ESCOLA D'ENGINYERIA

DEPARTAMENT D'ENGINYERIA QUÍMICA

FACING CURRENT EBPR BOTTLENECKS IN VIEW OF FULL-SCALE IMPLEMENTATION

Memòria per obtenir el Grau de Doctor per

la Universitat Autònoma de Barcelona

sota la direcció de

Juan Antonio Baeza i Albert Guisasola

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El Dr. JUAN ANTONIO BAEZA LABAT, professor agregat, i el Dr. ALBERT GUISASOLA I CANUDAS, professor lector, del Departament d'Enginyeria Química de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que l'enginyera química CARLOTA TAYÀ CRISTELLYS ha realitzat sota la nostra direcció, el treball que amb títol "Facing current EBPR bottlenecks in view of full-scale implementation", es presenta en aquesta memòria, i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I per a què se'n prengui coneixement i consti als afectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a

Bellaterra, 29 d'abril de 2013

Dr. Juan Antonio Baeza Labat

Dr. Albert Guisasola i Canudas

Pels meus pares en Toni i la Paloma,
el meu germà en Jan
i pels meus,
els que hi van ser i els que hi són...

AGRAÏMENTS

Durant aquest anys de tesi sempre he volgut escriure aquesta part per fer un recordatori de tota aquella gent que m'ha ajudat, tant intel·lectualment com anímicament, en la realització d'aquesta tesi.

Primerament vull agrair als meus directors de tesi, en Juan i l'Albert, que han estat uns models a seguir. La seva direcció ha sigut excel·lent. Tot i l'esforç a nivell d'investigació i els mals moments a l'hora d'obtenir bons resultats, han fet que tot resultés molt més senzill. Amb ells he aprés moltíssimes coses però la més important és que la feina ben feta no té fronteres i per sort, sí que té futur.

En segon lloc i no menys important vull agrair de tot cor al meu company de P-Team. En Javi no tan sols m'ha ajudat moltíssim en aquesta tesi a nivell d'investigació sinó que m'ha recolzat en tot moment. Amb tu Javi, he passat uns moments increïbles, ja sigui al laboratori com fora de congrés o prenent una birra. Ets el millor company de P-Team que hagués pogut trobar!!!

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He passat molt bones estones amb els meus companys de despatx, l'Edu, en Miki, en Marc i la Kristin (que ara són un pack) i al final de la tesi amb la Marcel·la. Tots ells no només m'han ensenyat la duresa de la vida de l'investigador i ens hem explicat les penes típiques de la recerca sinó que hem fet un munt d'activitats extraescolars... M'enduc uns amics imprescindibles per totes aquelles activitats ocio-culturals, teatre, cates, concerts...

Dins d'aquests grups hi ha els *Rollings*, aquells que quan vaig arribar ja eren els veterans del departament i als que ara ho som. Tardes de selecta, microcerveseria, sushistime, divendres tontos al departament, sumertimes, esquiades i per tot el que calgui sempre n'estan disposats...

Amb *Vichys*, *despatxils* i *rollings* m'emporto uns grans amics!!!!

Vull agrair a tots els companys i amics del departament que m'han ajudat a arribar a bon port, *aldolaseros*, *lipaseros*, *compostatgeros*, *maperos* i *tòxics*...

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RESUM

El tractament biològic d'aigües residuals es considera des de fa dècades la manera més sostenible, i econòmicament viable, per a depurar l'aigua contaminada. L'eliminació biològica de nitrogen ha estat àmpliament estudiada i s'han proposat diverses estratègies i processos per a implementar-la en les estacions depuradores d'aigües residuals (EDARs) tan urbanes com industrials. De la mateixa manera, també s'han estudiat els processos d'eliminació biològica de fòsfor (EBPR) en aigües residuals, però la seva implementació a escala real ve associada moltes vegades a fallades no esperades. A més a més, en molts casos s'han detectat interaccions negatives amb el procés d'eliminació de nitrogen i matèria orgànica que no s'havien previst en l'estudi dels mateixos de manera individual.

Aquesta tesi aprofundeix en la caracterització, l'estudi i la solució de problemes existents a l'hora d'implementar l'eliminació biològica de fòsfor en aigües residuals conjuntament amb l'eliminació biològica de matèria orgànica i nitrogen.

La investigació duta a terme en aquesta tesi té dos enfocaments diferents dins d'aquest marc. D'una banda s'estudien les causes de la interacció negativa entre els processos d'eliminació biològica de nitrogen i de fòsfor. D'altra banda s'estudia la possibilitat d'utilitzar, per part dels organismes acumuladors de fòsfor (PAO), fonts de carboni alternatives, que també s'utilitzen en el procés d'eliminació de nitrogen.

En el primer lloc, s'estudia una nova estratègia per l'eliminació de nutrients (nitrogen i fòsfor) que consisteix en la bioaugmentació de microorganismes PAO dins d'un sistema de nitrificació i desnitrificació. Així doncs, un SBR anòxic-aerobi, on coexisteixen els microorganismes encarregats de dur a terme l'eliminació de nitrogen, es bioaugmenta amb microorganismes PAO per a poder eliminar simultàniament ambdós nutrients. Es va comprovar que una configuració de SBR amb una fase anòxica amb dues alimentacions i una fase aeròbica permet aconseguir nitrificació, desnitrificació i EBPR. Un punt clau per l'èxit d'aquesta estratègia és proporcionar condicions operacionals adequades per evitar els problemes de "rising" (flotació de fangs en la fase de sedimentació degut al N_2 generat en la desnitrificació). Per evitar-ho, es requereix una fase aeròbia amb la suficient durada per assegurar el consum del PHA acumulat pels PAO, el que evita la disponibilitat de font de carboni requerida per la desnitrificació.

En aquest mateix context també s'estudia la interacció de diferents espècies intermèdies del procés d'eliminació de nitrogen, com són el nitrit i el nitrat, en el procés EBPR. Segons la bibliografia, la coexistència d'un donador d'electrons (matèria orgànica) i un acceptor d'electrons (nitrit o nitrat) en la fase teòricament anaeròbia és perjudicial pels microorganismes PAO i, per tant, pel procés EBPR. Per aquest motiu també es realitzen uns estudis en discontinu per veure aquesta afectació amb diferents concentracions de nitrat i utilitzant diferents fonts de carboni. Amb aquest estudi es pot concloure que productes intermitjos derivats del procés d'eliminació de

nitrogen, com és el cas del nitrit, poden afectar al procés EBPR quan els microorganismes PAO no han estat aclimatats prèviament a aquestes condicions.

D'altra banda, s'ha estudiat un dels principals problemes per a implementar a nivell real l'EBPR, la manca d'àcids grassos volàtils a l'aigua residual d'entrada al procés. És per això que, en segon lloc, s'han estudiat diverses estratègies per a la utilització de fonts de carboni diferents a les convencionals en el procés EBPR. S'han provat fonts de carboni que actualment s'utilitzen en el procés d'eliminació de nitrogen, com són el metanol i el glicerol, i que, per tant, abaratirien els costos quan ens trobem aigües residuals amb poca DQO disponible per dur a terme l'eliminació simultània de nitrogen i fòsfor. Les estratègies que es proven també són dues: la primera és la substitució directa de la font de carboni convencional (àcid propiònic) per la font de carboni en qüestió dins d'un sistema enriquit en microorganismes PAO. La segona estratègia, més innovadora, és la formació d'un consorci microbià amb llots de digestió anaeròbia, prèviament seleccionat, amb microorganismes PAO on els primers seran capaços de fermentar la font de carboni més complexa cap a àcids grassos volàtils de cadena curta (àcid acètic i propiònic) que seran utilitzats pels PAO per dur a terme el procés EBPR. Per a aconseguir un bons resultats s'han dissenyat estratègies de selecció per a obtenir el consorci microbià desitjat i s'han hagut de modificar les configuracions inicials dels reactors.

Per complementar aquest darrer estudi també s'ha assajat amb diferents fonts de carboni cadascun dels consorcis amb els que s'han obtingut millors resultats. D'aquesta manera es pot arribar a conèixer l'aplicabilitat d'aquestes estratègies a l'hora de treballar amb diferents tipus d'aigües residuals.

En el darrer capítol de la tesi, s'estudia una nova estratègia per a aconseguir uns llots enriquits majoritàriament amb microorganismes PAO. Aquest procediment permet eliminar del sistema aquells microorganismes competidors (GAO) que capten la font de carboni que hi ha disponible pels PAO i perjudiquen al procés EBPR. Per aquesta estratègia s'ha treballat amb un llot enriquit amb aquests competidors i s'ha canviat la configuració del reactor, que ha passat d'una configuració anaeròbia/aeròbia a una configuració anaeròbia/anòxica amb addició de nitrit. Els resultats demostren que la combinació d'àcid propiònic en la fase anaeròbia i l'addició de nitrit en la fase anòxica afavoreix la competició dels microorganismes PAO en front dels GAO inclús en les condicions on la composició de l'aliment més desfavoreix als PAO. L'aplicació d'aquesta estratègia va resultar en un llot amb un 85% de PAO i en el rentat dels GAO del sistema.

ABSTRACT

Biological nutrient removal is considered the most economical and sustainable technology to meet the increasingly stricter discharge requirements in wastewater treatment. Biological nitrogen removal has been extensively studied and several strategies based on such approach have been successfully applied in wastewater treatment plants (WWTPs) dealing with both urban and industrial effluents. In the same way, enhanced biological phosphorus removal (EBPR) has been studied, but its implementation at full-scale is still associated to unpredictable failures. Furthermore, when EBPR is implemented simultaneously to nitrogen and organic matter removal, some negative interactions have been found, while individually does not occur.

This thesis aims to improve the understanding of EBPR and solve some of the issues reported when EBPR is implemented in wastewater treatments together with biological removal of nitrogen and organic matter.

The research conducted in this thesis has two different approaches within this framework. On the one hand, the negative interaction between the nitrogen and phosphorus removal processes has been studied. On the other hand, the possibility to use alternative carbon sources, also used in nitrogen removal, has been assessed by developing novel strategies focused on obtaining new syntrophic consortia for application in EBPR.

In chapter 4, new insights for simultaneous nitrogen and phosphorus removal are presented. These strategies are based on the bioaugmentation of PAO microorganisms in nitrification/denitrification systems. In this sense, an anoxic/aerobic SBR with nitrogen removal is bioaugmented with PAO microorganisms in order to remove simultaneously both nutrients. A cycle configuration with an anoxic phase with two feedings and an aerobic phase was used to achieve nitrification, denitrification and EBPR. A key point for the success of this strategy was to provide proper operational conditions to avoid rising problems. Enough aerobic phase length was required to ensure complete PHA depletion for PAO microorganisms, which avoided the carbon source availability required for denitrification.

In this context, the interaction of different intermediates of the nitrogen removal process, such as nitrite and nitrate, on the EBPR processes was studied. According to previous knowledge, the coexistence of electron donor (organic matter) and electron acceptor (nitrite or nitrate) in the anaerobic phase is detrimental for PAO microorganisms and, therefore, the EBPR process. For this reason, several batch studies with different concentrations of nitrate and using diverse carbon sources were carried out to study this effect. The conclusion of such experiments was that intermediate products of the nitrogen removal process, such as nitrate, can affect EBPR process when PAO microorganisms have not been previously acclimated to these conditions.

Low concentration of volatile fatty acid in wastewaters has been also reported to be one of the main hurdles problems to implement EBPR process in full-scale WWTPs. For this reason, Chapter 5 presents the studies done in order to use different carbon sources for EBPR. Two carbon sources commonly used in nitrogen removal processes, namely methanol and glycerol, were tested, resulting in a cost reduction when nitrogen and phosphorus were removed simultaneously in wastewaters with low COD content. Two different strategies were assessed: first, the direct replacement of conventional carbon source (propionic acid) to the desired carbon source in a PAO-enriched sludge system. The second strategy, a novel one, was to develop a consortium of anaerobic sludge, comprising previously selected microorganisms, and PAO where the first ferment the complex carbon source to short-chain volatile fatty acids (i.e. acetic and propionic acid) which are subsequently used by the PAO in the EBPR process. Microbial selection strategies were designed and applied to find efficient consortiums to perform the desired conversions. To finish up this chapter, the most promising consortium for each carbon source from the previous step were tested for the use of different complex carbon sources. These studies indicated the feasibility of such strategies when working with different kinds or wastewater.

Finally, Chapter 6 presents the study of a new strategy to achieve a predominantly PAO-enriched sludge, removing GAO, which uptake significant proportions of the carbon source available for EBPR. For this alternative process, a GAO-enriched sludge SBR was used, changing the operational configuration of the reactor from anaerobic/aerobic to anaerobic/anoxic - nitrite. The combination of propionic acid as electron donor in the anaerobic phase and the addition of nitrite as electron acceptor in the anoxic phase, promoted PAO outcompetition of GAO microorganisms even when the influent properties were not advantageous for PAO. The application of this strategy resulted in the 85% of PAO in the enriched sludge, washing out GAO of the system.

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Chapter 1

INTRODUCTION

1.1. WATER AND WASTEWATER

Water is essential for all socio-economic development and for maintaining healthy ecosystems. As population increases and development calls for increased allocations of groundwater and surface water for the domestic, agriculture and industrial sectors, the pressure on water resources intensifies, leading to tensions, conflicts among users, and excessive pressure on the environment. The increasing stress on freshwater resources brought about by ever rising demand and profligate use, as well as by growing pollution worldwide, is of serious concern (UN-Water, 2006).

These water resources are very deficiently distributed among human population, being one of the main causes of poverty. It has been estimated that in order to ensure the basics needs, every individual needs 20 to 50 liters per day of water free from harmful contaminants (UN-Water, 2006). However, while the poorest fraction of population consume about 20 to 70 $L \cdot d^{-1}$ per person, the water consumption in Catalunya per person is about 320 $L \cdot d^{-1}$ and in the United States is about 550 $L \cdot d^{-1}$ (UN-Water, 2006).

1.1.1. BIOLOGICAL PROCESSES IN WASTEWATER TREATMENT

The treatment of contaminated urban and industrial wastewater by means of biological and chemical processes has been widely implemented. From economical and operational points of view, biological treatment has proved to be a robust and more energy efficient way of treating biodegradable wastewater if good process control can be ensured (Grady et al., 1999). A wide range of contaminants, such as organic matter and nutrients (mostly nitrogen and phosphorus) can be biologically removed to reduce the discharge levels in water bodies (rivers, lakes and seas).

1.2. ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR)

EBPR is widely accepted as the most economical and sustainable process to remove phosphorus from wastewater (Metcalf and Eddy, 2003) but at the same time, it is a complex process when compared to organic matter (chemical organic demand, COD) or nitrogen removal (Pijuan, 2004a). EBPR aims at removing and recovering phosphorus using biological processes and thus, without the addition of chemicals, which increases the costs and produces am undesired chemical sludge. EBPR has motivated many studies in order to understand and improve the processes involved in biological phosphorus removal. A search of the topic “EBPR” in the ISI Web of Science (30/03/2013) finds 541 indexed articles, with 9930 sum of the times cited. Figure 1.1 shows the yearly number of publications about EBPR, demonstrating that the research interest on this topic is still very high, with an average of more than 35 publications per year during the last ten years. In addition, the number of citations of these articles is also high, an average citation per item of 18.35 and an h-index of 49, implying that there are 49 of these articles with at least 49 citations.

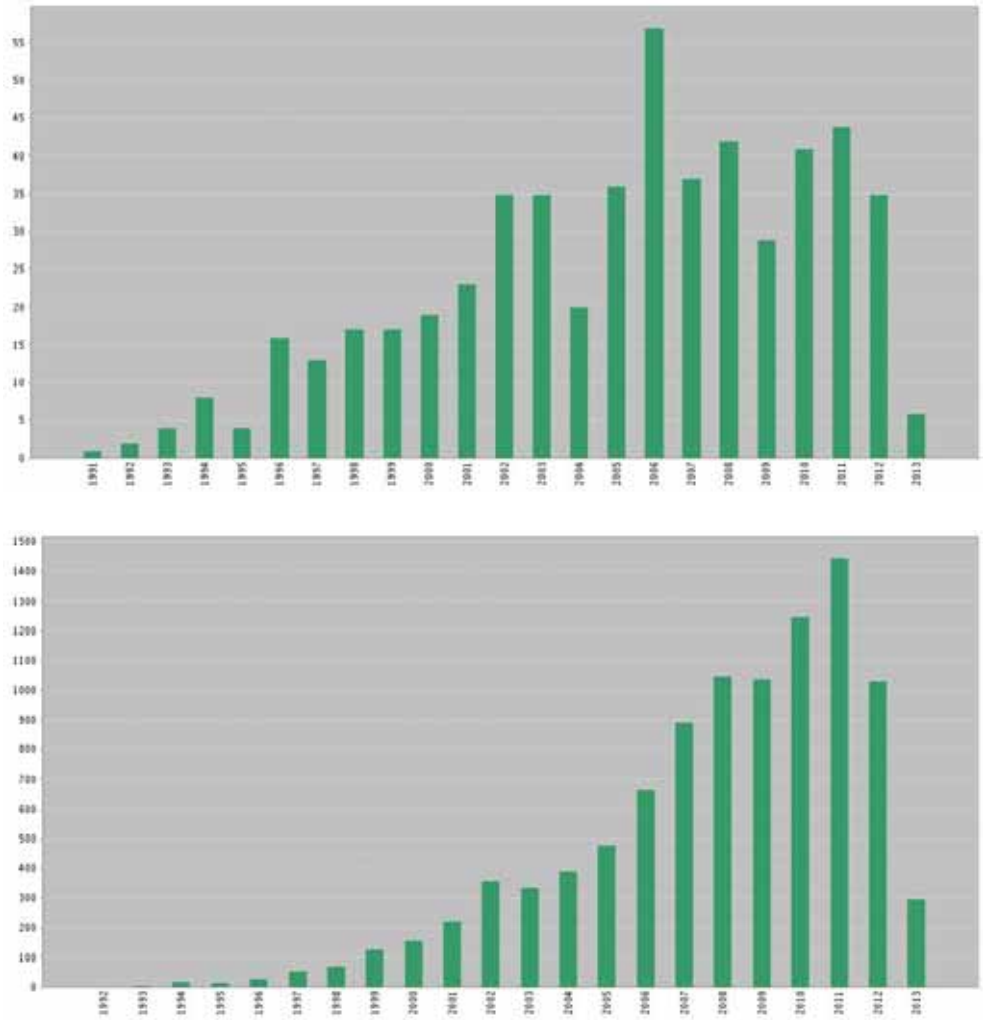

Figure 1.1. Results of the search “EBPR” in the ISI Web of Science (30/03/2013).
Up: Published Items in Each Year. Down: Citations in Each Year.

1.2.1. PHOSPHORUS

Phosphorus is indispensable to many life processes, and its role cannot be performed by any other element. Plants take up phosphorus in large quantities, along with other macronutrients such as nitrogen and potassium. Healthy and robust plant growth requires an assured supply of these elements, so it is no surprise that the green revolution of the last century relied on substantial inputs of fertilisers (Bondre, 2011).

To meet world demand for food, we uptake large amounts of nitrogen from the atmosphere to produce nitrogen fertilisers that can be used by plants. It is so abundant in the atmosphere that we will never run out of it. Phosphate-rich rocks formed slowly over geologic time and are not renewable on human timescales (Bondre, 2011).

Today it is acknowledged that addressing energy and water issues will be critical for meeting the future nutritional demands of a growing population (Smill, 2000; Pfeiffer, 2006) but the need to address the issue of limited phosphorus availability has not been widely recognized. Approximately 70% of the world’s demand for fresh water is for

agriculture (SIWI-IWMI, 2005) and about 90% of worldwide demand for rock phosphate is for food production (Rosmarin, 2004). It is predicted that demand for both resources will exceed supply in the coming decades. Experts suggest that a radical shift in the way we think about and manage water is required (Falkenmark and Rockström, 2002) the 'hydroclimatic realities' of water availability (SIWI-IWMI, 2005).

Moreover, phosphorus is one of the major nutrients contributing in the increased eutrophication of lakes and natural waters. Its presence causes many water quality problems including increased purification costs, loss of livestock and the possible lethal effect of algal toxins and drinking water so it has to be removed (Puig, 2007).

1.2.2. POLY-PHOSPHATE ACCUMULATING ORGANISMS (PAO) IN EBPR PROCESS

The EBPR process is based on the enrichment of activated sludge with poly-phosphate (poly-P) accumulating organisms (PAO) under alternating anaerobic/aerobic or anaerobic/anoxic conditions. Under anaerobic conditions, PAO can uptake organic substrates (preferably volatile fatty acids, VFA) and store them as a poly-hydroxyalkanoates (PHA). This storage requires energy provided by the hydrolysis of poly-P, which results in P-release into solution. The reduction power is provided mainly by the catabolism of stored glycogen. The release of phosphate produced under anaerobic conditions is compensated by its aerobic/anoxic P-uptake. In the aerobic/anoxic stage, PHA is used as a carbon and energy sources for growth, and for the replenishment of internal glycogen and poly-P pools (Mino et al., 1998). A schematic representation of the PAO metabolism is presented in Figure 1.2, while the typical profiles observed in conventional anaerobic/aerobic cycles are shown in Figure 1.3.

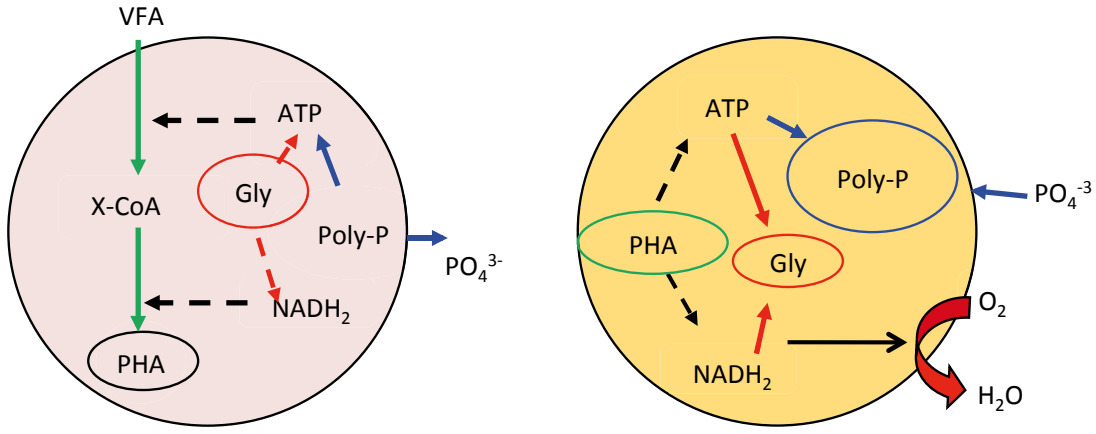

Figure 1.2. Scheme of PAO metabolism: left, anaerobic stage; right, aerobic stage.

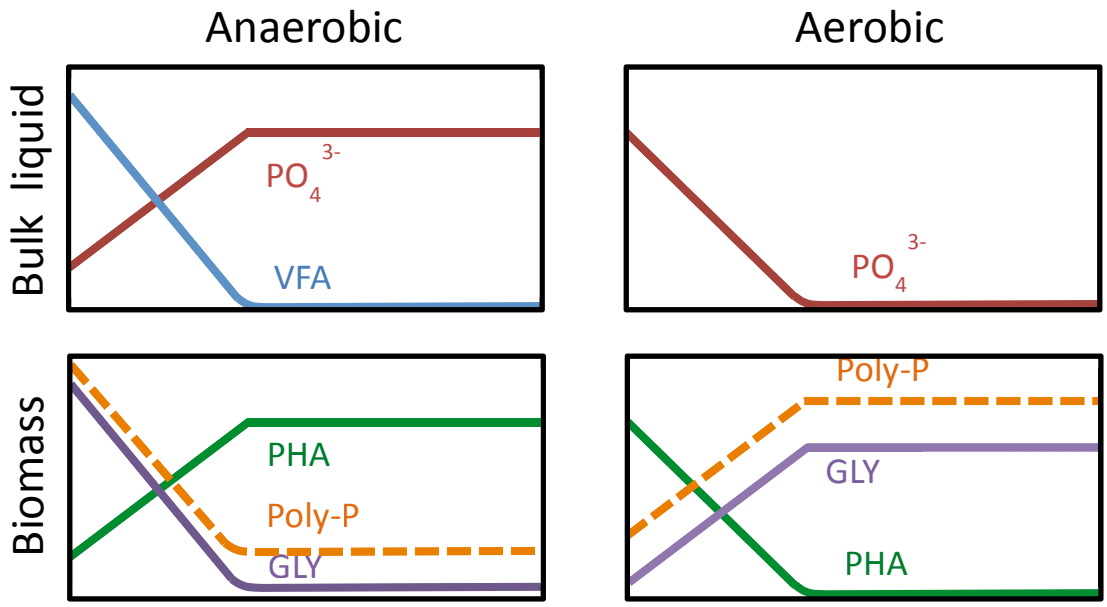

Figure 1.3. Schematic representation of the profiles of a typical anaerobic/aerobic cycle of a reactor with PAO-enriched sludge.

The two most common VFA present in municipal wastewater are acetate and propionate. The metabolism of acetate and propionate by PAO has been amply studied (Chen et al., 2004; Hood and Randall, 2001; Lemos et al., 2003; Lu et al., 2006; Oehmen et al., 2005a; Pijuan et al., 2004b; Thomas et al., 2003). Depending on the carbon source used, PHA polymers are produced with different composition. Pijuan et al. (2009) studied the effect of carbon sources on distribution of PHA obtained with acetate or propionate. The PHA composition was also strongly influenced by the carbon source. Acetate produced PHB-PHV copolymer with a much higher amount of PHB (70%), propionate produced PHV (53%) or PH2MV (49%) copolymer, while other non-identified PHA monomers were also detected with butyrate.

Isolating the bacteria responsible for P removal has been an item of research. First attempts were made based on the number of viable bacterial colonies that grew on defined media (Barker and Dold, 1996). Through these techniques, *Acinetobacter* was first proposed to be the primary organism responsible for P removal in EBPR (Fuhs and Chen, 1975), and was long believed to be the sole PAO present in EBPR plants. However, the use of microbiological techniques such as fluorescence in situ hybridisation (FISH), 16S rRNA-based clone libraries or denaturing gradient gel electrophoresis (DGGE), showed that a high diversity of phylogenetic groups are present in lab- and full-scale EBPR sludge (Seviour et al., 2003). It was with the use of specific FISH probes that *Acinetobacter* was shown to have little significance in full-scale plants when compared to members of other phylogenetic groups, such as the *Betaproteobacteria* and *Actinobacteria* (Bond et al., 1999; Wagner et al., 1994).

In spite of the difficulty of PAO isolation, the identification of this group of organisms has proceeded in the last decade through the use of molecular techniques. Bond et al. (1995) observed differences in community structure between phosphate and non-phosphate-removing sludge, as determined by phylogenetic analysis of 16S rRNA clone

libraries; in particular, the *Rhodocyclus* group from subclass 2 of the *Betaproteobacteria* was represented to a greater extent in the phosphate-removing community. Bond et al. (1999) later supported these conclusions through observing an abundance of *Rhodocyclus*-related bacteria by FISH in other sludges exhibiting good EBPR performance. Hesselmann et al. (1999) named the subclass 2 *Betaproteobacteria* closely related to *Rhodocyclus* as ''*Candidatus Accumulibacter phosphatis*''.

Hesselmann et al. (1999) and Crocetti et al. (2000) reported several FISH probes for *Accumulibacter* that targeted the organism at different areas of the 16S rRNA. These studies demonstrated that *Accumulibacter* corresponded to the characteristic PAO phenotype of anaerobic/aerobic cycling of poly-P and PHA through chemical staining. Since *Accumulibacter* was shown through FISH analysis to be a highly abundant PAO in many lab-scale systems (Hesselmann et al., 1999; Crocetti et al., 2000; Liu et al., 2001), a number of surveys were carried out to assess their presence in full-scale plants. Wastewater treatment plants with various process configurations and from different countries across four continents were evaluated (Zilles et al., 2002; Saunders et al., 2003; Kong et al., 2004; He et al., 2005; Gu et al., 2005; Wong et al., 2005). In all of these studies, *Accumulibacter* was present in relative abundance (4-22% of all Bacteria); considering the large diversity of organisms that is present in full-scale sludge, it was concluded to be an important organism contributing to P removal in EBPR plants.

However, it has been shown that other species can be involved in EBPR process in full-scale biological phosphorus removal (Nguyen et al., 2011). Four probe-defined groups in the three clades of *Tetrasphaera*-PAO constituted 18–30% of the total bacterial biomass present in five well-working EBPR plants with simultaneous nitrogen removal. *Tetrasphaera*-PAO seem to occupy a slightly different ecological niche than *Accumulibacter*. They are more diverse in substrate uptake, can take up glucose, and probably also ferment under anaerobic conditions. The novelty is the ability of *Tetrasphaera*- PAO to take up acetate and other substrates in the anaerobic phase, but their lack of ability to form PHA as a storage compounds (Nguyen et al., 2011). Nevertheless, these four new probes still need more experimental validation at the current stage.

Table 1.1 shows the classical and the recently developed probes that have been used for FISH identification in EBPR systems.

Table 1.1. 16S rRNA-targeted probes used for FISH detection in EBPR systems.

Probe	Sequence 5'-3'	Specificity	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter*	Crocetti et al. (2000)
PAO 651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter*	Crocetti et al. (2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter*	Crocetti et al. (2000)
PAO I	CCCAAGCAATTTCTTCCCC	*Accumulibacter* cluster I	Flowers et al. (2009)
PAO II	CCCGTGCAATTTCTTCCCC	*Accumulibacter* cluster II	Flowers et al. (2009)
Elo1-1250	CGCGATTTCGCAGCCCTT	*Tetrasphera elongata* cluster I	Nguyen et al. (2011)
Tet1-823	TGAGACCCGCACCTAGTT	*Tetrasphera elongata* cluster I	Nguyen et al. (2011)
Tet2-831	TCGTGAAATGAGTCCCAC	*Tetrasphera australiensis, veronensis* and uncultures *Tetrasphera*	Nguyen et al. (2011)
Tet2-87	TCGCCACTGATCAGGAGA	Uncultured *Tetrasphera* cluster II	Nguyen et al. (2011)
Tet2-174	GCTCCGTCTCGTATCCGG	*T. jenkinsii, T. australiensis, T. veronesis* and *Candidatus N.limicola*	Nguyen et al. (2011)
Tet3-19	GGTCTCCCCTACCATACT	Uncultured *Tetrasphera* cluster III	Nguyen et al. (2011)

1.3. BIOLOGICAL NITROGEN REMOVAL

Nitrogen (N) is an essential nutrient for plants and animals. Approximately 80% of the Earth atmosphere is composed of nitrogen and it is a key element of proteins and cells. The major contributors of nitrogen to wastewater are human activities such as food preparation, showering and waste excretion. Total nitrogen in domestic wastewater typically ranges from 20 to 70 $mg \cdot L^{-1}$ for low to high strength wastewater (Tchobanoglous et al., 2003). Factors affecting concentration include the extent of infiltration and the presence of industries. Influent concentration varies during the day and can vary significantly during rainfall events, as a result of inflow and infiltration to the collection system (EPA, 2010).

Nitrogen in untreated wastewater is principally in the form of ammonia or organic nitrogen, both soluble and particulate. Soluble organic nitrogen is mainly in the form of urea and amino acids. Untreated wastewater usually contains little or no nitrite or nitrate. A portion of organic particulate matter is removed by primary sedimentation. During biological treatment, part of the particulate organic nitrogen is transformed to ammonium and another inorganic forms. A portion of ammonium is assimilated into the cell of new biomass (Metcalf and Eddy, 2003).

Of the methods proposed for the removal of nitrogen, biological nitrification/denitrification is often the best for its high potential removal efficiency. Moreover, these processes are very stable and reliable and require low land area and consequently moderate cost. Additionally, they are relatively easy to control processes. The removal of nitrogen by nitrification/denitrification requires two steps.

In the first step, nitrification, ammonia is oxidized to nitrite (NO_2^-) by Ammonia Oxidizing Bacteria (AOB) and then, nitrite is oxidized to nitrate (NO_3^-) by Nitrite Oxidizing Bacteria (NOB).

Nitrification:
$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$
$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$

In the second step, denitrification, nitrite or nitrate are converted to nitrogen gas.

Denitrification:
$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

The removal of nitrogen in the form of nitrate by conversion to nitrogen gas is accomplished under anoxic conditions. The denitrification bacteria obtain energy for growth from the conversion but require a source of carbon source for cell synthesis (Metcalf and Eddy, 2003).

1.4. ACTIVATED SLUDGE SYSTEMS. PROCESS CONFIGURATION FOR BIOLOGICAL NUTRIENT REMOVAL

Treatment of wastewater by means of biological processes has been widely implemented for urban and industrial wastewater. Moreover, there are manyconfigurations for biological nutrient removal systems such as: Phoredox (A/O) (Figure 1.4), A^2/O (Figure 1.5), Five-stage Bardenpho (Figure 1.6), UCT (University of Cape Town) (Figure 1.7), modified UCT (Figure 1.8) and VIP (Virginia Initiative Plant) (Figure 1.9) (Metcalf and Eddy, 2003).

Figure 1.4 describes the A/O process, which is a patented version of Phoredox by *Air Products and Chemicals, Inc*. The main difference is the use of multiple-staged anaerobic and aerobic reactors. This process is used for combined carbon oxidation and phosphorus removal. In this process there is no nitrification, and the anaerobic retention time is 30 min to 1 h to provide the selective conditions for the biological phosphorus removal (Puig, 2007).

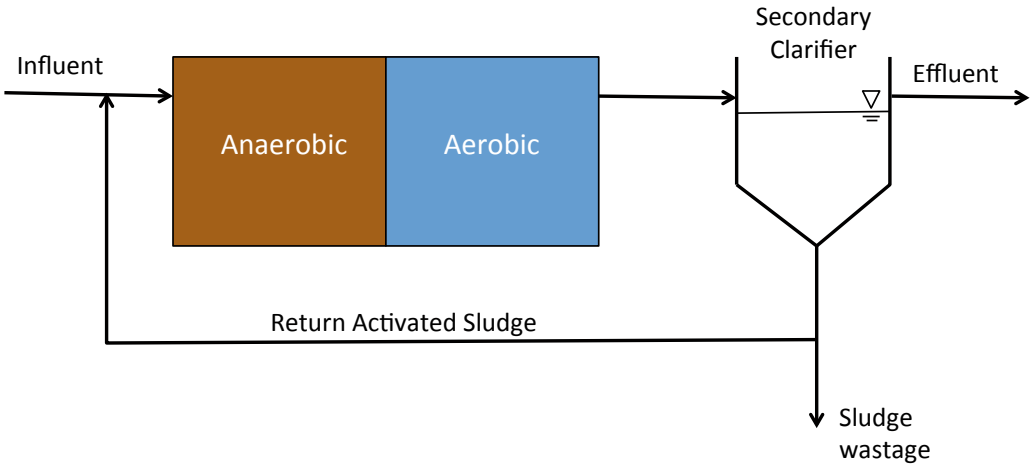

Figure 1.4. Phoredox or A/O process.

The A^2/O process (Figure 1.5) is patented by *Air Products and Chemicals, Inc*. It is a modified A/O process to provide an anoxic zone for denitrification. The operation is relatively simple compared to other processes. With this process it is achieved simultaneous nitrogen and phosphorus removal. In the anoxic zone there is a low oxygen concentration, but nitrite and nitrate can be used as electron acceptor from the recycle of the aerobic zone. However, it requires high COD/P ratios to reduce the potential nitrate/nitrite entering the anaerobic phase.

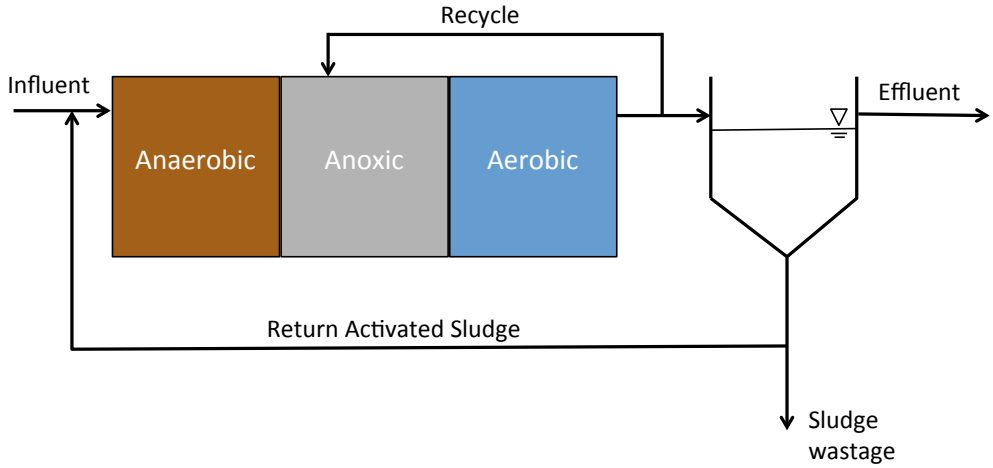

Figure 1.5. A^2/O process.

On the other hand, Barnard (1975) first achieved phosphorus removal in a mainstream process later called Bardenpho process. Figure 1.6 shows a 5-stage Bardenpho process, which provides anaerobic, anoxic and aerobic stages for phosphorus, nitrogen and carbon removal. A second anoxic stage is provided for additional denitrification using nitrate produced in the aerobic stage as the electron acceptor and the endogenous organic carbon as the electron donor. The final aerobic stage is used to strip residual nitrogen gas from solution to prevent rising problems and to minimize the P-release in the final clarifier (Metcalf and Eddy, 2003).

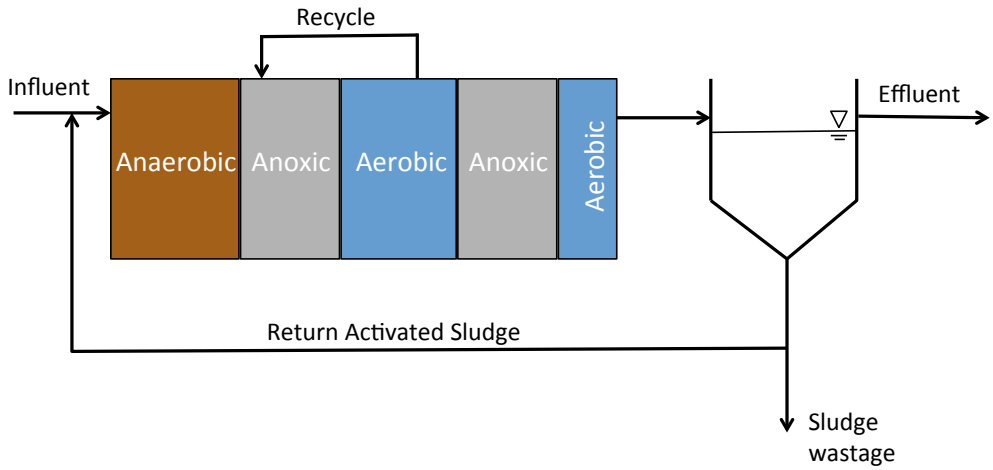

Figure 1.6. Modified Bardenpho (5-stage) process.

The UCT process (Figure 1.7) was developed in the University of Cape Town and is similar to the A^2/O process. There are two differences: (i) the return activated sludge is recycled to the anoxic reactor instead of the anaerobic stage, and (ii) there is an additional recycle from the anoxic reactor to the anaerobic reactor. This configuration decreases the amount of nitrate recycled to the anaerobic reactor, increasing the organic matter availability for EBPR.

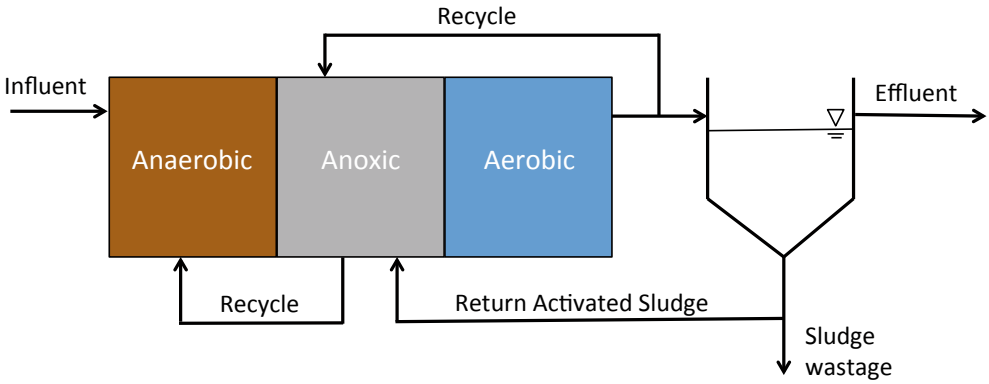

Figure 1.7. University of Cape Town (UCT) process.

The modified UCT process (Figure 1.8) splits the anoxic zone in two stages. The nitrate rich recycle from the aerobic zone is recycled to the head of the second anoxic stage. The nitrate contained in the return activated sludge is recycled to the fist anoxic stage where it is denitrified. Next, the denitrified returned activated sludge is recycled from the end of the first anoxic stage to the head of the anaerobic stage and mixed with the incoming wastewater.

