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

Unlike PAOs in traditional EBPR processes, the impacts of starvation on denitrifying polyphosphate-accumulating organisms (DPAOs) have been little reported. Denitrifying phosphorus removal (DPR) by DPAOs is a viable and sustainable technology, as N and P can be simultaneously removed with lower carbon source requirements, aeration costs and cell yields (Murnleitner et al., 1997). In particular, since DPAOs can use nitrite as an electron acceptor, DPR is naturally amenable to shortcut nitrification. By replacing nitrate with nitrite, the oxygen cost and carbon consumption of DPR can be reduced by approximately 25% and 40%, 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,
especially polysaccharides, are located in the core of aerobic granular sludge, and that this fraction of EPS can be depleted after long-term starvation (20 days), as evidenced by the void structure in the core of starved granules. Since most previous starvation investigations of EBPR sludge did not involve EPS, the contribution of EPS to the maintenance of metabolic activity of PAOs/DPAOs remains unclear.

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

2. Materials and methods

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

DPAOs sludge was enriched in two identical laboratory-scale sequencing batch reactors (SBR\textsubscript{NO3} and SBR\textsubscript{NO2}, using nitrate and nitrite as electron acceptors, respectively) with a working volume of 7.5 L as outlined by Wang et al. (2011). Both SBRs were independently operated in a cyclical anaerobic-anoxic-aerobic pattern with a cycle time of 8 h (15-min filling period, a 120-min anaerobic period, a 210-min 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). KNO₃ or KNO₂ solution was carefully added to the reactors during the anoxic phase to prevent nitrate or nitrite accumulation. Specifically, 34 mL KNO₃ solution was added to A-SBRₙₒ₃⁻, giving an initial NO₃⁻-N concentration of 34 mg/L; 11 mL KNO₂ solution was added into A-SBRₙₒ₂⁻ in three pulses (70 min of intervals), giving an initial NO₂⁻-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 CH₃CH₂COONa (300 mg chemical oxygen demand (COD)); 32.9 mg KH₂PO₄ (7.5 mg P); 55.3 mg K₂HPO₄·3H₂O (7.5 mg P); 38.2 mg NH₄Cl; 85.0 mg MgSO₄·7H₂O;
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$_2$S accumulated by the activity of
sulfate-reducing bacteria (Morgenroth et al., 2000; Yilmaz et al., 2007). All tests were carried out at 20 ± 1 °C, and the pH was manually controlled at 7.5 ± 0.1 by adding 0.3 M HCl or 0.3 M NaOH.

Liquid- and solid-phase samples were taken from each reactor on days 0, 1.5, 3, 5, 8, and 12. The mixed liquor was filtered through a Millipore filter unit (pore size = 0.45 µm), and the liquid portion was retained for analysis of VFA, NH$_4^+$-N, PO$_4^{3-}$-P, NO$_3^-$-N and NO$_2^-$-N. The solid portion (biomass) was centrifuged, freeze-dried and retained for analysis of intracellular polymers, including poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV), poly-3-hydroxy-2-methylvalerate (PH2MV), and glycogen. Samples were also taken for MLSS, mixed liquor volatile suspended solids (MLVSS) and EPS measurements.

2.4. Recovery batch experiments

After 12 days of starvation, the biomass activities of the starved nitrite/nitrate DPAO sludges were assessed in batch tests. The sludges in the four batch reactors were washed three times with 2.8 L propionate-free synthetic wastewater. At the beginning of the recovery test, 2.8 L of propionate-free synthetic wastewater was rapidly added to the reactors containing activated sludge from the end of the anaerobic phase (denoted AS$_{ANA.end}$) (A-R$_{NO3^{-}}$ and A-R$_{NO2^{-}}$). The anoxic reaction (3.5 h) was started by adding KNO$_3$/KNO$_2$ as described in Section 2.1, followed by a 0.5-h aerobic reaction. The reactors containing activated sludge from the end of the aerobic phase (denoted 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\textsubscript{NO3} and SBR\textsubscript{NO2}, respectively. Two recovery cycles were applied to each of these four reactors.

2.5. Analytical methods

Liquid- and solid-phase analyses of NH\textsubscript{4}\textsuperscript{+}-N, NO\textsubscript{3}\textsuperscript{-}-N, NO\textsubscript{2}\textsuperscript{-}-N, PO\textsubscript{4}\textsuperscript{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-\(\beta\)-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). \textit{Candidatus Accumulibacter phosphatis} (hereafter referred to as \textit{Accumulibacter}), \textit{Candidatus Competibacter phosphatis} (hereafter referred to as \textit{Competibacter}), \textit{Defluvicoccus}-related TFO, and \textit{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):

