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Nitrite and nitrate inhibition thresholds for a glutamate-fed bio-P sludge

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ABSTRACT

Enhanced biological phosphorus removal (EBPR) is an efficient and sustainable technology to remove phosphorus from wastewater. A widely known cause of EBPR deterioration in wastewater treatment plants (WWTPs) is the presence of nitrate/nitrite or oxygen in the anaerobic reactor. Moreover, most existing studies on the effect of either permanent aerobic conditions or inhibition of EBPR by nitrate or free nitrous acid (FNA) have been conducted with a "*Candidatus* Accumulibacter" or *Tetrasphaera*-enriched sludge, which are the two major reported groups of polyphosphate accumulating organisms (PAO) with key roles in full-scale EBPR WWTPs. This work reports the denitrification capabilities of a bio-P microbial community developed using glutamate as the sole source of carbon and nitrogen. This bio-P sludge exhibited a high denitrifying PAO (DPAO) activity, in fact, 56% of the phosphorus was uptaken under anoxic conditions. Furthermore, this mixed culture was able to use nitrite and nitrate as electron acceptor for P-uptake, being 1.8 μg HNO_2–N·L^{-1} the maximum FNA concentration at which P-uptake can occur. Net P-removal was observed under permanent aerobic conditions. However, this microbial culture was more sensitive to FNA and permanent aerobic conditions compared to "*Ca.* Accumulibacter"-enriched sludge.

1. Introduction

Phosphorus and nitrogen are the main nutrients to be removed from wastewater streams to avoid eutrophication of water bodies. Enhanced biological phosphorus removal (EBPR) is a cost-effective and environmentally friendly technology to remove phosphorus from wastewater (Nguyen et al., 2013). In EBPR, the proliferation of polyphosphate-accumulating organisms (PAO) occurs through the alternation of anaerobic and aerobic conditions (Comeau et al., 1987). "*Candidatus* Accumulibacter phosphatis" (hereafter "*Ca.* Accumulibacter") is one of the most widely known PAO, able to take up carbon source as volatile fatty acids (VFA) under anaerobic conditions and store them as polyhydroxyalkanoates (PHA). The required energy and reductive power are obtained through polyphosphate hydrolysis (and P-release) and through glycogen degradation. In a subsequent phase, phosphorus is taken up when an electron acceptor is supplied (normally oxygen, under aerobic conditions). Phosphorus uptake (P-uptake) is achieved through PHA oxidation, which is accompanied by biomass growth and regeneration of glycogen pools (Mino et al., 1998; Smolders et al., 1995). Alternatively to oxygen, nitrite or nitrate can also be used as electron acceptors (under anoxic conditions), which is advantageous because i) both nitrogen and phosphorus are simultaneously removed, ii) anoxic organic matter oxidation leads to lower aeration costs and iii) less biomass is produced (Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993). The organisms with the anaerobic-anoxic P-removal phenotype i.e., the microorganisms able to conduct anoxic P-uptake are known as denitrifying PAO or DPAO (Patel and Nakhla, 2006; Zeng et al., 2003).

The recent application of advanced molecular techniques has challenged the traditional PAO classification. On the one hand, "*Ca.* Accumulibacter" were divided into 14 clades based on the molecular analysis of the polyphosphate kinase 1 gene, ppk1 (Flowers et al., 2013; Mao et al., 2015; Song et al., 2019). While the observed phenotypic differences (i.e. denitrification capabilities, glycogen utilization characteristics and preference of certain VFA) are indicative of different ecological niches (Roy et al., 2021), the assumptions that the different clades have the same encoding for denitrification has been experimentally confronted (Rubio-Rincón et al., 2019). Thus, the accepted engineering classification of DPAO according this ppk gene (clade I: full denitrification capacity from nitrate onwards and clade II: from nitrite onwards) does not seem to be valid anymore (Rubio-Rincón et al., 2019). On the

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other hand, besides "*Ca.* Accumulibacter", other organisms have been identified as putative PAO in spite of not possessing the canonical PAO metabolism. For instance, *Tetrasphaera*-related organisms are also putative PAO present in full-scale EBPR systems and they were found to be more versatile in organic carbon usage (amino acids and sugars) despite their lack of the ability to store PHA (Marques et al., 2017). Likewise, *Dechloromonas*, *Micolunatus phosphovorus*, *Tessaracocus* and "*Candidatus* Obscuribacter" have also been identified as putative PAO in WWTPs by means of high-throughput sequencing technologies and advanced molecular tools (Roy et al., 2021).

Part of PAO have shown denitrification capabilities and the ability to uptake phosphorus under denitrifying conditions. Nitrite is an intermediate of both nitrification and denitrification processes and, thus, it can be accumulated under aerobic or anoxic conditions (Philips et al., 2002).

Despite nitrite-based P-uptake is feasible, high nitrite concentration may result in EBPR failure (Kuba et al., 1996; Zhou et al., 2012). Saito et al. (2004) showed that both the aerobic and anoxic phosphate uptake processes were inhibited by nitrite with a threshold at 0.4 mg N g^{-1} VSS and 2 mg N g^{-1} VSS, respectively, while Meinhold et al. (1999) reported that the full inhibition of both processes was at concentrations higher than 8 mg NO_2–N L^{-1}. Zhou et al. (2007) reported that FNA rather than nitrite was the true inhibitor of P-uptake and Ye et al. (2013) reported that, even at low FNA levels, the anaerobic metabolism of PAO was affected. Zeng et al. (2011) found that an FNA concentration of 2–3 μg HNO_2–N·L^{-1} inhibited anoxic P-uptake. Miao et al. (2018) studied the effect of FNA on N_2O production and on the anoxic phosphorus metabolism in a culture highly enriched in "*Ca.* Accumulibacter" and reported that PHA degradation was completely inhibited at 0.0923 mg HNO_2–N L^{-1}. Similarly, Zeng et al. (2014) showed that the aerobic P-uptake capability of a PAO-enriched sludge fed with VFAs was inhibited at 0.47 μg HNO_2–N L^{-1}, while at 2.25 μg HNO_2–N L^{-1}, the denitrifying P-removal was also inhibited. Wang et al. (2014) compared the resistance to FNA of two different PAO-enriched populations that had been operated with nitrate and nitrite as electron acceptor. The biomass adapted to nitrite showed higher FNA tolerance (up to 3.1 μg HNO_2–N L^{-1}), and thus alleviated the potential N_2O emissions during denitrification.

Another potential cause of EBPR deterioration in full-scale WWTPs is an increment of the oxygen amount entering to the anaerobic reactor through the external recycle (e.g. under excessive aeration or heavy rainfalls periods). These situations can be simulated at lab-scale when the electron donor (substrate) and the electron acceptor (nitrite, nitrate or oxygen) are present simultaneously (Pijuan et al., 2006; Vargas et al., 2009).

The two abovementioned causes of EBPR failure (i.e. nitrite/nitrate inhibition and oxygen presence under anaerobic conditions) have been widely studied in the literature at lab-scale with bio-P sludge. Lab-scale PAO-enriched sludge is usually grown and maintained under restricting operational conditions that may differ from those found in a real environment. Thus, it is practically impossible to maintain, under long-term lab-conditions, a complex microbiome similar to that found in real WWTPs. In fact, the recent developments of sequencing technology and bioinformatics have shown the huge diversity that can be found in real environments (Nierychlo et al., 2020). Wu et al. (2019) analysed the 16S ribosomal RNA gene sequences from samples taken from 269 WWTPs in 23 countries on 6 continents and estimated that activated sludge systems are globally inhabited by around 10^9 different bacterial species. Only about 10^4 species out of them have been cultivated and studied in detail. Thus, assuming that all cultivated species are present in real activated sludge, potentially 99.999% of the microbial taxa in activated sludge still remain uncultured. Despite this high diversity, they stated that activated sludge has a small, global core bacterial community (n = 28 operational taxonomic units) that is strongly linked to activated sludge performance.

