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Exploring the stability of an A-stage-EBPR system for simultaneous biological removal of organic matter and phosphorus

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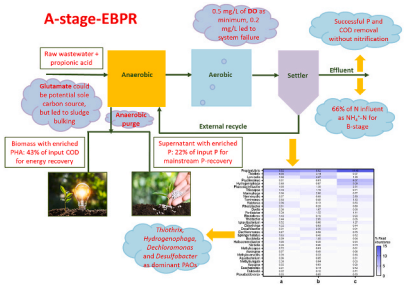
HIGHLIGHTS

- Long-term maintenance of an A-stage-EBPR under selected operational conditions.
- The system operated good at range 0.5–1 mg DO/L, but failed at 0.2 mg DO/L.
- Anaerobic purge led to 43% of the influent COD being sent to anaerobic digestion.
- Glutamate as carbon source was only feasible for 60 days due to settleability issues.
- Propionivibrio was the most abundant species during the whole operational process.

GRAPHICAL ABSTRACT



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ABSTRACT

This work evaluates the performance and stability of a continuous anaerobic/aerobic A-stage system with integrated enhanced biological phosphorus removal (A-stage-EBPR) under different operational conditions. Dissolved oxygen (DO) in the aerobic reactor was tested in the 0.2–2 mgDO/L range using real wastewater amended with propionic acid, obtaining almost full simultaneous COD and P removal without nitrification in the range 0.5–1 mgDO/L, but failing at 0.2 mgDO/L. Anaerobic purge was tested to evaluate a possible mainstream P-recovery strategy, generating a P-enriched stream containing 22% of influent P. COD and N mass balances indicated that about 43% of the influent COD could be redirected to the anaerobic digestion for methane production and 66% of influent NH_4^+-N was discharged in the effluent for the following N-removal B-stage. Finally, when the system was switched to glutamate as sole carbon source, successful EBPR activity and COD removal were maintained for two months, but after this period settleability problems appeared with biomass loss. Microbial community analysis indicated that *Propionivibrio*, *Thiothrix* and *Lewinella* were the most abundant species when propionic acid was the carbon source and *Propionivibrio* was the most favoured with glutamate. *Thiothrix, Hydrogenophaga, Dechloromonas* and *Desulfobacter* appeared as the dominant polyphosphate-accumulating organisms (PAOs) under different operation stages.

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1. Introduction

Biological nutrient removal (BNR) processes are widely investigated and applied in wastewater treatment plants (WWTPs) for removing phosphorus (P), nitrogen (N) and chemical oxygen demand (COD) from wastewater so that eutrophication caused by overloaded nutrient discharging to the water bodies is prevented (Welles et al., 2015; Yuan et al., 2012). Enhanced biological phosphorus removal (EBPR), based on the proliferation of polyphosphate-accumulating organisms (PAO), is an environmental-friendly and cost-effective technology for P removal in WWTPs (Oehmen et al., 2007; Tchobanoglous et al., 2014) PAO are promoted by alternating anaerobic and aerobic/anoxic conditions (Comeau et al., 1987). Under anaerobic conditions, PAO take up carbon sources (normally volatile fatty acids, VFA) and store them as polyhydroxyalkanoates (PHA). The required reductive power and energy for this process come from the degradation of glycogen and the hydrolysis of polyphosphate (poly-P) to phosphate, which is released into the mixed liquor. In the subsequent aerobic/anoxic condition, PAO oxidize their internal PHA reserves and obtain the required energy to grow, to regenerate their glycogen pools and to uptake phosphate as poly-P (Satoh et al., 1998; Smolders et al., 1994). Recent research on EBPR-related microorganisms show versatile metabolic ways of diverse putative PAOs (e.g. *Tetrasphaera*, *Dechloromonas* and *Thiothrix)* with various carbon source strategies. For example, *Tetrasphaera* were reported to use glycogen and free amino acids as possible intracellular substances for energy storage, rather than the conventional PHA economy when fed with glucose or amino acid as carbon source (Mielczarek et al., 2013; Singleton et al., 2022; Zhao et al., 2022). This versatile metabolic ways of PAO benefit for the full-scale EBPR implementation since the system becomes more resistant to a variable unstable environment.

Chemical dosing for P removal (aluminum and ferric salts) is widely accepted and it can face the problem of fluctuations in the influent COD/P ratio. However, when COD is not limited, EBPR outcompetes chemical P removal in terms of sustainability and costs at the expense of an increased degree of complexity of plant operation (Tchobanoglous et al., 2014). There is also the possibility of combining chemical precipitation to EBPR, which can have great potential (Bunce et al., 2018; Izadi et al., 2020; Kazadi Mbamba et al., 2019). The recent paradigm shift in the field of environmental engineering paves the way for the integration of novel P recovery strategies that are even more sustainable than EBPR, since P is a non-renewable resource that is expected to cause limitations in the next century (Cordell et al., 2009; Desmidt et al., 2015; Rittmann et al., 2011). Mainstream P-recovery from the anaerobic supernatant of EBPR systems is a promising methodology when compared to P-recovery from other side streams (e.g. effluent from anaerobic sludge digestion) because, theoretically, a higher percentage of P can be recovered (Zhang et al., 2022). Moreover, mainstream P-recovery can be linked to anaerobic biomass purging which would be beneficial in view of carbon recovery processes: i) sludge withdrawn from the anaerobic reactor shows higher biochemical methane potential when compared to sludge from the aerobic reactor and ii) anaerobic sludge (typical PAO with PHA storage as carbon source) contains a higher percentage of PHA and, thus, could also be a precursor of bioplastics after an extraction process (Chan et al., 2020; Larriba et al., 2020; Wang et al., 2016).

On the other hand, one of the latest configurations for energy recovery from wastewater is the two-stage A/B process (Boehnke and Diering, 1997; Wan et al., 2016; Wang et al., 2009). In a few words, the first A-stage is designed to capture as much COD as possible using a high-rate activated sludge (HRAS) system to redirect and concentrate carbon, rather than mineralization. 50–80% of COD can be recovered from the influent (Sancho et al., 2019) and can be redirected to anaerobic digestion for energy recovery as methane. Subsequently, autotrophic nitrogen removal is handled by a B-stage that includes, for example, partial nitritation (Isanta et al., 2015) combined with anammox (Jenni et al., 2014; Reino et al., 2018; Xu et al., 2015).

In our previous study (Zhang et al., 2021), the focus was on demonstrating the feasibility of the A-stage-EBPR concept, which integrates the traditional anaerobic/aerobic (A/O) EBPR configuration into an A-stage system to maximize the redirection of COD to anaerobic digestion, as well as to remove the P biologically. However, it was noted in that work that the A-stage-EBPR needed to be tested under a wider range of operating conditions to demonstrate its stability and performance, i.e. whether organic matter removal and PAO activity could be maintained and both the growth of nitrifying organisms and sedimentation problems (e.g. filamentous bulking) could be avoided.

On the one hand, the DO setpoint in the aerobic phase is essential. Lower DO can reduce the energy consumption of aeration at expenses of a decrease in PAO kinetics. Moreover, Izadi et al. (2021) also reported that PAO could outcompete glycogen accumulating organisms (GAO) under lower DO concentrations. It is therefore relevant to explore the effect of the DO setpoint on this novel A-stage-EBPR system.

