Long-term operation of a reactor enriched in *Accumulibacter* clade I DPAOs: performance with nitrate, nitrite and oxygen
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**ABSTRACT**

The microbiology of denitrifying enhanced biological phosphorus removal systems has been a subject of much debate. The question has centred on the affinities of different types of *Candidatus* Accumulibacter PAOs, type I and type II, towards different electron acceptors such as oxygen, nitrate and nitrite. This study used a propionate anaerobic/anoxic/aerobic lab-scale sequencing batch reactor where a microbial culture was successfully enriched in *Accumulibacter* type I organisms (approx. 90%). The culture was able to take up phosphorus using nitrate, nitrite and oxygen as electron acceptors, although experiments with oxygen led to the fastest P removal rate. The phosphorus uptake to nitrogen consumed ratio (P/N ratio), when using both nitrate and nitrite, was shown to be affected by pH in the range of 7–8.2, achieving higher values for lower pH values (7.0–7.5). The effect of pH on P removal seems to follow a similar trend for both nitrate and nitrite. To our knowledge, this is the first study where the impact of pH in the phosphate removal stoichiometry using the three most significant electron acceptors is shown for such a high enrichment in *Accumulibacter* type I.

**Key words** | *Candidatus* Accumulibacter phosphatis, denitrifying polyphosphate accumulating organisms, EBPR, nitrate

**INTRODUCTION**

Enhanced biological phosphorus removal (EBPR) has been suggested as an effective alternative to chemical precipitation methods for phosphorus removal. The process relies on alternating anaerobic/aerobic conditions which select for polyphosphate accumulating organisms (PAOs). These organisms have the capability of anaerobically storing carbon substrates under the form of polyhydroxyalkanoates (PHA) by using energy and reducing equivalents provided by the degradation of internal glycogen and internal polyphosphate chains. In aerobic conditions, PHA is then degraded and hence produces energy for glycogen and polyphosphate replenishment, for cell growth and maintenance. This cycling stimulates PAOs to accumulate all the available external phosphate, therefore contributing for the phosphorus removal in the effluent. This process is often coupled with simultaneous denitrification, which can be beneficial since it reduces carbon requirements as well as sludge production.

Although little is still known about the full diversity of the organisms bearing a PAO phenotype, until now most studies indicate that *Candidatus* Accumulibacter phosphatis, hereafter referred to as *Accumulibacter*, a non-isolated organism belonging to the *Rhodocyclales* order, is one of the main PAO associated with phosphorus removal (Oehmen et al. 2007). In the past years a discussion has subsisted on the denitrifying capabilities of *Accumulibacter* organisms since different authors, in studies using lab-scale EBPR reactors, obtained contradictory results. Some authors demonstrated that PAO cultures were able to fully denitrify using nitrate (Zeng et al. 2003; Kong et al. 2004), while others suggested the presence of two different populations, one with denitrification capabilities (Denitrifying PAOs – DPAOs) and another not able to denitrify (non-DPAOs) (Kerrn-Jespersen & Henze 1993; Meinhael et al. 1999; Ahn et al. 2002; Freitas et al. 2005).
However, in recent studies, two different clades were identified within *Accumulibacter* based on a differentiation of the polyphosphate kinase gene (ppk1) (He et al. 2007) and two new FISH probes were developed to distinguish between type IA (Acc-I-444) and type IIA, IIC and IID (Acc-II-444) (Flowers et al. 2009). This fact sustains the hypothesis that there were two different populations of *Accumulibacter* with different affinities to nitrate as suggested by Carvalho et al. (2007) based on different morphotypes and affinities for carbon and nitrogen substrates. Type I *Accumulibacter*, hereafter referred as PAOI, seems to have the ability to denitrify using nitrate whereas Type II *Accumulibacter*, hereafter referred as PAOII, cannot (Flowers et al. 2009). This correlates well with previous studies that found evidence of *Accumulibacter* communities being able to use nitrate for phosphorus removal (Carvalho et al. 2007) and *Accumulibacter* communities not able to use nitrate, but just nitrite (Martin et al. 2006; Guisasola et al. 2009).

This major breakthrough in the DPAO story puts forward new questions. It is still unclear what are the selecting conditions for either one of these *Accumulibacter* types. Although anoxic conditions using nitrate seem to favour PAOI over PAOII, they still seem to be able to co-exist (Carvalho et al. 2007; Oehmen et al. 2010). Additionally, PAOII’s affinity for nitrate has been demonstrated (Flowers et al. 2009), however its polyphosphate cycling performance with nitrite has not been shown.