Regarding the EBPR environment, one of the causes of the existing diversity is the carbon source. Usually, the preferred carbon sources at lab-scale have been VFAs (Oehmen et al., 2005a; Pijuan et al., 2004a; Welles et al., 2015). However, in full-scale WWTPs, other carbon sources than VFA can promote EBPR occurrence. Among them, amino acids, (accounting for 25–35% of the total COD) have been reported to be a potential carbon source for EBPR in WWTPs (Qiu et al., 2020). They reported that a strain of "*Ca.* Accumulibacter" clade IIF (SCELSE-1) was capable to metabolize amino acids (glutamate and aspartate among others) for EBPR. The concentration of free amino acids in raw wastewater is low, but their concentration in primary sludge or in the digestate can increase to the range of hundreds of mg/L. Therefore, the contribution of amino acids as carbon source for EBPR should not be overlooked since amino acids input to the secondary treatment can be expected from the recycling streams.

Therefore, in this work a bio-P sludge fed with the amino acid glutamate as the sole carbon and nitrogen source was developed to study the capabilities of the promoted bio-P microbial community, which will have different characteristics to the those usually found with VFA as sole carbon source. This is the first report on the potential toxic effects of FNA, nitrate and permanent aerobic conditions in a glutamate-fed EBPR community developed at pilot scale. Anaerobic-anoxic/aerobic batch tests with different carbon sources, with nitrite and nitrate and under permanent aerobic conditions were conducted to evaluate the denitrifying capabilities and contribution towards anoxic P-uptake.

2. Materials and methods

2.1. Continuous pilot plant description

The pilot plant (Rey-Martínez et al., 2019) consisted of three continuous stirred tank reactors with a total volume of 146 L and a 50 L settler. The plant was operated under the classical anaerobic-anoxic-aerobic (A^2/O) configuration for simultaneous carbon, nitrogen and phosphorus removal. The first reactor (R1, 28L) was anaerobic so that PAO were selected against other ordinary heterotrophic organisms (OHO). Nitrate, entering to the second reactor (R2, 28L) through the internal recycle was denitrified by either OHO or DPAO. The third reactor (R3, 90L) worked under aerobic conditions: organic matter was completely removed, phosphorus was taken up and nitrification took place. The sludge retention time (SRT) was maintained around 15 days with daily sludge wastage from the aerobic reactor. The influent flow rate was 240 L d^{-1} resulting in a hydraulic retention time of 14.6 h. The pH was controlled at 7.5 using an on-off controller with sodium carbonate (1 M) dosage in the aerobic reactor. Dissolved oxygen (DO) in the aerobic reactor was controlled at 1 ± 0.1 mg L^{-1}. The pilot plant operated at room temperature.

The starting point of this work was an A^2/O system fed with glutamate as sole carbon and nitrogen source aiming at simultaneous phosphorus and nitrogen removal. The plant was previously run for more than 480 days under different operational conditions as described in Rey-Martínez et al. (2019). Hence, the biomass was adapted to this new carbon source. Glutamate was entered from an individual storage tank where partial fermentation to VFA was detected and resulted in a gradual increase of the VFA content in the storage tank: up to 8.9% of the initial COD was converted to VFA after two days and up to 63.4% after five days. To avoid this excessive VFA/COD ratio, the feeding tank was replenished three times per week. Because of this strategy, VFA formation was highly minimized and it can be considered that glutamate was the only carbon source entering the anaerobic reactor. The results reported in this work show 261 days of operation after the change in the feeding strategy. The synthetic influent was prepared from a concentrated feed that was diluted with tap water as described in Rey-Martínez et al. (2019), with a final content of 10 mg PO_4^{-3}-P·L^{-1} and 400 mg COD·L^{-1} and using glutamate as sole carbon and nitrogen source (theoretical concentration of nitrogen provided by glutamate was 70.6 mg N·L^{-1}).

2.2. Batch experiments

Several batch tests were conducted in a magnetically stirred vessel (2 L), which was filled with fresh biomass withdrawn from the aerobic reactor of the A^2/O pilot plant without any pretreatment. Hence, the biomass from the batch experiments had the same characteristics as those in the pilot plant. Four different types of batch test configurations were used: i) 2 h anaerobic, 4 h anoxic and 2 h aerobic; ii) 2 h anaerobic and 4 h anoxic; iii) 2 h anaerobic and 3 h aerobic and iv) 2 h aerobic.

The anaerobic and anoxic phases were sparged with nitrogen at a constant flowrate with a mass flowmeter (EL-FLOW, Bronckhorst) to avoid oxygen leakage. pH (WTW Sentix 81) and DO (WTW CellOx 352) probes were connected to a multiparametric terminal (WTW INOLAB 3), which was in turn connected to a PC with a specific software allowing for data monitoring and manipulation of a high precision microdispenser (Crison Multiburette 2S) for pH control with acid/base addition (HCl and NaOH at 1 M, respectively). The aerobic phase was obtained by air sparging at a constant flowrate with a mass flowmeter (EL-FLOW, Bronckhorst) to guarantee a DO concentration above 3 mg L^{-1} during the whole experiment as in Guisasola et al. (2007).

Three sets of batch tests were performed to investigate the behaviour or the biomass:

i) Objective 1: assess carbon consumption under different electron donors (acetate, propionate and glutamate). The initial concentrations of carbon source used in the reactor were: 195 mg acetic acid·L^{-1} (208 mg COD·L^{-1}), 125 mg propionic acid·L^{-1} (189 mg COD·L^{-1}) or 270 mg COD·L^{-1} as glutamate.
ii) Objective 2: assess DPAO activity under different electron acceptors (nitrite or nitrate). The second set of experiments consisted of anaerobic-anoxic assays. The anoxic phase was obtained by adding a concentrated solution of nitrite (14.75–92.11 mg N·L^{-1} in the reactor) or nitrate (15–82.93 mg N·L^{-1} in the reactor). The batch experiments using nitrite as electron acceptor were performed at different pH values (7.5, 8 and 8.5) to evaluate the effect of different FNA concentrations in the biomass. Some of the nitrite and nitrate batch tests were followed by an aerobic phase.
iii) Objective 3: assess potential P-release under permanent aerobic conditions. Aerobic P-release was studied under different aeration flowrates.

The batch tests were sampled every 15 or 30 min for chemical analysis of the supernatant. Samples were filtered by 0.22 μm (Millipore) for the analysis of phosphate, nitrite, nitrate, ammonia, acetate, propionate and COD. The specific process rates were calculated as the slope of a linear regression including the first points of consumption or production, standardized against the biomass concentration.

The FNA concentration was calculated using the equation (1) and with K_a value determined using the equation (2) for a given temperature (°C) (Anthonisen et al., 1976).

$$[FNA] = \frac{S_{N-NO_2}}{K_a \cdot 10^{pH}} \quad (1)$$

$$K_a = e^{-\frac{2300}{(273+T)}} \quad (2)$$

2.3. Chemical and microbiological analyses

Phosphorus concentration in 0.22 μm filtered samples was measured by a phosphate analyser (PHOSPHAXsc, Hach Lange) which is based on vanadomolybdate yellow method. Ammonium nitrogen in 0.22 μm filtered samples was analysed by means of an ammonium analyser (AMTAXsc, Hach Lange), based on the potentiometric determination of ammonia. Nitrite and nitrate in filtered samples were analysed with ionic chromatography (DIONEX ICS-2000). Organic matter (chemical oxygen demand, COD), was measured using COD kits (Hach Lange LCK 314 and LCK 714) and a spectrophotomer (DR2800 Hach Lange). Total suspended solids (TSS) and volatile suspended solids (VSS) were analysed according to APHA, 1995.