$$b = -\ln \left( \frac{X_t}{X_0} \right) / t_d \quad (1)$$

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

3.2. Anaerobic starvation in nitrite/nitrate DPAOs biomass

3.2.1 Release of NH<sub>4</sub><sup>+</sup>-N and PO<sub>4</sub><sup>3-</sup>-P, and reduction of MLSS and MLVSS

Since the NH<sub>4</sub><sup>+</sup>-N profile reflects the biomass growth condition, it is a useful tool for determining the cell decay of DPAOs (Zeng et al., 2003b; Lu et al., 2007). In all reactors, the NH<sub>4</sub><sup>+</sup>-N concentrations increased gradually during the first 3 days of starvation and rapidly thereafter (Figure 1a), indicating the increasing extent of cell decay. Within 12 days of starvation, cell decay (including cell lysis and the respiration of intracellular materials) released 41.8 mg, 39.5 mg, 18.7 mg and 15.8 mg of NH<sub>4</sub><sup>+</sup>-N/L into the liquid phase of A-R<sub>NO3</sub>-, O-R<sub>NO3</sub>-, A-R<sub>NO2</sub>- and O-R<sub>NO2</sub>-, respectively (Figure 1a and Table 2). The ammonia release rates were clearly much higher in nitrate-DPAO sludges than those in nitrite-DPAO sludges (Table 2), whereas no appreciable difference was observed in the NH<sub>4</sub><sup>+</sup>-N release rates between AS<sub>ANA.end</sub> and AS<sub>AER.end</sub> (Figure 1a).

The starvation period in all reactors was also marked by P release, as 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\textsubscript{NO\textsubscript{2}-} and O-R\textsubscript{NO\textsubscript{2}-}) than those in nitrate-DPAO sludges (A-R\textsubscript{NO\textsubscript{3}-} and O-R\textsubscript{NO\textsubscript{3}-}) (Figure 2a). These findings are related to the lower P release rate in A-R\textsubscript{NO\textsubscript{2}-} and O-R\textsubscript{NO\textsubscript{2}-} (Table 3). Among these reactors, the MLSS and MLVSS concentrations were most heavily reduced in O-R\textsubscript{NO\textsubscript{3}-}, 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\textsubscript{NO\textsubscript{3}-} (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\textsubscript{ANA.end} were comparable to those in AS\textsubscript{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\textsubscript{ANA.end} and AS\textsubscript{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...
with the lower GAO percentage in nitrite-DPAO sludge (19 ± 0.6%) than in nitrate-DPAO sludge (27 ± 0.9%) (Table 1). Moreover, the amounts of glycogen degradation were much lower in AS$_{\text{ANA}}$ (A-R$_{\text{NO3}}$- and A-R$_{\text{NO2}}$-) than those in AS$_{\text{AER}}$ (O-R$_{\text{NO3}}$- and O-R$_{\text{NO2}}$-). Since glycogen had been partially degraded by anaerobic reactions to supply reducing equivalents before the starvation test, it is likely that less glycogen was available for AS$_{\text{ANA}}$ as compared to that for AS$_{\text{AER}}$. These findings agree with our previous observation (Wang et al., 2012) that glycogen degradation rate is approximately 48% lower for AS$_{\text{ANA}}$ than for AS$_{\text{AER}}$ after 7 days of anaerobic starvation at 15 °C.

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

### 3.2.3 Variations in EPS amounts and compositions

EPS production is essential for the survival of *Accumulibacter* in wastewater treatment systems (Martín et al., 2006). As a candidate carbon and energy source,
EPS degradation allows the microorganisms to rapidly adapt to varying influent composition, temperature (Martín et al., 2006) and substrate limitation (Zhang and Bishop, 2003). However, the contribution of EPS to the anaerobic endogenous metabolism of DPAOs has not been previously reported. We also report the first description of EPS variations during anaerobic starvation of DPAO sludges.

Figure 4 presents the EPS profiles after 3 and 12 days of starvation. The initial amounts of EPS in A-R_{NO2} and O-R_{NO2} sludges were approximately 17.0 and 11.5% lower, respectively, than those in their A-R_{NO3} and O-R_{NO3} counterparts (Figure 4d). This discrepancy is attributable to the different polysaccharides content in the EPS of different sludges. In particular, polysaccharides synthesis may be prevented by the presence of free nitrous acid (FNA) in nitrite-DPAOs (Wang et al., 2013a).

Specifically, the initial polysaccharides content of EPS was 60.8% and 71.3% higher in A-R_{NO3} and O-R_{NO3} than those in A-R_{NO2} and O-R_{NO2}, respectively (Figure 4b). Polysaccharides degradation in A-R_{NO3} and O-R_{NO3} was almost complete within 3 days of starvation (Figure 4b). Similarly, Wang et al. (2007) reported a sharp decrease (approximately 50%) in the polysaccharides content of EPS in highly resistant aerobic granules starved for 4 days. In A-R_{NO2} and O-R_{NO2}, polysaccharides degradation throughout the first 3 days was only 3.7 ± 0.2 mg/g MLVSS and 1.2 ± 0.0 mg/g MLVSS respectively, accounting for approximately 32% and 12% of the total polysaccharides content, respectively (Figure 4b). At the end of the starvation on day 12, the polysaccharides contents remained low in all reactors.
The proteins changes in the four reactors greatly differed from the polysaccharides changes (Figure 4a and b). The proteins content in all reactors slightly increased during the first 3 days of starvation and had decreased to low levels by day 12. It is speculated that, early in the starvation period, cell decay processes released a portion of the intracellular proteins into the extracellular space, where it was captured by EPS. Cellular decay is supported by the increased ammonia concentration in all four reactors during the first 3 days of starvation (Figure 1a). At the end of the starvation period, the proteins contents in all four reactors were heavily reduced, suggesting that proteins may also be hydrolyzed or degraded by the microorganisms as carbon and energy sources. The difference in EPS content variations was negligible between AS\textsubscript{ANA.end} and AS\textsubscript{AER.end} from the same parent (SBR\textsubscript{NO3-} or SBR\textsubscript{NO2-}).