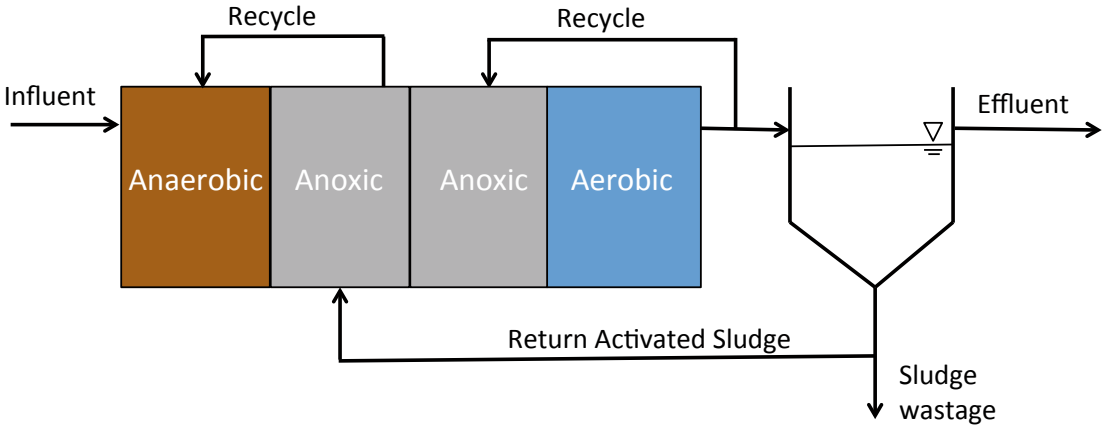

Figure 1.8. Modified UCT process.

The VIP process (Figure 1.9) (from Virginia Initiative Plant in Norfolk, US) is similar to A^2/O and UCT processes except for the methods used for recycling. The return of activated sludge is discharged to the inlet of the anoxic zone along with nitrified recycle from the aerobic zone. The mixed liquor from the anoxic zone is returned to the head end of the anaerobic zone. The organic matter in the process influent is stabilized through anaerobic mechanisms in the anaerobic zone, which reduces the oxygen requirements in the process (Metcalf and Eddy, 2003).

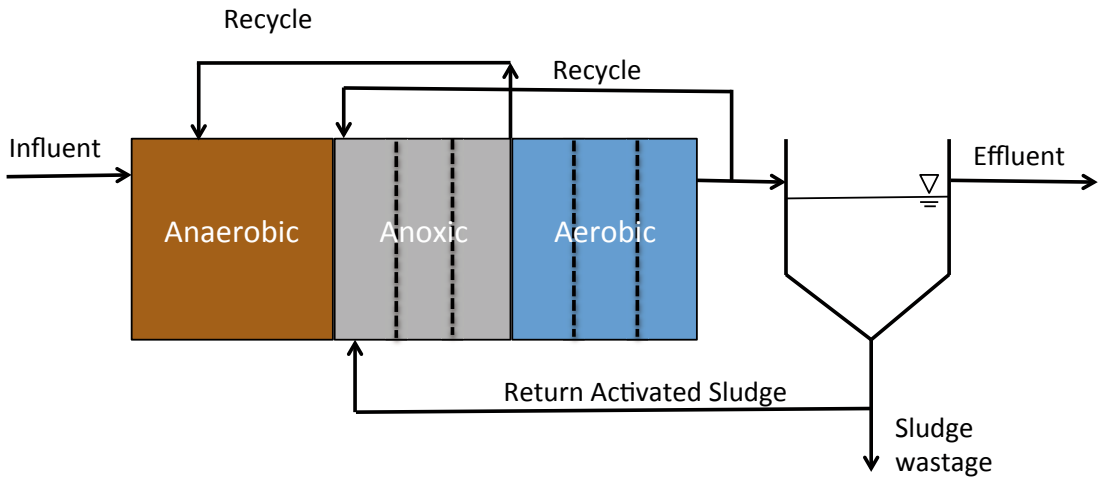

Figure 1.9. Virginia Initiative Plant (VIP) process.

1.4.1. SEQUENCING BATCH REACTOR (SBR) TECHNOLOGY

The Sequencing Batch Reactor (SBR) is the name given to a wastewater treatment system based on activated sludge and operated in a fill-and-draw cycle. The most important difference between SBR and the conventional activated sludge systems is that reaction and settle take place in the same reactor. Basically, all SBR have five phases in common, which are carried out in sequence as follows: fill, react, settle, extract and idle (Mace and Mata-Alvarez, 2002).

This configuration has a higher flexibility and controllability, allowing more rapid adjustment to changing influent characteristics (Baetens, 2000). Secondary settling tanks and sludge return system are not required and thus revert on lower investments and lower operational costs (Nowak and Lindtner, 2004). Furthermore, it is especially appropriate for places where there is significant flow variability (Metcalf and Eddy, 2003) or where space problems become a restriction.

SBRs are widely and commonly used in a biological wastewater treatment. SBR technology has been successfully applied in WWTPs treating urban (Lee et al., 2004; Puig et al., 2005) and industrial (Cassidy and Belia, 2005; Keller et al., 1997; Torrijos et al., 2001; Vives et al., 2003) wastewater. Additionally, SBRs have many other advantages such as achieved granular sludge and less bulking. In addition, the sequence of treatment cycles can be adjusted in various ways, modifying the length of the phases depending on the discharge requirements (Wilderer et al., 2001).

SBRs are especially preferred when nutrient removal is important, because enrichment in nitrifiers and denitrifiers and phosphorus removal bacteria may take place in the same vessel by simply changing the mixing and aeration conditions and time schedules. Nevertheless, SBRs also present many advantages for processes envisaging mainly carbonaceous load removal (Irvine et al., 1997; Wilderer et al., 2001).

The conditions applied during the fill and react phases must be adjusted according to the treatment objectives (organic matter, nitrogen or phosphorus removal). The classical operation of SBR is executing a sole filling during a cycle, but more than one

filling (two, three...) can be designed.

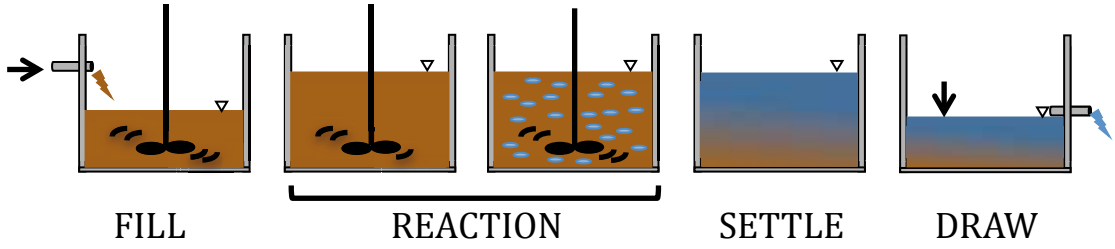

Figure 1.10. Sequence of phases in SBR operation.

The influent is mixed with the biomass (Figure 1.10), which then initiates biological reactions. During the react phase, usually under mixing conditions, the biomass consumes the substrates under controlled environmental conditions (aerobic, anoxic or anaerobic) depending on the required wastewater treatment. In the anaerobic phase, usually takes place the organic matter uptake by PAO microorganisms leading to P-release and in the aerobic react phases, the organic matter oxidation and nitrification occurs and phosphorus is uptaken. In the anoxic phase the classical heterotrophic denitrification process and again phosphorus uptake can occur.

Solids are allowed to separate from the liquid under quiescent conditions, resulting in a clarified supernatant that can be discharged as effluent. The settling time is based on subsidence of the sludge blanket layer and the concentration of the settled biomass (Ketchum Jr., 1997).

1.5. CURRENT EBPR BOTTLENECKS

EBPR is a widely studied and mostly understood technology. However, its full application is not achieved yet, partly because there are some issues that are still unknown. These issues don't prevent the technology from being feasible but result in reported failures, which sometimes, are very difficult to predict and to solve.

1.5.1. GLYCOGEN ACCUMULATING ORGANISMS (GAO)

One of the large known failures on EBPR systems is the presence of other microorganisms able to survive under anaerobic/aerobic conditions and compete with PAO for electron donor. These microorganisms are able to uptake the carbon source under anaerobic conditions without any P activity (Cech and Hartman, 1990, 1993; Crocetti et al., 2002; Liu et al., 1994; Nielsen, et al., 1999; Satoh et al., 1994; Wong et al., 2004). The term GAO (Glycogen accumulating organisms) was proposed by Mino et al. (1995), and is defined as the phenotype of organisms that store glycogen aerobically and consume it anaerobically as their primary source of energy for taking up carbon sources and storing them as PHA. Using a deteriorated EBPR sludge, Crocetti et al. (2002) generated two FISH probes (GAOQ431, GAOQ989), named ''*Candidatus Competibacter phosphatis*'' (henceforth referred to as *Competibacter*). Two other distinct subgroups of *Alphaproteobacteria* enriched in a propionate fed lab-scale reactor have been identified as GAO, both related to *Defluviicoccus vanus* (Meyer et

al., 2006; Wong et al., 2004). Two new probes for cluster I of *Defluviicoccus vanus* (TFO_DF218, TFO_DF618) were designed (Wong et al., 2004). Meyer et al. (2006) designed new probes for cluster II of *Defluviicoccus vanus* (DF988, DF1020) that comprised 33% of all bacteria in the original lab-scale reactor. Table 1.2 shows more details of the FISH probes designed for GAO detection.

Table 1.2. 16S rRNA-targeted probes used for FISH detection in GAO systems.

Probe	Sequence 5'-3'	Specificity	Reference
GAOQ431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	Nielsen et al. (1999)
GAOQ989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	Nielsen et al. (1999)
TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus-related* organisms cluster I	Wong et al. (2004)
TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus-related* organisms cluster I	Wong et al. (2004)
DF988	GATACGACGCCCATGTCAAGGG	*Defluviicoccus-related* organisms cluster II	Meyer et al. (2006)
DF1020	CCGGCCGAACCGACTCCC	*Defluviicoccus-related* organisms cluster II	Meyer et al. (2006)

1.5.1.1 Factors affecting PAO and GAO competition

Several factors influence the microbial competition in EBPR systems, such as the carbon source, pH, temperature and aeration (Brdjanovic et al.,1998a,b; Filipe et al., 2001; Lopez-Vazquez et al., 2008; 2009; Lu et al., 2006; Oehmen et al., 2005b; 2006a,b).

The ratio of organic carbon to P in the influent, known as COD/P ratio, is one factor that affects PAO-GAO competition. Several studies found that values higher than >50mg COD/mg P favour GAO instead of PAO (for a review see e.g. Mino et al., 1998), while lower values (between 10 and 20mg COD/mg P) favour PAO growth. However, a sufficient amount of VFA has to be provided in order to achieve good P removal (Oehmen et al., 2007).

Furthermore, the nature of the carbon source also affects the PAO-GAO competition. For example, while the use of acetate as a carbon source in EBPR systems has been often documented to yield robust and stable P removal performance, there are also many reported occasions where the P removal was deteriorated due to microbial competition of GAO with PAO (Oehmen et al., 2007). Several studies have suggested that propionate may be a more favourable substrate than acetate for successful EBPR performance (Chen et al., 2005; Oehmen et al., 2006a,b; Pijuan et al., 2004b; Thomas et al., 2003). Besides, other VFA such as butyrate, lactate, valerate and isovalerate can also be taken up by PAO or GAO (Hood and Randall, 2001; Lemos et al., 1998; Levantesi et al., 2002; Liu et al., 1996; Randall et al., 1997;) but the effect of these substrates on this competition should be studied in depth.

Otherwise, pH strongly influences the PAO–GAO competition, and an increase in pH can improve P-removal performance by selecting for PAO over GAO. This finding may

be a very useful control strategy for improving P-removal in EBPR systems. Naturally, excessive pH can be detrimental to EBPR process performance. In some studies, a decrease in the VFA uptake, P-release and P-uptake rates have been observed at pH values above 8.0 (Liu et al., 1996; Oehmen et al., 2005a; Schuler and Jenkins, 2002).

Temperature also appears to be a factor that has an impact on the PAO–GAO competition (Brdjanovic et al., 1998a). The experimental evidence obtained thus far suggests that GAO tend to become stronger competitors with PAOs at higher temperatures. This implies that competition by GAO with PAO in EBPR plants may be more problematic in warm climates, and during the summer months (Oehmen et al., 2007).

Other factors have been reported to affect PAO-GAO competition. Dissolved oxygen (DO) was studied as a possible selection factor (Brdjanovic et al., 1998b; Griffiths et al., 2002; Lemaire et al., 2006). Griffiths et al. (2002) observed a poor P removal and a high number of *Defluviicoccus vanus* at very high DO concentrations (4.5–5.0mg·L^{-1}), while DO concentrations around 2.5–3.0mg·L^{-1} seemed to favour a greater abundance of PAO. Additionally, Lemaire et al. (2006) observed an increase in the population of *Accumulibacter* and a decrease in *Competibacter* during SBR operation at a very low DO level (approximately 0.5 mg·L^{-1}). Furthermore, Saito et al. (2004) have observed that the accumulation of nitrite in the anoxic phase produced an increase in the *Competibacter* population, and suggested that it may be a factor that provides GAO an advantage over PAO. Table 1.3 summarises the most reported factors affecting this PAO-GAO competition.

Table 1.3. Summarized factors affecting PAO-GAO competition.

	PAO	GAO	Reference
Influent COD/P (mg COD/mg P)	10-20	>50	Mino et al., 1998
Carbon source more favourable	Propionate	Acetate	Chen et al., 2004; Oehmen et al., 2004, 2006b; Pijuan et al., 2004a; Thomas et al., 2003
pH	7.25-8	6.5-7.25	Oehmen et al., 2005b
Temperature (^{o}C)	<25	>25	Brdjanovic et al., 1998a; Lopez-Vazquez et al., 2006 Whang and Park et al., 2006
Dissolved oxygen (mg·L^{-1})	<2.5-3.5	4.5-5	Griffiths et al., 2002; Lemarie et al., 2006
Sludge age (days)	<10	>10	Whang and Park, 2006

Finally, the presence of nitrate or nitrite could be also a selecting factor in the PAO-GAO competition. Different groups of PAO and GAO have shown varying denitrification capacities (Carvalho et al., 2007; Wang et al., 2008; Zeng et al., 2003) that may have an important impact on their competition. It has been postulated that PAO I (*Accumulibacter* cluster I) are able to denitrify from nitrate and from nitrite, while PAOII (*Accumulibacter* cluster II) are able to denitrify just from nitrite (Carvalho et al.,

2007; Flowers et al., 2009; Oehmen et al., 2010). Kong et al. (2006) hypothesised that the different subgroups of *Competibacter* also display varying denitrifying capacities. Wang et al. (2008) showed that an enrichment of *Defluviicoccus* cluster I was able to reduce nitrate, but not nitrite, while Burow et al. (2007) suggested that *Defluviicoccus* cluster II was unable to denitrify.

Table 1.4. Comparison of the preferred VFA and the denitrification capabilities for the different PAO-GAO subgroups (adapted from Oehmen et al., 2010).

	Preferred VFA	**Denitrification capacity**	
		NO_3^-	**NO_2^-**
Accumulibacter PAO I	Acetate and Propionate	✓	✓
Accumulibacter PAO II		✖	✓
Competibacter Sub-groups 1, 4, 5	Acetate	✓	✖
Competibacter Sub-groups 3,7		✖	✖
Competibacter Sub-group 6		✓	✓
Defluviicoccus DFI	Propionate	✓	✖
Defluviicoccus DFII		✖	✖

1.5.2. CARBON SHORTAGE

Frequently, full-scale WWTPs influents are limited in available biodegradable carbon, particularly when aiming at simultaneous biological nutrient removal. Different solutions are proposed to increase this readily biodegradable content:
i) an external carbon source addition, which is usually not cost-effective because it increases the overall plant carbon footprint (Isaacs and Henze, 1995; Yuan et al., 2010).
ii) the utilization of sludge pre-fermentation to produce these compounds (Feng et al., 2009; Tong and Chen, 2007; Yuan et al., 2010).
iii) enriching the system in denitrifying PAO for achieving simultaneous phosphorus and nitrogen removal (Kerrn-Jespersen et al., 1993; Kuba et al., 1996). The use of nitrate or nitrite as electron acceptor instead of oxygen implies lower aeration costs and 30% less of sludge production.

Several external carbon sources have been studied to balance the abovementioned COD deficiency in wastewaters (Appeldoorm et al., 1992; Gerber et al., 1896; Hallin et al., 1996; Isaacs et al., 1994; Jones et al., 1987; Winter, 1989). Puig et al. (2008) used ethanol as external carbon source for EBPR and a recent study from Yuan et al. (2010) used glycerol, which is also used as external carbon source for denitrification, as a co-substrate with a previous prefermentation step to produce VFA that was used in a second step for EBPR. Thus, the utilisation of waste materials that could be converted somehow to VFA is an attractive alternative to overcome such carbon source deficiency.

1.5.3. NITRATE/NITRITE ENTERING IN THE ANAEROBIC PHASE

In full-scale WWTPs, EBPR has to coexist with biological nitrogen removal based on the aerobic nitrification and anoxic denitrification processes. Denitrifying and P-removal does occurs in full-scale WWTPs using UCT configuration (Kuba et al., 1997), and most of the reported WWTP configurations for simultaneous N and P removal have an aerobic zone before the secondary settler which may result in the presence of some nitrate or nitrite in the external recycle. Nitrate/nitrite would then enter in the anaerobic zone via the external recycle, leading to EBPR failure as reported for many WWTPs (Henze et al., 2008). The most commonly accepted hypothesis to describe this failure is that nitrate/nitrite presence triggers the competition for the electron donor (i.e. organic matter) between ordinary heterotrophic organisms (OHO) and PAO (Barker and Dold, 1996). The presence of nitrate is reported to avoid anaerobic P-release, which only occurred after nitrate depletion (<1 mg $N\text{-}NO_3^-\cdot L^{-1}$) (Kuba et al., 1994; Patel and Nakhla, 2006). On the other hand, van Van Niel et al. 1998 and Saito et al. 2004 suggested a possible inhibitory effect of some denitrification intermediates (nitrite or nitric oxide) on the EBPR process. Guerrero et al. (2011) experimentally observed that the nature of the carbon source rules such competition. Thereby, it was proved that OHO were able to outcompete PAO when the electron donor was a complex carbon source, while PAO won the competition when treating a wastewater with high VFA content.

1.6. REFERENCES

Appeldoorm, K. J., Kortstee, J. J., and Zehnder, A. J. B., 1992. Biological phosphate removal by activated sludge under defined conditions. Water Research, 26, 453-460.

Baetens, D., 2000. Enhanced biological phosphorus removal: modelling and experimental design. PhD Thesis, Universiteit Gent, Belgium.

Barker, P. S. and Dold, P. L., 1996. Denitrification behaviour in biological excess phosphorus removal activated sludge systems. Water Research, 30(4), 769–780.

Barnard, J. J., 1975. Biological nutrient removal without the addition of chemicals. Water Research, 9(5-6), 485–490.

Bond, P. L., Hugenholtz, P., Keller, J., and Blackall, L. L., 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Applied and Environmental Microbiology, 61(5), 1910–1916.

Bond, P. L., Erhart, R., Wagner, Mi, Keller, J., Blackall, L L, and Keller, R. G., 1999. Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. Applied and Environmental Microbiology, 65(9), 4077–4084.

Bondre, N., 2011. Phosphorus: How much is. Global Change, (76), 14–17.

Brdjanovic, D., Logemann, S., van Loosdrecht, M. C. M., Hooijmans, C. M., Alaerts, J. G., and Heijnen, J. J., 1998a. Influence of temperature on biological phosphorus removal: process and molecular ecological studies. Water Research, 32(4), 1035–1048.

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

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

Carvalho, G., Lemos, P. C., Oehmen, A., and Reis, M. A. M., 2007. Denitrifying phosphorus removal : Linking the process performance with the microbial community structure. Water Research, 41, 4383-4396.

Cassidy, D. P. and Belia, E., 2005. Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. Water Research, 39(19), 4817–4823.

Cech, J. S. and Hartman, P., 1990. Glucose induced break down of enhanced biological phosphate removal. Environmental Technology, 11, 651–656.

Cech, J. S. and Hartman, P., 1993. Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. Water Research, 27(7), 1219–1225.

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

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

Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D., and Blackall, L. L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Applied and Environmental Microbiology, 66(3), 1175–1182.

Crocetti, G. R., Banfield, J. F., Keller, J., Bond, P. L., and Blackall, L. L., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology, 148(11), 3353–64.

EPA, United States Environmental Protection Agency, 2010. Nutrient Control Design Manual. Cincinnati, Ohio

Falkenmark, M. and Rockström, J., 2002. "Neither water nor food security without a major shift in thinking: A water-scarcity close-up." in World Food Prize International Symposium, 1–10.

Feng, L., Chen, Y., and Zheng, X., 2009. Enhancement of waste activated sludge protein conversion and volatile fatty acids accumulation during waste activated sludge anaerobic fermentation by carbohydrate substrate addition: the effect of pH. Environmental Science and Technology, 43(12), 4373–80.

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

Flowers, J. J., He, S., Yilmaz, S., Noguera, D. R., and McMahon, K. D., 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different '*Candidatus Accumulibacter*' clades. Environmental Microbiology Reports, 1(6), 583-588.

Fuhs, G. W. and Chen, M., 1975. Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microbial Ecology, 2(2), 119–138.

Gerber, A., Mostert, E. S., Winter, C. T., and Villiers, R. H., 1986. The effect of acetate and other short-chain carbon compounds on the kinetics of biological nutrient removal. Water S.A., 12(1), 7-12.

Grady, J., Daigger, G., and Lim, H., 1999. *Biological wastewater treatment*, New York, Marcel Dekker.

Griffiths, P. C., Stratton, H. M., and Seviour, R. J., 2002. Environmental factors contributing to the "'G bacteria'" population in full-scale EBPR plants. Water Science and Technology, 46(4-5), 185–192.

Gu, A. Z., Saunders, A. M., Neethling, J. B., Stensel, H. D., and Blackall, L. L., 2005. "Investigation of PAOs and GAOs and their effects on EBPR performance at full-scale wastewater treatment plants in US" in WEFTEC. Washington, DC, USA.

Guerrero, J., Guisasola, A., and Baeza, J.A., 2011. The nature of the carbon source rules the competition between PAO and denitrifiers in systems for simultaneous biological nitrogen and phosphorus removal. Water Research, 45(16), 4793-4802.

Hallin, S., Rothman, M., and Pell, M., 1996. Adaptation of denitrifying bacteria to acetate and methanol in activated sludge. Water Research, 30, 1445-1450.

He, S., Gu, A Z, and McMahon, K. D., 2005. “The role of Rhodocyclus-like organisms in biological phosphorus removal: factors influencing population structureand activity” in WEFTEC.

Henze, M., van Loosdrecht, M., Ekama, G., and Brdjanovic, D., 2008. Biological Wastewater Treatment. IWA Publishing, London.

Hesselmann, R. P., Werlen, C., Hahn, D., van der Meer, J. R., and Zehnder, A. J., 1999. Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. Systematic and Applied Microbiology, 22(3), 454–65.

Hood, C. R. and Randall, A. A., 2001. A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. Water Research, 35(11), 2758–66.

Irvine, R L, Wilderer, P A, and Flemming, H. C., 1997. Controlled unsteady state processes and technologies - An overview. Water Science and Technology, 35(1), 1–10.

Isaacs, S. H., Henze, M., Soeberg, H., and Kummel, M., 1994. External carbon source addition as a means to control an activated sludge for nutrient removal process. Water Research, 25, 511-520.

Isaacs, S. H. and Henze, M., 1995. Controlled carbon source addition to an alternating nitrification-denitrification wastewater treatment process including biological P removal. Water Research, 29(1), 77–89.

Jones, P. H., Tadwalkar, A. D., and Hsu, C. L., 1987. Enhanced uptake of phosphorus by activated sludge: effect of substrate addition. Water Research, 21, 301-308.

Keller, J., Subramaniam, K., Gösswein, J., and Greenfield, P. F., 1997. Nutrient removal from industrial wastewater using single tank sequencing batch reactors. Water Science and Technology, 35(6), 335–362.

Kerrn-Jespersen, J. P., and Henze, M., 1993. Biological phosphorus uptake under anoxic and aerobic conditions. Water Research, 27(4), 617-624.

Ketchum Jr., L. H., 1997. Design and physical features of Sequencung Batch Reactors. Water Science and Technology, 35(1), 11-18.

Kong, Y., Nielsen, J. L., Halkjær, P., and Nielsen, P. H., 2004. Microautoradiographic study of *Rhodocyclus-related* polyphosphate-accumulating bacteria in full-scale Enhanced Biological Phosphorus Removal plants. Applied and Environmental Microbiology, 70(9), 5383–5390.

Kong, Q. X., Wang, X. W., Jin, M., Shen, Z. Q., and Li, J. W., 2006. Development and application of a novel and effective screening method for aerobic denitrifying bacteria. FEMS microbiology letters, 260(2), 150–155.

Kuba, T., Wachtmeister, A., van Loosdrecht, M. C. M., and Heijnen, J. J., 1994. Effect of nitrate on phosphorus release in biological phosphorus removal sustems. Water Science and Technology, 30(6), 263-269.

Kuba, T., van Loosdrecht, M. C. M., and Heijnen, J. J., 1996. Phosphorus and nitrogen removal with minimal COD requirement by integration of denitrifying dephosphatation and nitrification in a two-sludge system. Water Research, 30(7), 1702–1710.

Kuba, T., van Loosdrecht, M. C. M., Brandse, F. A., and Heijnen, J. J., 1997. Occurrence of denitrifying phosphorus removing bacteria in modified UCT-type wastewater treatment plants. Water Research, 31(4), 777-786.

Lee, H., Min, K. Y., Park, C. H., and Park, Y. H., 2004. Automatic control and remote monitoring system for biological nutrient removal on small wastewater treatment plants in Korea. Water Science and Technology, 50(6), 199–206.

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

Lemos, P. C., Viana, C., Salgueiro, E. N., Ramos, A. M., Crespo, J., and Reis, M, A, M., 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate accumulating mixed culture. Enzyme Microbilogy Technology, 22(8), 662-671.

Lemos, P. C., Serafim, L. S., Santos, M. M., Reis, M. a M., and Santos, H., 2003. Metabolic pathways for propionate utilisation by phosphorus accumulating organisms in activated sludge:13 C labelling and in vivo nuclear magnetic resonance. Applied and Environmental Microbiology, 69(1), 241–251.

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. Environmental Microbiology, 4(10), 559–569.

Liu, W T, Mino, T., Nakamura, K., and Matsuo, T., 1994. Role of glycogen in acetate uptake and polyhidroxyalkanoates synthesis in anaerobic-aerobic activated-sludge with minimized polyphosphate content. Journal of Fermentation and Bioengineering, 77(5), 535–540.

Liu, W T, Mino, T., Matsuo, T., and Nakamura, K., 1996. Biological phosphorus removal processes—effect of pH on anaerobic substrate metabolism. Water Science and Technology, 34(1-2), 25–32.

Liu, W. T., Nielsen, A. T., Wu, J. H., Tsai, C. S., Matsuo, Y., and Molin, S., 2001. *In situ* identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. Environmental Microbiology, 3, 110–122.

Lopez-vazquez, C. M, Song, Y., Hooijmans, C. M, Brdjanovic, D., Moussa, M. S., Gijzen, H. J., and van Loosdrecht, M. C. M., 2006. Short-term temperature effects on the anaerobic metabolism of Glycogen Accumulating Organisms. Biotechnology and Bioengineering, 97(3), 483-495.

Lopez-Vazquez, C. M., Song, Y., Hooijmans, C. M., Brdjanovic, D., Moussa, M. S., Gijzen, H. J., and van Loosdrecht, M. C. M., 2008. Temperature effects on the aerobic metabolism of glycogen-accumulating organisms. Biotechnology and Bioengineering, 101(2), 295–306.

Lopez-Vazquez, C. M, Oehmen, A., Hooijmans, C M, Brdjanovic, D., Gijzen, H J, Yuan, Z., and van Loosdrecht, M. C. M., 2009. Modeling the PAO-GAO competition: effects of carbon source, pH and temperature. Water Research, 43(2), 450–62.

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

Mace, S. and Mata-Alvarez, J. R., 2002. Utilisation of SBR technology for wastewater treatment: an overview. Industrial and Engineering Chemistry Research, 41(23), 5539–5553.

Metcalf and Eddy, 2003. Wastewater engineering: Treatment, disposal and reuse, New York.

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

Mino, T., Liu, W T, Kurisu, F., and Matsuo, T., 1995. Modeling glycogen- storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. Water Science and Technology, 31(2), 25–34.

Mino, T., van Loosdrecht, M. C. M., and Heijnen, J. J., 1998. Microbiology and biochemistry of enhanced biological phosphate removal process. Water Research, 32(11), 3193–3207.

Nguyen, H. T. T., Le, V. Q., Hansen, A. A., Nielsen, J. L., and Nielsen, P. H., 2011. High diversity and abundance of putative polyphosphate-accumulating *Tetrasphaera*-

related bacteria in activated sludge systems. FEMS Microbiology Ecology, 76(2), 256–67.

Nielsen, A. T., Liu, W. T., Filipe, C., Grady, L., Molin, S., and Stahl, D. A., 1999. Identification of novel group of bacteria in sludge of deteriorated biological phosphorus removal reactor. Applied and Environmental Microbiology, 65(3), 1251–1258.

Nowak, O. and Lindtner, S., 2004. "Investment and operating Costs of small wwtps – compared to larger Plants" in Fremantle WA (ed.), 6th Specialist conference on small water and wastewater systems. Autralia.

Oehmen, A., 2004. The competition between polyphosphate accumulating organisms and glycogen accumulating organisms in the enhanced biological phosphorus removal process. PhD Thesis. University of Queensland, Brisbane, Australia.

Oehmen, A., Vives, T. M., Lu, H., Yuan, Z., and Keller, J., 2005a. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Research, 39(15), 3727–37.

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

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

Oehmen, A., Zeng, R. J., Saunders, A. M., Blackall, L. L., Keller, J., and Yuan, Z., 2006b. Anaerobic and aerobic metabolism of glycogen-accumulating organisms selected with propionate as the sole carbon source. Microbiology, 152(9), 2767–78. .

Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L., and Reis, M. A. M., 2007. Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Research, 41(11), 2271–300.

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

Patel, J., and Nakhla, G., 2006. Interaction of denitrification and P removal in anoxic P removal systems. Desalination 201, 82-99.

Pfeiffer, D., 2006. Eating Fossil Fuels: Oil, Food and the Coming Crisis in Agriculture, Canada.

Pijuan, M., 2004a. Effect of different carbon sources and continous aereation on EBPR process. PhD Thesis, Universitat Autònoma de Barcelona. Catalunya.

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

Pijuan, M., Casas, C., and Baeza, J. A., 2009. Polyhydroxyalkanoate synthesis using different carbon sources by two enhanced biological phosphorus removal microbial communities. Process Biochemistry, 44(1), 97–105.

Puig, S., Corominas, L., Vives, M T, Colomer, J., Balaguer, M. D., and Colprim, J., 2005. Develpment and implementation of a real control system for nitrogen removal using PUR and ORP as endpoints. Industrial and Engineering Chemistry Research, 44(9), 3367–3373.

Puig, S., 2007. Operation and control of SBR processes for enhanced biological nutrient removal from wastewater. PhD Thesis, Universitat de Girona. Catalunya.

Puig, S., Coma, M., Monclús, H., van Loosdrecht, M C M, Colprim, J., and Balaguer, M. D., 2008. Selection between alcohols and volatile fatty acids as external carbon sources for EBPR. Water Research, 42(3), 557–66.

Randall, A. A., Benefield, L. D., and Hill, W. E., 1997. Induction of phosphorus removal in an enhanced biological phosphorus removal bacterial population. Water Research 31 (11), 2869–2877.

Rosmarin, A., 2004. The Precarious Geopolitics of Phosphorous Down to Earth (Science and Environment Fortnightly). June 30, 27-31.

SIWI-IWMI, 2005. *More nutrition per drop. Consumption Patterns in a Rapidly Changing World Note to Reader*, Stockholm.

Saito, T., Brdjanovic, D., and van Loosdrecht, M. C. M., 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. Water Research, 38(17), 3760–8.

Satoh, H., Mino, T., and Matsuo, T., 1994. Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation. Water Science and Technology, 30(6), 203–211.

Saunders, A. M., Oehmen, A., Blackall, L. L., Yuan, Z., and Keller, J., 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water science and technology : a journal of the International Association on Water Pollution Research, 47(11), 37–43.

Schuler, A. J. and Jenkins, D., 2002. Effects of pH on enhanced biological phosphorus removal metabolisms. Water Science and Technology, 46(4-5), 171–178.

Seviour, R. J., Mino, T., and Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiology Reviews, 27(1), 99–127.

Smill, V., 2000. *Feeding the World: A Challenge for the 21st Century*, Cambridge.

Tchobanoglous, G., Burton, F. L., and Stensel, H. D., 2003. Wastewater engineering: Treatment and reuse, New York.

Thomas, M., Wright, P., Blackall, L., Urbain, V., and Keller, J., 2003. Optimisation of Nossa BNR plant to improve prformance and reduced operating costs. Water Science and Technology, 47(12), 141–148.

Tong, J. and Chen, Y., 2007. Enhanced biological phosphorus removal driven by short-chain fatty acids produced from waste activated sludge alkaline fermentation. Environmental Science and Technology, 41(20), 7126–30.

Torrijos, M., Vuitton, V., and Moletta, R., 2001. The SBR process: an effiecient and economic solution for the treatment of wastewater at small cheesemaking diaries in the Jura Montains. Water Science and Technology, 43(3), 373–380.

UN-Water, 2006. *Coping with water scarcity: A strategic issue and priority for system-wide action*. International Decade for Action Water for Life 2005-2015.

Van Niel, E. W. J., Appeldoorn, K. J., Zehnder, A. J. B., and Kortstee, G. J. J., 1998. Inhibition of anaerobic phosphate release by nitric oxide in activated sludge. Applied Environmental Microbiology, 64, 2925-2930.

Vives, M T, Balaguer, M. D., García, S., García, R., and Colprim, J., 2003. Textile dyeing wastewater treatment in a sequencing batch reactor system. Journal of environmental science and health Part A-Toxic/Hazardous substances and environmental engineering, A38(10), 2089–2099.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedl, D., and Schleifer, K. H., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinetobacter and its application for in situ monitoring in activated sludge. Applied and Environmental Microbiology, 60(3), 792–800.

Wang, X., Zeng, R. J., Dai, Y.,Peng, Y., and Yuan, Z., 2008. The denitrification capability of cluster 1 *Defluviicoccus vanus*-related glycogen-accumulating organisms. Biotechnology and Bioengineering 99(6), 1329-1336.

Whang, L. M. and Park, J. K., 2006. Competition between polyphosphate and glycogen-accumulating organisms in enhanced-biological-phosphorus-removal systems: effect of temperature and sludge age. Water Environment Research, 78(1), 4–11.

Wilderer, P. A., Irvine, R. L., and Goronszy, M. C., 2001. Sequencing batch reactor technology. IWA Scientific and technical report, 10. London, UK.

Winter, C. T., 1989. The role of acetate in denitrification and biological phosphate removal in modified Bardenpho systems. Water Science and Technology, 21, 375-385.

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

Wong, M. T., Mino, T., Seviour, R. J., Onuki, M., and Liu, W. T., 2005. In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Research, 39(13), 2901–14.

Yuan, Q., Sparling, R., Lagasse, P., Lee, Y. M., Taniguchi, D., and Oleszkiewicz, J. A., 2010. Enhancing biological phosphorus removal with glycerol. Water science and technology : a journal of the International Association on Water Pollution Research, 61(7), 1837–43.

Zeng, R. J., Saunders, A. M., Yuan, Z., Blackall, L. L.,and Keller, J., 2003. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnology and Bioengineering, 83 (2), 140–148.

Zilles, J. L., Peccia, J., Kim, M., Hung, C., and Noguera, D. R., 2002. Involvement of Rhodocyclus-related organisms in phosphorus removal in full-scale wastewater treatment plants. Applied and Environmental Microbiology, 68(6), 2763–2769.

Chapter 2

OBJECTIVES

Enhanced Biological Phosphorus Removal (EBPR) is considered as the most economical and environmentally sustainable alternative for phosphorus removal from wastewaters since it does not need chemical addition and reduces the costs. EBPR fundamentals have been thoroughly studied and EBPR operation is fairly well understood at lab, pilot and real scale. Nevertheless, several problems still arise when integrating EBPR in a full-scale WWTP aiming at simultaneous carbon, nitrogen and phosphorus removal such as i) the integration of an anaerobic phase in the anoxic/aerobic process for N and COD removal, ii) the interaction of nitrification products with the EBPR process, iii) the limitation of carbon sources available in EBPR for wastewaters with non-favourable C/N/P ratios and iv) the recognized but not totally solved yet competition between PAO and GAO for the carbon source.

For these reasons, the aim of this thesis is to understand and propose solutions for these current bottlenecks in view of upgrading current WWTP to include EBPR process. Thus, the thesis is divided into three different goals:

- Study of the interaction between products and by-products of the biological nitrogen removal processes and EBPR in view of its simultaneous implementation.

- Development and implementation of new strategies to treat carbon shortage wastewaters through the utilisation of alternative carbon sources other than the typical VFA. These alternative carbon sources should be chosen considering its availability and price.

- Extension of the current knowledge on the PAO-GAO competition by proposing a new methodology to enrich an activated sludge in PAO disfavouring, at the same time, GAO growth.

Chapter 3

MATERIALS AND METHODS

This chapter comprises the chemical and microbiological analyses performed in the present research. All the experiments presented in this work were conducted at the UAB facilities in a pilot plant consisting of two identical fully monitored SBRs and a third SBR that was slightly different. Since the reactor configuration, synthetic media and monitoring tools used in each of the experiments were different, a specific section devoted to materials and methods is available in each chapter.

3.1 EBPR-SBRs PILOT PLANTs

3.1.1 EQUIPMENTS AND SOFTWARE

The pilot plant was located in the Department d'Enginyeria Química at UAB and consisted of three different SBRs. Figure 3.1 shows the picture of two identical SBRs (10 L) fully monitored. Both SBRs were controlled by a PLC (Siemens SIMATIC S7-226), which was on top of the control system. It controlled the inlet and the outlet pumps, the air and nitrogen electrovalves, the mechanical stirring, the pumps for acid and base dosage and received all the analogic measurements signals. The PLC was connected via RS-232 to a PC, which monitored and stored the data received through a software programed with Visual Basic 6.0 (Figures 3.2 and 3.3).

Figure 3.1. EBPR-SBRs Pilot Plant.

For a typical PAO-enriched sludge configuration, the SBRs were operated with 4 cycles per day with a controlled temperature of 25°C by a water bath. Each cycle consisted of 2 h anaerobic phase, 3.5 h of aerobic phase, 25 min of settling and extraction of 5 L of supernatant during the last 5 min of the cycle. A volume of 5 L of synthetic wastewater was added during the first 5 min of the anaerobic phase, producing a hydraulic retention time (HRT) of 12 h. The sludge retention time (SRT) was kept at 15 days by periodic sludge wastage at the end of the aerobic phase. For each SBR, the pH was

measured with a Crison 5203 probe and controlled at 7.5±0.1 with an on/off controller programed in the PLC acting over two peristaltic pumps (Watson Marlow 400 FS/M1) dosing HCl (1M) or NaOH (1M). During the anaerobic phase, nitrogen was sparged 30 seconds of each 5 min to avoid oxygen transfer from the surface. The dissolved oxygen was measured with a Hamilton Oxyferm 120 probe and was controlled during the aerobic phase from 3.5 to 4.5 $mg \cdot L^{-1}$ using an on/off controller acting on an electrovalve which allowed the opening/closing of the aeration.

The software of the PC was in charge of data acquisition from the PLC, monitoring of the system and storage of all the analogical data (Figures 3.2 and 3.3). It was also in charge of calculating and monitoring OUR and titrimetry data.

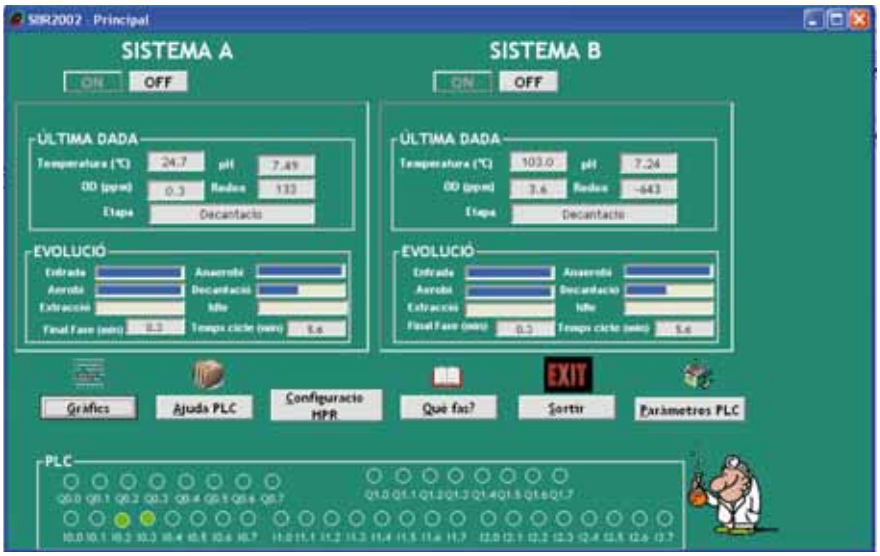

Figure 3.2. Screenshot of the SBRs state in the monitoring program.