PFA-fixed activated sludge samples were washed with 1 x phosphate-buffered saline (PBS) solution and resuspended in a mixed solution of 1 × PBS and 100% ethanol (50:50). Samples were stored at −20 °C before fluorescence in-situ hybridization (FISH) analysis.

Biomass was characterized using FISH techniques to quantify the amount of PAO and GAO present in the reactors as described in Amann (1995) using the following probes: Cy5-labelled EUBMIX for most bacteria (Daims et al., 1999), Cy3-labelled PAOMIX for "*Ca.* Accumulibacter", comprising PAO462, PAO651 and PAO846 (Crocetti et al., 2000) and Cy3-labelled GAOMIX for "*Candidatus* Competibacter phosphatis", comprising GAOQ431 and GAOQ989 probes (Crocetti et al., 2002). PAOMix FISH probe set was the conventional way to estimate the abundance of "*Ca.* Accumulibacter". However, Albertsen et al. (2016) discovered that *Propionivibrio* (a putative GAO) closely related to "*Ca.* Accumulibacter" is also targeted by the PAOMix probes, so the results generated applying these probes should be interpreted with care.

A Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) using a Plan-Apochromatic 63x objective (NA 1.4, oil) was used to quantify biomass analysing 30–40 fields and following an automated image analysis procedure as described in Jubany et al. (2009).

Identification of bacterial population was performed using the Illumina platform for one sample collected on day 158 of operation. The sample was washed three times with PBS and the sedimentation after centrifugation was stored at −20 °C for further analysis. Genomic DNA was extracted from biomass samples using Powersoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. A pellet from 3 mL of mixed liquor was used. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the quantity and quality of extracted DNA. A minimum of 20 ng μL^{-1} of extracted DNA was guaranteed to perform the analysis. Purified DNA was stored at −20 °C for further analysis. Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by an external service (Scsie UV, Valencia, Spain). For library preparation, the V4 region of the 16S rRNA gene was amplified and sequenced using the primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT), which has high sequence coverage for both bacteria and archaea and produces an appropriately sized amplicon for Illumina sequencing (Wu et al., 2015). The database used for the classification of organisms was based on the Greengenes database. The sequence reads were processed through Usearch software. OTUs were generated with the open reference methodology.

3. Results and discussion

3.1. Pilot plant performance and microbial community composition

The experimental profiles related to N- and P-removal during the 261 days of operation of the A^2/O pilot plant are shown in Fig. 1. The plant had previously been operated under similar conditions but with a higher influent VFA/COD ratio because of fermentation in the storage tank. High N- and P-removal efficiencies (85% and 99% respectively) were obtained during the entire operation. The solids concentration was maintained around 1.85 ± 0.29 g VSS·L^{-1} during the entire operation (Fig. S1, Supplementary Information).

Between days 208 and 215, technical problems with the external recirculation pump led to an increase in its flowrate, which in turn, caused a high nitrate input to the anaerobic reactor. That led to a decrease of the anaerobic PAO activity (i.e. lower P-release) but it did not affect the P-removal efficiency, being the concentration of phosphorus in the effluent 0.16 ± 0.08 mg P·L^{-1} during these days (Fig. 1).

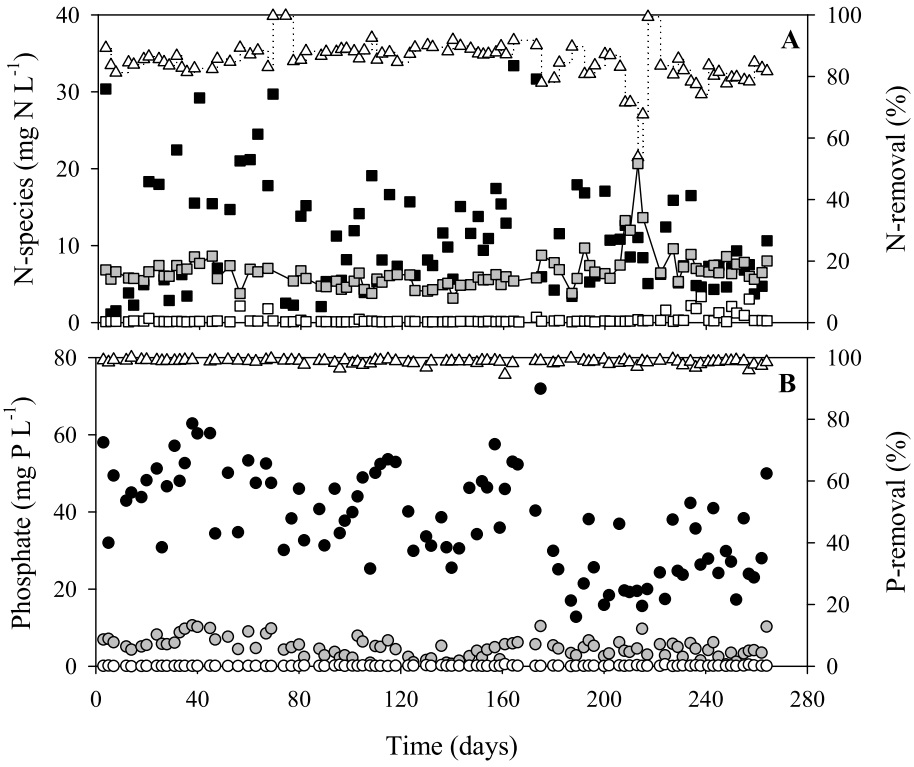

Fig. 1. Experimental profiles during the 261 d of operation of the A^2/O plant. A) Ammonium concentration in the influent (■) and effluent (■), nitrate concentration in the effluent (■) and N-removal efficiency (Δ). B) Phosphorus concentration in the anaerobic reactor (●), anoxic reactor (●) and effluent (○) and P-removal efficiency (Δ).

Nitrate presence in the anaerobic reactor resulted in lower COD available for denitrification in the anoxic reactor, and caused an increase of nitrate concentration in the effluent (15 ± 4 mg NO_3^--N·L^{-1}). Complete nitrification was achieved during the whole operational period since no nitrite was ever observed.

The P/C ratio was very low (0.15 ± 0.07 mol P·mol C^{-1}) particularly when compared to the P/C ratio proposed for conventional electron donors such as acetate (0.5 mol P·mol C^{-1}, Smolders et al., 1994) or propionate (0.42 mol P·mol C^{-1}, Oehmen et al., 2005b) for "*Ca.* Accumulibacter". However the P/C ratio is very similar to the obtained by Marques et al. (2018) in an SBR enriched in *Tetrasphaera*. Lower P/C ratios suggest that "*Ca.* Accumulibacter" are the dominating PAO and fermentable substrates are used as carbon source: glycerol (0.2 mol P·mol C^{-1}) as obtained by Lv et al. (2014) and Guerrero et al. (2012). Part of the COD is used during the fermentation process, resulting in less COD available for PAO. Glutamate is also a complex substrate that has to be firstly hydrolysed by fermentative bacteria during the anaerobic phase to produce VFA. In fact, when the influent VFA/COD ratio was lowered, it led to a severe increase of fermentative bacteria in the reactor (results shown below).

The P/C ratio and the P-release rate were lower to those values obtained in the previous operational study: 0.14 mol P·mol C^{-1} (Rey-Martínez et al., 2019) (Table S1). This decrease was due to lower influent VFA/COD ratio that led to a decrease of the amount of "*Ca.* Accumulibacter" in the system. The FISH percentages show that the amount of "*Ca.* Accumulibacter" was 21% in contrast to the 49% obtained during the previous operation. In any case, since P-removal was still complete during the entire operation, other PAO besides "*Ca.* Accumulibacter" could have been promoted.