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

3.3.1 Recovery of nitrite- and nitrate-DPAOs in AS\textsubscript{ANA.end}

Nitrite denitrification was completed during the first recovery cycle in both A-R\textsubscript{NO3-} and A-R\textsubscript{NO2-}. The P uptake efficiency of nitrite-DPAOs was actually slightly increased relative to the pre-starvation efficiency (Figure 5c and d; Figure S1 d). This increase is attributed to the relatively lower “secondary” P release after nitrite depletion by starved microorganism (1.6 mg PO\textsubscript{4}^{3-}-P/L vs. 10.8 mg PO\textsubscript{4}^{3-}-P/L in a typical pre-starvation cycle). In contrast, the efficiencies of nitrate denitrification and anoxic P uptake in starved nitrate-DPAOs sludge were decreased by 42.9% and 47.2%,
respectively, relative to their pre-starvation levels in a typical cycle (Figure 5a and b; Figure S1c and d). The rapid recovery of nitrite-DPAOs activities indicates a higher ability of these organisms to overcome starvation shock. The strong anoxic denitrification and P uptake efficiency during starvation was also partially contributed by PHA synthesis (Figure 3), which provides an electron donor and energy source for the DPAOs. Moreover, the higher amounts of PHA synthesis by nitrite-DPAOs than those by nitrate-DPAOs during the endogenous period (Table 3) may accelerate the anoxic metabolism of nitrite-DPAOs during the recovery period, further benefitting the stable operation of shortcut denitrification P removal systems.

In the second recovery batch test of AS\textsubscript{ANA.end}, the concentration of the released P reached 48.0 mg PO_4^{3-}-P/L and 47.3 mg PO_4^{3-}-P/L in A-R\textsubscript{NO3} and A-R\textsubscript{NO2} respectively, with a respective recovery percentage of 91.7% and 82.3%.

3.3.2 Recovery of nitrite- and nitrate-DPAOs in AS\textsubscript{AER.end}

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

The total amount of released P also sharply declined in O-R\textsubscript{NO3} and O-R\textsubscript{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\textsubscript{NO3} and O-R\textsubscript{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\textsubscript{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):

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

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

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

### 4.1.3 Energy production from intracellular polymers and EPS

Maintenance energy is critical for the survival of nutrient-limited bacteria. PAOs generally use poly-P and glycogen as maintenance energy sources under anaerobic starvation conditions (Lopez et al., 2006; Lu et al., 2007). However, the main source between them has yet to be clarified. Lu et al. (2007) found that, early in the
starvation period, PAOs prefer glycogen to poly-P as an energy source. In contrast, Lopez et al. (2006) found that PAOs sequentially consume poly-P and glycogen, which is supported by lack of any P released during the first day of starvation. No glycogen degradation was observed during an 8-hour anaerobic starvation batch test (Oehmen et al., 2005), supporting the assumption that PAOs use their stored poly-P as the sole source of maintenance energy. Therefore, in the present study, we examined the energy produced by both glycogen and poly-P. Moreover, the polysaccharides in EPS were considered as a potential additional maintenance energy source when calculating the maintenance ATP (Text S4).