In this study, a Sequencing Batch Reactor (SBR) was run for a period of over 2 years with an enriched population of PAOI in anaerobic-anoxic-aerobic conditions. During this period, the reactor was characterised in terms of its microbial population and in terms of its capacity for utilising different electron acceptors to perform phosphorus uptake. These results intended to contribute to the discussion on the efficiency of PAOI regarding different electron acceptors (nitrate, nitrite and oxygen) and also to shed some clues on what could be the main factors affecting PAOI selection.

**MATERIALS AND METHODS**

**Reactor setup and operation**

A 900 mL reactor was inoculated with sludge from another anoxic EBPR reactor (Carvalho et al. 2007). It was operated for a period of 880 days at 22°C (±2°C), with pH being controlled in the range of 7–8.2 with acid dosing (HCl, 0.2 M). The reactor was fed with a synthetic medium containing propionate as described by Carvalho et al. (2007) except for the phosphorus concentration that started at 30 mg-P/L, was increased to 60 mg-P/L at day 83. The reactor was operated in 8-h cycles that comprised a 2 h anaerobic phase, a 4 h anoxic phase with a nitrate pulse that averaged 35 mg N-NO₃/L, a 1 h aerobic phase, a 30 min settling phase, a 20 min purging phase and a 10 min feeding phase. The hydraulic retention time and the sludge retention time were maintained at 1 and 10 days respectively. The reactor was monitored throughout the operation time by chemical analysis but also by following the pH profile as has been suggested in a similar way by Vargas et al. (2008). The anaerobic and anoxic phases were maintained by sparging argon and in the aerobic phase compressed air was sparged so that oxygen levels were close to saturation.

**Batch tests**

During the operation of the reactor several batch tests were carried out in a 600 mL reactor inoculated with 300 mL of sludge from the parent reactor, washed with mineral medium and diluted with synthetic wastewater as described for the normal reactor’s operation. Batch tests were carried out by promoting an anaerobic phase by sparging argon gas until the carbon had been fully consumed (<2 h). The anoxic or aerobic phase were introduced by injecting a concentrated solution of nitrate (25–55 mg-N/L in the reactor), nitrite (25–65 mg-N/L in the reactor) or by sparging the reactor with air (oxygen level close to saturation). Most of the nitrate or nitrite batch tests were followed by an aerobic phase by sparging compressed air. The batch tests were sampled at regular intervals for chemical analysis of the supernatant (P, N, NO₃, NO₂, ammonia, propionate) and of intracellular compounds (PHA and glycogen). Rates were obtained by calculating the slope of a linear regression including the first points of consumption/production. Yields were calculated as the ratio of slopes from linear regression. The error bars represent the standard deviation of the slope obtained through the linear regression.

**Chemical Analysis**

Phosphate, nitrate and nitrite were analysed using the IonPac® AS9-HC High Pressure Liquid Chromatography (HPLC) column (Dionex, CA, USA) (Na₂CO₃ 0.9 mM, 50°C, 1 mL/min) coupled with an electrochemical detector (Dionex, CA, USA). Propionate was analysed by using an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA) and a Refractive Index detector (Merck, Germany). Samples were run using H₂SO₄ 0.01 N as eluent (0.6 mL/min, 50°C). Glycogen
was extracted from lyophilised cells (approximately 2–3 mg) by an acidic digestion (1 mL HCl 0.6 M, 2 hours, 100 °C). Samples were analysed by HPLC using an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA) and a Refractive Index detector (Merck, Germany), using H2SO4 0.01 N as eluent (0.5 mL/min, 30°C). PHAs were analysed using the method described in Lemos et al. (2006). Dry cell weight was assumed as being equal to Volatile Suspended Solids (VSS) and was determined according to Standard Methods (APHA 1995). Ammonia was quantified using an ammonia gas sensing combination electrode ThermoOrion 9512.