Regarding the P-uptake activity, Fig. 1 shows a high decrease of phosphorus concentration from the anaerobic reactor (38 ± 13 mg P·L^{-1}) to the anoxic reactor (5 ± 2 mg P·L^{-1}) and then a lower decrease to the aerobic reactor (0.16 ± 0.08 mg P·L^{-1}). Mass balances of phosphorus in the pilot plant considering these concentrations and the internal and external recirculation ratios (4 and 0.5 respectively) showed that more phosphorus was uptaken under anoxic conditions (56%) than under aerobic conditions (44%). The specific anoxic P-uptake rate was 2.2 times higher than that under aerobic conditions (Table S1), confirming the high DPAO activity of this culture. The specific anoxic and aerobic P-uptake rates were also lower than those of the previous operational period.

The Illumina technique was used to examine the microbial community developed in the A^2/O pilot plant on day 158. As shown in Fig. S2-up, at family level, *Rhodocyclaceae* (23.4%) was the most dominant family, which is closely related to "*Ca.* Accumulibacter" (Zhou et al., 2015). Three genera were identified within *Rhodocyclaceae* family, a 13.6% correspond to "*Ca.* Accumulibacter", 2.44% to *Propionivibrio* and 1.51% to *Dechloromonas* (Fig. 2S-down). "*Ca.* Accumulibacter" is broadly recognized as the model PAO (Oehmen et al., 2007) and, although it has not been formally categorized, their reference sequences were classified as belonging to *Rhodocyclaceae* family (Coats et al., 2017). *Propionivibrio* are fermentative bacteria and certain species could be postulated as putative PAO (Coats et al., 2017), but also Albertsen et al. (2016) reported the discovery of GAO belonging to this genus. Finally, *Dechloromonas* are a genus frequently reported as PAO in EBPR reactors (Liu et al., 2005; Terashima et al., 2016). *Chitinophagaceae*, *Saprospiraceae* and *Cytophagaceae* families accounted for 14.7%, 10.1% and 9.8% of the total bacteria. These families exhibit the ability to hydrolyse complex carbon sources, like proteins and polysaccharides (Rosenberg et al., 2014), and hence, they could be involved in the glutamate degradation. The *Comamonadaceae* family, 4.5% of the microbial community, has been identified in short SRT-EBPR systems treating protein-rich wastewater (Ge et al., 2015). Moreover, some members have been recognized as important denitrifiers in activated sludge systems (Khan et al., 2002). *Thiothrichaceae* family has an abundance of 2.9% and the genus associated to this family was *Thiothrix*. In the previous operation of the pilot plant, *Thiothrix* represented 38% of the total bacteria; hence, they experienced a sharp decline in the new

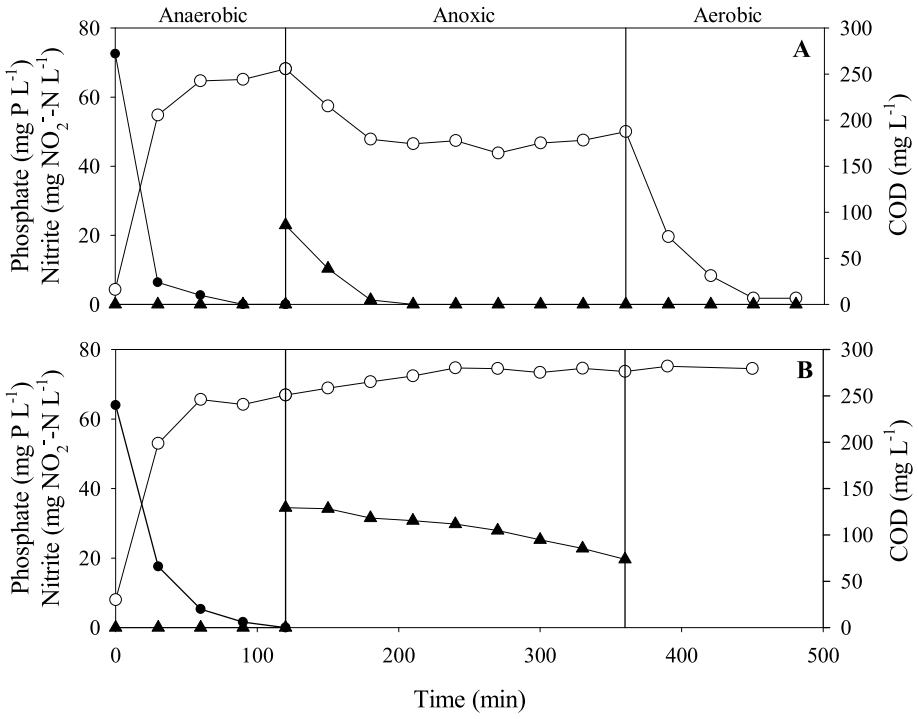

Fig. 2. Monitoring of two anaerobic/anoxic/aerobic batch experiments with nitrite as sole electron acceptor and pH = 7.5 A) With a spike of nitrite of 23 mg NO_2^--N L^{-1} and FNA concentration of 1.63 μg HNO_2–N L^{-1}. B) With a spike of nitrite of 34.51 mg NO_2^-- N L^{-1} and FNA concentration of 2.45 μg HNO_2–N L^{-1}. Phosphorus (○), COD (●) and nitrite (▲) concentrations.

operation. In fact, this decrease was corroborated by the lower sludge volume index (SVI): 158 ± 55 mL·$gVSS^{-1}$, which was almost half of that obtained in the previous operation (around 300 mL·$gVSS^{-1}$). *Nitrospiraceae* family accounted for 2.7% corresponding totally to *Nitrospira* genus, which are NOB generally adapted to low substrate concentrations and can exploit a low amount of nitrite (Kim and Kim, 2006).

Fig. S2-down, shows the microbial distribution at genera level. The major genera besides the already mentioned above, were *Runella* (9.84%) and *Lewinella* (9.79%). A comprehensive 16S rRNA gene reference database, MiDAS 3 developed by Nierychlo et al. (2020) was consulted and both genera, *Runella* and *Lewinella*, do not possess PAO metabolism and very low abundances (<0.05%) of both genera have been found in WWTPs in Denmark. Finally, it is worth to be mentioned that a recent study developed an analysis of commonly used 16S rRNA primer sets (the primer set used in this study 515F-806R among others) revealing extremely low specificity for *Micropruina* and *Microlunatus*, while it is greater than 90% for other organisms characteristic of EBPR microbial communities such as "*Ca.* Accumulibacter", "*Ca.* Competibacter", *Defluviicoccus*, *Dechloromonas*, and *Propionivibrio*. (Roy et al., 2021). Therefore, more than one primer set has to be used to know the reliable microbial distribution in activated sludge samples.

The microbial distribution developed in this work with glutamate as sole carbon and nitrogen source is more diverse than that obtained in other reports where the carbon source was VFA. Glutamate utilization promoted, among other issues, the proliferation of fermentative bacteria and potential scavengers of the fermentation products.

3.2. Denitrification capabilities of the culture

Batch tests with different electron acceptors were performed to study the denitrifying capabilities of the mixed microbial culture. The electron acceptors, i.e. nitrite and nitrate, were dosed at different concentrations.

3.2.1. Nitrite as electron acceptor

The objective of these batch tests was to understand the denitrifying capabilities of our bio-P sludge and to compare the inhibition threshold for FNA with the reported values for *Acccumulibacter*-enriched sludge. Several batch tests were conducted under different initial nitrite concentrations and at different pH values (7.5, 8 and 8.5). It has been reported that FNA-adapted and non FNA-adapted PAO have different response to FNA inhibition, being the non FNA-adapted PAO more sensible to the FNA presence (Meinhold et al., 1999; Saito et al., 2004; Zhou et al., 2012). It is worth mentioning that the sludge used in the current study was obtained from an A^2/O plant where simultaneous P and N removal was achieved. Hence, this sludge was adapted to nitrate but not necessarily to high concentrations of nitrite, since nitrification was complete and no nitrite accumulation was ever observed during the entire operation. On the other hand, Zhou et al. (2007) suggests that adaptation to nitrite/FNA does not necessarily increase the tolerance of "*Ca.* Accumulibacter" PAO to FNA, so there is not a consensus in this need of acclimatization to FNA.