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

In all reactors, most of the ATP was produced during the first 5 days of starvation. Glycogen was the primary maintenance energy source throughout the first 1.5 days, contributing more than approximately 70% to the total energy production in all four
reactors (Figure 6a-d). During day 1.5 – 3, poly-P was increasingly used as an alternative maintenance energy source, and its use dominated (contributed over 50% of the total ATP production) on day 5 in all four reactors (Figure 6a-d). Similar observation was also reported by Lu et al. (2007), who found that glycogen degradation provided the majority of the energy on day 1, after which there was a transition of the primary energy source from glycogen to poly-P. Whereas, Lopez et al. (2006) observed a sequential utilization of poly-P and glycogen by PAOs for maintenance energy production under anaerobic conditions. The reasons for this discrepancy have not been presented. Moreover, as these two studies did not present the data of the GAOs percentage, it is not easy to explain this discrepancy from the microbial point of view (e.g., PAOs vs. GAOs). In the present study, we doubt that this result might arise from the relatively high proportion of GAOs in both SBR\textsubscript{NO3-} (27 ± 0.9% of the total biomass) and SBR\textsubscript{NO2-} (19 ± 0.6% of the total biomass) (Table 1). The major energy source of GAOs is glycogen, while poly-P hydrolysis supplies energy for the dominant PAOs. In the nitrate-DPAOs sludges, polysaccharides provided additional maintenance energy during the first 3 days of starvation, with values of 16.2 and 20.3 ATP mmol/C-mol biomass for AS\textsubscript{ANA,end} and AS\textsubscript{AER,end}, accounting for 27% and 19% of the total energy production, respectively (Figure 6a and b). After day 5, a marked decline in energy production was observed (Figure 6a-d). Such relatively low energy production may not meet the minimal energy demands of DPAOs. The resulting cell lysis is evidenced in a sharp increase in
NH$_4^+$-N concentration (Figure 1). During the 12-day starvation, energy production of nitrite-DPAO sludges in AS$_{ANA,.end}$ and AS$_{AER,.end}$ were estimated to be 31% and 34% lower, respectively, than those of nitrate-DPAO sludges (Figure 6a-d). The relatively low requirement for maintenance energy of nitrite-DPAOs is further supported by the constant amounts of EPS polysaccharides within the first 3 days (Figure 4). This result may reflect a robust starvation survival response in nitrite-DPAOs. Following each nitrite addition, the parent SBR$_{NO2-}$ was exposed to episodes of famine (no nitrite was available during the first 30 – 70 min of the anoxic period; see Figure S1b). Consequently, nitrite-DPAOs relied on their internal poly-P and/or glycogen reserves for maintenance energy, as evidenced by the irregular P release and glycogen consumption during the anoxic phases of typical cycles (Figure S1d and f). Because of the frequent starvation episodes, and the high number of nitrite/FNA shocks in the parent SBR$_{NO2-}$, the nitrite-DPAOs were primed to adjust their endogenous mechanisms by slowing down their self-oxidation rate and lowering their maintenance energy, i.e., consuming less glycogen and poly-P, to survive under the imposed starvation conditions. Thus, acclimation of nitrite-DPAOs to the starved conditions may allow them higher adaptability to starvation shock than their nitrate-DPAO counterparts. In addition, AS$_{AER,.end}$ required more energy than AS$_{ANA,.end}$ (Figure 6a-d), primarily owing to the 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\textsubscript{NO3-}, O-R\textsubscript{NO3-}, A-R\textsubscript{NO2-} and O-R\textsubscript{NO2-} were estimated to be 0.022, 0.022, 0.008 and 0.007 day\textsuperscript{-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\textsuperscript{-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\textsubscript{ANA.end} and AS\textsubscript{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 ASANA.End rapidly recovered their intracellular polymer transformation ability, especially for the nitrite-DPAOs in A-RNO2-, 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-RNO3- 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.
The metabolism of intracellular materials in AS$_{AER, end}$ was severely inhibited by long-term starvation, as evidenced by the decrease in P uptake and release. Conversely, both reactors achieved complete denitrification, probably because the abundant propionate (2.89 and 3.22 mmol C/L for O-R$_{NO3-}$ and O-R$_{NO2-}$, respectively) derived from the preceding anaerobic phase stimulated the denitrification performance of OHOs. However, the low effluent P concentration in O-R$_{NO2-}$ confirmed that a fully functional system could be restored by the relatively high fraction of PAOII (Table 1).

In summary, the relatively low consumption of intracellular polymers, slow cell decay and rapid recovery of activity in nitrite-DPAO sludges (especially originating from the anaerobic end phase of the DPR process) demonstrate a strong ability to cope with starvation shock. These endogenous process characteristics of nitrite-DPAO sludges, combined with 25% reduction in the oxidation cost and 40% reduction in carbon consumption, may be exploited in efficient shortcut denitrification P removal.

5. Conclusions

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

(2) The estimated cell decay rates in A-R$_{NO3-}$, O-R$_{NO3-}$, A-R$_{NO2-}$ and O-R$_{NO2-}$ were
0.022, 0.022, 0.008, and 0.007 day$^{-1}$, respectively. Clearly, the cell decay rates were lower in nitrite-DPAOs than those in nitrate-DPAOs, indicating a better stringent starvation response by nitrite-cultivated DPAOs than by their nitrate-cultivated counterparts.

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

Acknowledgements

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Figure captions

Fig. 1. Variations in (a) NH$_4^+$-N and (b) PO$_4^{3-}$-P concentrations in A-R$_{NO3}$-, O-R$_{NO3}$-, A-R$_{NO2}$-, and O-R$_{NO2}$- during the 12 days of anaerobic starvation.

Fig. 2. Variations in (a) MLSS, (b) MLVSS, and (c) MLVSS/MLSS ratio in A-R$_{NO3}$-, O-R$_{NO3}$-, A-R$_{NO2}$-, and O-R$_{NO2}$- during the 12 days of anaerobic starvation.

Fig. 3. Variations in glycogen and PHA during the 12 days of anaerobic starvation: (a), A-R$_{NO3}$-, (b), O-R$_{NO3}$-, (c), A-R$_{NO2}$-, and (d), O-R$_{NO2}$-.

