# New insights in the metabolic behaviour of PAO under negligible Poly-P reserves

**Brenda Acevedo[a], Mónica Murgui[b], Luis Borrás[b], Ramón Barat[a*]**

[a]Instituto de Ingeniería del Agua y Medio Ambiente. Universitat Politècnica de València. Camino de Vera s/n, 46022. Valencia. Spain (e-mail: breacju@posgrado.upv.es, rababa@dihma.upv.es)

[b]Departamento de Ingeniería Química, Universitat de València. Avda. de la Universidad, s/n. 46100 – Burjassot. Valencia. Spain (e-mail: monica.murgui@uv.es, luis-borras-falomir@uv.es)

*Corresponding author. Tel.: +34 963879618, Fax: +34 963877618, E-mail: rababa@dihma.upv.es

## ABSTRACT

In a previous study the authors confirmed the ability of PAOs to perform GAO metabolism in short-term experiments. However, what happens when PAOs are exposed to poly-P shortage for an extended period of time? The answer to this question was the aim of this work from a macroscopic and microscopic point of view. Therefore, the poly-P was removed from a PAO enriched SBR and maintained without poly-P during five solid retention time. The PAOs were found to quickly change their metabolism to a clear GAO performance and remained without GAO colonization for the entire experimental period, even though GAO was present (around 5%) at the beginning of the experiment. Unlike the results obtained in the short-term experiments, in this case PAO Type I performed the GAO metabolism at the end of the experimental period.



## KEYWORDS

Polyphosphate accumulating metabolism (PAM), Glycogen accumulating metabolism (GAM), Enhanced biological phosphorus removal (EBPR), PAO Type I, PAO Type II.

## 1. INTRODUCTION

Polyphosphate accumulating organisms (PAOs) are responsible for enhanced biological phosphorus removal (EBPR). In order to promote the growth of these organisms and consequently remove phosphorus (P), anaerobic conditions are first required, followed by aerobic or anoxic conditions. Compared with chemical phosphorus removal, the EBPR process can be a very good choice when it comes to high levels of phosphorus removal and represents an opportunity for P recovery in the Wastewater Treatment Plants (WWTP). However, under certain circumstances EBPR systems may deteriorate due to factors that are not completely understood (Oehmen et al., 2007). Cech and Hartman (1993) observed EBPR deterioration attributed to changes in the microbial population. These authors detected groups of Gram-negative bacilli and gram-positive cocci, usually grouped in tetrads, which accumulated glycogen but not polyphosphate (poly-P). This group is known as *glycogen accumulating organisms* (GAOs) (Erdal et al., 2003). It is well known that the difference between PAO and GAO is the phosphate release due to the degradation of intracellular poly-P, under anaerobic conditions and the subsequent up-take of phosphates (stored as poly-P) under aerobic/anoxic conditions. Anaerobically, both microorganisms accumulate polyhydroxyalkanoates (PHA), which is used to synthesize glycogen under aerobic/anoxic conditions.

Several studies have focused on the factors that promote the growth of PAOs over GAOs to improve the efficiency of EBPR systems. Some of these factors are: pH (Oehmen et al., 2005b; Zhang *et al*. 2007), temperature (Brdjanovic et al., 1998; Erdal et al., 2003), sludge retention time (Whang and Park, 2006), oxygen concentration (Griffiths et al., 2002; Carvalheira et al., 2014) and C/P ratio in the influent (Liu et al., 1996; Schuler and Jenkins, 2003a).

The successful identification of the organisms present in activated sludge systems requires the combination of molecular and microscopic techniques (such as phylogenetic studies of the 16S rDNA gene or measurements of ppk1 gene expression levels, fluorescence *in situ* hybridization (FISH) and staining of poly-P). However, the FISH technique alone can give us a good approximation of the presence and the amount of a particular organism within the biomass. The GAO population quantified by FISH can also be reflected in the chemical analysis measuring the ratio $P_{release}/HAc_{uptake}$ and other ratios such as $Gly_{degraded}/HAc_{uptake}$, $PHA_{synthesized}/HAc_{uptake}$, and $P_{uptake}/PHA_{degraded}$. When $P_{release}/HAc_{uptake}$ ratio is low it is correlated with a high GAO population and low PAO population. Incomplete removal of phosphorus is another indicator of low quantities of PAOs and it has been suggested that the relative activity between PAOs and GAOs can be estimated by analyzing the $P_{release}/HAc_{uptake}$ ratio (Saunders et al., 2003; Schuler and Jekins, 2003b).

Short-term studies have postulated that PAOs are able to behave metabolically like GAOs, showing low ratios of $P_{release}/HAc_{uptake}$ when intracellular poly-P content is reduced. This change was firstly studied by Liu *et al*. (1997), who found that the influent P-mg/C-mg ratio was a key factor influencing the competition between PAO and GAO. In their study, they showed that a reduction of the ratio to 2/100 caused the depletion of the polyphosphate content in PAO, leading to a replacement by GAO. Later, Schuler and Jenkins (2003a) defined the concepts of PAM and GAM to refer to the metabolisms performed by PAO and GAO respectively. They found that low P/C ratios favour GAM metabolism, but not necessarily the growth of GAO. Results related with the metabolic change from PAM to GAM were also found by other authors (Erdal et al., 2008; Zhou et al., 2008; Acevedo et al., 2012). In 2013 Tian *et al*. (2013) concluded that under P-limiting conditions, PAO type I were unable to perform GAM, but it could not be discarded that PAO type II could switch to a GAM as a survival strategy. Recently, Welles *et al*. (2015) found similar results, showing

75 that under P-limiting conditions, both, PAO type I and type II, are able to shift their
76 metabolism form PAM to GAM, but PAO type II are more favoured because of their higher
77 HAc uptake rate. Acevedo et al., (2014) incorporated this PAO metabolic versatility in
78 previously proposed metabolic models (Smolders et al., 1994a, b; Lopez-Vazquez et al.,
79 2009).

80 However, what happens when PAOs are exposed to poly-P shortage for a long time? The
81 answer to this question was the aim of this work from both macroscopic and microscopic
82 points of view. Furthermore, this knowledge will provide new insights in the metabolic
83 behaviour of PAOs and the possible effect over the biological phosphorus removal
84 performance in a WWTP, when poly-P is extracted from PAO for further phosphorus
85 recovery.

86

87 **2. MATERIALS AND METHODS**

88 A laboratory scale sequencing batch reactor (SBR) with a working volume of 7 l was set to
89 carry out the phosphorus removal process under anaerobic-aerobic conditions and four 6 h
90 cycles per day. Each cycle consisted of five phases: a 5-min filling period; 90 min anaerobic
91 phase; 180 min aerobic phase; 80 min settling phase and 5 min withdrawing period.
92 Hydraulic retention time was 12 h. Biomass was taken daily from the system to keep Sludge
93 Retention Time (SRT) around 8 days. The reactor was equipped with conductivity, ORP, pH,
94 temperature and dissolved oxygen electrodes. The dissolved oxygen (DO) concentration in
95 the aerobic phase was controlled between 1.5 and 2.5 $O_2$ mg/1. Temperature was maintained
96 at 20 ±1 °C. pH was not controlled; the initial pH for each cycle was kept around 7.5 and
97 ranged from 7.0 to 9 during the different phases of each cycle. The synthetic wastewater used
98 consisted of two separate solutions as follows: one solution contained mineral compounds,

99 including $K_2HPO_4$, and the other contained acetate and $NH_4Cl$ (see Barat et al., 2008 for a
100 detailed description). Thiourea was added to the synthetic media (20 mg/1) to inhibit
101 nitrification. Synthetic wastewater was used with a COD/P ratio of 15 COD-mg/ P-mg (150
102 COD-mg /1 and 10 P-mg /1).

