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Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage

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ARTICLE INFO

Article history:
Received 14 October 2011
Received in revised form
30 December 2011
Accepted 6 January 2012
Available online 15 January 2012

Keywords:
Polyphosphate-accumulating organisms (PAO)
Glycogen-accumulating organisms (GAO)
Accumulibacter Type I
Accumulibacter Type II
Polyphosphate (poly-P)
Polyphosphate-accumulating metabolism (PAM)
Glycogen-accumulating metabolism (GAM)
Wastewater

ABSTRACT

Previous studies have shown that polyphosphate-accumulating organisms (PAOs) are able to behave as glycogen-accumulating organisms (GAOs) under different conditions. In this study we investigated the behavior of a culture enriched with *Accumulibacter* at different levels of polyphosphate (poly-P) storage. The results of stoichiometric ratios $Gly_{degraded}/HAc_{uptake}$, $PHB_{synthesized}/HAc_{uptake}$, $PHV_{synthesized}/HAc_{uptake}$ and $P_{release}/HAc_{uptake}$ confirmed a metabolic shift from PAO metabolism to GAO metabolism: PAOs with high poly-P content used the poly-P to obtain adenosine tri-phosphate (ATP), and glycogen (Gly) to obtain nicotinamide adenine dinucleotide (NADH) and some ATP. In a test where poly-P depletion was imposed on the culture, all the acetate (HAc) added in each cycle was transformed into polyhydroxyalkanoate (PHA) despite the decrease of poly-P inside the cells. This led to an increase of the $Gly_{degraded}/HAc_{uptake}$ ratio that resulted from a shift towards the glycolytic pathway in order to compensate for the lack of ATP formed from poly-P hydrolysis. The shift from PAO to GAO metabolism was also reflected in the change in the PHA composition as the poly-P availability decreased, suggesting that polyhydroxyvalerate (PHV) is obtained due to the consumption of excess reducing equivalents to balance the internal NADH, similarly to GAO metabolism. Fluorescence in situ hybridization analysis showed a significant PAO population change from Type I to Type II *Accumulibacter* as the poly-P availability decreased in short term experiments. This work suggests that poly-P storage levels and GAO-like metabolism are important factors affecting the competition between different PAO Types in enhanced biological phosphorus removal systems.

1. Introduction

Enhanced Biological Phosphorus Removal (EBPR) is widely accepted as one of the most economical and sustainable processes to remove phosphorus from wastewater. PAOs are the group of microorganisms primarily responsible for the EBPR process. PAOs are equipped with a polyphosphate-accumulating metabolism (PAM) under alternating anaerobic-aerobic/anoxic conditions. Under anaerobic conditions, PAOs take up volatile fatty acids (VFAs) (e.g. acetate) and store them as PHAs (Comeau et al., 1986; Mino et al., 1987; Wentzel et al., 1986). ATP is required for HAc transport (Comeau et al., 1986; Smolders et al., 1994) and conversion to acetyl coenzyme A (Ac-CoA) inside the cell. The source of ATP

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doi:10.1016/j.watres.2012.01.003

is thought to be provided primarily by the hydrolysis of intracellularly stored inorganic poly-P. A reducing equivalent source, such as reduced NADH, is required under anaerobic conditions for the synthesis of PHA. Many researchers have been studying biochemical pathways of PAOs (Schuler and Jenkins, 2003a,b; Erdal et al., 2005; Martin et al., 2006; Zhou et al., 2009). The major differences in the proposed metabolic pathways are related to the source of the reducing equivalents for PHA synthesis (Oehmen et al., 2007). Comeau et al. (1986) and Wentzel et al. (1986) suggested the anaerobic operation of the tricarboxilic acid cycle (TCA) as a reducing equivalent source. Mino et al. (1987) suggested that anaerobic glycogen degradation through glycolysis was a possible source of the reducing equivalents. It is suggested in many experimental studies (Pereira et al., 1996; Lemos et al., 1998; Hesselmann et al., 2000; Martin et al., 2006; Zhou et al., 2009) that both, TCA and glycolysis, provide the reducing equivalents. PAOs use oxygen or NOx (aerobic or anoxic conditions) to take up the phosphate in order to recover the poly-P level (Oehmen et al., 2007). Different species of PAOs have been reported, among which include Type I and Type II *Candidatus Accumulibacter phosphatis*, which are able to denitrify. The Type I are thought to be able to denitrify from nitrate and/or nitrite while Type II are able to denitrify from nitrite (Carvalho et al., 2007; Flowers et al., 2009). Under aerobic/anoxic conditions, the source of energy and carbon is obtained from stored PHAs, to regenerate the glycogen and recover the poly-P.

In EBPR processes, the GAOs compete with PAOs for VFA uptake under anaerobic conditions. GAOs have a glycogen-accumulating metabolism (GAM) which is similar to PAM but without phosphorus release and phosphorus uptake; therefore, GAOs do not remove phosphorus from wastewater. GAOs use glycolysis to obtain energy and reducing equivalents for VFA uptake and PHA storage. Under aerobic conditions, glycogen is regenerated from PHAs.

Microbiological studies identify PAOs and GAOs as different microorganisms with similar metabolisms. Given this observation, Schuler and Jenkins (2003a) hypothesized that GAM was most likely the dominant process in PAO cultures when subjected to certain operational conditions. It has been observed in recent studies that PAOs are able to behave as GAOs under different conditions (Barat et al., 2006, 2008; Erdal et al., 2008; Zhou et al., 2008).

Erdal et al. (2008) and Zhou et al. (2008) observed that under low poly-P conditions, PAOs are able to take up acetate anaerobically, suggesting that acetate can be stored as PHA using glycogen as the primary energy source. Barat et al. (2006, 2008) observed that the calcium ion clearly affects the PAOs performance in a Sequencing Batch Reactor (SBR) operated for EBPR. These authors detected a clear effect of influent Ca concentration on the amount of phosphate released per unit of acetate taken up during the anaerobic phase. They also observed a metabolic shift from PAM to GAM.

The aim of this work is to study the PAOs behavior with different levels of poly-P storage from a macroscopic to a microscopic point of view. For this purpose we have reduced the internal poly-P at different levels (from a highly enriched PAOs with poly-P to a system starved of poly-P) in a SBR reactor operated for EBPR in order to study the evolution of the internal polymers and the population dynamics of PAOs (including PAO types I and II) and GAOs. The results of this work could help for a better understanding of PAO metabolism and for a future modification of the current models adding the metabolic shift into PAO models.

2. Materials and methods

2.1. Reactor setup and operation

A laboratory scale sequencing batch reactor (SBR) enriched in *Accumulibacter* was setup in this study. The SBR was operated under anaerobic-aerobic conditions for biological phosphorus removal during 134 days. The SBR was fed with HAc as the sole carbon source. The working volume of the SBR was 7 l and the volume of synthetic wastewater added in each cycle was 3.5 l (overall hydraulic retention time was 12 h). Biomass was wasted daily from the system to maintain the sludge retention time at 8 days. The SBR was operated with four 6 h cycles per day: filling period 4 min; anaerobic phase 1.5 h; aerobic phase 3.5 h; settling phase 52 min and withdrawing period 4 min.