Fig. 4. EPS contents profile during the 12 days of anaerobic starvation in A-R$_{NO3}$-, O-R$_{NO3}$-, A-R$_{NO2}$-, and O-R$_{NO2}$-: (a) proteins, (b) polysaccharides, and (c) EPS (calculated as the sum of proteins, polysaccharides and humics). Data are the averages and their standard deviations in triplicate tests.

Fig. 5. Variations in NOx-$N$, PO$_4^{3-}$-$P$, glycogen and PHA in the recovery batch tests after 12 days of anaerobic starvation: (a), (e), A-R$_{NO3}$-; (b), (f), O-R$_{NO3}$-; (c), (g), A-R$_{NO2}$-; and (d), (h), O-R$_{NO2}$-.

Fig. 6. ATP production profile during the 12 days of anaerobic starvation: (a), A-R$_{NO3}$-, (b), O-R$_{NO3}$-, (c), A-R$_{NO2}$-, and (d), O-R$_{NO2}$-.
Table 1. Comparison of the anaerobic carbon transformations, $\text{PO}_4^{3-}$-P release, and biomass compositions (± standard error) with propionate supplied as carbon source in a typical cycle

<table>
<thead>
<tr>
<th></th>
<th>FISH quantification</th>
<th>$\text{PO}_4^{3-}$-P</th>
<th>Gly/ VFA</th>
<th>PHA/ VFA</th>
<th>PHB/ VFA</th>
<th>PHV/ VFA</th>
<th>PH2MV/ VFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Ac}_{\text{Tot}}$ (%)</td>
<td>$\text{Ac}_1$ (%)</td>
<td>$\text{Ac}_2$ (%)</td>
<td>$\text{De}_2$ (%)</td>
<td>$\text{Co}_2$ (%)</td>
<td>/ VFA$^f$</td>
<td>VFA$^g$</td>
</tr>
<tr>
<td>This study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBR$_{\text{NO}_3}$ (nitrate-based)</td>
<td>66 ± 1.5</td>
<td>28 ± 1.9</td>
<td>38 ± 1.4</td>
<td>27 ± 0.9</td>
<td>&lt;1</td>
<td>0.32</td>
<td>0.44</td>
</tr>
<tr>
<td>SBR$_{\text{NO}_2}$ (nitrite-based)</td>
<td>75 ± 1.1</td>
<td>21 ± 1.2</td>
<td>54 ± 1.7</td>
<td>19 ± 0.6</td>
<td>&lt;1</td>
<td>0.36</td>
<td>0.31</td>
</tr>
<tr>
<td>Previous studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oehmen et al. (2005)</td>
<td>63 ± 1.3</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.42</td>
<td>0.32</td>
</tr>
<tr>
<td>Carvalho et al. (2007)</td>
<td>75</td>
<td>44</td>
<td>31</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.40</td>
<td>0.32</td>
</tr>
<tr>
<td>Tayà et al. (2013)</td>
<td>85</td>
<td>55</td>
<td>30</td>
<td>&lt;10</td>
<td>0.34</td>
<td>0.49</td>
<td>1.47</td>
</tr>
</tbody>
</table>

$^a$ $\text{Ac}_{\text{Tot}}$: Total $\text{Accumulibacter}$. $^b$ Ac I: Type I $\text{Accumulibacter}$. $^c$ Ac II: Type II $\text{Accumulibacter}$. $^d$ De: $\text{Defluvicoccus}$. $^e$ Co: $\text{Competibacter}$.

$^f$ Units $\text{PO}_4^{3-}$-P mmol/C mmol. $^g$ Units C mmol/C mmol. $^h$ No data.
Table 2. Comparison of cell decay of PAOs/DPAOs in this study and previous studies during the 12 days of anaerobic starvation.

<table>
<thead>
<tr>
<th>Starvation condition</th>
<th>Initial MLVSS (g/L)</th>
<th>Temperature (°C)</th>
<th>Starvation period (d)</th>
<th>SRT (d)</th>
<th>NH₄⁺-N release (mg/L)</th>
<th>Cell decay rate (1/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-R &lt;sub&gt;NΟ₃&lt;/sub&gt;-</td>
<td>anaerobic</td>
<td>2.5</td>
<td>20 ± 2</td>
<td>12</td>
<td>20</td>
<td>41.8&lt;sup&gt;a&lt;/sup&gt; 0.022&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-R &lt;sub&gt;NΟ₃&lt;/sub&gt;-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.5&lt;sup&gt;b&lt;/sup&gt; 0.022&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A-R &lt;sub&gt;NΟ₂&lt;/sub&gt;-</td>
<td>anaerobic</td>
<td>18.7</td>
<td>20 ± 2</td>
<td>12</td>
<td></td>
<td>18.7&lt;sup&gt;a&lt;/sup&gt; 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-R &lt;sub&gt;NΟ₂&lt;/sub&gt;-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15.8&lt;sup&gt;b&lt;/sup&gt; 0.007&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Previous studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu et al. (2007)</td>
<td>anaerobic</td>
<td>1.6</td>
<td>22 ± 2</td>
<td>8</td>
<td>24</td>
<td>3.2&lt;sup&gt;b&lt;/sup&gt; 0.006&lt;sup&gt;b&lt;/sup&gt; (day 4– 8)</td>
</tr>
<tr>
<td>Hao et al. (2010a)</td>
<td>anaerobic</td>
<td>-</td>
<td>22 ± 0.5</td>
<td>7</td>
<td>12</td>
<td>0.036 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wang et al. (2012)</td>
<td>anaerobic</td>
<td>2.4</td>
<td>10 – 15</td>
<td>7</td>
<td>20</td>
<td>1.12&lt;sup&gt;a&lt;/sup&gt; 0.0006&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vargas et al. (2013)</td>
<td>aerobic-anaerobic</td>
<td>2.6</td>
<td>20 ± 2</td>
<td>21</td>
<td>46.8</td>
<td>14&lt;sup&gt;b&lt;/sup&gt; 0.029&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All results represent the sludge from the anaerobic end phases.

