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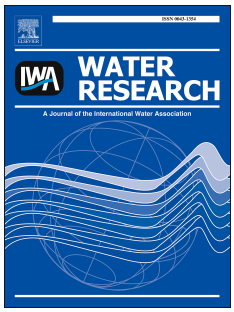

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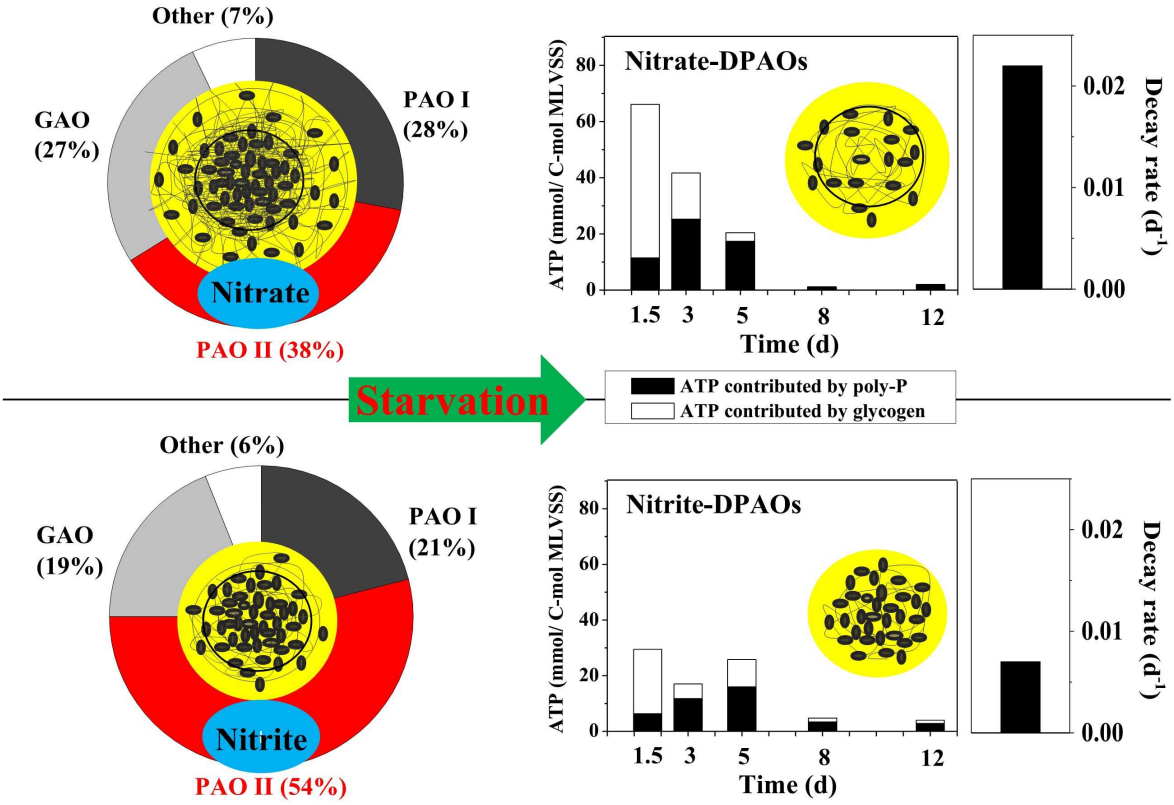

Comparison of endogenous metabolism during long-term anaerobic starvation of nitrite/nitrate cultivated denitrifying phosphorus removal sludges

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Abstract: Denitrifying phosphorus removal (DPR) by denitrifying phosphorus-accumulating organisms (DPAOs) is a promising approach for reducing energy and carbon usage. However, influent fluctuations or interruptions frequently expose the DPAOs biomass to starvation conditions, reducing biomass activity and amount, and ultimately degrading the performance of DPR. Therefore, a better understanding of the endogenous metabolism and recovery ability of DPAOs is urgently required. In the present study, anaerobic starvation (12 days) and recovery were investigated in nitrite- and nitrate-cultivated DPAOs at 20 ± 1 °C. The cell decay

rates in nitrite-DPAO sludges from the end of the anaerobic and aerobic phase were 0.008 day^{-1} and 0.007 day^{-1}, respectively, being 64% and 68% lower than those of nitrate-DPAO sludges. Nitrite-DPAO sludges also recovered more rapidly than nitrate-DPAO sludge after 12 days of starvation. The maintenance energy of nitrite-DPAO sludges from the end of the anaerobic and aerobic phase were approximately 31% and 34% lower, respectively, than those of nitrate-DPAO sludges. Glycogen and polyphosphate (poly-P) sequentially served as the main maintenance energy sources in both nitrite-and nitrate-DPAO sludges. However, the transformation pathway of the intracellular polymers during starvation differed between the sludges. Nitrate-DPAO sludge used extracellular polymeric substances (EPS) (mainly polysaccharides) as an additional maintenance energy source during the first 3 days of starvation. During this phase, EPS appeared to contribute to 19 – 27% of the ATP production in nitrate-DPAOs, but considerably less to the cell maintenance of nitrite-DPAOs. The high resistance of nitrite-DPAOs to starvation might be attributable to frequent short-term starvation and exposure to toxic substances such as nitrite/free nitrous acids in the parent nitrite-fed reactor. The strong resistance of nitrite-DPAO sludge to anaerobic starvation may be exploited in P removal by shortcut denitrification processes.

Keywords: Starvation; Denitrifying phosphorus-accumulating organisms; Maintenance; Intracellular polymers; Extracellular polymeric substances; Decay

1. Introduction

Owing to large fluctuations in the flow and composition of wastewater, the microorganisms responsible for biological wastewater treatment plants are frequently exposed to long-term famine conditions (days and sometimes weeks) (Lu et al., 2007). During sludge storage, by which large influent variations can be adjusted and flexible plant operation can be achieved, microorganisms may experience starvation (Morgenroth et al., 2000). Starvation significantly reduces the amount and activity of active microorganisms, and risks degrading the capacity, efficiency and robustness of wastewater treatment systems (Hao et al., 2010b; Wang et al., 2013b). Starvation is crucially important in enhanced biological phosphorus removal (EBPR) processes, since it alters the levels of intracellular storage compounds in the functional microbes (i.e., polyphosphate-accumulating organisms, PAOs) (Vargas et al., 2013). Indeed, excessive consumption of intracellular polymers (Yilmaz et al., 2007) or excessive decay of both PAOs and intracellular polymers (Miyake and Morgenroth, 2005) has been implicated in EBPR failure.

In the absence of external sustenance, starved microorganisms primarily undergo the endogenous processes consisting of cell maintenance and cell decay (Lu et al., 2007; Wang et al., 2012). The impacts of starvation on PAOs and endogenous processes have been extensively investigated (Lopez et al., 2006; Yilmaz et al., 2007; Lu et al., 2007; Hao et al., 2010a; Wang et al., 2012; Vargas, et al., 2013), and effective strategies for maintaining the biomass activity have been accordingly

64 proposed. Lopez et al. (2006) examined the effects of long-term (weeks) anaerobic
65 and aerobic starvation on the composition and activity of PAOs. They concluded that,
66 under aerobic starvation conditions, PAOs are notably attenuated by endogenous
67 processes, whereas no significant PAOs decay occurs under anaerobic starvation. Lu
68 et al. (2007) proposed an intermittent aerobic–anaerobic strategy for the long-term
69 storage of EBPR sludge. In this strategy, the PAOs decay more slowly than in aerobic
70 storage, and glycogen and poly-P are used at a slower rate than in anaerobic and
71 anoxic storage. A similar recovery strategy was recommended by Yilmaz et al. (2007),
72 who found that alternating anoxic/anaerobic and aerobic operation effectively
73 maintains the biomass activity of activated sludge used for biological nitrogen (N) and
74 phosphorus (P) removal, thereby enabling quick activity recovery (i.e., full recovery
75 within 4 days).

76 Unlike PAOs in traditional EBPR processes, the impacts of starvation on
77 denitrifying polyphosphate-accumulating organisms (DPAOs) have been little
78 reported. Denitrifying phosphorus removal (DPR) by DPAOs is a viable and
79 sustainable technology, as N and P can be simultaneously removed with lower carbon
80 source requirements, aeration costs and cell yields (Murnleitner et al., 1997). In
81 particular, since DPAOs can use nitrite as an electron acceptor, DPR is naturally
82 amenable to shortcut nitrification. By replacing nitrate with nitrite, the oxygen cost
83 and carbon consumption of DPR can be reduced by approximately 25% and 40%,
84 respectively (Abeling et al., 1992). Therefore, DPR by nitrite could be used for

innovative biological nutrient removal (BNR) systems where energy and carbon savings are a priority, for example, linking nitrite pathways (i.e., partial nitrification + nitrite-based denitrification) to EBPR (Guisasola et al., 2009; Marcelino et al., 2011; Zhou et al., 2011; Tayà et al., 2013). Moreover, as nitrite enriched PAOs need less carbon source (i.e., intracellular PHA) for P-uptake, and eventually they might have higher PHA accumulation which can be used to speed up their anoxic metabolism after the endogenous period.

Recently, identifying the inhibitory effects of nitrite and the feasibility of nitrite-based DPR has received increasing attention (Guisasola et al., 2009; Marcelino et al., 2011; Zhou et al., 2011). However, DPAOs metabolism, especially their endogenous metabolism, has not been properly elucidated. To our knowledge, the endogenous characteristics of nitrite metabolism of DPAOs have not been assessed. A better understanding of the mechanism of the impact of starvation on nitrite- and nitrate-DPAOs, and endogenous metabolism of DPAOs may favor the development of strategies for improvement of the robustness and performance of DPR processes, the resuscitation of DPR systems after famine scenarios, and the storage of DPAOs sludge.

Increasing evidence shows that extracellular polymeric substances (EPS) can serve as carbon and energy sources for active biomass growth under starvation conditions (Zhang and Bishop, 2003; Wang et al., 2005; Wang et al., 2007; Liu et al., 2007; Flemming et al., 2010). Wang et al. (2005) found that most biodegradable EPS,

106 especially polysaccharides, are located in the core of aerobic granular sludge, and that
107 this fraction of EPS can be depleted after long-term starvation (20 days), as evidenced
108 by the void structure in the core of starved granules. Since most previous starvation
109 investigations of EBPR sludge did not involve EPS, the contribution of EPS to the
110 maintenance of metabolic activity of PAOs/DPAOs remains unclear.

111 The purpose of this study is to identify the differences between the endogenous
112 characteristics of nitrite- and nitrate-DPAO sludges during 12-day anaerobic
113 starvation, and to better understand the endogenous metabolism of nitrite-DPAOs. The
114 transformation of intracellular polymers and post-starvation activity recovery are also
115 compared between nitrite and nitrate-DPAO sludges. We highlight the different
116 transformation pathways of intracellular polymers in nitrite- and nitrate-DPAOs
117 biomass. We also attempt to clarify the role of EPS (especially polysaccharides) in
118 nitrite/nitrate-DPAOs under anaerobic starvation conditions.

