



Benefits and drawbacks of integrating a side-stream sludge fermenter into an A₂O system under limited COD conditions

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ABSTRACT

The implementation of a side-stream sludge fermenter (SSSF) has been identified as a possible solution to improve the performance of an anaerobic/anoxic/aerobic (A₂O) configuration when treating low COD wastewater. This study systematically evaluated the effects of incorporating a SSSF into an A₂O configuration (side-stream enhanced biological phosphorus removal, S2EBPR) for P/N/COD removal under a limited influent COD (COD_{INF}) condition. The performance of the S2EBPR (with the SSSF receiving 6% of the recycled activated sludge and operating with a hydraulic retention time (HRT) of 2.4 d) and A₂O were compared under the same limited COD_{INF} (350 mg/L) condition. S2EBPR improved the amount of P removed (26.6%) under a low influent COD/P of only 26.3 compared with A₂O of 32.6, and enhanced denitrification (11%) without compromising full ammonium and COD removal. However, the P_{LOAD} to the plant increased due to the P-release in SSSF, resulting in higher effluent P concentration. The methane and energy recovery indexes were around 45% lower than those of A₂O. Sequencing analysis revealed a high abundance of PAO in accordance to its higher P removal. This study represents a comprehensive evaluation of the S2EBPR configuration and provides an assessment of its suitability.

1. Introduction

The increasingly serious eutrophication problem led by over-discharging of phosphorus (P) and nitrogen (N) drives the research for an efficient biological nutrient removal from wastewaters. Among the different wastewater treatment plants (WWTPs) configurations, the anaerobic/anoxic/aerobic (A₂O) configuration is the most common for simultaneous biological nutrient and organic matter removal [42]. P is removed by promoting the proliferation of polyphosphate-accumulating organisms (PAOs) with alternative anaerobic and aerobic/anoxic conditions in the so-called enhanced biological phosphorus removal (EBPR) process [11].

Low influent COD concentration and nitrate intrusion through the external recycle are common causes of A₂O failure [6,17,28]. Additional COD dosage can improve EBPR performance under these conditions at expense of higher cost and higher carbon footprint [39]. Besides that, the integration of a side-stream sludge fermenter (SSSF) into a conventional EBPR process (also known as S2EBPR) has been suggested as a potential strategy to overcome these issues since the volatile fatty acids (VFAs) production from sludge fermentation could cover these extra

COD requirements [10]. The influent of the SSSF can be a fraction of the anaerobic mixed liquor or the return activated sludge (RAS) (4%-30%) [7,24,31,49]. There are more than 80 full-scale applications of S2EBPR facilities worldwide [12,44,46,48], and most of them are implemented in Europe (60) and United States (12) [31]. Contrasting results exist in the literature regarding the effectiveness of S2EBPR vs conventional EBPR processes. Some studies have shown that the S2EBPR configuration has improved the P removal performance and stability compared to traditional EBPR configurations [22,31,49]. However, it has also been reported that S2EBPR showed high fluctuations of effluent P (0.6 ± 1.0 mgP/L) [46,49]. It has yet to be established how the S2EBPR configuration impacts WWTPs from a holistic perspective, especially when the influent COD is limited.

PAOs in S2EBPR systems can be promoted independently of the nature of the influent carbon source. For example, Vollertsen et al. [48] reported P concentrations about 40 mg/L and considerable COD in the SSSF of two S2EBPR-WWTPs, which made the plant less dependent on the input wastewater quality compared with a traditional EBPR-WWTP. The reasons are:

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- i) the biomass fermentation products are mostly VFA, a preferred electron donor for PAOs [24,31],
- ii) the extended anaerobic phase has been hypothesised to provide a competitive advantage for PAOs with respect to glycogen accumulating organisms (GAOs, i.e. PAOs competitors) or other heterotrophic organisms [7,6,49]. Wang et al. [49] showed a higher PAO activity in S2EBPR (higher PAOs and lower GAOs abundance). Nevertheless, there were no high differences between the abundance of putative PAOs, such as *Ca. Accumulibacter* and *Tetrasphaera*, and
- iii) besides VFAs, the SSSF can contain extra readily biodegradable COD (rbCOD), which could be further fermented to VFAs in the anaerobic reactor by fermenting-PAO such as *Tetrasphaera* [7,15,30].

Coats et al., [10] showed that the integration of an SSSF could promote GAO formation which could hinder EBPR activity. They studied this integration in an A/O EBPR sequencing batch reactor (i.e. without biological nitrogen removal). This contradiction on the results needs further investigation in plants aiming at simultaneous C/N/P removal.

S2EBPR systems seem a promising option in certain scenarios, particularly under limited COD conditions. However, the effectiveness of S2EBPR vs traditional EBPR has not before been systematically evaluated at limited influent COD/P ratios, nor how its operation impacts adjoining unit processes such as anaerobic digesters.

In this work, the EBPR performance when a SSSF reactor was integrated into an A₂O system under a low influent COD (COD_{INF}) scenario was comprehensively evaluated in a pilot-scale plant and on a long-term basis. A comprehensive understanding of the benefits and drawbacks of these systems is essential in view of identifying the scenarios where its industrial adoption would be positive.