**Microbial Characterization**

Sludge samples were periodically fixed in a 4% formaldehyde solution and stored at −18 °C. FISH was performed at regular intervals to assess the microbial composition as proposed by Amann (1995). An estimate of the microbial composition was obtained by observing the cell biovolume of a specific probe in Cy3 against a broad probe covering all bacteria in FITC. The generic probe used was EUBmix containing a mixture of EUB358, EUB388II and EUB388III (Amann et al. 1999; Daims et al. 1999). The specific probes used in Cy3 were ALF1b, BET42a, GAM42a for the identification of α-, β- and γ-Proteobacteria (Manz et al. 1992); PAOmix containing a mixture of PAO462, PAO651 and PAO846 to target Accumulibacter PAOs (Crocetti et al. 2000); GAOMix containing a mixture of GAOQ431, GAOQ989 and GB_G2 to target Competibacter Glycogen Accumulating Organisms (GAOs) (Crocetti et al. 2002; Kong et al. 2002); TFOmix containing a mixture of TFO_DF218 and TFO_DF618 targeting Defluvibacter vanus related GAOs cluster I (Wong et al. 2004); DEFmix containing a mixture of DF988 and DF1020 targeting D. vanus related GAOs cluster II (Meyer et al. 2006); Acc-I-444 and Acc-II-444 targeting Type IA and Type IIA, IIC and IID Accumulibacter respectively (Flowers et al. 2009). Samples were viewed using an Olympus BX51 epifluorescence microscope.

**RESULTS AND DISCUSSION**

**Acclimatization to an enriched reactor of PAOI**

The SBR was inoculated with sludge from a parent denitrifying EBPR reactor and was first operated for 53 days under normal anaerobic/aerobic EBPR conditions. Then, progressively until day 173 the sludge was acclimatized to anaerobic/anoxic/aerobic conditions by introducing and increasing the anoxic phase duration from 0 to 4 h and by increasing the nitrate concentration fed to the reactor from 0 to 40 mg-N/L (Figure 1). An example of a normal operation cycle is depicted in Figure 2, an example taken from the last step of the acclimatization phase (day 138). A typical PAO phenotype was observed with the cycling of phosphate, PHA and glycogen. Phosphorus was removed in both anoxic and aerobic conditions, although the rate was significantly faster in aerobic conditions.

During the acclimatization stage, the microbial community composition was estimated by using the FISH technique. Throughout the whole process, Accumulibacter cells dominated the reactor as shown by a hybridization of approximately 90% to the PAOmix probes. Throughout the operation time of the reactor, there were no GAOs present, neither Competibacter nor D. vanus related GAOs since no hybridization was observed with either GAOMix, TFOmix or DEFmix probes. There were however some unidentified filamentous bacteria structuring the flocs, as well as some small rod-shaped bacteria belonging to α- and γ-Proteobacteria that accounted for less than 10% of the total bio-volume and whose fraction was constant throughout most of the reactor’s operation time.

Although PAOI were always the dominant Accumulibacter type, over the acclimatization phase there was a shift from a significant quantity of PAOII in the system, to an accentuated decrease of PAOII population with very few clusters being observed at the end of the acclimatization phase and eventually to their disappearance below detection limit with the long term operation. This observation showed that the selective pressure applied was enough to allow PAOI to out-compete PAOII, even though there was still an aerobic phase.

![Figure 1](https://iwaponline.com/wst/article-pdf/63/2/352/445256/352.pdf) Description of the acclimatization process showing the nitrate and the phosphate concentration in the reactor for each acclimatization step as well as the duration of the anoxic and aerobic phases.
All *Accumulibacter* types can use oxygen as an electron acceptor, however, PAOI organisms are also able to use nitrate as opposed to PAOII organisms, that do not seem to be able to use nitrate (Flowers et al. 2009) although they have been suggested to possibly using nitrite, as indicated in the clade IIA metagenome obtained by Martín et al. (2006). Therefore, the high anoxic:aerobic ratio (4 h:1 h) seemed a sufficiently strong selective pressure for PAOI. Furthermore, not only was the aerobic phase not enough to allow PAOII population to remain in the system, but, because phosphorus removal with nitrate is slower than with oxygen (Figure 3a), it allowed for a final efficient polishing step which increased the overall efficiency of the process. Additionally, the kinetics of glycogen replenishment and PHA degradation are also faster in aerobic conditions than in anoxic conditions (results not shown) and so an aerobic zone, even a small one, favours the full recovery of the glycogen pool as has also been discussed by Carvalho et al. (2007). Maintaining of the aerobic phase was probably the decisive factor to achieve such high enrichment in PAOII organisms in this study, and could also be linked to the stability in the reactor’s operation over time.