The reactor was equipped with conductivity, ORP, pH, temperature and dissolved oxygen electrodes. The dissolved oxygen (DO) concentration in the aerobic phase was controlled between 1.5 and 2.5 O_2 mg l^{-1}. The temperature was maintained at 20 ± 1 °C. The initial pH for each cycle was kept around 7.5 and ranged from 7.0 to 8.9 during the different phases of each cycle. Synthetic wastewater was used during the pseudo-steady state and experimental period with a COD/P ratio of 10.5 COD mg P mg^{-1} (110 mg COD l^{-1} and 10.5 mg P l^{-1}). The wastewater consisted of two separate solutions as follows: one solution contained mineral compounds, including K_2HPO_4, and the other one contained acetate and NH_4Cl (see Barat et al., 2008 for a detailed description). Thiourea was added to the synthetic media (20 mg l^{-1}) in order to inhibit nitrification.

2.2. Experimental design

The SBR was seeded with sludge from a wastewater treatment plant (WWTP) operated for biological phosphorus removal that is located in Valencia (Spain). Once the process was stabilized and the biomass was enriched in PAOs, six experiments were carried out. All the experiments were carried out keeping constant all the factors known to favor the population of PAOs over GAOs: a low COD/P ratio in the influent of about 10–20 COD mg P mg^{-1} (Mino et al., 1998; Oehmen et al., 2007); a pH above 7 (Smolders et al., 1994; Liu et al., 1996; Bond et al., 1999; Filipe et al., 2001a, 2001b); and a temperature of 20 °C (Oehmen et al., 2007). Each experiment consisted of intensively monitoring the concentrations of HAc, phosphorus, ammonia, nitrate, PHA and glycogen during one whole SBR cycle. Moreover, COD, P_T, TSS and VSS were measured at the end of the aerobic phase. Samples for fluorescence in situ hybridization were also collected.

The 1st and 2nd experiments were carried out at the same operational conditions to characterize the process performance with high poly-P content. The 3rd, 4th and 5th experiments were carried out at decreasing intracellular poly-P

content. In order to reach these conditions, it was necessary to carry out a conditioning cycle before each of these experiments (one conditioning cycle in the experiments 3, and 4, and two conditioning cycles in the experiment 5). A period of time after each experiment was used for the system to recover the initial conditions of phosphorus release (see section 3). The 6th experiment was performed when the poly-P content in the biomass was recovered. During the course of the experiments the influent wastewater was the same, with the exception of the conditioning cycles prior to the 4th and 5th experiment, where the HAc concentration was increased to 170 and 400 COD mg l^{-1} respectively, in order to induce higher poly-P hydrolysis and phosphate release (Fig. 1A). The conditioning cycle consisted of removing the supernatant highly enriched with phosphate at the end of the anaerobic phase and replacing it with synthetic media without phosphate, reducing the poly-P content at the end of the cycle. Fig. 1B shows the different stages during the conditioning cycle.

2.3. *Analytical methods and microbiological techniques*

VFAs were measured by the method proposed by Moosbrugger et al. (1992) using a Metrhom 716 DMS tritino. The analyses of phosphorus, ammonia and nitrite were carried out using a Lachat QuikChem800 flow injection analyzer. COD, P_T, TSS and VSS were performed in accordance with Standard Methods (APHA, 2005). PHA was analyzed by the method proposed by Oehmen et al. (2005). For the determination of glycogen, 30 mg of biomass was mixed with 4 ml of a 0.6 M hydrochloric acid solution kept in a capped tube for 2 h at 100 °C. The resulting solution was centrifuged at 2600×*g* for 10 min. The glucose concentration in the solution was determined using a commercial enzymatic kit (GAHK20-1 KT Sigma–Aldrich).

FISH was performed to study the population dynamics of PAOs (including Type I and Type II *Accumulibacter*) and GAOs in the reactor. Cell hybridization was performed as described by Amann et al. (1990). The rRNA oligonucleotide probes used for FISH are listed in Table 1. Some probe associations were made for covering the adequate ranges: PAOmix (PAO462, PAO651, PAO846), DEFmix (TFO_DF218, TFO_DF618), DEF2mix (DF1020, DF988, H966, H1038) and EUBmix (EUB338, EUB338 II, and EUB338 III). All probes were used at a 35% formamide concentration. Hybridized cells were enumerated by means of capturing images with a confocal microscope Leica TCS SP (for PAO Types I and II signal over EUBmix probe), and with an epifluorescence microscope Leica DM2500 and a Leica DFC420c digital camera (for PAOmix, GB, DEFmix and DEF2-mix signals over EUBmix signal), using the Matlab software for image analysis. A minimum of 20 randomly chosen microscopic fields were quantified from each sample. Each of the images was examined to determine the optimum threshold values for each fluorochrome. The countable pixel area of the specific probe-fluorochrome signal (Type I and Type II PAO, PAOmix, GB, DEFmix or DEF2mix probes) was expressed as a mean percentage of the pixel area count from the EUBmix probe signal. Error of the quantification was calculated by dividing the standard deviation by the square root of "*n*", where "*n*" is the number of fields examined.

3. Results and discussion

The SBR was working in steady state and performing >99% phosphorus removal when the batch experiments were carried out. The monitoring of the ratio $\Delta Cond_{AN}$/VSS (Eq. (1)) provides valuable information for the EBPR process, because the amount of phosphorus released is highly related to the conductivity increase during the anaerobic stage (Aguado et al., 2006). The conductivity monitoring has facilitated the operation of the SBR, and has permitted the detection of steady state conditions. Fig. 2 shows a period (A) where the ratio $\Delta Cond_{AN}$/VSS increased with time until it stabilized, which corresponds to stable phosphorus release conditions

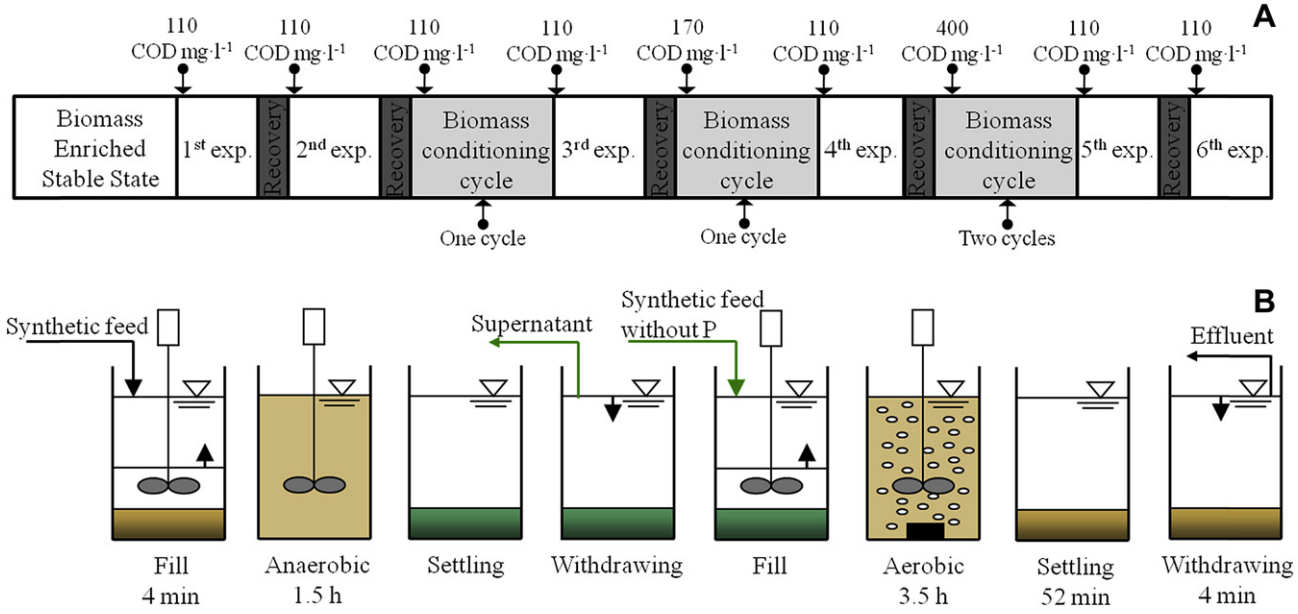

Fig. 1 – Experimental design (A) and phases of the biomass conditioning cycle for removing poly-P in the SBR (B).