103 **2.1. Experimental design**

104 The SBR was seeded with sludge from a wastewater treatment plant (WWTP) in Valencia
105 (Spain) that removes phosphorus biologically. Different parameters were monitored
106 throughout the experimental period in order to study the long term effect of poly-P shortage
107 over the biological process. The experimentation was divided in two periods. The Period 1
108 corresponds to the PAO enrichment period in order to obtain a reactor highly enriched with
109 PAO and Period 2 corresponds to the experimental period with low poly-P concentration. The
110 first characterization of the process (C1) took place after stabilizing and enriching the
111 biomass with PAOs at the end of Period 1. The poly-P concentration in the reactor was then
112 drastically reduced at the beginning of Period 2. This consisted of removing the phosphate-
113 enriched supernatant at the end of the anaerobic phase for 3 consecutive cycles. In each of the
114 three cycles the supernatant removed at the end of the anaerobic phase was replaced with
115 synthetic wastewater without phosphate (for further details of the P removal cycles see
116 Acevedo et al., 2012).

117 After the poly-P extraction cycles, the biological process was again characterized (C2). The
118 reactor was fed with synthetic wastewater with a low phosphate concentration (only enough
119 to supply P requirements as nutrient) for a long period of time (40 days, equivalent to 5 SRT),
120 at the end of which a third characterization (C3) was performed. Each characterization
121 consisted of an intensive monitoring of: HAc, phosphate, ammonium, nitrate, PHA and
122 glycogen during one operation cycle. PT, TSS and VSS were also measured at the end of the
123 aerobic phase in each characterization cycle. Between C2 and C3, HAc, phosphate,

ammonium, nitrate, PT, TSS, VSS, glycogen and PHA were regularly measured at the end of the aerobic and anaerobic phases.

## 2.2. Analytical methods and microbiological techniques

VFAs were measured by the method proposed by Moosbrugger *et al.* (1992) using a Metrhom 716 DMS tritino. Phosphorus, ammonia and nitrite were measured by 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*. (2005a). Glycogen determination was analyzed as described in Acevedo *et al*. (2012).

Microbiological analyses were carried out using FISH to identify the specific taxonomic group of bacteria found in the system. Cell hybridization was performed as described by Amann *et al.* (1990). The rRNA oligonucleotide probes used for FISH are those described in Table I of Acevedo *et al.* (2012). 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 numbered by capturing images with a Leica TCS SP confocal microscope (for PAO Types I and II signal over EUBmix probe), a Leica DM2500 epifluorescence microscope and a Leica DFC420c digital camera (for PAOmix, GB, DEFmix and DEF2- mix signals over EUBmix signal), with 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. The quantification error was calculated by

149 dividing the standard deviation by the square root of "n", where "n" was the number of fields
150 examined (Borrás L., 2008).

151

152 **3. RESULTS AND DISCUSSION**

153 The $\Delta Cond_{AN}/VSS$ ratio is a simple indicator of PAOs activity because the amount of
154 phosphorus released is closely related to the rise in conductivity during the anaerobic phase
155 (Acevedo, et al., 2012; Aguado, et al., 2006). Figure 1 shows the $\Delta Cond_{AN}/VSS$ ratio,
156 percentage of phosphorus removal, pH and VFA concentrations at the beginning and end of
157 the anaerobic phase of the experiment. The two periods studied can be distinguished
158 according to the parameters monitored: (Period 1) the PAO enrichment period in which the
159 $\Delta Cond_{AN}/VSS$ ratio increased with time until stabilization and (Period 2) the experimental
160 stage without poly-P.

161 The high $\Delta Cond_{AN}/VSS$ ratio values achieved at the end of Period 1 (around 0.3
162 mS/cm$^2$/VSS-g) indicate high phosphorus concentrations in the anaerobic phase. After poly-P
163 extraction (Period 2), $\Delta Cond_{AN}/VSS$ values dropped drastically to 0.05 mS/cm$^2$/VSS-g,
164 indicating low phosphorus release during the anaerobic phase.

165 As can be seen in Figure 1, the biological process achieved high P removal efficiencies
166 (>90%) during Period 2. Furthermore, the pH was maintained above 7.0 during the whole
167 experimental period in order to maintain pH conditions favouring PAO over GAO (Smolders
168 et al., 1994b; Liu et al., 1996; Bond et al., 1999; Filipe et al., 2001a, 2001b). However, there
169 were significant differences in the pH values at the end of the anaerobic phase between the
170 non limited (Period 1) and limited (Period 2) poly-P periods, which could affect to the
171 observed results regarding the population dynamics as will be discussed later. Before poly-P
172 extraction (Period 1) the pH at the end of the anaerobic stage dropped to 7 due to the release

of phosphate and its associated protons (around 3.42 P-mmol/l), inducing a significant pH decrease during the anaerobic stage. After poly-P extraction (Period 2) anaerobic phosphorus release was drastically reduced (concentrations < 0.4 P-mmol/l) with no effect on pH. However, pH rose during the anaerobic phase due to acetate consumption. For a detailed description of the pH trend through one operation cycle of a SBR operated for EBPR see Serralta *et al*. (2004).

Therefore, the pH variation during the anaerobic phase jointly the conductivity variation could be an indication of poly-P involvement in the reactor performance.

The amount of poly-P was calculated mathematically by Equation (1). $P_T$ was measured at the end of the aerobic stage, when $P_T$ is assumed to be the sum of poly-P and organic phosphorus. According to Metcalf and Eddy (2003) around 2% of the VSS is considered organic phosphorus.

$$PolyP = P_T - 2\%VSS \quad \text{(Eq. 1)}$$

In order to study the effect of poly-P shortage over PAO metabolism, different anaerobic ($P_{release}/HAc_{uptake}$, $Gly_{degraded}/HAc_{uptake}$, $PHV_{synthesized}/HAc_{uptake}$, $PHB_{synthesized}/HAc_{uptake}$ and $PHA_{synthesized}/HAc_{uptake}$) and aerobic ($Gly_{synthesized}/PHB_{degraded}$ and $P_{uptake}/PHB_{degraded}$) ratios were calculated throughout the experimental period.

