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Cooperation between *Candidatus* Competibacter and *Candidatus* Accumulibacter clade I, in denitrification and phosphate removal processes

F.J. Rubio-Rincón [a, b, *], C.M. Lopez-Vazquez [a], L. Welles [a], M.C.M. van Loosdrecht [b], D. Brdjanovic [a, b]

[a] Department of Environmental Engineering and Water Technology, UNESCO-IHE Institute for Water Education, Westvest 7, 2611AX, Delft, The Netherlands
[b] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ, Delft, The Netherlands

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ABSTRACT

Although simultaneous P-removal and nitrate reduction has been observed in laboratory studies as well as full-scale plants, there are contradictory reports on the ability of PAO I to efficiently use nitrate as electron acceptor. Such discrepancy could be due to other microbial groups performing partial denitrification from nitrate to nitrite. The denitrification capacities of two different cultures, a highly enriched PAO I and a PAO I-GAO cultures were assessed through batch activity tests conducted before and after acclimatization to nitrate. Negligible anoxic phosphate uptake coupled with a reduction of nitrate was observed in the highly enriched PAO I culture. On the opposite, the PAO I-GAO culture showed a higher anoxic phosphate uptake activity. Both cultures exhibited good anoxic phosphate uptake activity with nitrite (8.7 ± 0.3 and 9.6 ± 1.8 mgPO_4-P/gVSS.h in the PAO I and PAO I-GAO cultures, respectively). These findings suggest that other microbial populations, such as GAOs, were responsible to reduce nitrate to nitrite in this EBPR system, and that PAO I used the nitrite generated for anoxic phosphate uptake. Moreover, the simultaneous denitrification and phosphate removal process using nitrite as electron acceptor may be a more sustainable process as can: i) reduce the carbon consumption, ii) reduce oxygen demand of WWTP, and iii) due to a lower growth yield contribute to a lower sludge production.

1. Introduction

Phosphate is a key nutrient to remove from wastewater streams to avoid eutrophication of water bodies (Yeoman et al., 1988). One of the ways to remove phosphate is biologically, through the use of polyphosphate accumulating organisms (PAOs) in wastewater treatment plants (WWTP). *Candidatus* Accumulibacter phosphatis are one of the main PAOs performing the biological removal of phosphate in WWTP (Hesselmann et al., 1999). Under anaerobic conditions, PAOs are able to store volatile fatty acids (VFAs) as polyhydroxyalkanoates (PHAs), generating the required reduction equivalents ($NADH_2$) by converting glycogen to PHA. This fermentation pathway also generates some energy in the form of ATP, the remainder of the energy needed is supplied by polyphosphate consumption. Under aerobic or anoxic conditions, PAOs oxidize stored PHA to generate energy which is used to replenish polyphosphate and glycogen, to grow, and for maintenance purposes (Smolders et al., 1994a, 1994b; Kuba et al., 1996). PAOs proliferate in WWTP's by recirculating the activated sludge mixed liquor through anaerobic and aerobic/anoxic conditions, and directing the influent rich in VFAs to the anaerobic tank (Barnard, 1975).

The ability to store VFAs as PHAs under anaerobic conditions is not restricted to PAOs. Glycogen accumulating organisms (GAOs) such as *Candidatus* Competibacter phosphatis and *Defluvicoccus* are normally observed to grow in enhanced biological phosphorus removal (EBPR) systems. Nevertheless, contrary to PAO's metabolism, GAOs rely solely on the consumption of glycogen to produce the required ATP and $NADH_2$ needed for uptake of VFA and storage as PHA (Cech et al., 1993). Thus, GAOs do not contribute to the biological removal of phosphorus (Cech et al., 1993). Therefore,

* Corresponding author. Department of Environmental Engineering and Water Technology, UNESCO-IHE Institute for Water Education, Westvest 7, 2611AX, Delft, The Netherlands.
E-mail addresses: f.rubiorincon@unesco-ihe.org, F.J.RubioRincon@tudelft.nl (F.J. Rubio-Rincón), c.lopezvazquez@unesco-ihe.org (C.M. Lopez-Vazquez), l.welles@unesco-ihe.org (L. Welles), m.c.m.vanloosdrecht@tudelft.nl (M.C.M. van Loosdrecht), d.brdjanovic@unesco-ihe.org, d.brdanovic@tudelft.nl (D. Brdjanovic).

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their existence is generally associated to the failure of P-removal performance in EBPR systems (Lopez-Vazquez et al., 2009). Nevertheless, the ecological role of GAO in EBPR communities may be more diverse than just being a competitor of PAO.

Most of the WWTP that perform EBPR also remove nitrogen through nitrificationdenitrification processes. Early studies suggested that PAO could perform the anoxic phosphate uptake using nitrate or nitrite as electron acceptors, minimising the requirements of carbon and sludge production (Vlekke et al., 1988; Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993). Such anoxic phosphate uptake was also observed in the anoxic stages of full-scale WWTPs, confirming the existence and role of denitrifying polyphosphate accumulating organisms (DPAOs) (Kuba et al., 1997a, 1997b; Jeong Myeong et al., 2013). Later studies indicated that the ability to use nitrate or nitrite in denitrification differs according to the type of PAO clade (Ahn et al., 2001).

Using both the 16SrRNA and the polyphosphate kinase gene (ppk1) as a genetic marker, past research indicated that "*Ca.* Accumulibacter" is organized in two main clades: *Ca.* Accumulibacter phosphatis clade I (PAO I) and *Ca.* Accumulibacter phosphatis clade II (PAO II). Both clades composed of several distinct sub-clades (McMahon et al., 2002). Interestingly, a metagenomic analysis of different EBPR sludge indicated that the metagenome of PAO II lacked the respiratory nitrate reductase enzyme (*nar*), but contained the mechanisms to denitrify from nitrite onwards suggesting that PAO II could not use nitrate as electron acceptor (García Martín et al., 2006).

