as PHB and propionate was mostly stored as PHV although in some experiments performed with propionate, PH2MV was also present in a similar amount as PHV. When butyrate was used, another monomer unit was detected but could not be quantified.
- The response of two full scale sludges to acetate and propionate was studied. The results obtained were similar to the values described in the literature when these substrates were tested with enriched cultures. The main difference was that slightly less glycogen was degraded with full scale sludge than the amount measured with enriched cultures.
- The amount of total PHA production in full scale sludge was close to the values observed in the literature with enriched cultures. Nevertheless, the PHA composition seems to strongly depend on the history of the sludge (types of substrate, concentration that usually receive). Although general trends are observed in all the cases depending on the substrate used, the percentages of the different monomers were difficult to predict and differ from experiment to experiment.

Part of this thesis is focused on the effect of continuous aerobic conditions on the EBPR process. Chapter VII, VIII and IX described the results obtained about this topic. The main conclusions obtained are summarised next.

- Under aerobic conditions, an EBPR microbial community can take up acetate linked with phosphorus release, PHA storage and glycogen degradation. An analogous response of the results obtained in the feast/famine process and the results obtained in the anaerobic/aerobic process of an EBPR microbial community was found. Two different hypotheses were formulated and simulated to describe this aerobic acetate consumption linked to PHA production. The PAO-hypothesis considered that PAO stored acetate as PHB linked to P-release and glycogen degradation. The OHO-hypothesis considered that PAO stored acetate as PHB linked to P-release and glycogen degradation, and that OHO were the only responsible for the additional acetate consumption and PHB storage. Nevertheless, none of them could be completely rejected.

- The effect of intracellular storage polymers in the aerobic acetate uptake by an EBPR microbial community was assessed. Two experiments using the same amount of acetate were performed with EBPR biomass withdrawn from the end of the anaerobic period of a SBR and from the end of the aerobic period. A higher P-release *vs* C-uptake and glycogen degradation *vs* PHA formation ratios were observed in the experiment performed with biomass from the end of the aerobic period of the SBR. These ratios suggested a higher PAOs activity in this experiment, although the ratio of PHA produced per acetate consumed did not change between both aerobic experiments.

- Some experiments were performed using an enriched PAO population submitted under aerobic conditions during more than one SRT (11 days). Net phosphorus removal was achieved in the system at the beginning of the study, but after 4 days of operation under aerobic conditions, net phosphorus removal was not achieved anymore. Although the system lost its phosphorus removal capacity, a similar percentage of PAOs respect the total amount of bacteria was present in the sludge at the beginning of the study and at the end, after working 11 days in aerobic cycles.

- The sharp pointed peak in the OUR profile obtained in these experiments suggested that some famine processes occurred simultaneously to the feast process.

10.2. FUTURE WORK

This PhD dissertation presents the first results obtained in a new research line. There is still a lot of work to be done in this field in order to understand the EBPR process and the characteristics of the bacteria involved on it. At this point and with the knowledge acquired along this PhD thesis, I would suggest continue this work with studying in depth the different PHA monomers produced when different substrates are added. It would be interesting to know the effect of the composition of the different PHA monomers in the net phosphorus removal. It seems a key aspect in the EBPR systems. This knowledge will contribute to clarify the response of EBPR systems to real wastewater, where the composition and concentration of different substrates can be variable along the time.

Although in this PhD thesis no studies have been performed with an enriched GAO population, it is clear the need in studying the metabolism of these microorganisms. It has to be taken into account that most of the EBPR systems failures are consequence of the proliferation of these microorganisms. It is not clear yet which kind of microorganisms can behave as a GAO and how the competition between PAOs and GAOs work.

More research is also needed in the net phosphorus removal ability of EBPR microbial population subjected to continuous aerobic conditions and in the metabolic pathways that PAOs use to consume acetate under aerobic conditions. It should be assess also if EBPR sludge can recover the phosphorus uptake capacity after a long period with aerobic cycles.