The first characterization (C1) was performed in the pseudo-steady state of the phosphorus removal process. In Figure 2 (A) typical PAO phenotype profiles can be observed during the anaerobic (acetic acid uptake, phosphate release, glycogen degradation and PHA production) and aerobic (PHA degradation, glycogen synthesis and phosphate uptake) phases. Table I shows the main stoichiometric parameters of this study and the values proposed in the literature when using acetate as carbon source. A $P_{release}/HAc_{uptake}$ ratio of 0.67 P-mmol/C-

198 mmol and a poly-P content of 0.44 P-mg/VSS-mg were obtained in C1. As can be seen in
199 Table I, the stoichiometric ratios obtained in C1 are similar to those obtained by other authors
200 using PAO enriched cultures. In this case the acetate uptake rate was 0.095 C-mg/VSS-mg h

201 The second characterization (C2) was performed after extracting the poly-P content of the
202 PAOs. Poly-P content was reduced from 0.44 to 0.03 P-mg/VSS-mg. The cycle profiles
203 obtained are shown in Figure 2 (B). This poly-P extraction induced a drop in the
204 $P_{release}/HAc_{uptake}$ ratio to 0.09 P-mmol/C-mmol in the anaerobic phase. These stoichiometric
205 ratios are similar to those found in GAO enriched systems (Table I). In the second
206 characterization it was observed that the rate of acetic acid uptake dropped from 0.095 to
207 0.024 C- mg/VSS-mg h in relation to C1.

208 After reducing the poly-P content, phosphorus release dropped to almost zero. Figure 3
209 shows the evolution of poly-P content, total volatile and suspended solids and phosphorus in
210 the influent. Initially the acetic acid concentration stayed around 150 COD-mg/l, however,
211 after poly-P removal some acetate began to remain at the end of the anaerobic phase. The
212 influent acetic acid concentration was therefore reduced to 96 COD-mg/l to ensure that it
213 would be totally consumed and to avoid competition for VFA with other heterotrophic
214 bacteria. As can be seen in Figure 3, influent phosphate was drastically reduced after the
215 poly-P extraction cycles and was maintained around 1 P-mg/l to ensure P nutrient
216 requirements without significant poly-P accumulation. During this period of poly-P shortage
217 the poly-P/VSS ratio stayed between 0.01 and 0.04 P-mg/VSS-mg with a $P_{release}/HAc_{uptake}$
218 ratio of nearly 0.1 P-mmol/C-mmol.

219 After 5 SRT (40 days) with low poly-P content, the third characterization (C3) was
220 performed (Figure 2 C). The $P_{release}/HAc_{uptake}$ ratio obtained was 0.12 P-mmol/C-mmol with a
221 poly-P concentration of 0.04 P-mg/VSS-mg. The rate of acetic acid uptake remained low, at
222 0.03 C-mg/VSSmg h.

As previously mentioned, the poly-P content of the biomass and the $P_{release}/HAc_{uptake}$ ratio in the supernatant decreased significantly from C1 to C2. The following changes in the anaerobic ratios were also observed (see Table I): $Gly_{degraded}/HAc_{uptake}$ increased from 0.34 to 0.88 C-mmol/C-mmol, $PHB_{synthesized}/HAc_{uptake}$ and $PHV_{synthesized}/HAc_{uptake}$ changed from 1.03 to 1.31 and 0.17 to 0.44 C-mmol/C-mmol respectively. It was also observed that when PAOs were enriched in poly-P, the PHB percentage was between 85-90% and PHV was 10-15%, while at low poly-P concentrations the PHB percentage was reduced to around 66-70%, and PHV increased to 30-34%. The aerobic ratios also showed changes: the $P_{uptake}/PHB_{degraded}$ ratio decreased from 0.6 P-mmol/C-mmol in C1 to 0.1 P-mmol/C-mmol in C2 and aerobically synthesized glycogen also increased ($Gly_{synthesized}/PHB_{degraded}$ 0.41 C-mmol/C-mmol in C1 and 1.18 C-mmol/C-mmol in C2).

Figure 4 shows the stoichiometric ratios obtained in the present study and those obtained in short-term experiments (Acevedo et al., 2012). It can be seen that the stoichiometric ratios obtained in this work follow the same trend as in the short-term experiments. These results showed a clear correlation of the stoichiometric ratios with poly-P concentration. The ratios commonly registered for PAO and GAO cultures are shown by red and green lines, respectively, in Figure 4. With poly-P concentrations lower than 0.1 P-mg/VSS-mg the ratios obtained tend towards those reported for GAO culture (Liu et al., 1994, Zeng et al., 2002), while at higher poly-P values the ratios are similar to those obtained for PAO culture (Smolders et al., 1994a,b and Zhou et al., 2008). These results suggest that poly-P/VSS values lower than 0.1 P-mg/VSS-mg indicate that there is not enough poly-P for ATP production, enhancing the glycolytic pathway to supply the energy deficit.

The FISH technique made it possible to identify the PAO cluster as *Candidatus Accumulibacter phosphatis* bacteria, which are related to *Rhodocyclus*-like bacteria (Crocetti et al., 2000) and their clades PAO Type I and PAO Type II (Flowers *et al*. 2009). Figure 5

shows the results of the long-term microbial population monitoring in the SBR. At steady state (C1) the biomass in the reactor was composed of 82 ± 3 % of PAOs and less than 5 % of GAOs. During the poly-P shortage phase, the P/C ratio in the influent was around 0.01 P-mmol/C-mmol. Despite this low P/C ratio, the population of GAOs did not increase, but remained below 5%, and the PAO population remained the same throughout the experiments (between 70-83%), both with and without poly-P. These results suggest that the metabolic versatility of PAO to use glycogen as the main energy source without poly-P, jointly with the high pH maintained during the Period 2 (between 7.5 and 8.5, see Figure 1) are the main factors affecting the domination of PAO over GAO in the reactor.

Just after the extraction of poly-P (C2) the PAO Type I population remained at 72±5% and PAO Type II at 25±5%. During the reduction of poly-P content (from C1 to C2) a change in microbial populations occurred: Type I PAOs decreased by 12% in relation to Type II PAOs (Figure 5). However, in the present study, after maintaining a low P/C ratio (0.01 P-mmol/C-mmol) for a long period (132 to 169 day), the percentage of Type II PAO dropped below 5% and Type I PAO remained as the predominant group for this long-term experiment with low poly-P content. At the end of this period PAO Type I represented 76% of all bacteria detected with probe EUBmix, and 96% of PAO detected with PAOmix probe.

Both results, the short-term study (Acevedo et al., 2012) and the long-term study (the present study), are in accordance with Welles *et al*. (2015). These authors observed that when the poly-P content decreased, both '*Candidatus Accumulibacter phosphatis*' Type I and II could shift their metabolism from a PAO metabolism to a GAO metabolism and have the ability to solely rely on glycogen as energy source for HAc uptake.