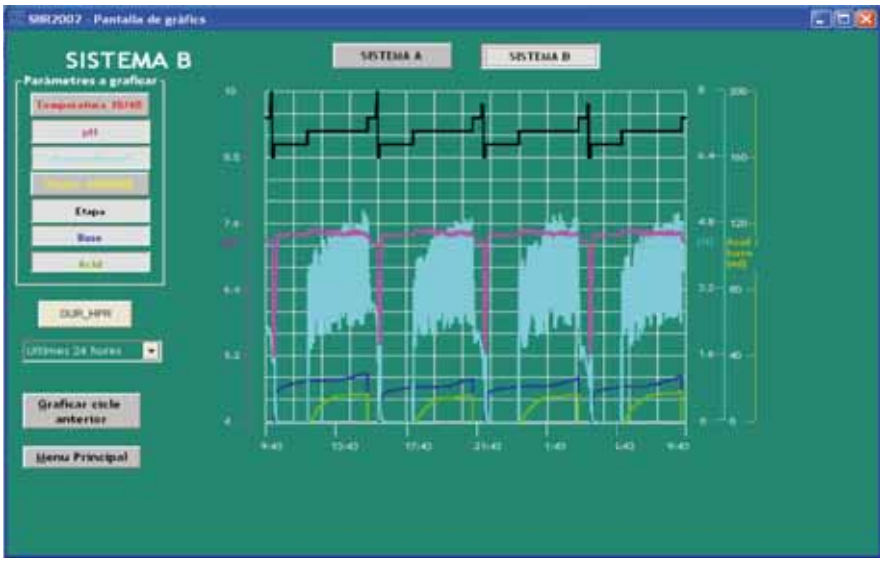

Figure 3.3. Screenshot of the graphical monitoring of SBR-B.

The third SBR was different from the others. It had a volume of 13 L and the liquid interchanged was usually 4 L. The pH probe was a Hamilton polilyte Pro120, and the dissolved oxygen was measured with a WTW Cellox 325 probe connected to a WTW Oxi-340 multiparametric equipment. The length and the configuration of the cycle depended on the experiment performed.

The software for this SBR (AddControl, Figure 3.4) was programed in Labwindows/CVI 2010 and it monitored and controlled all the equipment connected to an Advantech PCI 1711 data acquisition card.

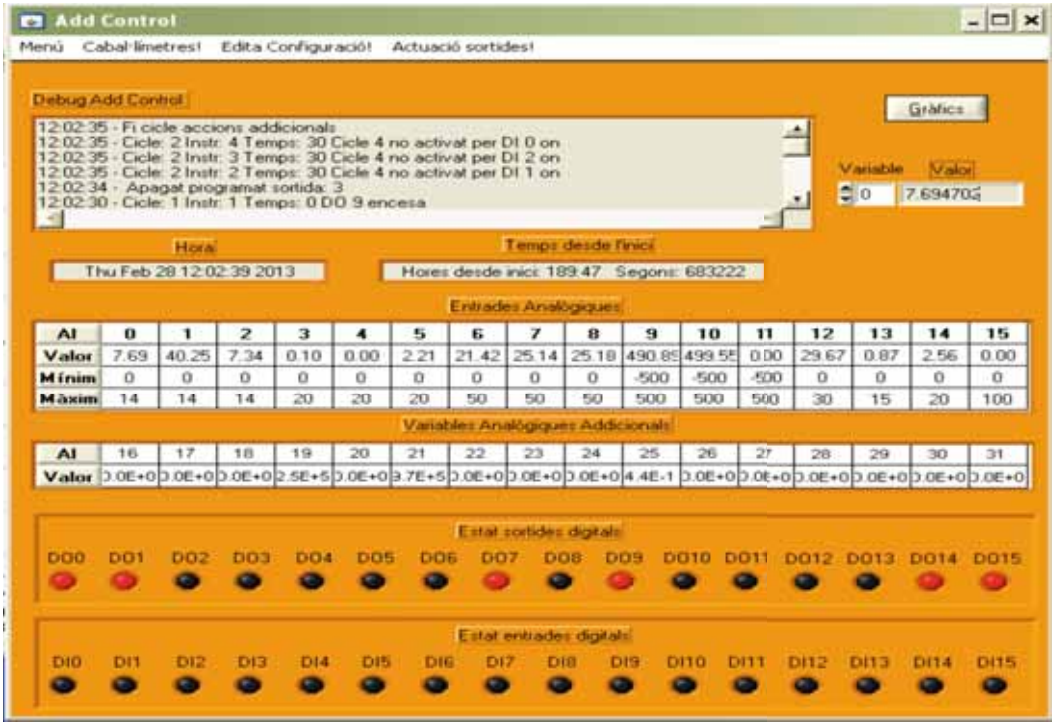

Figure 3.4. Screenshot for the control software of the third SBR.

3.1.2. TITRIMETRIC MEASUREMENTS

Titrimetric techniques were implemented in all the reactors with an accurate monitoring of the acid and base dosage to control the pH at 7.5. The proton production (HP, mmol H^+) was calculated according to equation 3.1.

$$HP = V_{Base} \cdot C_{Base} - V_{Acid} \cdot C_{Acid} \quad (3.1)$$

Where V_{Base} and V_{Acid} stand for the accumulated base and acid dosage (mL), C_{Base} and C_{Acid} for base and acid concentration (M).

3.2. CHEMICAL AND BIOCHEMICAL ANALYSES

3.2.1. PHOSPHORUS AND NITROGEN SPECIES (AMMONIA, NITRATE AND NITRITE)

All chemical samples were filtered with 0.22 μm Milex GP units (Millipore).

Orthophosphate-phosphorus ($P\text{-}PO_4^{-3}$) concentration samples were measured by a phosphate analyser (115 VAC PHOSPHAX sc, Hach-Lange) based on the Vanadomolybdate yellow method, where a two-beam photometer with LEDS measured the phosphate specific yellow colour. The detection limits were from 0.05 $mg{\cdot}L^{-1}$ to 15 $mg{\cdot}L^{-1}$ of $P\text{-}PO_4^{3-}$. It had an accuracy and a reproducibility of 2% ± 0.05 $mg{\cdot}L^{-1}$. Ammonia ($N\text{-}NH_3$) was analysed by a continuous flow analyser (CFA) based on a potentiometric determination of ammonia (Baeza et al., 1999).

In some experiments, phosphate and ammonia nitrogen were also measured using Dr. Lange kits: TNT 844, TNT 845, TNT 846 for phosphorus and LKC 303 for ammonium, using the DR2800 Hach-Lange spectrophotometer.

Nitrate ($N\text{-}NO_3^-$) and nitrite ($N\text{-}NO_2^-$) were quantified by ionic chromatography (ICS-2000 Dionex) with an IonPac AS9-HC column and Anion Self-Regenerating suppressor in autosuppression recycle mode (ASR ULTRA II 4mm). Eluent solution consisted of KOH 10mM. The conditions of the analyses were 30°C, 25 μL of volume injection, $1mL{\cdot}min^{-1}$ of flow injection, and 33 min of analysis time. The same equipment and methods were used in some experiments for the measurement of phosphate ($P\text{-}PO_4^{3-}$).

3.2.2. VOLATILE FATTY ACIDS (VFA)

All chemical samples were filtered with 0.22 μm Milex GP units (Millipore).

Acetic and propionic acid were measured by gas chromatography (GC) in an Agilent Technologies 7820A apparel equipped with a BP21 SGE column (30 m x 0.25 mm x 0.25mm; length x internal diameter x film thickness) and a flame ionisation detector (FID). A sample of 1 μL was injected at a temperature of 275°C under pulsed split conditions (29 psi). The carrier gas was helium with a split ratio of 10:1 at 2.9 ml/ min, the column temperature was set at 85°C for 1 min, followed by an increase of $3°C{\cdot}min^{-1}$ to reach 130°C. A second ramp of $35°C{\cdot}min^{-1}$ was maintained to reach 220°C. A cleaning step at 230°C during 5 min was used to remove any residue in the column. The run time was 20 min and the detector temperature was set at 275°C.

3.2.3. TOTAL SUSPENDED SOLIDS AND VOLATILE SUSPENDED SOLIDS

Total suspended solids (TSS) represents the total amount of suspended inorganic and organic matter in the mixed liquor sample. Volatile suspended solids (VSS) correspond to the volatile organic matter, which is a rough estimation of the biomass present. Both were analysed according to Standard Methods (APHA, 1995): 25 mL of mixed liquor were filtered through glass fibre filter (Whatman GF/C) previously dried up and weighed (W_0). The paper filter was introduced into the oven at 100°C overnight and placed in the desiccator 2h before being weighed again (W_1). After that, the filter was

placed in the furnace using a ceramic bowl during 30 min at 550°C and then in the desiccator for 2h before being weighed again (W_2). TSS was the difference between W_0 and W_1. VSS were quantified by the difference between W_1- W_2. Both results were expressed as mass per volume of filtered sample (i.e. $mg \cdot L^{-1}$). Triplicate analyses were done for each sample.

3.2.4. GLYCOGEN

Glycogen was determined by a modification of the method of Smolders et al. (1994) detailed in Pijuan (2004). This method consisted in the centrifugation of a certain volume of the mixed liquor of the sludge at 6000 rpm for 10 min and then the extraction of the supernatant. Afterwards, this sludge was frozen at -80°C before being lyophilised. A volume of 5 mL of HCl 0.6 M was added to each 20 mg of lyophilised sludge sample in screw topped glass tube, and then was heated at 100°C for 6 h. After cooling and filtering through 0.22 µm filter (Milipore), the concentration of glucose was measured using an YSI Model 2700 Select Biochemistry Analyser (Yellow Spring Instrument). Triplicates of each sample were done.

3.2.5. POLY-HYDROXYALKANOATES (PHA)

The different fractions of PHA (PHB, PHV and PH2MV) were quantified according to Oehmen et al. (2005). 40 mg of the lyophilized sludge sample was digested and methylated with 4 mL of acidified methanol with sulphuric acid, and 4 mL of chloroform, during 20 h at 100°C. The percentage of sulphuric acid depended on the carbon substrate used in the experiments (e.g. if propionic acid was used as a sole carbon source, the percentage was 10%). Benzoic acid was used as internal standard. The standards for the calibration were performed using 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).

After cooling, 1 mL of Mili-Q water was added and the continent was shaken vigorously for 1 min. One hour of settling was allowed for phase separation. The chloroform phase was then extracted from the samples, mixed with 1 g of sodium sulphate, and then separated from the solid phase and transferred to CG vials. A volume of 1 µL of sample was injected at GC system (Hewlett Packard 5890), operated with an INNOVAX column (30 m length x 0.53 mm internal diameter x 1 µm film thickness, Agilent), at 220°C and a flame ionisation detector (FID) at 275°C. The carrier gas was helium with a split ratio of 1:2, the column temperature was set at 70°C for 2 min, followed by an increase of $10°C \cdot min^{-1}$ to reach 160°C and was maintained 2 min at this temperature. Triplicates of each sample were done.

3.3. MICROBIAL ANALYSES

3.3.1. FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

In situ hybridization with rRNA-targeted oligonucleotide probes has become a widely applied tool for direct analysis of microbial population structures. This technique consists of oligonucleotides (short strands of nucleic acids) complementary to 16S rRNA sequence regions. They may form stable associations with 16S rRNA ribosomal

from fixed bacterial cells. 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 out from the cell. Otherwise, if the complementary sequence is present, the oligonucleotide hybridises the cell jointly with a fluorochrome. Cells with the fluorocesntly-labelled oligonucleotide (which have been hybridized with the 16S rRNA in the ribosome) can be directly visualised in a epifluorescent microscope or Confocal Laser Scanning Microscope (CLSM).

Sludge samples from the reactors were taken in order to use the FISH technique described by Amann (1995). The probes used in this thesis are shown on table 3.1. The mixed probes are: Cy5-labelled EUBMIX for most of bacteria (Daims et al., 1999); Cy3-labelled PAOMIX for *"Candidatus Accumulibacter phosphatis"*, comprising equal amounts of probes PAO462, PAO651 and PAO846 (Crocetti et al., 2000); Cy5-labelled PAO I for cluster I of *"Candidatus Accumulibacter phosphatis",* using Acc-I-444 probe; Cy5-labelled PAO II for cluster II of *"Candidatus Accumulibacter phosphatis",* using Acc-II-444 (Flowers et al., 2009)*;* Cy3-labelled GAOMIX for *"Candidatus Competibacter phosphatis"*, comprising equal amounts of probes GAOQ431 and GAOQ989 (Crocetti et al., 2002); Cy3-labelled DFIMIX for cluster I of "*Defluvicoccus vanus"* GAO, comprising TFO_DF218 and TFO_DF618 probes (Wong et al.,2004); and Cy3-labelled DFIIMIX for cluster II of *"Defluvicoccus vanus"* GAO, comprising DF988 and DF1020 probes plus helper probes H966 and H1038 (Meyer et al., 2006).

Table 3.1. Oligonucleotide probes used in this thesis.

Probe	Specificity	Fluorochrome	Reference
EUB338	Many but not all Bacteria	Cy5	Amann et al. (1995)
EUB338-II	Planctomycetales	Cy5	Daims et al. (1999)
EUB338-III	Verrucomicrobiales	Cy5	Daims et al. (1999)
PAO462	*"Candidatus Accumulibacter phosphatis"*	Cy3	Crocetti et al. (2000)
PAO651	*"Candidatus Accumulibacter phosphatis"*	Cy3	Crocetti et al. (2000)
PAO846	*"Candidatus Accumulibacter phosphatis"*	Cy3	Crocetti et al. (2000)
Acc-I-444	*"Candidatus Accumulibacter phosphatis"* cluster I	Cy5	Flowers et al. (2009)
Acc-II-444	*"Candidatus Accumulibacter phosphatis"* cluster II	Cy5	Flowers et al. (2009)
GAOQ431	*"Candidatus Competibacter phosphatis"*	Cy3	Crocetti et al. (2002)
GAOQ989	*"Candidatus Competibacter phosphatis"*	Cy3	Crocetti et al. (2002)
TFO_DF218	*"Defluviicoccus-related TFO"*	Cy3	Wong et al. (2004)
TFO_DF618	*"Defluviicoccus-related TFO"*	Cy3	Wong et al. (2004)
DF988[1]	*"Defluviicoccus-vanus"* cluster II	Cy3	Meyer et al. (2006)
DF1020[2]	*"Defluviicoccus-vanus"* cluster II	Cy3	Meyer et al. (2006)
H966	Helper probe	-	Meyer et al. (2006)
H1038	Helper probe	-	Meyer et al. (2006)

[1] DF988 used in conjunction with helper probes H966 and H1038
[2] DF1020 used in conjunction with helper probe H1038

The samples were fixed by 4% paraformaldehyde solution in a phosphate buffer 0.03M. All these probes were treated in a formamide solution (35%) at 46°C for 2h. FISH slides were visualised with a microscope Olympus Fluoview 1000 CLSM. The quantification of the different cells hybridized as a proportion of all bacteria was done using image analysis techniques as described in Jubany et al. (2009). 40 randomly chosen CLSM fields from different x, y and z coordinates were treated using the Matlab Image Processing Toolbox. Ten images obtained with the same sludge and procedures, but without addition of the probes were used for the evaluating the autofluorescence of the sample. The threshold in pixel intensity for removing autofluorescence was selected so that 99.99% of pixels with positive signal of these images were not considered.

3.3.1.1. FISH protocol

A) Sample fixation:

The sample fixation solution was a paraformaldehyde (PFA) solution prepared with 4 g PFA in 65 mL of Milli-Q water heated to 60°C. Then, 2M NaOH were added drop by drop and stirred rapidly until the solution was nearly clarified (1-2 min). The solution was removed from the heat source and 33 mL of 0.03 M PBS solution were added. Afterwards, pH was adjusted to 7.2 with HCl. Any remaining crystal was removed by sterile filtration (0.22 µm). The solution was cooled to 4°C and stored at this temperature for no longer than 2 days or stored at -20 °C in 1.5 mL aliquots (in a 2mL centrifuges tubs).

For sample fixation, 3 volumes of PFA solution (1.5 mL) were added to 1 volume of sample (0.5 mL) and it was held at 4 °C for 1-3 h (the sample could be centrifuged in case a high biomass concentration was required). Subsequently, the cells were pelleted by centrifugation (5000g) and the fixative was removed. The cells were washed twice with 1 M PBS, centrifuging each time. Then, the cells were resuspended and mixed in one volume (0.5 mL) of 1 M PBS per one volume (0.5 mL) of ice-cold ethanol.

B) Application of samples to the slides:

Depending on the sample concentration, around 5-20 µL of fixed sample was added to each well in the glass slide and let air dry or in a heater (max. 60°C). Then, samples were dehydrated in ethanol series (3 min each): 50%, 80% and 98% and let air dry.

C) Probe hybridization:

The hybridization buffer (Table 3.2) was prepared in 2 mL microcentrifuge tubes. 8 µL of this hybridization buffer were added to each well on the slide. The remainder was poured in the 50 mL hybridization falcon tube that contained cellulose tissue. 1 µL of probe working solution was added and mixed carefully on each well. The slide was placed into the 50 mL tube containing the moistened tissue, and was closed and putted in the hybridization oven at 46°C for 2 h. The working formamide concentration was 35% but generally it depends on the probe used.

Table 3.2. Composition of hybridization buffer.

Component	Volume to prepare 2 mL (microcentrifuge tube)
5M NaCl (autoclaved)	360 µL
1M Tris/HCl (autoclaved)	40 µL
10% SDS (not autoclaved)	2 µL
Formamide	700 µL
MilliQ water	898 µL

D) Washing:

The composition of the washing buffer solution is shown on Table 3.3. This solution was heated at 48**°**C in a water bath during the hybridization process. After 2 h of hybridation, the slides were carefully removed from the tube and were immersed into the washing buffer tube and into the water bath at 48°C for 10-15 min. Rapid transfer of slides prevents cooling and avoids non-specific probe binding.

Table 3.3. Washing buffer composition.

Component	Volume to prepare 50 mL (falcon tube)
5M NaCl (autoclaved)	80 µL
0.5M EDTA (autoclaved)	500 µL
1M Tris/HCl (autoclaved)	1 mL
MilliQ water	43.8 mL
10% SDS (not autoclaved)	50 µL

After the warm washing, the slide was gently rinsed with cold Milli-Q water. Water was directed above wells and allowed to flood over them. Both sides of the slide were washed to remove any salt, which is highly autofluorescent. After the washing step, all water droplets had to be removed from the wells by applying compressed air direct at the side of the slide.

E) Embedding:

A few drops of anti-bleaching reagent (Citifluor AF1) were applied to the wells on slides. Slides were covered with a large coverslip that had to be pressed down gently to remove excess reagent. Slides, at this point, could be kept at -20**°**C for some weeks without fluorescent losing.

3.4. REFERENCES

Amann, R. I., 1995. In situ identification of microrganisms by whole cell hybridation with rRNA targeted nucleic acid probes. Molecular microbial ecology manual. Edited by A. D. L. Akkermans, J. D. van Elsas and F. J. de Bruijn. London: Kluwer Academic Publications.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater Part 9000 Microbiological examination Standard Methods for the Examination of Water and Wastewater. Water and Wastewater, 19th ed. Port City Press, Baltimore, (1).

Baeza, J., Gabriel, D., and Lafuente, J., 1999. An expert supervisory system for a pilot WWTP. Environmental Modelling & Software, 14(5), 383–390.

Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D., Blackall, L. L, 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Applied and Environmental Microbiology, 66(3), 1175–1182.

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

Daims, H., Bruhll, A., Amann, R., Schleifer, K., and 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. Systematic and applied microbiology, 444, 434–444.

Flowers, J., He, S., Yilmaz, S., Noguera, D., McMahon, K.D., 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different '*Candidatus Accumulibacter*' clades. Environmental Microbiology Reports. 1 (6), 583-588.

Jubany, I., Lafuente, J., Carrera, J., and Baeza, J. A., 2009. Automated thresholding method (ATM) for biomass fraction determination using FISH and confocal microscopy. Journal of Chemical Technology and Biotechnology, 84(8), 1140–1145.

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

Oehmen, A., Lehmann, B. K., Zeng, R. J., Yuan, Z., and Keller, J., 2005. Optimisation of poly-β-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. Journal of Chromatography A, 1070(1-2), 131–136.

Pijuan, M., 2004. Effect of different carbon sources and continous aereation on EBPR process. PhD Thesis. Universitat Autònoma de Barcelona. Catalunya

Smolders, G. J. F., Meij, J., van Loosdrecht, M. C. M., and Heijnen, J. J., 1994. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnology and Bioengineering, 44, 837–849.

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

Chapter 4

INTERACTIONS BETWEEN BIOLOGICAL NITROGEN AND PHOSPHORUS REMOVAL

Part of this chapter has been published as:

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Guerrero, J., Tayà, C., Guisasola, A., Baeza, J. A., (2012). Understanding the detrimental effect of nitrate presence on EBPR systems: effect of the plant configuration. Journal of Chemical Technology and Biotechnology, 87, 1508–1511.

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Different configurations and strategies for simultaneous biological removal of organic matter and nutrients (N and P) in wastewater have been proposed. One of the major drawbacks of these configurations is the need of three different phases (anaerobic/anoxic/aerobic). This work demonstrates a new successful strategy to bring in enhanced biological phosphorus removal (EBPR) to a conventional nitrification/denitrification system by means of bioaugmentation with an enriched culture of phosphorus accumulating organisms (PAO). This strategy was tested in a sequencing batch reactor (SBR), where an 8 h configuration with 3 h anoxic, 4.5 h aerobic and 25 min of settling confirmed that nitrification, denitrification and PAO activity could be maintained for a minimum of 60 days of operation after the bioaugmentation step. The successful bioaugmentation strategy opens new possibilities for retrofitting full-scale WWTP originally designed for only nitrification/denitrification since the inclusion of an anaerobic phase would not be needed

4.1. INTRODUCTION

To meet more stringent requirements for discharge and reuse, most WWTP will need to undergo significant modifications and improvements (Tchobanoglous et al., 2003). The individual fundamentals of biological nitrogen removal or EBPR are nowadays well known. However, achieving simultaneous carbon, nitrogen and phosphorus removal requires alternating anaerobic, anoxic and aerobic phases, which can be obtained in conventional continuous systems (A^2/O or UCT) or in sequencing batch reactor (SBR) configurations.

EBPR requires alternating anaerobic and aerobic/anoxic conditions (Comeau et al., 1986), being the objective of the anaerobic phase to favour the PAO versus other heterotrophic organisms when competing for the electron donor (i.e. organic matter). It means that the effectiveness of the enrichment strongly depends on the nature of the electron donor: high percentage of volatile fatty acids (VFA) in the influent COD is critical to obtain a bacterial population with high P-removal capacity (Guerrero et al., 2011; Randall et al., 1997). As mentioned in chapter 1, under anaerobic conditions, PAO are able take up organic substrates (preferably short chain volatile fatty acids, VFA) and store them as polyhydroxyalkanoates (PHA). The competitors of PAO, GAO, are also capable of anaerobic VFA uptake but without contributing to P removal. Therefore, GAO are a major cause of EBPR failure as they are selected under similar conditions. The competition between PAO–GAO has been intensively studied and modelled due to its impact on P removal performance and efficiency for a long time (Cech et al., 1993; McIlroy and Seviour, 2009; Mino et al. 2004; Oehmen et al., 2007). Several key factors have been identified in the PAO–GAO competition (see chapter 1, section 1.5.1.1).

Understanding the wide EBPR scenario becomes harder when the interaction with biological nitrogen removal is considered (Patel and Nakhla, 2006). A fraction of PAO, called denitrifying PAO (DPAO), are able to use nitrate or nitrite as electron acceptor in addition to oxygen. This denitrification ability is highly beneficial since it leads to simultaneous denitrification and anoxic-P uptake resulting in a reduction of carbon requirements and sludge production (Murnleitner et al., 1997). However, experimental research with PAO and GAO has shown different denitrification capabilities on both populations. In the case of PAO, two different clades have been identified according to the presence of polyphosphate kinase gene (ppk1) (He et al., 2007) and each clade shows different denitrification capabilities. PAO able to reduce nitrate and nitrite in

addition to oxygen correlate well with cluster I of *Accumulibacter* (PAO I), while PAO unable to reduce nitrate but able to reduce nitrite have been correlated with cluster II *Accumulibacter* (PAO II) (Carvalho et al., 2007; Flowers et al., 2009; Guisasola et al., 2009). Additionally, GAO also display different denitrifying capacities. Wang et al. (2008) showed that *Defluviicoccus* cluster I was able to reduce nitrate but not nitrite, whereas Burow et al. (2007) suggested that *Defluviicoccus* cluster II was unable to denitrify neither nitrite nor nitrate (see Table 1.3 from chapter 1).

Regarding full-scale EBPR implementation, recent studies have cast doubts on the necessity of the anaerobic phase for achieving net P-removal. It has been proved that EBPR could be achieved in a single SBR-cycle under strictly aerobic conditions (Guisasola et al., 2004; Pijuan et al., 2006; Vargas et al., 2009) despite the fact that textbook knowledge indicates that an excessive aeration may deteriorate the efficiency of the EBPR process (Brdjanovic et al., 1998). Moreover, Ahn et al. (2007) proposed a different aerobic process for low COD wastewater where the addition of acetate is temporally separated from the addition of phosphate. The authors showed that this process was capable of obtaining net P-removal over an extended period. Finally, Wang et al. (2009) showed another single-stage oxic process capable of good P-removal using glucose as a carbon source. Hence, these studies may suggest that EPBR could be maintained, at least for a short period of time, without a mandatory anaerobic phase as long as a PAO-enriched population is present (i.e. PAO are not selected under these conditions but once there, they can survive).

Although, EBPR fundamentals are currently understood, unpredictable EBPR failures still occur in practice when simultaneous P and N removal is required. The presence of nitrate is reported to avoid anaerobic P-release, which only occurred after nitrate depletion (<1 mg $N\text{-}NO_3^- \cdot L^{-1}$) (Akin and Ugurlu, 2004; Patel and Nakhla, 2006). On the one hand, several reports suggested a possible inhibitory effect of some denitrification intermediates (nitrite or nitric oxide) on the EBPR process (Saito et al., 2004; Van Niel et al., 1998). On the other hand, the presence of nitrate or nitrite may also trigger the activity of ordinary heterotrophic organisms (OHO), which reduce nitrate or nitrite using COD as electron donor and result in less COD available for PAO growth (section 1.5.3 chapter 1).

Bioaugmentation is the inoculation of particular microorganisms (either pure cultures or microbial communities) in contaminated environments or bioreactors to accelerate the removal of an undesirable compound (El Fantroussi and Agathos, 2005). This technique is used in WWTPs to improve its performance (e.g., to treat specific contaminants or to reduce start-up periods). The bioaugmentation strategy for PAO has been successfully applied to lab-scale SBR systems, speeding-up the installation of good and stable EBPR in the bioaugmented reactor (Belia and Smith, 1997; Oerther et al., 1998; Dabert et al., 2001, 2005), but always using the classical anaerobic/aerobic configuration.

Considering all this previous background, bioaugmentation of a conventional activated sludge system with PAO-enriched sludge seems to be a possible alternative to achieve simultaneous C/N/P removal for a certain period of time without the necessity of an

anaerobic phase. If this strategy is proved to be successful, it would result in an alternative procedure to implement EBPR in full-scale WWTPs originally designed for only nitrification/denitrification. Thus, the WWTP could be bioaugmented with waste purge of a relatively small anaerobic/aerobic SBR operated in parallel and treating part of the influent wastewater. Within this context, this work aims at testing a novel bioaugmentation strategy of a conventional activated sludge system for C/N removal with PAO-enriched sludge and, during this period, monitoring the biological nutrient removal efficiency and the possible loss of PAO activity.

4.2. MATERIALS AND METHODS

4.2.1. LAB-SCALE SEQUENCING BATCH REACTORS (SBR)

Two SBR (10 L each) were used in this work (Figure 4.1). The PAO-enriched SBR (SBR_{PAO}) had operated as described in section 3.1.1, on chapter 3. The feeding consisted of two different synthetic wastewater solutions, the first solution (4.97 L) consisted of ($mg \cdot L^{-1}$ in reverse osmosis water): 110.5 KH_2PO_4, 83.7 K_2HPO_4, 100 NH_4Cl, 43.9 $MgSO_4 \cdot 7H_2O$, 160 $MgCl_2 \cdot 6H_2O$, 42 $CaCl_2 \cdot 2H_2O$, 50 allylthiourea (ATU) to inhibit nitrification and 30 mL of micronutrient solution consisted of ($g \cdot L^{-1}$): 1.5 $FeCl_3 \cdot 6H_2O$, 0.15 H_3BO_3, 0.03 $CuSO_4 \cdot 5H_2O$, 0.18 KI, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 4H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 \cdot 6H_2O$, and 68.5 mL EDTA 0.5 M. The initial phosphorus concentration was 20 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$. The carbon source solution (usually 0.025 L) was added from a separate concentrated solution (58 $g \cdot L^{-1}$) in order to obtain the desired concentration in the reactor. The temperature was controlled at 25 ± 1°C. HCl (1M) and NaOH (1M) were used to control the pH in the reactive phases at 7.50 ± 0.05. Acid and base were pumped using peristaltic dosage pumps (Watson Marlow 400 FS/M1 OEM).

The second SBR, SBR_N, was enriched with nitrifying and denitrifying organisms. This reactor had an 8 h cycle with two different feedings to distribute the load. Two different configurations were tested in this work. The first one had 4 h of anoxic phase (with two 2-min filling phases at t = 0 h and 2 h), 3 h of aerobic phase followed of 55 min of sedimentation and 5 min of extraction of 3 L of supernatant. The second configuration was distributed as 3 h of anoxic phase (with two 2-min filling phases at t = 0 h and 1.5 h), 4.5 h of aerobic phase, 25 min of sedimentation, and 5 min to extract 3L of supernatant. HRT was 24 h for both configurations of SBR_N. SRT was kept also around 15 days in both configurations. SBR_N was fed with synthetic wastewater that consisted of two different sources. For every 1.5 L of synthetic feed, the carbon source came from a concentrated feed (12 mL) with 72 $g \cdot L^{-1}$ of ethanol and 108 $g \cdot L^{-1}$ of propionic acid. The rest of micro and macronutrients were prepared in a diluted solution (in $g \cdot L^{-1}$): 0.384 NH_4Cl, 0.182 K_2HPO_4, 0.0072 $MgCl_2 \cdot 7H_2O$, 0.00016 $FeSO_4 \cdot 7H_2O$, 0.00010 $MnSO_4 \cdot H_2O$, 0.00016 $ZnSO_4 \cdot 7H_2O$, 0.00008 $CuSO_4 \cdot 7H_2O$, 0.00001 H_3BO_3 and 0.0032 $CaCl_2 \cdot 2H_2O$. The synthetic wastewater composition was then COD/N/P 1300/100/30 ($mg \cdot L^{-1}$). pH was controlled at 7.50 ± 0.05 using acid and base dosage: HCl (1 M) or NaOH (1 M), respectively. Both SBRs were fully monitored as described in chapter 3.

Figure 4.1. SBRs in the Department d'Enginyeria Química at UAB.

4.2.2. SBR_N BIOAUGMENTATION WITH PAO

SBR_N was bioaugmented using 2 L of biomass withdrawn from SBR_{PAO} (6200mg $TSS \cdot L^{-1}$). Once bioaugmented, SBR_N was monitored during a period of 23 days with the first configuration and during 63 days with the second configuration. The reasons for this configuration change are discussed below. A significant nitrification reduction was observed 8 days after the bioaugmentation and it was decided to add 1 L of nitrifying sludge from another SBR of the research group, which was operating for complete nitrification at high ammonia influent levels.

4.2.3. BATCH EXPERIMENTS

Batch experiments were performed in a magnetically stirred vessel (2L) that could be operated either under anaerobic/anoxic or aerobic conditions by sparging nitrogen gas or air respectively. This gas was supplied through a microdiffuser, which ensured good gas transfer to the liquid phase and was controlled with a mass flow meter (HiTec 825, Bronckhorst) to ensure a constant flow. The pH (Sentix 81,WTW) and DO (CellOx 325, WTW) probes were connected to a multiparametric reception equipment (INOLAB 3, WTW) which was in turn connected via RS232 to a PC with Visual Basic 6.0 software, allowing for data monitoring and storage in a Microsoft Excel sheet. Moreover, the software was also capable of manipulating a microdispenser equipment (Microburete 25 Crison) in order to keep the pH constant at 7.50 ± 0.01 with HCl (1M)/NaOH (1M) dosage. The respirometer vessel was thermally controlled at 25.0 ± 0.1°C.

4.2.4. MICROBIAL AND CHEMICAL ANALYSES

Fluorescence in situ hybridization (FISH) technique (Amann et al., 1995) coupled with confocal microscopy was used to quantify the biomass distribution as in Jubany et al. (2009). Hybridizations were performed using at the same time a Cy3-labelled specific probe and Cy5-labelled EUBMIX for most bacteria (Daims et al., 1999). Specific probe used for ammonium oxidising bacteria (AOB) detection was Nso190 (Mobarry et al., 1996) and for nitrite oxidising bacteria (NOB) was NIT3 (Wagner et al., 1996). PAO were hybridized with PAOMIX probes and glycogen accumulating organisms (GAO) with GAOMIX, DFIMIX and DFIIMIX probes according to the methodology described in chapter 3. Finally, PAO I and PAO II were detected with the probes described by Flowers et al. (2009).

Nitrite, nitrate and phosphate were measured using ionic chromatography (DIONEX ICS-2000). Ammonia nitrogen was measured using ammonium kits (Dr. Lange LKC 303) and a spectrophotometer (DR2800 Hach Lange). Mixed COD (with ethanol and propionic) and volatile and total suspended solids were measured according to the standard procedures proposed in APHA (1995).

4.3. RESULTS AND DISCUSSION

4.3.1. BIOAUGMENTATION EXPERIMENTS RESULTS

Figure 4.2 shows a typical SBR_{PAO} cycle where 20 mg $P\text{-}PO_4^{3-}\cdot L^{-1}$ were successfully removed under alternating anaerobic and aerobic conditions. The influent COD was taken up in the first 25 min linked to P-release, which was totally removed under aerobic conditions. SBR_{PAO} microbial community quantification with FISH revealed that PAO were 82% of the microorganisms present in this sludge and that around 90% of PAO were classified as PAO I and 10% as PAO II.

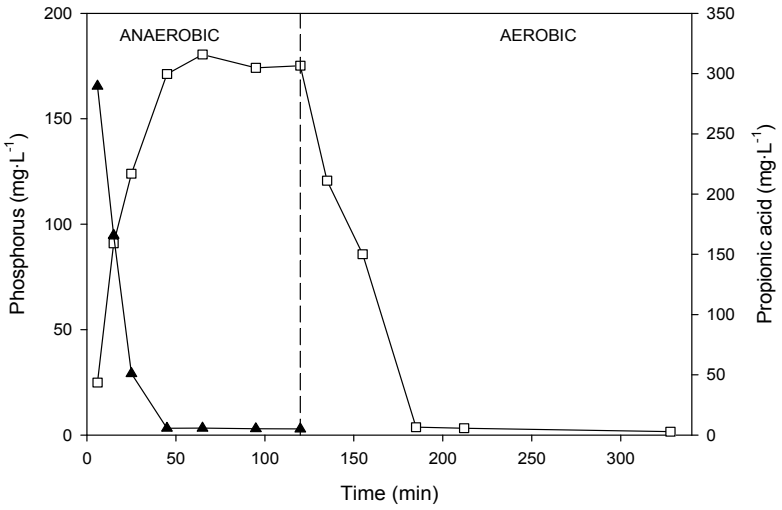

Figure 4.2. Evolution of propionic acid and phosphate in a conventional SBR_{PAO} cycle. Propionic acid (▲) and phosphorus (□).

Figure 4.3 shows the evolution of the main compounds of the SBR_N before and after the bioaugmentation. Figure 4.3a displays a cycle before the bioaugmentation step, when it was operating under stable conditions for biological nitrogen removal with alternating anoxic/aerobic phases. The nitrate produced in the aerobic phase of the previous cycle was used as electron acceptor during the anoxic operation. Regarding phosphorus, a minor increase in the anoxic phase and a slight decrease in the aerobic phase were observed. The profiles of ammonia and nitrate the day after bioaugmentation (Figure 4.3b) were similar to those of day 0, except for the fact that nitrification was not complete at the end of the aerobic stage. P-release was observed after both feeding steps as a consequence of COD storage as PHA, since nitrate levels were very low and the phase was practically anaerobic. Finally, despite phosphorus was taken up during the aerobic stage, net phosphorus removal was not achieved, probably because of the low PAO concentration and the limited length of the aerobic phase.

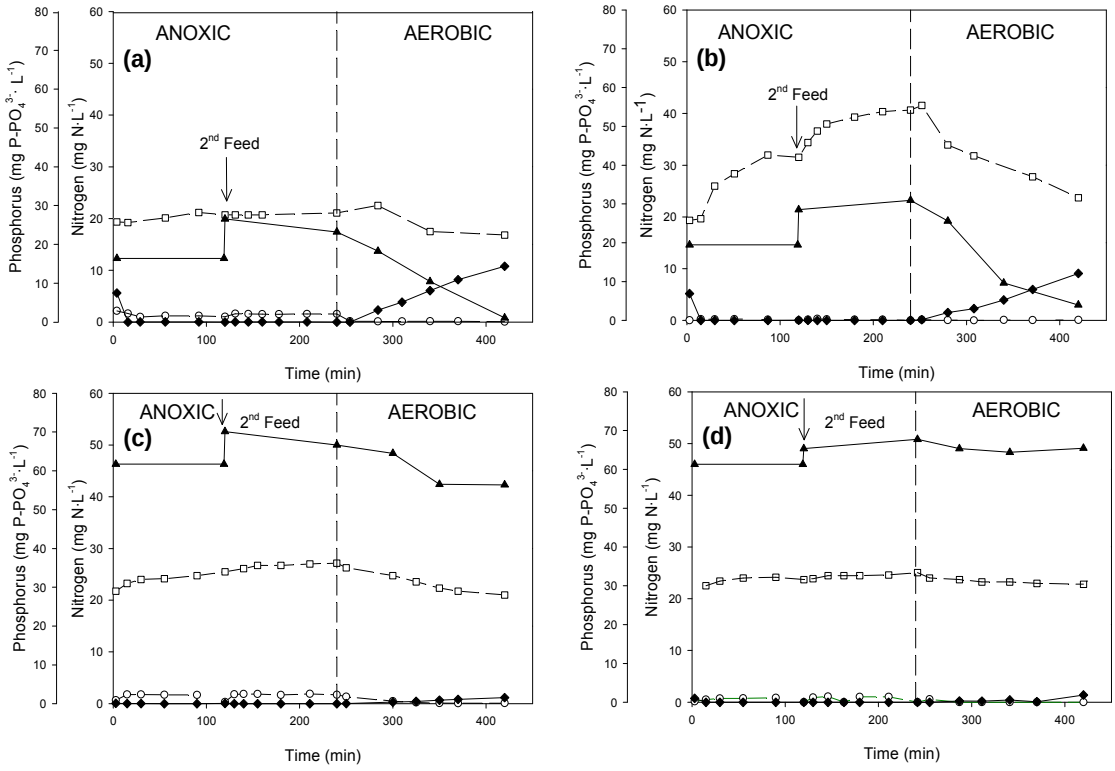

Figure 4.3. Evolution of ammonium, nitrite, nitrate and phosphate in SBR_N cycles during the initial configuration tested for days 0 (a), 1 (b), 11 (c) and 23 (d). The bioaugmentation was performed after the cycle monitored on day 0. Ammonium (▲), nitrate (◆), nitrite (○) and phosphorus (□).

Figure 4.3c–d show two SBR_N cycles on days of operation 11 and 23. Both figures show similar trends. Surprisingly, both nitrogen removal and EPBR processes failed. Ammonia concentration in the reactor increased as a result of a decrease in the nitrifying activity. Nitrification failure resulted in low nitrate production under aerobic conditions and hence, anoxic conditions disappeared. It should be noted that the nitrogen balance was not closed due to some ammonia loss because of stripping occurrence in the aerobic phase. This failure in biological nitrogen removal due to PAO bioaugmentation was unexpected. In addition, the loss of P-release and uptake activity

was also observed (Figure 4.3d). EBPR failure when coexisting with biological nitrogen removal has been previously reported, being nitrate presence under anaerobic conditions the major reported cause (Henze et al., 2008). However, EBPR failure in the present study was also surprising since nitrogen removal activity was lost and the theoretically anoxic phase was in fact anaerobic and thus, PAO favourable conditions were obtained.

4.3.2. RISING SLUDGE PHENOMENON

The cause of the loss of activity seems to be the result of some processes occurring in the settler. The fact that phosphorus uptake was incomplete under aerobic conditions lead to the presence of PAO with significant internal PHA levels after the aerobic phase (i.e. at the beginning of the settling phase). Stored PHA can be used as electron donor for denitrification reducing the nitrate produced under aerobic conditions, resulting in nitrogen gas formation. This gas enhances biomass flotation and hence, a severe loss of biomass settling capacity. Then, part of the biomass would be slowly washed away. This event has been widely described in the literature as rising sludge phenomenon (Henze et al., 1993), a major failure of biological nitrogen removal systems when nitrate is present in the settler and some COD is available. Hence, PAO denitrification using their internal carbon sources and the nitrate produced was the cause of the system loss. Similar problems were detected when combining nitrogen removal with denitrifying PAO in anaerobic/anoxic SBR configurations (Spagni et al., 2001). Rising sludge occurrence was initially unexpected in our configuration since the sludge from SBR_{PAO} had never seen nitrate before, as ATU was used to inhibit nitrification in this reactor. Hence, we observed denitrifying PAO activity with nitrate as electron acceptor even when our PAO were not acclimated to it.

