THE SELECTIVE ROLE OF NITRITE IN THE PAO/GAO COMPETITION

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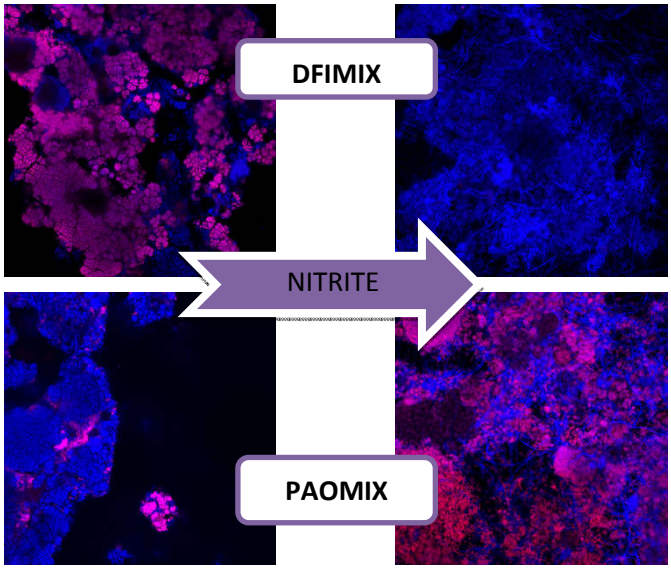
HIGHLIGHTS

- Combining EBPR-nitrite pathway with propionate as carbon source selects PAO vs GAO
- *Defluviicocus* GAO are washed out using nitrite as sole electron acceptor
- Nitrite is not a selection factor between PAOI and PAOII

GRAPHICAL ABSTRACT



ABSTRACT

Proliferation of Glycogen Accumulating Organisms (GAO) accounts as one of the major bottlenecks in biological phosphorus removal systems. GAO outcompeting Polyphosphate Accumulating Organisms (PAO) results in lower P-removal. Thus, finding optimal conditions that favour PAO in front of GAO is a current focus of research. This work shows how nitrite can provide a novel strategy for PAO enrichment. A propionate-fed GAO-enriched biomass (70 % *Defluviicoccus* I, 18% *Defluviicoccus* II and 10 % PAO) was subjected during 50 d to anaerobic-anoxic conditions with nitrite as electron acceptor. These operational conditions led to a PAO-enriched sludge (85 %) where GAO were washed out of the system (< 10%), demonstrating the validity of the new approach for PAO enrichment. In addition, the presented suppression of *Defluviicocus* GAO with nitrite represents an add-on benefit to the nitrite-based systems since the proliferation of non-desirable GAO can be easily ruled out and added to the other benefits (i.e. lower aeration and COD requirements).

KEYWORDS

Enhanced biological phosphorus removal (EBPR), nitrite, selection, polyphosphate accumulating organisms (PAO), glycogen accumulating organisms (GAO)

1. INTRODUCTION

Nowadays, enhanced biological phosphorus removal (EBPR) has become a widely accepted wastewater treatment technology because of its high sustainability and efficiency. In addition, EBPR is expected to be a viable source of phosphate in the future rather than a technology for biological P removal (Elser and Bennett, 2011). In the EBPR process, biomass is subjected to alternating anaerobic and aerobic/anoxic conditions so that polyphosphate accumulating organisms (PAO) are favoured against other ordinary heterotrophic bacteria. PAO survival capacity relies on their potential to take up organic substrates under anaerobic conditions to synthesize polyhydroxyalkanoates (PHA). The proliferation of several PAO competitors, known as Glycogen Accumulating Organisms (GAO), has become a significant bottleneck in the EBPR process. GAO presence in EBPR systems increases the carbon and chemical requirements, the sludge production and the total overall costs of the plant (Oehmen *et al.*, 2010c; Saunders *et al.*, 2003). Thus, many works (*e.g.*, Oehmen *et al*., 2010b, López-Vazquez *et al*., 2009) aim at understanding the competition between PAO and GAO in view of finding the optimal conditions that favours PAO growth against GAO in EBPR systems.

PAO and GAO are not single microorganisms but common terms to describe the observed response of several groups with respect to biological P removal. The latest microbiological studies have revealed the existence of several subgroups of PAO/GAO with different phenotypes (Table 1). Regarding PAO, the *Candidatus Accumulibacter phosphatis*, hereafter referred as *Accumulibacter*, are the most widely known PAO in contrast to others as for example Tetrasphera-related or Dechloromonas-related (Oehmen *et al.*, 2010b). Two different types of *Accumulibacter* (PAOI and PAOII) have been described using the polyphosphate kinase gene as genetic marker (He *et al.*, 2007;

Peterson *et al.*, 2008) with different denitrification capacity (i.e. PAOI can denitrify nitrate and nitrite whereas PAOII can only use nitrite). Regarding GAO, significant diversity has also been detected, being the *Gammaproteobacteria Candidatus Compectibacter phosphatis* (from this point on, *Competibacter*) and the *Alfaproteobacteria Defluviicoccus Vanus* (from this point on, *Defluviicoccus*), the most abundant in full-scale plants. The major difference between both groups is their affinity for propionic and acetic acids. *Defluviicoccus* can be divided into four different clusters (from DFI to DFIV), which have different denitrification capabilities. The denitrification capacities of *Defluviicoccus* DFI and DFII are shown in Table 1, while capacities of DFIII and DFIV, which are only found in some Waste Water Treatment Plants (WWTP) (Mcilroy and Seviour, 2009), have not been studied yet to the best of our knowledge.

The response of the microorganisms involved in bio-P removal to different electron acceptors and donors (Table 1) is the starting point of the present work. According to this information, none of the reported groups of GAO could survive in an EBPR system with propionate as sole electron donor and nitrite as sole electron acceptor. Hence, these two simultaneous conditions should lead to the washout of the GAO typically found in WWTP and thus, to PAO-enriched sludge. Moreover, as nitrite is a common electron acceptor for PAOI and PAOII, the distribution of these two organisms in the sludge should be similar.