However, the dominance of PAO type I observed in the present study is apparently in contradiction with the results obtained by Welles *et al*. (2015). These authors observed that under poly-P depleted conditions, the kinetic rates of PAO II were four times higher than

273 those of PAO I, suggesting that PAO II had a strong competitive advantage over PAO I.
274 Comparing the results obtained in the present study and the ones obtained by Welles *et al*.
275 (2015), there are some differences in both studies that could explain these results. The first
276 one is related with the operation time of the system under the poly-P depleted conditions
277 affecting to the acclimation period of bacteria to the new conditions. Welles *et al*. (2015)
278 carried out experiments at short-term in contrast with the long-term of the present study
279 where the reactor was operated during 5 SRT. Therefore, in this work the bacteria were
280 acclimated to generate energy and reducing power without poly-P, meanwhile in Welles *et al*.
281 (2015) the bacteria were not acclimated to work without poly-P. The second difference
282 consists on the pH during the operation cycle, which it is well know its affection over the
283 kinetics of the processes (Zhang et al., 2007; Weissbrodt et al., 2013). The experiments in
284 Welles *et al*. (2015) were conducted at pH 7.0, meanwhile in the present study the pH was
285 maintained between 7.5 and 8.5 during the anaerobic and aerobic phases (see Figure 1).
286 Therefore, the pH and the acclimation period could be factors that affect to the PAO type
287 performing the GAO metabolism and inducing the prevalence of PAO Type I in this study.
288 However, further research is needed to confirm these hypotheses.

289 FISH also revealed that after extracting poly-p (C2), the sum of PAO Types I and II was
290 significantly lower than the PAOs detected with the PAOmix probe, suggesting the
291 development of another type of PAO different of Types I and II. Therefore, it would be
292 convenient to apply other molecular techniques, (e.g. measurements of ppk1 gene expression
293 levels as a marker gene for DNA amplicon sequencing and phylogenetic analysis (He et al.,
294 2011; Peterson et al., 2008)) in order to study the whole microbial population and its changes.
295 These techniques could also reveal the identity and the role of the rest of bacteria not targeted
296 with the PAOmix probe. Tu and Schuller (2013) found, using molecular techniques such as
297 454 pyrosequencing combined with FISH, that *Dechloromonas* and *Tetrasphaera* spp. could

298 be also PAOs, as previously suggested by Goel et al., (2005) and Kong et al., (2005).
299 Although in our study the PAO (detected with PAOmix probe) ranged from 73% to 82% of
300 all bacteria throughout the experiment, it would be interesting to see if *Dechloromonas* and
301 *Tetrasphaera* spp (or any other potential PAO) were present and its involvement in the
302 metabolic change.

303 Differences were also found between the values of the $PHV_{synthesized}/HAc_{uptake}$ and
304 $PHB_{synthesized}/ HAc_{uptake}$ ratios obtained in the present study and those obtained in the short-
305 term study (see Figure 4, C and D). These differences could be due to variations in the PAO
306 population (Types I and II) in both studies. Another important factor observed in the long-
307 term experiments was the decrease in the acetic acid uptake rate when the poly-P content was
308 reduced. This behaviour was also reported by Zhou *et al*. (2008) and Welles *et al*. (2015).
309 However, despite the reduced acetate uptake rate, the GAO population in the reactor did not
310 grow significantly, as expected.

311 To sum up, the results obtained in this study are in agreement with those obtained by other
312 authors (Barat et al., 2008; Zhou et al., 2008; Acevedo et al., 2012; Welles *et al.* 2015) in
313 short-term experiments, and confirm the ability of PAOs to behave like GAOs when poly-P is
314 not available for energy production, even over long periods of time. Regarding the population
315 dynamics, the short term experiments (Acevedo et al., 2012 and Welles et al., 2015)
316 confirmed the ability of PAO Type II to quickly change to GAO metabolism. However,
317 although apparently PAO type I perform a slow transition from typical poly-P metabolism,
318 once established the metabolic change, the Type I dominate the PAO culture at long term,
319 comprising 96% of PAO detected with PAOmix probe. Therefore, PAO Type I are supposed
320 to be the responsible of the metabolic changes observed, probably due to the acclimation
321 period and the high pH maintained in the reactor. Nevertheless, further research is needed to
322 confirm this hypothesis.

Another important aspect to be considered in future researches is about what happens with the PAO-GAO competition in a system with an initial population distribution of PAO-GAO around 50-50% when poly-P is reduced, instead of the highly PAO enriched reactor used in this study.

## 4. CONCLUSIONS

Under P-limiting conditions, *Accumulibacter* has the ability of maintaining the metabolic behaviour of GAO for long-term (5 SRT) in an EPBR process. The anaerobic and aerobic stoichiometric ratios show the same metabolic change (from PAM to GAM). The results suggest that when poly-P/VSS is bellow 0.1 P-mg/VSS-mg, poly-P content is not high enough to produce ATP, and therefore, the glycolytic pathway is enhanced in order to supply the energy deficit. FISH proved that the metabolic shift was not due to a population change from PAO to GAO. Once the metabolic change was established, PAO Type-I dominate the long-term PAO culture.

## 5. ACKNOWLEDGEMENTS

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 the Consejo Nacional de Ciencia y Tecnología de México (CONACYT) No. 207966.

## 6. REFERENCES

345 Acevedo, B.; Oehmen, A.; Carvalho, G.; Seco, A.; Borrás, L., Barat, R., 2012. Metabolic
346 shift of polyphosphate-accumulating organisms with different levels of polyphosphate
347 storage. Water Research 46 (6), 1889-1900.

348 Acevedo, B.; Borrás, L.; Oehmen, A.; Barat, R., 2014. Modelling the metabolic shift of
349 polyphosphate-accumulating organisms. Water Research, 65 235-244

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

353 Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990.
354 Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for
355 analyzing mixed microbial populations. Applied and Environmental Microbiology 56 (6),
356 1919 -1925.

357 Barat, R., Montoya, T., Borras, L., Ferrer, J., Seco, A., 2008. Interactions between calcium
358 precipitation and the polyphosphate-accumulating bacteria metabolism. Water Research 42
359 (13), 3415-3424.

360 Bond, P.L., Erhart, R., Wagner, M., Keller, J., Blackall, L.L., 1999. Identification of some of
361 the major groups of bacteria in efficient and non efficient biological phosphorus removal
362 activated sludge systems. Applied Environmental Microbiology 65 (9), 4077-4084.

363 Borrás, L., 2008. Técnicas microbiológicas aplicadas a la identificación y cuantificación de
364 microorganismos presentes en sistemas EBPR (Microbial techniques applied to the
365 identification and quantification of microorganisms present in EBPR system). Ph.D. thesis,
366 Polythecnic University of Valencia.

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

370 Carvalheira, M., Oehmen, A., Carvalho, G., Eusébio, M., Reis, M.A.M., 2014. The impact of
371 aeration on the competition between polyphosphate accumulating organisms and glycogen
372 accumulating organisms. Water Research, 66, 296-307.

373 Cech, J. and Hartman, P., 1993. Competition between polyphosphate and polysacharide
374 accumulating bacteria in enhanced biological phosphate removal systems. Water Research 27
375 (7), 1219-1225.

376 Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L.,
377 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-
378 directed probes for their detection and quantitation. Applied and Environmental
379 Microbiology 66 (3), 1175-1182.

380 Erdal U. G., Erdal Z. K., Randall C. W. 2003. The competition between PAOs (phosphorus
381 accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced
382 biological phosphorus removal) systems at different temperatures and the effects on systems
383 performance. Water Science and Technology 47 (11), 1-8.

384 Erdal, U., Erdal, Z., Daigger, G., Randall, C., 2008. Is it PAO-GAO competition or metabolic
385 shift in EBPR system? Evidence from an experimental study. Water Science and Technology
386 58 (6), 1329-1334.