On the other hand, the nature of the influent carbon source is also essential when determining the minimum retention time needed under anaerobic conditions. Different carbon sources exert great influence during this process for the growth and metabolism of PAO and GAO (Nittami et al., 2017; Shen and Zhou, 2016). The application of VFA (e.g. acetate and propionate) is usual to promote PAO growth, obtaining microbial communities highly enriched in *Candidatus* Accumulibacter phosphatis (hereafter "*Accumulibacter*"). Then, a fermentation step may be needed for complex carbon sources (thus, enough anaerobic retention time) so that *Accumulibacter* PAO can live off the fermentation products. However, the different conditions and organic matter compounds in real wastewater may lead to the proliferation of fermentative types of PAO (e.g. *Tetrasphaera* or *Microlunatus*), GAO (e.g. *Micropruina*) or other facultative anaerobic bacteria which could produce substrate for PAO and GAO (Nielsen et al., 2019; Singleton et al., 2022). Proteins are also a kind of significant carbon source, accounting for example 25–35% of COD in real wastewater entering Danish EBPR plants (Nielsen et al., 2010). Hydrolysates of protein-amino acids have been applied in lab-scale EBPR studies (Marques et al., 2017; Shon et al., 2007; Zengin et al., 2011), where *Accumulibacter*, *Tetrasphaera*-related PAOs and *Thiothrix* were favoured. Glutamate, as a potential carbon source for EBPR has been specifically investigated (Dionisi et al., 2004; Kong et al., 2005; Marques et al., 2017; Rey-Martínez et al., 2021b), and it favors the growth of *Actinobacterial* PAOs and family *Comamonadaceae* (Chua et al., 2006; Kristiansen et al., 2013; Rey-Martínez et al., 2019). Glutamate contains a high fraction of nitrogen that is released to the medium when glutamate is fermented. Rey-Martínez et al. (2019) showed successful P and N removal with glutamate as sole carbon and nitrogen source in an anaerobic/anoxic/oxic continuous pilot system. However, the feasibility of using glutamate as sole carbon source in an A-stage-EBPR system and its effect on the microbial community have not been reported yet.

Therefore, this work aims to evaluate the performance of a continuous A-stage-EBPR system under different operational conditions. The main objectives of this work are: i) to study the effect of different DO setpoint in the aerobic reactor to maintain long-term successful organic matter and P removal without nitrification, ii) to investigate the possibility to recover P by purging from the anaerobic reactor, iii) to gain insight of the performance of the system with different carbon sources (propionic acid and glutamate), and iv) to evaluate the changes in the microbial community under the different operational conditions.

2. Materials and methods

2.1. Equipment

The A/O configuration consisted of two completed stirred reactors, the first anaerobic (V = 19 L) and the second one aerobic (V = 23 L), and a settler (25 L) (Fig. S1). The A-stage-EBPR system was controlled with an on-line system based on an Advantech PCI-1711 I/O card and an industrial PC running the Addcontrol software developed in the research

group. The aerobic reactor was equipped with a DO probe (HACH-CRI6050), a pH probe (HACH CRI5335) and a temperature probe (Axiomatic Pt1000). The DO in the aerobic reactor was controlled by a proportional-integral algorithm manipulating the aeration flow rate with a mass flow controller (F–201CV-RGD-22-V, Bronkhorst, Holland). The system was inoculated with sludge collected from a municipal WWTP (Manresa, Spain) and the raw wastewater used was from the primary settler effluent of the same plant. The average characteristics of the raw wastewater are shown in Table 1 (period I and II). Because of the low concentration of COD in this wastewater, additional propionic acid was added from a concentrated solution (46,000 mg/L COD) to increase it up to around 410–430 mg COD/L. Thus, the influent was composed of raw wastewater (90 L/d) with propionic acid solution (0.45 L/d). Synthetic wastewater was applied in period III using glutamate sodium as sole carbon and nitrogen source, with a theoretical concentration of 41 mgN/L. The external recycle flowrate (45 L/d) was set at 0.5 times the influent. Hydraulic retention time was 11.2 h considering only the reactors and 17.9 h also considering the settler. The sludge retention time (SRT) was controlled in different periods based on equation (1), considering the solids lost in the effluent and selecting the proper flow rate of wasted sludge.

$$SRT = \frac{V_{ana} \cdot X_{ana} + V_{aer} \cdot X_{aer}}{Q_{pur} \cdot X_{aer} + Q_{eff} \cdot X_{eff}} \quad (1)$$

where V_{ana} and V_{aer} (L) represent the volume of the anaerobic and aerobic reactors, X_{ana}, X_{aer} and X_{eff} (g/L) mean concentration of the biomass in both reactors and the effluent, Q_{pur} and Q_{eff} (L/d) are the flow rate of purge and effluent.

The pH during the reported period was in the range 6.2–7.8. The system was operated at room temperature (21±2 °C). The operational conditions of DO, purge position and carbon source used for the different periods are shown in Table 2.

2.2. Chemical and biochemical analyses

Samples for analysis of phosphate, COD, ammonium, nitrate, and nitrite were taken from the anaerobic reactor and the settler and filtered with 0.22 μm filters (Millipore) to separate the biomass. The concentration of phosphate was determined by a phosphate analyser (115 VAC PHOSPHAX sc, HACH) based on the Vanadomolybdate yellow method. COD was analysed by kits and a spectrophotometer (HACH Lange LCK 314 and LCK 714). Ammonium was analysed by an ammonium analyser (AMTAXsc, HACH) based on potentiometry. The concentrations of nitrite and nitrate were detected by strips (A029835 MACHEREY-NAGEL, A029985 MACHEREY-NAGEL), nitrate kits (LCK 339 HACH) and nitrite kits (LCK 342 HACH). Mixed liquor volatile suspended solids (VSS) and total suspended solids (TSS) from the anaerobic reactor, aerobic reactor and effluent were analysed according to Standard Methods (APHA, 1998). The sludge volume index (SVI) was obtained with the ratio of the observed volume (mL) of sludge (settling for 30 min) and TSS (g/L) from the aerobic reactor.

Table 1
Average compositions of the real wastewater amended with propionic acid used in period I and II (0-56d) and synthetic wastewater applied in Period III (57-142d).

Components	Units	Period I	Period II	Period III
PO_4^{3-}	mgP/L	6.0 ± 1.0	6.2 ± 0.6	6.4 ± 0.6
NH_4^+	mgN/L	58 ± 9	50 ± 9	41
COD_S[a] (raw wastewater)	mgCOD/L	186 ± 68	197 ± 30	-
COD_S[a] (external carbon source)	mgCOD/L	230 (propionic acid)	230 (propionic acid)	380 ± 30 (glutamate)
COD_S (total)	mgCOD/L	414 ± 67	425 ± 29	380 ± 30

[a] Soluble COD concentrations.

Table 2
DO, purge position and carbon source used for the different operational periods.

Period	Duration (d)	DO (mg/L)	Purge position	Carbon source
Ia	0–9	1.0	Aerobic	Raw wastewater + propionic acid
Ib	10–18	0.5	Aerobic	Raw wastewater + propionic acid
Ic	19–21	0.2	Aerobic	Raw wastewater + propionic acid
Id	22–26	0.5	Aerobic	Raw wastewater + propionic acid
IIa	27–39	1.0	Aerobic	Raw wastewater + propionic acid
IIb	40–56	1.0	Anaerobic	Raw wastewater + propionic acid
IIIa	57–88	1.0	Anaerobic	Synthetic wastewater (glutamate)
IIIb	89–122	1.0	Anaerobic	Synthetic wastewater (glutamate)
IIIc	123–142	1.0	Anaerobic	Synthetic wastewater (glutamate)

2.3. Performance indicators and fate of COD and nitrogen

The calculation of the P removal efficiency (PRE), the total COD removal efficiency (CRE), and the fate of COD and N are shown in the supplementary information (Table S1).