Table 1 – Oligonucleotide probes used in this study.

Probe	Sequence (5′ → 3′)	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Eubacteria*	Amann et al., 1990
EUB338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*	Daims et al., 1999
EUB338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	
PAO 462	CCGTCATCTACWCAGGGTATTAAC	*Rhodocyclus tenuis subgroup*	Crocetti et al., 2000
PAO651	CCCTCTGCCAAACTCCAG	*Candidatus Accumulibacter phosphatis*	
PAO846	GTTAGCTACGGCACTAAAAGG	*Rhodocyclus tenuis subgroup*	
Acc-I-444	CCCAAGCAATTTCTTCCCC	Clade IA and other Type I clades	Flowers et al., 2009
Acc-II-444	CCCGTGCAATTTCTTCCCC	Clade IIA, IIC and IID as Type II clades	
GB	CGATCCTCTAGCCCACT	*Gammaproteobacterial* GAO group	Kong et al., 2002
TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus*-related (cluster 1)	Wong et al., 2004
TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus*-related (cluster 1)	
DF1020	CCGGCCGAACCGACTCCC	*Defluviicoccus*-related (cluster 2)	Meyer et al., 2006
DF988	GATACGACGCCCATGTCAAGGG	*Defluviicoccus*-related (cluster 2)	
H966 (DF988 helper)	CTGGTAAGGTTCTGCGCGTTGC		
H1038 (DF988 helper)	AGCAGCCATGCAGCACCTGTGTGGCGT		

($P_{release}$). In period (B), experiments were carried out under steady state conditions as indicated by the conductivity measurements. In the 4th and 5th experiments, it is observed that the ratio $\Delta Cond_{AN}/VSS$ decreased considerably, pointing out that there was a reduction of the poly-P content. All the experiments were carried out when the system recovered the initial conditions of phosphorus release. Fig. 2 also shows that the value of pH at the beginning of the anaerobic phase was kept around 7.5 throughout the study. This pH value favors PAOs growth over GAOs (Filipe et al., 2001b).

$$\Delta Cond_{AN}/VSS = (\text{final conductivity} - \text{initial conductivity})_{\text{anaerobic stage}}/VSS \quad (1)$$

As previously indicated, the 1st and the 2nd experiments were carried out at the same operational conditions in order to characterize the steady state. The results were similar in both experiments. Fig. 3 shows the evolution of HAc, GLY, PHA (including polyhidroxybutirate (PHB) and PHV) and phosphate in the 2nd experiment. During the anaerobic phase the HAc was fully taken up. Degradation of glycogen and accumulation of PHA took place during the anaerobic phase. PHB was 95% of all the PHA, a small amount of PHV was produced and the PH2MV production was not observed. This composition is usually observed in similar phosphorus removal systems fed with acetate (Oehmen et al., 2007; Zhou et al., 2008, 2009). The stoichiometry obtained in the anaerobic phase from the 1st and 2nd experiments is summarized in Table 2. During the aerobic phase, PHA was degraded forming glycogen, and phosphorus in the supernatant was totally eliminated.

The 3rd, 4th and 5th experiments were carried out under low intracellular poly-P content (see Table 3). The measured results under these conditions are shown in Fig. 4. The amount of phosphate released in the anaerobic phase

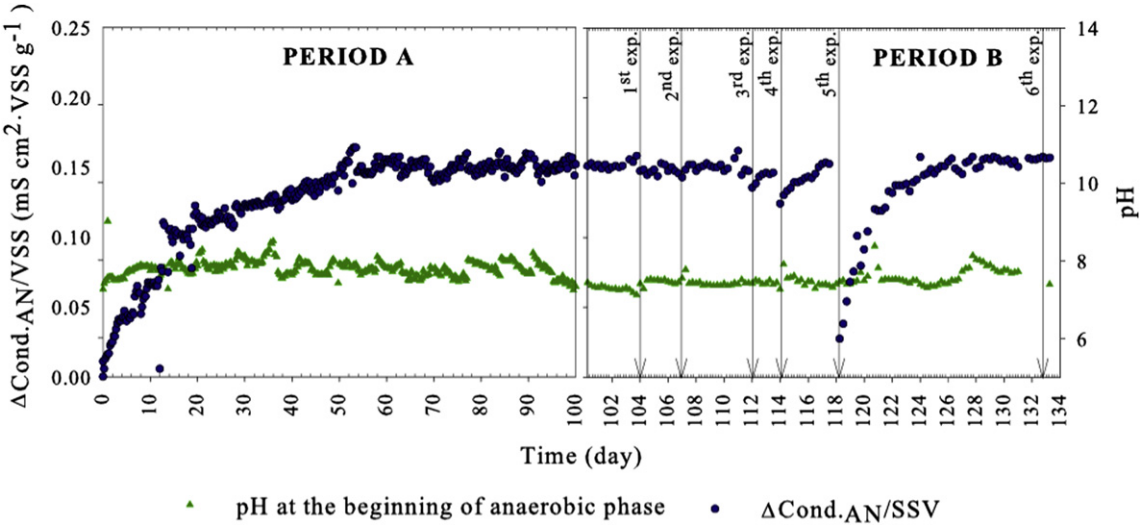

Fig. 2 – Monitoring of $\Delta Cond._{AN}/VSS$ and initial pH of the anaerobic phase. A: start up period, B: experimental period.

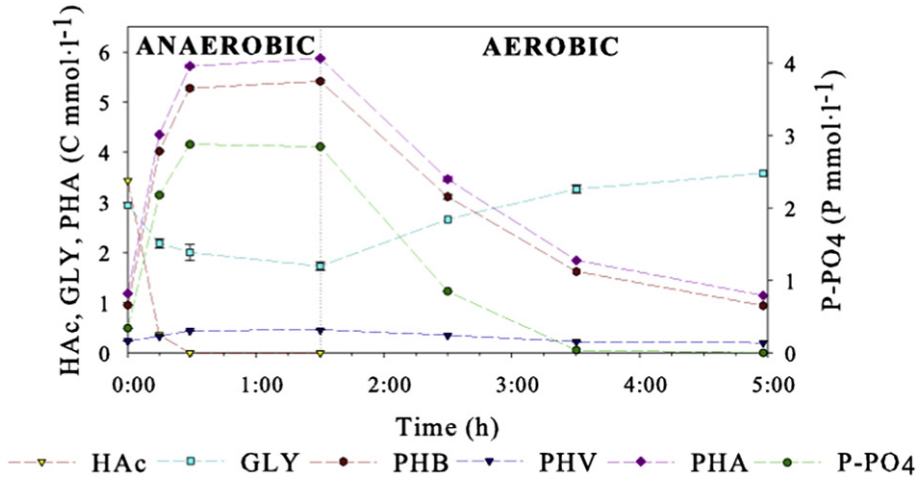

Fig. 3 – Evolution of carbon sources and phosphorus during the 2nd experiment. Each value is represented including its standard deviation.