119 **2. Materials and methods**

120 **2.1. Set up and long-term operation of parent reactors**

121 DPAOs sludge was enriched in two identical laboratory-scale sequencing batch
122 reactors (SBR_{NO3-} and SBR_{NO2-}, using nitrate and nitrite as electron acceptors,
123 respectively) with a working volume of 7.5 L as outlined by Wang et al. (2011). Both
124 SBRs were independently operated in a cyclical anaerobic-anoxic-aerobic pattern
125 with a cycle time of 8 h (15-min filling period, a 120-min anaerobic period, a 210-min
126 anoxic period, a 30-min aerobic period, a 20-min settling period, a 15-min effluent

discharging period, and a 70-min idle period). During the filling period, each reactor was fed with 5.5 L synthetic wastewater (***Section 2.2***). KNO_3 or KNO_2 solution was carefully added to the reactors during the anoxic phase to prevent nitrate or nitrite accumulation. Specifically, 34 mL KNO_3 solution was added to A-SBR_{NO3-}, giving an initial NO_3^--N concentration of 34 mg/L; 11 mL KNO_2 solution was added into A-SBR_{NO2-} in three pulses (70 min of intervals), giving an initial NO_2^--N concentration of 11 mg/L per pulse. Effluent was withdrawn from the port at 30 cm above the bottom, leaving 2.0 L of mixed liquor in the reactor.

The temperature was maintained at 20 ± 1 °C, and the rotation speed was controlled at 150 ± 10 rpm during the reaction phases. Nitrogen gas was introduced through the headspace for 5 min to ensure anaerobic condition at the beginning of the anaerobic phase of each cycle. In the post-aerobic phase, the dissolved oxygen (DO) concentration was controlled at 2 – 4 mg/L. The hydraulic retention time (HRT) was 10.9 h. To maintain the concentration of the mixed liquid suspended solids (MLSS) at 4000 ± 200 mg/L, 125 mL of mixed liquor was periodically discarded at the end of each aerobic period. The solid retention time (SRT) under these conditions was approximately 20 days.

2.2. Synthetic wastewater

The synthetic wastewater used in this study contained (per L): 257.1 mg CH_3CH_2COONa (300 mg chemical oxygen demand (COD)); 32.9 mg KH_2PO_4 (7.5 mg P); 55.3 mg $K_2HPO_4 \cdot 3H_2O$ (7.5 mg P); 38.2 mg NH_4Cl; 85.0 mg $MgSO_4 \cdot 7H_2O$;

10.0 mg $CaCl_2$. Therefore, the influent volatile fatty acid (VFA, i.e., propionate) to P ratio was 6.4 mg C/mg P. PAOs were preferentially selected by adding propionate as the sole carbon source (Oehmen et al., 2005). Trace salt solution (0.3 mL/L) and allylthiourea (4 mg/L) were added as described by Wang et al. (2011). The pH of the synthetic wastewater was maintained at 7.5 ± 0.1 by adding $NaHCO_3$.

2.3. Starvation batch experiments

Once the SBRs had reached steady-state operation, batch experiments were conducted in four identical sealed reactors, each with a working volume of 3.8 L and an overhead space of 0.2 L. The long-term nitrate or nitrite cultivated sludge in SBR_{NO3-} and SBR_{NO2-} was divided into two equal portions at the end of the decanting phase, and was then transferred to one of the four batch reactors. For each starvation test, 2.8 L synthetic wastewater was rapidly added to each reactor, maintaining the MLSS level at 4000 ± 200 mg/L. One of the two reactors incubated with nitrate-DPAOs sludge was operated with a 2-h anaerobic reaction (A-R_{NO3-}), the other was operated with a 2-h anaerobic reaction, a 3.5-h anoxic reaction and a 0.5-h aerobic reaction (O-R_{NO3-}). The corresponding nitrite-DPAOs sludges (operated under the same conditions) from the end of the anaerobic and aerobic phases are called A-R_{NO2-} and O-R_{NO2-}, respectively. After one operation cycle, each reactor was sparged with nitrogen gas for 10 min to maintain anaerobic conditions, and was left idle for the next 12 days. During the starvation experiments, each reactor was sparged with nitrogen gas for 10 min per day to remove any H_2S accumulated by the activity of

169 sulfate-reducing bacteria (Morgenroth et al., 2000; Yilmaz et al., 2007). All tests were
170 carried out at 20 ± 1 °C, and the pH was manually controlled at 7.5 ± 0.1 by adding
171 0.3 M HCl or 0.3 M NaOH.

172 Liquid- and solid-phase samples were taken from each reactor on days 0, 1.5, 3, 5,
173 8, and 12. The mixed liquor was filtered through a Millipore filter unit (pore size =
174 0.45 µm), and the liquid portion was retained for analysis of VFA, NH_4^+-N, PO_4^{3-}-P,
175 NO_3^--N and NO_2^--N. The solid portion (biomass) was centrifuged, freeze-dried and
176 retained for analysis of intracellular polymers, including poly-β-hydroxybutyrate
177 (PHB), poly-β-hydroxyvalerate (PHV), poly-3-hydroxy-2-methylvalerate (PH2MV),
178 and glycogen. Samples were also taken for MLSS, mixed liquor volatile suspended
179 solids (MLVSS) and EPS measurements.

180 **2.4. Recovery batch experiments**

181 After 12 days of starvation, the biomass activities of the starved nitrite/nitrate DPAO
182 sludges were assessed in batch tests. The sludges in the four batch reactors were
183 washed three times with 2.8 L propionate-free synthetic wastewater. At the beginning
184 of the recovery test, 2.8 L of propionate-free synthetic wastewater was rapidly added
185 to the reactors containing activated sludge from the end of the anaerobic phase
186 (denoted $AS_{ANA.end}$) (A-R_{NO3-} and A-R_{NO2-}). The anoxic reaction (3.5 h) was started by
187 adding KNO_3/KNO_2 as described in ***Section 2.1***, followed by a 0.5-h aerobic reaction.
188 The reactors containing activated sludge from the end of the aerobic phase (denoted
189 $AS_{AER.end}$) (O-R_{NO3-} and O-R_{NO2-}) were rapidly supplemented with 2.8 L of synthetic

wastewater at the beginning of the recovery test, and then were directly operated through the typical cycle for SBR_{NO3-} and SBR_{NO2-}, respectively. Two recovery cycles were applied to each of these four reactors.

2.5. Analytical methods

Liquid- and solid-phase analyses of NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, MLSS and MLVSS were performed by the standard method (APHA, 1998). DO and pH were measured online using oxygen and pH meters (oxi 3310 and pH 3310, WTW Company, Germany), respectively. Glycogen, poly-β-hydroxyalkanoates (PHA), VFA, and EPS were determined by the procedure detailed in the Supplementary Information (SI) (Text S1). PHA content in the sludge sample was defined as the sum of the measured PHB, PHV and PH2MV. EPS was extracted by the formaldehyde-NaOH method, and was calculated as the sum of polysaccharides, proteins and humic substances.

The relative PAOs and glycogen-accumulating organisms (GAOs) abundances in both parent SBRs were estimated by 16S rRNA fluorescence in situ hybridization (FISH), as described in Wang et al. (2013b). *Candidatus Accumulibacter phosphatis* (hereafter referred to as *Accumulibacter*), *Candidatus Competibacter phosphatis* (hereafter referred to as *Competibacter*), *Defluvicoccus*-related TFO, and *Defluvicoccus*-related DF were targeted by appropriate oligonucleotide probes (Text S2 and Table S1).

2.6. Determination of cell decay rate

The decay rate of DPAOs was estimated from the measured NH_4^+-N release rate based on the activated biomass composition ($CH_{2.09}O_{0.54}N_{0.20}P_{0.015}$) (Smolders et al., 1994), i.e., primarily based on the reduction in the amount of bacteria. The MLVSS is assumed as the sum of PHA, glycogen, and active biomass (Smolders et al., 1995). Thus, the active biomass is estimated by subtracting the PHA and glycogen content from the MLVSS. Accordingly, the decay rate is calculated based on Eq. (1) (Lesouef et al., 1992):

$$b = -\ln (X_t/X_0)/t_d \quad (1)$$

where b is the decay death rate of the PAOs, X_0 and X_t denote the active biomass concentration (without glycogen and PHA) before and after starvation respectively, and t_d is the duration of the starvation period.

3. Results

3.1. Performance of SBR_{NO2-} and SBR_{NO3-} and relevant microbial populations

The SBR_{NO2-} and SBR_{NO3-} steadily operated for 210 days. Typical cycle tests were conducted during steady-state operation (Figure S1) and the DPAOs activities were estimated from the biochemical reaction rates. The maximum rates of P release and uptake, denitrification, and intracellular polymer transformations were higher for nitrite DPAOs than those for nitrate-DPAOs (Table 1), indicating that DPAOs activities are greater in the nitrite-fed than in the nitrate fed reactors.

FISH results show that *Accumulibacter*, bound to the PAOMIX probe, were the

231 dominant organisms in both reactors, comprising 66 ± 1.5% and 75 ± 1.1% of total
232 biomass in SBR_{NO3-} and SBR_{NO2-}, respectively (Table 1). The ratio of PAOII (unable
233 to use nitrate as an electron acceptor) to total PAOs was 57.6% and 72% in SBR_{NO3-}
234 and SBR_{NO2-}, respectively. *Defluvicoccus*-related GAOs were approximately 27 ± 0.9%
235 and 19 ± 0.6% in SBR_{NO3-} and SBR_{NO2-}, respectively, while *Competibacter*-related
236 GAOs were nearly undetectable (< 1 %) in both SBRs (Table 1).

237 **3.2. Anaerobic starvation in nitrite/nitrate DPAOs biomass**

238 ***3.2.1 Release of NH_4^+-N and PO_4^{3-}-P, and reduction of MLSS and MLVSS***

239 Since the NH_4^+-N profile reflects the biomass growth condition, it is a useful tool for
240 determining the cell decay of DPAOs (Zeng et al., 2003b; Lu et al., 2007). In all
241 reactors, the NH_4^+-N concentrations increased gradually during the first 3 days of
242 starvation and rapidly thereafter (Figure 1a), indicating the increasing extent of cell
243 decay. Within 12 days of starvation, cell decay (including cell lysis and the respiration
244 of intracellular materials) released 41.8 mg, 39.5 mg, 18.7 mg and 15.8 mg of
245 NH_4^+-N/L into the liquid phase of A-R_{NO3-}, O-R_{NO3-}, A-R_{NO2-} and O-R_{NO2-},
246 respectively (Figure 1a and Table 2). The ammonia release rates were clearly much
247 higher in nitrate-DPAO sludges than those in nitrite-DPAO sludges (Table 2), whereas
248 no appreciable difference was observed in the NH_4^+-N release rates between
249 $AS_{ANA.end}$ and $AS_{AER.end}$ (Figure 1a).

250 The starvation period in all reactors was also marked by P release, as
251 intracellular poly-P was degraded to obtain energy for maintenance processes.