2.4. Batch tests

Two batch tests were carried out to investigate the EBPR activity at different stages: a) day 52 in period IIb, with DO = 1 mg/L, raw wastewater amended with propionic acid and anaerobic purge; and b) day 88 in period IIIa, with DO = 1 mg/L, glutamate as sole carbon source and anaerobic purge.

The tests were performed in a magnetically stirred vessel (2 L) monitored with pH (Sentix 81, WTW) and DO (Cellox 325, WTW) probes. The sludge for these tests was withdrawn from the aerobic reactor. Sodium propionate was used as sole carbon source. Anaerobic conditions were maintained for the first 3 h by supplying nitrogen gas, and aerobic conditions were achieved with a constant air flowrate during the following 3 h in batch a). In batch b), 2 h anaerobic and 2 h of aerobic phase were enough for the full P release and uptake. The pH was not controlled and was around 7.6 ± 0.4 during the tests. The temperature was maintained (25 °C) with a water bath. Samples were taken every 30 min and filtered with 0.22 μm Millipore filters immediately for the analysis of phosphate, ammonium, nitrate, nitrite and COD.

2.5. Microbiological analyses

Sludge samples were collected in the aerobic reactor during different stable operation periods to identify the bacterial population by Illumina amplicon sequencing of the 16S rRNA gene: (a) propionic acid as carbon source under aerobic purge (period IIa), (b) propionic acid and anaerobic purge (period IIb) and (c) glutamate under anaerobic purge (period IIIa). Each sample was washed with PBS for three times, centrifugated, and stored at – 20 °C for further DNA extractions. Soil DNA isolation plus kits (Norgen Biotek CORP, Ontario., Canada) were used for the genomic DNA extraction process. The extracted DNA was quantified by a DNA NanoDrop 1000 Spectrophotometer (Waltham, MA, USA), and analysed by the "Genomic and Bioinformatics service center" at UAB. The universal primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify and sequence the V4 region of the 16S rRNA to ensure high sequence coverage of bacteria and archaea and to produce an appropriately sized amplicon for

Illumina sequencing (Wu et al., 2015). The Greengenes database was used to classify the organisms and the sequence reads were analysed through Usearch software.

3. Results and discussion

The experimental work conducted can be divided into three different periods. Period I (Section 3.1) aimed at finding the minimum DO setpoint to run the A-stage-EBPR system. Period II (Section 3.2) was set to explore the A-stage-EBPR performance when operated with anaerobic purge instead of the conventional aerobic purge. Finally, glutamate was used in period III (Section 3.3) to study the effect of this carbon source on the A-stage-EBPR performance and on the microbial community. The profiles of C, N and P during the whole experimental period are shown in Fig. 1 and the average values for each period are reported in Table 3.

3.1. Performance under different DO setpoints

The A-stage-EBPR system was operated under different DO setpoints in period I (Table 2) with raw wastewater amended with propionic acid. Whenever the operational conditions were changed, we waited the adequate time to reach a stable performance, which was around 3 SRTs. The starting value was a DO setpoint of 1 mg/L and it was gradually decreased (1–0.5-0.2 mg/L) when successful simultaneous P and COD removal were reached. Fig. 1a shows the experimental P concentration in the influent, anaerobic reactor and aerobic reactor and its removal efficiency PRE (defined in Table S1), while Fig. 1b shows COD concentration at the same sampling points. During period Ia (DO = 1 mg/L, purge of 6 L/d and SRT = 6 d), excellent P and COD removal efficiency were obtained (PRE = 98 ± 1% and CRE = 95 ± 7%, Table 3). Ammonia (Fig. 1c), nitrate and nitrite (Fig. 1d) profiles showed that after 6 days of operation there was almost no nitrate or nitrite was detected in the effluent, thus, the nitrifying activity was negligible. The A-stage-EBPR system aims at suppressing nitrification since nitrogen is supposed to be removed by the subsequent B-stage and, besides that, avoiding the nitrate entering the anaerobic reactor through the external recycle which may hinder PAO activity. VSS was about 2.63 ± 0.35 g/L (Table 4 and Fig. 2a) in the reactor and 0.03 ± 0.02 g/L (Table 4 and Fig. 2b) in the effluent. This low concentration of biomass in the effluent, in addition to a low SVI of 77 mL/g (Fig. 2c and Table 4) was an indication of the good settleability of the sludge. The ratio of VSS/TSS in the aerobic reactor was 0.91 at the end of this period (Fig. 2d), lower than the anaerobic ratio of 0.96. Thus, there was a significant change in poly-P concentration, a clear indication of good P-release and P-uptake activity. As a conclusion of period Ia, the system showed successful P and COD removal performance and good sludge settleability with the selected operational conditions and DO = 1 mg/L, which suggests that it is feasible for the system to step into the subsequent stage.

The DO setpoint was moved from 1 to 0.5 mg/L (Period Ib) to assess whether the same performance could be obtained with lower aeration requirements. Izadi et al. (2021) indicated that operating DO at 0.8 mg/L could remove 90% of P in an A/O SBR system with a high enrichment in PAO. Other studies also showed that lower DO values could promote the selection of PAO over GAO (Carvalheira et al., 2014; Chiu et al., 2007). In our work, PRE and CRE were maintained at 94 ± 3% and 95 ± 2%, while nitrification activity remained suppressed as expected. The solids showed a little decrease (around 2.3 gVSS/L in the reactor) and SVI slightly increased to 134 ± 29 mL/g. Then, the system was able to maintain a good performance under the DO = 0.5 mg/L condition.