Table 2 – Stoichiometric parameters observed in this study and proposed in literature, for processes that use acetate as C source.

	Anaerobic					Aerobic	
	P_{rel}/HAc_{upt} [a]	Gly_{degrad}/HAc_{upt} [b]	PHA_{synt}/HAc_{upt} [b]	PHB_{synt}/HAc_{upt} [b]	PHV_{synt}/HAc_{upt} [b]	Gly_{form}/PHA_{degrad} [b]	P_{upt}/PHA_{degrad} [a]
This study PAO							
1st	0.70	0.38	1.36	1.31	0.05	–[c]	–[c]
2nd	0.73	0.35	1.36	1.30	0.06	0.39	0.60
3rd	0.66	0.51	1.46	1.37	0.09	0.48	0.47
4th	0.50	0.66	1.61	1.53	0.08	0.55	0.37
5th	0.08	1.08	2.02	1.74	0.28	0.61	0.10
6th	0.67	0.35	1.31	1.20	0.10	0.45	0.71
PAO metabolic models							
Comeau et al., 1986; Wentzel et al., 1986	0.50	–	0.89	0.89	–		
Smolders et al., 1994	0.50[d]	0.50	1.33	1.33	0	0.42	0.41
Pereira et al., 1996	0.16	0.70	1.48	1.02	0.46		
Hesselmann et al., 2000	0.37	0.61	1.40	1.11	0.29		
Experimental studies PAO							
Smolders et al., 1995	0.48	0.34	1.30	1.20	0.14		
Kisoglu et al., 2000	0.80	0.31	0.83	–	–		
Filipe et al., 2001b	0.57	0.50	1.28	1.13	0.15		
Jeon et al., 2001		0.45	1.75	1.40	0.27		
Yagci et al., 2003		0.48	1.23	1.08	0.12		
Oehmen et al., 2005		0.60	1.58	1.31	0.26		
Lu et al., 2006		0.46	1.25	1.18	0.07		
Zhou et al., 2008		0.45	1.22	1.15	0.07		
GAO metabolic models							
Satoh et al., 1994	0	1.25	1.96	1.33	0.63		
Zeng et al., 2003	0	1.12	1.86	1.36	0.46	0.65	0
Experimental studies GAO							
Liu et al., 1994	0.010	1.20	1.51	1.10	0.41		
Jeon et al., 2001	0.015	1.21	2.04	1.50	0.49		
Filipe et al., 2001c	0.020	0.92	1.53	1.20	0.33		
Oehmen et al., 2005		1.17	1.83	1.30	0.54		

a Units P mmol C $mmol^{-1}$.
b Units C mmol C $mmol^{-1}$.
c No data.
d Calculated with pH = 7.

Table 3 – Phosphorus, poly-P, TSS and VSS content in the biomass and microbiological results in all the experiments.

		Experiment					
	Units	1	2	3	4	5	6
P_T/VSS	P mg·VSS mg^{-1}	0.3	0.32	0.21	0.09	0.03	0.25
poly-P/VSS	P mg·VSS mg^{-1}	0.28	0.3	0.19	0.07	0.01	0.23
TSS	TSS mg l^{-1}	2566	2262	2310	1558	1280	1560
VSS	%	45	42	46	58	92	44
Type I PAO	%[a]		66 ± 7	54 ± 9	32 ± 10	23 ± 5	73 ± 6
Type II PAO	%[a]		8 ± 3	27 ± 7	48 ± 12	36 ± 7	9 ± 6
PAO_{mix}	%[a]	88 ± 3	87 ± 2	90 ± 2	80 ± 4	86 ± 4	92 ± 1
$GB + DEF_{mix} + DEF2_{mix}$	%[a]	0	0	0	0	0	0

a Percentage of PAO_{mix} and GAOs (GB, DEF_{mix}, $DEF2_{mix}$) probes over the EUB_{mix} probes.

decreased in each experiment from 2.28 P mmol l^{-1} in the 3rd experiment to 0.23 P mmol l^{-1} in the 5th experiment. These results indicated that the poly-P stored in PAOs was gradually decreasing in the biomass. HAc was fully taken up in the 3rd and 4th experiments; while in the 5th experiment, the HAc was not completely taken up in the anaerobic phase. The degradation of glycogen increased in each experiment from 2.02 C mmol l^{-1} in the 3rd experiment to 2.98 C mmol l^{-1} in the 5th experiment. The PHA accumulated in the anaerobic phase increased from 4.00 C mmol l^{-1} in the 3rd experiment to 5.53 C mmol l^{-1} in the 5th experiment. The experimental stoichiometry in the anaerobic phase for the experiments 3, 4 and 5 is shown in Table 2.

The 6th experiment was carried out when the system was recovered after performing the experiments with low concentrations of poly-P. With this study it was intended to clarify whether the system would reproduce similar features to those observed in experiments 1 and 2. This hypothesis was confirmed. As can be observed in Fig. 4D, the HAc was fully consumed and the evolution of carbon and phosphorus was similar to that shown in the 1st and 2nd experiments. Stoichiometric values and content of poly-P obtained once the release of phosphorus was stabilized are shown in Tables 2 and 3, respectively.

The poly-P content in each experiment was estimated mathematically by means of equation (2). The P_T was measured at the end of the aerobic phase. We assume that at the end of the aerobic phase the P_T is the sum of poly-P and organic phosphorus (2% of VSS according to Metcalf and Eddy Inc., 2003). The results obtained are shown in Table 3. As can be seen, the concentration of poly-P was reduced up to 98% in the 5th experiment.

$$\text{poly} - \text{P} = \text{P}_\text{T} - 2\%\ \text{VSS} \quad (2)$$

As will be discussed later, the FISH results showed that the biomass was highly enriched in *Accumulibacter* in all the experiments while GAOs were not detected in any of the experiments (Table 3).

The anaerobic metabolism of PAOs has been widely described in stoichiometric models (Comeau et al., 1986; Wentzel et al., 1986; Smolders et al., 1994; Pereira et al., 1996; Hesselmann et al., 2000) and experimental studies (Smolders et al., 1995; Kisoglu et al., 2000; Filipe et al., 2001c; Jeon et al., 2001; Yagci et al., 2003; Oehmen et al., 2005; Lu et al., 2006; Zhou et al., 2008). The ratios of $Gly_{degraded}/HAc_{uptake}$, $PHB_{synthesized}/HAc_{uptake}$ and $PHV_{synthesized}/HAc_{uptake}$ obtained by these authors are shown in Table 2. Also included in this table are the ratios proposed in literature for GAO enriched populations.

Under anaerobic conditions, PAOs release phosphate due to the hydrolysis of intracellular poly-P to obtain ATP and metabolize the HAc, while GAOs use glycolysis for the same purpose. For this reason, the $P_{release}/HAc_{uptake}$ ratio is often used for indicating the PAM or GAM activity at a given pH in mixed PAO-GAO cultures (Filipe et al., 2001a). Values between 0.48 and 0.80 P mmol C $mmol^{-1}$ for PAO-enriched cultures and from 0 to 0.02 P mmol C $mmol^{-1}$ for GAO enriched cultures can be found in the literature (see Table 2).