Later studies carried out by Carvalho et al. (2007), Flowers et al. (2009) and Oehmen et al. (2010a,b) suggested that the PAO I clade was capable of a considerable anoxic phosphate uptake activity using nitrate and/or nitrite as an electron acceptor. Alternatively, the PAO II clade could solely use nitrite for anoxic uptake (Carvalho et al., 2007; Flowers et al., 2009; Oehmen et al., 2010a). Later studies of Lanham et al. (2011) validated the ability of PAO I to use nitrate and nitrite as electron acceptor, while using a highly enriched PAO I culture (approx. 90% PAO I bio-volume). In line with these results, Camejo et al., 2016 reported that PAO I had the enzymes necessary to denitrify from nitrate onwards. However, the bio abundance of PAO I with respect of all microbial community on the studies of Camejo et al. (2016) was 15–20%. On the contrary, other studies indicated that PAO I was not capable of using nitrate as electron acceptor for anoxic phosphate uptake (Saad et al., 2016). The latter authors demonstrated that a highly enriched PAO I culture (>95%) cultivated under anaerobic-oxic (AO) conditions were not able to perform an anoxic P-uptake activity when exposed to nitrate (Saad et al., 2016).

These contradictory findings on the anoxic P-uptake of PAO I using nitrate, suggest that factors other than the occurrence of a specific PAO clade affect the denitrification capacity in EBPR systems. Different studies on mixed PAO-GAO cultures suggest that GAO rather than PAO use nitrate as electron acceptor (Kong et al., 2006; Lemaire et al., 2006). However, it is not possible to reach conclusive remarks from such studies as the actual PAO and GAO fractions present in those systems were low (approx. 2–6% *Candidatus* Competibacter) and the PAO clade (PAO I or PAO II) present in those systems was not reported. In line with these observations, Ribera-Guardia et al. (2016) suggested that PAO (PAO I and PAO II) have a certain preference for nitrite over nitrate as electron acceptor. Nevertheless, contrary to Kong et al. (2006) and Lemaire et al. (2006), Ribera-Guardia et al. (2016) suggested that PAO I were responsible for the reduction of nitrate to nitrite. However, up to 30% of the bio-volume present in that DPAO reactor was not characterized via their microbial analysis, and the relative abundance of PAO I compared to all bacteria was around 26% (Ribera-Guardia et al., 2016).

Thus, it is still unclear whether PAO I are able to directly use nitrate for the uptake of phosphate or if PAO I rather uses the nitrite generated by side populations, such as GAO. This research aims to understand the role of GAO in denitrifying EBPR systems. For this purpose, the ability to use nitrate or nitrite as electron acceptor of an enriched PAO I and a PAO I-GAO culture was assessed and compared.

2. Materials and methods

2.1. Enrichment of the PAO I and PAO I-GAO mixed cultures

Two EBPR systems were enriched in two double jacketed reactors with a working volume of 2.5 L. Both reactors were inoculated with 500 mL of activated sludge from Nieuwe Waterweg WWTP (Hoek van Holland, The Netherlands). The reactors were automatically controlled as sequencing batch reactors (SBR) in cycles of 6 h, consisting of 5 min feeding, 2 h 10 min anaerobic phase, 2 h 15 min aerobic phase, 1 h settling time and 30 min for effluent withdrawal. During the effluent withdrawal phase, half of the working volume was removed providing an HRT of 12 h. At the end of the aerobic phase, 78 mL of mixed liquor were wasted in order to control the SBR at a solids retention time (SRT) of 8 d. The pH was controlled at 7.6 ± 0.1 in order to favour PAO over GAO (reactor 1) and at 7.0 ± 0.1 in order to obtain a mixed PAO-GAO community (reactor 2; Filipe et al., 2001), through the addition of 0.4 M HCl and 0.4 M NaOH. The temperature was controlled externally with a LAUDA system at 20 ± 1 °C, and the dissolved oxygen (DO) concentration at 20% of the saturation level during the aerobic phase. The DO and pH were continuously monitored online, and orthophosphate (PO_4-P), total suspended solids (TSS) and volatile suspended solids (VSS) were measured twice per week. When no significant changes in these parameters were observed for at least 3 SRT, it was assumed that the system was under pseudo steady-state conditions.

2.2. Synthetic medium

The medium was separated in two bottles containing the carbon source and mineral medium. In order to further favour the growth of PAO over GAO the reactor 1 was fed (per litre) with 0.63 g of $NaHAc•3H_2O$ and 0.06 mL of propionic acid (3:1 ratio COD basis) while for reactor 2 it contained (per litre) 0.85 g of $NaHAc·H_2O$, which should favour a mixed PAO-GAO community (Lopez-Vazquez et al., 2009). The carbon source solutions were fed to both systems to reach 396 mg COD/L in the influent of each reactor. The mineral medium was the same in both reactors and contained per litre 0.10 g NH_4Cl, 0.11 g $NaH_2PO_4·H_2O$, 0.09 g $MgSO_4·7H_2O$, 0.01 g $CaCl_2·2H_2O$, 0.03 g KCl, 0.01 g yeast extract, and 0.3 mL of trace element solution prepared according to Smolders et al. (1994a,b). In order to inhibit the nitrification during the aerobic phase 20 mg N-allylthiourea per litre (ATU) was added. After being fed and diluted inside the reactor, the initial phosphate and nitrogen concentrations were of 25 mg PO_4-P/L and 36 mg NH_4-N/L, respectively.

2.3. Biomass acclimatization to anoxic conditions

To assess the denitrification activity of the biomass cultures enriched in both systems, short-term activity tests were conducted in the parent reactors and in a separate batch set-up with 400 mL working volume. Assuming that the nitrite/nitrate reductase enzymes may only be expressed and become active after a period of exposure to anoxic conditions (Skennerton et al., 2014), the biomass in the reactors was acclimatized to such conditions prior to the execution of the tests. In order to acclimatize the biomass and at

the same time ensure the complete uptake of phosphate, the cycle of the reactors were modified from anaerobic-aerobic to anaerobic-anoxic-aerobic conditions. The anaerobic phase had a length of 45 min, the anoxic phase of 2 h, the aerobic phase of 2 h, settling time of 45 min and effluent withdrawal of 30 min. To ensure anoxic conditions, nitrate was fed as a pulse from a stock solution that contained 2 g NO_3-N/L (reaching up to 10 mg NO_3-N/L in the reactor). Nitrate was present during the two hours of anoxic phase. The acclimatization of the reactors was carried out for 8 cycles prior to the conduction of each batch activity test.