Specifically, the P release rate was elevated during day 1 – 5 in all reactors; thereafter, the P concentration in the bulk remained constant (Figure 1b). Correspondingly, the MLSS concentrations gradually decreased during the first 5 days (Figure 2a). Notably, the MLSS concentration decreased less in nitrite-DPAO sludges (A-R_{NO2-} and O-R_{NO2-}) than those in nitrate-DPAO sludges (A-R_{NO3-} and O-R_{NO3-}) (Figure 2a). These findings are related to the lower P release rate in A-R_{NO2-} and O-R_{NO2-} (Table 3). Among these reactors, the MLSS and MLVSS concentrations were most heavily reduced in O-R_{NO3-}, largely because the storage products (especially glycogen and poly-P) were most depleted in this reactor, accounting for approximately 51% and 33% of the decrease of MLVSS and MLSS, respectively, in O-R_{NO3-} (Table 3). Similarly, Lopez et al. (2006) reported that maintenance processes utilizing organic (PHA and glycogen) and inorganic (poly-P) storage products accounted for about 23% and 29% of the aerobic decrease of MLVSS and MLSS, respectively. The MLVSS, MLSS and MLVSS/MLSS variations in $AS_{ANA.end}$ were comparable to those in $AS_{AER.end}$ from the same parent SBR (Figure 2).

3.2.2 Variations in glycogen and PHA contents

In all four reactors, most of the glycogen was consumed within the first 5 days (Figure 3). The glycogen degradation was approximately 21% and 38% lower in the $AS_{ANA.end}$ and $AS_{AER.end}$ sludges, respectively, when compared with those in their nitrate-DPAO sludge counterparts (Figure 3 and Table 3). These findings indicate a relatively lower energy requirement for glycogen hydrolysis in nitrite-DPAOs, and may also correlate

273 with the lower GAO percentage in nitrite-DPAO sludge (19 ± 0.6%) than in
274 nitrate-DPAO sludge (27 ± 0.9%) (Table 1). Moreover, the amounts of glycogen
275 degradation were much lower in $AS_{ANA.end}$ (A-R_{NO3-} and A-R_{NO2-}) than those in
276 $AS_{AER.end}$ (O-R_{NO3-} and O-R_{NO2-}). Since glycogen had been partially degraded by
277 anaerobic reactions to supply reducing equivalents before the starvation test, it is
278 likely that less glycogen was available for $AS_{ANA.end}$ as compared to that for $AS_{AER.end}$.
279 These findings agree with our previous observation (Wang et al., 2012) that glycogen
280 degradation rate is approximately 48% lower for $AS_{ANA.end}$ than for $AS_{AER.end}$ after 7
281 days of anaerobic starvation at 15 °C.

282 PHA was synthesized during the 12 starvation days in all four reactors. Most of
283 the PHA was synthesized during the first 5 days (Figure 3), corresponding to the high
284 glycogen degradation. The main PHA components of nitrite-and nitrate-DPAO
285 sludges fed with propionate as the sole carbon source were PH2MV and PHV (Table
286 3). During the 12 days of starvation more PHA was synthesized by $AS_{ANA.end}$ in
287 A-R_{NO2-} (2.79 mmol-C/g-MLVSS) than that by $AS_{ANA.end}$ in A-R_{NO3-} (1.84
288 mmol-C/g-MLVSS) (Table 3), and the PHA synthesis rate in A-R_{NO2-} was almost
289 twice that in A-R_{NO3-}. Similar results were obtained for $AS_{AER.end}$, suggesting that
290 most of the degraded glycogen was converted to PHA in the nitrite-DPAO sludges.

291 ***3.2.3 Variations in EPS amounts and compositions***

292 EPS production is essential for the survival of *Accumulibacter* in wastewater
293 treatment systems (Martín et al., 2006). As a candidate carbon and energy source,

294 EPS degradation allows the microorganisms to rapidly adapt to varying influent
295 composition, temperature (Martín et al., 2006) and substrate limitation (Zhang and
296 Bishop, 2003). However, the contribution of EPS to the anaerobic endogenous
297 metabolism of DPAOs has not been previously reported. We also report the first
298 description of EPS variations during anaerobic starvation of DPAO sludges.
299 Figure 4 presents the EPS profiles after 3 and 12 days of starvation. The initial
300 amounts of EPS in A-R_{NO2-} and O-R_{NO2-} sludges were approximately 17.0 and 11.5%
301 lower, respectively, than those in their A-R_{NO3-} and O-R_{NO3-} counterparts (Figure 4d).
302 This discrepancy is attributable to the different polysaccharides content in the EPS of
303 different sludges. In particular, polysaccharides synthesis may be prevented by the
304 presence of free nitrous acid (FNA) in nitrite-DPAOs (Wang et al., 2013a).
305 Specifically, the initial polysaccharides content of EPS was 60.8% and 71.3% higher
306 in A-R_{NO3-} and O-R_{NO3-} than those in A-R_{NO2-} and O-R_{NO2-}, respectively (Figure 4b).
307 Polysaccharides degradation in A-R_{NO3-} and O-R_{NO3-} was almost complete within 3
308 days of starvation (Figure 4b). Similarly, Wang et al. (2007) reported a sharp decrease
309 (approximately 50%) in the polysaccharides content of EPS in highly resistant aerobic
310 granules starved for 4 days. In A-R_{NO2-} and O-R_{NO2-}, polysaccharides degradation
311 throughout the first 3 days was only 3.7 ± 0.2 mg/g MLVSS and 1.2 ± 0.0 mg/g
312 MLVSS respectively, accounting for approximately 32% and 12% of the total
313 polysaccharides content, respectively (Figure 4b). At the end of the starvation on day
314 12, the polysaccharides contents remained low in all reactors.

315 The proteins changes in the four reactors greatly differed from the
316 polysaccharides changes (Figure 4a and b). The proteins content in all reactors
317 slightly increased during the first 3 days of starvation and had decreased to low levels
318 by day 12. It is speculated that, early in the starvation period, cell decay processes
319 released a portion of the intracellular proteins into the extracellular space, where it
320 was captured by EPS. Cellular decay is supported by the increased ammonia
321 concentration in all four reactors during the first 3 days of starvation (Figure 1a). At
322 the end of the starvation period, the proteins contents in all four reactors were heavily
323 reduced, suggesting that proteins may also be hydrolyzed or degraded by the
324 microorganisms as carbon and energy sources. The difference in EPS content
325 variations was negligible between $AS_{ANA.end}$ and $AS_{AER.end}$ from the same parent
326 (SBR_{NO3-} or SBR_{NO2-}).

327 **3.3. Recovery of nitrite- and nitrate-DPAOs activities after 12 days of starvation**

328 ***3.3.1 Recovery of nitrite- and nitrate-DPAOs in $AS_{ANA.end}$***

329 Nitrite denitrification was completed during the first recovery cycle in both A-R_{NO3-}
330 and A-R_{NO2-}. The P uptake efficiency of nitrite-DPAOs was actually slightly increased
331 relative to the pre-starvation efficiency (Figure 5c and d; Figure S1 d). This increase
332 is attributed to the relatively lower "secondary" P release after nitrite depletion by
333 starved microorganism (1.6 mg PO_4^{3-}-P/L vs. 10.8 mg PO_4^{3-}-P/L in a typical
334 pre-starvation cycle). In contrast, the efficiencies of nitrate denitrification and anoxic
335 P uptake in starved nitrate-DPAOs sludge were decreased by 42.9% and 47.2%,

336 respectively, relative to their pre-starvation levels in a typical cycle (Figure 5a and b;
337 Figure S1c and d). The rapid recovery of nitrite-DPAOs activities indicates a higher
338 ability of these organisms to overcome starvation shock. The strong anoxic
339 denitrification and P uptake efficiency during starvation was also partially contributed
340 by PHA synthesis (Figure 3), which provides an electron donor and energy source for
341 the DPAOs. Moreover, the higher amounts of PHA synthesis by nitrite-DPAOs than
342 those by nitrate-DPAOs during the endogenous period (Table 3) may accelerate the
343 anoxic metabolism of nitrite-DPAOs during the recovery period, further benefitting
344 the stable operation of shortcut denitrification P removal systems.

345 In the second recovery batch test of $AS_{ANA.end}$, the concentration of the released P
346 reached 48.0 mg PO_4^{3-}-P/L and 47.3 mg PO_4^{3-}-P/L in A-R_{NO3-} and A-R_{NO2-}
347 respectively, with a respective recovery percentage of 91.7% and 82.3%.

348 ***3.3.2 Recovery of nitrite- and nitrate-DPAOs in $AS_{AER.end}$***

349 The VFA concentration in O-R_{NO3-} and O-R_{NO2-} ($AS_{AER.end}$) decreased from its initial
350 5.89 mmol C/L to 5.16 mmol C/L and 4.60 mmol C/L, respectively, at the end of the
351 anaerobic phases in the first recovery batch test (data not shown) , representing a
352 decrease of 87.6% and 78.1%, respectively, compared with the amount of VFA
353 assimilated by their parents (SBR_{NO3-} and SBR_{NO2-}) in the anaerobic phase. Indeed,
354 during the anaerobic phase of both parent SBRs, the added VFA were completely
355 depleted within 30 minutes (Figure S1).

356 The total amount of released P also sharply declined in O-R_{NO3-} and O-R_{NO2-}, by

86.9% and 79.4% respectively, relative to their corresponding values in the typical parent SBRs (Table 4). This result may be attributed to rapid degradation of poly-P during starvation, leaving minimal quantities of poly-P in DPAOs cells starved for 12 days. During the subsequent anoxic phases, the amount of P uptake in O-R_{NO3-} and O-R_{NO2-} was only 4.1 and 5.7 mg PO_4^{3-}-P/L respectively, indicating a severe deterioration of P removal ability (Table 4). Nevertheless, the effluent P concentration in O-R_{NO2-} remained at 1.3 mg PO_4^{3-}-P/L, suggesting that activity was recovered more rapidly in nitrite-DPAOs sludge than in nitrate-DPAOs sludge (Figure 5b and d). In contrast to the largely diminished P removal, the denitrification efficiencies during the anoxic phase were similar to those observed during typical cycles of the parent SBRs. This result is likely due to the presence of ordinary heterotrophs (OHOs) and denitrifying glycogen-accumulating microorganisms (DGAOs), which might use the residual VFA of the anaerobic phase for anoxic denitrification.

4. Discussion

The endogenous processes of PAOs can be differentiated into two aspects: (i) maintenance processes linked to utilization of intracellular polymers (mainly poly-P and glycogen) and EPS, which maintain cellular integrity and activity; and (ii) decay processes, which reduce the amount and/or activity of the active biomass (Lopez et al., 2006; Hao et al., 2010b).