This observation is not minor and should be seriously considered when extending systems for biological C/N removal with EPBR. Rising sludge can occur even when no soluble COD is measured in the influent of the settler. Nitrate presence in the settler is relatively common and PAO are able to accumulate organic matter as PHA and use nitrate as electron acceptor. The selecting factors between *Accumulibacter* cluster I and cluster II are not understood yet; however our results prove that nitrate presence in SBR_{PAO} was not necessary to obtain a microbial community with around 90% of clade IA *Accumulibacter* (i.e. the ones able to denitrify form nitrate) as measured with the FISH analysis.

Figure 4.4 shows two different experiments with sludge from the SBR_{PAO} confirming this hypothesis. A first batch experiment was conducted with sludge withdrawn at the end of the aerobic phase (Figure 4.4a). A pulse of nitrate and COD was added under nitrogen-sparging conditions and it was observed that PAO could denitrify from nitrate simultaneously to COD uptake and P-release. Figure 4.4b shows another experiment conducted in a 1L graduated cylinder. Nitrate was added to SBR_{PAO} sludge withdrawn after the anaerobic phase. This sludge had a high amount of PHA stored and the rising sludge phenomenon was clearly observed. This effect of rising sludge did not take place in the SBR_N before its bioaugmentation because the microbial community present did not have internal COD resources after the aerobic phase.

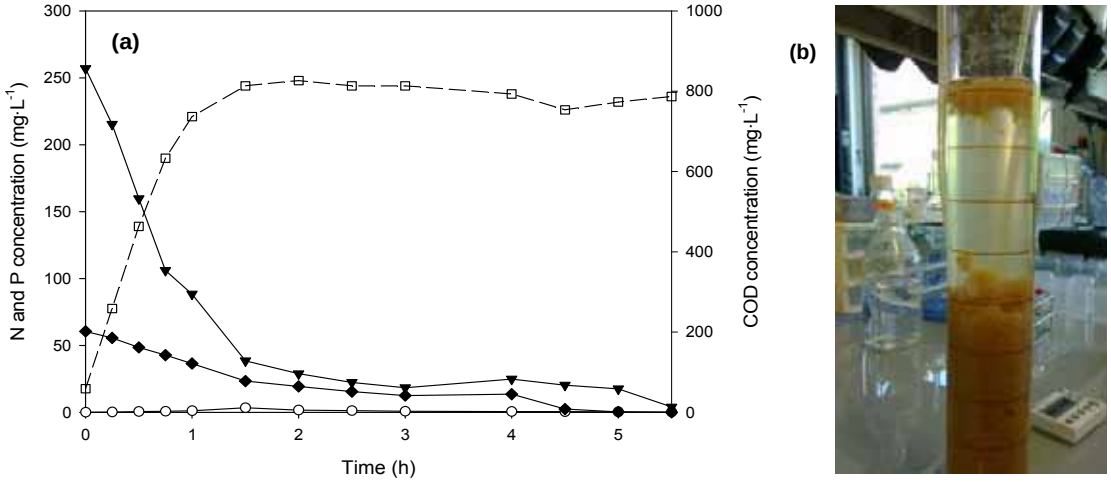

Figure 4.4. (a) Batch experiment under anoxic conditions with sludge withdrawn at the end of the aerobic phase of SBR_{PAO}. COD (▼), nitrate (◆), nitrite (○) and phosphorus (□). (b) Picture of sludge withdrawn at the end of the anaerobic phase of SBR_{PAO} after 2 h of a nitrate addition.

4.3.3. SYSTEM RECOVERY

According to the hypothesis proposed, the rising sludge occurrence could be avoided if the internal PHA levels were lowered at the end of the aerobic phase (i.e. the initial COD load was decreased or the aerobic phase was extended to achieve complete P removal). This would prevent the occurrence of denitrification/rising and the loss of biomass in the settling phase. Then, the aerobic phase was lengthened by 50% (from 3 to 4.5 h) while the anoxic phase was reduced. This minor cycle modification resulted in successful results. Figure 4.5 presents a SBR_N cycle after 37 days of the configuration change. In this cycle, the recovery of the nitrifying biomass can be observed, which entails higher ammonia oxidation in the aerobic phase, and consequently higher nitrate concentration in the anoxic phase of the subsequent cycle. Similarly, EBPR activity was recovered as can be observed in the P-release and P-uptake, resulting in net P-removal.

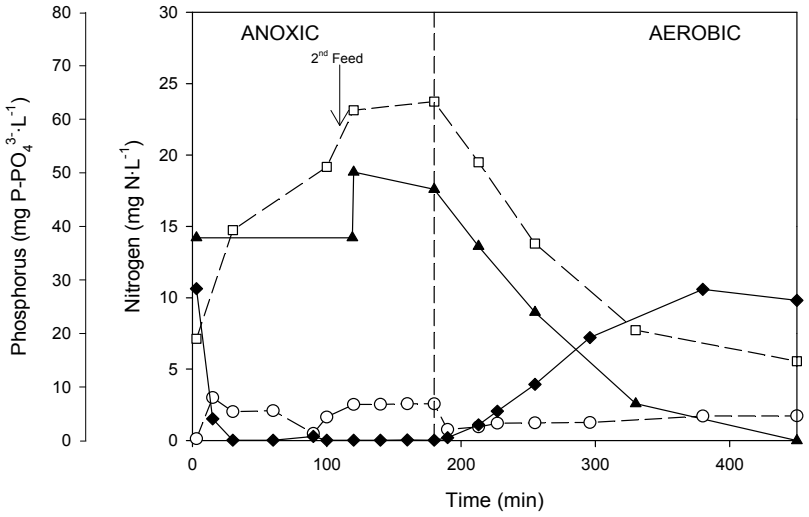

Figure 4.5. Experimental cycle of SBR_N 65 days after the bioaugmentation and 37 days after the change of configuration. Ammonium (▲), nitrate (◆), nitrite (○) and phosphorus (□).

Moreover, Figure 4.6 provides more information about the loss and recovery of the PAO activity due to the proposed configuration change. Figure 4.6a shows the anaerobic P-release and the aerobic P-uptake during this period and the differences among the first and second configuration clearly indicate the benefits of the small configuration change. Figure 4.6b shows the evolution of VSS/TSS ratio at the end of the aerobic phase in the bioaugmentation period. This ratio can be a good indication of the amount of PAO present in the sludge. When PAO are predominant, the VSS/TSS ratio is lower than the average ratio of ordinary heterotrophic and the nitrifying biomass due to their high content in poly-P. During the first configuration period, this ratio increased because PAO were consuming their internal poly-P reserves until the change of configuration. From then on, the ratio decreased again due to PAO growth. These results demonstrate that the first configuration in SBR_N did not allow maintaining EBPR activity due to the reduced duration of the aerobic stage.

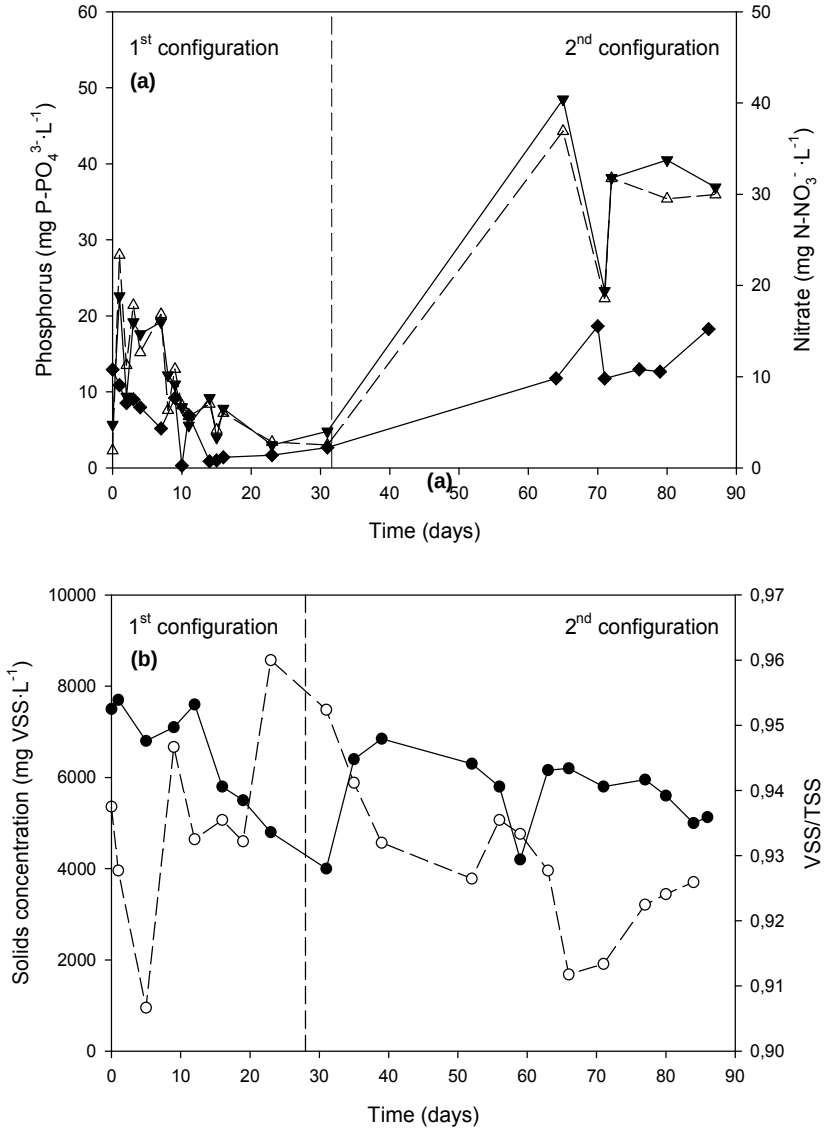

Figure 4.6. Evolution of some important parameters during 86 days of experiment in SBR_N. (a) P-release in the anoxic phase, P-uptake in the aerobic phase and nitrate at the end of the aerobic phase. P-release (△), P-uptake (▼) and nitrate (◆). (b) VSS and VSS/TSS at the end of the aerobic phase. VSS/TSS (○) and VSS(●).

Similarly, Figure 4.6a shows the evolution of nitrate at the end of the aerobic phase during the experiment. A significant decrease of nitrification activity is observed during the first configuration proposed, whereas nitrate concentration increases with the change of configuration. This graph shows the loss and recovery of nitrogen removal activity before and after the increase of the aerobic phase length. It should be noted that at day 7, the system was bioaugmented with nitrifying sludge (1 L) to avoid the nitrification loss; however this bioaugmentation was useless because of the rising sludge.

The successful bioaugmentation strategy obtained in the second configuration differs from the results of Belia and Smith (1997) and Dabert et al. (2005). These works diminished the start up period of conventional anaerobic/aerobic systems suitable for P-removal from 6 to 7 weeks or 29 days, respectively, to 14 days by means of the inoculation of PAO-enriched sludge. However, the presented anoxic/aerobic SBR configuration is a configuration where PAO could be outcompeted by ordinary heterotrophic organisms depending on the nitrate load during the anoxic phase (Hu et al., 2002).

4.3.4. FISH RESULTS

Table 4.1. FISH results from different steps of the bioaugmentation process.

%	Start first configuration	Start second configuration	End Second configuration
PAO (%PAO I)	20.5 (88)	25.6 (94)	16.5 (90)
GAO	1.4	3.1	5.4
AOB	3.7	<1	30
NOB	2.1	2.7	7.5
DFI	21.6	14.6	30
DFII	<1	50.6	6.4

Table 4.1 presents the results of FISH quantification obtained during the bioaugmentation study. The percentage of PAO in the sludge was almost constant during this period, which is in agreement with our hypothesis that the loss of EPBR activity was due to physical reasons (rising sludge phenomenon). The type of PAO detected (around 90% of PAO I, which is able to denitrify from nitrate) is also in accordance with the observed denitrification capability of the sludge. These results demonstrate that rising sludge is an expected problem that can be relevant for the design of future systems for biological C/N removal with EPBR because: (i) nitrate presence in the settler of WWTP is relatively common, (ii) PAO are able to accumulate organic matter as PHA and (iii) PAO I can use nitrate as electron acceptor even when they have not seen nitrate before.

Once the rising sludge phenomenon was solved with the extended aerobic phase, EBPR activity was rapidly recovered (Figure 4.5) since a significant percentage of PAO was still present in the sludge. The low abundance of *Competibacter* was also expected since the system was fed with propionate as carbon source, which is known to select

PAO in front of *Competibacter* (Oehmen et al., 2005). The results for the two groups of *Defluviicoccus* are also in agreement with recent literature findings and with the AOB and NOB presence. It has been reported that *Defluviicoccus* cluster I (DFI) are able to reduce nitrate (Wang et al., 2008) whereas *Defluviicoccus* cluster II (DFII) are unable to denitrify (Burow et al., 2007). In our study, DFI concentration was higher at the beginning of the first configuration and at the final sample of the second configuration, when nitrification activity was occurring and nitrification products (mostly nitrate) were present. Conversely, DFII concentration increased during the first configuration period, linked to the reduced nitrifying activity and low nitrate conditions, but was highly reduced during the second period of operation.

4.3.5. PRACTICAL IMPLICATIONS

Conventional anoxic/aerobic activated sludge reactors aiming at nitrogen removal are unable to perform EBPR, as anaerobic conditions are required to favour PAO growth against ordinary heterotrophs (Mino et al., 1998). In contrast, this work shows that when an anoxic/aerobic SBR was bioaugmented with PAO-enriched sludge and a proper aerobic phase length was selected, the system evolved to a new steady state where nitrogen removal via nitrification/denitrification and EBPR could coexist for a minimum of two months. These results open new possibilities for retrofitting WWTP designed for COD and N removal when stricter discharge limits require the implementation of biological P-removal. For example, a conventional continuous full-scale anoxic/aerobic WWTP configuration could be bioaugmented with PAO obtained in a relatively small anaerobic/aerobic SBR operating in parallel using part of the influent wastewater. Waste sludge of this SBR would be continuously inoculated to the conventional system where PAO would be active providing biological P-removal as demonstrated in this work. The new side-reactor would avoid the modification of the full-scale WWTP, although a careful economic assessment would be required for each specific application to implement the most economical option. Similar approaches based on side-reactors but for nitrification have been previously proposed and applied in full-scale WWTPs by Salem et al. (2004) in the BABE® process and by Kos (1998) in the InNITRI® process.

Regarding the initial configuration tested with shorter aerobic phase, it unexpectedly resulted in a progressive loss of both EBPR activity and nitrification performance due to rising sludge in the sedimentation phase. The presence of PAO I using nitrate as electron acceptor and their internal PHA polymers as electron donor was the cause of rising sludge. This problem is relevant for the design of future systems for biological C/N removal with EPBR. However, the increase of the aerobic phase length, as in the successful second configuration tested, avoided rising sludge by minimising the PHA levels at the end of this phase.

4.3.6. NITRATE PRESENCE IN THE ANAEROBIC PHASE

Nitrate presence on the anaerobic phase and its denitrification intermediates (nitrite or nitric oxide) is usually described in the literature as detrimental for EBPR systems (Akin and Ugurlu, (2004); Patel and Nakhla, (2006); Pijuan et al., (2010); Van Niel et al.,

(1998)). However, inhibition of PAO by nitrate was not detected in the operating conditions of the bioaugmented SBR_N or in the experiment detailed in Figure 4.4a, despite a nitrate presence up to 10 $mg \cdot L^{-1}$ was observed at the start of the anaerobic phase. Therefore, an additional study was performed with PAO biomass to test the effect of different carbon sources and nitrate availability during the supposedly anaerobic phase of the SBR cycle, in order to test if inhibition could appear under other operational conditions.

Several batch experiments were performed aiming at (i) studying the competition for COD between PAO and OHO when the electron donor and nitrate coexisted and (ii) assessing whether nitrate was also an inhibitory compound for EBPR. The procedure in this case was quite different than in previous batch tests. In these experiments, the vessel was filled with biomass extracted from the SBR_{PAO} and was aerated for 12h to ensure the depletion of PHA reserves. After 12h of aeration, a pulse of the electron donor (200 ± 25 mg $COD \cdot L^{-1}$ of acetic acid, propionic acid o sucrose) and nitrate were added under nitrogen-sparging conditions. Figure 4.7 shows the experimental profiles obtained with the PAO-enriched sludge from SBR_{PAO} using acetic acid, propionic acid and sucrose as sole carbon sources. These carbon sources were selected in view of the possibility to use other complex carbon sources for EBPR. Acetic and propionic acid are the typical carbon sources known effective for EBPR, but it could be useful to know if complex carbon sources as sucrose can be an alternative to conduct EBPR.

It is important to emphasize that these experiments were performed several months after the previous experiments, and hence the population in the reactor was not the same as during the bioaugmentation. For these batch experiments the FISH quantification resulted in a different microbial community: 68% of PAO (49% of PAO I and 51% of PAO II) and around 20% of GAO.

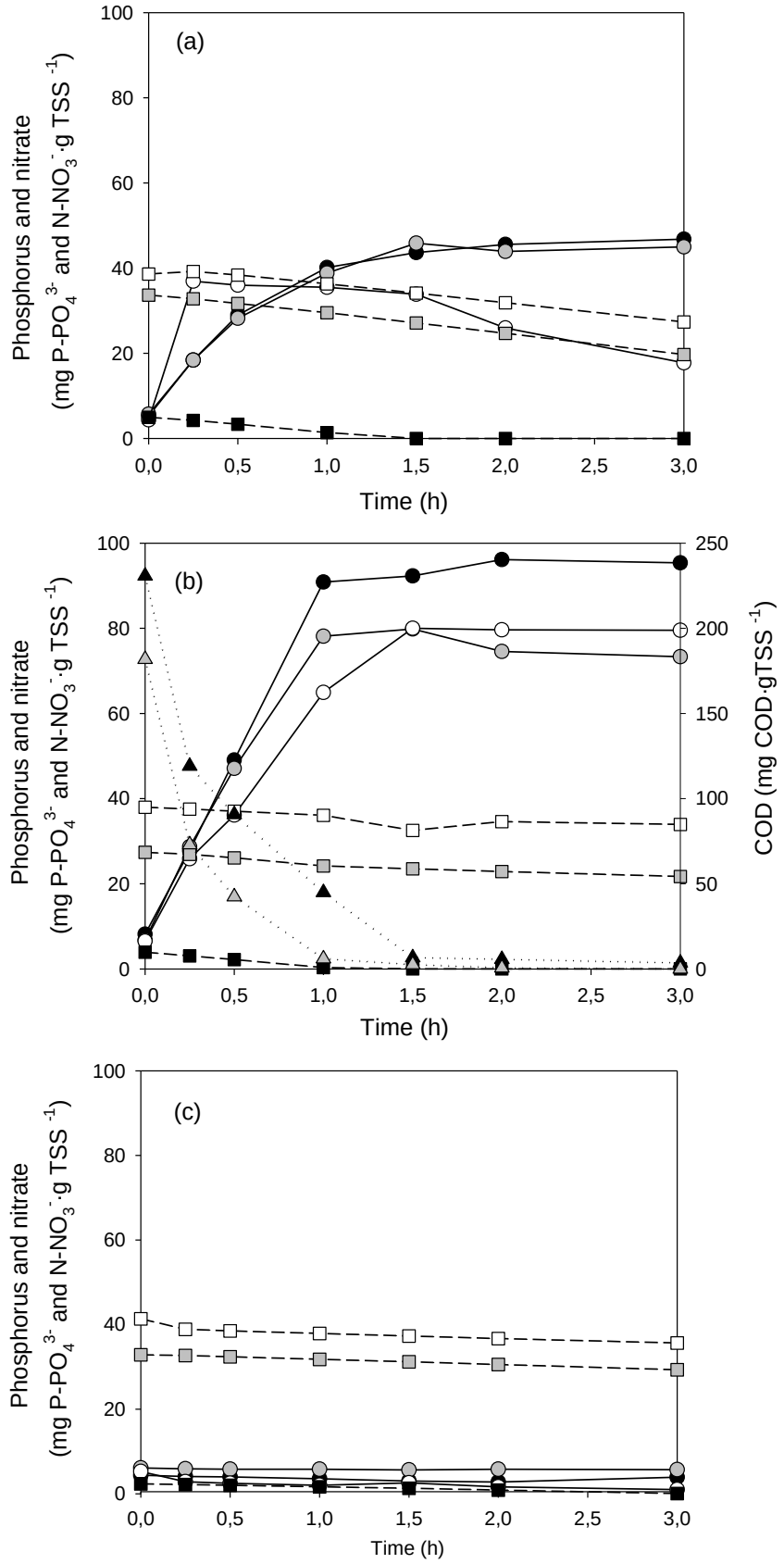

Figure 4.7. Batch tests results obtained with the PAO-enriched sludge from SBR_{PAO} by adding different carbon sources ((a) acetic acid, (b) propionic acid, (c) sucrose). Dotted line and (▲) represent COD, solid line and (●) Phosphorus and dashed line and (■) Nitrate. The symbol filling corresponds to the initial nitrate concentration: 0 $mg \cdot L^{-1}$ (black), 40 $mg \cdot L^{-1}$ (grey) and 60 $mg \cdot L^{-1}$ (white).

Table 4.2 summarises the major experimental conversions obtained. The main outcome of this table is that lower P/C ratios and lower P-release rates were obtained under nitrate presence with both acetic and propionic acids. The highest COD uptake rate was obtained with propionic acid followed by acetic acid and no COD uptake was achieved with sucrose due to the lack of fermentative bacteria able to produce VFA for EBPR. COD uptake rate increased with nitrate concentration when acetic acid was used probably indicating that denitrification was enhanced. If the hypothesis of detrimental effect on EBPR process of some denitrification intermediates as nitrite or nitric oxide was correct, nitrate uptake rate (NUR) should decrease in parallel with nitrate concentration increase. This was observed, e.g. for the case of propionic acid, the NUR was reduced 41% when the nitrate concentration was increased from 40 to 60 $mg \cdot L^{-1}$. The feed of the SBR_{PAO} contained ATU to avoid nitrification and, therefore, the SBR_{PAO} sludge was never in contact with nitrate. Hence, it could be expected that a high nitrate addition results in some inhibition, as opposite to the usual operating conditions in the bioaugmented SBR, where the initial nitrate concentration was lower and no inhibition was observed. In any case, this inhibition effect was considerably lower than in other studies (Patel and Nakhla, 2006; Akin and Ugurlu, 2004) where P-release was only observed when nitrate concentration was below 1 $mg \cdot L^{-1}$.

Table 4.2. Major transformations obtained in the batch studies with different carbon sources.

	Initial $N\text{-}NO_3^-$ concentration ($mg \cdot L^{-1}$)	Acetic acid	Propionic acid	Sucrose
P-release rate ($g\ P\text{-}PO_4^{3-} \cdot g\ TSS^{-1} \cdot d^{-1}$)	0	0.82	1.99	0.00
	40	0.78	1.69	0.00
	60	0.59	1.36	0.00
Nitrate Uptake Rate ($g\ N\text{-}NO_3^- \cdot g\ TSS^{-1} \cdot d^{-1}$)	0	-	-	-
	40	0.10	0.08	0.07
	60	0.06	0.05	0.03
COD uptake rate ($g\ COD \cdot g\ TSS^{-1} \cdot d^{-1}$)	0	2.19	5.45	0.00
	40	4.01	4.36	0.00
	60	4.20	4.23	0.00
P-release/C-uptake (P mmol/C mmol)	0	0.47	0.45	0.00
	40	0.26	0.38	0.00
	60	0.16	0.38	0.00

If we compare the batch experiment with propionic acid and the batch experiment performed during the bioaugmentation (Figure 4.4a), the results were clearly different. The reason can be attributed to the different microbial community of both systems. On the batch experiment done during the bioaugmentation (Figure 4.4a), the population of PAO was 82% with 90% of PAO I, the *Accumulibater* able to use nitrate and nitrite as electron acceptor. In the current experiments (Figure 4.7b), the population of PAO were 68% with only 49% of PAO I. According to the differences of

the population in batch experiments, it could be explained that low PAO I concentration favoured the inhibition for nitrate presence. Moreover, the existing differences between the experimental profiles of Figure 4.4a and 4.7b (i.e. denitrification is only observed in the first) are consequence of the different initial COD. The experiment in Figure 4.4a has enough organic matter to denitrify the initial nitrate and to use the remaining COD in the PAO metabolism (i.e. storing it as PHA linked to P-release).

4.4. CONCLUSIONS

This study demonstrates that the bioaugmentation strategy of a SBR for C/N removal with PAO-enriched sludge led the system to evolve to a new steady state where nitrification, denitrification and biological phosphorus removal could be maintained, as opposite to the previous steady state where EBPR was not observed. Rising sludge problems with progressive loss of both N and P removal activity were initially detected, but the increase of the aerobic phase length minimised the PHA levels at the end of this phase and, consequently, successful operation without rising sludge and with stable C/N/P removal was maintained for 2 months.

PAO response to nitrate depends on the usual operational conditions of each system. The PAO-enriched sludge developed in the anaerobic/aerobic SBR, which had never worked with nitrate before, showed a distribution of almost 50% of PAO I and 50% of PAO II. A decrease in EBPR activity was observed in parallel with an increase in nitrate concentration. Propionic acid and acetic acid as carbon sources supported simultaneous denitrification and P-release but at the expense of lower P-release rates. Sucrose was never taken up by this sludge.

The batch experiments suggested that the nitrate inhibitory effect could be overcome if PAO population was acclimated to nitrate. In this sense, P-release rate did not decrease when nitrate was present with propionic acid or acetic acid. In contrast, when sucrose was used as a carbon source, P-release did not occur. For complex carbon sources (i.e. sucrose), it can be argued that nitrate presence has an inhibitory effect in EBPR, not to inhibit the P-release process itself but to prevent VFA production by fermentative bacteria.

4.5. REFERENCES

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

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R. C., May, J. W., Vasiliadis, G., Seviour, R. J., 2007. Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor. Applied and Environmental Microbiology, 73(7), 2257–70.

Akin, B. S. and Ugurlu, A., 2004. The effect of an anoxic zone on biological phosphorus removal by a sequential batch reactor. Bioresource Technology, 94(1), 1–7.

Amann, R. I., 1995. Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. Molecular Ecology, 4, 543–554.

Belia, E. and Smith, P. G., 1997. The bioaugmentation of sequencing batch reactor sludges for biological phosphorus removal. Water Science and Technology, 35(1), 19–26.

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

Burow, L. C., Kong, Y., Nielsen, J. L., Blackall, L. L., and Nielsen, P. H., 2007. Abundance and ecophysiology of Defluviicoccus spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. Microbiology, 153(1), 178–85.

Carvalho, G., Lemos, P. C., Oehmen, A., and Reis, M. A. M., 2007. Denitrifying phosphorus removal: Linking the process performance with the microbial community structure. Water Research, 41, 4383–4396.

Cech, J. S. and Hartman, P., 1993. Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. Water Research, 27(7), 1219–1225.

Comeau, Y., Hall, K., Hancock, R., and Oldham, W., 1986. Biochemical model for enhanced biological phosphorus removal. Water Research, 20, 1511–1521.

Dabert, P., Fleurat-Lessard, A., Mounier, E., Delgenès, J. P., Moletta, R., and Godon, J. J., 2001. Monitoring of the microbial community of a sequencing batch reactor bioaugmented to improve its phosphorus removal capabilities. Water Science and Technology, 43(3), 1–8.

Dabert, P., Delgenes, J. P., and Godon, J. J., 2005. Monitoring the impact of bioaugmentation on the start up of biological phosphorus removal in a laboratory scale activated sludge ecosystem. Applied Microbiology and Biotechnology, 66(5), 575–588.

Daims, H., Bruhll, A., Amann, R., Schleifer, K., and 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. Systematic and Applied Microbiology, 444, 434–444.

El Fantroussi, S. and Agathos, S. N., 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Current Opinion in Microbiology, 8(3), 268–75.

Flowers, J. J., He, S., Yilmaz, S., Noguera, D. R., and McMahon, K. D., 2009. Denitrification capabilities of two biological phosphorus removal sludges

dominated by different “Candidatus Accumulibacter” clades. Environmental Microbiology Reports, 1(6), 583–588.

Guerrero, J., Guisasola, A., and Baeza, J. A., 2011. The nature of the carbon source rules the competition between PAO and denitrifiers in systems for simultaneous biological nitrogen and phosphorus removal. Water Research, 45(16), 4793–802.

Guisasola, A, Pijuan, M., Baeza, J. A., Carrera, J., Casas, C., and Lafuente, J., 2004. Aerobic phosphorus release linked to acetate uptake in bio-P sludge: process modeling using oxygen uptake rate. Biotechnology and Bioengineering, 85(7), 722–33.

Guisasola, A., Qurie, M., Vargas, M., Casas, C., and Baeza, J. A., 2009. Failure of an enriched nitrite-DPAO population to use nitrate as an electron acceptor. Process Biochemistry, 44(7), 689–695.

He, S., Gall, D. L., and McMahon, K. D., 2007. “Candidatus Accumulibacter” population structure in enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes. Applied and Environmental Microbiology, 73(18), 5865–74.

Henze, M., Dupont, R., Grau, P., and de la Sota, A., 1993. Rising sludge in secondary settlers due to denitrification. Water Research, 27(2), 231–236.

Henze, M., van Loosdrecht, M. C. M., Ekama, G., and Brdjanovic, D., 2008. *Biological wastewater treatment*, IWA Publishing London.

Hu, Z. R., Wentzel, M. C., and Ekama, G. A., 2002. Anoxic growth of phosphate-accumulating organisms (PAOs) in biological nutrient removal activated sludge systems. Water Research, 36(19), 4927–37.

Jubany, I., Lafuente, J., Carrera, J., and Baeza, J. A., 2009. Automated thresholding method (ATM) for biomass fraction determination using FISH and confocal microscopy. Journal of Chemical Technology and Biotechnology, 84(8), 1140–1145.

Kos, P., 1998. Short SRT (solids retention time) nitrification process/flowsheet. Water Science and Technology, 38(1), 23–29.

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

Mino, T., van Loosdrecht, M. C. M., and Heijnen, J. J., 1998. Microbiology and biochemistry of enhanced biological phosphate removal process. Water Research, 32(11), 3193–3207.

Mino, T., Satoh, H., and Matsuo, T., 2004. Metabolisms of different bacterial populations in enhanced biological phosphorus removal processess. Water science and technology, 29(7), 67–70.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E., and Stahl, D. A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Applied and Environmental Microbiology, 62(6), 2156–2162.

Murnleitner, E., Kuba, T., van Loosdrecht, M. C. M., and Heijnen, J. J., 1997. An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnology and bioengineering, 54(5), 434–50.

Van Niel, E. W., Appeldoorn, K. J., Zehnder, A. J., and Kortstee, G. J., 1998. Inhibition of anaerobic phosphate release by nitric oxide in activated sludge. Applied and Environmental Microbiology, 64(8), 2925–30.

Oehmen, A., Yuan, Z., Blackall, L. L., and Keller, J., 2005. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnology and Bioengineering, 91(2), 162–8.

Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L., Reis, M. A. M., 2007. Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Research, 41(11), 2271–300.

Oerther, D. B., Danalewich, J., Dulekgurgen, E., Leveque, E., Freedman, D. L., and Raskin, L., 1998. Bioaugmentation of sequencing batch reactors for biological phosphorus removal: Comparative rRNA sequence analysis and hybridization with oligonucleotide probes. Water Science and Technology, 37(4-5), 469–473.

Patel, J. and Nakhla, G., 2006. Interaction of denitrification and P removal in anoxic P removal systems. Desalination, 201(1-3), 82–99.

Pijuan, M., Guisasola, Al., Baeza, J. A., Carrera, J., Casas, C., and Lafuente, J., 2006. Net P-removal deterioration in enriched PAO sludge subjected to permanent aerobic conditions. Journal of Biotechnology, 123(1), 117–126.

Pijuan, M., Ye, L., and Yuan, Z., 2010. Free nitrous acid inhibition on the aerobic metabolism of poly-phosphate accumulating organisms. Water Research, 44(20), 6063–6072.

Randall, A. A., Benefield, L. D., Hill, W. E., Nicol, J. P., Boman, G. K., and Jing, S. R., 1997. The effect of volatile fatty acids on enhanced biological phosphorus removal and population structure in anaerobic/aerobic sequencing batch reactors. Water Science and Technology, 35(1), 153–160.

Saito, T., Brdjanovic, D., and van Loosdrecht, M. C. M., 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. Water Research, 38(17), 3760–3768.

Salem, S., Berends, D. H., van der Roest, H. F., van der Kruij, R. J., and van Loosdrecht, M. C. M., 2004. Full-scale application of the BABE technology. Water Science and Technology, 50(7), 87–96.

Spagni, A., Stante, L., and Bortone, G., 2001. Selection of denitrifying phosphorus accumulating organisms in activated sludge. Environmental Technology, 22(12), 1429–1437.

Tchobanoglous, G., Burton, F. L., and Stensel, H. D., 2003. Wastewater engineering: Treatment and reuse, New York.

Vargas, M., Casas, C., and Baeza, J. A., 2009. Maintenance of phosphorus removal in an EBPR system under permanent aerobic conditions using propionate. Biochemical Engineering Journal, 43(3), 288–296.

Wagner, M., Rath, G., Koops, H. P., Flood, J., and Amann, R., 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. Water Science and Technology, 34(1-2), 237–244.

Wang, D. B., Zeng, R J, Dai, Y., Peng, Y., and Yuan, Z. G., 2008. the denitrification capability of cluster 1 *defluvicoccus* vanus-related glycogen-accumulating organisms. Bioresource Technology, 99(6), 1329–1336.

Wang, D., Li, X., Yang, Q., Zheng, W., Liu, Z., Liu, Y., Cao, J., Yuea, X., Shena, T., Zeng G., Denga, J., 2009. The probable metabolic relation between phosphate uptake and energy storages formations under single-stage oxic condition. Bioresource Technology, 100(17), 4005–11.

Zeng, R. J., Saunders, A. M., Yuan, Z., and Blackall, L. L., 2003. Identification and Comparison of Aerobic and Denitrifying Polyphosphate-Accumulating Organisms. Biotechnology and Bioengineering, 83(2), 140–148.

Chapter 5

OPENING UP NEW POSSIBILITIES IN EBPR: A SYNTROPHIC CONSORTIUM FOR THE UTILISATION OF A WIDER RANGE OF CARBON SOURCES

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Part of this chapter has been presented as oral presentation:

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The presence of suitable carbon sources for enhanced biological phosphorus removal (EBPR) plays a key role in phosphorus removal from wastewater in urban WWTP. For wastewaters with low volatile fatty acids (VFAs) content, an external carbon addition could be necessary. Methanol and glycerol are the most common external carbon sources used for denitrification and they could be a priori promising alternatives, but previous attempts to use them for EBPR have failed. The aim of this chapter was to study two different strategies: direct replacement of the usual carbon source for methanol or glycerol and a two-step consortium development with anaerobic degraders and PAO. An example using dairy wastewater with a low COD/P ratio confirms the feasibility of using glycerol as an external carbon source to increase P removal activity. The approach used in this work opens a new range of possibilities and, similarly, other fermentable substrates can be used as electron donors for EBPR.

5.1. INTRODUCTION

EBPR is considered the most economical and sustainable technology to meet the increasingly stricter discharge requirements of WWTP (Broughton et al., 2008). However the effectiveness of EBPR highly depends on the nature of the carbon source that plays the electron donor role, being the presence of volatile fatty acids (VFA) a key factor to obtain a high P removal capacity (Chu et al., 1994; Randall et al., 1997a; Merzouki et al., 2005; Guerrero et al., 2011).

VFA as acetic and propionic acid are the most suitable and commonly studied carbon sources for EBPR (Pijuan et al., 2004; Zeng et al., 2003), but other compounds such as glucose (Jeon and Park, 2000), ethanol (Puig et al., 2008), butyrate, lactate or valerate have also been reported as possible carbon sources (Oehmen et al., 2007).

For wastewaters with low VFA content, two different solutions are proposed: i) an external carbon addition, which is usually not cost-effective and it increases the overall carbon footprint (Isaacs and Henze, 1995; Yuan et al., 2010) and ii) the utilisation of sludge pre-fermentation to produce these compounds (Tong and Chen, 2007; Feng et al., 2009). When choosing a possible external organic matter, both, the economics and the selective use of the carbon source by PAO against GAO has to be considered (Puig et al., 2008). Thus, the utilisation of waste materials that could be converted somehow to VFA is a very attractive alternative to overcome such VFA deficiency.

Denitrification is another biological process that may require external carbon addition and hence, it would be very practical to find a carbon source suitable for both processes. Methanol is the most commonly used external carbon source for denitrification (Purtchert et al., 1996) and, as such, its utilization to make EBPR possible seems a priori a promising alternative. Moreover, the current research on methanol production from non-fossil sources (i.e., bio-methanol) may upgrade the process sustainability. However, so far, it has been shown that methanol is inappropriate for biological phosphorus removal. Randall et al. (1997a) showed that methanol could not be used as carbon source in a sequencing batch reactor (SBR) cultivated with glucose fermentation products. Cho and Molof (2004) investigated the use of a combined carbon source (methanol and acetic acid) for simultaneous nitrogen and phosphorus removal. They found the highest P removal when all the influent COD was acetic acid, indicating that methanol was not used for P removal purposes. Similarly, Louzeiro et al. (2002) studied the effect of methanol on the simultaneous biological removal of N and P and concluded that despite methanol was probably not

utilized by PAO, methanol addition was critical, since it depleted the available nitrate and thus allowed EBPR to take place. Puig et al. (2008) studied methanol utilization in an ethanol-acclimated EBPR sludge and observed that methanol was not a suitable carbon source in short-term tests. Given this limited research, further investigation is required to assess whether methanol could be used by PAO during long-term EBPR operation.

Glycerol has also been reported as a proper external carbon source for denitrification in WWTP (Grabinska-Loniewsa et al., 1985; Akunna et al., 1993; Bodík et al., 2009; Torà et al., 2011). Glycerol is a by-product of biodiesel fuel production: about 1 L of glycerol is generated for every 10 L of biodiesel fuel produced (Johnson and Taconi, 2007). The glycerol derived from biodiesel production has many impurities that together with the increase of its production have resulted in a drop of glycerol prices. As a consequence, glycerol has become a waste material with associated disposal costs (Yazdani and Gonzales, 2007; Escapa et al., 2009). Nowadays, glycerol demand only constitutes a 22% of the annual production capacity (Johnson and Taconi, 2007) and thus, it has grabbed the attention of the engineering community. Although the utilisation of glycerol for these purposes seems promising, few studies on its use as carbon source for EBPR have been reported. Yuan et al., 2010 investigated glycerol as a possible carbon source in EBPR with two configurations: 1) direct application of glycerol as a sole carbon source and 2) supplementing a VFA-enriched supernatant from glycerol co-fermentation with waste activated sludge. Despite the latter was successful, the authors reported that EBPR activity was not achieved when glycerol was used as a sole carbon source in a single-sludge system.

Anaerobic degradation of glycerol under certain conditions can yield significant propionate production (Barbirato et al., 1997; Himmi et al., 2000; Zhang and Yang, 2009), which is a good carbon source for EBPR (Oehmen et al., 2005a,b and 2007). In fact, Pijuan et al. (2004) proved that PAO could selectively consume propionate against glycogen accumulating organisms (GAO *Competibacter*, one of the competitors of PAO). Hence, an *in situ* generation of propionate or other VFA from glycerol would be a possible solution to achieve EBPR with glycerol as a sole carbon source.

Therefore, the idea proposed in this chapter is to develop a suitable *syntrophic consortium* with conventional anaerobic biomass and PAO in a single sludge system (Figure 5.1). Anaerobic microorganisms (acidogens/acetogens) would be selected to degrade the desired complex substrates (glycerol, methanol or other substrates) and PAO would live off the fermentation products enabling EBPR. In short, methanogens would be replaced by PAO in the conventional anaerobic degradation sequence. However, this replacement does not seem a straightforward issue and several strategies for promoting the “EBPR-sink” pathway against methanogenesis could be required (e.g. assuring the presence of required micro-nutrients or selecting proper anaerobic and aerobic lengths to guarantee PAO and acidogenic/acetogenic activities while avoiding methanogenesis). In addition, the proliferation of GAO in the system has to be considered, monitored and, if possible, avoided.

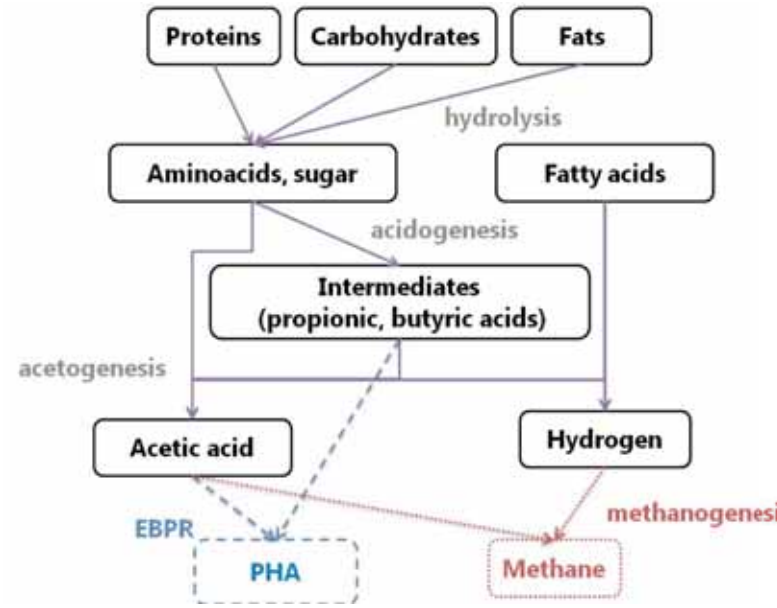

Figure 5.1. Basic scheme of the proposed idea. Dotted: conventional anaerobic methanogenesis, dashed: syntrophic consortium between anaerobic biomass and PAO, solid: overlapped pathways.