2.4. Batch activity tests

Once the activity of the reactors reached a pseudo steady-state conditions, batch tests were conducted before and after the anoxic acclimatization of the biomass. 200 mL of mixed liquor were taken after the aerobic phase, diluted to 400 mL and introduced into two 500 mL double-jacketed reactors. In order to identify the role of GAO in denitrifying EBPR, either propionate (which benefit the carbon uptake by PAO) or acetate was used as carbon source (Pijuan et al., 2004). Both carbon sources were tested in combination with three electron acceptors (oxygen, nitrate, and nitrite). The cycle was operated anaerobically for 2 h 5min and either anoxic or aerobic for 2 h 15 min. In each test, the initial organic concentration was of 396 mg COD/L. In the corresponding tests, DO was provided continuously by sparging compressed air at 10 L/h. Nitrate or nitrite were fed as pulse and kept at a concentration between 1 and 7 mg N/L during the whole time of the anoxic phase (2 h 15 min). The pH and temperature were controlled at 7.0 ± 0.1 and 20 ± 1 °C. The sludge was constantly stirred with a magnetic stirred operated at 300 rpm. The TSS and VSS concentrations were measured at the start and end of each phase (anaerobic, anoxic, or aerobic). Samples for the determination of acetate and propionate concentrations were collected at different time intervals during the anaerobic phase. Nitrate, nitrite, ammonia, and ortho-phosphate were measured throughout the anoxic or aerobic phase. These parameters were analysed as detailed below.

In order to verify the potential role of GAO in the denitrification of EBPR systems, an extra batch test was performed, aimed to enhance the activity of either PAO or GAO. The pH during the anaerobic phase was controlled either at pH 6.0 to favour carbon storage of GAO or at pH 8.0 to enhance the carbon storage of PAO (Filipe et al., 2001; Oehmen et al., 2005). This batch test was carried out using acetate as carbon source and biomass from the PAO I-GAO culture, which contained similar biomass fractions of PAO I and GAO.

2.5. Long-term exposure of PAO I to anoxic conditions (NO_3^-)

In order to enhance the development of a denitrifying EBPR culture that could use nitrate as electron acceptor, the reactor 1 was operated under an anaerobic-anoxic-aerobic (A^2O) configuration for 4 SRT. The 6 h cycle was performed in the same way as described for the acclimatization of biomass to the presence of nitrate, with the difference that the nitrate concentration at the start of the anoxic phase was gradually increased to 20 mg NO_3-N/L. Ortho-phosphate, ammonia, nitrate, nitrite and DO were measured at different time intervals during one cycle.

In order to assess the contribution of endogenous respiration and cell decay to denitrification, 250 mg of hydrolysed biomass was added in the anoxic phase. The biomass used was extracted from the same reactor. In order to enhance the biomass hydrolysis, it was left at room temperature for 1 month. To further increase the cell decay, the biomass was further exposed during 3 h at pH 2.0 and re-adjusted to pH 7.0 prior to addition in the reactor.

2.6. Analyses

Ortho-phosphate and nitrite were analysed according to methods 4500-P-C and 4500-NO_2-B, respectively as described in APHA et al. (2005). Nitrate and ammonia were measured according to ISO 7890/1 (1986) and NEN 6472 (1983), respectively. Acetate and propionate were measured using a Varian 430-GC Gas Chromatograph (GC) equipped with a split injector (200 °C), a WCOT Fused Silica column (105 °C) and coupled to a FID detector (300 °C). Helium gas was used as carrier gas and 50 μL of butyric acid as internal standard. TSS and VSS were measured in triplicate (APHA et al., 2005).

2.7. Fluorescence in situ hybridization (FISH)

To estimate the microbial populations distribution in the reactors, FISH analyses were performed according to Amman (1995). *Ca.* Accumulibacter phosphatis were targeted with the PAOMIX probe (mixture of probes PAO 462, PAO 651 and PAO 846) (Crocetti et al., 2000). The presence of *Ca.* Accumulibacter clade I and clade II was assessed with probes Acc-1-444 and Acc-2-444 (Flowers et al., 2009). *Ca.* Competibacter phosphatis was identified with the GB probe according to Kong et al. (2002). *Defluvicoccus* clusters 1 and 2 were identified with the TFO-DF215, TFO-DF618, DF988 and DF1020 probes (Wong et al., 2004; Meyer et al., 2006). Vectashield with DAPI was used to avoid the fading and stain all living organisms (Nielsen et al., 2009).

FISH quantification of each probe was performed by image analysis of 25 random pictures taken with an Olympus BX5i microscope and analysed with the software Cell Dimensions 1.5 (Hamburg, Germany). The relative abundance of bacteria was estimated based on the percentage of surface area positively stained with the corresponding probes with regard to the total area covered with DAPI (Flowers et al., 2009). The standard error of the mean was calculated as described by Oehmen et al. (2010b).

2.8. Stoichiometry and kinetics

The ratio P/VFA was calculated based on the observed net phosphate released at the end of the anaerobic phase per total organic carbon consumed. The rates of interest were:

i) $\boldsymbol{q_{NO_3}}$ – Nitrate uptake rate, in mg NO_3-N/gVSS.h
ii) $\boldsymbol{q_{NO_2}}$ – Nitrite uptake rate, in mg NO_2-N/gVSS.h
iii) $\boldsymbol{q_{PO_4,NO_3}}$ – Anoxic phosphate uptake rate in the presence of nitrate, in mg PO_4-P/gVSS.h
iv) $\boldsymbol{q_{PO_4,NO_2}}$ – Anoxic phosphate uptake rate in the presence of nitrite, in mg PO_4-P/gVSS.h
v) $\boldsymbol{q_{PO_4,Ox}}$ – Aerobic phosphate uptake rate, in mg PO_4-P/gVSS.h

All rates were calculated by linear regression based on the profiles observed as described in Smolders et al. (1995). All rates reported are the maximum observed with a R-squared value higher than 0.95 and taken into consideration around 5 measurements. The oxygen uptake rate (OUR) was measured in a separate biological oxygen monitoring (BOM) unit equipped with a WTW OXi 340i unit connected to the software Multilab as described in Lopez-Vazquez et al. (2008).