<sup>b</sup> All results represent the sludge from the aerobic end phases.
Table 3. Transformations of materials in the liquid- and solid-phase in A-R\textsubscript{NO3}, O-R\textsubscript{NO3}, A-R\textsubscript{NO2} and O-R\textsubscript{NO2} during the 12 days of anaerobic starvation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nitrate-sludge</th>
<th>Nitrite-sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-R\textsubscript{NO3}</td>
<td>O-R\textsubscript{NO3}</td>
</tr>
<tr>
<td>MLSS reduction (mg/L)</td>
<td>770</td>
<td>1650</td>
</tr>
<tr>
<td>MLVSS reduction (mg/L)</td>
<td>357</td>
<td>793</td>
</tr>
<tr>
<td>PO\textsubscript{4}-P release (mg/L)</td>
<td>87.2</td>
<td>150.8</td>
</tr>
<tr>
<td>Glycogen degradation (mmol-C/g-MLVSS)</td>
<td>3.03</td>
<td>5.56</td>
</tr>
<tr>
<td>Glycogen degradation rate\textsuperscript{a} (mmol-C/g-MLVSS/d)</td>
<td>0.61</td>
<td>1.11</td>
</tr>
<tr>
<td>PHB synthesis (mmol-C/g-MLVSS)</td>
<td>0.23 (0.12)</td>
<td>0.58 (0.19)</td>
</tr>
<tr>
<td>PHV synthesis (mmol-C/g-MLVSS)</td>
<td>1.07 (0.58)</td>
<td>1.42 (0.46)</td>
</tr>
<tr>
<td>PH2MV synthesis (mmol-C/g-MLVSS)</td>
<td>0.54 (0.29)</td>
<td>1.06 (0.35)</td>
</tr>
<tr>
<td>PHA synthesis (mmol-C/g-MLVSS)</td>
<td>1.84</td>
<td>3.06</td>
</tr>
<tr>
<td>PHA synthesis rate (mmol-C/g-MLVSS/d)</td>
<td>0.30\textsuperscript{a}</td>
<td>0.58\textsuperscript{a}</td>
</tr>
<tr>
<td>Polysaccharides consumption (mg/g-MLVSS)</td>
<td>36.6 ± 0.4</td>
<td>46.4 ± 0.5</td>
</tr>
<tr>
<td>Proteins consumption (mg/g-MLVSS)</td>
<td>144.0 ± 2.3</td>
<td>129.0 ± 13.3</td>
</tr>
<tr>
<td>Total EPS consumption (mg/g-MLVSS)</td>
<td>196.0 ± 0.1</td>
<td>200.1 ± 9.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All results obtained during the first 5 days.
\textsuperscript{b} All results obtained during the first 3 days.

Data in the brackets represent the fractions of the components in PHA.
Table 4. Comparison of denitrifying P-removal efficiency during anaerobic/anoxic phases in typical cycles and recovery batch tests.

<table>
<thead>
<tr>
<th>Items</th>
<th>Amount of (\text{PO}_4^{3-})-P release (^a)</th>
<th>Amount of (\text{PO}_4^{3-})-P uptake (^a)</th>
<th>Amount of (\text{NO}_x)-N reduction (^b)</th>
<th>P/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate-sludge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBR(_{\text{NO}_3})</td>
<td>52.33</td>
<td>36.69</td>
<td>33.37</td>
<td>1.10</td>
</tr>
<tr>
<td>A-R(_{\text{NO}_3})</td>
<td>47.98 (91.7) *</td>
<td>19.37 (52.8)</td>
<td>19.36 (58.0)</td>
<td>1.00 (63.7)</td>
</tr>
<tr>
<td>O-R(_{\text{NO}_3})</td>
<td>6.87 (13.1)</td>
<td>4.09 (11.1)</td>
<td>33.68 (100.9)</td>
<td>0.12 (7.6)</td>
</tr>
<tr>
<td>Nitrite-sludge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBR(_{\text{NO}_2})</td>
<td>57.48</td>
<td>26.44</td>
<td>33</td>
<td>0.80</td>
</tr>
<tr>
<td>A-R(_{\text{NO}_2})</td>
<td>47.30 (82.3) *</td>
<td>35.97 (136.0)</td>
<td>30 (90.9)</td>
<td>1.20 (69.0)</td>
</tr>
<tr>
<td>O-R(_{\text{NO}_2})</td>
<td>11.82 (20.6)</td>
<td>5.73 (21.7)</td>
<td>33 (100.0)</td>
<td>0.17 (9.8)</td>
</tr>
</tbody>
</table>

\(^a\) Units mg \(\text{PO}_4^{3-}\)-P/L.