387 Filipe, C., Daigger, G., Grady, C., 2001a. Effects of pH on the rates of aerobic metabolism of
388 phosphate-accumulating and glycogen-accumulating organisms. Water Environmental
389 Research 73 (2), 213-222.

390 Filipe, C., Daigger, G., Grady, C., 2001b. pH as a key factor in the competition between
391 glycogen-accumulating organisms and phosphorus-accumulating organisms. Water
392 Environmental Research 73 (2), 223-232.

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

396 Griffiths, P., Stratton, H., Seviour, R., 2002. Environmental factors contributing to the G
397 bacteria population in full-scale EBP plants. Water Science and Technology, 46 (4-5), 185-
398 192.

399 Goel, R.K., Sanhueza, P., and Noguera, D.R. 2005. Evidence of Dechloromonas sp.
400 participating in enhanced biological phosphorus removal (EBPR) in a bench-scale aerated-
401 anoxic reactor. WEFTEC Conf Proc 8: 3864–3871.

402 He, S., & McMahon, K. D. 2011. "Candidatus Accumulibacter" gene expression in response
403 to dynamic EBPR conditions. The ISME Journal, 5(2), 329–340.

404 Kong Y., Nielsen J.L. and Nielsen P.H., 2005. Identity and Ecophysiology of Uncultured
405 Actinobacterial Polyphosphate-Accumulating Organisms in Full-Scale Enhanced Biological
406 Phosphorus Removal Plants. Appl. Environ. Microbiol. vol. 71 (7), 4076-4085.

407 Liu, W. T., Mino, T., Nakamura, K., Matsuo, T. 1994. Role of glycogen in acetate uptake and
408 polyhydroxyalkanoate synthesis in anaerobic-aerobic activated sludge with a minimized
409 polyphosphate content. Journal of fermentation and bioengineering, 77 (5), 535-540.

410 Liu, W.T., Mino, T., Matsuo, T., Nakamura, K., 1996. Biological phosphorus removal
411 processes effect of pH on anaerobic substrate metabolism. Water Science and Technology 34
412 (1-2), 25-32.

413 Liu, W.-T.; Nakamura, K.; Matsuo, T. & Mino, T., 1997. Internal energy-based competition
414 between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus
415 removal reactors-Effect of P/C feeding ratio. Water Research 31 (6) 1430 – 1438.

416 Liu, Y., Chen, Y., Zhou, Q., 2007. Effect of initial pH control on enhanced biological
417 phosphorus removal from wastewater containing acetic and propionic acids. Chemosphere
418 66, 123–129.

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

422 Metcalf and Eddy Inc., 2003. Wastewater Engineering: Treatment and Reuse, fourth ed.
423 McGraw-Hill Inc., New York.

424 Moosbrugger, R.E., Wentzel, M.C., Ekama, G.A., Marais, G.V.R., 1992. Simple Titration
425 Procedures to Determine $H_2CO_3$ Alkalinity and Short-chain Fatty Acids in Aqueous
426 Solutions Containing Known Concentrations of Ammonium, Phosphate and Sulphide Weak
427 Acid/bases. University of Cape Town, Pretoria, Republic of South Africa. Water Research
428 Commission, Report No. TT 57/92, Research Report W 74.

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

432 Oehmen, A., Vives, M.T., Lu, H., Yuan, Z., Keller, J., 2005b. The effect of pH on the
433 competition between polyphosphate accumulating organisms and glycogenaccumulating
434 organisms. Water Res. 39, 3727–3737.

435 Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L., Reis, M. A. M.,
436 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale
437 (review). Water Research 41 (11), 2271-2300.

438 Peterson SB, Warnecke F, Madejska J, McMahon KD, Hugenholtz P. 2008. Environmental
439 distribution and population biology of Candidatus Accumulibacter, a primary agent of
440 biological phosphorus removal. Environ Microbiol. 10, 2692–2703.

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

445 Schuler, A.J., Jenkins, D., 2003(a). Enhanced biological phosphorus removal from
446 wastewater by biomass with different phosphorus contents, part I: Experimental results and
447 comparison with metabolic models. Water Environmental Research 75 (6), 485-498.

448 Schuler, A.J., Jenkins, D., 2003(b). Enhanced biological phosphorus removal from
449 wastewater by biomass with different phosphorus contents, part II: anaerobic adenosine
450 triphosphate utilization and acetate uptake rates. Water Environmental Research 75 (6), 512-
451 522.

452 Serralta, J., Borras, L., Blanco, C., Barat, R., Seco, A., 2004. Monitoring pH and electric
453 conductivity in an EBPR sequencing batch reactor. Water Science and Technology. 50 (10),
454 145–152.

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

458 Smolders, G. J. F., Vandermeij, J., van Loosdrecht, M. C. M., Heijnen, J. J., 1994b. Model of
459 the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and
460 pH influence. Biotechnology and Bioengineering, 43, (6) 461-470

461 Tian, W.-D., Lopez-Vazquez, C., Li, W.-G., Brdjanovic, D., Van Loosdrecht, M., 2013.
462 Occurrence of PAOI in a low temperature EBPR system. Chemosphere, 92 (10) , 1314-1320.

463 Tu, Y. and Schuler, A.J. 2013. Low Acetate Concentrations Favor Polyphosphate-
464 Accumulating Organisms over Glycogen-Accumulating Organisms in Enhanced Biological
465 Phosphorus Removal from Wastewater. Environmental Science Technology 47 (8),
466 3816−3824

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

470 Welles, L., Tian, W., Saad, S., Abbas, B., Lopez-Vazquez, C., Hooijmans, C., van
471 Loosdrecht, M. Brdjanovic, D., 2015. *Accumulibacter* clades Type I and II performing
472 kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate
473 uptake. Water Research 83, 354-366

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

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

480 Zeng, R. J.; van Loosdrecht, M. C. M.; Yuan, Z. & Keller, J., 2002. Metabolic Model for
481 Glycogen-Accumulating Organisms in Anaerobic/Aerobic Activated Sludge Systems.
482 Biotechnology and Bioengineering 81, (1) 92-105.

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

486

Figure 1. Profile of the $\Delta Cond_{AN}/VSS$ ratio, pH at the beginning and end of the anaerobic phase, and VFA concentration at the beginning of the anaerobic stage.

Figure. 2. Evolution of the main sludge components in an SBR cycle. (A) C1: in pseudo-steady state. (B) C2: after poly-P removal. (C) C3: after 40 days at low levels of poly-P.

Figure 3. Concentration of poly-P, TSS and VSS at the beginning and end of the anaerobic phase.

Figure 4. Anaerobic and aerobic ratios obtained in this long-term study compared to those reported in short-term experiments (Acevedo et al., 2012). Anaerobic ratios: A) $P_{release}/HAc_{uptake}$. B) $Gly_{degraded}/HAc_{uptake}$. C) $PHB_{synthesized}/HAc_{uptake}$. D) $PHV_{synthesized}/HAc_{uptake}$. E) $PHA_{synthesized}/HAc_{uptake}$. Aerobic ratios: F) $Gly_{synthesized}/PHB_{degraded}$ y G) $P_{uptake}/PHB_{degraded}$. Red and green lines indicate the typical values reported in the literature for PAO and GAO cultures.