4.1. Cell maintenance in the nitrite/nitrate-DPAOs sludge

4.1.1 Energy and reducing equivalent sources from intracellular polymers

While poly-P is consensually regarded as a maintenance source during anaerobic starvation, glycogen is an important energy pool as well as an equivalent reducing source for the maintenance of PAOs (Lopez et al., 2006; Lu et al., 2007; Wang et al., 2012; Vargas et al., 2013). Under anaerobic conditions, glycogen is usually processed for maintenance through a combination of glycolysis and the PHA production pathway (Lopez et al., 2006; Lu et al., 2007). The main reactions involved in glycogen degradation and PHA formation in nitrite- and nitrate-DPAOs were described in details in previous studies (Filipe et al. 2001; Zeng et al. 2003a). The overall reaction (in terms of the molar relationships) is given by Eq. (2):

$$-\text{glycogen} + 1/6\ \text{PHB} + 5/12\ \text{PHV} + 1/4\ \text{PH2MV} + 1/2\text{ATP} = 0 \qquad (2)$$

PHA synthesis in the present starvation test is summarized in Table 3. The ratios among PHB, PHV and PH2MV in the nitrate-DPAOs sludge reactors are well-predicted by Eq. (2). However, the proposed stoichiometry of anaerobic maintenance does not adequately describe the composition of the PHA synthesized by starved nitrite-DPAO sludges. The higher PH2MV content suggests that the investigated nitrite-DPAO sludges adopt different survival metabolic pathways in their endogenous processes. This may correlate with a larger fraction of propionyl-CoA (precursor of PH2MV) produced from acetyl-CoA through the methylmalonyl pathway, as reported by Yagci et al. (2003). Similarly, Oehmen et al.

(2006) analyzed the PHA composition in anaerobic test batch culture without propionate addition, and obtained 12% PHB, 41% PHV and 47% PH2MV. They attributed this result to enrichment of *Alphaproteobacteria* GAOs. The PHA synthesis mechanism in nitrite-DPAOs during starvation is detailed in the SI (Text S3**)**.

4.1.2 Maintenance substrate and energy sources from EPS

EPS is an important potential energy source that enables DPAOs to manage starvation shock. In all reactors, EPS were almost completely depleted after 12 days of starvation, and minimal non-biodegradable EPS remained in the sludge (Figure 4). Especially, the easily biodegradable components of EPS (i.e., polysaccharides) were rapidly utilized for maintenance during the initial starvation period, followed by utilization of proteins (Figure 4a and b). Indeed, various extracellular enzymes have been detected in activated sludge and biofilms, many of which can potentially degrade EPS components to low-molecular-mass compounds that can then be utilized by microorganisms as carbon and energy sources during starvation periods (Flemming et al., 2010; Zhang and Bishop, 2003; Liu et al., 2007). The main degraders of EPS polysaccharides are hydrolases and lyases (Laue et al., 2006), such as N-acetyl-β-hexosaminidase (Kaplan et al., 2004). Metagenomic analysis of two EBPR sludge communities has revealed the genes encoding enzymes for carbohydrate polymer hydrolysis and subsequent monomer degradation pathways in the dominant flanking species, namely, *Flexibacter*-like, *Xylella*-like and *Thiothrix*-like populations (Martín et al., 2006). Martín et al. (2006) also reported that one of the EPS gene

418 cassettes in *Accumulibacter* encodes UDP-glucose dehydrogenase, which catalyzes
419 the precursor of the glucoronic acid component of EPS. Therefore, the dominant
420 flanking *Xylella*-like population may be able to degrade the glucoronic acid in
421 *Accumulibacter* EPS as a carbon and energy source. Additionally, EPS extracted from
422 biofilms were also found to be effectively degraded by their own producing
423 microorganism (Zhang and Bishop, 2003).

424 After 3 days of starvation, at least 90% less polysaccharides were degraded by
425 nitrite-DPAO sludges than by nitrate-DPAO sludges (Figure 4b). This result may be
426 ascribed to the relatively low initial polysaccharides content in the SBR_{NO2-} sludge. It
427 should also be mentioned that because the degradation pathway of proteins is more
428 complicated than that of polysaccharides, it is not certain that the decreased proteins
429 (in EPS) contents had been used as substrate to produce energy by cells or only had
430 been hydrolyzed or broken down into its component, i.e., amino acids. Since amino
431 acids in the bulk were not analyzed during the starvation period, the exact amount of
432 EPS proteins being degraded as substrate cannot be accurately determined, and
433 requires further study.

434 ***4.1.3 Energy production from intracellular polymers and EPS***

435 Maintenance energy is critical for the survival of nutrient-limited bacteria. PAOs
436 generally use poly-P and glycogen as maintenance energy sources under anaerobic
437 starvation conditions (Lopez et al., 2006; Lu et al., 2007). However, the main source
438 between them has yet to be clarified. Lu et al. (2007) found that, early in the

439 starvation period, PAOs prefer glycogen to poly-P as an energy source. In contrast,
440 Lopez et al. (2006) found that PAOs sequentially consume poly-P and glycogen,
441 which is supported by lack of any P released during the first day of starvation. No
442 glycogen degradation was observed during an 8-hour anaerobic starvation batch test
443 (Oehmen et al., 2005), supporting the assumption that PAOs use their stored poly-P as
444 the sole source of maintenance energy. Therefore, in the present study, we examined
445 the energy produced by both glycogen and poly-P. Moreover, the polysaccharides in
446 EPS were considered as a potential additional maintenance energy source when
447 calculating the maintenance ATP (Text S4).

448 As shown in Figure 6, glycogen and poly-P were simultaneously utilized during
449 the first 3 days of starvation, similar to our previous observations (Wang et al., 2012).
450 Notably, the polysaccharides in EPS also contributed a sizeable fraction of the
451 maintenance energy, especially in nitrate-DPAO sludge (Figure 6a and b). Hydrolysis
452 of 1 mol poly-P presumably yields 1 mol ATP, while degradation of 1 mol-C glycogen
453 and 1 mol-C polysaccharides should each generate 0.5 mol ATP (Smolders et al.,
454 1994). Maintenance energy production by DPAOs under anaerobic conditions was
455 calculated from the amounts of glycogen consumed and P released (Table 3 and
456 Figure 4).

457 In all reactors, most of the ATP was produced during the first 5 days of starvation.
458 Glycogen was the primary maintenance energy source throughout the first 1.5 days,
459 contributing more than approximately 70% to the total energy production in all four

460 reactors (Figure 6a-d). During day 1.5 – 3, poly-P was increasingly used as an
461 alternative maintenance energy source, and its use dominated (contributed over 50%
462 of the total ATP production) on day 5 in all four reactors (Figure 6a-d). Similar
463 observation was also reported by Lu et al. (2007), who found that glycogen
464 degradation provided the majority of the energy on day 1, after which there was a
465 transition of the primary energy source from glycogen to poly-P. Whereas, Lopez et al.
466 (2006) observed a sequential utilization of poly-P and glycogen by PAOs for
467 maintenance energy production under anaerobic conditions. The reasons for this
468 discrepancy have not been presented. Moreover, as these two studies did not present
469 the data of the GAOs percentage, it is not easy to explain this discrepancy from the
470 microbial point of view (e. g., PAOs vs. GAOs). In the present study, we doubt that
471 this result might arise from the relatively high proportion of GAOs in both SBR_{NO3-}
472 (27 ± 0.9% of the total biomass) and SBR_{NO2-} (19 ± 0.6% of the total biomass) (Table
473 1). The major energy source of GAOs is glycogen, while poly-P hydrolysis supplies
474 energy for the dominant PAOs. In the nitrate-DPAOs sludges, polysaccharides
475 provided additional maintenance energy during the first 3 days of starvation, with
476 values of 16.2 and 20.3 ATP mmol/C-mol biomass for $AS_{ANA.end}$ and $AS_{AER.end}$,
477 accounting for 27% and 19% of the total energy production, respectively (Figure 6a
478 and b). After day 5, a marked decline in energy production was observed (Figure
479 6a-d). Such relatively low energy production may not meet the minimal energy
480 demands of DPAOs. The resulting cell lysis is evidenced in a sharp increase in

481 NH_4^+-N concentration (Figure 1).

482 During the 12-day starvation, energy production of nitrite-DPAO sludges in
483 $AS_{ANA.end}$ and $AS_{AER.end}$ were estimated to be 31% and 34% lower, respectively, than
484 those of nitrate-DPAO sludges (Figure 6a-d). The relatively low requirement for
485 maintenance energy of nitrite-DPAOs is further supported by the constant amounts of
486 EPS polysaccharides within the first 3 days (Figure 4). This result may reflect a robust
487 starvation survival response in nitrite-DPAOs. Following each nitrite addition, the
488 parent SBR_{NO2-} was exposed to episodes of famine (no nitrite was available during the
489 first 30 – 70 min of the anoxic period; see Figure S1b). Consequently, nitrite-DPAOs
490 relied on their internal poly-P and/or glycogen reserves for maintenance energy, as
491 evidenced by the irregular P release and glycogen consumption during the anoxic
492 phases of typical cycles (Figure S1d and f). Because of the frequent starvation
493 episodes, and the high number of nitrite/FNA shocks in the parent SBR_{NO2-}, the
494 nitrite-DPAOs were primed to adjust their endogenous mechanisms by slowing down
495 their self-oxidation rate and lowering their maintenance energy, i.e., consuming less
496 glycogen and poly-P, to survive under the imposed starvation conditions. Thus,
497 acclimation of nitrite-DPAOs to the starved conditions may allow them higher
498 adaptability to starvation shock than their nitrate-DPAO counterparts. In addition,
499 $AS_{AER.end}$ required more energy than $AS_{ANA.end}$ (Figure 6a-d), primarily owing to the
500 lower energy available from glycogen and poly-P in $AS_{AER.end}$.

4.2 Decay process of the nitrite/nitrate-DPAO sludges

Cell decay refers to any process that reduces the weight (negative growth) and the specific activity of biomass. The cell decay rates in A-R_{NO3-}, O-R_{NO3-}, A-R_{NO2-} and O-R_{NO2-} were estimated to be 0.022, 0.022, 0.008 and 0.007 day^{-1}, respectively (Table 2). The cell decay rates were much lower in nitrite-DPAO sludges than in nitrate-DPAO sludges, and were comparable to the value reported by Lu et al. (2007) (0.006 day^{-1}) for PAOs under anaerobic starvation conditions.

As mentioned in ***Section 4.1.3***, frequent starvation episodes in the parent nitrite-SBR might evoke stringent starvation responses in the nitrite-DPAOs sludge. In other words, nitrite-DPAOs possess a better survival strategy via a relatively low maintenance energy requirement to ensure their survival in nutrient-limited systems (Salem et al., 2006). The decay rates of DPAOs in $AS_{ANA.end}$ and $AS_{AER.end}$ did not appreciably differ in the present study.

4.3. Recovery of nitrite/nitrate-DPAOs activities

Transition from starvation to full functionality is essential for the survival of bacterial systems (Lu et al. 2007). Vargas et al. (2013) found that once wastewater is reintroduced, both PAOs and GAOs can recover their initial acetate uptake rates, indicating strong survival ability during the starvation period. Yilmaz et al (2007) also reported that the P-release and P-uptake in a starved culture were fully recovered within 4 days of gradual re-introduction of influent wastewater. In the present study, nitrite-DPAOs recovered from 12-day starvation within one day (Figure 5). These

findings well correspond with the lower maintenance energy requirement and decay rate in nitrite-DPAO sludges than those in nitrate-DPAO sludges. In particular, for the nitrite-DPAOs sludge, less (about 40%) PHA consumption for anoxic P uptake as well as high PHA synthesis during the endogenous period than that of the nitrate-DPAOs sludge (Table 3) may speed up their anoxic metabolism during the recovery period, which thus provides an additional benefit to the stable operation of shortcut denitrification P removal systems.