On the other hand, biological nitrogen removal and EBPR are nowadays integrated in activated sludge systems aiming at simultaneous nutrient removal. In these systems, the denitrification capacity of PAO gains a lot of relevance since PAO can reduce the nitrite/nitrate produced in the aerobic phase using the PHA stored in the anaerobic phase. N removal via the nitrite pathway, i.e. nitritation followed by denitritation, NH_4^+

→ NO_2^- → N_2 is a more cost effective alternative when treating low COD/N wastewaters due to the lower aeration and COD requirements (Turk and Mavinic, 1987). If the nitrite pathway was implemented, the role of nitrite would be very significant as well as its effect on the PAO/GAO competition. The implementation of the nitrite pathway and EBPR (nitritation and denitritation linked to phosphorus removal) has been reported both in suspended (Marcelino *et al.*, 2011; Zeng *et al.*, 2011) and particularly in granular systems (de Kreuk *et al.*, 2005; Mosquera-Corral *et al.*, 2005; Yilmaz *et al.*, 2008). Recently, Bassin *et al.* (2012) demonstrated that nitrite-based dephosphatation was the main pathway for achieving simultaneous P and N removal despite the GAO presence. They detected high PAOII proliferation and the role of GAO was to reduce the nitrate to nitrite.

The aim of this work is to demonstrate that an EBPR sludge operating with propionic acid and nitrite as a sole electron donor and acceptor, respectively, leads to the suppression of GAO activity and to the proliferation of PAO. For this purpose, a highly GAO-enriched sludge was operated under anaerobic/anoxic-nitrite configuration and with propionic acid as a sole carbon source for more than 50 d. The results would help not only for a better understanding of the underlying mechanisms of the PAO/GAO competition but also to understand the role of nitrite in biological nutrient removal systems.

2. MATERIALS AND METHODS

2.1 Experimental set-up

The experimental set-up consisted of a lab-scale Sequencing Batch Reactor (SBR) (V = 10 L) with oxygen, pH, ORP and temperature probes with a PLC (Siemens S7-226) on top of the control system. The SBR was initially operated for 90 d under anaerobic-

aerobic conditions with low influent P and high T to favour GAO growth. The cycle length was 6 h with the following sequence: anaerobic 2 h (initial feeding 5 min), aerobic 3.5 h, sedimentation 25 min and supernatant extraction 5 min (5 L). The synthetic wastewater used in the GAO-enrichment period consisted of the nutrient wastewater solution (4.96 L) (in $g \cdot L^{-1}$): 0.1 NH_4Cl, 0.044 $MgSO_4 \cdot 7H_2O$, 0.16 $MgCl_2 \cdot 6H_2O$, 0.042 $CaCl_2 \cdot 2H_2O$, 0.03 $NaHCO_3$, 0.0276 KH_2PO_4, 0.0209 K_2HPO_4 and 0.005 allylthiourea to inhibit nitrification and 0.30 mL of nutrient solution, based on Smolders *et al.* (1994). The carbon source solution (0.04 L) contained only propionic acid for an initial concentration of 225 mg L^{-1} of COD. The initial phosphate concentration was set to 5 mg P L^{-1}, resulting in a COD/P ratio of 45. The hydraulic retention time (HRT) was 12h. Sludge retention time (SRT) was kept at 15 d with periodic wastage at the end of the aerobic phase. The temperature was controlled at 25 ± 1 °C. HCl (1 M) and NaOH (1 M) were used to control the pH in the reactive phases at 7.50 ± 0.05. Nitrogen was bubbled during the anaerobic phase to avoid oxygen surface transfer. Dissolved oxygen (DO) was controlled during the aerobic phase between 3.5 and 4.5 mg L^{-1} to avoid oxygen limitations with an on/off controller.

During the GAO-washout period, the SBR configuration was moved to 2 h anaerobic, 3.5 h anoxic, 25 min of sedimentation and 5 min for the extraction. Nitrogen was bubbled during all the reactive period. Sodium nitrite was automatically added to the system from a concentrated solution (3.5 g N L^{-1}) to obtain anoxic conditions. The synthetic wastewater was switched to more realistic values (initial concentration of propionic acid of 100 mg $COD \cdot L^{-1}$ and P of 5 mg P L^{-1}) with an initial COD/P ratio of 20. HRT and SRT were kept at the previous values.

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

168 This technique consists of the indirect measurement of the proton production (HP)
169 through the monitoring of the amount of base (or acid) dosage necessary to maintain the
170 pH constant (Eq. 1).

171 $$HP = C_{BASE} V_{BASE} - C_{ACID} V_{ACID} \quad (1)$$

172 where V_{BASE} and V_{ACID} stand for the accumulated base and acid dosage (mL) and C_{BASE}
173 and C_{ACID} for base and acid concentration. Acid (HCl at 1 M) and base (NaOH at 1 M)
174 were added using peristaltic dosage pumps (8 $mL \cdot min^{-1}$).
175

176 **2.2 Chemical and microbiological analyses**

177 Phosphorus concentration in 0.22 μm filtered samples was measured by a phosphate
178 analyser (115 VAC PHOSPHAX sc, Hach-Lange) based on the Vanadomolybdate
179 yellow method. Nitrite in filtered samples was analysed with Ionic Chromatography
180 (DIONEX ICS-2000). Propionic acid was measured by Agilent Technologies 7820 A
181 GC as described in Guerrero *et al.* (2012). Glycogen and PHA were measured by
182 triplicate according to the methodology described in Guisasola *et al.* (2009). Total
183 suspended solids and volatile suspended solids (VSS) were analysed according to
184 APHA (1995).
185 Fluorescence in situ hybridization (FISH) technique (Amann, 1995) coupled with
186 confocal microscopy was used to quantify the biomass distribution as in Jubany *et al.*
187 (2009). Hybridizations were performed with Cy3-labelled specific probes (Crocetti *et*
188 *al.*, 2000; Crocetti *et al.*, 2002) and Cy5-labelled EUBMIX for most bacteria (Daims *et*
189 *al.*, 1999). Table SM-1 in Supplementary Material (SM) details the probes used in this
190 work.