Figure 5. Microbial monitoring in the SBR: PAO Type I, PAO Type II, PAOmix and GAOmix over EUBmix.

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

| | | | ANAEROBIC PARAMETERS | | | | | AEROBIC PARAMETERS | |
|---|---|---|---|---|---|---|---|---|---|
| **Description** | **Time** | **Poly-P/VSS mg/mg** | **$P_{rel}/HAc_{upt}$ P-mmol/C-mmol** | **$GLY_{degrad}/HAc_{upt}$ C-mmol/C-mmol** | **$PHB_{synt}/HAc_{upt}$ C-mmol/C-mmol** | **$PHV_{synt}/HAc_{upt}$ C-mmol/C-mmol** | **$PHA_{synt}/HAc_{upt}$ C-mmol/C-mmol** | **$GLY_{synt}/PHB_{degrad}$ C-mmol/C-mmol** | **$P_{upt}/PHB_{degrad}$ P-mmol/C-mmol** |
| **Acevedo et al., 2012** | **2nd experiment** | **0.35** | **0.73** | **0.35** | **1.3** | **0.06** | **1.36** | **0.41** | **0.64** |
| **Acevedo et al., 2012** | **4th experiment** | **0.09** | **0.5** | **0.66** | **1.53** | **0.08** | **1.61** | **0.58** | **0.39** |
| **Acevedo et al., 2012** | **5th experiment** | **0.01** | **0.08** | **1.08** | **1.74** | **0.28** | **2.02** | **0.7** | **0.11** |
| **Acevedo et al., 2012** | **6th experiment** | **0.25** | **0.67** | **0.35** | **1.2** | **0.10** | **1.31** | **0.48** | **0.76** |
| Smolders et al., 1994a, Smolders et al., 1994b | Experimental (pH=7.4) PAOs | _ | 0.6 | 0.5 | 1.32 | - | - | 0.45 | 0.34 |
| Zhou et al., 2008 | Experimental study PAOs | - | 0.58 | 0.45 | 1.15 | 0.07 | 1.22 | - | - |
| Liu et al., 1994 | Experimental study GAOs | - | 0.01 | 1.2 | 1.1 | 0.41 | 1.51 | - | - |
| Zeng et al., 2003 | Experimental study GAOs | - | 0 | 1.2 | 1.39 | 0.52 | 1.91 | 1.04 | 0 |
| C1. Steady state high content poly-P | 116 d | 0.44 | 0.67 | 0.34 | 1.03 | 0.17 | 1.2 | 0.41 | 0.6 |
| Cycles of removing poly-P | 118 d | 0.2 | 0.61 | 0.55 | 1.05 | 0.14 | 1.19 | - | NA |
| | 118 d | 0.08 | 0.50 | 0.61 | - | - | - | 0.58 | NA |
| | 118 d | 0.05 | 0.19 | - | - | - | - | - | NA |
| C2. Start low content of poly-P | 119 d | 0.03 | 0.09 | 0.88 | 1.31 | 0.44 | 1.75 | 1.18 | 0.1 |
| Low content of poly-P | 124 d | 0 | 0.02 | 1.87 | 1.72 | 0.77 | 2.50 | 1.87 | 0.01 |
| | 127 d | 0 | 0.01 | 1.51 | 1.39 | 0.77 | 2.17 | - | 0 |
| | 149 d | 0.01 | 0.09 | 1.48 | 1.42 | 0.72 | 2.14 | 1.08 | 0.07 |
| | 151 d | 0.01 | 0.05 | 1.19 | 1.31 | 0.63 | 1.94 | 0.98 | 0.04 |
| | 154 d | 0.01 | 0.08 | 1.1 | 1.3 | 0.56 | 1.86 | - | - |
| C3. Steady state low content poly-P | 159 d | 0.04 | 0.12 | 0.71 | 0.98 | 0.43 | 1.41 | 0.85 | 0.13 |

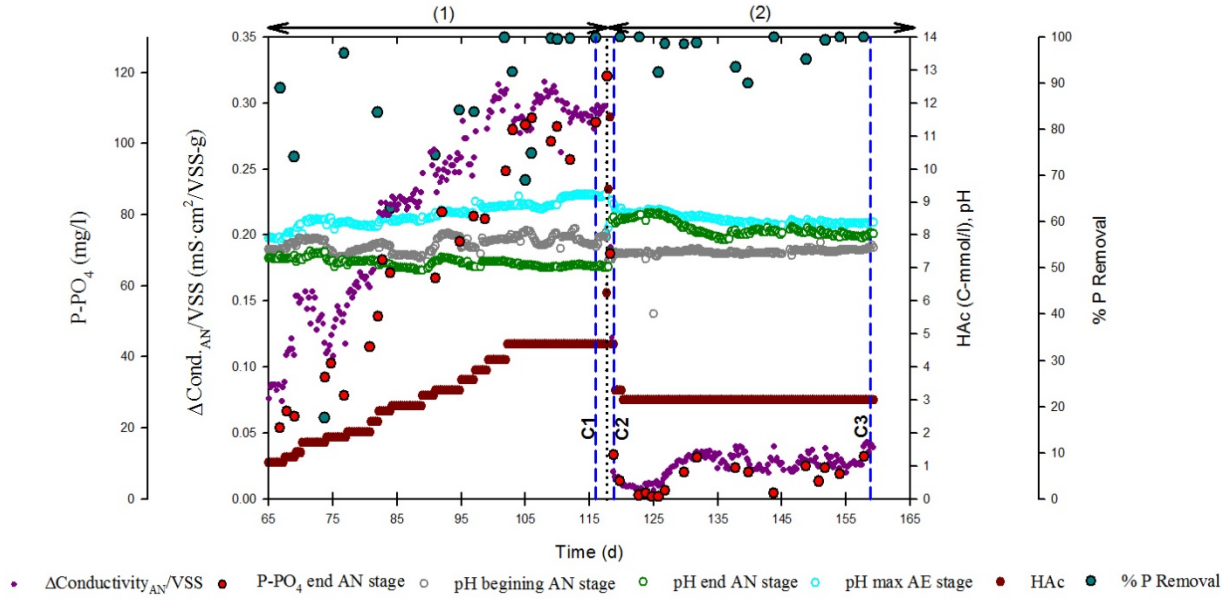


Figure 1. Profile of the $\Delta Cond_{AN}/VSS$ ratio, phosphates end of the anaerobic stage, pH at the beginning and end of the anaerobic stage, pH max aerobic and VFA concentration at the beginning of the anaerobic stage.