Fig. 2 displays the experimental profiles of two typical batch tests. In Fig. 2A, 90% of initial glutamate was consumed in the first 30 min of the anaerobic phase linked to P-release. In the anoxic phase, nitrite was spiked (initial concentration of 23 mg NO_2^--N L^{-1}) and simultaneous P-uptake and nitrite consumption was observed. When nitrite was depleted, a slight P-release occurred. Finally, under aerobic conditions, P-uptake took place again until complete P-removal. The batch test presented in Fig. 2A, was performed at pH of 7.5 resulting in an initial concentration of FNA of 1.63 μg HNO_2–N L^{-1}. Zhou et al. (2007), found that FNA inhibition on anoxic P-uptake started at these low levels (1–2 μg HNO_2–N L^{-1}) using a "*Ca.* Accumulibacter"-enriched sludge. Total inhibition occurred at 20 μg HNO_2–N L^{-1}. In contrast, Fig. 2B shows a batch test where the initial nitrite concentration (34.5 mg NO_2^--N L^{-1}) resulted in an FNA concentration of 2.45 μg HNO_2–N L^{-1} that completely inhibited the uptake process. A low nitrite consumption rate 1.9 mg NO_2–N g^{-1}VSS·h^{-1} compared to the 10.9 mg NO_2–N g^{-1}VSS·h^{-1} obtained for the batch test of Fig. 2A was observed. Moreover, P-release instead of P-uptake was detected.

At the beginning of the aerobic phase, nitrite concentration was 20

mg NO_2^--N L^{-1} (1.34 μg HNO_2–N L^{-1}). This FNA concentration was even lower than the maximum in Fig. 2A (1.63 μg HNO_2–N L^{-1}), where no inhibition was observed for the anoxic P-uptake. Surprisingly, no P-uptake was observed despite nitrite and oxygen were present, even though it has been reported that some DPAO can use both electron acceptors simultaneously leading to a higher metabolic rate (Zhou et al., 2012). Lanham et al. (2011) also observed a failure in aerobic P-uptake after a nitrite spike for concentrations ranging 25–65 mg NO_2^--N L^{-1}, which were equivalent to 1.1–5.4 μg HNO_2–N L^{-1}. Our results show that when the biomass was subjected to an inhibitory FNA concentration, P-uptake was affected even after restoring aerobic conditions. On the other hand, other possible explanation for this phenomenon could be that the aerobic P-uptake was more sensitive to FNA inhibition than the anoxic P-uptake. This is in agreement with the report of Saito et al. (2004), who working at a pH of 7.47 ± 0.1 observed that less than 0.4 mg NO_2^--N g^{-1}VSS and around 2 mg NO_2^--N g^{-1}VSS were determined as threshold for aerobic and anoxic P-uptake, respectively. In our case, the anoxic phase tolerated up to 13 mg NO_2^--N g^{-1}VSS at a pH of 7.5. In the case of the aerobic phase, we never observed P-uptake if the nitrite concentration resulted inhibitory in the previous anoxic phase. This observation is somehow controversial since other studies stated that the anoxic P-uptake was more sensitive to nitrite/FNA inhibition (Meinhold et al., 1999; Zhou et al., 2012).

The threshold of FNA inhibition depends on the microbial population, its FNA adaptation and on the type of microbial culture (suspended or attached biomass) (Jabari et al., 2016; Zhou et al., 2011). Zhou et al. (2012) observed that anoxic P-uptake was 100% inhibited at FNA concentration of 5 μg HNO_2–N L^{-1} with a FNA-adapted sludge with 45% of "*Ca.* Accumulibacter". In another study, Zhou et al. (2007) stated that anoxic P-uptake was totally inhibited when the FNA concentration was greater than 20 μg HNO_2–N L^{-1} with a "*Ca.* Accumulibacter"-enriched sludge (85%).

Several batch tests were performed at different FNA concentrations to determine the maximum FNA at which anoxic P-uptake could occur. Different nitrite concentrations at different pHs were used to obtain a wide range of FNA concentrations. Fig. 3 shows the phosphorus (A) and nitrite (B) uptake rates for each FNA concentration tested. A decrease in both rates was observed with the increase in FNA concentration, resulting in 2 μg HNO_2–N L^{-1} the concentration that completely stopped the P-uptake. When our bio-P sludge was subjected to an inhibitory FNA concentration, P-release instead of P-uptake was observed during anoxic phase, indicating that FNA concentration directly affects P-uptake mechanisms rather than P-release. Three batch tests performed with different inhibitory FNA concentrations are shown in Fig. S3. A P-release of 15.7, 13.1 and 16.6 mgP·L^{-1} was observed during the anoxic phase in figures S3A, S3B and S3C respectively. Moreover, denitrititation by DPAO was also inhibited by FNA. However, the inhibitory effect was lower than that on P-uptake, since nitrite uptake rates around 2 mg N·g^{-1} VSS·h^{-1} were obtained for FNA concentrations between 2 and 2.5 μg HNO_2–N L^{-1}. This represents 76.5% of inhibition in the nitrite uptake rate when the FNA concentration increased from 1.05 to 2.11 μg HNO_2–N L^{-1}. Nevertheless, these FNA values led to a complete P-uptake inhibition.

A summary of the FNA and nitrite concentrations used and the nitrite, nitrate and P-uptake rates and P/N ratios obtained for each batch test is presented in Table 1. The anoxic P-uptake rates were in the range 6.8–12.0 mg P·g^{-1}VSS·h^{-1} under FNA concentrations around 1.80–0.25 μg HNO_2–N L^{-1} respectively. The maximum FNA concentration that the biomass could tolerate while maintaining P-uptake was 1.8 μg HNO_2–N L^{-1}, as P-release instead of P-uptake was observed for higher FNA values. This threshold is lower than in other works with bio-P sludge, where P-uptake was observed with higher FNA concentrations (2.45 and 2.88 μg HNO_2–N L^{-1}) (Guisasola et al., 2009; Saito et al., 2004) despite the P-uptake rates obtained in our work in the range of no FNA inhibition were higher than some values reported in the literature (Table 2). Zhou et al. (2007) obtained anoxic P-uptake rates around 1 mg P·g^{-1} VSS·h^{-1} for a "*Ca.* Accumulibacter"-enriched sludge and for FNA concentrations ranging 0.85–38.8 μg HNO_2–N L^{-1}. Moreover, Jabari et al. (2016) obtained anoxic P-uptake rates of 7.6–8.7 mg P·g^{-1} VSS·h^{-1} with 0.2–1.8 μg HNO_2–N L^{-1} respectively in an integrated fixed-film activated sludge system. Guisasola et al. (2009) obtained an anoxic P-uptake rate of 3.42 mg P·g^{-1} VSS·h^{-1} when the FNA concentration was 2.45 μg HNO_2–N L^{-1} with sludge adapted to nitrite and enriched in PAOMIX (42%). Finally, Saito et al. (2004) obtained anoxic P-uptake rates between 11.5 and 7 mg P·g^{-1}VSS·h^{-1} for FNA concentrations ranging 0.72–2.88 μg HNO_2–N L^{-1}. From these results, our bio-P sludge possesses a lower FNA inhibition threshold; nevertheless, the P-uptake rates obtained were, together with the presented by Saito et al. (2004), the higher anoxic P-uptake rates reported in the literature. Finally, Marques et al. (2018) obtained a low P-uptake rate in an SBR enriched in *Tetrasphaera*, even though the FNA concentration was very high (12.69 μg HNO_2–N L^{-1}). The authors concluded that *Tetrasphaera* has the capability of nitrite-based denitrification, but the specific P-uptake rate in anoxic conditions was very low when compared to "*Ca.* Accumulibacter" and the values obtained in this study.