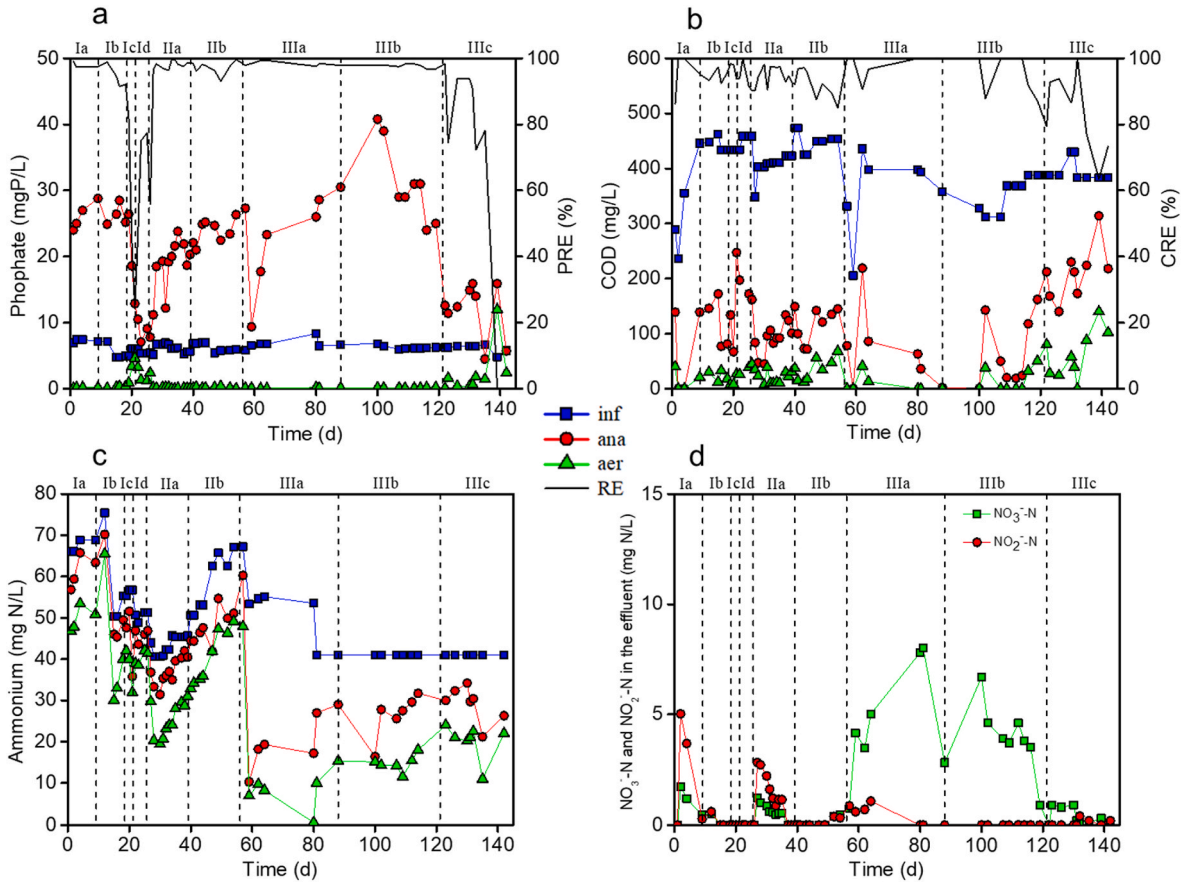

Fig. 1. Evolution for different operational periods of the removal efficiencies and concentrations in the influent, the anaerobic reactor and the aerobic reactor. a) Phosphorus, b) COD, c) ammonium and d) nitrate and nitrite.

Table 3
P and COD concentrations and removal performance obtained during different periods.

Period	Duration (d)	P_{INF}[a] (mgP/L)	P_{ANA}[b] (mgP/L)	P_{AER}[c] (mgP/L)	PRE[d] (%)	COD_{INF}[a] (mgCOD/L)	COD_{ANA}[b] (mgCOD/L)	COD_{AER}[c] (mgCOD/L)	CRE[e] (%)
Ia	0–9	7.2 ± 0.9	26 ± 2	0.2 ± 0.1	98 ± 1	331 ± 91	70 ± 80	15 ± 19	95 ± 7
Ib	10–18	5.4 ± 1.2	26 ± 2	0.3 ± 0.1	94 ± 3	445 ± 14	119 ± 47	23 ± 10	95 ± 2
Ic	19–21	5.7 ± 0.7	19 ± 7	3.0 ± 1.9	50 ± 29	434 ± 0	149 ± 91	14 ± 11	97 ± 3
Id	22–26	5.5 ± 0.4	9 ± 2	2.0 ± 1.0	64 ± 15	452 ± 12	177 ± 18	37 ± 10	94 ± 4
IIa	27–39	6.1 ± 0.6	19 ± 4	0.1 ± 0.1	98 ± 1	407 ± 21	91 ± 27	21 ± 11	95 ± 3
IIb	40–56	6.2 ± 0.6	24 ± 2	0.2 ± 0.1	97 ± 2	450 ± 19	117 ± 32	36 ± 20	92 ± 4
IIIa	57–88	6.8 ± 0.8	26 ± 4	0.1 ± 0.1	98 ± 1	360 ± 76	69 ± 74	8 ± 15	98 ± 4
IIIb	89–122	6.3 ± 0.3	29 ± 8	0.1 ± 0.0	98 ± 1	358 ± 32	76 ± 79	22 ± 29	94 ± 8
IIIc	123–142	6.2 ± 0.6	12 ± 4	2.4 ± 3.9	65 ± 41	396 ± 21	210 ± 53	60 ± 47	85 ± 12

[a] INF: concentration in the influent.
[b] ANA: concentration in the anaerobic reactor.
[c] AER: concentration in the aerobic reactor.
[d] PRE: P removal efficiency.
[e] CRE: COD removal efficiency.

Table 4
Evolution of SRT, solids concentration, VSS/TSS ratio and settleability in the A-stage-EBPR system for different periods.

Period	Duration (d)	SRT (d)	VSS_{AER} (g/L)	VSS_{EFF} (g/L)	VSS/TSS_{ANA}	VSS/TSS_{AER}	SVI (mL/g)
Ia	0–9	6.3 ± 0.1	2.63 ± 0.35	0.03 ± 0.02	0.89 ± 0.06	0.85 ± 0.05	77 ± 20
Ib	10–18	5.4 ± 0.6	2.29 ± 0.28	0.05 ± 0.03	0.94 ± 0.04	0.91 ± 0.01	134 ± 29
Ic	19–21	2.9 ± 0.9	1.42 ± 0.35	0.14 ± 0.04	0.96 ± 0.03	0.93 ± 0.00	617 ± 169
Id	22–26	0.7 ± 0.4	0.84 ± 0.01	0.60 ± 0.31	1.00 ± 0.01	1.00 ± 0.01	1143 ± 24
IIa	27–39	6.9 ± 0.2	2.70 ± 0.10	0.03 ± 0.01	0.93 ± 0.02	0.91 ± 0.01	91 ± 12
IIb	40–56	6.4 ± 0.3	2.45 ± 0.15	0.03 ± 0.01	0.94 ± 0.02	0.93 ± 0.02	178 ± 64
IIIa	57–88	6.7 ± 1.8	2.34 ± 0.27	0.07 ± 0.03	0.95 ± 0.03	0.92 ± 0.03	232 ± 62
IIIb	89–122	1.9 ± 1.0	1.72 ± 0.26	0.42 ± 0.20	0.97 ± 0.01	0.93 ± 0.05	518 ± 115
IIIc	123–142	0.5 ± 0.1	0.79 ± 0.22	0.71 ± 0.03	1.00 ± 0.00	0.99 ± 0.01	1139 ± 352

The DO setpoint in the aerobic reactor was further decreased to 0.2 mg/L (period Ic) and, subsequently, PAO activity was severely damaged. P concentration in the anaerobic reactor (P_{ANA}) decreased from 26.4 to 12.9 mg/L in the first 2 days, and the corresponding P concentration in the aerobic reactor (P_{AER}) increased from 0.4 to 4.6 mg/L. Conversely, COD removal performance was not affected (CRE about 97%). Anaerobic carbon concentration shortly increased in this period, indicating a slight decrease of anaerobic COD consumption. The reasons are either PAO reaching their maximum capacity for COD storage as PHA or its inability to restore the poly-P reserves under aerobic conditions due to oxygen limitation. Then, the excess of COD from the anaerobic phase was oxidized under limited aerobic conditions, which led to a severe decrease of the settleability (SVI increased to 617 ± 169 mL/g), probably due to the promotion of filamentous bacteria, which is expected under these operational conditions (Jenkins et al., 2003). The high effluent VSS (around 0.14 gVSS/L) led to a decrease of the SRT to 3 days. Our previous studies showed that 4 days SRT was the minimum threshold of this A-stage-EBPR system (Zhang et al., 2021). Then, the performance was severely affected in period Ic (DO = 0.2 mg/L).

After this unsuccessful operational period, efforts were made to recover lost activity by increasing the DO to 0.5 mg/L and drastically reducing the purge to increase SRT (period Id). Despite these changes, P removal performance showed no improvement, the biomass in the reactor decreased (0.84 ± 0.01 gVSS/L) and SVI increased to 1143 mL/g, indicating a high proliferation of filamentous bacteria. Moreover, an increasingly high ratio of VSS/TSS about 1 was observed (Table 4 and Fig. 2d), which showed the red flag of PAO washout (Chan et al., 2017; Oehmen et al., 2006; Zhang et al., 2021). As a summary of Period I, a setpoint of 0.2 mg/L DO was not able to support a stable performance of the A-stage-EBPR system, but operation at DO in a range 0.5–1 mg/L was feasible. Operating at a too low DO can lead to problems of poor settleability and loss of PAO activity.