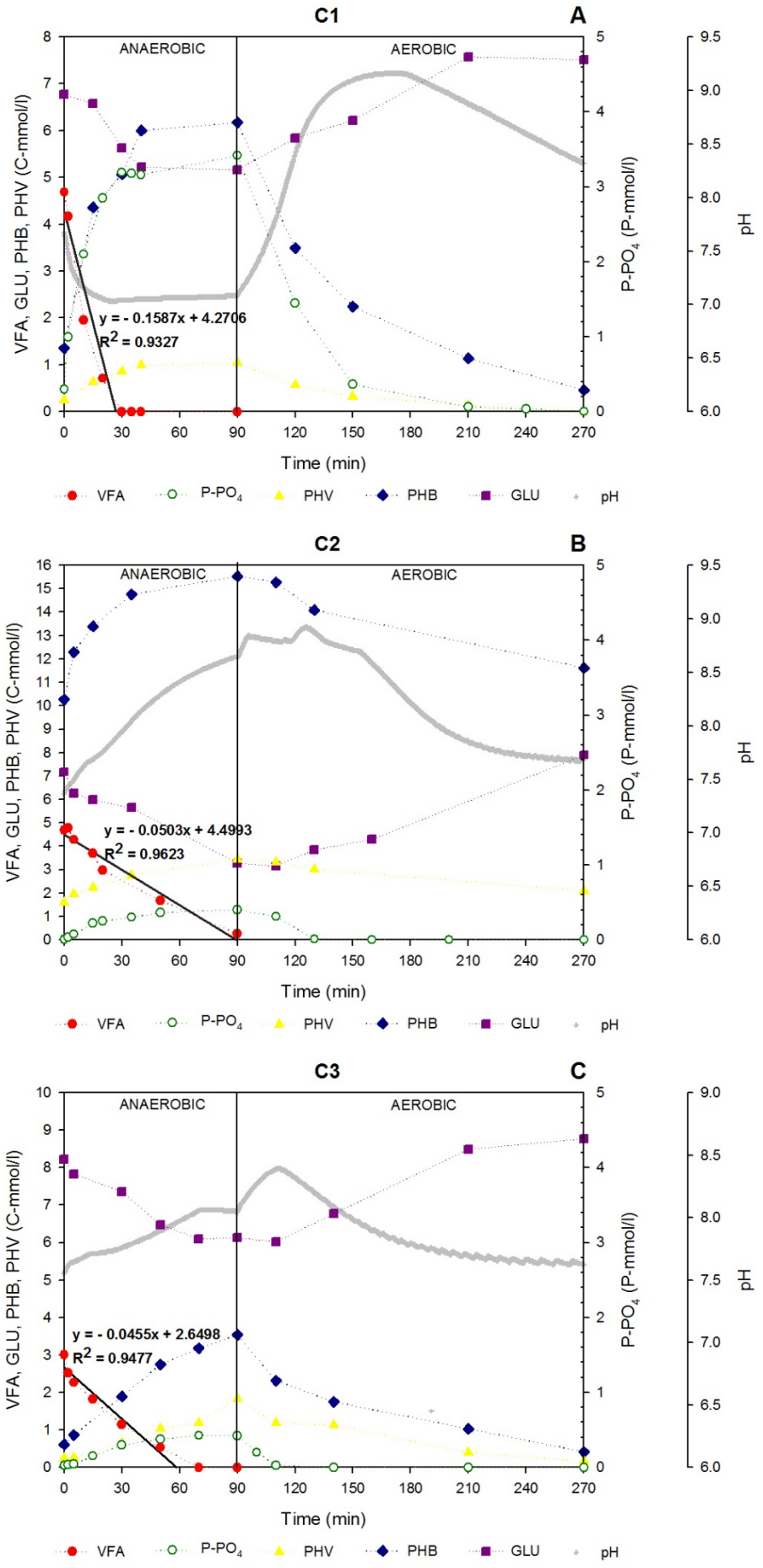


Figure. 2. Evolution of the main sludge components in an SBR cycle. (A) C1: in pseudo-steady state. (B) C2: after poly-P removal. (C) C3: after 40 days at low levels of poly-P.

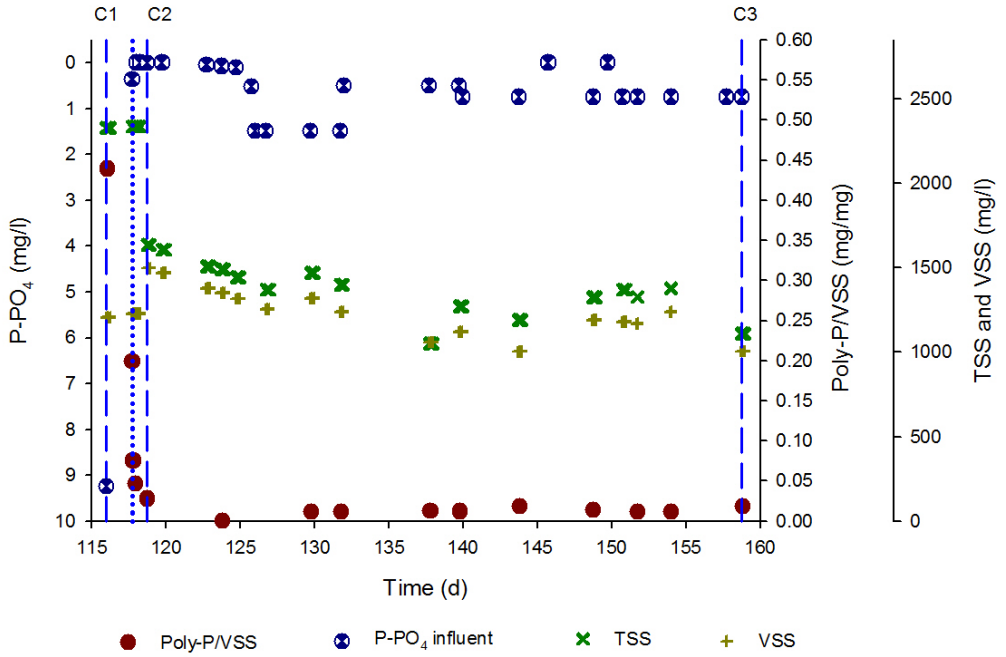


Figure 3. Concentration of poly-P, TSS and VSS at the beginning and end of the anaerobic stage.