Microorganisms in $AS_{ANA.end}$ rapidly recovered their intracellular polymer transformation ability, especially for the nitrite-DPAOs in A-R_{NO2-}, where glycogen synthesis and PHA degradation reached 179% and 99%, respectively, of their pre-starvation levels (Figure 5g and Figure S1f). This observation is consistent with the strong recovery of denitrifying P removal efficiency with the value of 136% (Table 4), indicating a high activity of nitrite-DPAOs. The post-starvation nitrate reduction rate in A-$R_{NO3-}$was approximately 35% less than the pre-starvation rate. Since no external carbon was added in the anoxic phase during the first recovery batch test, OHOs could not have been involved in nitrite/nitrate reduction. Thus, nitrate denitrification was accomplished by PAOI and/or DGAOs (Zeng et al., 2003b). PAOI can simultaneously reduce nitrate and remove anoxic P, but PAOII cannot use nitrate as an electron acceptor (Flowers et al., 2009). Therefore, the reduced nitrate denitrification rates suggest that starvation shock may inhibit the activity of PAOI and/or DGAOs.

543 The metabolism of intracellular materials in $AS_{AER.end}$ was severely inhibited by
544 long-term starvation, as evidenced by the decrease in P uptake and release. Conversely,
545 both reactors achieved complete denitrification, probably because the abundant
546 propionate (2.89 and 3.22 mmol C/L for O-R_{NO3-} and O-R_{NO2-}, respectively) derived
547 from the preceding anaerobic phase stimulated the denitrification performance of
548 OHOs. However, the low effluent P concentration in O-R_{NO2-} confirmed that a fully
549 functional system could be restored by the relatively high fraction of PAOII (Table 1).
550 In summary, the relatively low consumption of intracellular polymers, slow cell
551 decay and rapid recovery of activity in nitrite-DPAO sludges (especially originating
552 from the anaerobic end phase of the DPR process) demonstrate a strong ability to
553 cope with starvation shock. These endogenous process characteristics of nitrite-DPAO
554 sludges, combined with 25% reduction in the oxidation cost and 40% reduction in
555 carbon consumption, may be exploited in efficient shortcut denitrification P removal.

556 **5. Conclusions**

557 (1) The anaerobic maintenance energy was approximately 30% lower in
558 nitrite-DPAOs sludge than that in nitrate-DPAOs sludge. Glycogen and poly-P
559 sequentially served as the primary maintenance energy sources in starved
560 nitrite/nitrate-DPAOs. The polysaccharides in EPS were rapidly consumed by
561 nitrate-DPAOs sludge during the first 3 days of starvation; conversely, nitrite-DPAOs
562 sludge converted less of these polysaccharides into maintenance energy.

563 (2) The estimated cell decay rates in A-R_{NO3-}, O-R_{NO3-}, A-R_{NO2-} and O-R_{NO2-} were

564 0.022, 0.022, 0.008, and 0.007 day^{-1}, respectively. Clearly, the cell decay rates were
565 lower in nitrite-DPAOs than those in nitrate-DPAOs, indicating a better stringent
566 starvation response by nitrite-cultivated DPAOs than by their nitrate-cultivated
567 counterparts.

568 (3) After 12 days of starvation, nitrite-DPAO sludges recovered more rapidly than
569 nitrate-DPAO sludges. The denitrifying P removal efficiencies, as well as the
570 transformation rates of intracellular polymers in the nitrite-DPAO sludges (especially
571 that from the end of the anaerobic phase) were almost identical to their pre-starvation
572 values, indicating a rapid return (within 1 day) to full functionality.

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680 **Figure captions**

681 **Fig. 1.** Variations in (a) NH_4^+-N and (b) PO_4^{3-}-P concentrations in A-R_{NO3-}, O-R_{NO3-},

682 A-R_{NO2-}, and O-R_{NO2-} during the 12 days of anaerobic starvation.

683 **Fig. 2.** Variations in (a) MLSS, (b) MLVSS, and (c) MLVSS/MLSS ratio in A-R_{NO3-},

684 O-R_{NO3-}, A-R_{NO2-} , and O-R_{NO2-} during the 12 days of anaerobic starvation.

685 **Fig. 3**. Variations in glycogen and PHA during the 12 days of anaerobic starvation: (a),

686 A-R_{NO3-}, (b), O-R_{NO3-}, (c), A-R_{NO2-} , and (d), O-R_{NO2-}.

687 **Fig. 4.** EPS contents profile during the 12 days of anaerobic starvation in A-R_{NO3-},

688 O-R_{NO3-}, A-R_{NO2-}, and O-R_{NO2-}: (a) proteins, (b) polysaccharides, and (c) EPS

689 (calculated as the sum of proteins, polysaccharides and humics). Data are the averages

690 and their standard deviations in triplicate tests.

691 **Fig. 5.** Variations in NOx^--N, PO_4^{3-}-P, glycogen and PHA in the recovery batch tests

692 after 12 days of anaerobic starvation: (a), (e), A-R_{NO3-}; (b), (f), O-R_{NO3-}; (c), (g),

693 A-R_{NO2-}; and (d), (h), O-R_{NO2-}.

694 **Fig. 6.** ATP production profile during the 12 days of anaerobic starvation: (a), A-R_{NO3-},

695 (b), O-R_{NO3-}, (c), A-R_{NO2-}, and (d), O-R_{NO2-}.

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Table 1. Comparison of the anaerobic carbon transformations, PO_4^{3-}-P release, and biomass compositions (± standard error) with propionate supplied as carbon source in a typical cycle

	FISH quantification					PO_4^{3-}-P / VFA[f]	Gly/ VFA[g]	PHA/ VFA[g]	PHB/ VFA[g]	PHV/ VFA[g]	PH2MV/ VFA[g]
	Ac_{Tot}[a] (%)	*Ac_I*[b] (%)	*Ac_{II}*[c] (%)	*De*[d] (%)	*Co*[e] *(%)*						
This study											
SBR_{NO3-}(nitrate-based)	66 ± 1.5	28 ± 1.9	38 ± 1.4	27 ± 0.9	<1	0.32	0.44	1.54	0.08	0.66	0.80
SBR_{NO2-}(nitrite-based)	75 ± 1.1	21 ± 1.2	54 ± 1.7	19 ± 0.6	<1	0.36	0.31	1.46	0.11	0.61	0.74
Previous studies											
Oehmen et al. (2005)	63 ± 1.3	-	-	<1	<1	0.42	0.32	1.23	0.04	0.55	0.65
Carvalho et al. (2007)	75	44	31	<1	<1	0.40	0.32	0.97	0	0.40	0.57
Tayà et al. (2013)	85	55	30	-	<10	0.34	0.49	1.47	-	-	-

[a] *Ac_{Tot}*: Total *Accumulibacter*. [b] *Ac* I: Type I *Accumulibacter*. [c] *Ac* II: Type II *Accumulibacter*. [d]*De*: *Defluvicoccus*. [e]*Co*: *Competibacter*.

[f] Units PO_4^{3-}-P mmol/C mmol. [g] Units C mmol/C mmol. - No data.

Table 2. Comparison of cell decay of PAOs/DPAOs in this study and previous studies during the 12 days of anaerobic starvation.

	Starvation condition	Initial MLVSS (g/L)	Temperature (°C)	Starvation period (d)	SRT (d)	NH_4^+-N release (mg/L)	Cell decay rate (1/d)
This study							
A-R_{NO3-}	anaerobic	2.5	20 ± 2	12	20	41.8[a]	0.022[a]
O-R_{NO3-}						39.5[b]	0.022[b]
A-R_{NO2-}						18.7[a]	0.008[a]
O-R_{NO2-}						15.8[b]	0.007[b]
Previous studies							
Lu et al. (2007)	anaerobic	1.6	22 ± 2	8	24	3.2[b]	0.006[b] (day 4– 8)
Hao et al. (2010a)	anaerobic	-	22 ± 0.5	7	12	-	0.036 ± 0.003[b]
Wang et al. (2012)	anaerobic	2.4	10 – 15	7	20	1.12[a]	0.0006[a]
						1.59[b]	0.0008[b]
Vargas et al. (2013)	aerobic-anaerobic	2.6	20 ± 2	21	46.8	14[b]	0.029[b]

[a] All results represent the sludge from the anaerobic end phases.

[b] All results represent the sludge from the aerobic end phases.

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Table 3. Transformations of materials in the liquid- and solid-phase in A-R_{NO3-}, O-R_{NO3-}, A-R_{NO2-} and O-R_{NO2-} during the 12 days of anaerobic starvation.

Parameters	Nitrate-sludge		Nitrite-sludge	
	A-R_{NO3-}	O-R_{NO3-}	A-R_{NO2-}	O-R_{NO2-}
MLSS reduction (mg/L)	770	1650	670	910
MLVSS reduction (mg/L)	357	793	360	347
PO_4^{3-}-P release (mg/L)	87.2	150.8	45.3	102.7
Glycogen degradation (mmol-C/g-MLVSS)	3.03	5.56	2.41	3.44
Glycogen degradation rate[a] (mmol-C/g-MLVSS/d)	0.61	1.11	0.48	0.69
PHB synthesis (mmol-C/g-MLVSS)	0.23 (0.12)	0.58 (0.19)	0.19 (0.07)	0.54 (0.15)
PHV synthesis (mmol-C/g-MLVSS)	1.07 (0.58)	1.42 (0.46)	1.16 (0.42)	1.40 (0.39)
PH2MV synthesis (mmol-C/g-MLVSS)	0.54 (0.29)	1.06 (0.35)	1.44 (0.52)	1.66 (0.46)
PHA synthesis (mmol-C/g-MLVSS)	1.84	3.06	2.79	3.61
PHA synthesis rate (mmol-C/g-MLVSS/d)	0.30[a]	0.58[a]	0.76[b]	1.02[b]
Polysaccharides consumption (mg/g-MLVSS)	36.6 ± 0.4	46.4 ± 0.5	11.7 ± 0.8	10.1 ± 0.4
Proteins consumption (mg/g-MLVSS)	144.0 ± 2.3	129.0 ± 13.3	133.1 ± 9.3	146.8 ± 1.5
Total EPS consumption (mg/g-MLVSS)	196.0 ± 0.1	200.1 ± 9.0	162.4 ± 0.3	177.6 ± 7.2

[a] All results obtained during the first 5 days.

[b] All results obtained during the first 3 days.

Data in the brackets represent the fractions of the components in PHA.

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Table 4. Comparison of denitrifying P-removal efficiency during anaerobic/anoxic phases in typical cycles and recovery batch tests.

Items	Amount of PO_4^{3-}-P release [a]	Amount of PO_4^{3-}-P uptake [a]	Amount of NO_x-N reduction [b]	P/N ratio
Nitrate-sludge				
SBR_{NO3-}	52.33	36.69	33.37	1.10
A-R_{NO3-}	47.98 (91.7) *	19.37 (52.8)	19.36 (58.0)	1.00 (63.7)
O-R_{NO3-}	6.87 (13.1)	4.09 (11.1)	33.68 (100.9)	0.12 (7.6)
Nitrite-sludge				
SBR_{NO2-}	57.48	26.44	33	0.80
A-R_{NO2-}	47.30 (82.3) *	35.97 (136.0)	30 (90.9)	1.20 (69.0)
O-R_{NO2-}	11.82 (20.6)	5.73 (21.7)	33 (100.0)	0.17 (9.8)

706 [a] Units mg PO_4^{3-}-P/L.