The results obtained in this study show that cultures enriched in PAOs (without GAOs) could present $P_{release}/HAc_{uptake}$ ratios below 0.16 P mmol C $mmol^{-1}$, which are commonly associated with mixed cultures with high presence of GAOs. Fig. 5A shows that the $P_{release}/HAc_{uptake}$ ratio decreases from 0.73 P mmol C $mmol^{-1}$ to 0.08 P mmol C $mmol^{-1}$. The low values of the ratio $P_{release}/HAc_{uptake}$ obtained are due to the low amount of poly-P available. Similar results of $P_{release}/HAc_{uptake}$ ratios were observed in Zhou et al. (2008) when the anaerobically produced phosphates were washed out. Barat et al. (2008) observed a significant calcium phosphate precipitation, which reduces the amount of poly-P available as an energy source. However, despite the decrease of poly-P, all the HAc added in each cycle was consumed by PAOs and transformed into PHA. Therefore, these results could suggest an increase in the use of the glycolytic pathway in order to compensate for the lack of ATP formed from poly-P hydrolysis. This hypothesis is confirmed by the values obtained for the ratio $Gly_{degraded/HAcuptake}$ in the different experiments of this study (Fig. 5B).

In the literature, it can be found experimental and modeling values of the ratio $Gly_{degraded}/HAc_{uptake}$ between 0.31 and 0.5 C mmol C $mmol^{-1}$ for PAOs and from 0.92 to 1.25 C mmol C $mmol^{-1}$ for GAOs. In this study, using a culture enriched in PAOs, ratios of $Gly_{degraded}/HAc_{uptake}$ from 0.38 to 1.08 C mmol C $mmol^{-1}$ were obtained and this ratio increased as the ratio of poly-P/VSS decreased, as can be seen in Fig. 5B. The 5th experiment is clearly poly-P limited but HAc uptake is still observed and a $Gly_{degraded}/HAc_{uptake}$ ratio of 1.08 C mmol C $mmol^{-1}$ was obtained. These results suggest that the energy required for the uptake of the HAc is not only

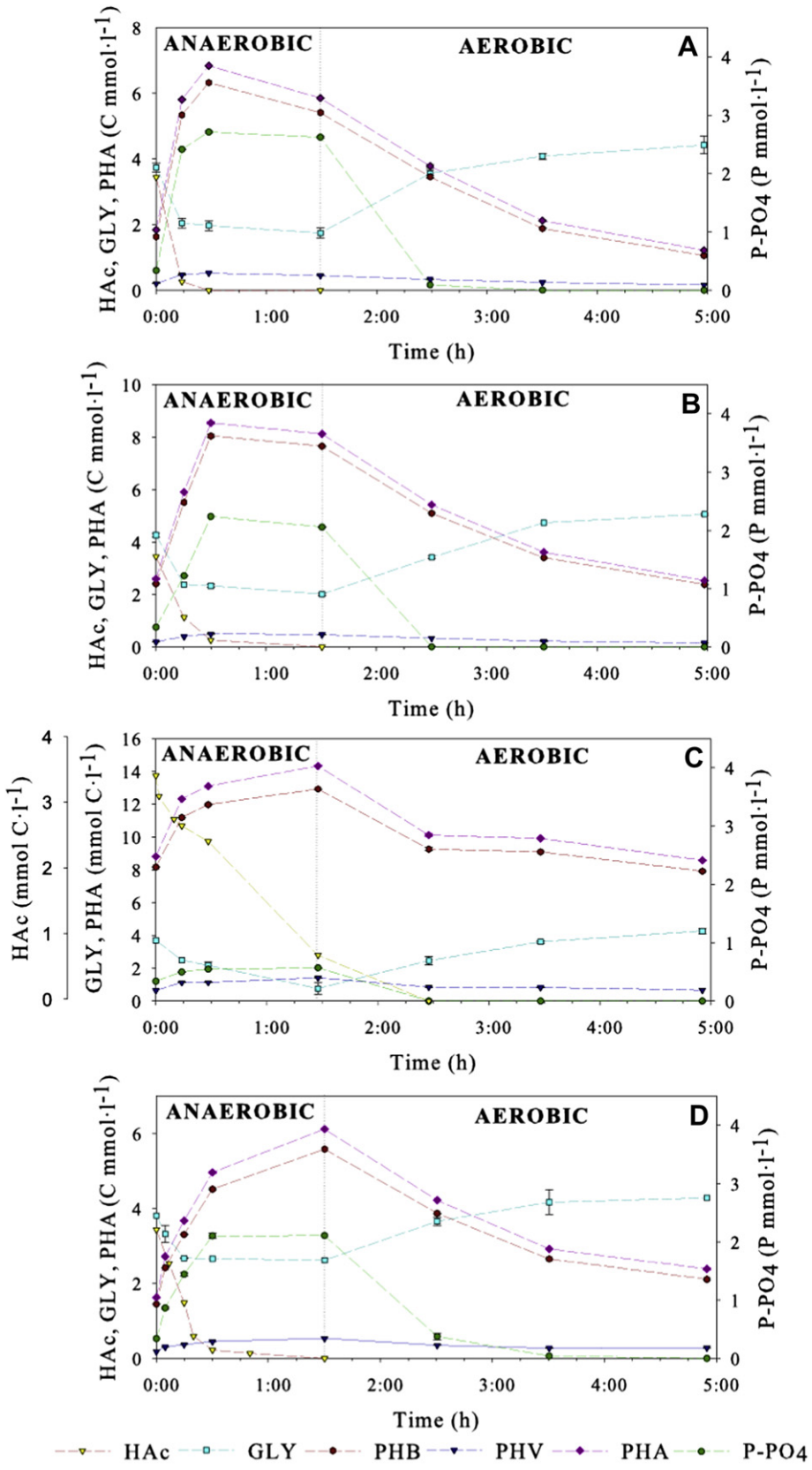

Fig. 4 – Evolution of carbon compounds and phosphorus during the 3rd (A), 4th (B), 5th (C) and 6th (D) experiment.

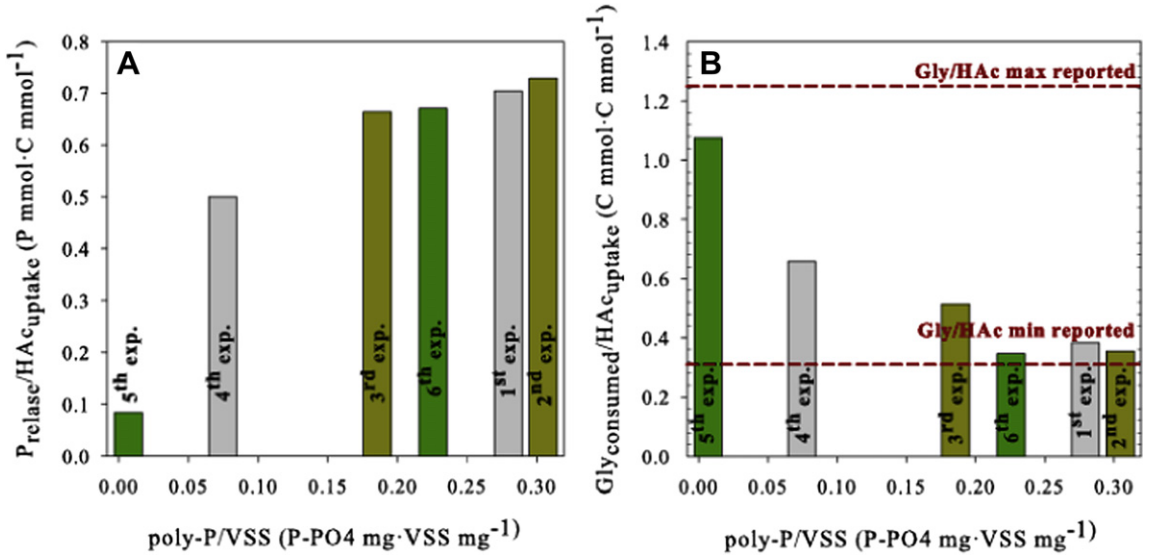

Fig. 5 – Ratios of: $P_{release}/HAc_{uptake}$ (A) and $Gly_{degraded}/HAc_{uptake}$ (B) observed in the sludge with different amounts of poly-P.

derived from the poly-P degradation, but also from the glycogen degradation. The low amount of glycogen at the end of the anaerobic phase could have caused HAc not to be completely taken up during the anaerobic phase (Fig. 4C).