191 **2.3 Batch experiment**

A batch experiment to test whether PAO could anaerobically store VFA as PHA under limiting poly-P conditions was designed in a 1 L vessel. This vessel could be operated either under anaerobic/anoxic or aerobic conditions by sparging nitrogen gas or air through a microdiffuser, which ensured good gas transfer to the liquid phase. Gas flow was controlled with a mass flow meter (HiTec 825, Bronckhorst). The pH (Sentix 81,WTW) and DO (CellOx 325, WTW) probes were connected to its multiparametric terminal (INOLAB 3, WTW) which was in turn connected via RS232 to a PC allowing for data monitoring and storage. The PC also manipulated a high precision microdispenser (Crison Multiburette 2S) to keep the pH constant at 7.50 ± 0.01 with HCl (1 M)/NaOH (1 M) dosage. The reactor was thermally controlled at 25.0 ± 0.1 °C. The sludge was taken from the SBR at the end of the anoxic phase and placed in the vessel under anaerobic conditions. Two pulses of 100 mg L^{-1} of propionic acid were added (at 0 and 150 min) to exhaust most of internal P reserves. Then, after a settling period, the supernatant was withdrawn and the biomass was carefully washed to remove the remaining propionic acid and phosphorus in the medium. Next, an aerobic phase was conducted to deplete most of the internally stored PHA. Finally, an anaerobic/nitrite-anoxic cycle was conducted with the addition of 200 mg L^{-1} of propionic acid. After all P was released, a settling period to wash the supernatant was carried out to ensure that no propionic acid was present in the next step. Finally, an anoxic phase was performed by adding two pulses of 30 mg NO_2^-N L^{-1}.

3. RESULTS

A GAO-enriched EBPR sludge was promoted based on the guidelines proposed by the work of Lopez-Vazquez *et al.* (2009) under anaerobic/aerobic conditions with propionate as sole carbon source: COD/P in the influent was fixed to 45 with very low

217 initial P concentration in the reactor (5 mg·L^{-1}). Once the sludge was enriched in GAO,
218 the ratio was moved to a more reasonable value (COD/P = 20). Figure 1 (LEFT) shows
219 the results obtained from the last of the anaerobic-aerobic cycles, performed with the
220 new COD/P ratio. Propionate was rapidly depleted and linked to some phosphate
221 release, although the P/C ratio obtained was 0.20 indicating a significant presence of
222 GAO in the sludge. Nevertheless, P depletion was observed during the aerobic phase,
223 showing that despite the low P/C ratio, the PAO content in the sludge was enough to
224 achieve complete aerobic P-uptake due to the low initial concentration of P. The FISH
225 results (Fig. 1 RIGHT) supported these observations, indicating a low percentage of
226 PAO (10%) and a significant amount of GAO (70% DFI and 18% DFII), while
227 *Compectibacter* GAO were scarcely detected, in accordance to the lack of acetate in the
228 feed.
229 Then, oxygen was replaced for nitrite as sole electron acceptor. Figure 2a - 2d shows
230 some examples of the experimental profiles obtained during the 50 d of anaerobic-
231 anoxic operation. At first glance, the results indicate a fast adaptation to nitrite without
232 any significant inhibition and a significant increase of the anoxic P uptake activity. This
233 observation is corroborated with the evolution of the maximum nitrite uptake rate
234 (NUR_{MAX}) estimated in several cycles, which shows a clear increasing trend (Fig. 2e).
235 Figure 3 shows the evolution of the FISH quantifications during the experimental
236 period. As can be observed, the objective of this work, i.e. proof that nitrite is a strong
237 selection factor in the PAO/GAO competition in a propionic-fed environment, was
238 achieved and all the GAO species initially present in the sludge were removed. The
239 percentage of PAO increased in parallel to the GAO depletion. It is also worth
240 mentioning that both PAOI and PAOII were equally favoured in this system. With
241 respect to these FISH measurements, Fig. SM-1 shows selected images for all the

probes tested and provides a better visualisation of the evolution of each biomass population.

Finally, Fig. 4 shows a batch cycle conducted at day 41 where the internal storage polymers (glycogen and PHA) were measured. The experimental profiles are in agreement with those found in the literature. Most of the PHA was polyhydroxyvalerate (PHV) (66 ± 9 %) in accordance to the use of propionate as sole carbon source (Pijuan *et al.* 2009). Table 2 compares the experimental ratios obtained with other similar experimental works and with those estimated theoretically with metabolic modelling. As can observed, the experimental values are unexpectedly far from those typical of PAO if one takes into account the high enrichment showed by the FISH images. These results will be discussed in the following section.

4. DISCUSSION

The major outcome of this work is the demonstration that nitrite can be used as the selection factor in the PAO/GAO competition when *Defluviicocus* GAO are predominant in the sludge. The operation of an enriched culture of *Defluviicocus* under anaerobic/anoxic-nitrite conditions with propionate as sole carbon source resulted in the washout of *Defluviicocus* in parallel to the growth of PAO showing the feasibility to obtain a highly enriched culture of PAO (85%) even from an enriched culture of *Defluviicocus*. GAO enrichment was initially forced with an unusually high COD/P and then, this ratio was moved it into a conventional value (COD/P = 20). While this decrease may have contributed towards the enrichment of PAO over GAO, using this ratio as sole selection factor has not been reported as a successful strategy for obtaining a highly PAO-enriched sludge without GAO.