Thus, the main objectives were: (1) to explore the COD_{INF} limits for the A₂O vs S2EBPR configurations, (2) to study the impact on the plant performance of integrating a SSSF reactor in different zones of an A₂O plant, (3) to assess how S2EBPR affects downstream biogas production, if employed instead of an A₂O process, (4) to investigate the difference of microbial communities in the A₂O and S2EBPR processes under different operational conditions and COD/P ratios.

2. Materials and methods

2.1. Equipment and operation parameters

The initial pilot-scale A₂O configuration consisted of three continuous stirred tank reactors for simultaneous C/N/P removal, with anaerobic reactor (R1, 28 L), anoxic reactor (R2, 28 L), aerobic reactor (R3, 90L) and settler (50 L). The feeding solution introduced to the anaerobic reactor consisted of tap water (144 L/d) and a concentrated solution (7 L/d). The internal recycle (IR) from R3 to R2 (450 L/d) was used to keep the anoxic condition in R2. Waste sludge was discharged from R3 automatically with a flowrate selected to maintain the desired sludge retention time (SRT). The settler produced the effluent stream and an enriched biomass stream (external recycle, ER) which was recycled to R1 in a flow of 140 L/d. The percentage of ER with respect to the influent was in the recommended range of 0.5–1 [42].

The three reactors were monitored on-line with DO (HACH CRI6050), pH (HACH CRI5335) and temperature probes (Axiomatic Pt1000) connected to multimeters (HACH CRI-MM44). On-line data was acquired with a data acquisition card (Advantech PCI-1711), which was connected to a PC with the AddControl software [5] developed in the research group for process monitoring and control. DO in R3 was controlled with a proportional-integral algorithm manipulating the aeration flow rate with a mass flow controller (MFC F-201CV, Bronkhorst) using a DO setpoint of 2 or 3 mg/L in different periods. pH in R3 was controlled with an on-off controller dosing a sodium carbonate solution to adjust the pH about 7.5. The system was operated at room

temperature (22 ± 2 °C).

The composition of the concentrated solution is shown in Table S1, which contained 1.4 g P-PO₄³⁻/d and 5.6 gN-NH₄⁺/d. The initial organic matter concentration was 585 mgCOD/L during the start-up period, then decreased to 500 mgCOD/L and progressively decreased in steps of 50 mgCOD/L for each operational period, in order to determine the COD-limited A₂O operation (Table 1).

The S2EBPR configuration was implemented by installing a SSSF (20 L) reactor treating part of the external recycle from the settler. The SSSF reactor was mixed with a magnetic stirrer at 200 rpm and was kept under anaerobic conditions to favour fermentation processes. The flowrate to the SSSF was set to 6% of the RAS flowrate (8.4 L/d), which led to an HRT of 2.4 d in the SSSF. The percentage was selected based on preliminary experiments and a literature review showing typical values of RAS to SSSF in the range 4%–30%. The effluent of the SSSF was fed to R1 most of the time, although it was connected to R2 or R3 for some shorter periods to investigate its effect. The relative configurations and diagrams are shown in Figure S1 and S2. HRT in the A₂O was about 23 h considering only the reactors and 31 h when considering the settler. In the case of S2EBPR, HRT was about 26 h considering only the reactors and 34 h with the settler.

SRT was calculated with equation (1) for the A₂O plant and equation (2) for the S2EBPR:

$$SRT = \frac{V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER}}{Q_{PUR} \cdot X_{AER} + Q_{EFF} \cdot X_{EFF}} \quad (1)$$

$$SRT = \frac{V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER}}{Q_{PUR} \cdot X_{AER} + Q_{EFF} \cdot X_{EFF} + Q_{SSSF} \cdot \Delta X_{SSSF}} \quad (2)$$

where V_{ANA} , V_{ANOX} and V_{AER} (L) are the volume of the anaerobic, anoxic and aerobic reactors, X_{ANA} , X_{ANOX} and X_{AER} (g/L) the biomass concentration in these reactors, Q_{PUR} , Q_{EFF} and Q_{SSSF} mean the flow rate (L/d) of waste activated sludge (WAS), effluent and SSSF. X_{EFF} is the biomass concentration in the effluent. ΔX_{SSSF} was the decay of biomass in the SSSF reactor, which was calculated as the input biomass concentration minus the biomass concentration of SSSF, where the input biomass concentration is the theoretical concentration from the external recycle ($X_{SETTLER}$) determined with equation (3):

Table 1

Operational parameters for each period: COD concentration in the influent, SSSF connection, waste activated sludge (WAS) flow, and DO setpoint.