3. Results

3.1. Biomass characterization in the EBPR systems

Two EBPR systems (hereafter EBPR1 and EBPR2 for the PAOI and PAO I-GAO cultures, respectively) were operated for more than 150

days. They reached pseudo steady-state conditions before the activity tests were carried out (supplementary information A). Both systems consumed all carbon source within the first 15 min of the anaerobic phase and showed complete P-removal. The anaerobic P-release/VFA-uptake ratio in EBPR1 was substantially higher than in EBPR2 (0.65 and 0.45 P-mmol/C-mmol, respectively), indicating that EBPR1 had a higher PAO fraction. Under aerobic conditions, EBPR1 took up phosphate at a faster rate than EBPR2 (47 $mgPO_4$-P/gVSS.h *versus* 23 $mgPO_4$-P/gVSS.h, respectively).

In order to assess the dominant microbial communities involved in each system, FISH analyses (Fig. 1) showed that the sludge in EBPR1 contained 97± 4% *Candidatus* Accumulibacter phosphatis of which more than 99% belonged to *Ca.* Accumulibacter phosphatis Clade I. The sludge in EBPR2 was composed of a mixed culture of *Ca.* Accumulibacter phosphatis (47± 3%) and *Candidatus* Competibacter phosphatis (47± 5%). The fraction of *Ca.* Accumulibacter phosphatis in EBPR2 consisted mainly of *Ca.* Accumulibacter phosphatis clade I (>94%). *Defluvicoccus* was not detected in either reactor while, in contrast to EBPR2, *Ca.* Competibacter phosphatis was not detected in EBPR1. Both systems remained in pseudo steady state conditions during this research, ie. no changes in rates and stoichiometry of the conversions. Thus, it is unlikely that a microbial change occurred in this short period of time.

3.2. Batch activity tests before acclimatization to nitrate

A profile of the different batch tests conducted during this research can be found in the supplementary material B. Three control tests executed before the acclimatization of the biomass to the presence of nitrate were performed with sludge from the PAO I-GAO culture (EBPR2). The addition of three different electron acceptors (oxygen, nitrate, and nitrite) was assessed in each batch test. In the three tests, all the carbon source (acetate) was consumed in the anaerobic phase, resulting in anaerobic P-release concentrations of up to 108 ± 12 mgP/L. In the experiment conducted with oxygen, a complete removal of phosphate was observed with a maximum OUR of 47 mgO_2/gVSS.h and a phosphate uptake rate of 29.8 $mgPO_4$-P/gVSS.h. During the experiment with nitrite as electron acceptor, the system was able to take up 14.3 $mgPO_4$-P/gVSS.h and 4.8 mg NO_2-N/gVSS.h. In the experiment conducted with nitrate as electron acceptor, neither phosphate nor nitrate removal was observed.

3.3. Batch activity tests after acclimatization to nitrate

3.3.1. Effect of acetate as carbon source

Under anaerobic conditions, acetate was fully consumed in all tests. However, as expected, the anaerobic P-release was substantially higher in the batch tests performed with the PAO I culture (EBPR1) than in the tests performed with the PAO I-GAO culture (EBPR2): 151 ± 11 $mgPO_4$-P/L and 115 ± 1 $mgPO_4$-P/L, respectively.

Table 1 shows the aerobic/anoxic phosphate uptake and nitrate/nitrite reduction rates observed in the PAO I and PAO I-GAO cultures. The PAO I culture had a 49% faster aerobic phosphate uptake rate than the PAO I-GAO culture (Table 1). The maximum OUR was slightly higher in the mixed PAO I-GAO culture than in the PAO I enriched culture (51 mgO_2/gVSS.h and 44 mgO_2/gVSS.h, respectively). The net ammonia consumption (assumed to be directly associated to grow) was 4 $mgNH_4$-N/L in the PAO I culture and 5 $mgNH_4$-N/L in the PAO I-GAO culture.

When nitrate was used as electron acceptor, a considerable anoxic P-uptake in the PAO I culture was not observed (Table 1). On the other hand, the PAO I-GAO culture removed 8.7 $mgPO_4$-P/gVSS.h together with 3.2 $mgNO_3$-N/gVSS.h. Nitrite never accumulated in any test as a potential intermediate product of the denitrification process (Supplementary information B).

On the contrary, when nitrite was used as electron acceptor the PAO I culture displayed a faster P-uptake rate of 9.6 $mgPO_4$-P/gVSS.h compared to 7.7 $mgPO_4$-P-gVSS.h observed in the PAO I-GAO culture. Likewise the observed nitrite uptake rate of the PAO I culture was faster than the observed nitrite uptake rate of the PAO I-GAO culture (11.4 $mgNO_2$-N/gVSS.h and 6.5 $mgNO_2$-N/gVSS.h, respectively).

3.3.2. Effect of propionate as carbon source

In all of the tests, propionate was fully consumed under anaerobic conditions. During the aerobic period, both cultures were able to remove all phosphate completely but at different rates. The PAO I culture was capable of taking up phosphate and oxygen at a rate of 32.5 $mgPO_4$-P/gVSS.h and 58 mgO_2/gVSS.h, respectively. The P-uptake rate and maximum OUR of the PAO I-GAO culture was around half of those obtained with the PAO I culture: 13.6 $mgPO_4$-P/gVSS.h and 27 mgO_2/gVSS.h, correspondingly.

Interestingly when nitrate was used as an electron acceptor, the P-removal activity of the PAO I-GAO culture was substantially higher than that of the PAO I culture. The anoxic phosphate uptake and nitrate reduction rate observed in the PAO I culture were 45% of those observed in the PAO I-GAO culture (Table 1). On the contrary, when nitrite was added as electron acceptor the PAO I culture had a faster nitrite reduction rate than the PAO I-GAO culture (11.1 and 7.5 $mgNO_2$-N/gVSS.h, respectively). However, the anoxic P-uptake over nitrite of the PAO I-GAO culture was substantially higher than the anoxic P-uptake over nitrite of the PAO I culture (11.5 and 7.7 $mgPO_4$-P/gVSS.h, respectively).