Table 5
COD mass balance during periods I and II. All COD items are represented as a percentage of the influent COD.

Period	COD_{EFF}[a] (%)	COD_{PUR}[b] (%)	COD_{EFFB}[c] (%)	COD_{PURB}[d] (%)	COD_{OUT}[e] (%)	COD_{MINER}[f] (%)
Ia	4 ± 6	0.2 ± 0.2	21 ± 12	32 ± 17	58 ± 12	42 ± 12
Ib	5 ± 2	0.3 ± 0.3	10 ± 8	53 ± 2	68 ± 5	32 ± 5
IIa	6 ± 6	0.3 ± 0	10 ± 2	54 ± 2	70 ± 4	30 ± 4
IIb	7 ± 4	0.4 ± 0.2	8 ± 3	43 ± 2	58 ± 5	42 ± 2

[a] COD in the effluent after filtration.
[b] COD in the purge stream after filtration.
[c] COD contained in the biomass of the effluent.
[d] COD contained in the biomass of the purge stream.
[e] Total COD output obtained as the sum of the previous four items: e = a+b + c + d.
[f] Percentage of input COD mineralized to CO_2: f = 100-e %.

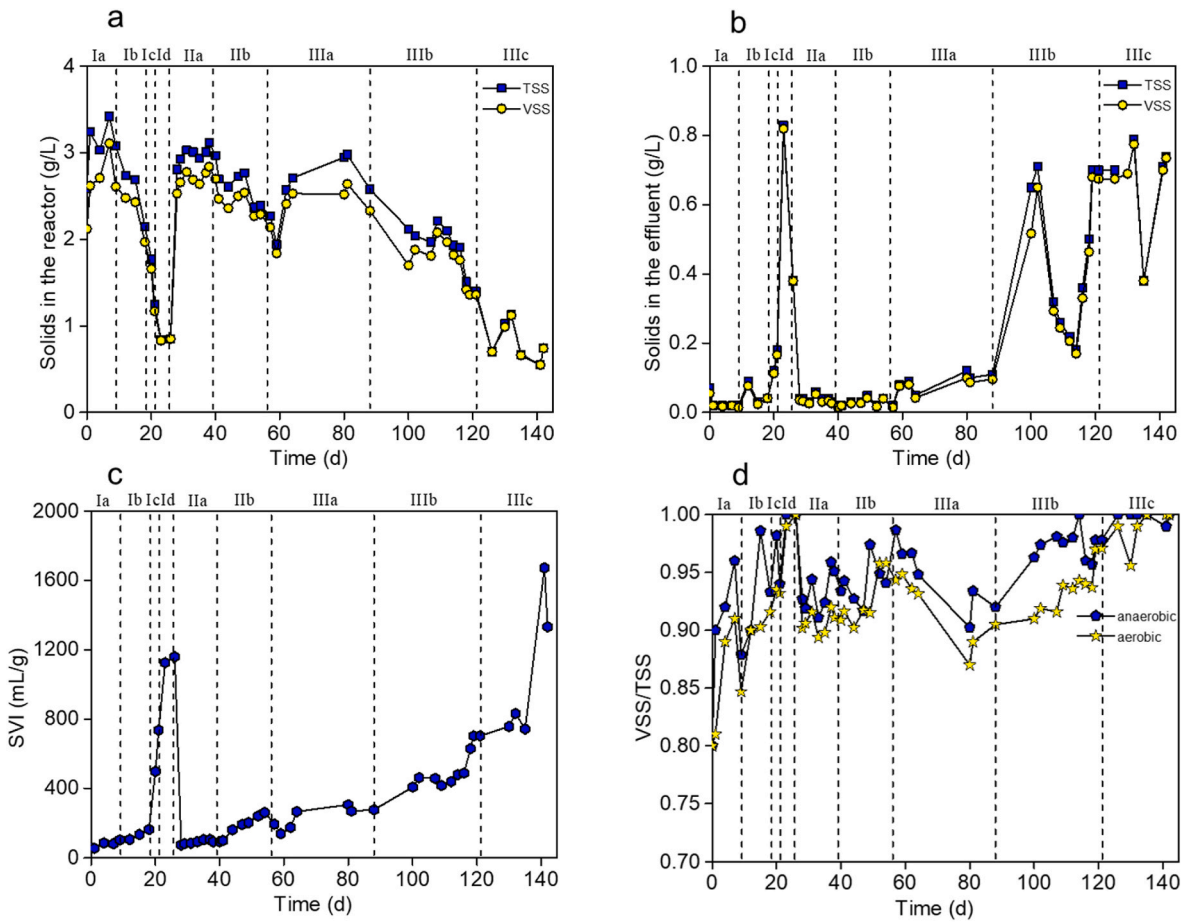

Fig. 2. Solids related evolution for different operational periods. Solids concentration in (a) the reactor, (b) effluent, (c) SVI and (d) VSS/TSS ratio.

3.2. System performance under different purge positions

The sludge from the anaerobic reactor could be an opportunity to increase energy recovery because of its higher PHA content and therefore higher potential for biomethane production (Chan et al., 2020). In addition to that, the anaerobic liquor contains a high P concentration which could be adequate for P recovery by precipitation. However, the stability of the A-stage-EBPR operation under these operational conditions should be demonstrated.

To study this operation, the plant was reinoculated on day 27 in period IIa. The same inoculation biomass, and operation conditions (DO setpoint, purge position and carbon source) were kept for a better agreement with the previous results. In any case, the discussion on the microbial community evolution was all based in samples withdrawn after this reinoculation. The plant was operated with a DO setpoint of 1 mg/L and a reduced purge of 1 L/d from the aerobic reactor to achieve a good P removal performance. The concentration of P in the effluent was about 0.1 ± 0.1 mg/L (PRE = 98 ± 1% and CRE = 95 ± 3%). Some nitrification appeared at the start of period IIa due to the biomass reinoculation and a long SRT. The purge flowrate was gradually increased to decrease the SRT, operating at 6 days from day 36. Then, PRE remained stable between 97% and 100% and similarly, CRE was around 98% and nitrification activity ceased. The VSS in the reactor was stable at 2.7 ± 0.1 g/L (Fig. 2a) and SVI was 91 ± 12 mL/g showing good settleability.

The purge position was moved from the aerobic to the anaerobic reactor on day 40 (period IIb), and the system was maintained at an SRT of 6 days. The performance of the plant was maintained using the anaerobic purge (Fig. 1): PRE remained stable with 97 ± 2% and CRE was around 92 ± 4% even though there were insignificant fluctuations probably due to the changeable influent COD of the real wastewater. However, the effluent COD was always less than 40 mg/L. Neither nitrate nor nitrite was detected in the effluent, which implied that the nitrification was avoided. VSS in the reactor experienced a little decrease with a concentration of 2.5 ± 0.2 gVSS/L and SVI slightly increased from 161 to 260 mL/g.