The operation with propionate led to the selection of *Defluviicocus* GAO versus *Competibacter* GAO, which have been found to be favoured with acetate as carbon source (Oehmen *et al.,* 2005b). *Competibacter* are able to use nitrite as electron acceptor (Kong *et al.,* 2006b) and hence, the strategy of using acetate as sole carbon source under anaerobic/anoxic-nitrite conditions would not have led to an enriched culture of PAO. The observed *Defluviicocus* GAO washout is somehow in contrast to the findings of Liu *et al.* (2010) with a highly-enriched culture of *Competibacter* GAO obtained using acetate as sole carbon source. They found that nitrite dosage was more adverse for the aerobic PAO metabolism rather than for the aerobic GAO metabolism and suggested that nitrite could provide a competitive advantage of *Competibacter* over PAO. In our case, *Defluviicocus* were much more abundant (DFI 70% and DFII 18 %) than *Competibacter* and the anaerobic/anoxic-nitrite conditions were demonstrated to be more favourable for PAO than for *Defluviicocus*. Based on these results, it seems clear that nitrite-pathway EBPR systems should focus on achieving a carbon source with high propionate fractions over acetate to combine the positive effects of nitrite and propionate on PAO selection. In this sense, Chen *et al*. (2013) proposed recently a novel strategy to increase the propionate content in the wastewater.

With respect to the utilization of nitrite by *Defluviicocus*, it was reported that DFI cannot use nitrite as an electron acceptor (Wang *et al.,* 2007) whereas DFII is unable to denitrify (Kong *et al.*, 2006a). DFIII were not found in the system and the denitrification capacities of DFIV are not reported so far. This GAO washout contrasted with the experimental ratios (PHA/C and Gly/C) of Table 2, which are in general far from the theoretical ones widely accepted for PAO. The explanation for such high values would lay on the low amount of P in the influent used, which resulted in a low poly-P content of the biomass. Under these conditions, PAO can take up propionate

using glycogen as primary energy source (a typical GAO behaviour) for survival purposes. The fact that PAO can activate the glycolytic pathway in order to balance the lack of energy derived from low poly-P hydrolysis has already been reported in the literature (Acevedo *et al.*, 2012; Erdal *et al.*, 2008; Zhou *et al.*, 2008). This ability of PAO to store volatile fatty acid (VFA) as PHA anaerobically under limiting poly-P conditions and use it afterwards was observed in a batch experiment (Fig. 5). The experiment, conducted at the end of the experimental period, aimed at forcing PAO to take up propionate anaerobically under scarce poly-P conditions. For this aim, most of the internal poly-P was depleted under anaerobic conditions with an excess of carbon addition, showing a P/C ratio of 0.21. Then, the medium was replaced for a phosphate-free medium and the system was left overnight under aerobic conditions. Propionate was added again under anaerobic conditions and P-release was very low despite the high amount of COD taken up (P/C < 0.01), indicating that PAO (85% of PAO in the sludge) had changed their behaviour from PAO to GAO. Finally, the medium was replaced to remove any propionate remaining and nitrite was added. Successful denitritation was observed showing that PAO were responsible for this nitrite reduction since *Defluviicocus* are not able to reduce nitrite.

Moreover, when dealing with nitrite-based EBPR systems, pH should be also considered. High pH favours PAO against GAO due to kinetics (Oehmen et al., 2005a), but when nitrite is present in the system it has an additional positive effect because it results in less free nitrous acid (FNA) concentration, a reported P-uptake inhibitor (Pijuan et al., 2010). Hence, the combination of nitritation with PAO denitritation should be carefully performed to avoid high FNA concentration that would be detrimental for EBPR stability. In our experimental study, the utilization of a controlled

315 pH of 7.5, linked to the careful addition of nitrite in several pulses was probably helpful
316 to avoid the observance of any FNA inhibition.
317 This work also shows how a conventional anaerobic/aerobic EBPR system can be
318 directly adapted to anaerobic/anoxic conditions with nitrite as electron acceptor from
319 day 1 without any intermediate anaerobic/anoxic/aerobic configuration as previously
320 reported (Vargas *et al.*, 2011). The nitrite-based anoxic P uptake rate was, however,
321 much slower than that under aerobic conditions and hence, nitrite should be wisely
322 dosed to avoid its presence at the end of the anoxic phase. Nitrite entering the anaerobic
323 phase can be very detrimental for the PAO growth (see discussion below). Despite these
324 limitations, the SBR got rapidly acclimatised to nitrite as an electron acceptor and
325 anoxic-P uptake was complete in all the period. The last cycles of the period were very
326 similar (Fig. 2) and typical of an EBPR cycle with complete P removal. P-uptake was
327 usually higher than P-release and net P removal was usually achieved.
328 The fast adaptation to nitrite is in agreement with the fact that both PAOI and PAOII
329 initially present in the sludge are able to reduce nitrite (Oehmen *et al.*, 2010b), and
330 hence nitrite utilization as sole electron acceptor should not be a selection factor
331 between PAOI and PAOII. This observation was confirmed with the final FISH
332 measurements (65 % PAOI *versus* 35 % PAOII, Fig. 3) and with a batch experiment
333 with nitrate as electron acceptor (Fig. SM-2). Nitrate utilisation did not need an
334 acclimatisation period at all and complete anoxic P uptake was achieved. These results
335 contrast with previous works where nitrite-based selections led to systems without
336 nitrate-reducing capabilities (Guisasola *et al.*, 2009; Jiang *et al.*, 2006). In this sense, a
337 complete understanding on the competition between PAOI and PAOII in nitrite-
338 reducing EBPR environments is still needed. Selecting PAOI in front of PAOII would
339 be more interesting in view of increasing flexibility (i.e. both nitrate and nitrite could be