3.4. PAO I-GAO anoxic activity according to a different anaerobic pH

Previous results showed that the PAOI-GAO culture performed a higher anoxic phosphorus uptake using nitrate than the PAO I culture. In order to assess if this difference was caused by the presence of GAO, the pH of the anaerobic phase was set either at 8.0 or 6.0 which according to Filipe et al. (2001) should benefit the acetate uptake rate of either PAO or GAO, respectively. The anaerobic P/VFA ratio was higher at pH 8.0 than at pH 6.0 (0.76 and 0.47 P-mmol/C-mmol, respectively) in line with previous observations on the pH effect on PAO cultures (Smolders et al., 1994b; Filipe et al., 2001).

The maximum anoxic P-uptake rate observed was higher when the acetate uptake of GAO was favoured (6.5 $mgPO_4$-P/gVSS.h; Fig. 2). On the other hand, when the acetate uptake of PAO was favoured the anoxic P-uptake rate showed two different trends, an initial anoxic P-uptake rate of 3.7 $mgPO_4$-P/gVSS.h followed by a slower anoxic P-uptake rate of only 1.2 $mgPO_4$-P/gVSS.h (Fig. 2). A similar trend was observed in the nitrate uptake rate, decreasing by 43% from 3.6 to 2.1 $mgNO_3$-N/gVSS.h when the acetate uptake of GAO or PAO was favoured, respectively. In both experiments, nitrate was added in excess and nitrite was not detected.

3.5. Long-term anaerobic-anoxic-aerobic (A_2O) performance

To assess if the long-term exposure to nitrate could enhance the anoxic P-removal performance, the SBR enriched with the PAO I culture was operated in anaerobic-anoxic-aerobic (A_2O) cycles for 4 SRT. A microbial characterization was performed to study if the long-term exposure could favour the growth of other microorganisms in addition to PAO I. No change in the dominant microbial populations was observed (Fig. 1). The fraction of PAO I remained above 90% and that of GAO below 5%. 21 $mgPO_4$-P/L were anoxically removed with a P-uptake rate of 4.7 $mgPO_4$-P/gVSS.h. The nitrate

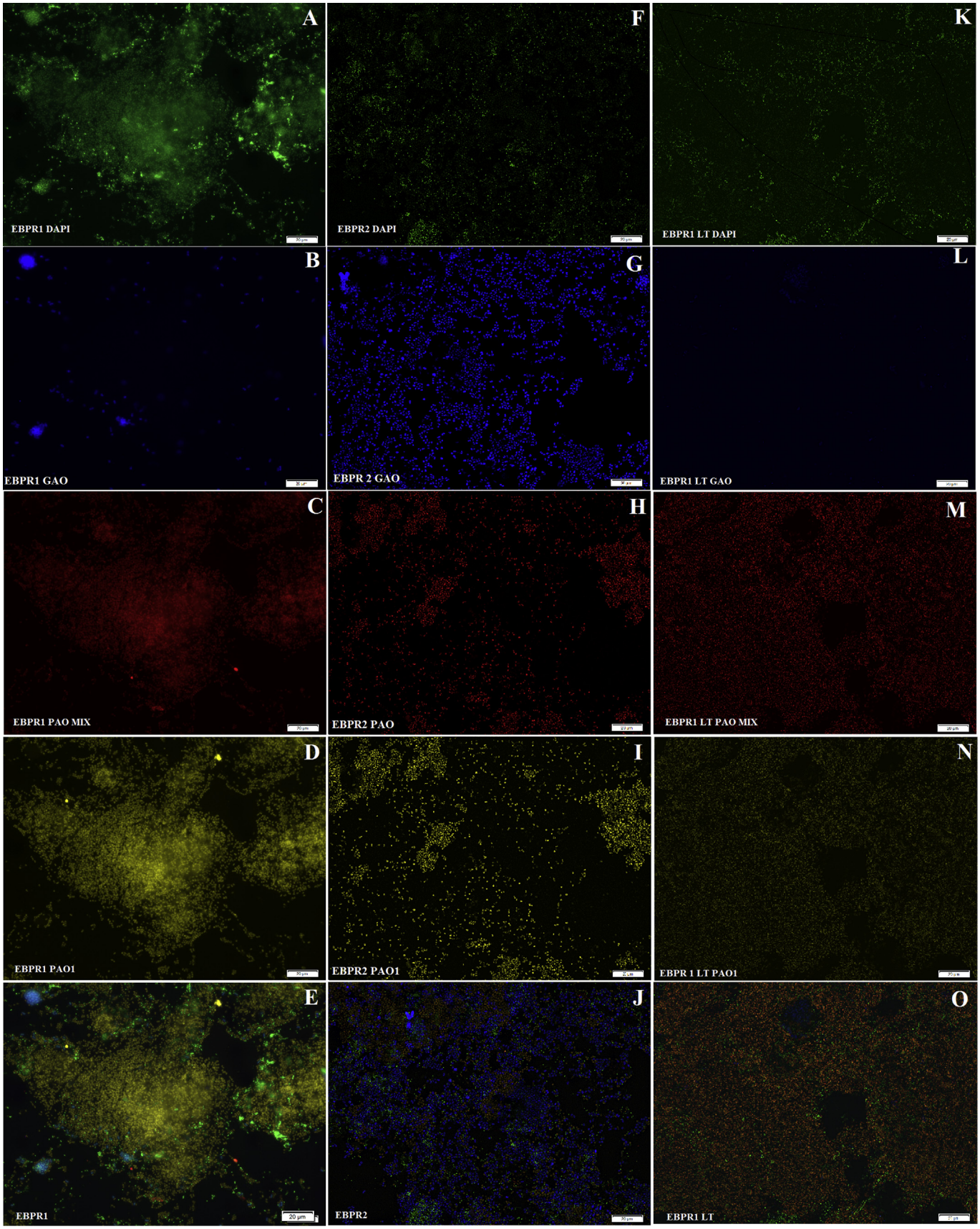

Fig. 1. Microbial identification analyses performed by fluorescence *in situ* hybridization (FISH) in the biomass cultures cultivated in reactor EBPR 1 (A to E), EBPR 2 (F to J), and after the long-term exposure to nitrate in EBPR 1 (K to O). The green color indicates DAPI staining, blue GAO mix, red PAO mix and yellow PAO I. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Maximum specific phosphate uptake rates of the PAO I and mixed PAO I-GAO cultures with three different electron acceptors (oxygen, nitrate or nitrite) and two different carbon sources (acetate, and propionate).