707 [b] Units mg NO_x^--N/L.

708 * Results obtained from the second recovery batch tests.

709 Data in brackets represent the percentage recovery relative to values obtained in typical cycles of

710 the corresponding parent SBRs (%).

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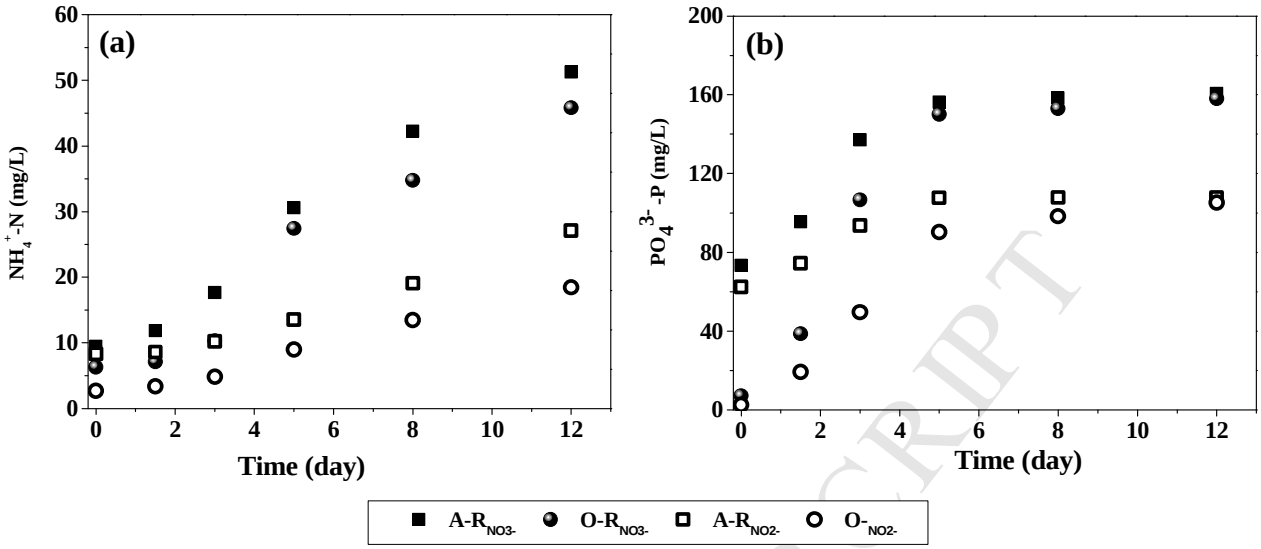
715 **Fig. 1**



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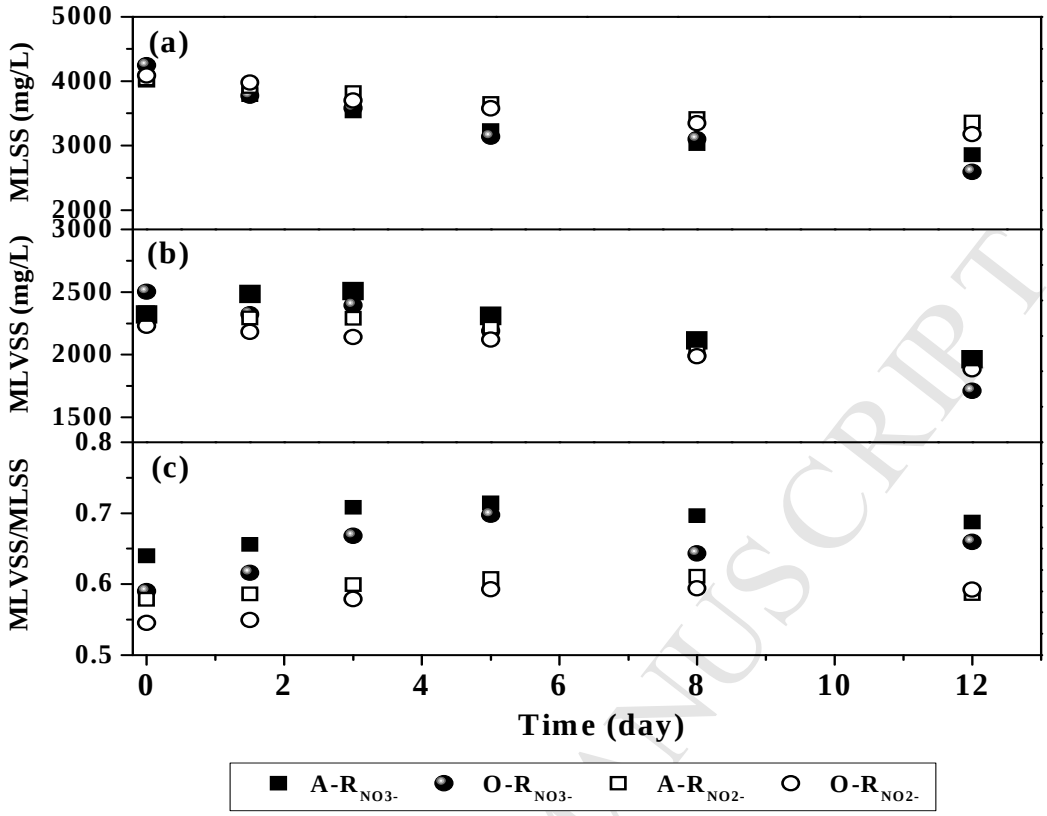
719 **Fig. 2**



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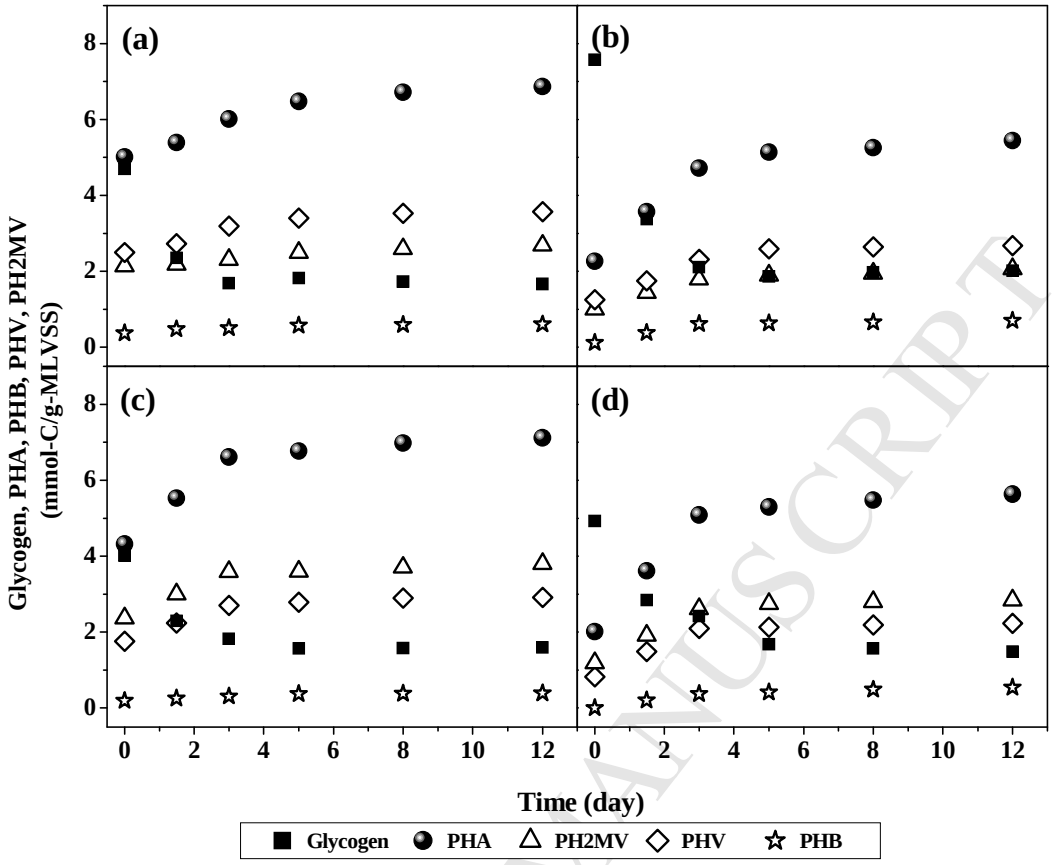
728 **Fig. 3**



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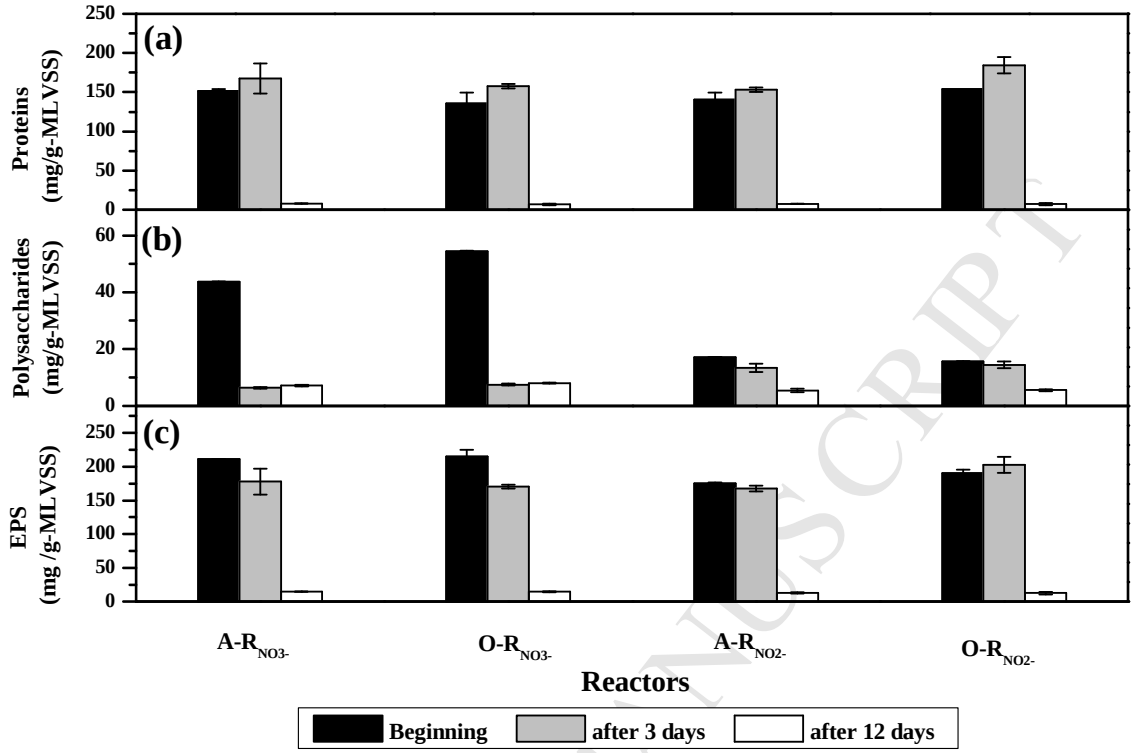
733 **Fig. 4**