340 treated). Otherwise, a PAOII-enriched sludge would be desirable to integrate partial
341 nitrification and EBPR provided the specific nitrite reduction rate of PAOII seems to be
342 higher than PAOI.
343 Two different practical implications can be drawn from this work. On the one hand,
344 nitrite and propionate could be used as strategy to enrich a bio-P sludge with PAO. PAO
345 have yet to be isolated and novel strategies for its enrichment are very interesting for
346 fundamental studies on PAO physiology and biochemistry (Lu *et al.*, 2006). On the
347 other hand, the suppression of *Defluviicocus* GAO when nitrite is the electron acceptor
348 represents an add-on to the nitrite-based EBPR systems since the proliferation of non-
349 desirable GAO can be easily ruled out and added to the other benefits (i.e. lower
350 aeration and COD requirements).
351 Finally, these results provide useful recommendations for improving the EBPR activity
352 in full-scale WWTP. The implementation of nitrite pathway for nitrogen removal and
353 the utilization of external carbon sources fermentable to propionic acid, as glycerol
354 (Guerrero et al., 2012) or food waste (Chen et al., 2013), should favour PAO in front of
355 both *Competibacter* and *Defluviicocus* GAO and hence would provide a more stable
356 and efficient P removal.
357

358 **5. CONCLUSIONS**

359 A bio-P sludge enriched in *Defluviicocus* GAO (70 % DFI, 18% DFII and 10 % PAO)
360 was operated under anaerobic/anoxic-nitrite configuration, achieving the washout of
361 these microorganisms in parallel to the growth of PAO (up to 85%) and demonstrating
362 that nitrite is a key selection factor in the PAO/GAO competition.
363 This novel strategy not only allows the achievement of a highly PAO-enriched activated
364 sludge, but also demonstrates that the integration of denitritation with EBPR favours the

suppression of *Defluviicocus* GAO and represents an additional advantage for the EBPR configurations using anoxic-nitrite conditions, in addition to lower aeration and COD requirements.

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512

513 **Table 1** Comparison of the preferred VFA and the denitrification capabilities for the

514 different PAO/GAO subgroups (adapted from Oehmen et al., 2010b)

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

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518 **Table 2** Comparison of the typical PAO/GAO ratios of this work with others found in

519 the literature for propionic acid as sole carbon source.

		Gly/VFA (mmolC. mmol^{-1}C)	PHA (mmolC. mmol^{-1}C)	P/C (mmolP. mmol^{-1}C)
Chen et al., 2005	PAO		1.02	0.45
Pijuan et al., 2009	PAO	0.45	0.64	0.27
Oehmen et al., 2010a	PAO	0.33	1.22	0.3
	GAO	0.67-1.0	1.50- 1.78	
This Study	PAO	0.49	1.47	0.34

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523 **Table S1** Summary of the probes used in the FISH measurements

Short name	Specificity	Reference
EUBMIX	Most bacteria	Daims *et al.* 1999
PAOMIX	*Accumulibacter phosphatis*	Croccetti *et al.* 2000
PAO I	Cluster I of *Accumulibacter phosphatis*	Flowers *et al.* 2009
PAO II	Cluster II of *Accumulibacter phosphatis*	
GAOMIX	*Competibacter phophatis*	Croccetti *et al.* 2002
DFIMIX	Cluster I of *Defluviicoccus vanus*	Wong *et al.* 2004
DFIIMIX	Cluster II of *Defluviicoccus vanus*	Meyer *et al.* 2006
DFIII	Cluster III of *Defluviicoccus vanus*	Mcilroy and Seviour (2009)

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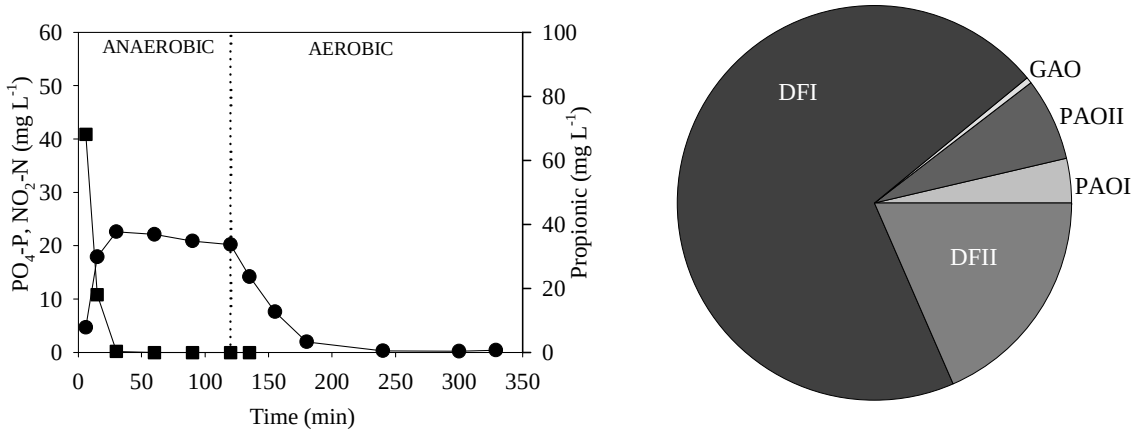

Figure 1 (LEFT) Experimental propionic acid (■) and phosphate (●) profiles for the GAO-enriched sludge. (RIGHT) Microbial distribution according to FISH images.