The shift from PAM to GAM was also observed in the higher production of PHA as shown in Fig. 6. These results could be due to the increase in the use of the glycolytic pathway previously commented. The increase in the glycogen degradation to supply the extra energy implies a higher production of reducing equivalents and acetyl-CoA that will be reduced, resulting in more PHA production. Therefore, the increase of the glycolysis activity causes an increase of the amount of PHA produced. Furthermore, an important variation on the PHA composition was also observed as the poly-P availability decreased. The ratios of $PHV_{synthesized}/HAc_{uptake}$ varied from 0.05 C mmol C $mmol^{-1}$ in the 2nd experiment to 0.28 C mmol C $mmol^{-1}$ in the 5th experiment. These results suggest that the PHV is obtained through propionyl-CoA produced by consuming excess reducing equivalents through pyruvate-succinate-propionate to balance the internal NADH. This is in accordance with the GAO metabolism.

It can be clearly noticed that the anaerobic ratios obtained in this study in the 1st, 2nd, 3rd and 6th experiment are within the ranges described in literature for PAO metabolism. However, in the 5th experiment, where the available poly-P was limited, the ratios obtained were close to those obtained for GAO metabolism (Table 2). The ratios obtained in 4th experiment are not in the range of PAO or GAO metabolisms but between them. Therefore, this 4th experiment can be described as a transition behavior between PAM and GAM.

The aerobic ratio $Gly_{synthesis}/PHA_{degraded}$ was also studied. For experiments 2, 3 and 6, the ratios obtained were between 0.38 and 0.48 C mmol C $mmol^{-1}$, which are similar to the values reported by Smolders et al. (1994) for PAO-enriched cultures (see Table 2). However, in the 5th experiment the ratio

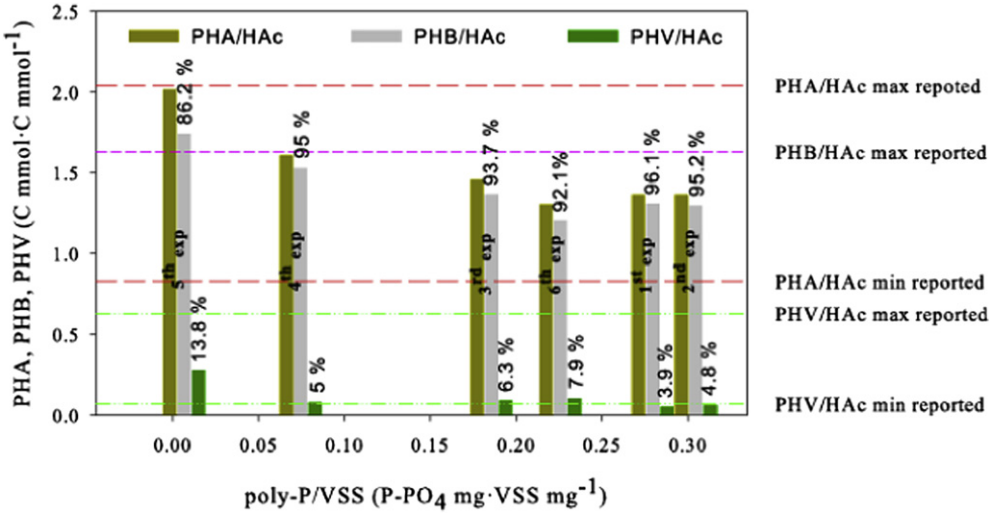

Fig. 6 – Experimental values obtained for the ratios $PHA_{synthesized}/HAc_{uptake}$, $PHB_{synthesized}/HAc_{uptake}$ and $PHV_{synthesized}/HAc_{uptake}$ at different amounts of intracellular poly-P.

$Gly_{synthesis}/PHA_{degraded}$ obtained was 0.61 C mmol C $mmol^{-1}$, which is similar to the ratio obtained by Zeng et al. (2003) for GAO enriched cultures. The $Gly_{synthesis}/PHA_{degraded}$ ratio obtained in the 4th experiment was between the values for PAO and GAO cultures reported in literature (Table 2). These results point out that the metabolic shift from PAM to GAM can be observed not only in the anaerobic phase but also in the aerobic phase. This metabolic change could be due the low phosphate concentration available during the aerobic phase.

As previously pointed out, FISH results showed that the biomass was highly enriched in *Accumulibacter* in all the experiments (Table 3). *Competibacter*, *Defluviicoccus*-cluster 1 and *Defluviicoccus*-cluster 2 were not detected in any of the experiments. However, there was an interesting change in the population dynamics of *Accumulibacter* species. In this study, it was observed the presence of two types of PAOs: Type I and Type II (Carvalho et al., 2007). Notice also that, meanwhile in most of the experiments sum of both type of PAOs was close to the total amount of *Accumulibacter* detected with the PAOmix probe, the 5th experiment does not follow this trend. In this experiment there is a discrepancy that could be due to a partial growth of other PAO clades not included in the probes used in this study. It was observed that *Accumulibacter* Type I dominated the biomass when PAOs were highly enriched with poly-P. By decreasing the poly-P content, a shift in the population was observed: Type I PAO decreased while Type II PAO increased (Fig. 7). Another important aspect to pay attention is that this population change between PAOs Type I and II took place within few operational cycles. For example, it was observed a clear increase in PAO Type II and a decrease in PAO Type I from experiment 2 to experiment 3. However, the difference between both experiments is that experiment 2 was performed at steady state in a highly enriched PAO culture with high poly-P content (0.3 P g·VSS g^{-1}) and experiment 3 was performed after one conditioning cycle in which poly-P content was reduced until 0.19 P g·VSS g^{-1}. Therefore, the FISH analysis confirms that only two cycles at low poly-P (the conditioning cycle plus the experiment cycle) are enough to significantly change the PAO population between experiment 2 and experiment 3. This trend is repeated along the experiments and, in the last experiment (experiment 6 carried out once the PAOs recovered the poly-P: 0.27 P g·VSS g^{-1}) it was observed that the PAOs distribution reverted to the population observed in the first experiments once the system was enriched again with poly-P. This work shows that not only does nitrate and anoxic phase length affect the Type I to Type II ratio (Oehmen et al., 2010), but also the polyphosphate content and GAO-like metabolism influences this competition between different PAO Types. These results could explain why in some cases PAO cannot switch to a GAO-like metabolism and other times they can (Brdjanovic et al., 1998; López-Vázquez et al., 2008; Zhou et al., 2008).