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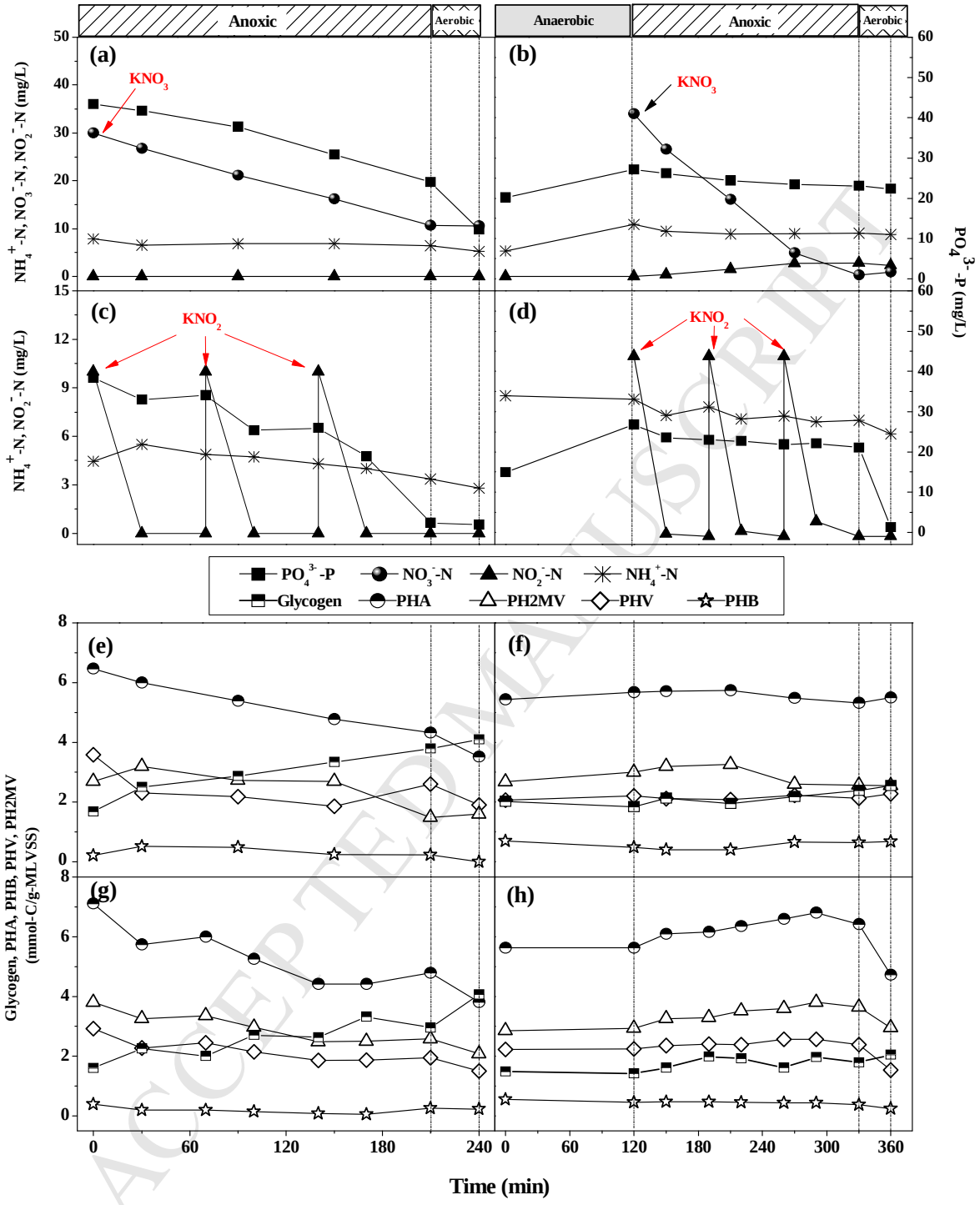
737 **Fig. 5**



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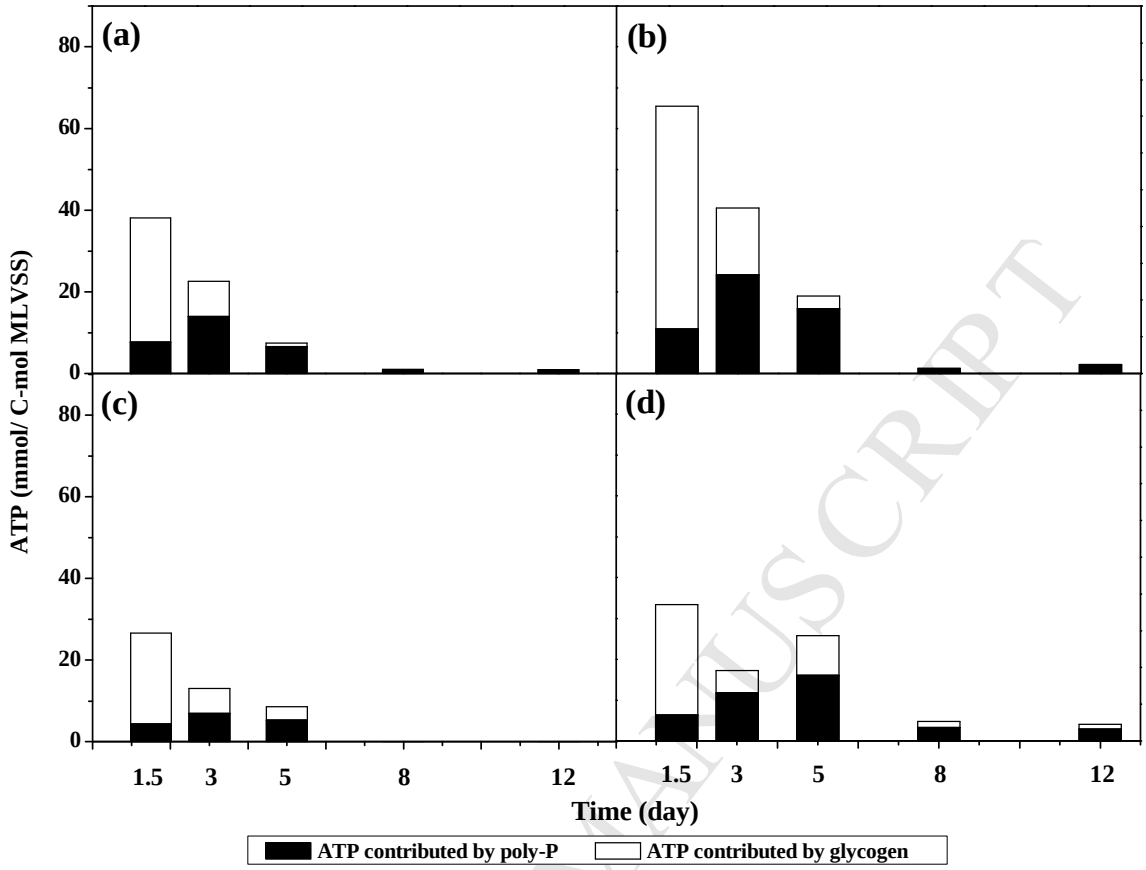
741 **Fig. 6**



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Supplementary Information

Title: Comparison of endogenous metabolism during long-term anaerobic starvation of nitrite/nitrate cultivated denitrifying phosphorus removal sludges

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Text S1. The detailed descriptions of analysis of VFA, PHA, glycogen, and EPS

a) VFA

VFA (propionate) was measured using an Agilent 6890N gas chromatograph (GC) equipped with a 30 m×0.53 mm×1 μm (length×ID×film) DB-WAXetr column and a flame ionization detector (FID) at 220 °C.

b) PHA

PHA, including poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV), poly-3-hydroxy-2-methylvalerate (PH2MV), was measured according to a method described by Oehmen et al. (2005). Briefly, approximately 20 mg freeze-dried samples of biomass were put into screw-topped glass tubes, and 2 mL of chloroform, 2 mL of an acidified methanol solution (10% H_2SO_4) and 0.1mL benzoic acid methanol solution (2 g of benzoic acid dissolved in 100mL methanol used as an internal standard) were subsequently added. The samples were then digested for 4h at 100 °C. After cooling, 1 mL of distilled water was added and mixed vigorously with each sample. Thereafter, 1 h of settling time was allowed to achieve phase separation. When the phases were separated, approximately 1 mL of the bottom organic layer was transferred to the GC vials for analysis. 3 μL of the chloroform phase was analyzed with a gas chromatograph (Thermo Focus GC). The chromatograph was operated with a HP-5 column (30 m length×0.32 mm ID×0.25 μm film), a split injection ratio of 1:15 and helium as the carrier gas (1.5 mL/min). A flame ionisation detection (FID) unit was operated at 250 °C with an injection port temperature of 230 °C. The oven temperature was set to 80 °C for 4 min, increased at 8 °C /min to120 °C, and then to 220 °C at 30 °C /min and held for 2 min.

c) Glycogen

Glycogen was determined by the anthrone method (Frølund et al., 1996). A 5-ml volume of 0.6 M HCl was added to weighed (approximately 10 mg), freeze-dried biomass in screw-topped glass tubes, and then heated at 100 °C for 3 h. After cooling

788 to room temperature, samples were sheared by a vortex mixer (XW-80A,
789 Shanghai Qingpu Huxi Instrument Co., Ltd, China) for 1 min, and were transferred to
790 10-mL tubes, followed by centrifugation (CT15RT, Techcomp, China) for 5 min at
791 10 000 g. About 1 mL supernatant was added to 4 mL of anthrone-H_2SO_4 reagent (0.2%
792 anthrone (w/v) in 80% (v/v) H_2SO_4) in 10-mL colorimetric tubes. All tubes were
793 placed in a water bath at 100 °C for 10 min. After cooling at 4 °C for 5 min in cold
794 water, samples were measured by a UV/VIS spectrophotometer (UV765, Shanghai
795 Precision & Scientific Instrument Co., Ltd, China) at 625 nm. Glucose was used as
796 the standard.

797 **d) EPS**

798 A heat extraction method (Li et al., 2007) was applied to extract the EPS (including
799 loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS)) in the sludge. 30 mL
800 sludge suspension was first dewatered by centrifugation (CT15RT, Techcomp, China)
801 in a 50-mL tube at 4 000 g for 5 min. The supernatant was recovered for water quality
802 analysis. The sludge pellet in the tube was re-suspended into 15 mL of 0.05% NaCl
803 solution. The sludge mixture was then diluted with the NaCl solution (pre-heating to
804 70 °C) to its original volume of 30 mL. Immediately, the sludge suspension was
805 sheared by a vortex mixer (XW-80A, Shanghai Qingpu Huxi Instrument Factory,
806 China) for 1 min, followed by centrifugation at 4 000 g for 10 min. The organic
807 matter in the supernatant was readily extractable EPS, and was regarded as the
808 LB-EPS of the biomass. For the extraction of the TB-EPS, the sludge pellet left in the

809 centrifuge tube was re-suspended in 0.05% NaCl solution to its original volume of 30
810 mL. The sludge suspension was heated to 60 °C in a water bath for 30 min. After
811 cooling to room temperature, the sludge mixture was centrifuged at 4 000 g for 15
812 min. The supernatant collected was regarded as the TB-EPS of the sludge.