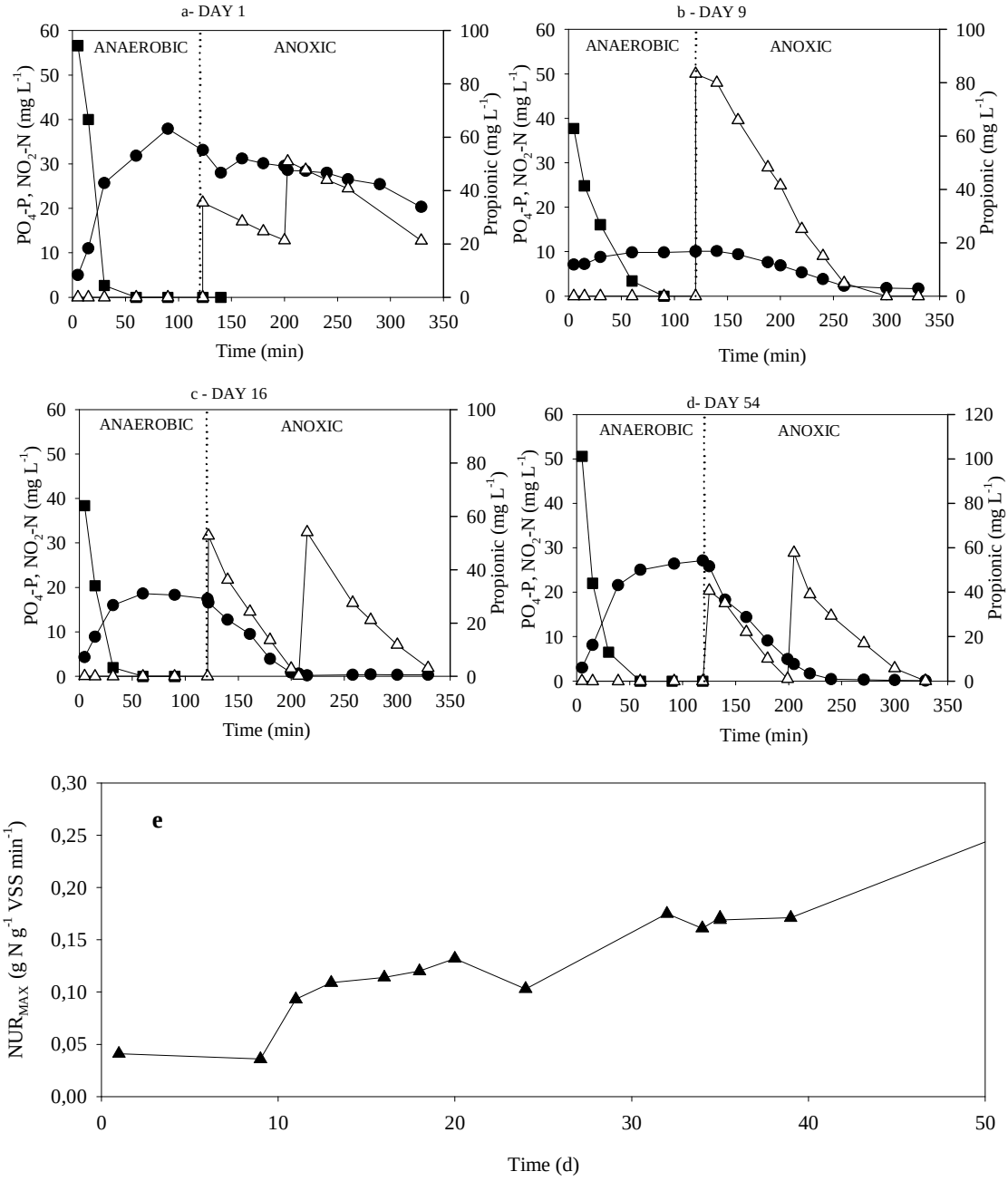

Figure 2 (a to d) Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for some cycles during the experimental period. (e) NUR_{MAX} evolution during the experimental period.

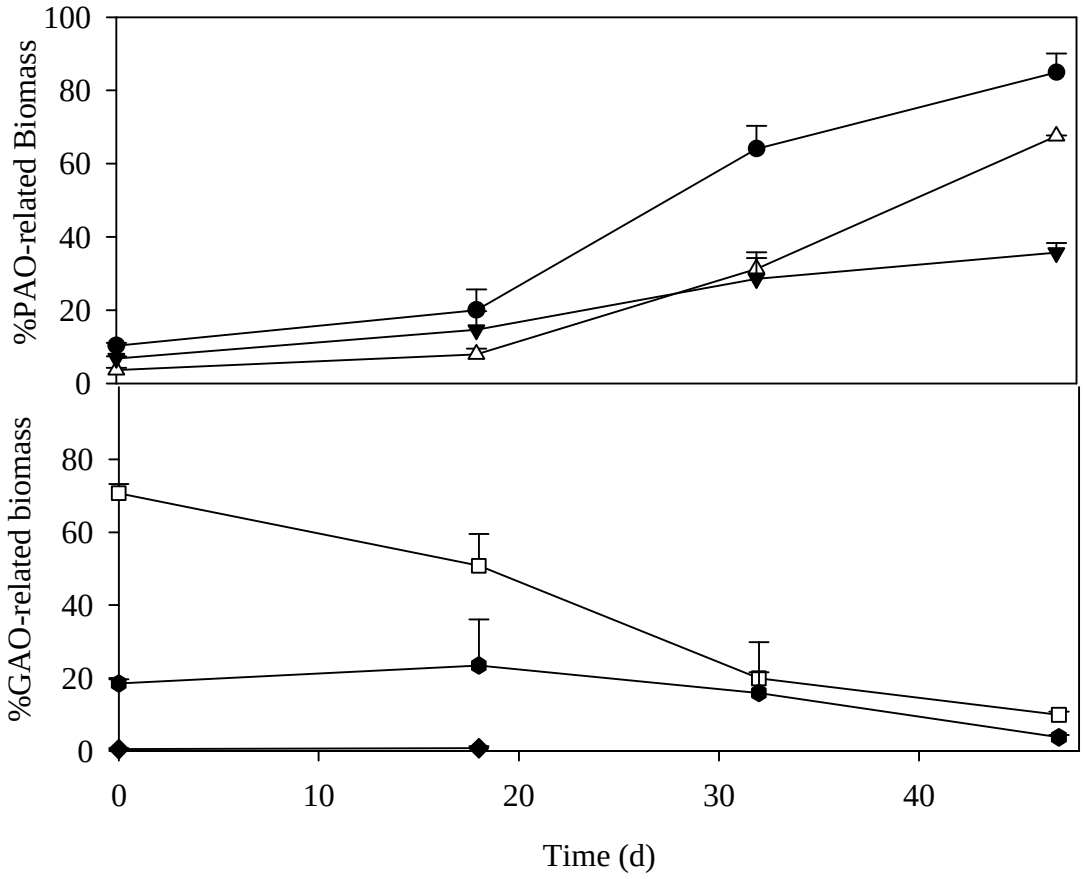

Figure 3 FISH distribution percentages of the biomass. PAO-related biomass (UP): PAOMIX (●), PAO I (△) and PAO II (▼). GAO-related biomass (DOWN): GAOMIX (◆), DF1MIX (□) and DF2MIX (⬢).