Culture	C-source	$q_{PO_4,Ox}$	q_{PO_4,NO_3}	q_{PO_4,NO_2}	q_{NO_3}	q_{NO_2}
		mgP/gVSS.h	mgP/gVSS.h	mgP/gVSS.h	mgN/gVSS.h	mgN/gVSS.h
PAO I	Acetate	37.8	0.6	9.6	1.8	11.4
	Propionate	32.5	3.4	7.7	2.6	11.1
PAO I-GAO	Acetate	25.4	8.7	7.7	3.2	6.5
	Propionate	13.6	7.7	11.5	5.7	7.5
PAO I[a]	Acetate	29.7	4.7	N.A	3.9	N.A

[a] Observed rate after 4 SRT under anaerobic-anoxic-oxic (A_2O) operation conditions.

uptake rate increased to 3.9 $mgNO_3$-N/gVSS.h removing up to 17 $mgNO_3$-N/L. To explain if such nitrate consumption could be the result of endogenous respiration by ordinary heterotrophic organisms (OHOs), 250 mg VSS of hydrolysed biomass was added at the start of the anoxic phase and an additional anoxic batch activity test was conducted. The net P and nitrate uptake concentrations did not increase and remained around 20 $mgPO_4$-P/L and 15 $mgNO_3$-N/L, respectively. This indicated that the addition of partially digested biomass did not have any effect on the anoxic P-uptake activity.

4. Discussion

4.1. Anoxic P-uptake activity of PAO on nitrate and nitrite

The PAO I-GAO culture had a considerably higher anoxic P-uptake on nitrate than the PAO I culture. However, the anoxic P-uptake with nitrite as electron acceptor was not considerably different between both cultures (8.7 ± 0.3 and 9.6 ± 1.8 $mgPO_4$-P/gVSS.h in the PAO I and PAO I-GAO cultures, respectively). This suggests that PAO I are able to denitrify using nitrite, but that they may rely on other microbial communities to perform the first part of denitrification process from nitrate to nitrite. In line with this hypothesis, Tayà et al. (2013) proposed to use nitrite as a selective measure for PAO I in the so called PAO-GAO competition since the GAO present in their system were unable to denitrify over nitrite. On the contrary, Lanham et al. (2011), using an enriched PAO I (90%) culture, concluded that PAO I is able to use nitrate for anoxic P-uptake (approximately 9 $mgPO_4$-P/gVSS.h). Interestingly, once the maximum anoxic phosphate uptake was reached in their studies, they observed a moderate anoxic phosphate release and glycogen consumption, whereas the biomass still contained PHA (around 0.5 C-mmol/gVSS) and nitrate was still present. Thus, it seems that the PAO in the system of Lanham et al. (2011) was not capable to generate the required energy for maintenance processes from the oxidation of PHA with nitrate. Instead, as suggested by Kerrn-Jespersen and Henze (1993), when PAO cannot generate energy from the oxidation of PHA they hydrolyse polyphosphate and consume glycogen as source of energy. According to Kerrn-Jespersen and Henze (1993) an anoxic P- release when PAO still contain PHA and nitrate is still present is a clear indication that the concerned PAO community cannot reduce nitrate. In the studies of Lanham et al. (2011) around 10% of the biomass was composed of rod shaped bacteria belonging to alpha- and gamma-proteobacteria (possibly GAO), which might be able to reduce nitrate into nitrite which could have been used for the anoxic P-uptake observed in their systems, supporting the observations drawn in this study.

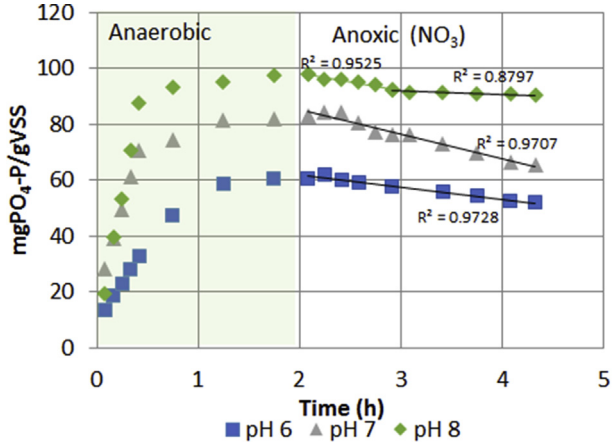

Fig. 2. Anoxic phosphate uptake profiles at pH 7.0 observed in the mix PAO I-GAO culture (EBPR2) after different anaerobic stages performed at pH 6.0, 7.0 and 8.0 using acetate as carbon source.

The results obtained in this research indicate that the PAO I culture enriched in our system cannot efficiently use nitrate as electron acceptor and therefore is unable to perform an efficient anoxic P-uptake activity using nitrate as electron acceptor (Table 1), which is in agreement with previous studies (Saad et al., 2016). Saad et al. (2016) failed to show which organism was responsible for the denitrification observed in their system (0.56–1.4 mg NO_3-N/gVSS.h), whereas based on the kinetics observed in our study it seems that GAO were responsible for the reduction of nitrate to nitrite in EBPR systems.