5.2. MATERIALS AND METHODS

5.2.1. EQUIPMENTS AND SYNTHETIC WASTEWATER

Five different SBRs were used (Table 5.1). In this chapter, the experiments presented were not conducted in parallel but will be presented in this way in view of simplifying the text. Actually, the experiments with methanol were carried out in a first stage while glycerol experiments were conducted in a second stage. Prior to this study, SBR-A, SBR-B and SBR-D (10 L each) were used for obtaining and maintaining PAO-enriched sludge. The activated sludge, obtained from a municipal WWTP (Granollers, Catalonia), was enriched in PAO using propionic acid to avoid *Competibacter* GAO growth (Pijuan et al., 2004) and thus, to obtain a highly PAO-enriched sludge. Moreover, propionic-fed PAO can easily switch to consume other carbon sources as acetic acid without any acclimation period (Pijuan et al., 2009). SBR-A was used as source of PAO-enriched sludge for a bioaugmentation strategy, while SBR-B and SBR-D were used to test the feasibility of a direct replacement of propionic acid for methanol and glycerol respectively. All SBRs were fully monitored for oxygen, pH, and temperature as described on chapter 3 secction 3.1. A PLC controlled the equipment and phase configuration. The SBRs were operated with different configurations as described in Table 5.2. The temperature was controlled with a thermostatic bath. The pH was controlled at 7.50±0.05. The SBRs that has been used for PAO-enriched sludge and for the direct replacement strategy were operated with a TRH of 12h, by extracting 5 L of each cycle. A fixed nitrogen gas flow was sparged during the anaerobic phase to maintain strict anaerobic conditions. DO was maintained from 3.5 to 4.5 $mg \cdot L^{-1}$ in the aerobic phase to avoid oxygen limitations.

A volume of 5 L of feeding (synthetic wastewater solution plus concentrated carbon source solution) was added during the first 5 min of the cycle. The synthetic wastewater solution (4.97 L) consisted of ($mg \cdot L^{-1}$ in reverse osmosis water): 110.5

KH_2PO_4, 83.7 K_2HPO_4, 100 NH_4Cl, 43.9 $MgSO_4 \cdot 7H_2O$, 160 $MgCl_2 \cdot 6H_2O$, 42 $CaCl_2 \cdot 2H_2O$, 50 allylthiourea (ATU) to inhibit nitrification, and 30 mL of nutrient solution. The initial phosphorus concentration was 20 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$. The nutrient solution ($g \cdot L^{-1}$) consisted of: 1.5 $FeCl_3 \cdot 6H_2O$, 0.15 H_3BO_3, 0.03 $CuSO_4 \cdot 5H_2O$, 0.18 KI, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 4H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 \cdot 6H_2O$, and 68.5 mL EDTA 0.5 M. The concentrated carbon source solution (0.03 L of concentrated solution of propionic acid, methanol or glycerol) was added to obtain the desired initial concentration in the reactor.

Table 5.1. Description of the strategy developed in each reactor.

Name	Strategies	Carbon source
SBR-A	PAO-enriched sludge stock	Propionic acid
SBR-B	Direct Replacement	Methanol
SBR-C	Syntrophic Consortium	Methanol
SBR-D	Direct Replacement	Glycerol
SBR-E	Syntrophic Consortium	Glycerol

Table 5.2. Description of the different periods for the proposed strategies.

SBR	Period	Days	Cycle configuration (h)*	T[a] (°C)	Base used	Feed Cobalt ($mg \cdot L^{-1}$)
SBR-A	A1	>125	6 (2 + 3.5 + 0.5)	25	NaOH (1M)	0
SBR-B	B1	30	6 (2 + 3.5 + 0.5)	25	NaOH (1M)	0
SBR-C	C1	0-8	6 (4.5 + 0 +1.5)	35	$NaHCO_3$ (1M)	0.2
	C2	9-22	6 (4 + 0.5 + 1.5)	35	$NaHCO_3$ (1M)	0.2
	C3	23-60	8 (5 + 2 + 1)	25	$NaHCO_3$ (1M)	0.2
SBR-D	D1	0-25	6 (2 + 3.5 + 0.5)	25	NaOH (1M)	0
	D2	26-52	8 (5 + 2.5 + 0.5)	25	NaOH (1M)	0
	D3	53-100	8 (4 + 3.5 + 0.5)	25	NaOH (1M)	0
SBR-E	E1	0-7	6 (5 + 0 + 1)	35	$NaHCO_3$ (1M)	0.2
	E2	8-40	6 (5 + 0.5 + 0.5)	35	$NaHCO_3$ (1M)	0.2
	E3	41-50	8 (5 + 2.5 + 0.5)	25	$NaHCO_3$ (1M)	0.2
	E4	51-68	8 (5 + 2.5 + 0.5)	25	$NaHCO_3$ (1M)	0.2
	E5	69-125	8 (4 + 3.5 + 0.5)	25	$NaHCO_3$ (1M)	0.2

* Total (Anaerobic+Aerobic+Settling)

On the other hand, SBR-C was used to grow the syntrophic consortium for simultaneous methanol and phosphorus removal, while SBR-E was used to grow the consortium for glycerol and phosphorus removal. SBR-C and SBR-E (13 L) had only minor differences with respect to SBR-A, SBR-B and SBR-D. Initial anaerobic sludge inoculum was obtained from the anaerobic digester of a municipal WWTP (Granollers, Catalonia). During the anaerobic phase, no nitrogen was sparged in SBR-C or SBR-E to avoid possible methanol losses due to stripping. When aerobic conditions were required, DO was maintained from 3.5 to 4.5 $mg \cdot L^{-1}$ with an on/off controller. pH was

also controlled at 7.5±0.1 using HCl (1M) and bicarbonate (1M). Bicarbonate was used as pH-increaser instead of NaOH because it is a required co-substrate in the acetogenic breakdown of methanol (Florencio et al., 1993a). A volume of 4 L of synthetic wastewater was added during the first 5 min of each cycle. A settling phase took place at the end of each cycle, followed by 10 min to extract 4 L of the supernatant. The SBRs were operated under different configurations during this work, which are summarized in Table 5.2. The synthetic media was similar to the media described above with the only difference of cobalt addition (0.2 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$) to avoid its limitation for acetogenic bacteria (Florencio et al., 1993a).

Finally, the feasibility of using glycerol as external carbon source for real wastewaters with a low COD/P content was studied. For this aim, dairy-processing wastewater (Table 5.3) was used with a COD/P ratio that ranged between 10 and 13. SBR-B was inoculated with 5 L of PAO-enriched sludge from SBR-A and it was operated under the same conditions as period D3 (Table 5.2). Dairy wastewater was fed during the first week and afterwards it was supplemented with a concentrated solution of glycerol (around 200 $mg \cdot L^{-1}$ of glycerol expressed as COD were added in each cycle).

Table 5.3. Dairy-processing wastewater average composition.

Composition	Concentration ($mg \cdot L^{-1}$)
Total COD	355-455
$N\text{-}NH_4^+$	20
$P\text{-}PO_4^{-3}$	35
TSS	127
pH	5.0-7.6

5.2.2 BATCH EXPERIMENTS

Off-line batch experiments were performed in a magnetically stirred vessel (2 L) (Figure 5.2), that is known as LFS respirometer (Spanjers et al., 1996). Each batch experiment mimicked a SBR cycle of interest, with a first anaerobic phase (by nitrogen sparging) and a subsequent aerobic phase (by oxygen sparging). pH (WTW Sentix 81) and DO (WTW CellOx 325) probes were connected to a multiparametric terminal (WTW INOLAB 3). It was in turn connected via RS232 to a PC with specific software allowing for data monitoring and manipulation of a high precision microdispenser (Crison Multiburette 2S) for pH control with acid/base addition. Biomass (2 L) was withdrawn at the end of the aerobic phase of a SBR and was placed in the stirred vessel under anaerobic conditions. The experiment started with a pulse of concentrated feed with the desired COD and P concentration. Figure 5.2 shows the batch experiments set-up.

Figure 5.2. Experimental set-up of the LFS Respirometer-titrimeter used for batch experiments.

5.2.3. CHEMICAL AND MICROBIOLOGICAL ANALYSES

Chemical analyses were performed with 0.22µm filtered samples.

Methanol concentration was determined by using an Agilent Technologies 7820 A GC equipped with Innowax column (30 m x 0.53 mm x 1.0 mm; length x internal diameter x film thickness) and a flame ionization detector (FID). A sample of 1 µL was injected at a temperature of 50°C under pulsed split conditions (15 psi). The carrier gas was helium with a split ratio of 20:1 at 1.2 psi, the column temperature was set at 45°C for the first 5 min, followed by an increase of 20°C·min^{-1} until the stable value of 110°C was reached. A cleaning step at 230°C during 5 min was used to remove any residue in the column. The run time was 20 min and the detector temperature was set at 300°C.

Glycerol concentration was determined by HPLC (Dionex Ultimate 3000) analysis using an ionic exchange column (ICSep ICE-COREGEL 87H3, Trans- genomic). The mobile phase was 6 mM sulphuric acid. The injection volume was 20 µL and the chromatogram was quantified with the Cromeleon software (Dionex).

Phosphorus, propionic and acetic acid concentrations were analysed according to the methods described in chapter 3.

Fluorescence in situ hybridization (FISH) technique (Amann et al., 1995) coupled with confocal microscopy was used to quantify the biomass distribution as described in chapter 3.

5.3 RESULTS AND DISCUSSION

5.3.1 DIRECT REPLACEMENT OF PROPIONIC ACID FOR METHANOL (SBR-B)

Methanol could not be used as an extra carbon source for EBPR purposes in previous studies in the literature. However, our first approach was to test if a careful direct replacement of the usual carbon source of SBR-B (200 mg COD·L^{-1} of propionic acid) by methanol, avoiding any other carbon source, was a proper strategy. A low initial concentration of 50 mg·L^{-1} of methanol was used to ensure its consumption under anaerobic conditions, because methanol presence during the aerobic phase could affect the composition of the microbial community in a mid-term basis, as it could favor other aerobic methanol degraders. In this sense, minimizing the electron donor presence in the aerobic phase is a key factor to achieve a sludge highly enriched in PAO. Given the low amount of COD added, SBR-B was not purged during this period to avoid biomass washout. Figure 5.3 summarizes the experimental results obtained during this period (30 days): the upper figures show the experimental phosphate (left) and methanol (right) profiles obtained in nine different cycles. Methanol consumption was negligible in the first day but anaerobic and aerobic methanol degradation was already observed on day 3, indicating that the microbial community needed an adaptation period. Increased anaerobic methanol consumption was observed during the first 12 days of operation, although some methanol was still available under aerobic conditions. The initial concentration of methanol was decreased to 20 mg·L^{-1} to ensure complete anaerobic methanol depletion.

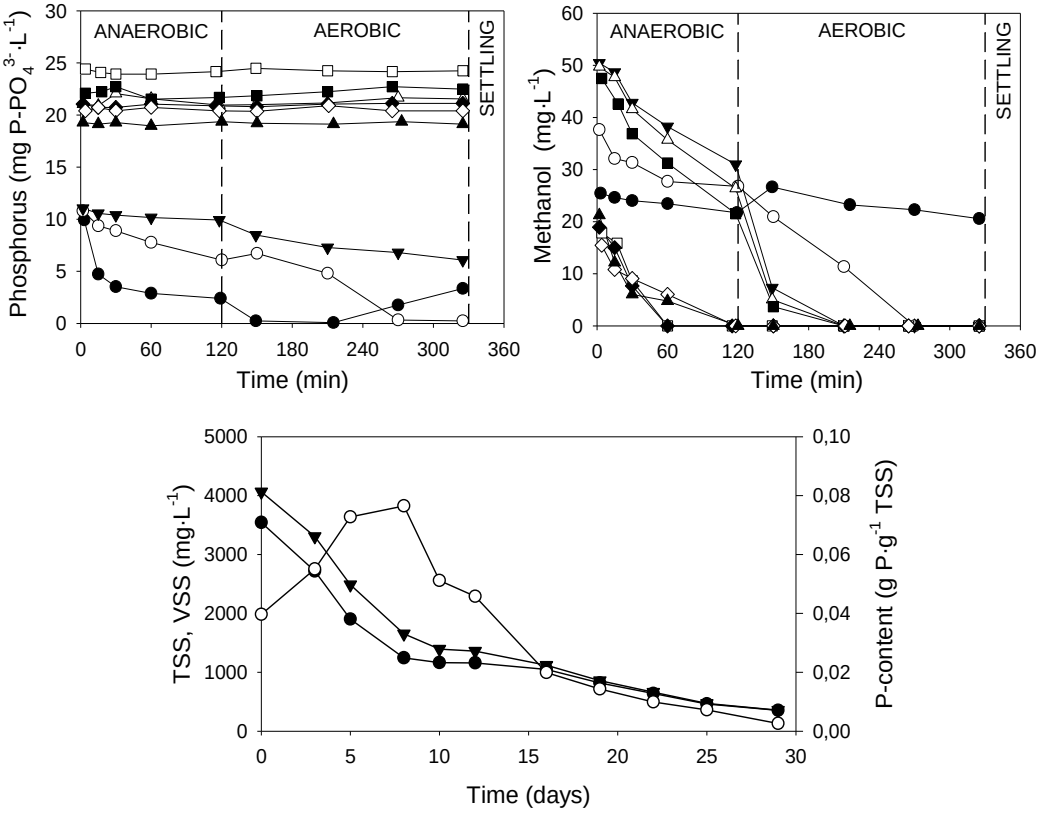

Figure 5.3. Experimental profiles obtained during the *direct replacement* experiment for methanol. Up: Phosphorus and methanol profiles for 9 cycles conducted in this period (day 1 (●), day 3 (○), day 5 (▼), day 8 (△), day 10 (□), day 12 (■), day 16 (◆), day 19 (◇) and day 25(▲)). Down: VSS (●), TSS (▼) and P-content (○) profiles.

With respect to the experimental P profiles, anaerobic P-release was not observed despite the methanol consumption. However, some aerobic P-uptake was still maintained the first 5 days of operation. From day 8, neither anaerobic P-release nor aerobic P-uptake occurred, despite the existing anaerobic methanol consumption. This lack of EBPR activity proved that a propionate-fed PAO-enriched community was not able to grow on methanol as sole carbon source under the usual operating conditions of SBR-B. These results corroborated the reported data that stated that methanol could not be directly taken up by bio-P sludge (Louzeiro et al., 2002; Randall et al., 1997a) even if the sludge was previously acclimated to another short chain alcohol as ethanol (Puig et al., 2008).

Figure 5.3 (Down) shows a gradual VSS decrease during this period, indicating that biomass was decaying as a consequence of the lack of a suitable carbon source. Over the 29 days of this experiment, the TSS and VSS decreased by 91% and 90%, respectively. The VSS profile was fitted to Equation (5.1) obtaining a decay coefficient of b=0.074 day^{-1} (R^2 = 0.95).

$$\frac{dX}{dt} = -bX \tag{5.1}$$

The comparison of this decay rate with the values reported in the literature confirms that PAO biomass was virtually under starvation conditions due to the negligible utilization of methanol during this period. Lopez et al. (2006) studied the endogenous processes of PAO during long-term (3–4 weeks) aerobic and anaerobic starvation. They obtained an aerobic decay value of 0.15 day^{-1}, which is higher than the one obtained in our study, where starvation conditions alternate anaerobic and aerobic conditions. This observation agrees with the results observed in (Lu et a., 2007), who studied the endogenous PAO metabolism under intermittent aerobic/anaerobic conditions for a period of 8 days. They concluded that predominantly anaerobic conditions (15 min aeration every 6 h) resulted in negligible cell decay rates and slower glycogen and poly-P utilization. Yilmaz et al. (2007) concluded that an alternating anaerobic and aerobic phase was more effective in maintaining biomass activity than aerobic conditions since PAO were given suitable conditions to perform their normal anaerobic and aerobic metabolism.

The phosphorus content in the solids (g $P \cdot g^{-1}$ TSS, Figure 5.3 Down) was approximated using Equation (5.2), which assumes that the cell salts content is mainly poly-P ($Mg_{0.33} \cdot K_{0.33} \cdot PO_3$, Brdjanovic et al., 1996; Marcelino et al., 2009). The constant value of 3.23 comes from dividing the poly-P molecular weight (100 $g \cdot mol^{-1}$) by the phosphorus molecular weight (31 $g \cdot mol^{-1}$). The P-cell content started at 0.04 g $P \cdot g^{-1}$ TSS and increased to 0.076 g $P \cdot g^{-1}$ TSS 8 days after methanol introduction as sole carbon source. This increase is possibly due to the absence of an adequate carbon source and to the still active aerobic P-storage processes, as observed in the P profile (Figure 5.3 left). When the maximum P-cell content was reached, it started to decrease rapidly because PAO processes were no longer active, as observed in the flat profiles of P (Figure 5.3 up-left). After 29 days, internal phosphorus content was almost depleted (0.003 g $P \cdot g^{-1}$ TSS).

$$P - cell\ content = \frac{TSS - VSS}{TSS}\frac{1}{3.23} \quad (5.2)$$

In order to gain more insight into the process, EBPR activity was monitored using batch tests with propionic acid as carbon source (Figure 5.4). After 12 days of methanol feeding, the P-release rate was only 20% lower than the reference experiment (conducted before methanol feeding started). In contrast, aerobic P-uptake rate decreased 67% after 12 days of starvation. After 22 days of methanol-feed conditions, no P-uptake took place and only a small amount of P was released. At day 33, neither P-release nor P-uptake was observed, as a consequence of the important decrease in PAO biomass. Figure 5.4 shows that the aerobic P-uptake metabolism was more affected than the P-release, probably because the remaining active PAO still had an important amount of stored Poly-P.

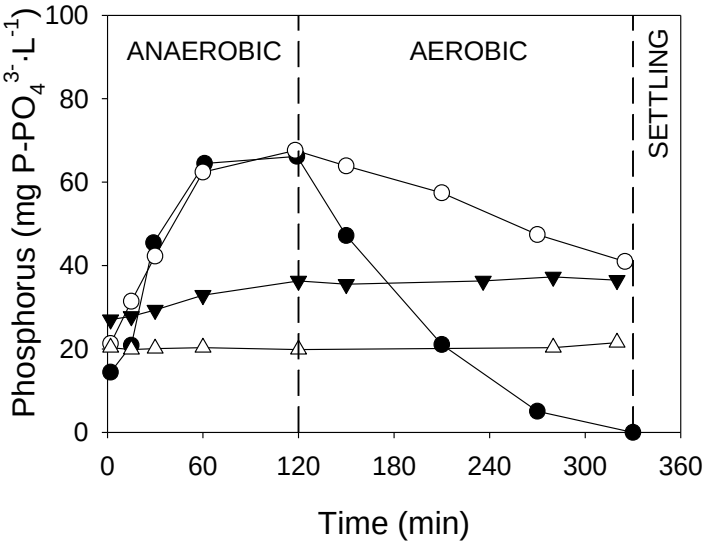

Figure 5.4. Batch experiments conducted during the *direct replacement* experiment using propionic acid as carbon source to assess PAO activity (day 1 (●), day 12 (○), day 22 (▼), day 33 (△)).

These results confirm that the direct application of methanol to a PAO-enriched sludge was not a proper strategy to achieve simultaneous methanol and P removal. Nevertheless, this strategy was successfully applied using glycerol as a sole carbon source (results below). Whether direct application of fermentable substrates could work with bio-P sludge depends on the presence in the initial microbial community of the required anaerobic microorganisms able to transform anaerobically those substrates into VFA ready to be used by PAO. When direct application is not feasible, as in the case of methanol, a syntrophic consortium would be another strategy to be tested.

5.3.2. METHANOL-DRIVEN EBPR USING A SYNTROPHIC CONSORTIUM (SBR-C)

The purpose of this consortium was to obtain a methanol-based EBPR sludge with methanol-degrading acetogens converting methanol to VFA, which in turn would be used by PAO as carbon source for biological removal purposes. This syntrophic consortium was obtained in two steps. In a first step, an anaerobic sludge was subjected to conditions for favouring anaerobic methanol degradation via

acetogenesis against methanogenesis. Then, in a second step, this acetogenic-enriched sludge system was bioaugmented with PAO-enriched sludge so that PAO live off the acetogenesis products.

5.3.2.1. Selection of methanol-degrading acetogens

The initial consortium from a conventional anaerobic WWTP digester contains the classical anaerobic digestion microfauna (fermentative, acidogens, acetogens, methanogens, etc.). Among these, we wanted to enrich our sludge with methanol-consuming acetogens in order to produce VFA. Methanol-degrading acetogenesis produces H_2/CO_2 or acetate (Paulo et al., 2003). We aimed at PAO growing on this acetate produced. Conversely, several species of methanogens and acetogens are able to utilize methanol as carbon and energy source for growth without a previous step of acetogenesis (Jarrel and Kalmokoff, 1988), which is not desired in this study. In our case, methanogenic activity needs to be suppressed for three reasons: (i) methanogens could compete for methanol with acetogens; (ii) methanogens could compete for acetate with PAO; and (iii) methanogens could use hydrogen as electron donor. This would indirectly decrease acetate formation from methanol since conversion of methanol to H_2/CO_2 is very sensitive to the H_2 partial pressure (Conrad et al., 1986).

If methanol is added as carbon source, substrate competition between methanogens and acetogens during the degradation of methanol will occur and several factors such as inorganic carbon, cobalt, methanol concentration, or undissociated VFA concentration can play a key role in this competition. The works of Florencio et al. (1993a,b; 1995) set the guidelines to understand this competition. In short, acetogenesis is expected to predominate if the methanol concentration is high (>1,000 $mg \cdot L^{-1}$), exogenous inorganic carbon is supplied, cobalt (at least 0.2 $mg \cdot L^{-1}$) is available and methanogens are inhibited. This inhibition can be caused either by a high amount of undissociated VFA, by the presence of inhibitors like bromoethanesulfonic acid (BESA) or by specific toxics of methanogens such as dichloromethane and chloroform. Temperature is also an important feature (Kotsyurbenko et al., 1996,2001; Speece et al., 1996), while acetogens and methanogens are equally active at a medium temperature range (around 25°C), acetogenic activity dominates at lower temperatures (<15°C) and methanogens are more active at higher temperatures (>30°C). Kalle and Menon (1984) observed the distribution of VFA in a biogas digester at different temperatures. At 25°C it contained 87–88% VFA, in comparison to 38% observed in the digester operating at 35°C. In view of a posterior symbiotic coexistence with PAO, temperatures around 20–25°C can be also beneficial to avoid the presence of GAO, which are favoured at high temperatures (Lopez-Vazquez et al., 2009).

Finally, oxygen was also a key parameter in this selection process, since an aerobic phase was required for PAO to grow. The fact that oxygen is required for EBPR activity can be also helpful when suppressing methanogenic activity. Despite acetogens were considered strict anaerobes, it has been proved that they can grow under oxygen presence and are even capable to consume small amounts of oxygen (Karnholz et al., 2002). On the other hand, Ferry (1993) or Kato et al. (1997) stated that despite

methanogenic activity ceases in presence of oxygen, methanogens can be fairly tolerant to oxygen exposure. In any case, experimental reports suggest that acetogens are more resistant to oxygen than methanogens and hence, the aerobic phase may be more detrimental to the latter (Hungate, 1969; Martin and Savage, 1988; Whitman et al., 1992).

Then, SBR-C was inoculated with anaerobic sludge under configuration C1 (Table 5.2). The average biomass concentration during this selection phase was 4,770 mg VSS·L^{-1} (5,930 mg TSS·L^{-1}). High temperature (35°C) was initially chosen to favour complete anaerobic methanol degradation. Figure 5.5 shows the experimental methanol profiles during this enrichment period. During the first 8 days, the system was working under C1 conditions to favour anaerobic methanol degradation. Next, at day 8, the last 30 min of anaerobic phase were changed to aerobic conditions (configuration C2). Methanol degradation decreased with this configuration, suggesting that partial inhibition took place when oxygen was introduced in the system. However, configuration C2 was maintained and, at day 22, the results indicated that the biomass was adapted to anaerobic methanol consumption without being inhibited by the final aeration phase of 30 min. Then, configuration C3 was applied extending the cycle length to 8 h with longer aeration time (2h) and using lower temperature (25°C). These changes resulted in slower substrate uptake and consequently, the initial methanol concentration was lowered to 150 mg·L^{-1} to ensure complete COD consumption during the anaerobic phase.

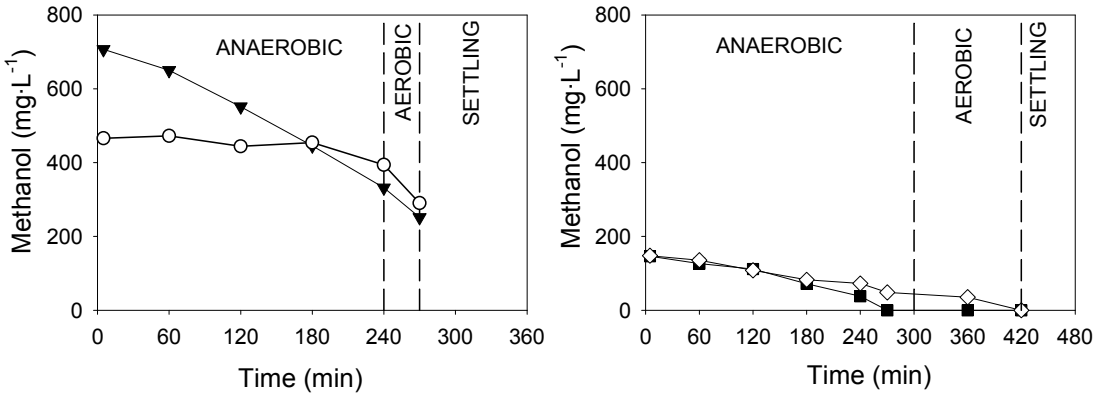

Figure 5.5. Different cycles from the acetogenic selection period with methanol as sole carbon source and anaerobic/aerobic/settling configuration. Left: period C2 (day 9 (○), day 22(▼)). Right: period C3 (day 24 (◇), day 26 (■)). Note: aerobic phase (C2 configuration) started at day 8.

During the anaerobic phase of all this period, base was dosed to maintain pH at the desired setpoint. According to Equation (5.3) (Heijthuijsen and Hansen, 1986), it was a clear indication of VFA production from methanol, because 0.25 mol of protons are produced for each mol of methanol consumed for acetogenesis.

$$4CH_4OH + 2HCO_3^- \rightarrow 3CH_2COO^- + H^+ + 4H_2O \quad (5.3)$$

Moreover, it should be noted that, as indicated by Florencio et al. (1995), around 10% of the initial methanol is used for growth purposes in the acetogenic metabolism and

thus it could be assumed that at least 10% of the initial methanol would be lost for growth purposes and not converted to acetate.

Several experiments were conducted to corroborate the anaerobic production of acetic acid from methanol. A pulse of 600 $mg \cdot L^{-1}$ of methanol was added to anaerobic sludge that had been daily aerated during 30 min (Figure 5.6). Around 10% in weight basis of acetic acid production was observed after 24 h. This production was lower than the theoretical one if methanogens were totally inhibited (i.e., Equation 5.3) indicating that either methanogenesis had not been totally inhibited or that some other methanol-consuming microorganisms were present.

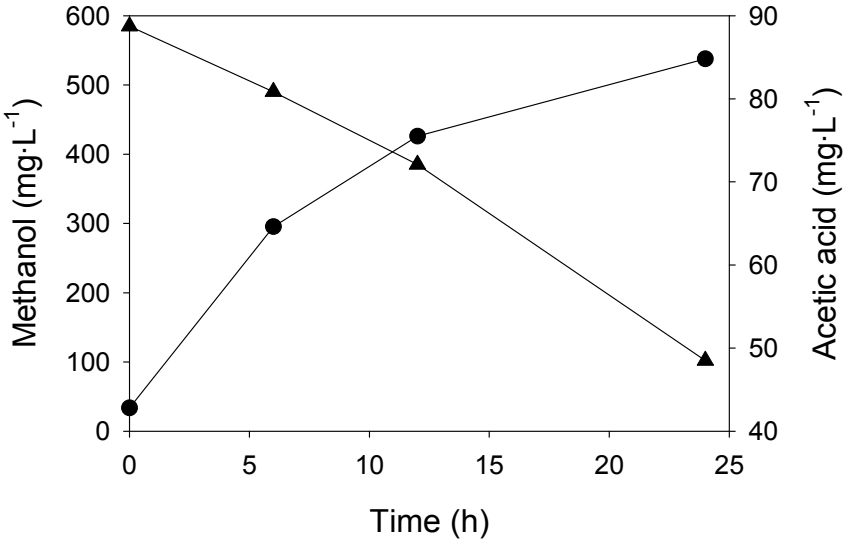

Figure 5.6. Batch experiment with addition of 600 $mg \cdot L^{-1}$ of methanol to anaerobic sludge subjected to 30 min aeration every 24 h. Methanol (▲) and acetic acid (●).

The extent of methanogenesis was assessed with another batch experiment where the acetic acid uptake rate obtained with a periodically aerated anaerobic sludge was compared to that obtained with an anaerobic sludge never exposed to aerobic conditions. Anaerobic acetic acid uptake rate was evaluated for both experiments obtaining 30.06 $mg \cdot L^{-1}$ h^{-1} for the non-aerated sludge and 21.24 $mg \cdot L^{-1} \cdot h^{-1}$ for the periodically aerated sludge. These values indicate that (i) part of the methanogenic activity was reduced; and (ii) part of the acetic acid from anaerobic methanol degradation observed during the consortium build-up was likely converted to CO_2 and methane. Nevertheless, it was expected a progressive decrease of methanogenic activity after PAO bioaugmentation due to its competition for acetate under anaerobic conditions.

5.3.2.2. PAO bioaugmentation, simultaneous P and methanol removal

Negligible P-release or uptake was observed in SBR-C before PAO bioaugmentation, despite the anaerobic/aerobic phases. A significant amount of PAO or GAO was not expected in the inoculum sludge from the WWTP anaerobic digester. PAO and GAO can store VFA under anaerobic conditions, but they need an electron acceptor

(oxygen, nitrate, or nitrite) to grow, and these alternate conditions are not found in an anaerobic digester.

PAO were bioaugmented into SBR-C to promote EBPR after 25 days of methanol-degrading acetogens growth under sequential anaerobic/aerobic-settling cycles. This bioaugmentation was conducted with 4 L of enriched-PAO sludge from SBR-A (conventional anaerobic/aerobic configuration with propionic acid as carbon source). The initial biomass concentration in the bioaugmented system was 5,290 mg VSS·L^{-1} (6,135 mg TSS·L^{-1}, VSS/TSS=0.86). Figure 5.7 shows the experimental profiles of one cycle from the first 3 days. As observed, net-P removal (P-release and P-uptake) was achieved from the first day and was effectively maintained during the first 3 days with similar experimental profiles. In fact, P-release rate increased up to 25% when comparing day 3 and day 1. Assuming that methanol is not directly consumed by propionic acid-fed PAO (as previously determined in the direct replacement strategy), EBPR activity indicated that PAO were growing on the acetic acid generated by acetogens in the methanol degradation. Regarding the methanol consumption (Figure 5.8 right), it was observed that around 50 mg·L^{-1} of methanol were consumed during the first days of operation, but this amount increased along the operation to 90 mg·L^{-1} on day 17. To reduce the methanol remaining at the end of the aerobic phase, the initial amount was reduced to 90 mg·L^{-1}, achieving a concentration around 20 mg·L^{-1} at the end of the anaerobic phase.

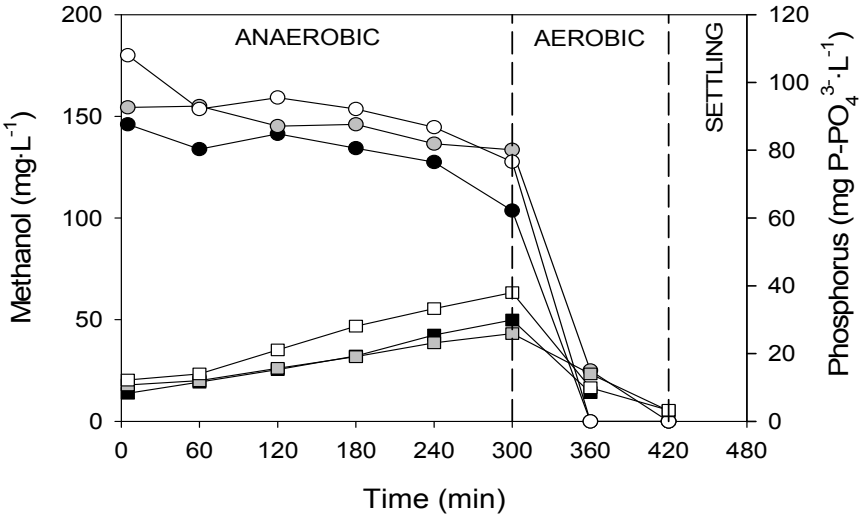

Figure 5.7. Experimental phoshorus (□) and methanol (○) profiles of the first cycles with the acetogenic-PAO consortium period (black: day 1, dark gray: day 2, white: day 3).

The system was run during more than 30 days and several cycles were monitored during this period. As can be observed (Figure 5.8 left), net-P removal was achieved most of the time, indicating that the syntrophic consortium was able to use methanol as sole carbon source for EBPR purposes. The biomass concentration decreased at the end of this period to 3,784 mg VSS·L^{-1} (4,540 mg TSS·L^{-1}) but maintaining a VSS/TSS ratio of 0.83. The phosphorus content of the biomass was estimated with Equation (5.2) and a high value around 0.06 g P·g^{-1} VSS was obtained supporting the observed EBPR activity.

Table 5.4. Summary of different FISH measurements for the syntrophic consortium with methanol.

	SBR-A (PAO-enriched sludge)	SBR-C (start bioaugmentation)	SBR-C (end bioaugmentation)
GAO, DFIMIX	1.9 ± 0.3	0.6 ± 0.1	0.06 ± 0.01
GAO, DFIIMIX	5.1 ± 1.0	1.6 ± 0.3	0.17 ± 0.03
GAO, GAOMIX	3.8 ± 1.0	1.2 ± 0.3	5.4 ± 2.1
PAOMIX	61 ± 6	19 ± 2	11 ± 3

However, both the average of the experimental P/C_{MET} ratio (mol $P \cdot mol^{-1}$ C-methanol) and the FISH measurements indicated a possible proliferation of GAO under the operating conditions. Table 5.4 shows that both DFI and DFII almost disappeared from the system, while GAOMIX-binding cells increased up to 5.4%. The presence of GAOMIX type GAO with acetate as carbon source has been extensively reported (Oehmen et al., 2007). The increase of GAOMIX linked to the decrease of DFI and DFII is consistent with the hypothesis of methanol degradation mostly to acetate rather than propionate. The experimental P/C_{MET} ratio was 0.54±0.11 (n = 9), during the first 15 days and it decreased up to 0.38±0.08 (n = 8) during the last period.

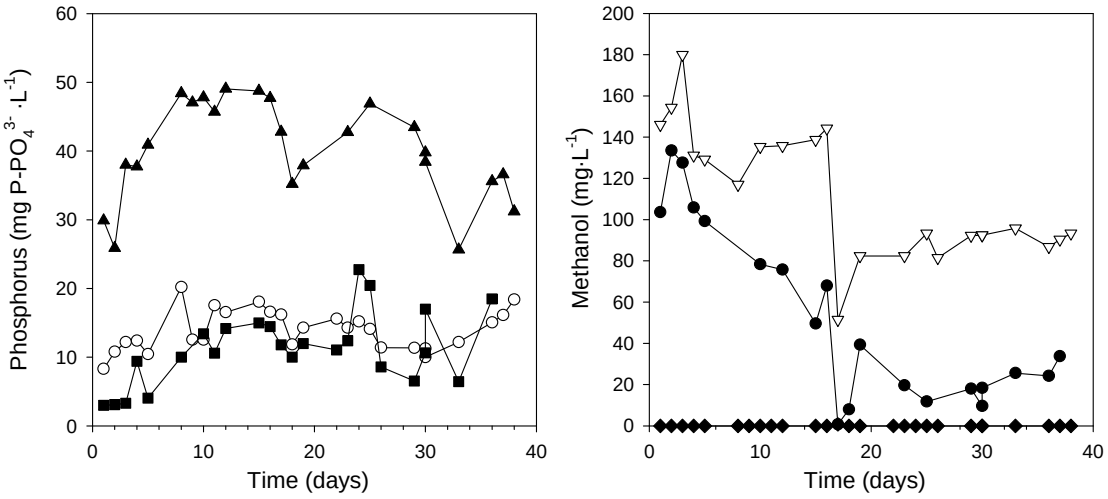

Figure 5.8. Experimental phosphorus and methanol profiles during the syntrophic acetogenic-PAO consortium period. Left: P start of cycle (○), P end of anaerobic phase (▲) and end of aerobic phase P (■) Right: methanol start of cycle (▽), methanol end of anaerobic phase (●) and end of aerobic phase methanol (◆).

The theoretical maximum P/C_{MET} ratio should be around 0.75 assuming that (i) the initial methanol was all converted to acetic acid (for every 4 mol of methanol 3 mol of acetic were formed); and (ii) the theoretical P/C_{ACET} ratio was 0.5. Three different plausible hypotheses can explain the fact that the experimental P/C ratio was lower than the theoretical one: (i) part of the methanol was used for growth purposes; (ii) part of the methanol was anaerobically degraded in a different pathway than desired (e.g., acidogenic butyrate formation, Heijthuijsen and Hansen, 1986; Kerby et al., 1983, formaldehyde or methane production Florencio et al., 1993a; Gonzalez-Gil et al., 1999); or (iii) part of the acetate was used by other microorganisms than PAO (e.g., GAO). The first hypothesis would explain only a 10% decrease in the P/C ratio, as 10% is the approximate amount of methanol devoted to growth and not transformed to acetate (Florencio et al., 1995).

Regarding the second hypothesis, no other intermediate apart from acetic acid (e.g., propionic acid or formaldehyde) was detected in any chromatographic analysis performed in the anaerobic samples. Nevertheless, a batch experiment with an enriched PAO-sludge was performed to exclude the possible use of formaldehyde as intermediate of the observed methanol-driven EBPR. It was tested whether PAO could uptake formaldehyde and, as expected, the results indicate that it was toxic or inhibitory (Figure 5.9). Formaldehyde concentration slightly decreased in this experiment, but it was linked to a high P-release, even under aerobic conditions. No P-uptake was observed and hence formaldehyde was likely more a toxic or inhibitor of PAO rather than a suitable carbon source.

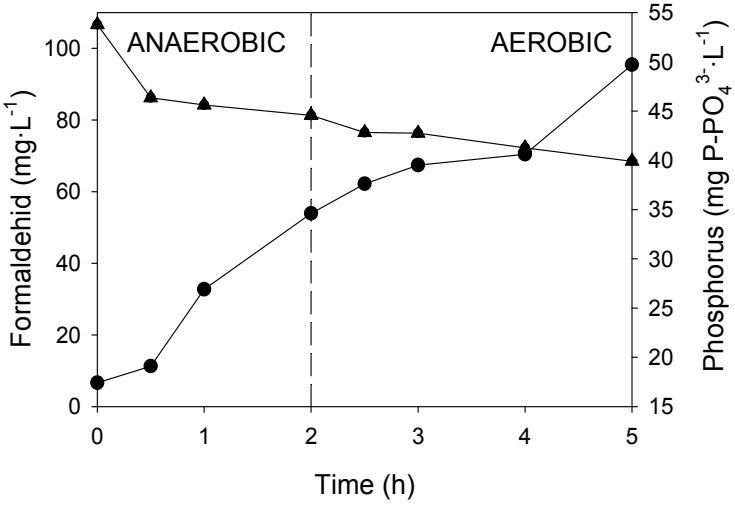

Figure 5.9. Experimental phosphorus and formaldehyde profiles obtained in a conventional batch experiment with a PAO-enriched sludge. Formaldehyde (▲), Phosphorus (●).

On the other hand, the hypothesis of acetate utilization by GAO is confirmed by FISH results. Although EBPR activity was maintained for 1 month, the PAO percentage decreased from 19% to 11%, while the total amount of GAO (sum of DFI, DFII, and GAOMIX) simultaneously increased from 3.4% to 5.6%. Considering these values, the percentage of PAO with respect the sum of GAO and PAO decreased from 85% to 66%. The P/C ratio calculated at the end of the period considering the initial P/C ratio of 0.54 and the change in these percentages is 0.42, which fairly agrees with the experimental ratio of 0.38±0.08. This result would exclude an important contribution of the second hypothesis. Nevertheless, the possible presence of other GAO-phenotype microorganisms not labeled with GAOMIX, DFIMIX, or DFIIMIX probes cannot be ruled out. In this sense, the identification and elucidation of the role of other microorganisms present in this novel syntrophic microbial community seems to be an interesting further research work for the whole understanding of this novel strategy for P-removal.