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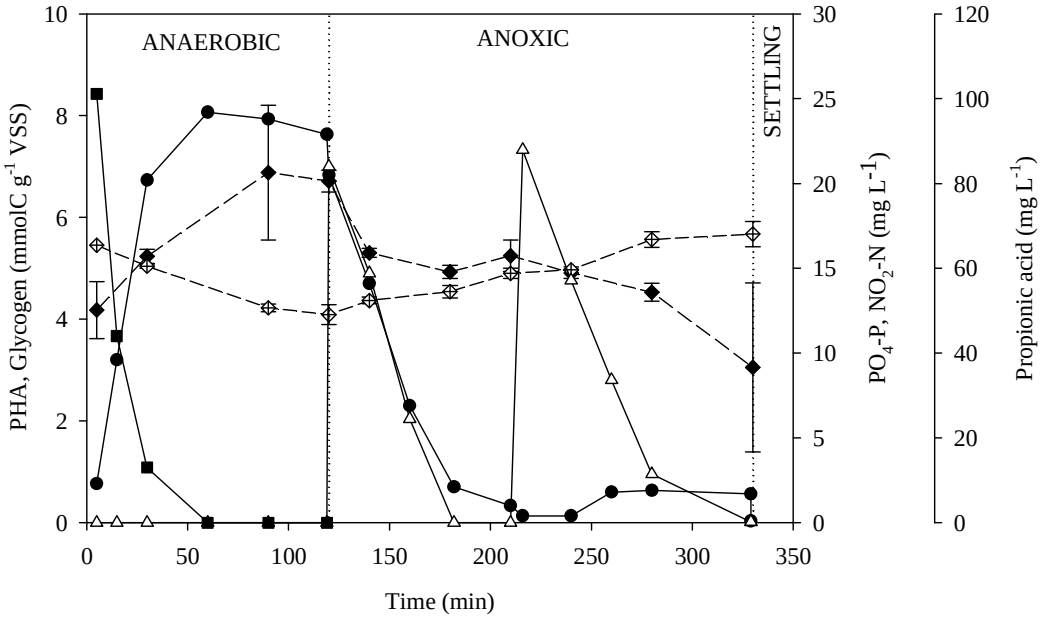

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551 **Figure 4** Experimental PHA (◆), glycogen (◇), propionic acid (■), phosphate (●) and

552 nitrite (△) profiles for a cycle from day 41.

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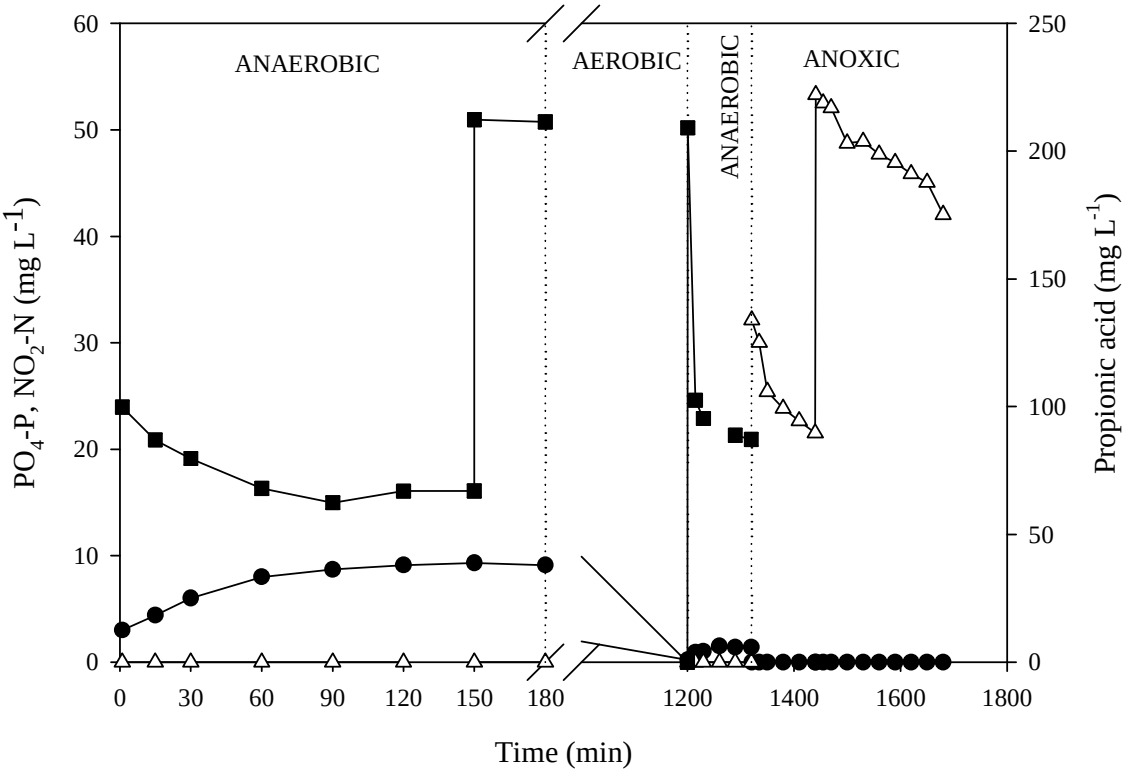

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556 **Figure 5** Experimental propionic acid (■), phosphate (●) and nitrite (△) profiles for the

557 batch experiment of PAO behaving like GAO.