3.2.1. The potential for P recovery under anaerobic purge condition

Anaerobic purging is not only beneficial for carbon recovery due to the higher biomethane production potential of the anaerobic sludge, but also provides an opportunity for mainstream P-recovery due to the enriched P concentration in the anaerobic supernatant (Acevedo et al., 2015; Guisasola et al., 2019; Zhang et al., 2022). The potential for P recovery under anaerobic purge condition in this work can be calculated based on P_{ANA} during period IIb (24 ± 2 mgP/L) and the purge flowrate (5 L/d). Considering the influent flowrate of 90 L/d and an average P concentration of 6.2 mg/L, about 22% of P in the influent was contained in the anaerobic supernatant. P concentration in this mixed liquid was increased by a factor of four compared to the input, which should favour its precipitation and recovery as struvite or vivianite after a separation step for the biomass. In a previous work, Larriba et al. (2020) obtained an average recovery of 45% of the influent P by struvite precipitation from the anaerobic supernatant in a demo-scale pilot plant for a long period operation (with higher SRT about 10–15 d). However, this higher percentage was obtained by redirecting 8.6% of the influent flow to the P-recovery stream, whereas in the present work only 5.6% was proposed for redirection. A higher P-recovery percentage would be possible, but at the expense of adding a biomass separation and recycling stage able to separate the P-recovery stream from the anaerobic purge extraction. Otherwise, the SRT would be too low.

3.2.2. Mass balances of carbon and nitrogen

Table 5 shows the results of the COD mass balance in period I and II. The potential COD outlets are: 1) effluent, as dissolved COD or biomass, 2) purge, as dissolved COD or biomass and 3) COD mineralization (i.e. the formation of CO_2). Mineralized COD decreased from 42 ± 12% to 32 ± 5% with the DO decrease from 1 mg/L (period Ia) to 0.5 mg/L (period Ib). These values are comparable to the mineralization observed in a non-EBPR A-stage reported by Jimenez et al. (2015) for SRT = 0.5 d (37%) and lower to that observed at SRT = 2 d (67%). They are close to the range of a continuous A-stage (41–58%) and an A-stage SBR (20–48%) reported in a previous work (Rey-Martínez et al., 2021a) and slightly higher than those reported for SRT = 1.0 d (22%) and SRT = 2.1 d (27%) in a pilot-scale continuous HRAS system (Carrera et al., 2022).

Regarding the COD content in the biomass of the purge, a much higher fraction of the input COD could be redirected to the anaerobic digestion under DO = 0.5 mg/L (53 ± 2%) compared to that of DO = 1 mg/L (32 ± 17%). When the purge was moved to the anaerobic reactor with DO = 1 mg/L (period IIb), the input COD stored in the biomass was increased up to 43 ± 2%, showing the positive effect of the anaerobic purge. In the work of (Jimenez et al., 2015), the COD redirection in their A-stage system increased from 23 to 48% when decreasing the SRT from 2 to 0.3 d, while Rey-Martínez et al. (2021a) reported values of 30 and 34% for the continuous A-stage at SRT = 1 and 2 d and up to 62% for the A-stage SBR at SRT = 1d. Carrera et al. (2022) in their HRAS system showed about 24% of COD stored in the biomass at SRT = 2.1 d and increasing to 29% at SRT = 1.0 d with an additional percentage of COD adsorption in the range 25–30%.

Comparing the results of our work with previous results obtained in non-EBPR A-stage systems, it can be concluded that with the A-stage-EBPR system operating at SRT = 6 d and low oxygen concentration in the range 0.5–1 mg/L, it is possible to obtain COD redirection results to purged biomass and COD mineralization percentages that are comparable to non-EBPR A-stage systems operating at much lower SRTs even below 2 d.

A minimum amount of influent COD is required for successful P and N removal. Textbook recommends the threshold ratios for the design of WWTP configurations (A2/O and UCT) with a minimum of readily biodegradable carbon source (rbCOD) for simultaneous C, N and P removal: 10 g rbCOD/gP and 6.6 g rbCOD/gNO_3^--N (Tchobanoglous et al., 2014). The A/B system proposed in this work requires a lower COD amount since N is allegedly removed at the B stage under autotrophic conditions. Despite this, the water was still deficient in COD. In that case, integrating in situ fermentation for rbCOD production could be a solution for the required COD (e.g. fermentation of primary sludge or waste solid by side-stream sludge fermenter) (Barnard et al., 2017; Fan et al., 2022, Fan et al., 2021; Wang et al., 2019). In addition, increasing the anaerobic retention time to promote the fermentation of influent complex carbon sources could also be a feasible strategy (Guerrero et al., 2015; Yuan et al., 2009).

Table 6
N mass balance during periods I and II. All N items are represented as a percentage of the influent N.

Period	N_{EFF}[a] (%)	N_{PUR}[b] (%)	N_{EFFB}[c] (%)	N_{PURB}[d] (%)	N_{OUT}[e] (%)	NH_4^+-N_{EFF}[f] (%)
Ia	76 ± 7	3 ± 2	8 ± 4	15 ± 12	101 ± 8	71 ± 3
Ib	67 ± 12	5 ± 1	8 ± 7	36 ± 5	115 ± 4	66 ± 11
IIa	62 ± 2	4 ± 0	7 ± 1	42 ± 1	115 ± 2	62 ± 2
IIb	66 ± 3	4 ± 1	7 ± 3	26 ± 5	102 ± 9	66 ± 2

[a] Total N in the effluent after filtration.
[b] Total N in the purge stream after filtration.
[c] N contained in the biomass of the effluent.
[d] N contained in the biomass of the purge stream.
[e] Total N output obtained as the sum of the previous four items: e = a+b + c + d.
[f] Ammonium nitrogen in the effluent after filtration.

Table 6 shows the results of the N mass balance. Assuming no denitrification, the fate of the inlet N that is not in the effluent can be either biomass assimilation or nitrification (i.e. nitrite/nitrate). As can be observed, the total outlet N (N_{OUT}) covered all the input N in period I and II when the system was under stable operation, which means no significant nitrification occurred, and it was in accordance with the result of the system performance. All the N_{OUT} values higher than 100% are probably due to the hydrolysis of some organic N in the feed that was not considered in the influent. The percentage of N_{PURB} was 15 ± 12% under the DO of 1 mg/L in period Ia, and it increased to 36 ± 5% under the DO of 0.5 mg/L.

N mass balances reveal that an average value of 66% of the influent nitrogen was present as ammonium in the effluent, showing a relatively high fraction of ammonium left for the following B-stage. The rest of N (34%) was mostly contained in the biomass due to growth and the rest was soluble ammonium in the purge stream. The 34% was higher than the 7–21% range obtained in the A-stage SBR reported by Rey-Martínez et al. (2021a), probably due to the higher SRT in the A-stage-EBPR that could lead to higher biomass growth instead of other adsorption processes that can occur at lower SRT.

3.3. System performance with glutamate as carbon source

The anaerobic retention time is key when operating an A-stage-EBPR system. This value should be as low as possible to operate the system under low SRT conditions but high enough to maintain EBPR activity. Since PAO mainly use short-chain fatty acids, different processes coexist under anaerobic conditions: hydrolysis and fermentation of complex organic substrates to simple organic compounds and the posterior anaerobic uptake of these simple compounds by PAO. The rate of the limiting step will determine the minimum anaerobic residence time needed and, therefore, the nature of the carbon source (i.e. its biodegradability) is very important. Period III was operated with glutamate to better understand the link between the fractionation of the influent organic matter and the operation of A-stage-EBPR systems.