\(^b\) Units mg \(\text{NO}_x\)-N/L.

* Results obtained from the second recovery batch tests.

Data in brackets represent the percentage recovery relative to values obtained in typical cycles of the corresponding parent SBRs (%).
Fig. 1

(a) NH$_4^+$-N (mg/L) over time

(b) PO$_4^{3-}$-P (mg/L) over time

Legend:
- ■ A-R$_{NO3}$
- ○ O-R$_{NO3}$
- □ A-R$_{NO2}$
- ▲ O-R$_{NO2}$
Fig. 2

(a) MLSS (mg/L) vs. Time (day)

(b) MLVSS (mg/L) vs. Time (day)

(c) MLVSS/MLSS vs. Time (day)

Key:
- ■ A-NO₃
- ○ O-NO₃
- □ A-NO₂
- ○ O-NO₂
Fig. 3

(a) Glycogen, PHA, PH2MV, PHV

(b) Glycogen, PHA, PH2MV, PHV, PHB

(c) Glycogen, PHA, PH2MV, PHV

(d) Glycogen, PHA, PH2MV, PHV, PHB

Glycogen, PH2MV, PHV, PHB (mmol-C/g-MLVSS) vs. Time (day)
Fig. 4

(a) Proteins (mg/g-MLVSS)
(b) Polysaccharides (mg/g-MLVSS)
(c) EPS (mg/g-MLVSS)

A-R$_{NO_3}$  O-R$_{NO_3}$  A-R$_{NO_2}$  O-R$_{NO_2}$

Reactors

Beginning  after 3 days  after 12 days
Fig. 5

Anoxic

Aerobic

KNO$_3$

NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N (mg/L)

Glycogen, PHA, PHB, PHV, PH2MV (mmol-C/g-MLVSS)

PO$_4^{3-}$-P NO$_3^-$-N NO$_2^-$-N NH$_4^+$-N

Time (min)

PO$_4^{3-}$-P NO$_3^-$-N NO$_2^-$-N NH$_4^+$-N Glycogen PHA PH2MV PHV PHB

Glycogen, PHA, PHB, PHV, PH2MV (mmol-C/g-MLVSS)
Fig. 6

ATP (mmol/C-mol MLVSS)

Time (day)

ATP contributed by poly-P
ATP contributed by glycogen

(a) (b) (c) (d)
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% H₂SO₄) and 0.1 mL benzoic acid methanol solution (2 g of benzoic acid dissolved in 100 mL methanol used as an internal standard) were subsequently added. The samples were then digested for 4 h 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 to 120 °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
to room temperature, samples were sheared by a vortex mixer (XW-80A, Shanghai Qingpu Huxi Instrument Co., Ltd, China) for 1 min, and were transferred to 10-mL tubes, followed by centrifugation (CT15RT, Techcomp, China) for 5 min at 10,000 g. About 1 mL supernatant was added to 4 mL of anthrone-H$_2$SO$_4$ reagent (0.2% anthrone (w/v) in 80% (v/v) H$_2$SO$_4$) in 10-mL colorimetric tubes. All tubes were placed in a water bath at 100 °C for 10 min. After cooling at 4 °C for 5 min in cold water, samples were measured by a UV/VIS spectrophotometer (UV765, Shanghai Precision & Scientific Instrument Co., Ltd, China) at 625 nm. Glucose was used as the standard.

d) EPS

A heat extraction method (Li et al., 2007) was applied to extract the EPS (including loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS)) in the sludge. 30 mL sludge suspension was first dewatered by centrifugation (CT15RT, Techcomp, China) in a 50-mL tube at 4,000 g for 5 min. The supernatant was recovered for water quality analysis. The sludge pellet in the tube was re-suspended into 15 mL of 0.05% NaCl solution. The sludge mixture was then diluted with the NaCl solution (pre-heating to 70 °C) to its original volume of 30 mL. Immediately, the sludge suspension was sheared by a vortex mixer (XW-80A, Shanghai Qingpu Huxi Instrument Factory, China) for 1 min, followed by centrifugation at 4,000 g for 10 min. The organic matter in the supernatant was readily extractable EPS, and was regarded as the LB-EPS of the biomass. For the extraction of the TB-EPS, the sludge pellet left in the
A centrifuge tube was re-suspended in 0.05% NaCl solution to its original volume of 30 mL. The sludge suspension was heated to 60 °C in a water bath for 30 min. After cooling to room temperature, the sludge mixture was centrifuged at 4,000 g for 15 min. The supernatant collected was regarded as the TB-EPS of the sludge.