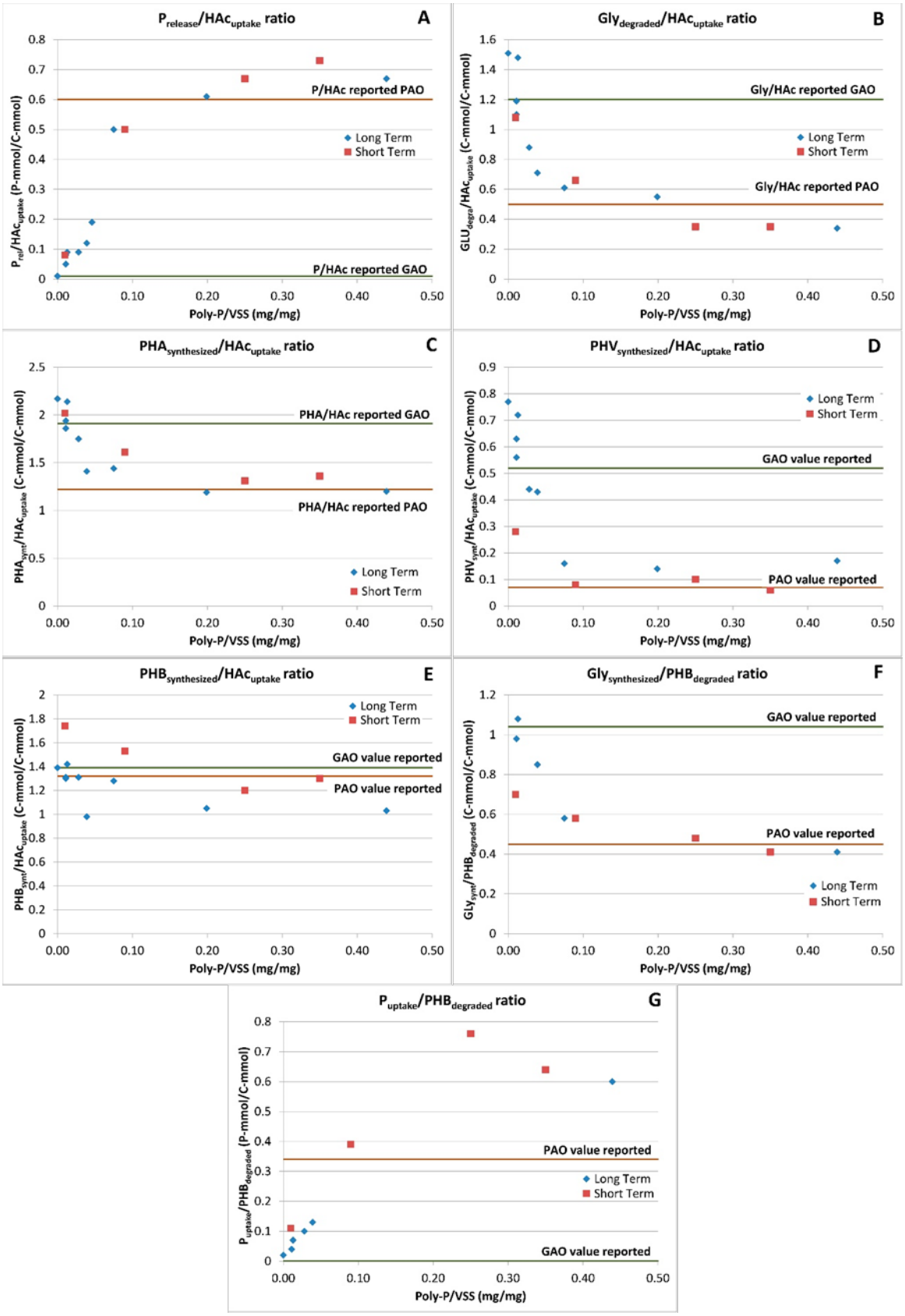


Figure 4. Anaerobic and aerobic ratios obtained in this long-term study compared to those reported in short-term experiments (Acevedo *et al.*, 2012). Anaerobic ratios: A) $P_{release}/HAc_{uptake}$. B) $Gly_{degraded}/HAc_{uptake}$. C) $PHB_{synthesized}/HAc_{uptake}$. D) $PHV_{synthesized}/HAc_{uptake}$. E) $PHA_{synthesized}/HAc_{uptake}$. Aerobic ratios: F) $Gly_{synthesized}/PHB_{degraded}$ y G) $P_{uptake}/PHB_{degraded}$. Red and green lines indicate the typical values reported in the literature for PAO and GAO cultures.

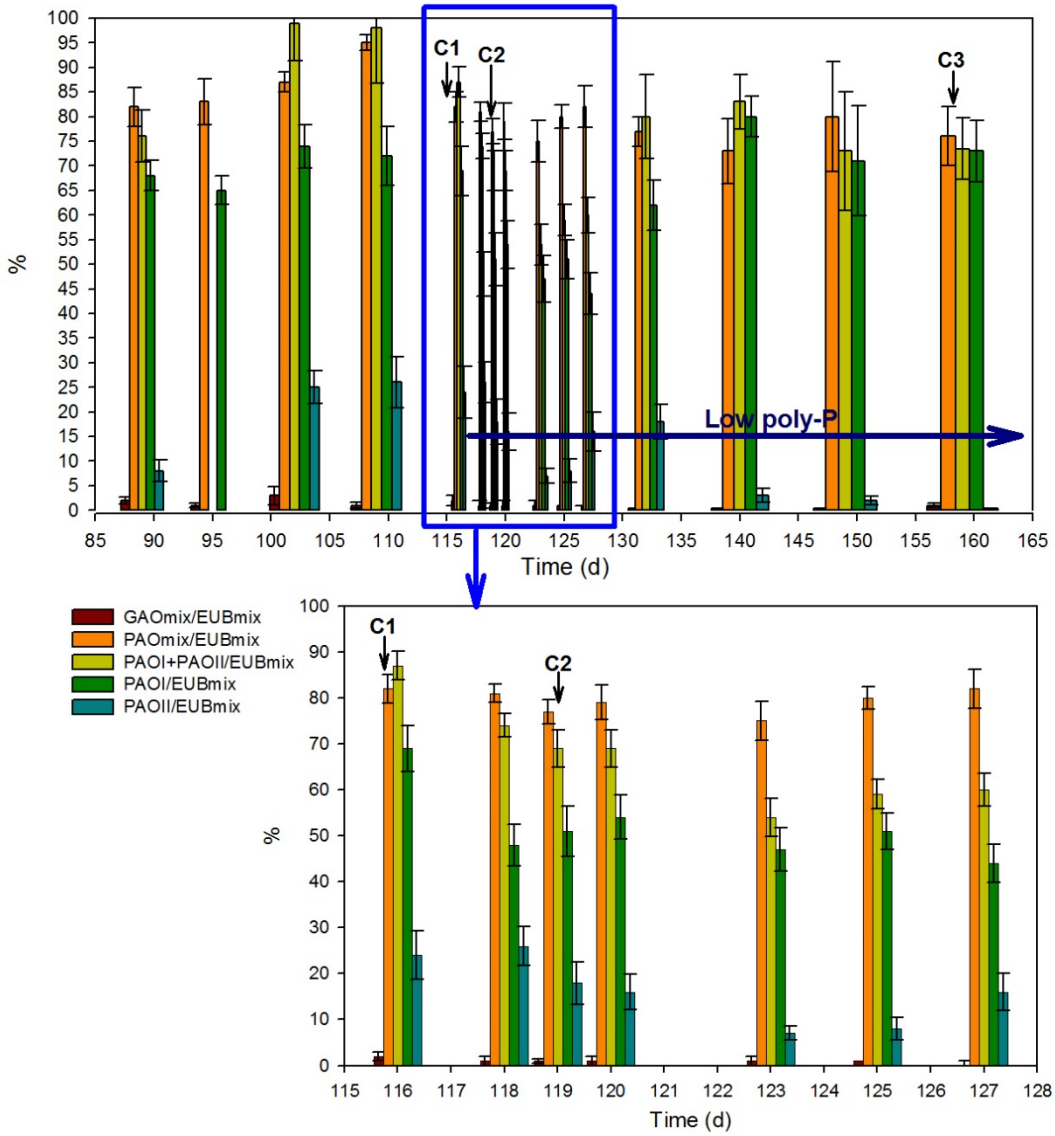


Figure 5. Microbial monitoring in the SBR: PAO Type I, PAO Type II, PAOmix and GAOmix over EUBmix.