On day 57, the carbon source was switched from propionic acid to sodium glutamate under a DO of 1 mg/L and anaerobic purge (period IIIa). Glutamate contains nitrogen that is released as ammonium when hydrolysed, thus it acted both as carbon and nitrogen source. Unexpectedly, the use of glutamate led to nitrification and hence the appearance of nitrate and nitrite in the aerobic reactor (Fig. 1d). However, both PRE and CRE were very high, around 98% (Table 3). VSS was around 2.34 ± 0.27 g/L and, despite SVI was around 232 ± 62 mL/g, the effluent VSS concentration was low. The SRT was 6.7 ± 1.8 days and the A-stage-EBPR system showed successful P and COD removal performance in spite of the effluent nitrate (5.0 mg/L) and nitrite (1.1 mg/L). In fact, this period showed higher P-release and uptake rates than that with propionic acid as carbon source (Table 7). Glutamate as the sole carbon source seemed to be responsible for the overgrowth of filamentous bacteria. Similar problems of settleability were observed in a previous work, indicating that filamentous bacteria could be clearly favoured with a high content of glutamate in the feed (Rey-Martínez et al., 2019). This period showed successful removal of P and COD

Table 7
PAO activity and relative stoichiometric ratio in the anaerobic/aerobic batch tests carried out with the sludge from the aerobic reactor in two different periods.

Period	Carbon source	PO_4^{3-}-P_{max} (mgP/L)	PO_4^{3-}-P_{min} (mgP/L)	P release rate (mgP/gVSS min)	P uptake rate (mgP/gVSS min)	P/C (mol P/mol C)
IIb	Propionic acid	20	0.8	0.11	0.12	0.119
IIIa	Glutamate	24	0.4	0.19	0.16	0.121

despite detecting nitrate and nitrite, indicating that PAO, filamentous bacteria and nitrifiers could coexist for more than 30 days in this A-stage-EBPR system.

Severe bulking issues happened in period IIIb, leading to low SRT (2 d) and a high SVI 518 ± 115 mL/g. The purge was reduced from 7 to 3 L/d and bleach was stepwise dosed as recommended (Jenkins et al., 2003) to decrease sludge bulking, but this problem persisted. In any case, PRE and CRE could be maintained about 98% and 94% even under the bad settleability condition. From that moment onwards, the bulking problem increased (with SVI = 1139 mL/g) and caused a high concentration of biomass in the effluent (0.71 g VSS/L) and a big loss of biomass in the reactor (decreasing down to 0.79 g VSS/L) in period IIIc. The ratio of VSS/TSS increased up to 1 and the SRT decreased to 0.5 d which led to the system failure.

As a summary of period III, the use of glutamate as the only carbon source allowed to maintain successful EBPR activity and COD removal for 2 months (periods IIIa and IIIb), but with a progressive loss of biomass settleability, causing a decrease in biomass concentration in the reactor. EBPR was lost when the VSS concentration decreased below 1 g/L. In addition, the undesired occurrence of some nitrifying activity could not be avoided. Undesirable SVI increase due to poor settleability has already been reported in previous EBPR works at low SRT (Valverde-Pérez et al., 2016; Zhang et al., 2021). The change of the PAO microbial community due to the change of carbon source to glutamate, linked to the operation at low SRT may be the reason for this poor sedimentation. Therefore, the next step in the research would be achieving a more stable EBPR performance with glutamate as additional carbon source, and exploring the way to alleviate the bulking problem. Nevertheless, the use of glutamate should only be problematic if it is the only carbon source, or if a significant glutamate concentration is maintained for long periods. In any case, a pilot-scale study using the real influent would be desirable before the implementation of this type of system in a full-scale plant.

Two batch tests were performed to study the PAO activity with different carbon sources at the end of period IIb on day 52 (a) and period IIIa on day 88 (b) with the sludge from the aerobic reactor (Table 7 and Fig. 3a under propionic acid and 3b under glutamate as carbon source). P concentration in these tests reached 20 and 24 mg/L at the end of the anaerobic phase and less than 1 mg/L at the end of the aerobic phase, which indicates robust P removal activity. P-release and uptake rate showed higher values under glutamate as carbon source than propionic acid, with P-release and uptake rate 0.19 vs 0.11 and 0.16 vs 0.12 mgP/gVSS·min, respectively. The ratio of P/C (mol P/mol C, where C means the COD provided by propionate dosage expressed as mol of C) didn't exhibit major differences, with a value around 0.12. This value is much lower than those reported by Shen and Zhou (2016) (0.23–0.44) and than the theoretical value for propionic acid reported by Oehmen et al. (2005) of 0.42. The low values may indicate that our system had a fraction of GAO (which agrees with the results presented in the next section). There was some nitrite at the start of batch b, accompanied with denitrification during the anaerobic phase and some nitrification during the aerobic phase. However, the activity of PAO seemed to be unaffected, which is consistent with the high PRE and CRE values obtained in the plant with glutamate as carbon source and indicates the coexistence of PAO, nitrifiers and denitrifies in our system. The profiles of ammonium and COD showed similar trends.

3.4. Evolution of the microbial community

The variations and relative abundances of the functional bacteria were analysed by 16S rRNA gene sequencing at genus levels (Fig. 4). *Propionivibrio* exhibited the higher percentage in all the conditions, increasing from 8.8 to 9.5% with propionic acid to 14.5% when using glutamate. *Propionivibrio* has been commonly referred as a GAO (Albertsen et al., 2016; Roy et al., 2021). Thrash et al. (2010) indicated that *Propionivibrio* militaris has a strictly respiratory metabolism, and they can utilize various substrates that include acetate and propionate. In addition, *Propionivibrio* was also reported to have the fermentation ability (Albertsen et al., 2016). As a result, the high EBPR activity in period IIIa could be probably related to the ability of *Propionivibrio* to ferment glutamate to VFA. A high percentage of *Propionivibrio* in a glutamate-fed system was also observed in the work of Rey-Martínez et al. (2019) since they hold the most abundant species (11.6%) in an A/O SBR system with glutamate and aspartate as carbon source. However, the real metabolic activities are still unknown and need further exploration. Some investigations even tended to consider certain strains of *Propionivibrio* as putative PAO (Coats et al., 2017) and Li et al. (2019) proposed that *Propionivibrio* may harbour new strains belonging to PAOs due to the dominant position (48.9%) in a successful system for simultaneous N and P removal with propionate as carbon source.

Thiothrix has also been recognized as a putative PAO in some reports (Meng et al., 2020; Rey-Martínez et al., 2019; Rubio-Rincón et al., 2017) and it grows at low COD concentration as well as in a sulfur-reducing environment (Rubio-Rincón et al., 2017). It was observed in the A-stage-EBPR process in high proportions (about 4.6%, 3.1% and 0.37%). However Rey-Martínez et al. (2019) showed that *Thiothrix* ranked the most abundant position (37%) in a glutamate-fed anaerobic/anoxic/aerobic EBPR system, but in that case the system was operated at a higher SRT (10-15d), which could be the reason for the difference in abundance. *Lewinella* was also detected to have high proportions in all conditions, with 1.9%, 4.5% and 1.3%. *Lewinella* was shown to hold a 9.8% percentage in the glutamate-fed A2O system by Rey-Martínez et al. (2021b), though no investigation demonstrated *Lewinella* to possess PAO metabolism.