Both the LB-EPS and TB-EPS extractions were analyzed for proteins (PN), polysaccharides (PS) and humic-like substances (HS). The PN and HS were analyzed by a UV/VIS spectrophotometer (UV765, Shanghai Precision & Scientific Instrument Co., Ltd, China) following the modified Lowry method (Frølund et al., 1995) using bovine serum albumin (Sigma) and humic acid (Fluka) as the standards, respectively. The PS content was determined by the anthrone method described by Frølund et al. (1996) using glucose as the standard.
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 Alphaproteobacteria (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
the mean ($SE_{\text{mean}}$) was calculated as the standard deviation divided by the square root of the number of images.

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

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe sequence (5’-3’)</th>
<th>Specificity</th>
<th>FA (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338</td>
<td>GCTGCTCCCCGATTAGGAGT</td>
<td>Most Bacteria</td>
<td>35</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>EUB 338-II</td>
<td>GCAGCCACCCGATAGGAGT</td>
<td>Planctomycetales and other Bacteria</td>
<td>35</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>EUB 338-III</td>
<td>GCTGCCACCCGGAGGAGT</td>
<td>Verrucomicrobiales and other Bacteria</td>
<td>35</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>PAO462</td>
<td>CCGTCATCTACWCAAGGTATTAAC</td>
<td>Most Accumulibacter spp.</td>
<td>35</td>
<td>Crocetti et al., 2000</td>
</tr>
<tr>
<td>PA0651</td>
<td>CCC TCT GCC AAA CTC CAG</td>
<td>Most Accumulibacter spp.</td>
<td>35</td>
<td>Crocetti et al., 2000</td>
</tr>
<tr>
<td>PA0846</td>
<td>GTTAGCTACGGCAGTAAAAGG</td>
<td>Most Accumulibacter spp.</td>
<td>35</td>
<td>Crocetti et al., 2000</td>
</tr>
<tr>
<td>Acc-I-444</td>
<td>CCCAAGCAATTCTTCCCC</td>
<td>Most Accumulibacter I</td>
<td>35</td>
<td>Flowers et al., 2009</td>
</tr>
<tr>
<td>Acc-II-444</td>
<td>CCCGTGCAATTTCTTCCCC</td>
<td>Most Accumulibacter IIA, IIC and IID</td>
<td>35</td>
<td>Flowers et al., 2009</td>
</tr>
<tr>
<td>ALF969</td>
<td>TGGTAAGGTTCTGCGCGT</td>
<td>Most Alphaproteobacteria</td>
<td>35</td>
<td>Crocetti et al., 2002</td>
</tr>
<tr>
<td>GAOQ431</td>
<td>TCCCCCGCTAAAGGGCTT</td>
<td>Some Competibacter spp.</td>
<td>35</td>
<td>Crocetti et al., 2002</td>
</tr>
<tr>
<td>GAOQ989</td>
<td>TTCCCCGGATGCTAAGGC</td>
<td>Some Competibacter spp.</td>
<td>35</td>
<td>Crocetti et al., 2002</td>
</tr>
<tr>
<td>GB_G2</td>
<td>TTCCCCAGATGCTAAGGC</td>
<td>Some Competibacter spp.</td>
<td>35</td>
<td>Kong et al., 2002</td>
</tr>
<tr>
<td>TFO_DF218</td>
<td>GAAGCCTTTGGCCCCTCAG</td>
<td>Some Defluvicoccus vanus</td>
<td>35</td>
<td>Wong et al., 2004</td>
</tr>
<tr>
<td>TFO_DF618</td>
<td>GCCTCACTTGTCTAACCAG</td>
<td>Some Defluvicoccus vanus</td>
<td>35</td>
<td>Wong et al., 2004</td>
</tr>
<tr>
<td>DF988</td>
<td>GATACGGCAGCCCATGTCAAGGG</td>
<td>Some Defluvicoccus vanus spp.</td>
<td>35</td>
<td>Meyer et al., 2006</td>
</tr>
<tr>
<td>DF1020</td>
<td>CCGGCCGAAACCGACTCCC</td>
<td>Some Defluvicoccus vanus spp.</td>
<td>35</td>
<td>Meyer et al., 2006</td>
</tr>
</tbody>
</table>
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).
Figure captions

**Figure S1** Variations in nutrients and carbon profiles during a typical cycle in SBR$_{NO_3}$ and SBR$_{NO_2}$.
Fig. S1. Variations in nutrients and carbon profiles during a typical cycle in SBR$_{NO_3}$ and SBR$_{NO_2}$. 

(a) NH$_4$-N, NO$_3$-N, and NO$_2$-N concentrations.

(b) Nitrite additions and nitrite oxidation.

(c) PO$_4$-P concentrations.

(d) P release during the cycle.

(e) PHA, PHB, PHV, and PH$_2$MV concentrations.

(f) Gly and Pro concentrations.
References


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