The ratio of phosphorus removed to nitrogen denitrified during anoxic conditions (P/N ratio) describes whether the energy obtained from nitrite denitrification is used for P-uptake. The average P/N ratio was 0.39 ± 0.03 mol P·mol^{-1} N for all the batch tests except for the inhibited batch tests (Table 1). However, a decrease in the P/N ratios and even negative values were obtained for inhibitory FNA concentrations, therefore, it seems that the FNA had a negative impact on P/N ratio, in accordance with the results obtained in other studies (Jabari et al., 2016).

3.2.2. Nitrate as electron acceptor

Different nitrate concentrations were used to study its effect on the process kinetics and stoichiometry (Table 1, Fig. 4). Simultaneous P-

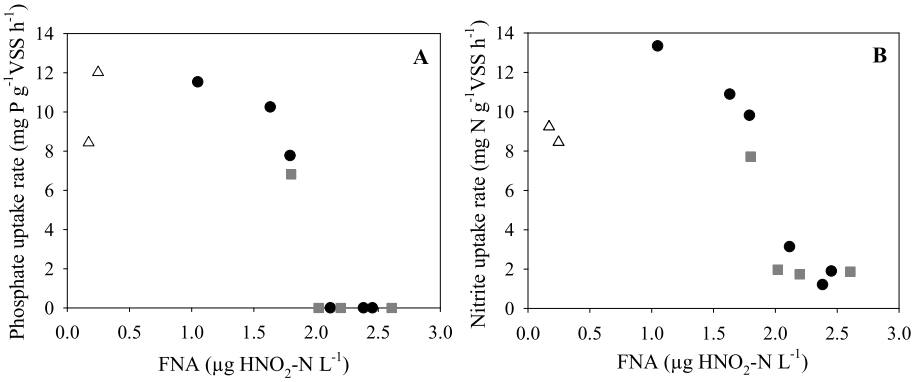

Fig. 3. Correlation between the P-uptake (A) and N-uptake (B) rates with FNA concentration at different pH values: 7.5 (●), 8 (■) and 8.5 (Δ).

Table 1
Summary of batch tests results with nitrite and nitrate as electron acceptors.

Electron acceptor	pH	NO_X dosed (mg NO_X-N L^{-1})	FNA concentration (μg HNO_2–N L^{-1})	NO_X uptake rate (mg NO_X-N g^{-1} VSS·h^{-1})	Anoxic P-uptake rate (mg P·g^{-1} VSS·h^{-1})	P/N ratio (mol P·mol^{-1} N)
NO_2^-	7.5	14.8	1.05	13.3	11.5	0.39
		23.0	1.63	10.9	10.3	0.43
		25.2	1.79	9.8	7.8	0.36
		29.8	2.12	3.1	−1.7	−0.55
		33.5	2.38	1.2	−2.4	−2.00
		34.5	2.45	1.9	−1.9	−1.00
	8	50.5	1.80	7.7	6.8	0.40
		65.0	2.02	2.0	−2.7	−1.35
		77.6	2.20	1.7	−2.2	−1.29
		92.1	2.61	1.9	−1.9	−1.00
	8.5	24.1	0.17	9.2	8.4	0.41
		34.8	0.25	8.5	12.0	0.64
NO_3^-	7.5	15.0		5.0	9.8	0.89
		23.6		3.4	5.3	0.71
		27.7		4.0	6.7	0.75
		41.6		3.2	5.6	0.79
		61.9		2.6	4.7	0.83
		70.4		2.7	3.0	0.49
		82.9		2.5	2.7	0.49

removal and nitrate denitrification were observed in all batch tests. The experimental profiles of carbon, phosphorus and nitrate in a conventional cycle are presented in Fig. 4A. Glutamate was completely taken up during the anaerobic phase linked to P-release. Then, in the subsequent anoxic phase, an initial amount of nitrate (23.6 mg NO_3^--N L^{-1}) was supplied and simultaneous phosphorus and nitrate uptake was observed. P-uptake ceased when nitrate was fully reduced and no nitrite accumulation was observed when nitrate was dosed in any of the batch tests performed in this work. This lack of nitrite build-up could suggest that complete nitrate reduction was conducted by a single population (Carvalho et al., 2007) or it could be hypothesized the coexistence of bacteria capable only to reduce nitrate to nitrite, while DPAO could denitrify from nitrite onwards (Guisasola et al., 2009). The absence of nitrite was also observed by Lanham et al. (2011) and Carvalho et al. (2007) in propionate-fed systems. However, Carvalho et al. (2007) observed nitrite accumulation when the carbon source was acetate. In the same way, Zhou et al. (2010) observed a low nitrite accumulation in all experiments with high initial concentrations of nitrate (30, 60 and 120 mg L^{-1}). On the other hand, Flowers et al. (2009a,b) did not observe nitrite accumulation during the batch tests with a sludge that had not been exposed to nitrate.

Fig. 4B shows the phosphorus and nitrate uptake rates for each of the initial nitrate concentrations tested. The higher the initial concentration of nitrate, the lower both rates were. Nevertheless, contrarily to the case of nitrite, nitrate caused a decrease in the consumption rates but not a complete inhibition. In fact, P-uptake occurred even at the highest concentration tested (82.9 mg NO_3^--N L^{-1}), which was also observed by Zhou et al. (2010). P-uptake rate experimented a reduction of 72% when the initial nitrate concentration increased from 15 to 82.9 mg NO_3^--N L^{-1} whereas the nitrate uptake rate decreased 50% (Fig. 4B). The lowest nitrate concentration tested was 15 mg NO_3^--N L^{-1}, which led to the highest P-uptake and nitrate uptake rates. In the case of Zhou et al. (2010), both uptake rates were higher with nitrate concentration in a range of 5–30 mg NO_3^--N L^{-1}. Then, higher concentrations such as 60 and 120 mg NO_3^--N L^{-1} caused a decrease in the uptake rates. It is worth to be mentioned that the P-uptake rate was an average of 30% faster when nitrite was used as electron acceptor than with nitrate. The same trend was observed by Lee et al. (2001) when nitrite concentrations up to 10 mg NO_2^--N L^{-1} (2.57 μg HNO_2–N L^{-1}) were supplied. A comparison of the P-uptake rates using nitrate obtained in this work with some values reported in literature are shown in Table 2. The values obtained by Flowers et al. (2009a,b) were lower than the values observed in this study even for higher nitrate concentrations. Nevertheless, other authors reported higher values (Zeng et al., 2003; Zhou et al., 2010). The low P-uptake rates could be explained by the microbial community developed using complex substrates, since in both, our work and the study developed by Flowers et al. (2009a,b), the carbon source were glutamate and a mixture of acetate and casaminoacids respectively. In the other studies, simple carbon sources such as acetate or propionate were used. Nevertheless, the concentrations of nitrate used in this work were very high in comparison with the typical values observed in real WWTPs. For instance, in the work developed by Vieira et al. (2019), the maximum nitrate concentration (13 mg NO_3^--N L^{-1}) was obtained in the aeration tank of a conventional activated sludge in the WWTP of Lisbon. However, and depending on the type of configuration used, higher concentrations could be obtained. For example, when operating batch systems, nitrate/nitrite concentrations at the start of the anoxic phase can be much higher depending on the influent ammonium load.

The average P/N ratio (Table 1) was 0.79 ± 0.07 mol P·mol N^{-1} for initial nitrate concentrations ranging 15.0–61.9 mg NO_3^--N L^{-1}. For the highest range of nitrate concentrations tested (70.7 and 82.9 mg NO_3^--N L^{-1}) the P/N ratio decreased down to 0.49 mol P·mol N^{-1}. Lv et al. (2014) conducted a study consisted on three SBRs using acetate, propionate and glycerol as sole carbon sources, obtaining P/N ratios of 0.75, 0.76 and 0.57 mol P·mol^{-1} N, respectively, with an initial nitrate concentration of 20 mg N·L^{-1}. The P/N ratio that they obtained for acetate and propionate were in the range of the average P/N ratio observed in this study. Finally, Carvalho et al. (2007) obtained a P/N value of 0.82 mol P·mol^{-1}N in a PAOI-enriched sludge, which is in agreement with the values obtained in this study for nitrate concentrations ranging 15–61.9 mg NO_3^--N L^{-1}.