The increase of both the anoxic phase length as well as the nitrate concentration over time affected the phosphorus uptake rate in the anoxic (rP anoxic) and the aerobic phase (rP aerobic) (Figure 3a). With the increase of the anoxic phase length, the phosphorus uptake rate in anoxic conditions increased, as opposed to the aerobic phosphorus uptake rate, which decreased. This is consistent with the evolution of the amount of phosphorus taken up in anoxic (P<sub>anox</sub>) and aerobic conditions (P<sub>aero</sub>). The ratio between P<sub>anox</sub> and P<sub>aero</sub> increased showing that the sludge responded positively to the new anoxic conditions and that the selective pressure reflected successfully on the system’s performance (Figure 3b).

**Phosphate uptake under different electron acceptors**

Throughout the two years of operation time after the initial acclimatization period, several batch tests were conducted with different electron acceptors: nitrate, nitrite and oxygen. For nitrate batch tests, no nitrite accumulation was ever observed. For nitrite batch tests, the free nitrous acid concentration (FNA) was calculated using the equation suggested by Zhou et al. (2007). The concentrations obtained were between 1.1 and 5.4 μg HNO<sub>2</sub>-N/L which was below the range indicated as potentially inhibitory. No significant correlation was observed between FNA concentration and either phosphorus uptake rate (rP) and nitrogen uptake rate (rN) or the P/N ratio. Phosphorus uptake was observed with all three electron acceptors throughout the whole time of operation. Oxygen was the electron acceptor that promoted a faster rP, even after phosphorus uptake under anoxic conditions. In nitrite batch tests followed by an aerobic phase, aerobic P uptake was never observed, despite PHA still being available (results not shown), which indicates that there seemed to be an inhibitory factor remaining that affected the cells’ capacity to function correctly.
Furthermore, two batch tests were performed at days 781 and 849 with nitrate and oxygen respectively and phosphate and/or nitrate spikes were supplied until no phosphate uptake was observed. The phosphorus uptake ceased when PHA levels were depleted (Figure 4). The overall PHA consumed per mol of P stored was much higher in anoxic conditions with nitrate (3.1 C-mol/P-mol) than in aerobic conditions (1.3 C-mol/P-mol) which supports the observation that phosphorus removal is less efficient in anoxic conditions than in aerobic conditions. For the batch test with nitrate, after the maximum P uptake was reached, a very moderate P release occurred concomitant with a slight glycogen consumption. When an aerobic phase was introduced there was no variation of either phosphate, glycogen or PHA confirming that the PAOI organisms completely exhausted their poly-P accumulation capacity under nitrate-anoxic conditions.

Throughout the different batch tests, pH was not constant but rather controlled in the range of 7–8.2. This resulted in different batch tests conducted at different pH values in between that range which led to interesting results. The effect of pH on PAO metabolism has often been discussed and most results seem to point pH values of 7–7.5 as optimum for PAO activity, as opposed to GAOs who would be favoured by lower pH values 6.5–7 (Serafim et al. 2002; Oehmen et al. 2005; Oehmen et al. 2007). There are fewer results on the impact of pH on P uptake rates, both in aerobic or anoxic conditions, in particular with highly enriched cultures. Still, some studies point towards an inhibitory or negative effect on P uptake at pH 8.5 (Oehmen et al. 2005).

An interesting correlation was found between pH and the P/N ratio in anoxic conditions, both for nitrite and nitrate (Table 1). Between 7 and 8 the P/N values seem to decrease slightly and for nitrite, above pH 8, they decreased more significantly suggesting that there would be an energy shift from phosphorus uptake to other metabolic functions such as glycogen production or growth as discussed in Oehmen et al. (2010). A similar inhibitory effect was observed on the rP under aerobic conditions for pH 8.5 by Oehmen et al. (2005). In fact, in nitrite batch tests where pH values were 8 and...
higher, an initial lag-phase with a slower rP was also observed. The fact that the same trend was observed with nitrate and nitrite, suggests that the efficiency of the phosphorus removal with both electron acceptors can only be compared under the same pH values.