PAOs and GAOs are different organisms; however, this research proved that similar metabolic behaviors can be observed when PAOs have low concentrations of poly-P, suggesting that both organisms use similar metabolic pathways. Fig. 8 shows a scheme of PAO metabolism as proposed by different authors. PAOs use mainly glycolysis for the production of NADH. However, when they are low in poly-P, glycolysis is used to produce the ATP needed to take up HAc and synthesize PHA. It is possible that the use of the glycolytic pathway to supply the needs of ATP generates an excess of NADH. This NADH is used for the transformation of acetyl-CoA and propionyl-CoA to PHA, which comprises monomeric units of HV (hidroxyvalerate), 3H2MB (3-hydroxy-2-methylbutyrate) and 3H2MV (3-hydroxy-2-methylvalerate). The production of propionyl-CoA is carried out by the succinate-propionate pathway as suggested by Hesselmann et al., 2000, also consuming NADH. This scheme may represent the potential metabolic pathways that PAOs follow when their metabolic shift occurs.

The present research confirmed a metabolic change of a PAO-enriched culture at the macroscopic level, as other authors have observed under different operational conditions (Barat et al., 2006, 2008; Erdal et al., 2008 and Zhou et al., 2008). It was also observed, at the microscopic level, a population shift between Type I and Type II PAOs. These results suggest that Type II PAOs have a greater ability to adapt to changes in poly-P storage conditions, possibly because they are able to use different metabolic pathways. However, it is not clear whether the metabolic change is due to a population shift in the biomass, or that Type II PAO are able to change their metabolism from PAM to GAM when they have a low internal

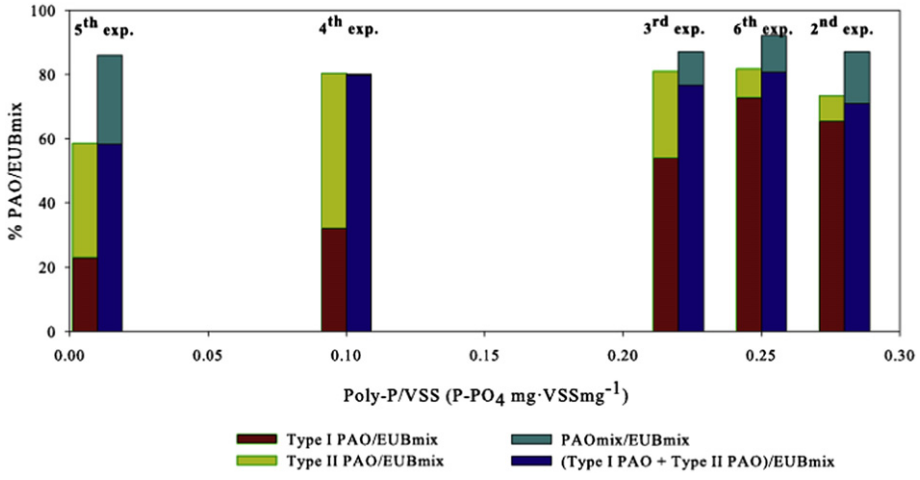

Fig. 7 – PAOs population dynamics: PAOmix, PAO Type I and PAO Type II.

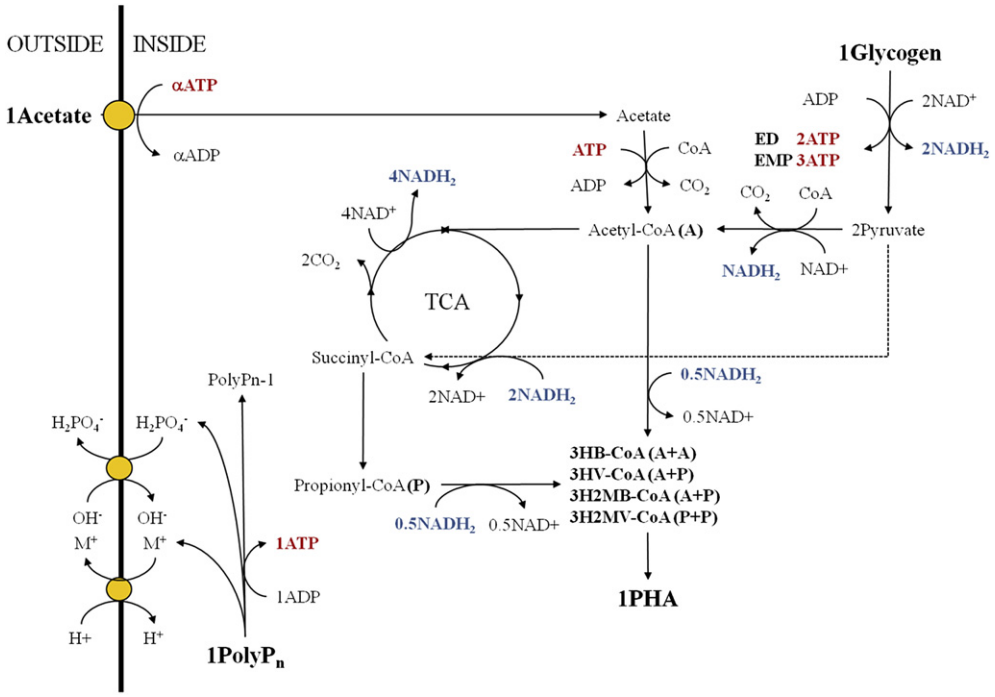

Fig. 8 – Schematic anaerobic metabolism for PAOs (Hesselmann et al., 2000; Reis et al., 2003; Martin et al., 2006 and Zhou et al., 2009).

poly-P content, whereas Type I are unable to use the glycolytic and succinate-propionate pathways as efficiently. For this reason, future research is needed to study both PAOs (Type I and II) metabolism and its behavior at long term experiments under low poly-P content.

4. Conclusions

At a macroscopic level, the anaerobic and aerobic stoichiometric ratios of a biomass enriched in *Accumulibacter* presented a metabolic shift from PAM to GAM as the poly-P was gradually decreased and from GAM to PAM when the poly-P was recovered. The results observed showed that the decrease of poly-P stimulate an increase in the use of the glycolytic pathway in order to compensate for the lack of ATP formed from poly-P hydrolysis. The shift from PAM to GAM was also observed in the higher production of PHA due to the increase in the use of the glycolytic pathway. Furthermore, an important variation on the PHA composition ($PHV_{synthesized}$/HAc_{uptake}) was also observed as the poly-P availability decreased. These results suggest that the PHV is obtained by consuming excess reducing equivalents through the pyruvate-succinate-propionate pathway to balance the internal NADH, which is in accordance with the GAO metabolism.

At a microscopic level, FISH analyses revealed that *Accumulibacter* Type I dominated the biomass when PAOs were highly enriched with poly-P and that the population changed to *Accumulibacter* Type II when the poly-P was gradually decreased. These results suggest that Type II PAOs have a greater ability to adapt to changes in poly-P storage conditions, possibly because they are able to use different metabolic pathways, whereas Type I are unable to use the glycolytic and succinate-propionate pathways as efficiently. Furthermore, it was observed a shift from Type II to Type I when the poly-P was recovered. These observations suggested that not only does nitrate and anoxic phase length affect the Type I to Type II ratio, but also the polyphosphate content and GAO-like metabolism influences this competition between different PAO Types.

Acknowledgments

This research work has been supported by the Generalitat Valenciana (GVPRE/2008/044) and the Polytechnic University of Valencia (PAID-06-08-3227), which are gratefully acknowledged. Special acknowledgements to Consejo Nacional de la Ciencia y la Tecnología de México (CONACYT) No. 207966 and the Fundação para a Ciência e Tecnologia for PEst-C/EQB/LA0006/2011, PEst-OE/EQB/LA0004/2011 and SFRH/BPD/30800/2006.