Different P/N ratios for nitrite and nitrate were obtained in the present study. This difference can be explained by the different oxygen equivalence of both species, being 1.67 times higher for nitrate than for nitrite, and hence, lower amount of nitrogen is required per unit of phosphorus uptake. The average P/N ratio for nitrate was 0.79 ± 0.07 mol P·mol^{-1}N in the range 15–61.9 mg NO_3^--N L^{-1}, while the average P/N ratio for nitrite was 0.44 ± 0.10 mol P·mol^{-1} N when no FNA inhibition was observed. Hence, our experimental nitrate/nitrite ratio was 0.79/0.44 = 1.81, which was similar to the theoretical value of 1.67.

The maximum P-uptake rate in our work (9.8 mg P·g^{-1} VSS·h^{-1}) was obtained with the lowest initial nitrate concentration tested, 15 mg NO_3^--N L^{-1}. On the other hand, Zhou et al. (2010), observed an increase in the P-uptake rate with the increase in the initial nitrate concentration until 30 mg NO_3^--N L^{-1}, which was considered as the optimal concentration in their study.

Table 2
Comparison of P-uptake rates with FNA and nitrate found in this work with some values reported in the literature.

Study	pH	*FNA concentration ($\mu g\ HNO_2$–N L^{-1})*	*P-uptake rate (mg P·g^{-1} VSS·h^{-1})*	*FNA-adaptation biomass*
Saito et al. (2004)	7	0.24	0.5	Non-reported
		0.48	3.0	
		0.72	11.5	
		1.68	10.5	
		2.88	7.0	
Zhou et al. (2007)	8	0.86	1.06	Non-reported
Guisasola et al. (2009)	7.5	2.45	3.42	Adapted
Jabari et al. (2016)	7.8	0.2	7.6	Adapted
		0.4	7.7	
		1.2	8.3	
		1.8	8.7	
		0.55	6.0	Non-adapted
		1.35	3.89	
Marques et al. (2018)	7.1	12.69	0.93	Adapted
This work	8.5	0.25	12.0	Non-adapted
	8	1.80	6.8	
	7.5	1.79	7.8	
		1.63	10.3	
		1.05	11.5	

Study	*Initial nitrate concentration (mg NO_3^--N L^{-1})*	*P-uptake rate (mg P·g^{-1} VSS·h^{-1})*	*NO_3^-*	*Acclimatization (carbon source)*
Zeng et al. (2003)	53.2	16.90	Yes	(Acetate)
Carvalho et al. (2007)	10	8.36	Yes	(Acetate)
	50	19.51	Yes	(Propionate)
Flowers et al. (2009a,b)	25	1.42	No	(Acetate + casaminoacids)
	25	2.94	Yes	
Zhou et al. (2010)	20	14.61	No	(Acetate)
	30	18.29		
	60	8.44		
	120	2.67		
Marques et al. (2018)	88.6	1.55	Yes	(Casein hydrolysate)
This work	15	9.8	Yes	(Glutamate)
	23.6	5.3		
	61.9	4.7		
	82.9	2.7		

3.2.3. Different electron donors

Textbook knowledge states that a high VFA/COD ratio is a key factor to achieve efficient P-removal (Guerrero et al., 2012; Lv et al., 2014; Merzouki et al., 2005). However, in a glutamate-fed system, the effect of VFA is not clear a priori, as some microorganisms may be able to use glutamate without a fermentation step, while others as typically considered for "*Ca.* Accumulibacter" need a previous fermentation of glutamate to VFA for a good EBPR performance. Considering that acetic and propionic acids are the two most common VFA present in domestic wastewaters (Pijuan et al., 2004b), batch experiments with acetic acid, propionic acid and glutamate as sole carbon sources were conducted to study the behaviour of our glutamate-fed bio-P sludge. Fig. S4 shows the experimental profiles for three batch experiments under sequential anaerobic and aerobic conditions. Similar trends were observed in all batch tests: the carbon source was anaerobically consumed linked to P-release and phosphorus was taken up in the subsequent aerobic phase.

The carbon uptake rate for glutamate (57.81 mg C·g^{-1} VSS·h^{-1}) was higher in comparison to that found with acetic and propionic (27.4 and 41.0 mg C·g^{-1} VSS·h^{-1} respectively) (Table 3), probably because the biomass was acclimatized to this carbon source. The P/C ratio observed for acetic acid (0.55 mol P·mol^{-1} C) was in accordance with the literature values for "*Ca.* Accumulibacter" PAO: 0.32 to 0.66, being 0.5 the most accepted value (Oehmen et al., 2004, Zeng et al., 2003 and Mcmahon et al., 2002). The P/C ratio obtained for propionic acid was 0.45, which was similar to the value obtained by Pijuan et al. (2004b). Finally, the P/C ratio observed for glutamate in the batch tests (0.21 mol P·mol^{-1} C) was slightly higher than that obtained in the continuous operation (0.15 ± 0.07 mol P·mol C^{-1}). It is a low value in the range of similar ratios obtained using complex carbon sources such as glycerol (0.2 mol P·mol C^{-1}, Guerrero et al. (2012)) or casein hydrolysate (0.35 mol P·mol C^{-1}, Marques et al. (2017)). Regarding P, the highest P-release rate was obtained for propionic acid; however, the P-uptake rate was the highest for acetic acid. This is in contrast with the results obtained by Patel and Nakhla (2006), where the P-release and P-uptake rates were higher in the case of acetic acid than for propionic acid and other carbon sources such as butyric acid and primary effluent. For propionic acid and glutamate, the P-uptake rates were similar between them. In the case of the batch test with glutamate, no VFA were detected during the anaerobic phase, which indicate that the VFA consumption rate was probably limited by the VFA formation rate. The fast glutamate consumption could indicate that some microorganisms are able to use glutamate directly without a fermentation step. However, unavoidably, some glutamate is fermented to VFA, which would explain the presence of some "*Ca.* Accumulibacter" and hence, a high P-release with acetate and propionate (Figs. S4A and S4B). On the other hand, it has recently been reported that a strain of "*Ca.* Accumulibacter" clade IIF has been shown to directly metabolize glutamate among others amino acids (Qiu et al., 2020).

Finally, Qiu et al. (2019) studied the anaerobic P-release in three full-scale WWTPs in Singapore with different carbon sources: VFA, sugars, alcohols, amino acids and carboxylic acids. VFA induced the highest P-release 24.0–39.4 mg P L^{-1} for acetic and 17.0–34.6 mg P L^{-1} for propionate. P-release for amino acids was around 23.2 mg P L^{-1}. In our study, P-release for VFA was higher than that obtained for glutamate, however, the values of P-release (99.50 mg P L^{-1} for acetic, 72.10 mg P L^{-1} for propionic and 63.6 mg P·L^{-1} for glutamate) were higher than those reported by Qiu et al. (2019) and we had similar biomass concentration (around 2 g L^{-1}).

3.3. Phosphorus removal under permanent aerobic conditions

Four batch experiments were performed using different aeration flowrates to assess the behaviour of the mixed culture when the electron donor (glutamate) and the electron acceptor (nitrate and/or oxygen) were present simultaneously. P-release under aerobic conditions is observed in Fig. S5A and the presence of nitrate is due to the carbon source used. Glutamate contains nitrogen, which is degraded to ammonia, and this ammonia is oxidized to nitrate when oxygen is present. The DO profile is shown in Fig. S5B, oxygen was present during the entire experiment. As observed in previous studies (Guisasola et al., 2004; Pijuan et al., 2005), two different phases (feast and famine) could be distinguished, despite the aeration flowrate was not changed along the experiment. In the feast phase, glutamate was consumed linked to P-release and oxygen consumption. Moreover, nitrate concentration at the end of the feast phase was 15.7 mg NO_3^--N L^{-1}. When glutamate was depleted, oxygen concentration started to increase and P-uptake took place.