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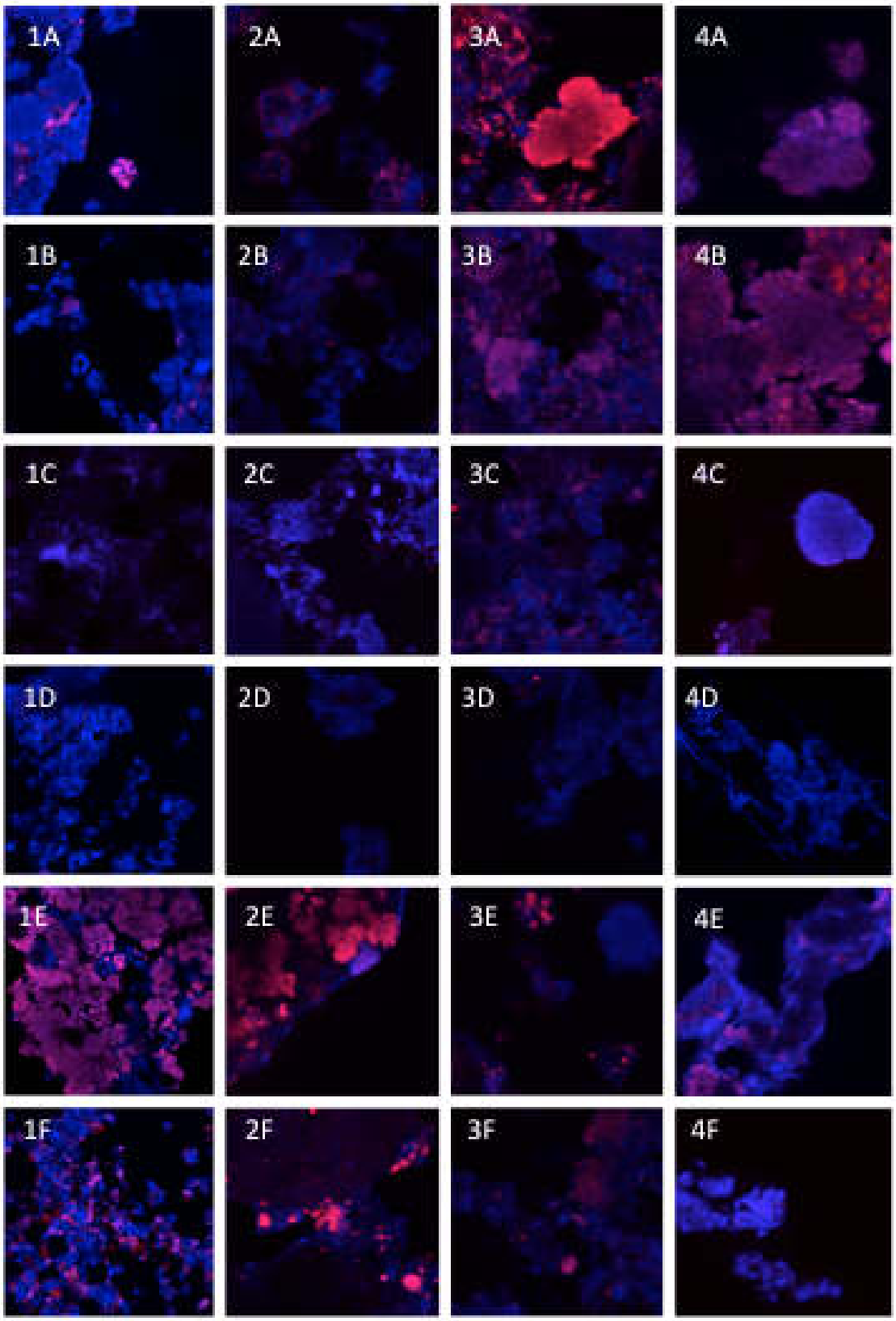
559 Day 0 Day 18 Day 32 Day 47



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561 **Figure S1** FISH/CLSM representative images of the sludge from the SBR reactor
562 during the enrichment. A. PAOMIX, B. PAO clade I, C. PAO clade II, D. GAOMIX, E.
563 DF1MIX, F. DF2MIX. Specific probe is shown in pink and EUBMIX probe in blue.

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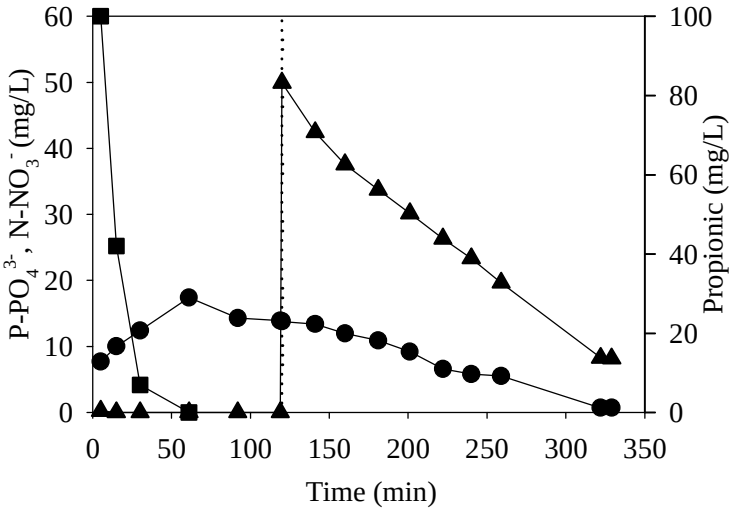

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566 **Figure S2** Experimental propionic (■), P (●) and NO_3^- (▲) profiles for a cycle at day

567 30.

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