REFERENCES

Aguado, D., Montoya, T., Ferrer, J., Seco, A., 2006. Relating ions concentration variations to conductivity variations in a sequencing batch reactor operated for enhanced biological phosphorus removal. Environmental Modelling and Software 21 (6), 845–851.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing

mixed microbial populations. Applied and Environmental Microbiology 56 (6), 1919–1925.
American Public Health Association, 2005. Standard methods for the Examination of Water and wastewater, 21th ed. American Water Works Association and Water Environmental Federation, Washington, DC, USA.
Barat, R., Montoya, T., Borras, L., Seco, A., Ferrer, J., 2006. Calcium effect on enhanced biological phosphorus removal. Water Science and Technology 53 (12), 29–37.
Barat, R., Montoya, T., Borras, L., Ferrer, J., Seco, A., 2008. Interactions between calcium precipitation and the polyphosphate-accumulating bacteria metabolism. Water Research 42 (13), 3415–3424.
Bond, P.L., Erhart, R., Wagner, M., Keller, J., Blackall, L.L., 1999. Identification of some of the major groups of bacteria in efficient and non efficient biological phosphorus removal activated sludge systems. Applied Environmental Microbiology 65 (9), 4077–4084.
Brdjanovic, D., Logemann, S., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998. Influence of temperature on biological phosphorus removal: process and molecular ecological studies. Water Research 32 (4), 1035–1048.
Carvalho, G., Lemos, P.C., Oehmen, A., Reis, M.A., 2007. Denitrifying phosphorus removal: linking the process performance with the microbial community structure. Water Research 41 (19), 4383–4396.
Comeau, Y., Hall, K., Hancock, R., Oldham, W., 1986. Biochemical-model for enhanced biological phosphorus removal. Water Research 20 (12), 1511–1521.
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.
Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 22 (3), 434–444.
Erdal, Z.K., Erdal, U.G., Randall, C.W., 2005. Biochemistry of enhanced biological phosphorus removal and anaerobic COD stabilization. Water Science Technology 52 (10–11), 557–567.
Erdal, U., Erdal, Z., Daigger, G., Randall, C., 2008. Is it PAO-GAO competition or metabolic shift in EBPR system? Evidence from an experimental study. Water Science and Technology 58 (6), 1329–1334.
Filipe, C., Daigger, G., Grady, C., 2001a. Effects of pH on the rates of aerobic metabolism of phosphate-accumulating and glycogen-accumulating organisms. Water Environmental Research 73 (2), 213–222.
Filipe, C., Daigger, G., Grady, C., 2001b. pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. Water Environmental Research 73 (2), 223–232.
Filipe, C., Daigger, G., Grady, C., 2001c. A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH. Biotechnology and Bioengineering 76 (1), 17–31.
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.
Hesselmann, R.P.X., Von Rummell, R., Resnick, S.M., Hany, R., Zehnder, A.J.B., 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Research 34 (14), 3487–3494.
Jeon, C.O., Lee, D.S., Lee, M.W., Park, J.M., 2001. Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: effect of pH. Water Environmental Research 73 (3), 301–306.
Kisoglu, Z., Erdal, U., Randall, C., 2000. The effect of CODTP ratio on intracellular storage materials, system performance and kinetic parameters in a BNR system. In: Proceedings of the 73rd annual. Water Environment Federation Technical Exposition and Conference Anaheim, California.
Kong, Y., Ong, S., Ng, W., Liu, W., 2002. Diversity and distribution of a deeply branched novel proteobacteria group found in anaerobic–aerobic activated sludge processes. Environmental Microbiology 4 (11), 753–757.
Lemos, P.C., Viana, C., Salgueiro, E.N., Ramos, A.M., Crespo, J., Reis, M.A.M., 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. Enzyme and Microbial Technology 22 (8), 662–671.
Liu, W.T., Mino, T., Nakamura, K., Matsuo, T., 1994. Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic–aerobic activated-sludge with a minimized polyphosphate content. Journal of Fermentation and Bioengineering 77 (5), 535–540.
Liu, W.T., Mino, T., Matsuo, T., Nakamura, K., 1996. Biological phosphorus removal processes–effect of pH on anaerobic substrate metabolism. Water Science and Technology 34 (1–2), 25–32.
López-Vázquez, C.M., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Van Loosdrecht, M.C., 2008. Factors affecting the microbial populations at full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plants in The Netherlands. Water Research 42 (10–11), 2349–2360.
Lu, H., Oehmen, A., Virdis, B., Keller, J., Yuan, Z., 2006. Obtaining highly enriched cultures of Candidatus *Accumulibacter* phosphates through alternating carbon sources. Water Research 40 (20), 3838–3848.
Martin, H.G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K., McHardy, A.C., Yeates, C., He, S., Salamov, A., Szeto, E., Dalin, E., Putnam, N., Shapiro, H.J., Pangilinan, J.L., Rigoutsos, I., Kyrpides, N.C., Blackall, L.L., McMahon, K.D., Hugenholtz, P., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nature Biotechnology 24 (10), 1263–1269.
Meyer, R., Saunders, A., Blackall, L., 2006. Putative glycogenaccumulating organisms belonging to Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology 152 (2), 419–429.
Mino, T., Tsuzuki, Y., Matsuo, T., 1987. Effect of Phosphorus Accumulation on Acetate Metabolism in the Biological Phosphate Removal Processes. Pergamon Press. 27–38.
Mino, T., Van Loosdrecht, M.M.C., Heijnen, J.J., 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Research 32 (11), 3193–3207.
Moosbrugger, R.E., Wentzel, M.C., Ekama, G.A., Marais, G.V.R., 1992. Simple Titration Procedures to Determine H2CO3 Alkalinity and Short-chain Fatty Acids in Aqueous Solutions Containing Known Concentrations of Ammonium, Phosphate and Sulphide Weak Acid/bases. University of Cape Town, Pretoria, Republic of South Africa. Water Research Commission, Report No. TT 57/92, Research Report W 74.
Metcalf and Eddy Inc., 2003. Wastewater Engineering: Treatment and Reuse, fourth ed. McGraw-Hill Inc., New York.
Oehmen, A., Yuan, Z., Blackall, L., Keller, J., 2005. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnology and Bioengineering 91 (2), 162–168.

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 (review). Water Research 41 (11), 2271–2300.

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

Pereira, H., Lemos, P.C., Reis, M.A.M., Crespo, J., Carrondo, M.J.T., Santos, H., 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo C-13-NMR labelling experiments. Water Research 30 (9), 2128–2138.

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., Van Loosdrecht, M.C.M., 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess and Biosystems Engineering 25 (6), 377–385.

Satoh, H., Mino, T., 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.

Schuler, A.J., Jenkins, D., 2003a. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part II: anaerobic adenosine triphosphate utilization and acetate uptake rates. Water Environmental Research 75 (6), 512–522.

Schuler, A.J., Jenkins, D., 2003b. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part III: anaerobic sources of reducing equivalents. Water Environmental Research 75 (6), 499–511.

Smolders, G., Vandermeij, J., Van Loosdrecht, M., Heijnen, J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process stoichiometry and pH influence. Biotechnology and Bioengineering 43 (6), 461–470.

Smolders, G.J.F., Klop, J.M., Van Loosdrecht, M.C.M., Heijnen, J.J., 1995. A metabolic model of the biological phosphorus removal process: I. Effect of the sludge retention time. Biotechnology and Bioengineering 48 (3), 222–233.

Wentzel, M., Lotter, L., Loewenthal, R., Marais, G., 1986. Metabolic behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal - a biochemical model. Water SA 12 (4), 209–224.

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

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

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

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

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