Literature values obtained by Carvalho et al. (2007) and Flowers et al. (2009) from enriched systems in PAOI, obtained with propionate and acetate fed cultures respectively, fit the trend observed in this study. However, Flowers et al. (2009) also reported a P/N value with nitrate for an enriched PAOII system still containing 40% PAOI, much lower than the one for the PAOI enriched system. This low value could be explained by a secondary P release from the PAOII organisms that dominated the reactor and that masked the real P uptake by the PAOI present. Guisasola et al. (2009) also reported a lower value than expected with nitrite in a 46% nitrite-acclimatized Accumulibacter culture, possibly a PAOII enriched culture. This could be the result of the 36% D. vanus cluster 1 population also present, although D. vanus were suggested to denitrify but not from nitrite (Wang et al. 2008). It could also indicate that PAOI organisms seem to be more efficient to take up phosphorus in anoxic conditions than PAOII organisms, even with nitrite as an electron acceptor. Anyhow, since PAOI are able to denitrify using nitrate and nitrite, as opposed to PAOII, in order to ensure a good denitrifying phosphorus removal, the selection for PAOI organisms should be favoured. Additionally, a higher P/N removal will be achieved for pH values between 7 and 7.5.

**CONCLUSIONS**

This study achieved one of the highest enrichments in PAOI Accumulibacter so far reported through a propionate-fed reactor operated under anaerobic/anoxic/aerobic conditions with nitrate pulses. The enrichment process produced a culture displaying both anoxic and aerobic phosphorus removal. The high anoxic:aerobic ratio was suggested as a favourable selecting and stabilizing factor for PAOI.

### Table 1

Anoxic P/N ratios for different batch tests

<table>
<thead>
<tr>
<th>pH</th>
<th>P/N</th>
<th>stdev</th>
<th>time</th>
<th>Acc.</th>
<th>PAOI</th>
<th>PAOII</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃</td>
<td>7.1</td>
<td>1.0</td>
<td>0.1</td>
<td>781*</td>
<td>± 90%*</td>
<td>± 90%</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>1.3</td>
<td>0.5</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.75</td>
<td>0.05</td>
<td>264</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>0.63</td>
<td>0.02</td>
<td>468</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.58</td>
<td>0.03</td>
<td>575</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.41</td>
<td>0.03</td>
<td>341</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂</td>
<td>7.2</td>
<td>0.89</td>
<td>ND</td>
<td>183</td>
<td>± 90%*</td>
<td>± 90%</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.65</td>
<td>0.03</td>
<td>809*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.1</td>
<td>0.20</td>
<td>0.04</td>
<td>483</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>8.2</td>
<td>0.33</td>
<td>0.01</td>
<td>327</td>
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<tr>
<td>NO₃</td>
<td>7.6</td>
<td>0.82</td>
<td>.</td>
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<td>75%</td>
<td>44%</td>
<td>31%</td>
</tr>
<tr>
<td>NO₂</td>
<td>7.5</td>
<td>0.29</td>
<td>.</td>
<td>.</td>
<td>46%</td>
<td>ND</td>
<td>46%**</td>
</tr>
<tr>
<td>NO₃</td>
<td>7.3</td>
<td>0.63</td>
<td>.</td>
<td>.</td>
<td>72%</td>
<td>93%</td>
<td>7%</td>
</tr>
<tr>
<td>NO₂</td>
<td>7.3</td>
<td>0.29</td>
<td>.</td>
<td>.</td>
<td>82%</td>
<td>39%</td>
<td>61%</td>
</tr>
</tbody>
</table>

*Batch tests done at days 781 and 809 had a lower enrichment in Accumulibacter due to the increase of a rod population of x- and y-Proteobacteria non-correlated with the EBPR function

** This study obtained a 46% enrichment in Accumulibacter cocci-shaped cells able to use nitrite but not nitrate which suggests the presence of PAOII organisms, however this was not demonstrated with the Acc-II-444 probe.
organisms: the anoxic phase length was sufficiently long to eliminate PAOII organisms, the short aerobic phase provided enough oxygen for a faster and more efficient replenishment of the PAOI glycogen and polyphosphate pool. In several batch tests, it was shown that although phosphorus removal was significantly faster with oxygen, the microbial culture was able to remove phosphorus using two other electron acceptors: nitrate and nitrite. Concerning the efficiency of the removal process with nitrate and nitrite, the P/N ratio observed was impacted by pH and varied in a similar trend for nitrate and nitrite in the range of pH values of 7–8.2: a lower pH value led to higher P/N values. PAOI seem to be more efficient in P removal in anoxic conditions and therefore the favoring of their selection in any denitrifying system is recommended in detriment of PAOII organisms.

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