The phosphate profiles obtained in the batch tests using different aeration flowrates are shown in Fig. S6. The maximum P-release value (44.5 mg P L^{-1}) was obtained with the lowest aeration flowrate tested (50 mL min^{-1}), that resulted in the lowest oxygen concentration (DO concentration in the bulk liquid was near zero during the experiment). However, when the aeration flowrate was increased to 500 mL min^{-1} (with a DO concentration between 4 and 1.5 mg L^{-1}), the P-release resulted in 21.3 mg P L^{-1} (Fig. S6). In this sense, Pijuan et al. (2005) obtained higher values (ca. 49 mg P L^{-1}) in the feast phase, at 2 mg L^{-1}

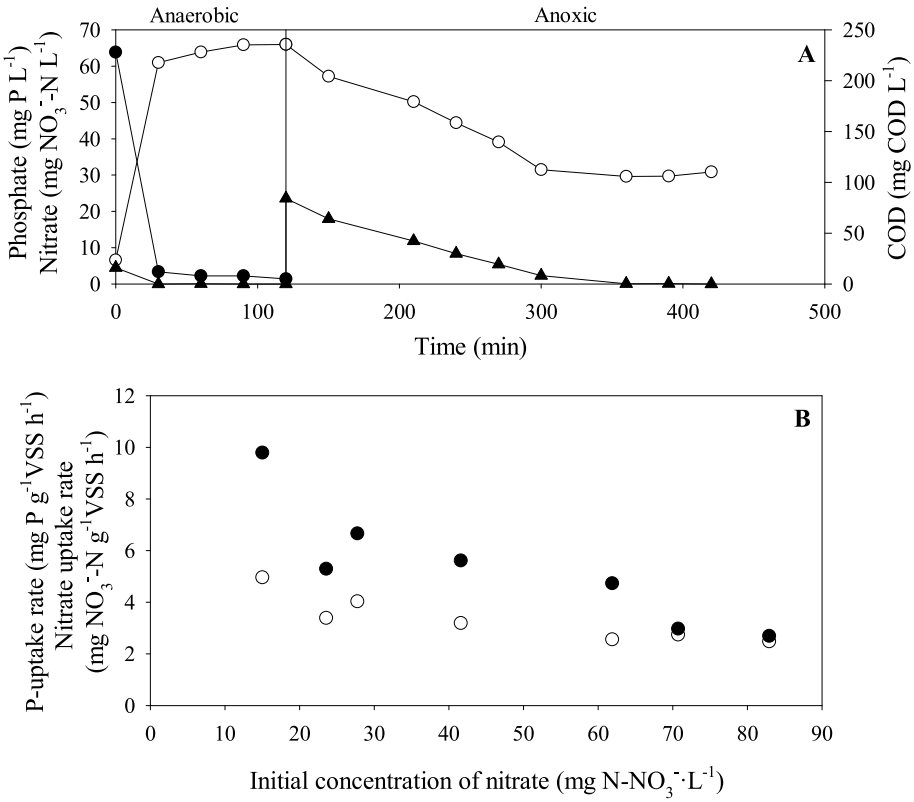

Fig. 4. Anaerobic/anoxic batch experiments with nitrate as sole electron acceptor. A) Concentrations of phosphorus (○), COD (●) and nitrate (▲). B) Effect of the initial concentration of nitrate in the different batch experiments on nitrate uptake rate (○) and P-uptake rate (●).

Table 3
Observed uptake and release rates for different carbon sources.

Carbon source	Specific P release rate (mg P·g VSS^{-1}·h^{-1})	Carbon uptake rate (mg C·g VSS^{-1}·h^{-1})	Specific P uptake rate (mg P·g VSS^{-1}·h^{-1})	P/C ratio (mol P·mol C^{-1})
Acetic acid	24.3	27.4	31.5	0.55
Propionic acid	36.1	41.0	21.3	0.45
Glutamate	30.6	57.8	21.4	0.21

of oxygen concentration. Therefore, the mixed culture used in this study was able to release phosphorus when the carbon source and oxygen coexisted and net P-removal was observed in the whole range of DO applied. The lower the aeration flowrate, the higher P-release was (Fig. S6). Nevertheless, some oxygen limitation when the aeration flowrates were 50, 150 and 250 mL min^{-1} could contribute to such P-release. In fact, DO concentration reached zero values even during the feast phase when the mentioned low aeration flowrates were used. This might have limited oxygen penetration in the biomass, creating anaerobic conditions inside the floccules. Contrarily, the minimum DO concentration achieved during the feast phase was 1.35 mg L^{-1} when the aeration flowrate was 500 mL min^{-1}. Another explanation to the behaviour observed in Fig. S6 can be the glutamate fermentation degree, i.e. the higher the oxygen presence during the feast phase, the lower glutamate fermentation occurred. Therefore, "*Ca.* Accumulibacter", which live off fermentation products, could not contribute to the P-release. Izadi et al. (2021) reported that lower DO concentrations may be more effective for EBPR performance providing advantages for PAO over GAO, while excessive aeration repeatedly demonstrated instability in P-removal as a result of high GAO competition.

Vargas et al. (2009) showed that it was possible to maintain EBPR activity under permanent aerobic conditions during a long period with propionate as sole carbon source. Nittami et al. (2011) operated successfully an SBR under alternating anaerobic-aerobic conditions with strictly aerobic conditions for one cycle every three or four days with acetate as carbon source. There are other studies reporting that the simultaneous presence of electron donor and acceptor does not affect the long-term operation of the EBPR (Ahn et al., 2007; Yadav et al., 2016). Nevertheless, Pijuan et al. (2006) observed that net P-removal was achieved under permanent aerobic conditions only during the first 4 days of operation when acetate was used as carbon source. In the same line, Zafiriadis et al. (2017) reported that the simultaneous presence of electron donor and acceptor in the mixed liquor inhibited P-uptake. These discrepancies may be attributed to differences in the biomass community structure and functionality, which was affected by the carbon source used (Nittami et al., 2017).

4. Conclusions

This is the first report on the denitrifying capabilities of a bio-P sludge fed with glutamate at pilot scale. Biomass shows successful EBPR performance with a more diverse microbial community than most lab-scale studies with VFA as the sole carbon source. This microbial community was capable to use both nitrite and nitrate as electron acceptors for P-uptake. P-uptake was affected by the FNA concentration and the process completely stopped at FNA values of 2 μg HNO_2–N L^{-1}. This value is lower than the inhibitory concentrations reported for "*Ca.* Accumulibacter"-enriched cultures. In the case of nitrate, P-uptake rate did not show a complete inhibition even at high nitrate values. However, 72% reduction on the P-uptake rate was observed when the initial nitrate concentration was increased from 15.0 to 82.9 mg N–NO_3 L^{-1}. The P/N ratios were higher when nitrate was the electron acceptor (0.49–0.89 mol P/mol N for nitrate vs 0.36–0.43 mol P/mol N for nitrite), however, the anoxic P-uptake rates were higher using nitrite.

The microbial culture developed in this work was also able to

perform EBPR under permanent aerobic conditions. However, the amount of P-release under these conditions was lower than that observed by Pijuan et al. (2006) with a "*Ca.* Accumulibacter"-enriched culture.

Credit author statement

Natalia Rey-Martínez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – Original; Gökçe Merdan: Methodology, Validation, Investigation, Data curation; Albert Guisasola: Conceptualization, Validation, Writing – review & editing. Juan Antonio Baeza: Conceptualization, Validation, Resources, Software, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131173.

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