Finally, despite that the feasibility of EBPR with methanol using this syntrophic consortium of methanol-degraders and PAO is clearly shown in this work, future research in this field should aim at avoiding the long-term presence of GAO or different anaerobic pathways.

5.3.3. DIRECT REPLACEMENT OF PROPIONIC ACID FOR GLYCEROL (SBR-D)

Our first strategy to achieve glycerol-based EBPR was to feed a PAO-enriched sludge with glycerol as a sole carbon source. SBR-D was inoculated with PAO-enriched sludge withdrawn from SBR-A, which had been fed for several months with propionic acid as sole carbon source under conventional anaerobic/aerobic conditions. The major characteristics of this sludge were: 61±6% PAO, a P/C ratio of 0.44 mol P/mol C_{PROP} (typical of propionic-fed PAO, Oehmen et al., 2005a), a P-release rate of 31.4 mg $P \cdot g^{-1}$ $VSS \cdot h^{-1}$, 2.7 g $VSS \cdot L^{-1}$ and a VSS/TSS ratio at the end of the aerobic phase of 0.71. All these values were indicative of a sludge highly enriched in PAO.

Three periods (D1, D2, D3) with different SBR configurations were used (Table 5.2). Figures 5.10 and 5.11 summarise the results of this strategy. Period D1 consisted of a direct replacement of propionic acid for glycerol with the standard cycle configuration used with propionic acid in SBR-A. The initial glycerol concentration was set to a low value of 60 $mg \cdot L^{-1}$ to avoid its presence under aerobic conditions. From the first day on, most of the glycerol was consumed under anaerobic conditions (Figure 5.11).

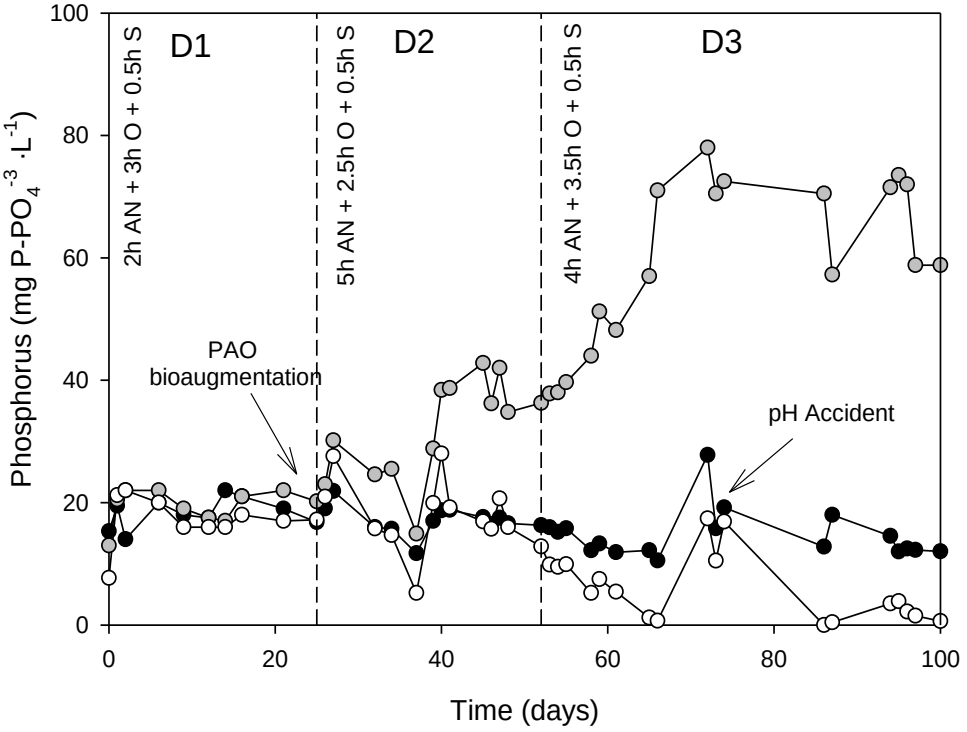

Figure 5.10. Phosphorus (●) evolution during the direct replacement of propionic acid for glycerol strategy. Initial (black), end anaerobic phase (grey), end aerobic phase (white).

However, this anaerobic COD consumption was not linked to any EBPR activity as neither P-release nor P-uptake was observed (Figure 5.10). During this period, PAO activity was periodically assessed through batch experiments with propionic acid as carbon source and PAO activity was progressively lost. Figure 5.12 (left) displays four of these batch tests conducted during the direct replacement strategy. The comparison of the batch tests at the start and at the end of period D1 (i.e. black vs white triangles) clearly shows this EBPR activity loss. These results were in agreement with the results

of Yuan et al. (2010), who introduced glycerol in an acetate-fed PAO- enriched sludge and the process failed.

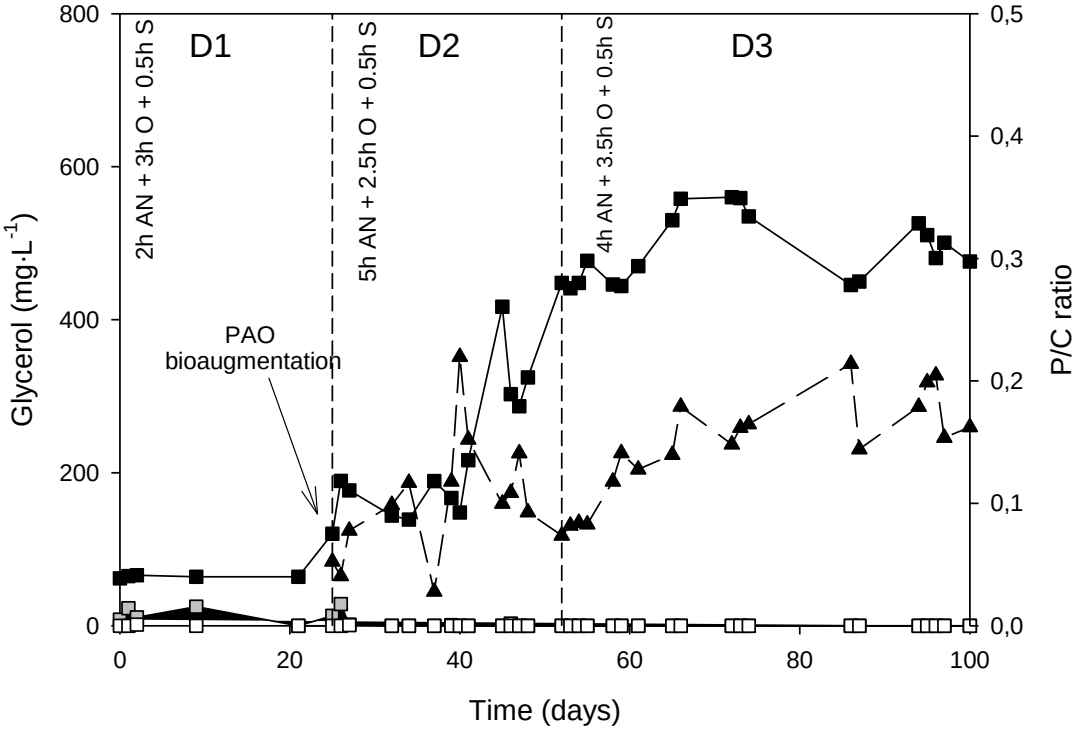

Figure 5.11. Glycerol (■) and P/C ratio (▲) during the direct replacement of propionic acid for glycerol strategy. Concentration of glycerol: Initial (black), end anaerobic phase (grey), end aerobic phase (white).

The initial amount of glycerol in period D1 was very low and, given the fact that it was not totally converted to PAO-utilisable products (see discussion below), the system might have been carbon limited. Then, it was decided to increase the amount of the initial glycerol up to 200 mg·L^{-1} and, consequently, increase the length of the anaerobic phase to 5 h (period D2, Table 5.2) so all glycerol could be anaerobically consumed (the total cycle length was in consequence extended to 8 h in period D2). Due to the decrease of PAO activity during period D1 (Table 5.2), SBR-D was bioaugmented with 5 L of PAO-enriched sludge from SBR-A to have a similar initial PAO population as period D1. This glycerol load increase and new configuration was beneficial and, after two weeks, P-release was already observed (Figure 5.10). Despite the periodic increase of the P-released, net-P removal was never achieved in period D2 (Figure 5.10), which prevented successful PAO enrichment. Initial glycerol was increased (Figure 5.11) so that P-uptake rate also increased as a consequence of higher PHA storage. However, EBPR activity did not improve in period D2. Periodical batch tests with propionic acid indicated a certain recovery of EBPR activity with respect to period D1 (Figure 5.12 left). However, the experimental P profiles of the cycles (Figure 5.13) indicated that, whereas the anaerobic phase was longer than needed to take up all the initial COD, the aerobic phase was too short for complete P-removal. Then, it was decided to test a last cycle configuration (period D3) with longer aerobic phase (Table 5.2).

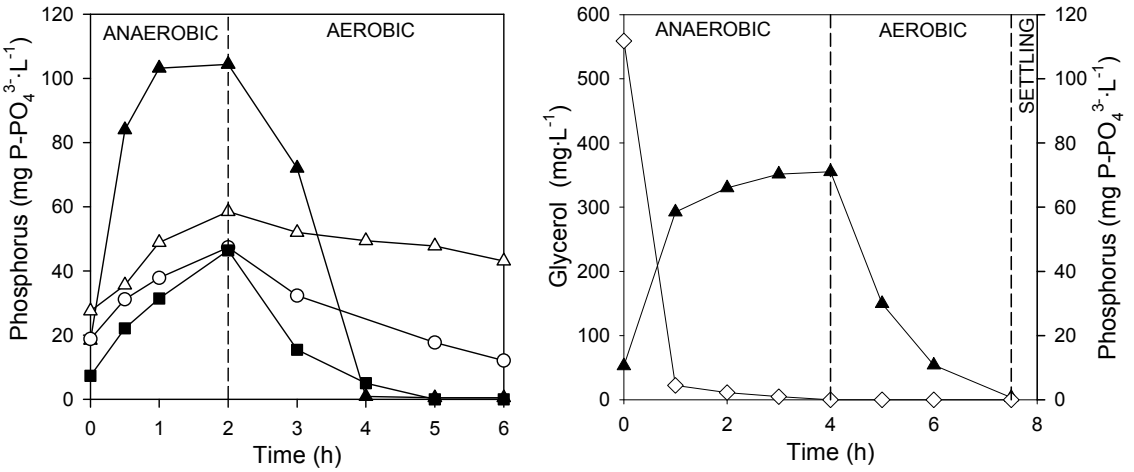

Figure 5.12. Left: Phosphorus profiles in four different batch tests with propionic acid during the direct replacement strategy: start of period D1 (▲), end of period D1 (△), . o. . (○) and mid period D3 (■). Right: Experimental P (▲) and glycerol (◇) profiles from the last cycle of period D3.

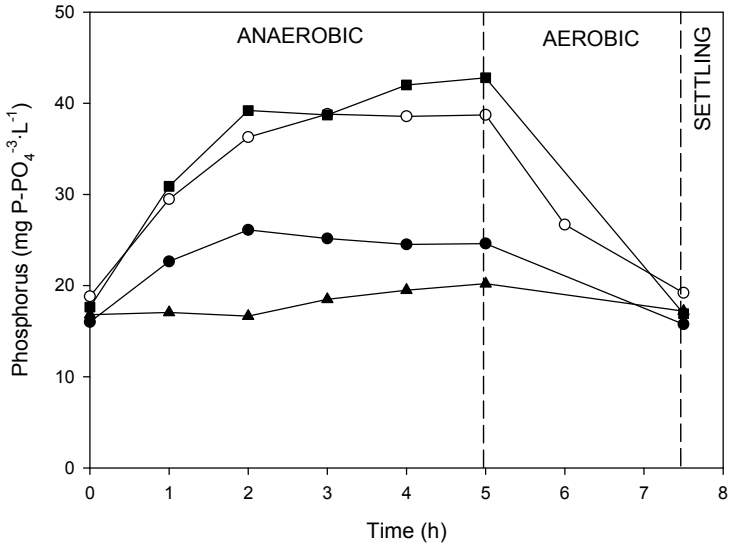

Figure 5.13. Phosphorus profiles in four different cycles with glycerol in period D2 during the direct replacement strategy. Day 26 (▲), day 32 (●), day 40 (○) and day 44 (■).

The configuration in period D3 was proved to be very successful and net-P removal was rapidly achieved (Figure 5.10). Consequently, PAO growth was favoured and EBPR activity was clearly observed. It was decided to push the system to its limits and the amount of initial glycerol was step-wise increased up to 500 mg·L^{-1} (Figure 5.11). Figure 5.12 (left) shows that the EBPR activity (tested with propionic acid) improved after period D3. Moreover, Figure 5.12 (right) illustrates a typical EBPR test with glycerol as a sole carbon source during this period, demonstrating for the first time glycerol-driven EBPR in a single-sludge system. The major causes of the success were that the SBR phases were sufficiently long for anaerobic glycerol transformation to VFA, VFA-uptake and posterior aerobic P-uptake.

Table 5.5. Summary of different FISH measurements for the direct replacement with glycerol.

Period	D1	D2	D3
GAO, DFIMIX	n.d	39±4.0	34±7.0
GAO, DFIIMIX	n.d	1±0.5	1.7±0.3
GAO, GAOMIX	n.d	1±0.5	1±0.5
PAOMIX	< 5 %	35.0±6.0	48.6±6.9

n.d: not detected

Two extra indications of the PAO increase during this period D3 are the FISH measurements and the P/C ratio evolution (i.e. amount of P-released per mol of carbon-glycerol taken up). The percentage of PAO was estimated at the end of each period using the FISH methodology (Table 5.5). The obtained results, i.e. sharp increase of % PAO in period D3, are in clear agreement with the experimental P profiles obtained. Figure 5.11 displays the P/C ratio evolution throughout the experiment. As can be observed, the ratio tends to 0.2 mol P/mol $C_{GLYCEROL}$. At first glance, this value may seem very low, particularly when compared to P/C ratios for conventional PAO electron donors: acetate (0.5 mol P/mol C_{AC}, Smolders et al., 1994) or propionate (0.42 mol P/mol C_{PROP}, Oehmen et al., 2005a). However, an explanation could be given: we hypothesise that glycerol was not directly used by PAO but it was firstly anaerobically degraded to products that could be used by PAO, essentially propionate. This would explain why glycerol was not directly used for PAO in the period D1 and also the long time required for EBPR obtainment in our system, i.e. the time required for anaerobic glycerol degraders or fermenters to grow. The anaerobic glycerol metabolism is widely described in the literature and propionate is known as the major fermentation product of its metabolism with respect to other compounds such as acetate, butyrate or propanol (Barbirato et al., 1997; Himmi et al., 2000; Yuan et al., 2010). The ratio of propionate to glycerol depends on the microbial culture used, being 0.6-0.8 mol/mol an average yield found in the literature with pure cultures, for example, *Propionibacterium freudenreichii* or *Propionibacterium acidipropionici* (Barbirato et al., 1997; Himmi et al., 2000; Zhang and Yang, 2009). However, this yield may be lower with mixed cultures. The real value is difficult to predict since the selectivity of fermentation products from a single substrate in mixed culture fermentations is, nowadays, a research issue (Temudo et al., 2008; Forrest et al., 2010). Hence, the maximum P/C ratio that could be obtained from glycerol could be calculated assuming i) the abovementioned propionic to glycerol yields and ii) that only PAO used the fermentation products. This would result in theoretical maximum P/C ratios around 0.25-0.33 mol P/mol $C_{GLYCEROL}$, which are closer to the ones experimentally observed (Figure 5.11). The lower values obtained could be probably linked to the fact that the propionate yield from glycerol was lower. In fact, the results obtained in the next section with a mixed culture (Figure 5.16) show a yield around 0.5 mol of propionate per mol of consumed glycerol (see discussion below) that would result in a theoretical P/C ratio of 0.21 mol P/mol $C_{GLYCEROL}$, which agrees with the experimental P/C ratio obtained (Figure 5.11). Another reason for this low P/C ratio could be the simultaneous consumption of part of glycerol or propionate by other microorganisms as for example GAO. Table 5.5 shows the percentage of GAO after periods D2 and D3. Figure 5.14 show an example of the FISH images obtained after period D3. A significant amount of

GAO, mostly DFIMIX-binding GAO were present. These microorganisms were already present in the initial inoculum (SBR-A) and are commonly observed in propionate-fed EBPR systems (Oehmen et al., 2010). Thus, the fact that DFIMIX-binding bacteria were not removed during this period can also be an indicator of propionate-based EBPR metabolism. Moreover, PHA and glycogen evolution was monitored in a batch experiment at the end of period D3. The results obtained were in agreement with the discussion above. The ratio of glycogen degraded to glycerol consumed in the anaerobic phase was around 0.25 mol $C_{GLYCOGEN}$/mol $C_{GLYCEROL}$ which, is lower than the theoretical value reported in the propionate metabolism of PAO (0.33 Oehmen et al., 2005a). This is in agreement with the fact that only a fraction of glycerol turns into PAO-utilisable products. Regarding PHA, its distribution at the end of the anaerobic phase was also distinctive of propionate-fed EBPR systems due to the high presence of PHV and PH2MV (Pijuan et al., 2009). In our case, the average distribution of the PHA at the end of the anaerobic phase was 25% PHB, 45% PHV and 30% PH2MV. The ratio of PHA produced per glycerol taken up in molar basis (PHA/$C_{GLYCEROL}$) was 0.31, which is also lower than the theoretical value of 1.22 reported in Oehmen et al. (2005a).

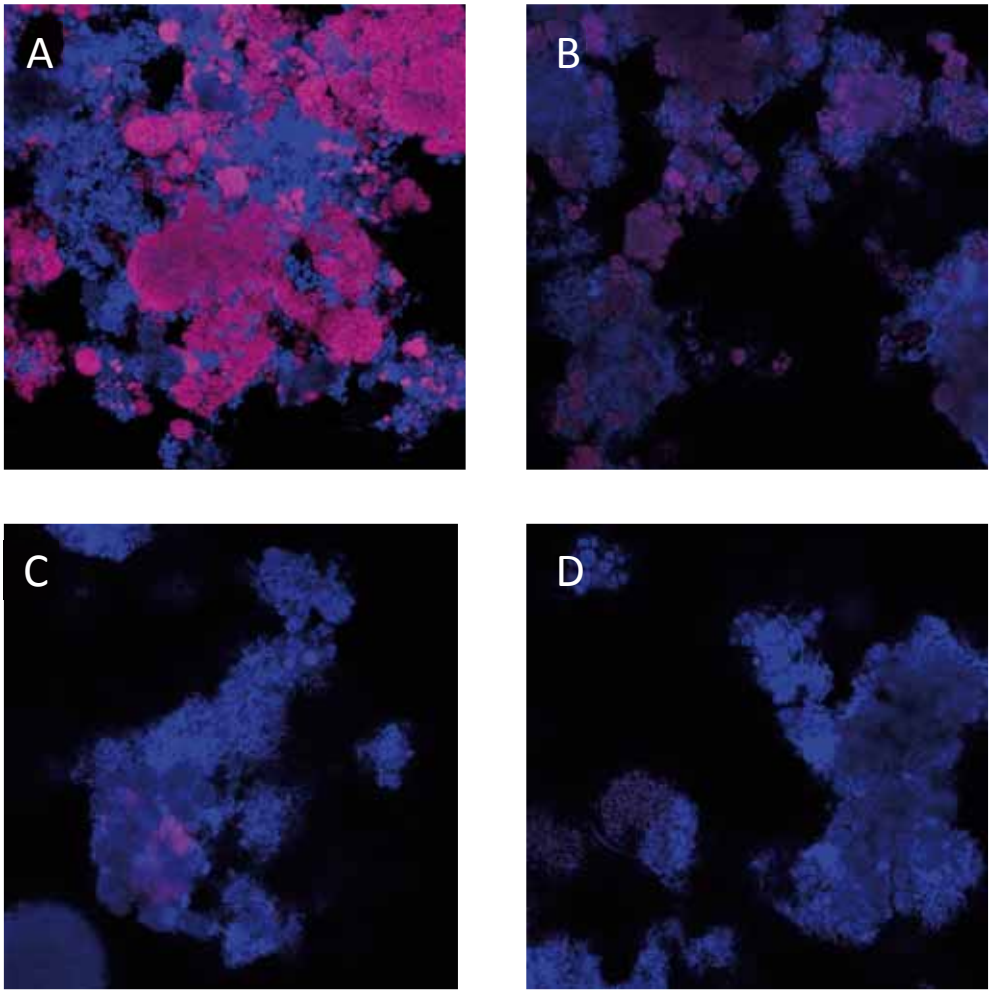

Figure 5.14. FISH/CLSM representative images of the sludge from the direct replacement strategy (SBR-D) at the end of period D3. EUBMIX is shown in blue, while specific probe is shown in pink; (A) PAOMIX, (B) DFIMIX, (C) DFIIMIX and (D) GAOMIX.

Assuming that a fermentation step was required in order to obtain glycerol-based EBPR, a new strategy was designed for a faster obtainment of the consortium sludge (i.e. anaerobic glycerol degraders + PAO). This two-step strategy consisted on bioaugmenting a glycerol-fed anaerobic sludge with PAO. The anaerobes (essentially, acidogens) would anaerobically degrade glycerol and PAO would live off the fermentation products enabling thus, simultaneous glycerol and phosphorus removal.

5.3.4. TWO-STEP CONSORTIUM DEVELOPMENT STRATEGY WITH GLYCEROL (SBR-E)

This strategy aimed at obtaining simultaneous glycerol and P removal using a syntrophic consortium between glycerol-degrading anaerobes and PAO in a two-step basis. The first should degrade glycerol to fermentation products, which in turn would be used by PAO for biological phosphorus removal purposes. This syntrophic consortium was obtained using a two-step procedure. In a first step (see Table 5.2, SBR-E periods E1-E3), anaerobic sludge inoculated in SBR-E was subjected to conditions so that glycerol acidogenesis was favoured against methanogenesis. Then, in a second step (periods E4-E5 from Table 5.2), SBR-E was bioaugmented with PAO-enriched sludge from SBR-A so that PAO live off the anaerobic glycerol degradation products.

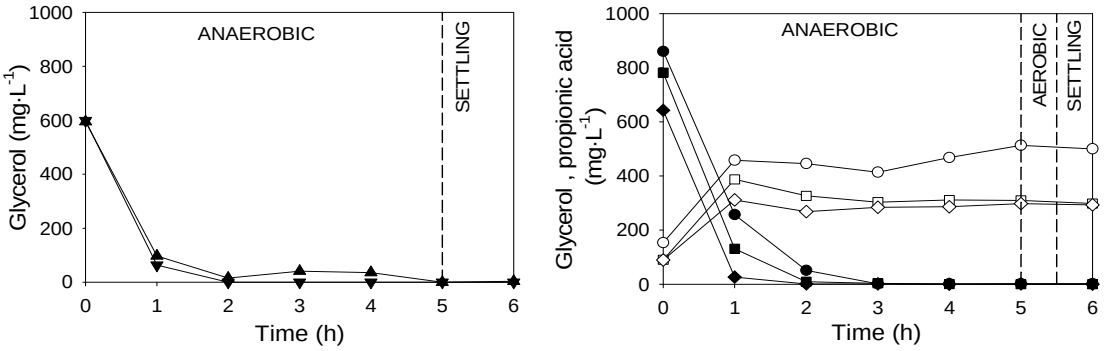

Figure 5.15. Left: Glycerol profiles for two cycles of period E1 of the two-step development strategy: day 1 (▲); day 5 (▼). Right: Glycerol (black) and propionic acid (white) profiles for three different cycles during period E2 of the two-step development strategy: day 13 (○), day 21 (□) and day 26 (◇).

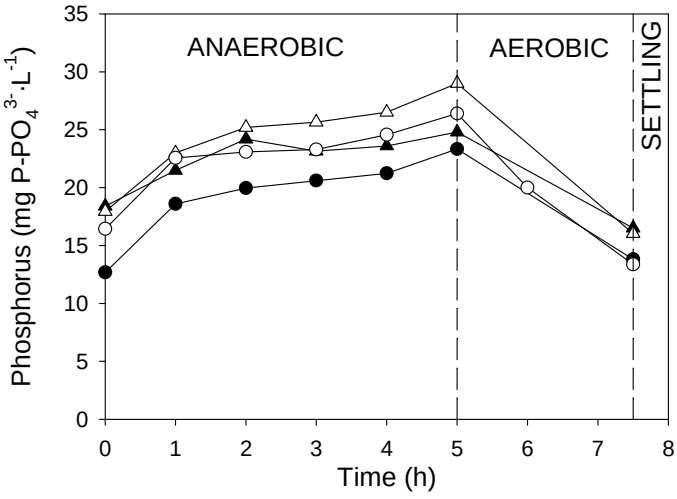

Figure 5.16. P profiles in four different cycles with glycerol in period E3 during the two-step consortium strategy. Day 44 (△), day 48 (●) and day 50 (○).

Period E1 corresponded to the acclimation period where a conventional anaerobic community was subjected to glycerol under strict anaerobic conditions (SBR-E). Figure 5.15 left shows the glycerol profiles of two cycles from this period. As can be observed, anaerobic glycerol utilization was obtained from the very first day. During this period, glycerol was likely converted to methane since fermentation products were scarcely present at the end of the reactive phase. In period E2, a short aerobic phase (30 min) was introduced to suppress the possible methanogenic activity. Figure 5.15 right shows three monitored cycles during period E2. As can be observed, the introduction of intermittent aeration (0.5 h of every 6 h) resulted in propionate production due to the expected suppression of methanogenic activity, which is in agreement with textbook knowledge (Hungate, 1969; Martin and Savage, 1988; Whitman et al., 1992). Glycerol was mostly degraded to propionate with an average ratio of 0.50±0.05 (n = 5) mol propionate/mol glycerol. As abovementioned, this value was lower than the ones reported in the literature for pure cultures and seems to be in agreement with the experimental results found in the previous section (section 5.3.3). The aerobic phase was extended 2 h during period E3 to assess whether long aerobic phases (typical of EBPR systems) were detrimental to anaerobic glycerol degraders. The results on period E3 (Figure 5.16) were very similar to the ones obtained in period E2, indicating that the conventional EBPR configuration was not harmful for this anaerobic sludge and that PAO bioaugmentation was feasible.

The system was bioaugmented with PAO at the start of period E4 (Figures 5.17) and EBPR linked to glycerol degradation was observed from the first day on. However, the extent of P release and P uptake was not as high as expected and the amount of net-P removed was very low. Similarly to the direct replacement strategy, the experimental P profiles indicated that EBPR activity was hindered by an aerobic phase that was too short. Then, aerobic phase was extended in period E5 (Table 5.2) resulting in the same configuration as in period D3 of SBR-D. The results obtained in period E5 were more satisfactory than in period D3; however, the EBPR activity observed with the direct replacement strategy was never achieved. The major cause of this difference can be found in the experimental ratios that indicate that part of the initial glycerol was not used for EBPR purposes: $P/C_{GLYCEROL}$ ratio (always lower than 0.15, Figure 5.17 down), $Gly_{DEG}/C_{GLYCEROL}$ (0.27 mol $C_{GLYCOGEN}$/mol $C_{GLYCEROL}$ in a batch experiment at the end of period E5) and $PHA/C_{GLYCEROL}$ (0.47 mol C_{PHA}/mol $C_{GLYCEROL}$ in a batch experiment at the end of period E5). These values were lower than the theoretical values proposed for propionate-fed PAO (Oehmen et al., 2005b). The PHA distribution in these experiments was 8% PHB, 35% PHV and 57% PH2MV, distinctive again of propionate-based EBPR metabolism. The growth of some glycerol degraders (inoculated with the anaerobic sludge) that did not produce VFA and the promotion of anaerobic VFA scavengers other than PAO (as for example GAO, see Table 5.6) could be a possible explanation for the low $P/C_{GLYCEROL}$ ratio. Figure 5.18 show an example of the FISH images obtained after period E5, where a significant fraction of DFIMIX-binding GAO can be observed. In any case, it should be noted that this strategy also resulted in a significant PAO growth (Table 5.6) achieving PAO percentages similar to the direct replacement strategy. As a conclusion, despite the fact that glycerol-based EBPR was obtained, this strategy failed to be faster (or even better) than the previous strategy and, hence, according to our experimental results, the direct replacement strategy is recommended for glycerol.

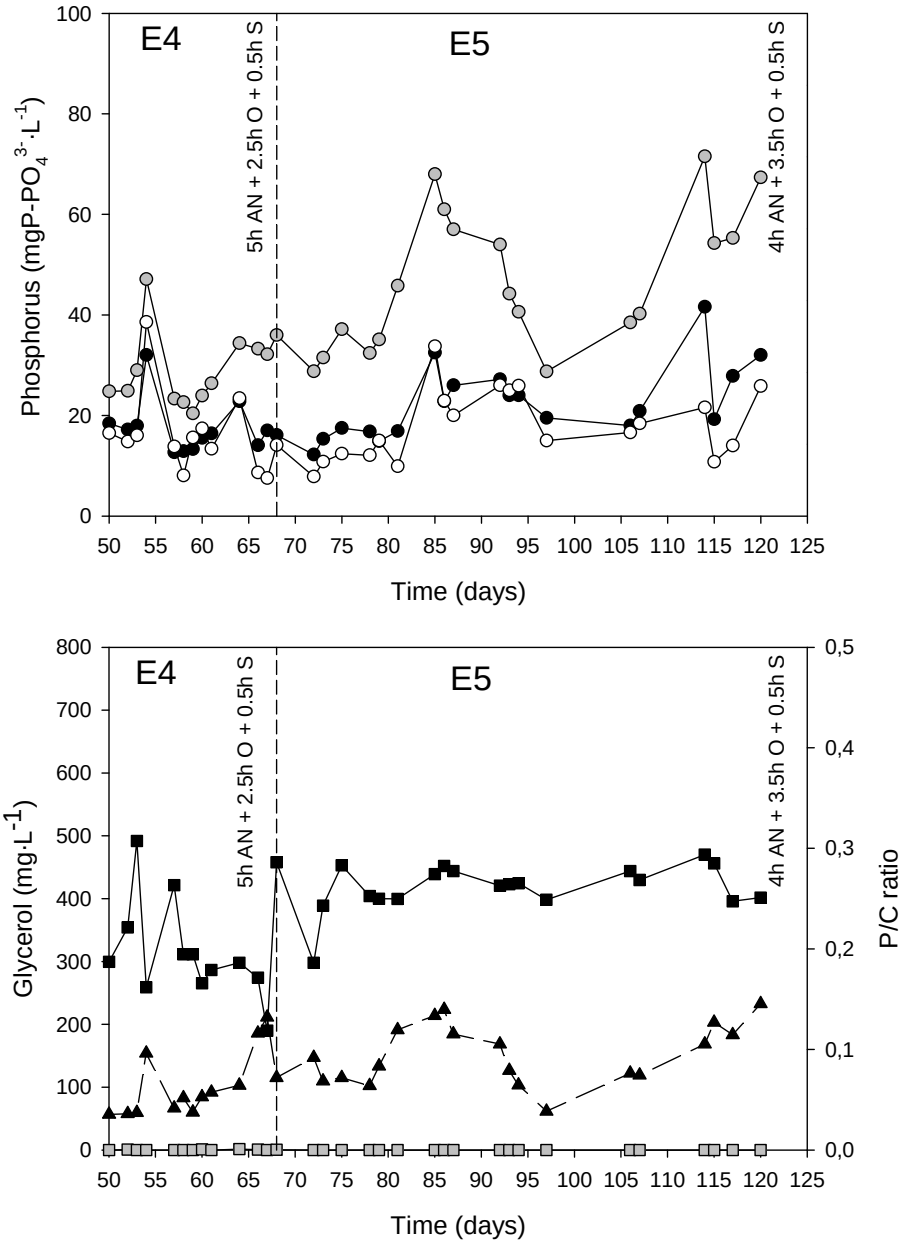

Figure 5.17. Up: Phosphorus profiles during two-step consortium strategy for glycerol. Initial (black), end anaerobic phase (grey), end aerobic phase (white). Down: Glycerol (■) and P/C ratio (▲). Concentration of glycerol: Initial (black), end anaerobic phase (grey).

Using long anaerobic retentions times, the growth of anaerobic glycerol degraders which produce PAO-utilisable products are favoured due to the presence of a highly active EBPR sludge that rapidly uses these fermentation products. However, if anaerobic glycerol degraders are previously selected without PAO, different anaerobic glycerol degradation routes could be favoured, including bacteria (other than PAO) able to use fermentation products.

Table 5.6. Summary of different FISH measurements for the two-step consortium strategy with glycerol.

Period	E4	E5
GAO, DFIMIX	20±3	30±4
GAO, DFIIMIX	1.3±0.2	0.7±0.2
GAO, GAOMIX	1.9±0.4	n.d
PAOMIX	8.3±3.4	46.4±11.0

n.d: not detected

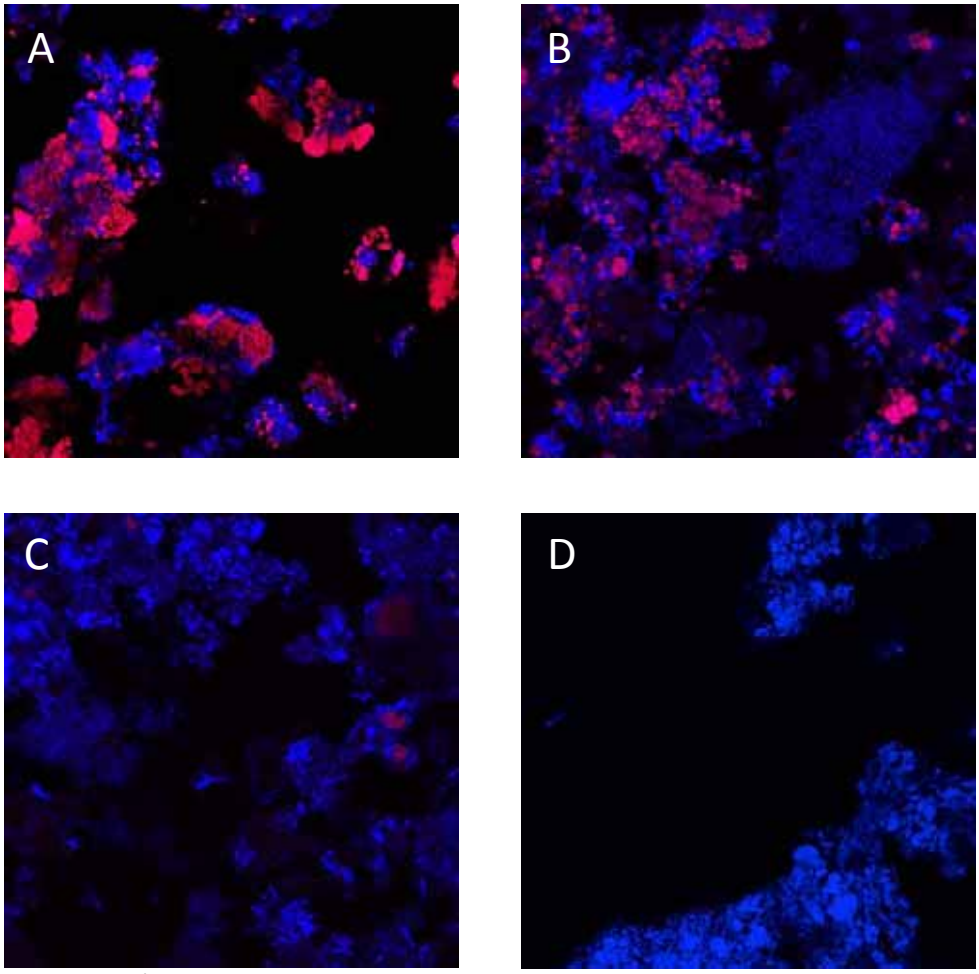

Figure 5.18. FISH/CLSM representative images of the sludge from two-step consortium strategy for glycerol (SBR E) at the end of period E5. EUBMIX is shown in blue, while specific probe is shown in pink; (A) PAOMIX, (B) DFIMIX, (C) DFIIMIX and (D) GAOMIX.

A SBR was run using dairy wastewater as sole carbon source which, led to a low COD/P ratio (around 11.5±1.5) (Figure 5.19). According to Broughton et al. (2008), 13 is the minimum COD/P ratio in order to have successful P removal in similar wastewaters. As can be observed, both net-P removal and the percentage of P removal were very low during the first days. Then, from day 7 on, a concentrated solution of glycerol was added together with dairy wastewater resulting in a COD/P ratio increase up to 16±1. This modification led to successful results since both net-P removal and the percentage of P removal were significantly increased.

This study completes previous works where glycerol was shown as a good carbon source for denitrification. Consequently, glycerol could be recommended as an external carbon source for both nitrogen and phosphorus removal. This would simplify WWTP management avoiding the utilization of more dangerous carbon sources as methanol, which must be diluted to reduce its fire hazard and would also allow the utilization of a waste material with a disposal associated costs. However, the interaction between denitrifiers and PAO and their competition for the carbon source must be further studied in view of its utilisation in biological nutrient removal systems. These studies, which were out of the scope of the present work, are nowadays being conducted as a continuation of this research line.

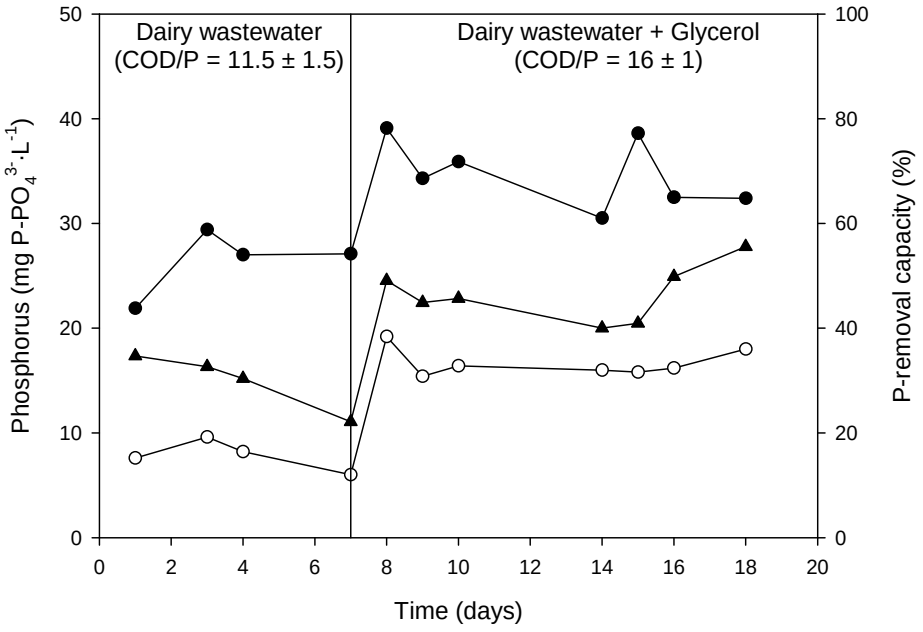

Figure 5.19. Experimental net-P removal activity variation when glycerol is supplemented to a dairy wastewater feed. P influent (●), P net-removal (○) and P-removal capacity (▲).

5.3.5. RESPONSE OF THE SYNTROPHIC CONSORTIUM TO OTHER CARBON SOURCES

The response of the methanol-fed consortium (SBR-C) and the direct replacement glycerol-fed consortium (SBR-D) to different carbon sources was studied and compared to the response with a propionate-fed PAO-enriched sludge (SBR-A). Different carbon sources were tested in single batch tests and the evolution of phosphorus was monitored. Figure 5.20 shows the P release, P uptake and net-P removal for each of the batch experiments.