In Table 2 the stoichiometry ratios observed during different anoxic P-uptake studies as function of pH, carbon source and biomass fraction are presented. Similar to the observations obtained in our research (Fig. 2), Lanham et al. (2011) reported that a pH increase led to a lower anoxic P-uptake per mol of nitrate (Table 2). According to Filipe et al. (2001), a pH above 7.2 benefits the acetate uptake performed by PAO. Thus, if PAO I is able to efficiently use nitrate a pH above 7.2 would lead to a higher anoxic P-uptake whereas the opposite should occur at pH values lower than 7.2. The limited anoxic P-uptake activity at pH 8.0 but higher anoxic P-removal performance at pH 6.0 suggests that GAO carry out the denitrification process from nitrate to nitrite, and that PAO I denitrify from nitrite onwards. The two different anoxic P-uptake rates observed at pH 8.0 in this study (Fig. 2) support this hypothesis as a pH increase slows down the acetate uptake of GAO (Filipe et al., 2001). Thus, the PHA stored by GAO at pH 8 might have been used at the start of the anoxic phase to denitrify and supply nitrite to PAO I for anoxic P-uptake (3.7 $mgPO_4$-P/gVSS.h) but became limiting after 1 h (1.2 $mgPO_4$-P/gVSS.h) once the carbon source of GAO got exhausted. These are strong indications that organisms such as GAO could have an essential role in the simultaneous denitrification and phosphate removal processes.

4.2. Effect of the carbon source on the anoxic P-uptake activity

Similar maximum specific anoxic P-uptake rates with nitrite

Table 2
Comparative stoichiometric ratios reported in literature from different EBPR systems performing anoxic P-uptake activities as a function of pH and *Accumulibacter* fractions.

Source	pH	System	Carbon	P/VFA	VSS/TSS	molO_2/mol P	molNO_3-N/molP	molNO_2-N/molP	% Acc.	% PAOI	% Com.	% Def.
This study	7	EBPR2	Acetate	0.54 ± 0.01	0.62	0.65	0.73–0.88	0.94–1.58	50 ± 3%	47 ± 3%	47 ± 5%	N.R.
This study	7	EBPR2	Propionate	0.58 ± 0.06	0.62	0.54	0.86–0.97	1.11–1.35	50 ± 3%	47 ± 3%	47 ± 5%	N.R.
This study	6	EBPR2	Acetate	0.40	0.62	N.R.	0.76–1.12	N.R.	50 ± 3%	47 ± 3%	47 ± 5%	N.R.
This study	8	EBPR2	Acetate	0.70	0.62	N.R.	0.83–5.00	N.R.	50 ± 3%	47 ± 3%	47 ± 5%	N.R.
This study	7	EBPR1	Acetate	0.72 ± 0.06	0.6	0.45	5.16[a]	1.18–3.8	98 ± 3%	97 ± 4%	4 ± 1%	N.R.
This study	7	EBPR1	Propionate	0.52 ± 0.04	0.6	0.54	0.84–1.53[a]	1.19–1.95	98 ± 3%	97 ± 4%	4 ± 1%	N.R.
Carvalho et al. (2007)	7.0–8.2	Step 0	Acetate	0.52			5.00		64 ± 2%		1%	5%
Carvalho et al. (2007)	7.0–8.2	Step 1	Acetate	0.16			1.67					
Carvalho et al. (2007)	7.0–8.2	Step 2	Acetate	0.16			1.67		37 ± 2%		NO	5%
Carvalho et al. (2007)	7.0–8.2	Step 0	Propionate	0.4			0.80		89 ± 2%		1%	5%
Carvalho et al. (2007)	7.0–8.2	Step 1	Propionate	0.37			1.20					
Carvalho et al. (2007)	7.0–8.2	Step 2	Propionate	0.29			1.85					
Carvalho et al. (2007)	7.0–8.2	Step 3	Propionate	0.32			1.22		76 ± 2%			1%
Kuba et al. (1993)			Acetate	0.58	0.65–0.70	1.15	1.07					
Lanham et al. (2011)	7.1–7.2	Day 183	Propionate	0.53			1	1.12	90%		N.D	N.D
Lanham et al. (2011)	7.5	Day 264	Propionate	0.53			1.33	N.R.	90%		N.D	N.D
Lanham et al. (2011)	7.9	Day 468	Propionate	0.53			1.59	N.R.	90%		N.D	N.D
Lanham et al. (2011)	8	Day 57	Propionate	0.53			1.72	1.53	90%		N.D	N.D
Flowers et al. (2009)	7.3	BR1-N	Acetate	0.62			1.59		72 ± 11%	93 ± 1%		
Flowers et al. (2009)	7.3	BR2-N	Acetate	0.62			3.45		82 ± 11%	39 ± 1%		
Vargas et al. (2011)	7.5		Acetate	0.55 ± 0.07				2.07 ± 0.40	40 ± 7%	NR	ND	ND
Vargas et al. (2011)	7.5		Propionate	0.38 ± 0.08				2.96 ± 0.34	60 ± 4%	NR	ND	27%
Zeng et al. (2003)	7.3		Acetate	0.34	0.84		2.00		38%	NR	NR	NR

were observed in the PAO I and PAO I-GAO culture independently of the organic carbon used either acetate or propionate that ranged on average around 8.7 ± 0.3 and 9.6 ± 1.8 mg PO_4-P/gVSS.h, for the PAO I and PAOI –GAO cultures, respectively (Table 1). This suggests that the addition of acetate or propionate as carbon source did not play an important role on the anoxic P-uptake with nitrite. Similar suggestions were made by Vargas et al. (2011) as they observed similar anoxic P-uptake rates with nitrite when either propionate or acetate was used as carbon source (12.7 and 14.8 mgPO_4-P/gVSS.h, respectively).

On the contrary, the addition of propionate and nitrate to the PAO I culture increased the anoxic P-uptake from 0.6 to 3.4 mgPO_4-P/gVSS.h (Table 1). This is in agreement with the observations of Carvalho et al. (2007) who reported a higher anoxic P-uptake rate when propionate instead of acetate was used as carbon source and nitrate as electron acceptor (19.5 and 8.4 mgPO_4-P/gVSS.h, respectively). Carvalho et al. (2007) suggested that the replenishment of glycogen played an important role in the stability and performance of the acetate and propionate fed reactors. However, our experiments were executed at short term and performed with similar initial biomass composition and intracellular fractions. The use of propionate would generate a higher PHV fraction as compared when acetate is used as carbon source (Satoh et al., 1992). Thus, the fraction of PHA (PHV/PHB) might play an important role, and during energy limiting conditions PAO could have preferential pathways depending on which storage polymer is more essential to be restored. The use of acetate as carbon source requires more reducing equivalents (provided by glycogen) than when propionate is fed (Satoh et al., 1992). This might result in a preferential pathway to restore glycogen under energy limiting conditions when acetate is used, resulting in the lower anoxic phosphate uptake with nitrate observed in these systems.