Rhodobacter (Hirais et al., 1991) has been reported as an important bacteria in conventional EBPR systems and percentages about 2.0%, 0.3% and 0.6% were observed. *Hydrogenophaga* is assumed to be a putative PAO by some investigations, and is presented in systems using

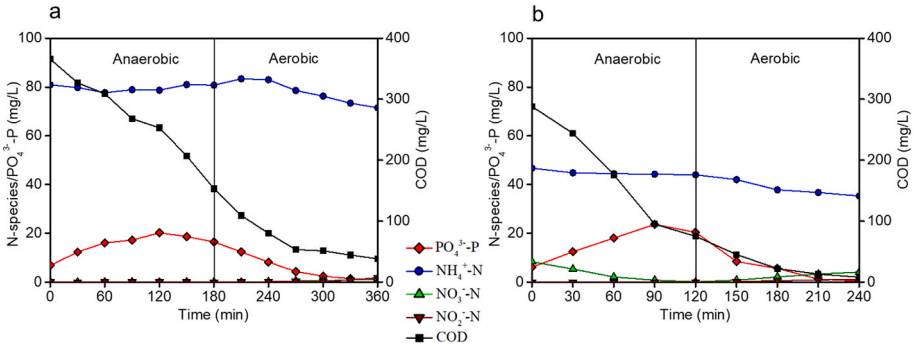

Fig. 3. Anaerobic/aerobic batch tests in terms of P, N, and COD with sludge withdrawn from the aerobic reactor on (a) day 52 (period IIb) and (b) day 88 (period IIIa).

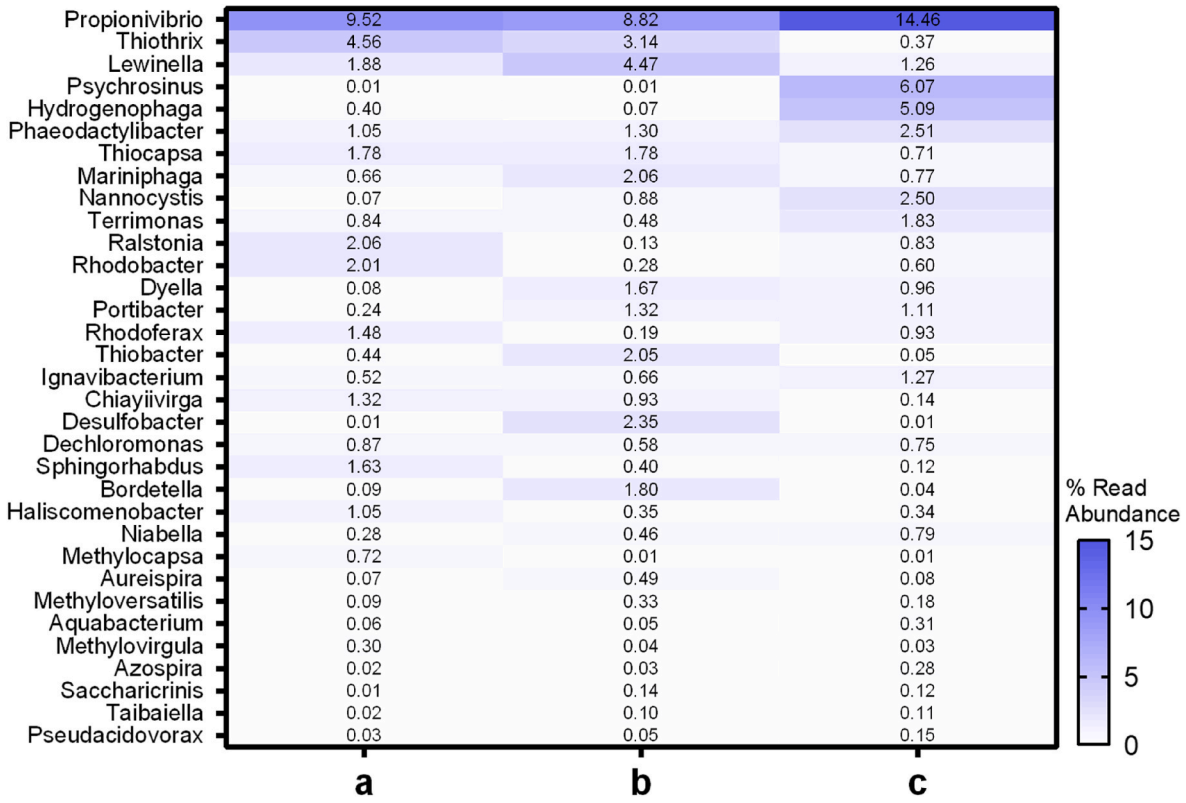

Fig. 4. Microbial communities in the level of genus in the A-stage-EBPR system during stable operation: with (a) propionic acid as carbon source under aerobic purge on day 38 (period IIa) and (b) anaerobic purge on day 56 (period IIb), and (c) with glutamate as carbon source under anaerobic purge on day 81 (period IIIa). The microorganisms are ranked according to the sum of their abundance during the three periods.

acetic, ethanol or real wastewater as carbon source (Ge et al., 2015; Iannacone et al., 2021, Iannacone et al., 2020). The highest abundance (5.1%) was observed in the glutamate system, being less than 0.5% with propionic acid. Ge et al. (2015) showed that *Hydrogenophaga* was promoted when treating a protein-rich wastewater system, and the work of Rey-Martínez et al. (2019) also detected its presence with glutamate and aspartate as carbon source. Thus, it is not surprising that *Hydrogenophaga* could be favoured in the glutamate-fed A-stage-EBPR system. *Dechloromonas*, which can use oxygen or NO_X as electron acceptors, has been reported as a functional PAO and appears extensively in full-scale WWTP (Petriglieri et al., 2021). The relative percentage of *Dechloromonas* in the propionic-fed periods was about 0.9% and 0.6% and about 0.8% for the glutamate-fed period. *Desulfobacter* is closely related to organisms implicated in the sulfur-EBPR studies (Zhang et al., 2017), and appeared at a high percentage of 2.4% in period IIb with anaerobic purge for treating the real wastewater. Finally, in contrast to other investigations, *Tetrasphaera*-related organisms were only detected in very low concentration (not shown) throughout the operation. All in all, it can be observed that the appearance of different putative PAO assured the efficient utilization of the carbon sources for a successful operation of the A-stage-EBPR system.

4. Conclusions

This work explored the performance of an A-stage-EBPR system under different operational conditions, showing situations where the system operates stable but also some cases where stability problems can appear. High P and COD removal was reached (94–98% and 95%) under DO setpoints of 0.5 and 1 mg/L when treating raw wastewater amended with propionic acid. However, decreasing the DO setpoint to 0.2 mg/L led to the deterioration of the system.

Changing the purge position from the aerobic to the anaerobic reactor maintained good COD and P removal performance and stable settleability without nitrification. About 22% of the influent P could be recovered using the anaerobic purge and about 43% of the influent COD could be captured and recovered (rather than mineralized) according to the mass balances. Nitrogen mass balance showed that 66% of the input N was in the effluent as ammonium for further treatment in the B-stage.

Employing glutamate as sole carbon and nitrogen source allowed simultaneous COD and P removal but with a slight nitrification build-up. After two months of operation with glutamate, biomass settleability was progressively lost and EBPR activity disappeared, indicating that it may be only a suitable carbon source for short periods. The microbial community analysis showed that *Propionivibrio, Thiothrix* and *Lewinella* exhibited the highest abundances. *Propionivibrio* percentage seemed to be correlated to high P-removal. The dominance of *Thiothrix, Hydrogenophaga, Dechloromonas* and *Desulfobacter* was observed in the different stages during the operation process.

Author statement

This work is part of the PhD Thesis of Congcong, who was in charge of the experimental work presented in the manuscript. She was in charge of data curation, investigation, methodology and writing – original draft. Juan A. Baeza and Albert Guisasola were the supervisors of this work. Albert was in charge of conceptualization, formal analysis, investigation, supervision, visualization and Writing – review & editing. Juan Antonio was in charge of conceptualization, funding acquisition, investigation, project administration, software, supervision, validation, visualization and writing – review & editing. The three authors participated in the discussion and writing of the manuscript.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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