813 Both the LB-EPS and TB-EPS extractions were analyzed for proteins (PN),
814 polysaccharides (PS) and humic-like substances (HS). The PN and HS were analyzed
815 by a UV/VIS spectrophotometer (UV765, Shanghai Precision & Scientific Instrument
816 Co., Ltd, China) following the modified Lowry method (Frølund et al., 1995) using
817 bovine serum albumin (Sigma) and humic acid (Fluka) as the standards, respectively.
818 The PS content was determined by the anthrone method described by Frølund et al.
819 (1996) using glucose as the standard.
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Text S2. Fluorescent in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was performed as described by Amann (1995) using Cy5-labelled EUBMIX probes, which are specific for most Bacteria (Daims et al., 1999). The Cy3-labelled PAOMIX probe (PAO462, PAO651 and PAO846) were used to target *Candidatus Accumulibacter phosphatis* (Crocetti et al., 2000), a known PAO; the Cy3-labelled GAOMIX probe (GAOQ431, GAOQ989 and GB_G2), DFMIX probe (TFO_DF 218 and TFO_DF 618, and DF 988 and DF 1020) were used to target *Candidatus Competibacter phosphatis*, *Defluvicoccus*-related TFO (Cluster I) in *Alphaproteobacteria*, and *Defluvicoccus*-related DF (Cluster II) in *Alphaproteobacter*ia (Crocetti et al., 2002; Kong et al., 2002; Wong et al., 2004) to determine the microbial population distributions of the PAOs and GAOs (Table S1). All oligonucleotide probes were commercially synthesized, and all hybridization buffer contained 35% (v/v) formamide. Cy5-labeled Acc-I-444 was used to target PAOI *Accumulibacter*, while FAM-labeled Acc-II-444 was used to target PAOIIA, IIC and IID *Accumulibacter* (Flowers et al. 2009).

FISH preparations were visualized using a Zeiss LSM 510 Meta confocal laser-scanning microscope (CLSM) using a Plan-Apochromat 63×oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The percentage of *Accumulibacter*, *Competibacter* and *Defluvicoccus* in the entire bacterial population (EUBMIX) was determined via FISH image analysis using image analyzing software (Image-Pro Plus, V6.0, Media Cybernetics). The standard error of

842 the mean (SE_{mean}) was calculated as the standard deviation divided by the square root

843 of the number of images.

Supplementary Table S1. Oligonucleotide probes used for fluorescence in situ hybridization (FISH)

Probe	Probe sequence (5'-3')	Specificity	FA (%)	Reference
EUB 338	GCTGCCTCCCGTAGGAGT	Most Bacteria	35	Daims et al., 1999
EUB 338-II	GCAGCCACCCGTAGGTGT	Planctomycetales and other Bacteria	35	Daims et al., 1999
EUB 338-III	GCTGCCACCCG AGGAGT	Verrucomicrobiales and other Bacteria	35	Daims et al., 1999
PAO462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter spp.*	35	Crocetti et al., 2000
PAO651	CCC TCT GCC AAA CTC CAG	Most *Accumulibacter spp.*	35	Crocetti et al., 2000
PAO846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter spp.*	35	Crocetti et al., 2000
Acc-I-444	CCCAAGCAATTTCTTCCCC	Most *Accumulibacter* I	35	Flowers et al., 2009
Acc-II-444	CCCGTGCAATTTCTTCCCC	Most *Accumulibacter* IIA, IIC and IID	35	Flowers et al. , 2009
ALF969	TGGTAAGGTTCTGCGCGT	Most Alphaproteobacteria	35	Crocetti et al., 2002
GAOQ431	TCCCCGCCTAAAGGGCTT	Some *Competibacter spp.*	35	Crocetti et al., 2002
GAOQ989	TTCCCCGGATGTCAAGGC	Some *Competibacter spp.*	35	Crocetti et al., 2002
GB_G2	TTCCCCAGATGTCAAGGC	Some *Competibacter spp*.	35	Kong et al., 2002
TFO_DF218	GAAGCCTTTGCCCCTCAG	Some *Defluvicoccus vanus*	35	Wong et al., 2004
TFO_DF618	GCCTCACTTGTCTAACCG	Some *Defluvicoccus vanus*	35	Wong et al., 2004
DF988	GATACGACGCCCATGTCAAGGG	Some *Defluvicoccus vanus spp*.	35	Meyer et al., 2006
DF1020	CCGGCCGAACCGACTCCC	Some *Defluvicoccus vanus spp*.	35	Meyer et al., 2006

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Text S3. The detailed explanation for the PHA synthesis mechanism during anaerobic starvation

The PHA composition depends greatly on whether PAOs selectively or randomly condense activated acetyl-CoA and propionyl-CoA to form PHA. Oehmen et al. (2006) proposed that the stoichiometry of PAOs fed with propionate were closely correlated with the model based on selective condensation of activated acetyl-CoA and propionyl-CoA, while GAOs tend to randomly condense activated acetyl-CoA and propionyl-CoA in PHA formation. Lemos et al. (2003) concluded that the notable difference in the components of PHA in various studies is probably due to different populations and/or metabolisms. Because there were more GAOs (27 ± 0.5%) in nitrate-DPAOs, a better prediction was obtained by Eq. (2) (***Section 4.1.1***), which was based on the random condensation pattern. Conversely, the relatively high PH2MV content means a larger amount of activated propionyl-CoA production and/or a more preferentially selective binding together of activated propionyl-CoA molecules. The exact underlying reason for this requires further investigation.

Text S4. The maintenance energy calculation of poly-P, glycogen and polysaccharides in the EPS

The maintenance energy production by DPAOs was calculated from the amounts of glycogen consumption and poly-P hydrolysis, based on the assumption of hydrolysis of 1 mol poly-P yielding 1 mol ATP, and degradation of 1 mol-C glycogen producing 0.5 mol ATP (Smolders et al., 1994).

The content of polysaccharides in EPS was also measured using the anthrone method (Frølund et al., 1996), the same as that of glycogen, which is determined by total carbohydrates using glucose as the standard. 1 mol-C polysaccharides in EPS is deduced to produce 0.5 mol ATP, according to the existed conclusion that degradation of 1 mol-C glycogen produces 0.5 mol ATP (Smolders et al., 1994).

874 **Figure captions**

875 **Figure S1** Variations in nutrients and carbon profiles during a typical cycle in

876 SBR_{NO3-} and SBR_{NO2-}

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879 **Fig. S1**

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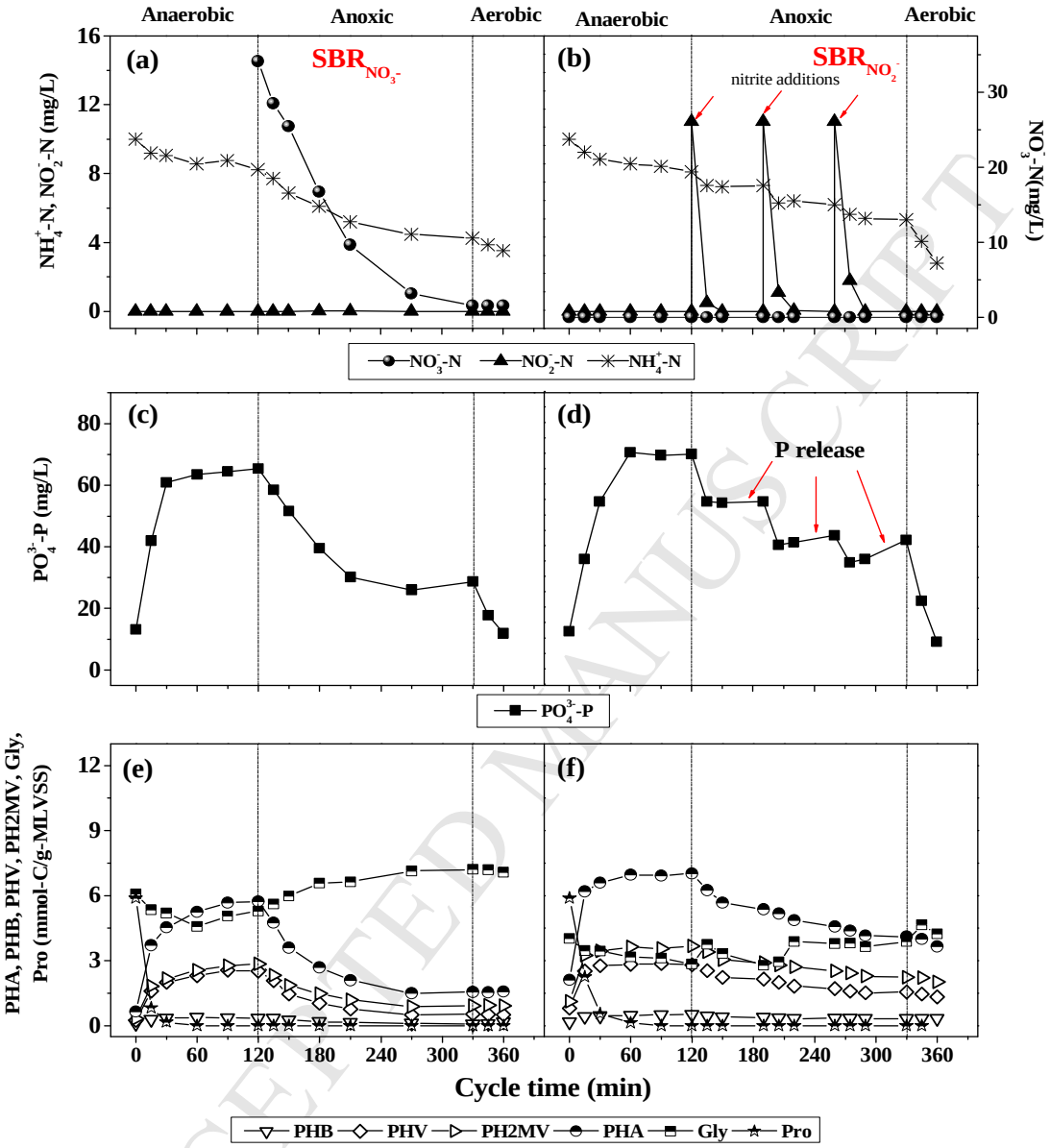

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883 **Fig. S1.** Variations in nutrients and carbon profiles during a typical cycle in SBR_{NO3-} and SBR_{NO2-}.

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Highlights (85 characters)

► Anaerobic starvation (12d) and recovery of nitrite-DPAO was studied for the first time

► EPS polysaccharides were an additional maintenance energy source for DPAO' survival

► Maintenance energy and cell decay were lower for nitrite- than nitrate-DPAO sludge

► Nitrite-DPAO had better stringent response to the starvation than nitrate-DPAO

► Nitrite-DPAO sludge had faster starvation recovery than nitrate-DPAO sludge