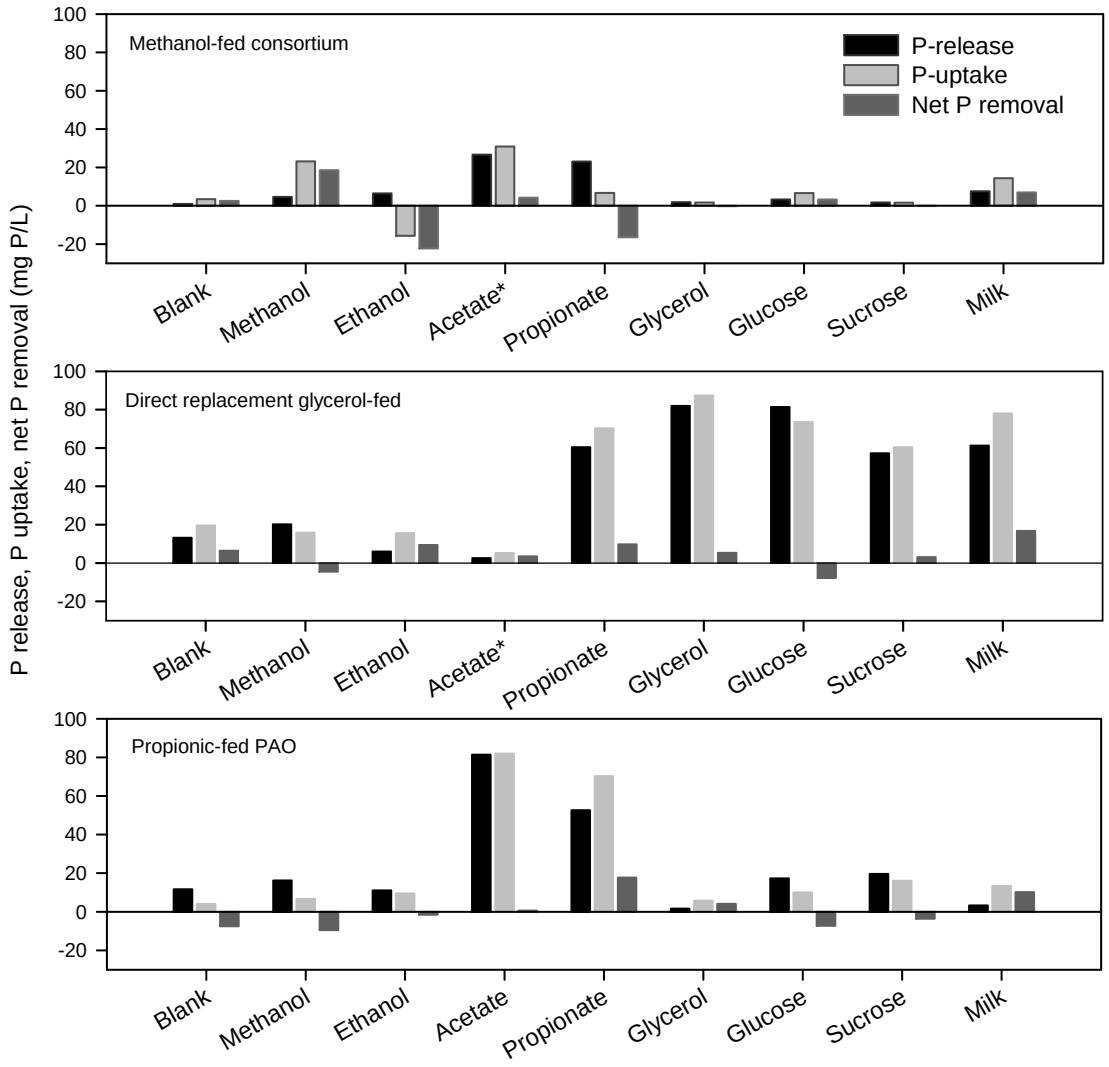

Figure 5.20. P-release, P-uptake and net-P removal for methanol-fed consortium (SBR-B), direct replacement glycerol-fed (SBR-D) and propionic-fed PAO (SBR-A) using different carbon sources (initial concentration 600 mg COD·L^{-1} except for acetate* which was 100 mg COD·L^{-1}).

As can be observed, the activity with the direct replacement glycerol-fed was much higher than in the other cases indicating that this consortium could successfully operate with other similar complex carbon sources as glucose, sucrose or milk. On the other hand, the methanol-fed consortium shows much less activity and significant P activity was only observed with methanol, acetic and propionic acid. The reason is that the anaerobic biomass distribution is very different in both cases. In the methanol-fed consortium case, anaerobic methanol acetogens are selected and they are unable to degrade complex substrates with higher number of carbons. On the other side, the anaerobic biomass selected during glycerol degradation is also able to degrade other carbon sources using the same pathway.

5.4.CONCLUSIONS

Syntrophic consortia comprising conventional anaerobic biomass and PAO were developed in single sludge configurations. The major outcome of these consortiums is the utilisation of complex fermentable substrates as carbon source for EBPR in single sludge systems. Then, these substrates could be an alternative carbon source for WWTP facing carbon shortages and avoiding EBPR failures.

Methanol-driven EBPR (P-removal using methanol as sole carbon source) was obtained using a syntrophic consortium between methanol-degrading acetogens and PAO. The first would be in charge of converting methanol to by-products, which could be used by PAO as carbon source for biological P-removal. This syntrophic consortium was obtained using a two-step procedure: an anaerobic sludge was subjected to conditions favouring methanol degradation via acetogenesis and then it was bioaugmented with PAO-enriched sludge. Conversely, a previous strategy with direct replacement of propionic acid by methanol in a conventional anaerobic/aerobic configuration demonstrated that only enriched PAO biomass was unable to survive using methanol as sole carbon source.

On the other hand, this work also demonstrates the feasibility of using glycerol as a sole carbon source for EBPR. The best results were obtained with the direct replacement strategy with a longer anaerobic phase than the default configuration. Using long anaerobic retentions times, anaerobic glycerol degradation to PAO-utilisable products are favoured due to the presence of a highly active EBPR sludge. The single-sludge SBR with 4 h anaerobic, 3.5 h aerobic and 0.5 settling was shown as a proper configuration to achieve net-P removal using only this carbon source. Therefore, glycerol is a promising external carbon source in full-scale WWTP facing carbon source shortages when enough anaerobic hydraulic retention time is provided.The development of a microbial community able to use glycerol for EBPR opens a new range of possibilities. With a similar approach, other fermentable substrates could also be used as carbon sources for EBPR.

Finally, the response of each consortium (methanol-fed or glycerol-fed) to other complex sources was studied and it was concluded that for long-chain complex carbon (i.e. glucose, sucrose and milk) the glycerol-fed consortium was the best when compared to the methanol-fed consortium. In this case, the fermentation from complex substrates to propionic acid was favoured instead of acetic acid.

5.5. REFERENCES

Akunna, J. C., Bizeau, C., and Moletta, R., 1993. Nitrate and nitrite reductions with anaerobic sludge using various corbon sources: glucose, glycerol, acetic acid lactic acid. Water Research, 27(8), 1303–1312.

Barbirato, F., Chedaille, D., and Bories, A., 1997. Propionic acid fermentation from glycerol: comparison with conventional substrates. Applied Microbiology and Biotechnology, 47(4), 441–446.

Bodík, I., Blstáková, a, Sedlácek, S., and Hutnan, M., 2009. Biodiesel waste as source of organic carbon for municipal WWTP denitrification. Bioresource Technology, 100(8), 2452–6.

Brdjanovic, D., Hooijmans, C. M., van Loosdrecht, M. C. M., Alaerts, G. J., and Heijnen, J. J., 1996. The dynamic effects of potassium limitation on biological phosphorus removal. Water Research, 30(10), 2323–2328.

Broughton, A., Pratt, S., and Shilton, A., 2008. Enhanced biological phosphorus removal for high-strength wastewater with a low rbCOD:P ratio. Bioresource Technology, 99(5), 1236–1241.

Cho, E. and Molof, A. H., 2004. Effect of sequentially combining methanol and acetic acid on the performance of biological nitrogen and phosphorus removal. Journal of Environmental Management, 73(3), 183–187.

Chu, A., Mavinic, D S, Kelly, H. G., and Ramey, W. D., 1994. Volatile fatty acid production in thermophilic aerobic digestion of sludge. Water Research, 28(7), 1513–1522.

Conrad, R., Schink, B., and Phelps, T., 1986. Thermodynamics of H2-consuming and H2-producing metabolic reactions in diverse methanogenic environments under in situ conditions. FEMS, 38(1986), 353–360.

Escapa, A., Manuel, M. F., Morán, A., Gómez, X., Guiot, S. R., and Tartakovsky, B., 2009. Hydrogen Production from Glycerol in a Membraneless Microbial Electrolysis Cell. Energy & Fuels, 23(9), 4612–4618.

Feng, L., Chen, Y., and Zheng, X., 2009. Enhancement of waste activated sludge protein conversion and volatile fatty acids accumulation during waste activated sludge anaerobic fermentation by carbohydrate substrate addition: the effect of pH. Environmental Science andTechnology, 43(12), 4373–4380.

Ferry, J. G., 1993. *Methanogenesis: Ecology, physiology, biochemistry, & genetics*, New York, USA.

Florencio, L., Jenicek, P., Field, J. A., and Lettinga, G., 1993a. Effect of cobalt on anaerobic degradation of methanol. Journal of Fermentation and Bioengineering, 75(5), 368–374.

Florencio, L., Nozhevnikova, A., van Langerak, A., Stams, A. J. M., Field, J. A., and G., L., 1993b. Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiology Letters, 109, 1–6.

Florencio, L., Field, J. A., and Lettinga, G., 1995. Substrate competition between methanogens and acetogens during the degradation of methanol in UASB reactors. Water Research, 29(3), 915–922.

Forrest, A. K., Sierra, R., and Holtzapple, M. T., 2010. Effect of biodiesel glycerol type and fermentor configuration on mixed-acid fermentations. Bioresource Technology, 101(23), 9185–9189.

Gonzalez-Gil, G., Kleerebezem, R., and Lettinga, G., 1999. Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH4 monitoring. Applied and Environmental Microbiology, 65, 1789–1793.

Grabinska-Loniewsa, A., Slomczynski, T., and Kanska, Z., 1985. Denitrification sludge with glycerol as a sole carbon source. Water Research, 19(12), 1471–1477.

Guerrero, J., Guisasola, A., and Baeza, J. A. 2011. The nature of the carbon source rules the competition between PAO and denitrifiers in systems for simultaneous biological nitrogen and phosphorus removal. Water Research, 45(16), 4793–4802.

Heijthuijsen, J. H. F. G. and Hansen, T. A., 1986. Interspecies hydrogen transfer in cocultures of methanolutilizing acidogens and sulfate-reducing or methanogenic bacteria. FEMS Microbiology Letters, 38, 57–64.

Himmi, E. H., Bories, a, Boussaid, a, and Hassani, L., 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii ssp. shermanii*. Applied Microbiology and Biotechnology, 53(4), 435–440.

Hungate, R. E., 1969. “A roll tube method for cultivation of strict anaerobes.” in J. R. Norris and D. W. Ribbons (eds.), Modern Methods in the Study of Microbial Ecology. Academic Press, New York, USA, 117–132.

Isaacs, S. H. and Henze, M., 1995. Controlled carbon source addition to an alternating nitrification-denitrification wastewater treatment process including biological P removal. Water Research, 29(1), 77–89.

Jarrel, K. F. and Kalmokoff, M. L., 1988. Nutritional requirements of the methanogenic archaebacteria. Canadian Journal of Microbiology, 34(5), 557–576.

Jeon, C. O. and Park, J. M., 2000. Enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose as a sole carbon source. Water Research, 34(7), 2160–2170.

Johnson, D. T. and Taconi, K. A., 2007. The Glycerin Glut: Options for the Value-Added Conversion of Crude Glycerol Resulting from Biodiesel Production. Enviromental progress, 26(4), 338–348.

Kalle, G. P. and Menon, K. K. G., 1984. Inhibition of methanogenesis and its reversal during biogas formation from cattle manure. Journal of Biosciences, 6(3), 315–324.

Karnholz, A., Küsel, K., Gößner, A., Schramm, A., and Drake, H. L., 2002. Tolerance and Metabolic Response of Acetogenic Bacteria toward Oxygen. Applied and Environmental Microbiology, 68(2), 1005–1009.

Kato, M. T., Field, J. A., and Lettinga, G., 1997. Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. Brazilian Journal of Chemical Engineering, 14(4).

Kerby, R., Niemczura, W., and Zeikus, J. G., 1983. Single-carbon catabolism in acetogens: Analysis of carbon flow in acetobacterium woodii and butyribacterium methylotrophicum by fermentation and 13C nuclear magnetic resonance measurement. Journal Bacteriology, 155(3), 1208–1218.

Kotsyurbenko, O. R., Nozhevnikova, A. N., Soloviova, T. I., and Zavarzin, G. A., 1996. Methanogenesis at low temperatures by microflora of tundra wetland soil. Anthonie van Leewuenhoek, 69(1), 75–86.

Kotsyurbenko, O. R., Glagolev, M. V., Nozhevnikova, A. N., and Conrad, R., 2001. Competition between homoacetogenic bacteria and methanogenic archaea for hydrogen at low temperature. FEMS Microbiology Ecology, 38(2-3), 153–159.

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

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

Louzeiro, N. R., Mavinic, D. S., Oldham, W. K., Meisen, A., and Gardner, I. S., 2002. Methanol-induced biological nutrient removal kinetics in a full-scale sequencing batch reactor. Water Research, 36(11), 2721–2732.

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

Marcelino, M., Guisasola, A., and Baeza, J. A., 2009. Experimental assessment and modelling of the proton production linked to phosphorus release and uptake in EBPR systems. Water Research, 43(9), 2431–2440.

Martin, J. H. and Savage, D. C., 1988. Degradation of DNA in cells and extracts of the obligately anaerobic bacterium Roseburia cecicola upon exposure to air. Applied and Environmental Microbiology, 54(6), 1619–1621.

Merzouki, M., Bernet, N., Delgenès, J. P., and Benlemlih, M., 2005. Effect of prefermentation on denitrifying phosphorus removal in slaughterhouse wastewater. Bioresource Technology, 96(12), 1317–1322.

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

Oehmen, A., Lehmann, B. K., Zeng, R J, Yuan, Z., and Keller, J., 2005b. Optimisation of poly-β-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. Journal of Chromatography A, 1070(1-2), 131–136.

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

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

Paulo, P. L., Stams, A. J. M., Field, J. A., Dijkema, C., van Lier, J. B., and Lettinga, G., 2003. Pathways of methanol conversion in a thermophilic anaerobic (55 degrees C) sludge consortium. Applied Microbiology and Biotechnology, 63(3), 307–14.

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

Pijuan, M., Casas, C., and Baeza, J. A., 2009. Polyhydroxyalkanoate synthesis using different carbon sources by two enhanced biological phosphorus removal microbial communities. Process Biochemistry, 44(1), 97–105.

Puig, S., Coma, M., Monclús, H., van Loosdrecht, M. C. M., Colprim, J., and Balaguer, M. D., (2008). Selection between alcohols and volatile fatty acids as external carbon sources for EBPR. Water Research, 42(3), 557–66.

Purtchert, I., Siegrist, H., and Gujer, W., 1996. Enhanced denitrification with methanol at WWTP Zürich-Werdhölzli. Water Science and Technology, 33(12), 117–126.

Randall, A. A., Benefield, L. D., and Hill, W. E., 1997a. Induction of phosphorus removal in an enhanced biological phosphorus removal bacterial population. Water Research, 31(11), 2869–2877.

Randall, A A, Benefield, L D, Hill, W E., Nicol, J. P., Boman, G. K., and Jing, S. R., 1997b. The effect of volatile fatty acids on enhanced biological phosphorus removal and population structure in anaerobic/aerobic sequencing batch reactors. Water Science and Technology, 35(1), 153–160.

Smolders, G. J. F., Meij, J., van Loosdrecht, M. C. M., and Heijnen, J. J., 1994. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnology and Bioengineering, 44, 837–849.

Spanjers, H., Vanrolleghem, P., Olsson, G., and Dold, P., 1996. Respirometry in control of the activated sludge process-principles. Water Science and Technology, 34(3-4), 117–126.

Speece, R. E. 1996. Anaerobic biotechnology for industrial wastewaters. New York, USA.

Temudo, M. F., Poldermans, R., Kleerebezem, R., and van Loosdrecht, M. C. M., 2008. Glycerol fermentation by (open) mixed cultures: a chemostat study. Biotechnology and Bioengineering, 100(6), 1088–1098.

Tong, J. and Chen, Y., 2007. Enhanced biological phosphorus removal driven by short-chain fatty acids produced from waste activated sludge alkaline fermentation. Environmental Science and Technology, 41(20), 7126–7130.

Torà, J. A., Baeza, J A, Carrera, J., and Oleszkiewicz, J. A., 2011. Denitritation of a high-strength nitrite wastewater in a sequencing batch reactor using different organic carbon sources. Chemical Engineering Journal, 172(2-3), 994–998.

Whitman, W. B., Bowen, T. C., and Boone, D. R., 1992. *The Prokaryotes, vol. 1* (A. Balows, H. G. TruEper, M. Dworkin, W. Harder, and K. H. Schleifer, eds.), Springer Verlag, New York, USA.

Yazdani, S. S. and Gonzales, R., 2007. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Current Opinion in Biotechnology, 18(3), 213–219.

Yilmaz, G., Lemaire, R., Keller, J., and Yuan, Z., 2007. Effectiveness of an alternating aerobic, anoxic/anaerobic strategy for maintaining biomass activity of BNR sludge during long-term starvation. Water Research, 41(12), 2590–8.

Yuan, Q., Sparling, R., Lagasse, P., Lee, Y. M., Taniguchi, D., and Oleszkiewicz, J. A., 2010. Enhancing biological phosphorus removal with glycerol. Water science and technology , 61(7), 1837–43.

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

Zhang, A. and Yang, S. T., 2009. Propionic acid production from glycerol by metabolically engineered *Propionibacterium acidipropionici*. Process Biochemistry, 44(12), 1346–1351.

Chapter 6

THE SELECTIVE ROLE OF NITRITE IN THE PAO-GAO COMPETITION

Part of this chapter has been submitted as:

Tayà, C., Garlapati, V. K., Guisasola, A., and Baeza, J.A., (2013). The selective role of nitrite in the PAO-GAO competition. Chemosphere.

Proliferation of Glycogen Accumulating Organisms (GAO) accounts as one of the major bottlenecks in biological phosphorus removal systems. GAO outcompeting Polyphosphate Accumulating Organisms (PAO) results in lower P-removal and in a significant cost increase. Thus, finding optimal conditions that favour PAO in front of GAO is a current focus of research. Two strategies were designed with this purpose: i) an anaerobic/anoxic (A2) configuration using propionic acid as electron donor in the anaerobic phase and nitrite as electron acceptor in the anoxic phase; and ii) periodic alternation of anaerobic/anoxic and anaerobic/aerobic cycle configurations, to alternate oxygen and nitrite as electron acceptors. The first strategy shows how nitrite can provide a novel strategy for PAO enrichment. A propionate-fed GAO-enriched biomass (70% DFI, 18% DFII and 10% PAO) was operated more than 50 days under anaerobic/anoxic conditions with nitrite as electron acceptor. These operational conditions led to a PAO-enriched sludge (85%) where GAO were washed out of the system (<10%), demonstrating the validity of the new approach for PAO enrichment even with low P influent. Moreover, the second strategy confirms the results obtained in the first strategy achieving a PAO-enriched sludge (from 18% to 70%) and washing out GAO of the system (<2%). In addition, the presented suppression of Defluviicocus GAO with nitrite represents an add-on to the nitrite-based systems since the proliferation of non-desirable GAO can be easily ruled out and added to the other benefits (i.e. lower aeration and COD requirements).

6.1. INTRODUCTION

The proliferation of several PAO competitors, especially GAO, has become a significant bottleneck in the EBPR process. GAO presence in EBPR systems increases the carbon and chemical requirements, the sludge production and the total overall costs of the plant (Oehmen et al., 2010a; Saunders et al., 2003). Thus, many works (*e.g.*, Lopez-Vazquez et al., 2009; Oehmen et al., 2010b) aim at understanding the competition between PAO and GAO in view of finding the optimal conditions that favours PAO growth against GAO in EBPR systems. PAO and GAO are not single microorganisms but common terms to describe the observed response of several groups with respect to biological P removal. The latest microbiological studies have revealed the existence of several subgroups of PAO-GAO with different phenotypes (Table 6.1). Regarding PAO, the *Candidatus Accumulibacter phosphatis*, hereafter referred as *Accumulibacter*, are the most widely known PAO in contrast to others as for example *Tetrasphera*-related or *Dechloromonas*-related (Oehmen et al., 2010b). Two different types of *Accumulibacter* (PAO I and PAO II) have been described using the polyphosphate kinase gene as genetic marker (He et al., 2007; Peterson et al., 2008) with different denitrification capacity (i.e. PAO I can denitrify nitrate and nitrite whereas PAO II can only use nitrite). Regarding GAO, significant diversity has also been detected, being the *Gammaproteobacteria Candidatus Compectibacter phosphatis* (from this point on, *Competibacter*) and the *Alfaproteobacteria Defluviicoccus Vanus*, the most abundant in full-scale plants (from this point on, *Defluviicoccus*). The major difference between both groups is their affinity for propionic and acetic acids. *Defluviicoccus* can be divided into four different clusters (from DFI to DFIV), which have different denitrification capabilities. The denitrification capacities of *Defluviicoccus* DFI and DFII are shown in Table 6.1, while capacities of DFIII and DFIV, which are only found in some WWTP (McIlroy and Seviour, 2009), have not been described yet to the best of our knowledge.

The response of the microorganisms involved in bio-P removal to different electron acceptors and donors (Table 6.1) is the starting point of the present work. According to this information, none of the reported groups of GAO could survive in an EBPR system

with propionate as sole electron donor and nitrite as sole electron acceptor. Hence, these two simultaneous conditions should lead to the washout of the GAO typically found in WWTP and thus, to PAO-enriched sludge. Moreover, as nitrite is a common electron acceptor for PAO I and PAO II, the distribution of these two organisms in the sludge should be similar.

Table 6.1. Comparison of the preferred VFA and the denitrification capabilities for the different PAO-GAO subgroups (adapted from Oehmen et al., 2010b).

	Preferred VFA	**Denitrification capacity**	
		NO_3^-	**NO_2^-**
Accumulibacter PAO I	Acetate and Propionate	✓	✓
Accumulibacter PAO II		✖	✓
Competibacter Sub-groups 1, 4, 5	Acetate	✓	✖
Competibacter Sub-groups 3,7		✖	✖
Competibacter Sub-group 6		✓	✓
Defluviicoccus DFI	Propionate	✓	✖
Defluviicoccus DFII		✖	✖

On the other hand, biological nitrogen removal and EBPR are nowadays integrated in activated sludge systems aiming at simultaneous biological nitrogen removal. In these systems, the denitrification capacity of PAO gains a lot of relevance since PAO can reduce the nitrite/nitrate produced in the aerobic phase using the PHA stored in the anaerobic phase. N removal via the nitrite pathway, i.e. nitritation followed by denitritation, $NH_4^+ \rightarrow NO_2^- \rightarrow N_2$ is a more cost effective alternative when treating low COD/N wastewaters due to the lower aeration and COD requirements (Turk and Mavinic, 1987). If the nitrite pathway was implemented, the role of nitrite would obviously be very significant as well as its effect on the PAO-GAO competition. The implementation of the nitrite pathway and EBPR (nitritation and denitritation linked to phosphorus removal) has been reported both in suspended (Marcelino et al., 2011; Zeng et al., 2011) and particularly in granular systems (de Kreuk et al., 2005; Mosquera-Corral et al., 2005; Yilmaz et al., 2008). Recently, Bassin et al. (2012) demonstrated that nitrite-based dephosphatation was the main pathway for achieving simultaneous P and N removal despite the GAO presence. They detected high PAO II proliferation and the role of GAO was to reduce nitrate to nitrite.

The aim of this work is to demonstrate that an EBPR sludge operating with propionic acid and nitrite as a sole electron donor and acceptor, respectively, leads to the suppression of GAO activity and to the proliferation of PAO. The results would help not only for a better understanding of the underlying mechanisms of the PAO-GAO competition but also to understand the role of nitrite in biological nutrient removal systems.

6.2. MATERIALS AND METHODS

6.2.1. EQUIPMENTS

The experimental set-up consisted of a lab-scale SBR (V=10 L) with oxygen, pH, ORP and temperature probes with a PLC controlling the system. The SBR was initially operated during 90 days under anaerobic/aerobic conditions with low influent P and high temperature (30°C) to favour GAO growth. The SBR was operated as a typical PAO-enriched sludge configuration (described on chapter 3, section 3.1.1). The synthetic wastewater used in the GAO-enrichment period consisted of the nutrient wastewater solution (4.96 L) (in $g \cdot L^{-1}$): 0.1 NH_4Cl, 0.044 $MgSO_4 \cdot 7H_2O$, 0.16 $MgCl_2 \cdot 6H_2O$, 0.042 $CaCl_2 \cdot 2H_2O$, 0.03 $NaHCO_3$, 0.0276 KH_2PO_4, 0.0209 K_2HPO_4, 0.005 allylthiourea (ATU) to inhibit nitrification and 0.30 mL of nutrient solution, based on Smolders et al. (1994). The carbon source solution (0.04 L) contained only propionic acid for an initial concentration of 225 $mg \cdot L^{-1}$ of COD into the SBR. The initial phosphate concentration was set to 5 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$, resulting in a COD/P ratio of 45. The HRT was 12h, while SRT was kept at 15 d with periodic wastage at the end of the aerobic phase. During the experiments the temperature was controlled at 25 ± 1 °C. HCl (1 M) and NaOH (1 M) were used to control the pH in the reactive phases at 7.50 ± 0.05. Nitrogen was bubbled throughout the anaerobic period.

During the anaerobic/anoxic SBR configuration for GAO-washout sodium nitrite was automatically added to the system from a concentrated solution (3.5 g $N\text{-}NO_2 \cdot L^{-1}$) to obtain anoxic conditions. Nitrogen was bubbled during all the reactive periods. The synthetic wastewater was switched to more realistic values (initial concentration of propionic acid of 100 mg $COD \cdot L^{-1}$ and $P\text{-}PO_4^{3-}$ of 5 mg $P \cdot L^{-1}$) with an initial COD/P ratio of 20. HRT and SRT were kept at the previous values.

Titrimetric techniques were used not only to control the pH but to detect the nitrite depletion point in the anoxic phase as defined in previous works (Vargas et al., 2008).

6.2.2 CHEMICAL AND MICROBIOLOGICAL ANALYSES

Chemical and microbial analyses were done as described in chapter 3. FISH technique (Amann et al., 1995) coupled with confocal microscopy was used to quantify the biomass distribution. Table 6.2 details the probes used in this work.

Table 6.2. Summary of the probes used in the FISH measurements.

Short name	Specificity	Reference
EUBMIX	Most bacteria	Daims *et al.* 1999
PAOMIX	*Accumulibacter phosphatis*	Croccetti *et al.* 2000
PAO I	Cluster I of *Accumulibacter phosphatis*	Flowers *et al.* 2009
PAO II	Cluster II of *Accumulibacter phosphatis*	
GAOMIX	*Competibacter phophatis*	Croccetti *et al.* 2002
DFIMIX	Cluster I of *Defluvicoccus vanus*	Wong *et al.* 2004
DFIIMIX	Cluster II of *Defluvicoccus vanus*	
DFIII	Cluster III of *Defluvicoccus vanus*	Mcilroy and Seviour (2009)

6.3. RESULTS AND DISCUSSION

6.3.1 NITRITE AS SOLE ELECTRON ACCEPTOR

The purpose of this first set of experiments was to evaluate the effectiveness of an anaerobic/anoxic (nitrite-based) configuration on the GAO removal in a propionic-fed environment. For this purpose, a GAO-enriched EBPR sludge was promoted based on the guidelines proposed by the work of Lopez-Vazquez et al. (2009) under anaerobic/aerobic conditions with propionate as sole carbon source: COD/P in the influent was fixed to 45 with very low initial P concentration in the reactor (5 $mg \cdot L^{-1}$). Figure 6.1 (Left) shows the results obtained under steady state conditions. Propionate was rapidly depleted linked to some phosphate release, although the P/C ratio obtained was 0.20 indicating a significant presence of GAO in the sludge. Nevertheless, P depletion was observed during the aerobic phase, showing that despite the low P/C ratio, the PAO content in the sludge was enough to achieve complete aerobic P-uptake due to the low initial concentration of P. The FISH results (Figure 6.1 Right) supported these observations, indicating a low percentage of PAO (10%) and a significant amount of GAO (70% DFI and 18% DFII), while *Competibacter* GAO were scarcely detected, in accordance to the lack of acetate in the feed.

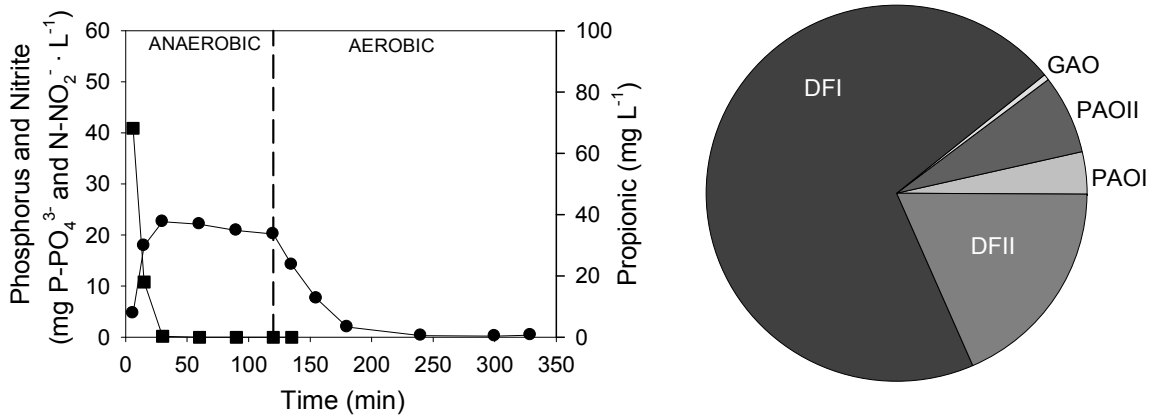

Figure 6.1. Left: Experimental propionic acid (■) and phosphate (●) profiles for the GAO-enriched sludge. Right: Microbial distribution according to FISH images.

Once the sludge was enriched in GAO, the electron acceptor was switched from oxygen to nitrite without any acclimation period and the COD/P ratio was moved to a more reasonable value (COD/P=20). Figure 6.2 (a to d) shows some examples of the experimental profiles obtained during the 50 days of operation. At first glance, the results indicate a fast adaptation to nitrite without any significant inhibition issues and a significant increase of the anoxic P uptake activity. This observation is corroborated with the evolution of the maximum nitrite uptake rate (NUR_{MAX}) estimated in several cycles, which shows a clear increasing trend (Figure 6.2e).

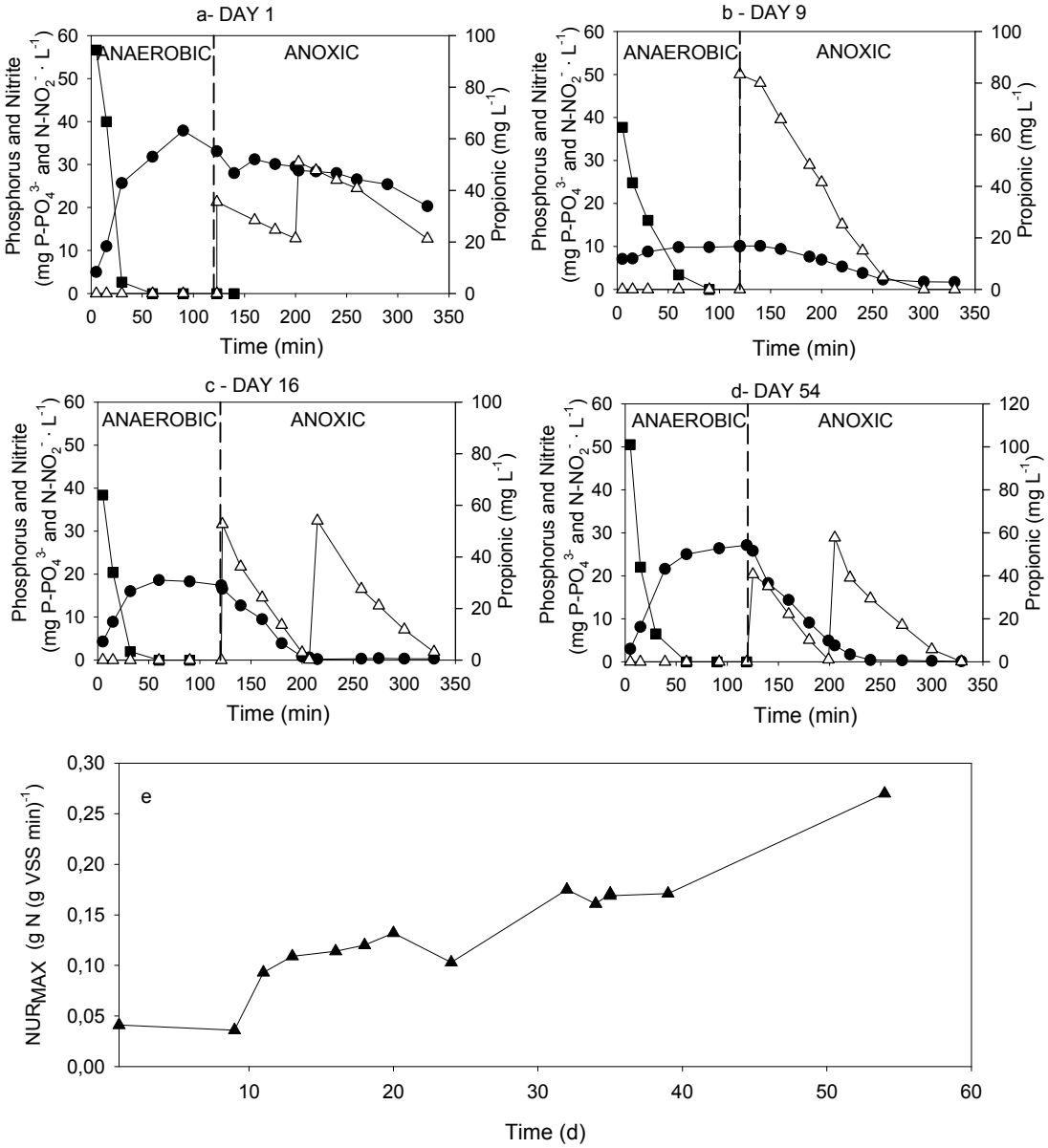

Figure 6.2. (a to d) Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for some cycles during the experimental period. (e) NUR_{MAX} evolution during the experimental period.

Figure 6.3 shows the evolution of the FISH quantifications during the experimental period. As can be observed, the objective of this work, i.e. proof that nitrite is a strong selection factor in the PAO-GAO competition in a propionic-fed environment, was fully achieved and all the GAO species initially observed in the sludge were removed. The percentage of PAO increased in parallel to the GAO depletion. It is also worth to

mention that both PAO I and PAO II were equally favoured in this system. With respect to these FISH measurements, Figure 6.4 shows selected images for all the probes tested and provides a better visualisation of the evolution of each biomass population.

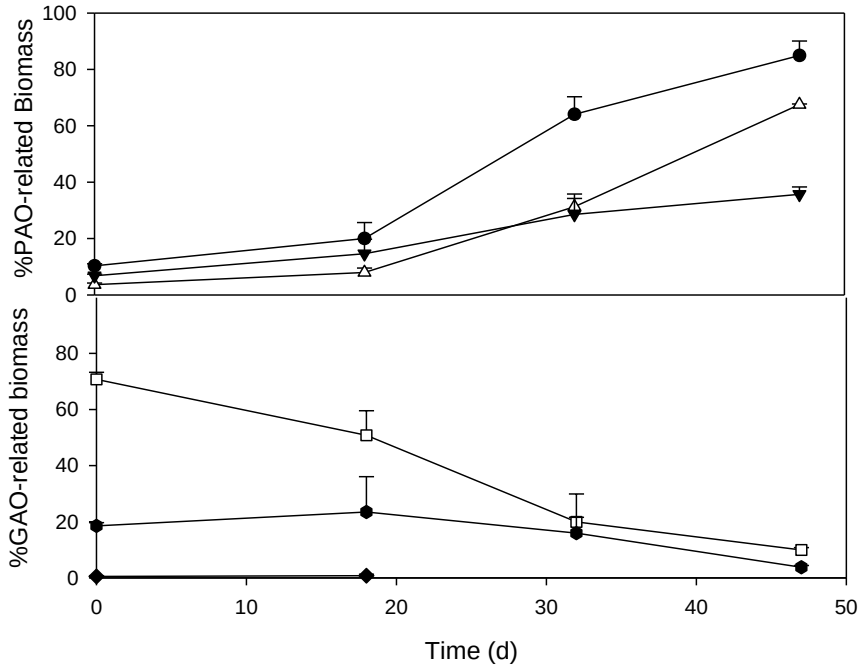

Figure 6.3. FISH distribution percentages of the biomass. PAO-related biomass Up: PAOMIX (●), PAO I (△) and PAO II (▼). GAO-related biomass Down: GAOMIX (◆), DFIMIX (□) and DFIIMIX (⬢).

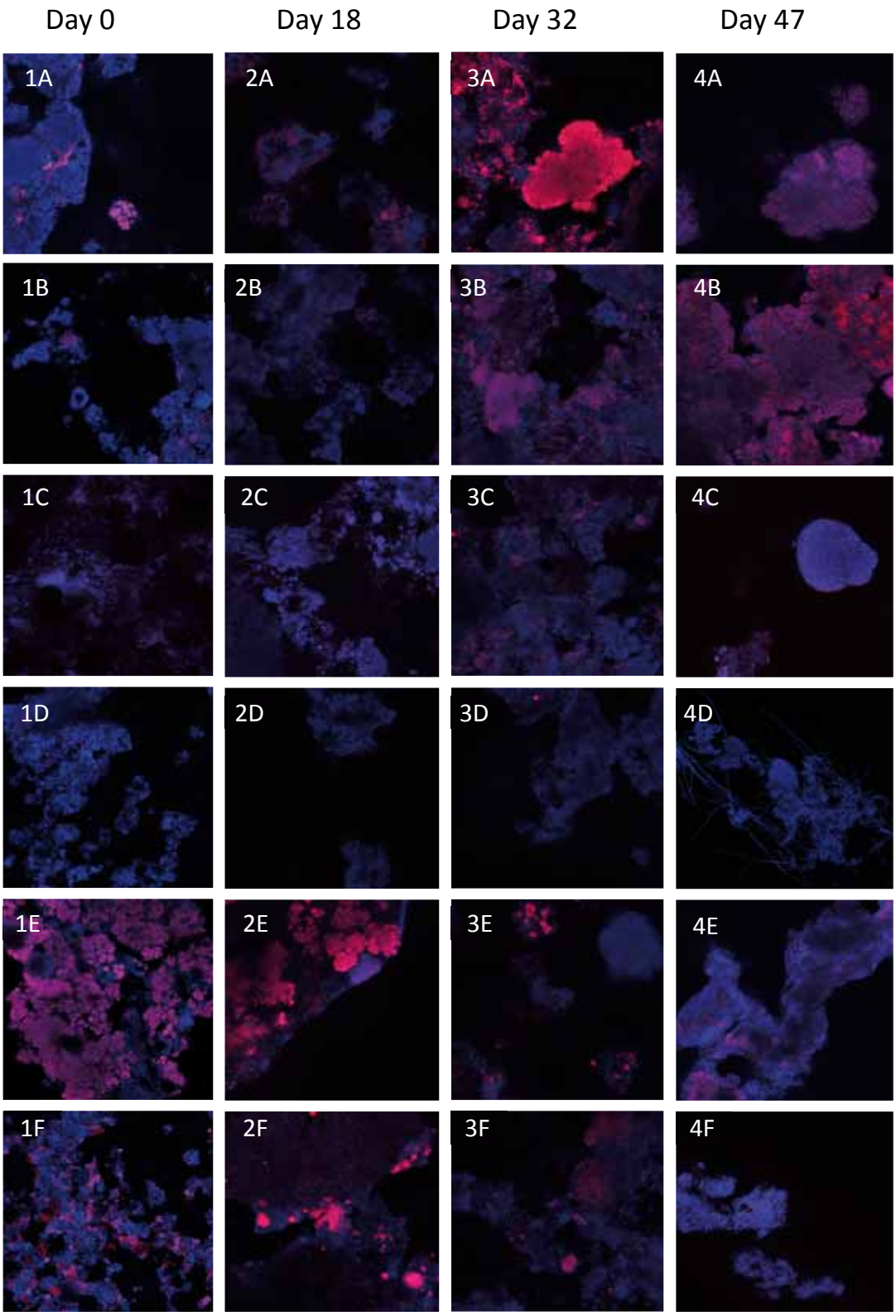

Figure 6.4. FISH/CLSM representative images of the sludge from the SBR reactor during the enrichment. A. PAOMIX, B. PAO I, C. PAO II, D. GAOMIX, E. DFIMIX, F. DFIIMIX. Specific probe is shown in pink and EUBMIX probe in blue.

Finally, Figure 6.5 shows a cycle conducted at day 41 where the internal storage polymers (glycogen and PHA) were measured. The experimental profiles are in agreement with those found in the literature. Most of the PHA was PHV (66 ± 9 %) in

accordance to the use of propionate as sole carbon source (Pijuan et al., 2009). Table 6.3 shows the experimental ratios obtained during the anaerobic phase for glycogen degraded, PHA produced and phosphate released per VFA taken up (Gly/VFA, PHA/VFA and P/C respectively). The experimental ratios are compared to other similar experimental works and with those estimated theoretically with metabolic modelling. As can observed, the experimental values of GLY/VFA and PHA/VFA are unexpectedly higher than those typical of PAO if one takes into account the high enrichment showed by the FISH images. These results will be deeply discussed in the following section.

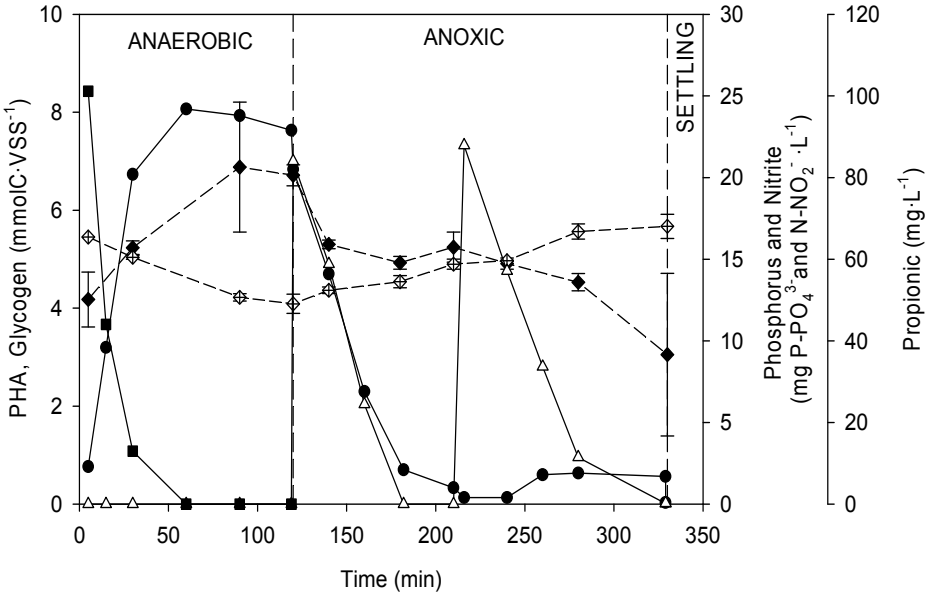

Figure 6.5. Experimental PHA (◆), glycogen (◇), propionic acid (■), phosphate (●) and nitrite (△) profiles for a cycle from day 41.