4.3. Role of flanking communities in denitrification

Compared to this study, Lanham et al. (2011) observed a similar P-release per VFA uptake (at pH 7.0) when using propionate as carbon source (Table 2). This suggests that both cultures had similar fractions of PAO I (on day 183 in the study performed by Lanham et al., 2011). However, as previously explained, a significant anoxic P-uptake on nitrate was not observed in our study. On the other hand, Skennerton et al. (2014) reported that the enzymes used for denitrification (nitrate reductase and periplasmic nitrate reductase enzymes) differ among the subclades of PAO I. Thus, the main differences observed between our studies and the ones of Lanham et al. (2011) might be due to either the fractions of flanking communities (side populations) or the subclades of PAO I present in both systems.

Flowers et al. (2009) suggested that PAO I was able to use nitrate as electron acceptor (Table 2). However, the uptake rates reported by Flowers et al. (2009) of 1.4 mgNO_3-N/gVSS.h and 2 mgPO_4-P/gVSS.h can be considered practically negligible (lower than the uptake rate of 3.4 mg PO_4-P/gVSS.h observed in this study in the enriched PAO I culture). Moreover, the flanking communities present in their sludge could account for up to 20–30% of the total microbial populations (based on their reported estimations) and therefore the presence of bacteria with a GAO phenotype cannot be discarded. The authors suggest that due to the anaerobic P/VFA ratio of 0.61 P-mol/C-mol it was unlikely that bacteria such as GAO were present. This ratio is lower than the one observed in our study (of 0.72 ± 0.05 P-mol/C-mol), making feasible the presence of GAO (or other organisms able to store PHA anaerobically) in their system. Moreover, as recently showed by Welles et al. (2015) that the P/VFA ratio observed on EBPR systems is affected by the Poly-P content of PAOs, hence a high P/VFA ratio (>0.5 P-mol/C-mol) should not be attributed solely to a high fraction of PAOs.

Interestingly, Kim et al. (2013) observed a decrease in the Accumulibacter fraction from 55 to 29% and an increase of *Dechloromonas* from 1 to 19% and Competibacter from 16 to 20% when decreasing the length of the aerobic phase and increasing the anoxic phase. Interestingly, the increase in the dose of nitrate resulted in nitrite accumulation and an increase in the anoxic P-uptake activity even though the fraction of *Ca* Accumulibacter had decreased. These observations are in agreement with the higher anoxic P-uptake activity observed in the mixed PAO I-GAO culture, supporting the hypothesis that GAO contribute to the anoxic P-uptake activity by denitrifying the available nitrate to nitrite for its further utilization by PAOI.

The hypothesis that GAO (or other side communities) are essential for the anoxic P-uptake of PAO on nitrate is in agreement with the observations drawn by García Martín et al. (2006). They suggested that the first part of the nitrate respiration might be carried out by flanking communities since the PAO culture of that study lacked the nitrate reductase enzyme but had the rest of the required enzymes to perform the denitrification process from nitrite onwards. Besides GAO, other flanking communities, even ordinary heterotrophs, could satisfy their carbon needs on dead biomass or ex. polymeric substances (Ichihashi et al., 2006). Fermentative PAO like *Tetrasphera* (Kristiansen et al., 2013) and autotrophic organisms able to use other electron donors and acceptors (inorganic carbon, methane or sulphide) (Brock et al., 2012; Rubio-Rincón et al., 2017) could also play a role in EBPR systems on the first denitrification step from nitrate to nitrite on the benefit of PAO.

4.4. Implications for full-scale systems

The PAO I community enriched in this study could take up phosphate efficiently under anoxic conditions using nitrite as electron acceptor but not using nitrate. When a complete nitrification occurs, it means that other organisms such as GAO have to perform the partial denitrification step to nitrite in order to sustain an efficient anoxic P-uptake and EBPR process. However, even if the proliferation of GAOs can provide the nitrite necessary to enhance the anoxic P-uptake, an excess of GAOs will limit the anaerobic PHA storage by PAOs which subsequently affect the net biological phosphorus removal. Thus, the dynamic GAO/PAO fractions on biological nutrient removal WWTP could be a reason for the regularly observed instabilities over the biological removal of phosphorus.

For carbon and energy efficient and stable EBPR processes, it might be beneficial to integrate EBPR with a partial nitritation process. The partial nitritation, and subsequent use of nitrite for the anoxic phosphorus uptake can help to: (i) reduce the carbon consumption (which could potentially be diverted to biogas production), (ii) reduce the oxygen supply, (iii) and due to the lower anoxic growth yield, contribute to a lower sludge production. Moreover, the anoxic P-uptake with nitrite can be seen as an alternative for a partial nitritation Anammox process (Mulder et al., 1995), which would rely on full BOD removal in the first treatment stage. With a first stage, removing stably a large fraction but not all BOD, the combination of EBPR over nitrite might be more attractive than only Anammox applications. In this way simultaneous nitrogen and phosphate removal (with nitrite) can be achieved as it is described in recent promising full-scale observations by Yeshi et al. (2016). Nevertheless, a drawback from this approach could be the accumulation of nitrite and subsequent inhibition of the metabolism of PAO due to free nitrous acid (FNA). Thus the nitrite concentration and pH would need to be closely monitored.

5. Conclusions

The enriched PAOI culture was not capable to perform a considerable anoxic P-uptake whit nitrate as electron acceptor (0.6 up to 3.4 mg PO_4P/gVSS.h). In contrast, the PAOI-GAO culture was capable to uptake phosphorus using nitrate (around 8 mg PO_4-P/gVSS.h). Moreover the anoxic P-uptake on nitrite as electron acceptor was not considerably different between both cultures (8.7 ± 0.3 and 9.6 ± 1.8 mgPO_4-P/gVSS.h in the PAO I and PAO I-GAO cultures, respectively). These findings strongly suggest that not all PAO I can fully denitrify and that GAO might not only compete with PAO for substrate in the anaerobic period, but also supply electron acceptors (nitrite) in anoxic environments to PAO in a partly competitive and partly syntrophic relationship.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.05.001.

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