The experimental results demonstrate that nitrite can be used as the selection factor in the PAO-GAO competition when *Defluviicocus* GAO are predominant in the sludge. The operation of an enriched culture of *Defluviicocus* under anaerobic/anoxic-nitrite conditions with propionate as sole carbon source resulted in the washout of *Defluviicocus* in parallel to the growth of PAO showing the feasibility to obtain a highly enriched culture of PAO (85%) even from an enriched culture of *Defluviicocus*.

Table 6.3. Typical EBPR performance ratios during the anaerobic phase. Comparison of experimental data in this study with others values found in the literature for propionic acid as sole carbon source.

		Gly/VFA (mmolC/mmolC)	PHA/VFA (mmolC/mmolC)	P/C (mmolP/mmolC)
Chen et al., 2005	PAO	0.45	0.98	0.41
Pijuan et al., 2009	PAO	0.45	0.64	0.27
Oehmen et al., 2010a	PAO	0.33	1.22	0.17-0.22
	GAO	0.67-1.0	1.50- 1.78	-
This Study	PAO	0.49	1.47	0.34

The initial operation with propionate led to the selection of *Defluviicocus* GAO versus *Competibacter* GAO, which are favoured with acetate as carbon source. *Competibacter* are able to use nitrite as electron acceptor (Table 6.1) and hence, the strategy of using acetate as sole carbon source under anaerobic/anoxic-nitrite conditions would not have led to an enriched culture of PAO.

The observed *Defluviicocus* GAO washout is somehow in contrast to the findings of Liu et al. (2010) with a highly-enriched culture of *Competibacter* GAO obtained using acetate as sole carbon source. They found that nitrite dosage was more adverse for the aerobic PAO metabolism rather than for the aerobic GAO metabolism and suggested that nitrite could provide a competitive advantage of *Competibacter* over PAO. In our case, *Defluviicocus* were much more abundant (DFI 70% and DFII 18 %) than *Competibacter* and the anaerobic/anoxic-nitrite conditions were demonstrated to be more favourable for PAO than for *Defluviicocus*. In addition, nitrite was carefully dosed in the anoxic phase and was totally consumed, avoiding nitrite leakage in the anaerobic phase. Based on these results, it seems clear that nitrite-pathway EBPR systems should focus on achieving a carbon source with high propionate fractions over acetate to combine the positive effects of nitrite and propionate on PAO selection. In this sense, Chen et al. (2013) proposed recently a novel strategy to increase the propionate content in the wastewater that can be of interest for the implementation of this GAO washout strategy in full-scale WWTPs.

With respect to the utilization of nitrite by *Defluviicocus*, it was reported that DFI cannot use nitrite as an electron acceptor (Wang et al., 2007) whereas DFII is unable to denitrify (Kong et al., 2006). DFIII were not found in the system and the denitrification capacities of DFIII and DFIV are not reported so far. This GAO washout contrasted with the experimental ratios (Gly/VFA, PHA/VFA and P/C) of Table 6.3, which are somehow far from the theoretical ones widely accepted for PAO. The explanation for such different values would lay on the low amount of P in the influent used, which resulted in a low poly-P content of the biomass. Under these conditions, PAO can take up propionate using glycogen as primary energy source (a typical GAO behaviour) for survival purposes. The fact that PAO can activate the glycolytic pathway in order to balance the lack of energy derived from low poly-P hydrolysis has already been reported in the literature (Erdal et al., 2008; Zhou et al., 2008; Acevedo et al., 2012). Acevedo et al. (2012) found values between 0.51 to 1.08 mmol C/mmol C for glycogen degradation/VFA uptake during the anaerobic phase in a PAO-enriched SBR where the influent had P limitations using acetic acid as a carbon source. These results are close to 1.12 mmol C/mmol C that Zeng et al. (2003) found for a metabolic model of GAO.

The ability of PAO to store VFA as PHA anaerobically under limiting poly-P conditions and use it afterwards was studied in a batch experiment conducted at the end of the experimental period (Figure 6.6). This experiment aimed at forcing PAO to take up propionate anaerobically under scarce poly-P conditions. For this aim, most of the internal poly-P was depleted under anaerobic conditions with an excess of carbon addition, showing a P/C ratio of 0.21. Then, the medium was replaced for a phosphate-free medium and the system was left overnight under aerobic conditions. Propionate was added again under anaerobic conditions and P-release was very low

despite the high amount of COD taken up (P/C<0.01), indicating that PAO (85% of PAO in the sludge) had changed their behaviour from PAO to GAO phenotype. Then nitrite was added and it was successfully denitrified showing that PAO were responsible for this nitrite reduction since *Defluviicocus* are not able to reduce nitrite.

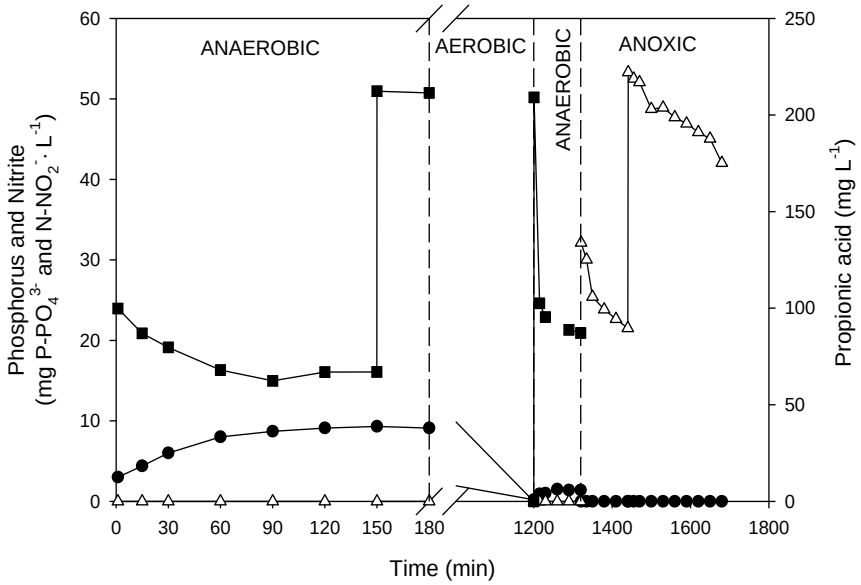

Figure 6.6. Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for the batch experiment of PAO behaving like GAO.

This work also shows how a conventional anaerobic/aerobic EPBR system can be directly adapted to anaerobic/anoxic conditions with nitrite as electron acceptor from day 1 without requiring any intermediate anaerobic/anoxic/aerobic configuration used in previous works (Vargas *et al.*, 2011). The nitrite-based anoxic P uptake rate was, however, much slower than that under aerobic conditions and hence, nitrite should be wisely dosed to avoid its presence at the end of the anoxic phase. Nitrite entering the anaerobic phase can be very detrimental for the PAO growth. Despite these limitations, the SBR got rapidly acclimatised to nitrite as an electron acceptor and anoxic-P uptake was complete in all the period. The last cycles of the period were very similar (Figure 6.2) and typical of an EBPR cycle with complete P removal. P-uptake was usually higher than P-release and net P removal was usually achieved.

The fast adaptation to nitrite is in agreement with the fact that both PAO I and PAO II initially present in the sludge are able to reduce nitrite (Table 6.1), and hence nitrite utilization as sole electron acceptor should not be a selection factor between PAO I and PAO II. This observation was confirmed with the final FISH measurements (65 % PAO I *versus* 35 % PAO II, Figure 6.3) and with a batch experiment with nitrate as electron acceptor (Figure 6.7). As can be observed, nitrate utilisation did not need an acclimatisation period at all and complete anoxic P uptake was achieved. These results contrast with previous works where nitrite-based selections led to systems without nitrate-reducing capabilities (Jiang et al., 2006; Guisasola et al., 2009).

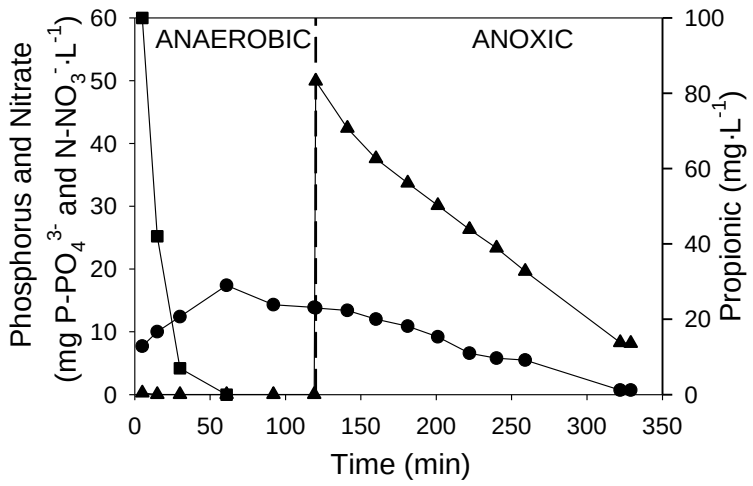

Figure 6.7. Experimental propionic acid (■), phosphorus (●) and nitrate (▲) profiles for a batch experiment at day 30 using nitrate as electron acceptor at the anoxic phase.

Finally, two different practical implications can be drawn from this work. On the one hand, nitrite and propionate could be used as strategy to enrich a bio-P sludge with PAO. PAO have yet to be isolated and novel strategies for its enrichment are very interesting for fundamental studies on PAO physiology and biochemistry (Lu et al., 2006). On the other hand, the suppression of *Defluviicocus* GAO when nitrite is the electron acceptor represents an add-on to the nitrite-based EBPR systems since the proliferation of non-desirable GAO can be easily ruled out and added to the other benefits (i.e. lower aeration and COD requirements).

6.3.2 COMBINATION OF NITRITE AND OXYGEN AS ELECTRON ACCEPTORS

At this point, a second experiment was planned aiming at i) confirming the results obtained in the first experiment and ii) proposing a more efficient technology based on these results. Three modifications were planned.

1) The initial P concentration was increased to 20 mg $P \cdot L^{-1}$ in order to avoid the unexpected PAO behaviour observed in the previous experiment under P limitation (i.e. using internal glycogen as if they were GAO).
2) The configuration was not anaerobic/anoxic during all the period but periods under classical anaerobic/aerobic conditions were periodically introduced. Nitrite as sole electron acceptor selects PAO versus GAO but at a slow growth rate and we believed that enclosing oxygen periods, where PAO grow faster, would enhance the PAO selection process. Moreover, and from a practical point of view, maintaining a nitrite-based EBPR configuration results in high risk of failure since achieving nitrite depletion under anoxic conditions is essential for the success of the process. Thus, an accurate monitoring of the nitrite dosage to the system is required.
3) The initial GAO-enriched biomass had been fed with a mixture of acetate and propionate as carbon sources to obtain an initial GAO-enriched sludge with a fair fraction of *Competibacter*. However, the enrichment was not as successful as desired and a poor fraction of *Competibacter* was obtained.

Figure 6.8 shows the experimental results of the GAO-enriched sludge fed with 50% of propionic acid and 50% of acetic acid. As expected, any P-release was observed during the anaerobic phase nor P-uptake.

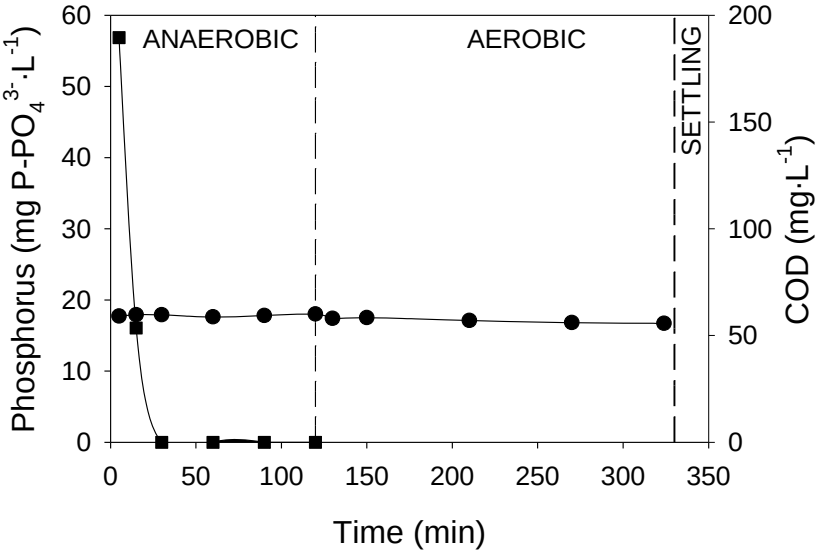

Figure 6.8. Experimental propionic acid (■) and phosphate (●) profiles for a GAO-enriched sludge cycle.

This reactor was bioaugmented with 2L of PAO-enriched sludge to have some PAO activity and was set under the proposed conditions. The reactor was operated for more than 90 days and Table 6.4 shows the different system configurations used. The alternation of anaerobic/anoxic or anaerobic/aerobic configurations was initially scheduled to be around 15 days and 7 days respectively. These durations were selected to ensure periods with GAO washout (anaerobic/anoxic) with other periods favoring PAO growth (anaerobic/aerobic). However, the initial schedule was modified based on a stepwise decision according to the experimental observations. When a decrease in EBPR performance was detected during the anaerobic/anoxic operation, the system was switched to the fail-safe conventional anaerobic/aerobic configuration for EBPR recovery. The titrimetric measurements, related to denitritation rates, were used to evaluate PAO activity, in addition to the conventional P-release and P-uptake rates.

Table 6.4. Summary results of the monitored cycles under different SBR conditions.

Days	Electron donor	Electron Acceptor		P-release	P uptake
	mg $COD \cdot L^{-1}$	class	mg $N\text{-}NO_2^- \cdot L^{-1}$	mg $P\text{-}PO_4^{3-} \cdot L^{-1}$	mg $P\text{-}PO_4^{3-} \cdot L^{-1}$
1	150	Oxygen	0	27.5	40.8
11	100	Nitrite	10	11.8	-
14	100	Nitrite	10	12.1	7.7
17	100	Nitrite	10	1.6	1.5
21	100	Oxygen	0	19.1	28.5
24	100	Oxygen	0	10.2	11.9
28	100	Oxygen	0	30.0	42.4
29	150	Oxygen	0	41.7	51.5
31	150	Oxygen	0	43,7	60.0
32	100	Nitrite	15	30.5	12.8
35	150	Nitrite	15	-	-
36	100	Oxygen	0	13.9	24.3
37	100	Oxygen	0	14.3	25.6
38	100	Nitrite	15	10.5	15.5
39	100	Nitrite	15	10.8	15.2
42	100	Nitrite	15	12.7	18.0
41	100	Nitrite	15	8.2	9.4
42	150	Oxygen	0	28.0	40.1
47	150	Oxygen	0	32.1	44.0
48	150	Oxygen	0	33.4	46.4
49	150	Oxygen	0	35.8	46.0
54	150	Oxygen	0	41.5	52.9
55	100	Nitrite	15	35.3	18.9
57	100	Nitrite	15	15.7	18.5
58	100	Nitrite	15	17.9	18.2
61	100	Nitrite	15	18.2	23.9
63	100	Nitrite	15	9.1	13.7
65	150	Oxygen	0	19.4	43.0
68	150	Oxygen	0	41.4	58.5
70	150	Oxygen	0	43.4	58.7
72	100	Nitrite	20	20.7	7.3
75	100	Nitrite	20	12.9	15.0
82	150	Oxygen	0	39.4	45.8
86	150	Oxygen	0	42.5	58.4
91	150	Oxygen	0	44.8	60.4

Figure 6.9 (a to d) shows four typical cycles of the reactor using different conditions. As can be observed, PAO selection versus GAO was satisfactory. Figure 6.9a shows the first cycle under anaerobic/aerobic conditions and when compared to Figure 6.9d (the last cycle conducted under anaerobic/aerobic conditions), the EBPR activity increased because of the enrichment of the sludge with PAO microorganisms. From the beginning to the end of the strategy the P/C ratio increased from 0.14 to 0.35, confirming the success of these strategies for the PAO selection. The P/C ratio was similar to the one obtained with the previous strategy (0.34).

During the operation with anaerobic/anoxic configuration (Figure 6.9b and 6.9c) EBPR activity was maintained, observing anaerobic P-release followed by a higher P-uptake linked to denitritation. A significant increase was also observed for the NUR during the experiments from 0.07 to 0.11 g N·(g VSS·min)$^{-1}$. However, the NUR obtained in the first strategy was from 0.05 to 0.26 g N·(g VSS·min)$^{-1}$ (Figure 6.2e), showing that the best strategy to achieve denitrifying-PAO was the first one, where nitrite was the sole electron acceptor. Table 6.5 compares the FISH results obtained during different phases of this strategy and the success was confirmed since PAO increased from 18.3% of PAO to 70.9% and both *Competibacter* and *Defluvicoccus* GAO were washed out from the system.

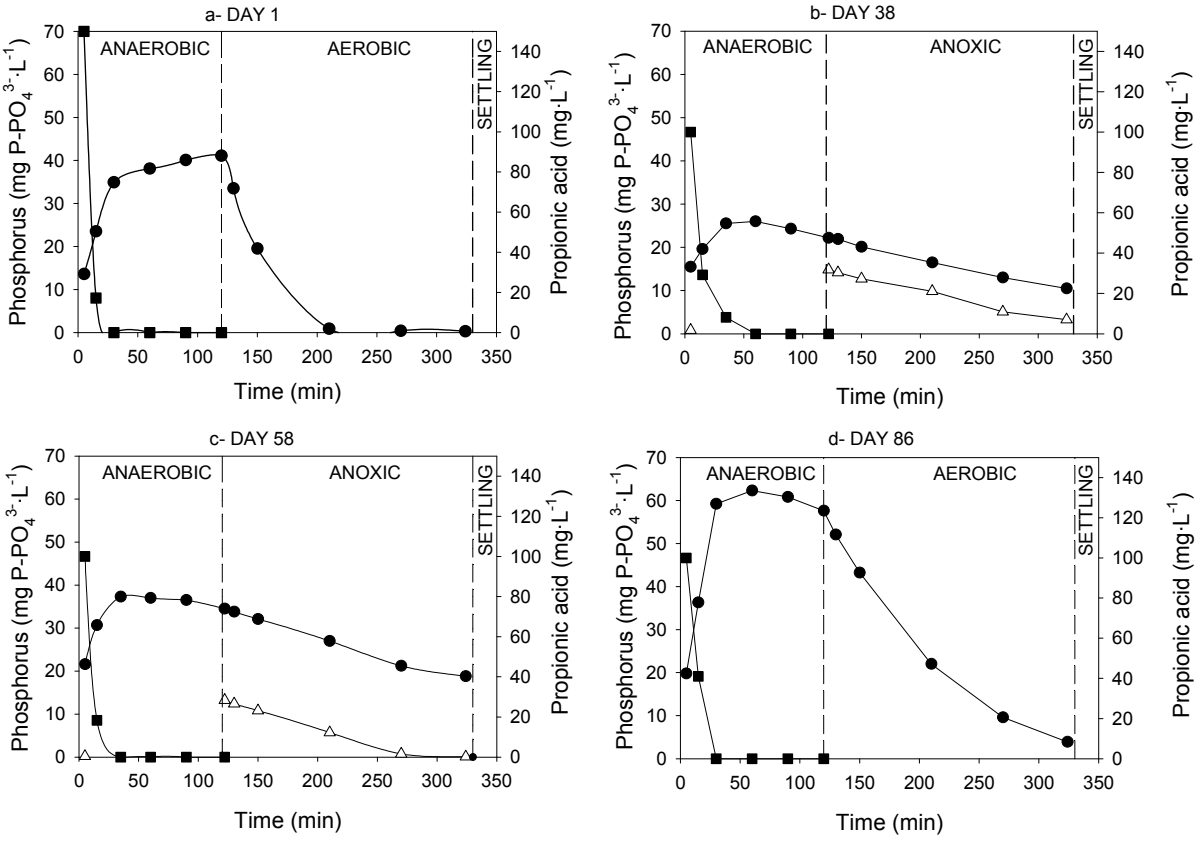

Figure 6.9. (a to d) Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for some cycles during the experimental period.

Table 6.5. Summary of different FISH measurements during the experiment with alternation of electron acceptor.

Day	1	30	78
	%	%	%
PAO	18.3±2.5	37.2±0.9	70.9±4.7
PAO I	0.6±0.1	28.5±3.9	22.3±6.4
PAO II	17.7±2.4	8.6±3.0	48.6±1.7
GAO	0.9±0.3	n.d	n.d
GAO, DFI	71.7±0.4	33.6±1.8	1.0±0.3
GAO, DFII	8.5±0.6	5.3±0.6	0.3±0.1

n.d: not detected

6.4. CONCLUSIONS

This work confirms the theoretical hypothesis not previously demonstrated that a system fed with propionate as sole electron donor and nitrite as sole electron acceptor leads to the washout of GAO and favours the PAO growth.

On a first strategy, a bio-P sludge enriched in *Defluviicocus* GAO (70 % DFI, 18% DFII and 10 % PAO) was operated under anaerobic/anoxic-nitrite configuration, achieving the washout of these microorganisms in parallel to the growth of PAO (up to 85%) and demonstrating that nitrite is a key selection factor in the PAO-GAO competition.

On a second strategy, a mixture of GAO microorganisms and PAO (71 % DFI, 8.5% DFII, 1% *Competibacter* and 18.3 % PAO) was operated alternating nitrite or oxygen as the electron acceptor, with good results since PAO population increased up to 70.9% and again, it achieved the GAO washout from the system. However, low values of NUR were obtained alternating anaerobic/aerobic or anoxic configurations compared with the fist strategy using anaerobic/anoxic conditions.

These novel strategies not only allow the achievement of a highly PAO-enriched activated sludge, but also demonstrate that the integration of denitritation with EBPR favours the suppression of GAO and represents an additional advantage for the EBPR configurations using anoxic-nitrite conditions, in addition to lower aeration and COD requirements.

6.5. REFERENCES

Acevedo, B., Oehmen, A., Carvalho, G., Seco, A., Borrás, L., and Barat, R., 2012. Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage. Water Research, 46(6), 1889–900.

Amann, R. I., Ludwig, W., and Schleifer, K. H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology and Molecular Biology reviews, 59(1), 143–169.

Bassin, J. P., Kleerebezem, R., Dezotti, M., and van Loosdrecht, M. C. M., 2012. Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors operated at different temperatures. Water Research, 46(12), 3805–3816.

Chen, Y., Chen, Y. S., Xu, Q., Zhou, Q., Gu, G., 2005. Comparison between acclimated and unacclimated biomass affecting anaerobic–aerobic transformations in the biological removal of phosphorus. Process Biocemistry 40(2), 723-734.

Chen, Y.G., Li, X., Zheng, X., Wang D.B., 2013. Enhancement of propionic acid fraction in volatile fatty acids produced from sludge fermentation by the use of food waste and Propionibacterium acidipropionici. Water Research 47(2), 615–622.

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

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

Daims, H., Bruhll, A., Amann, R., Schleifer, K., 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. Systematic Applied Microbiology 22, 434–444.

Elser, J. and Bennett, E., 2011. Phosphorus cycle : A broken biogeochemical cycle. Nature, 478, 29–31.

Erdal, U. G., Erdal, Z. K., Daigger, G. T., and Randall, C., 2008. Is it PAO-GAO competition or metabolic shift in EBPR system? Evidence from an experimental study. Water science and technology : a journal of the International Association on Water Pollution Research, 58(6), 1329–1334.

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

Guisasola, A., Qurie, M., Vargas, M. D. M., Casas, C., and Baeza, J. A., 2009. Failure of an enriched nitrite-DPAO population to use nitrate as an electron acceptor. Process Biochemistry, 44(7), 689–695.

He, S., Gall, D. L., and McMahon, K. D., 2007. “Candidatus Accumulibacter” population structure in enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes. Applied and environmental microbiology, 73(18), 5865–5874.

Jiang, Y., Wang, B., Wang, L., Chen, J., and He, S., 2006. Dynamic response of denitrifying poly-P accumulating organisms batch culture to increased nitrite concentration as electron acceptor. Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances and Environmental Engineering, 41(11), 2557–2570.

Kong, Q. X., Wang, X. W., Jin, M., Shen, Z. Q., and Li, J. W., 2006. Development and application of a novel and effective screening method for aerobic denitrifying bacteria. FEMS microbiology letters, 260(2), 150–155.

de Kreuk, M. K., Pronk, M., and van Loosdrecht, M. C. M., 2005. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Research, 39(18), 4476–4484.

Liu, Y., Pijuan, M., Yuan, Z., 2010. The effect of free nitrous acid on the anabolic and catabolic processes of glycogen accumulating organisms. Water Res. 44(9), 2901-2909.

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

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

Marcelino, M., Wallaert, D., Guisasola, A., and Baeza, J. A., 2011. A two-sludge system for simultaneous biological C, N and P removal via the nitrite pathway. Water Science and Technology, 64(5), 1142–1147.

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

Mosquera-Corral, A., de Kreuk, M. K., Heijnen, J. J., and van Loosdrecht, M. C. M., 2005. Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. Water Research, 39(12), 2676–2686.

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

Oehmen, A., Carvalho, G., Lopez-Vazquez, C. M., van Loosdrecht, M. C. M., and Reis, M. A. M., 2010b. Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Research, 44(17), 4992–5004.

Oehmen, A., Carvalho, G., Freitas, F., Reis, M. A. M. 2010c. Assessing the abundance and activity of denitrifying polyphosphate accumulating organisms through molecular and chemical techniques. Water Science and Tecnology 61(8), 2061-2068.

Peterson, S. B., Warnecke, F., Madejska, J., Mcmahon, K. D., and Hugenholtz, P., 2008. Environmental distribution and population biology of Candidatus Accumulibacter , a primary agent of biological phosphorus removal. Environmental Microbiology, 10(10), 2692–2703.

Pijuan, M., Casas, C., Baeza, J.A., 2009. Polyhydroxyalkanoate synthesis using different carbon sources by two enhanced biological phosphorus removal microbial communities. Process Biochemistry 44(1), 97–105.

Saunders, A. M., Oehmen, A., Blackall, L. L., Yuan, Z., and Keller, J., 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water science and technology : a journal of the International Association on Water Pollution Research, 47(11), 37–43.

Smolders, G. J. F., Meij, J., van Loosdrecht, M. C. M., and Heijnen, J. J., 1994. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnology and Bioengineering, 44, 837–849.

Turk, O., and Mavinic, D. S., 1987. Benefits of using selective inhibition to remove nitrogen from highly nitrogenous wastes. Environmental Technology Letters, 8(1-12), 419–426.

Vargas, M., Guisasola, A., Lafuente, J., Casas, C., Baeza, J.A., 2008. On-line titrimetric monitoring of anaerobic–anoxic EBPR processes. Water Science and Technology 57(8), 1149-1154.

Vargas, M., Guisasola, A., Artigues, A., Casas, C., Baeza, J. A., 2011. Comparison of a nitrite-based anaerobic–anoxic EBPR system with propionate or acetate as electron donors. Process Biochemistry 46(3), 714-720.

Wang, X., Zeng, R. J., Dai, Y., Peng, Y., and Yuan, Z., 2007. The denitrification capability of cluster 1 *Defluviicoccus* vanus-related glycogen-accumulating organisms. Biotechnology and Bioengineering, 99(6), 1329–1336.

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

Yilmaz, G., Lemaire, R., Keller, J., and Yuan, Z., 2008. Simultaneous nitrification , denitrification , and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. Biotechnology and Bioengineering, 100(3), 529–541.

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

Zeng, W., Yang, Y., Li, L., Wang, X., and Peng, Y., 2011. Effect of nitrite from nitritation on biological phosphorus removal in a sequencing batch reactor treating domestic wastewater. Bioresource technology, 102(12), 6657–6664.

Zhou, Y., Pijuan, M., Zeng, R. J., Lu, H., and Yuan, Z., 2008. Could polyphosphate-accumulating organisms (PAOs) be glycogen-accumulating organisms (GAOs)? Water Research, 42(10-11), 2361–2368.

Chapter 7

GENERAL CONCLUSIONS

The main conclusions that can be drawn from this PhD thesis are:

- A novel bioaugmentation strategy with PAO microorganisms for a conventional nitrogen removal system was designed and implemented to achieve biological nitrogen and phosphorus removal. A cycle configuration with an anoxic phase with two feedings and an aerobic phase was used to achieve nitrification, denitrification and EBPR. A key point for the success of this strategy is to provide proper operational conditions to avoid rising problems. Enough aerobic phase length is required to ensure complete PHA depletion for PAO microorganisms, which avoids the carbon source availability required for denitrification.

- The acclimation of the PAO-enriched sludge to the different compounds derivatives of the nitrogen removal processes should be a previous step to avoid the inhibition. The presence of an electron donor (nitrite or nitrate) and electron acceptor (carbon source) in the anoxic phase requires an acclimation period for PAO to achieve nitrogen and phosphorus removal.

- The presence of fermentative bacteria in the microbial community is required for achieving EBPR with complex carbon sources. These bacteria ferment the complex carbon sources to produce VFAs as acetic or propionic acids, which are the preferred carbon source for *Accumulibacter* PAO.

- Two different strategies to develop novel consortiums with fermentative bacteria and PAO were studied. EBPR was achieved by the first time with the utilization of carbon sources that nowadays are only used for denitrifying process (methanol and glycerol). This success could reduce the operational costs for wastewaters with low VFA content, allowing the utilization of the same carbon source than denitrification.

- A syntrophic consortium to use methanol, with anaerobic acetogens and PAO, was tested for more than 30 days with successful results. This novel consortium was also able to use ethanol performing EBPR.

- It was developed a new consortium with fermentative bacteria and PAO to use glycerol as carbon source. Glycerol was fermented and converted mainly to propionic acid, which was used by PAO achieving phosphorus removal in a one–sludge system for the first time. This consortium was operated for more than 60 days with simultaneous glycerol and phosphorus removal. The consortium successfully used other complex carbon sources as sucrose, glucose and milk with no acclimation period.

- To obtain a highly PAO-enriched sludge and to remove the different GAO competitors of *Accumulibacter* PAO, a new strategy was tested. Using propionic acid as electron donor in the anaerobic phase and nitrite as electron acceptor in the anoxic phase, the GAO microorganisms (GAOMIX, DFI and DFII) were washed out of the system obtaining a microbial community with 85% of PAO.

This enrichment was done even when feed conditions with low P influent concentration favoured GAO growth against PAO.

Figure 5.4. Batch experiments conducted during the *direct replacement* experiment using propionic acid as carbon source to assess PAO activity (day 1 (●), day 12 (○), day 22 (▼), day 33 (△)).

Figure 5.5. Different cycles from the acetogenic selection period with methanol as sole carbon source and anaerobic/aerobic/settling configuration. Left: period C2 (day 9 (○), day 22(▼)). Right: period C3 (day 24 (◇), day 26 (■)). Note: aerobic phase (C2 configuration) started at day 8.

Figure 5.6. Batch experiment with addition of 600 $mg \cdot L^{-1}$ of methanol to anaerobic sludge subjected to 30 min aeration every 24 h. Methanol (▲) and acetic acid (●).

Figure 5.7. Experimental phoshorus (□) and methanol (○) profiles of the first cycles with the acetogenic-PAO consortium period (black: day 1, dark gray: day 2, white: day 3).

Figure 5.8. Experimental phosphorus and methanol profiles during the syntrophic acetogenic-PAO consortium period. Left: P start of cycle (○), P end of anaerobic phase (▲) and end of aerobic phase P (■) Right: methanol start of cycle (▽), methanol end of anaerobic phase (●) and end of aerobic phase methanol (◆).

Figure 5.9. Experimental phosphorus and formaldehyde profiles obtained in a conventional batch experiment with a PAO-enriched sludge. Formaldehyde (▲), Phosphorus (●).

Figure 5.10. Phosphorus (●) evolution during the direct replacement of propionic acid for glycerol strategy. Initial (black), end anaerobic phase (grey), end aerobic phase (white).

Figure 5.11. Glycerol (■) and P/C ratio (▲) during the direct replacement of propionic acid for glycerol strategy. Concentration of glycerol: Initial (black), end anaerobic phase (grey), end aerobic phase (white).

Figure 5.12. Left: Phosphorus profiles in four different batch tests with propionic acid during the direct replacement strategy: start of period D1 (▲), end of period D1 (△), end of period D2 (○) and mid period D3 (■). Right: Experimental P (▲) and glycerol (◇) profiles from the last cycle of period D3.

Figure 5.13. Phosphorus profiles in four different cycles with glycerol in period D2 during the direct replacement strategy. Day 26 (▲), day 32 (●), day 40 (○) and day 44 (■).

Figure 5.14. FISH/CLSM representative images of the sludge from the direct replacement strategy (SBR D) at the end of period D3. EUBMIX is shown in blue, while specific probe is shown in pink; (A) PAOMIX, (B) DFIMIX, (C) DFIIMIX and (D) GAOMIX.

Figure 5.15. Left: Glycerol profiles for two cycles of period E1 of the two-step development strategy: day 1 (▲); day 5 (▼). Right: Glycerol (black) and propionic acid (white) profiles for three different cycles during period E2 of the two-step development strategy: day 13 (○), day 21 (□) and day 26 (◇).

Figure 5.16. P profiles in four different cycles with glycerol in period E3 during the two-step consortium strategy. Day 44 (△), day 48 (●) and day 50 (○).

Figure 5.17. Up: Phosphorus profiles during two-step consortium strategy for glycerol. Initial (black), end anaerobic phase (grey), end aerobic phase (white). Down: Glycerol (■) and P/C ratio. Concentration of glycerol: Initial (black), end anaerobic phase (grey).

Figure 5.18. FISH/CLSM representative images of the sludge from two-step consortium strategy for glycerol (SBR E) at the end of period E5. EUBMIX is shown in blue, while specific probe is shown in pink; (A) PAOMIX, (B) DFIMIX, (C) DFIIMIX and (D) GAOMIX.

Figure 5.19. Experimental net-P removal activity variation when glycerol is supplemented to a dairy wastewater feed. P influent (●), P net-removal (○) and P-removal capacity (▲).

Figure 5.20. P-release, P-uptake and net-P removal for methanol-fed consortium(SBR-B), direct replacement glycerol-fed (SBR-D) and propionic-fed PAO (SBR-A) using different carbon sources (initial concentration 600 mg $COD \cdot L^{-1}$ except for acetate* which was 100 mg $COD \cdot L^{-1}$).

Figure 6.1. Left: Experimental propionic acid (■) and phosphate (●) profiles for the GAO-enriched sludge. Right: Microbial distribution according to FISH images.

Figure 6.2. (a to d) Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for some cycles during the experimental period. (e) NUR_{MAX} evolution during the experimental period.

Figure 6.3. FISH distribution percentages of the biomass. PAO-related biomass Up: PAOMIX (●), PAO I (△) and PAO II (▼). GAO-related biomass Down: GAOMIX (◆), DFIMIX (□) and DFIIMIX (⬢).

Figure 6.4. FISH/CLSM representative images of the sludge from the SBR reactor during the enrichment. A. PAOMIX, B. PAO I, C. PAO II, D. GAOMIX, E. DFIMIX, F. DFIIMIX. Specific probe is shown in pink and EUBMIX probe in blue.

Figure 6.5. Experimental PHA (◆), glycogen (◇), propionic acid (■), phosphate (●) and nitrite (△) profiles for a cycle from day 41.

Figure 6.6. Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for the batch experiment of PAO behaving like GAO.

Figure 6.7. Experimental propionic acid (■), phosphorus (●) and nitrate (▲) profiles for a batch experiment at day 30 using nitrate as electron acceptor at the anoxic phase.

Figure 6.8. Experimental propionic acid (■) and phosphate (●) profiles for a GAO-enriched sludge cycle.

Figure 6.9. (a to d) Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for some cycles during the experimental period.

LIST OF TABLES

LIST OF ABBREVATIONS

AO	Anaerobic/Aerobic configuration
AOB	Ammonia Oxidizing Bacteria
A2O	Anaerobic/Anoxic/Aerobic configuration
BNR	Biological Nutrient Removal
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
DF1	*Defluviicoccus vanus* cluster I
DF2	*Defluviicoccus vanus* cluster II
DO	Dissolved Oxygen
DPAO	Denitrifying Poly-phosphate Accumulating Organisms
EBPR	Enhanced Biological Phosphorus Removal
EDTA	Ethylenediaminetetraacetic
FID	Flame Ionisation Detector
FISH	Fluorescence *In Situ* Hybridization
GAO	Glycogen Accumulating Organisms
GC	Gas Chromatography
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic Retention Time
LFS	Liquid Flow Static respirometer
NaHCO3	Sodium Hydrogen Carbonate
NaOH	Sodium Hydroxide
NOB	Nitrite Oxidizing Bacteria
NUR	Nitrogen Uptake Rate
OHO	Ordinary Heterotrophic Organisms
PAO	Poly-phosphate Accumulating Organisms
PAO I	*Accumulibacter* cluster I
PAO II	*Accumulibacter* cluster II
PBS	Phosphate Buffer Saline
PHA	Poly-HydroxyAlkanoates
PHB	Poly-β-HydroxyButyrate
PHV	Poly-β-HydroxyValerate
PH2MB	Poly-β-Hydroxy-2-MethylButyrate
PH2MV	Poly-β-Hydroxy-2-MethylValerate
PLC	Program Logic Control
Poly-P	Poly-phosphate
P-uptake	Phosphorus Uptake
P-removal	Phosphorus removal
PUR	Phosphorus Uptake Rate
RNA	RiboNucleic Acid
SBR	Sequencing Batch Reactor
SRT	Sludge Retention Time
TSS	Total Suspended Solids

UAB	Universitat Autònoma de Barcelona
UCT	University of Cape Town
VFA	Volatile Fatty Acids
VIP	Virginia Initiative Plant
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

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ACCEPTED PUBLICATIONS

- Gomà, A., Guisasola, A., *Tayà, C.,* Baeza, J. A., Baeza, M., Bartrolí, A., Lafuente, J., and Bartrolí, J., 2010. Benefits of carbon dioxide as pH reducer in chlorinated indoor swimming pools. *Chemosphere* 80(4), 428-432.

- Farreny, R., Morale-Pinzón, T., Guisasola, A., *Tayà, C.,* Rieradevall, J., and Gabarrell, X., 2011. Roof selection for rainwater harvesting: Quantity and quality assessments in Spain. *Water Research* 45(10), 3245-3254.

- *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2011. Assessment of a bioaugmentation strategy with polyphosphate accumulating organisms in a nitrification/denitrification sequencing batch reactor. *Bioresource Technology*, 102(17), 7678-7684.

- Guerrero, J., *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2011. Glycerol as a sole carbon source for enhanced biological phosphorus removal. *Water Research* 46(9), 2983-2991.

- Guerrero, J., *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2012. Understanding the detrimental effect of nitrate presence on EBPR systems: effect of the plant configuration. *Journal of Chemical Technology and Biotechnology*, 87, 1508–1511.

- *Tayà, C.,* Guerrero, J., Vanneste, G., Guisasola, A., and Baeza, J. A., 2012. Methanol-driven enhanced biological phosphorus removal with a syntrophic consortium. 2012. *Biotechnology and Bioengineering,* 110(2), 391-400.

SUBMITTED PUBLICATIONS

- *Tayà, C.,* Garlapati, V. K., Guisasola, A., and Baeza, J. A., 2013. The selective role of nitrite in PAO/GAO competition. *Submitted*.

CONFERENCES

INTERNATIONAL CONFERENCES:

- *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2010. Evaluación de una estrategia de bioaumentación para la eliminación biológica de fósforo en un sistema de nitrificación/desnitrificación. Young Water Professional (IWA). Barcelona 2012. Poster presentation.

- Guerrero, J., *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2011. Understanding the detrimental effect of nitrate presence on EBPR systems: effect of the plant configuration. Small Water and Wastewater Systems & 4th Conference on Decentralized Water and Wastewater International Network. Venice, Italy 2011. Oral presentation.

- *Tayà, C.,* Guerrero, J., Guisasola, A., and Baeza, J. A., 2012. Opening up new possibilities in EBPR: a syntrophic consortium for the utilisation of a wider range of carbon sources. Nutrient Removal and Recovery (IWA).Harbin, China 2012. Oral presentation.

NATIONAL CONFERENCES:

- *Tayà, C.,* Guerrero, J., Guisasola, A., and Baeza, J. A., 2010. Estudio de la interacción de nitrato en la eliminación biológica de fósforo. Mesa Española de Tratamiento de Agua. Bilbao, 2010. Poster presentation.

- Guerrero, J., *Tayà, C.,* Guisasola, A., and Baeza, J. A., 2012. Descifrando los mecanismos subyacentes al deterioro de EBPR por presencia de nitrato en la zona anaerobia de EDAR con eliminación biológica simultánea de nitrógeno y fósforo. Mesa Española de Tratamiento de Agua. Almería, 2012. Oral presentation.