Period	Day operation	COD _{INF} (mg/L)	SSSF connection	WAS flow (L/d)	DO (mg/L)
I a	1	—	—	0 to 10	2
I b	18	585	—	10	2
I c	32	500	—	10	2
I d	42	450	—	10	2
I e	50	400	—	10	2
I f	57	350	—	10	2
I g	70	300	—	10	2
I h	78	500	—	10	2
I i	84	400	—	10	2
I j	92	450	—	10	2
II a	107	450	anaerobic reactor	10	2
II b	119	350	anaerobic reactor	1	2
II c	133–175	350	anaerobic reactor	5	2
III a	206	350	anaerobic reactor	7	2
III b	217	350	disconnect SSSF	7	2
III c	232	350	anoxic reactor	7	2
III d	246	350	aerobic reactor	7	2
III e	255	350	aerobic reactor	7	3
III f	268–283	350	anaerobic reactor	7	3

$$X_{SETTLER} = \frac{(Q_{EFF} + Q_{ER} + Q_{SSSF}) \cdot X_{AER} - Q_{EFF} \cdot X_{EFF}}{Q_{SSSF} + Q_{ER}} \quad (3)$$

where Q_{ER} means the flow rate of external recycle.

SRT of the A₂O and S2EBPR was controlled around 13 ± 3 days by manipulating the WAS flowrate from the aerobic reactor. The actual SRT for each period is shown in Table S5.

Four different operational periods were run. Period I (days 1–106) aimed at assessing the lower limit of the COD_{INF} under an A₂O configuration. Period II (days 107–205) explored the S2EBPR performance under different operational conditions. Finally, period III (day 206–283) focused on studying the effect of connecting the SSSF effluent to different reactors of the S2EBPR. The micronutrients composition was adapted from Smolders et al. [40]. The biomass for inoculation was obtained from the municipal WWTP of Baix Llobregat (Barcelona, Spain).

2.2. Chemical and biochemical analyses

Samples for phosphate, COD, ammonium, nitrate and nitrite were withdrawn from R1, R2, R3 and SSSF almost daily and filtered with 0.22 µm filters (Millipore). The concentration of phosphate was analysed by a phosphate analyser (115 VAC PHOSPHAX sc, Hach-Lange) based on the Vanadomolybdate yellow method [23]. The concentration of ammonium was measured with an ammonium analyser (AMTAXsc, Hach Lange) based on a gas selective electrode potentiometric determination of ammonia [25]. Nitrite and nitrate were determined by Ion Chromatography (DIONEXICS-2000). COD was measured by kits (HACH LCK 314 and LCK 714) and a spectrophotometer.

Sludge samples were withdrawn from R1, R2, R3, SSSF and effluent, and evaluated by mixed liquor volatile suspended solids (VSS) and total suspended solids (TSS) according to Standard Methods [4]. The corresponding sludge volume index (SVI) was calculated as the observed volume (mL) of sludge from the aerobic reactor after settling for 30 min divided by the TSS (g/L) measured on the same day.

All the performance indicators are described in the Supplementary Information section SI-2.

2.3. Batch tests

2.3.1. PAOs batch activity tests

Four batch tests were conducted to estimate PAO activity. The biomass was obtained from the A₂O aerobic reactor when the system was at stable operation in period I c: (day 36), I e (day 57), I f (day 70), and I g (day 78). Batch tests were carried out in a system equipped with a magnetically stirred vessel of 2 L, DO probe (Cellox 325, WTW) and pH probe (Sentix 81, WTW). The anaerobic and anoxic conditions were maintained 2 h by nitrogen gas sparging, followed by 2 h of aerobic condition with a mass flowmeter (MFC F-201CV, Bronkhorst). The carbon source for the anaerobic phase was the same composition as the feed for the A₂O pilot plant to reach a concentration about 200 mg/L. Nitrate was dosed at the end of the anaerobic phase to reach 10 mg N/L. The temperature and pH were controlled about 25 °C and 7.5 ± 0.3 throughout the process. Samples for phosphate, nitrogen species and COD were taken every 30 min and filtered with 0.22 µm filters (Millipore).

2.3.2. Biochemical methane potential (BMP) batch tests in S2EBPR system

Three sets of BMP experiments as in [3] were conducted to investigate methane production from different sludge samples (anaerobic, aerobic and SSSF) under stable S2EBPR system performance. The inoculum sludge was from the anaerobic digester of an urban WWTP (Manresa, Barcelona) and degassed at 37 °C for at least 3 days before use. The anaerobic digestion tests were conducted in 160 mL serum bottles with 125 mL of effective volume and 35 mL headspace for biogas production. All the tests lasted for 42 days. Further details are provided

in Zhang et al. [51].

2.3.3. Microbiological analyses

Sludge samples were collected from the aerobic reactor on day 36 (Period I c), 56 (I e), 69 (I f), 77 (I g), 105 (I j), and biomass from the aerobic reactor and SSSF on day 175 (II c), 245 (period IIIc), 266 (period IIIe) and 283 (period IIIf) when the system reached stable operation. The bacterial population was identified by the Illumina amplicon sequencing of the 16S rRNA gene. The detailed process was as follows: the samples from the system were washed by PBS for three times and centrifugated for further DNA extractions. Soil DNA isolation plus kit (Norgen Biotek CORP, Ontario, Canada) were used for Genomic DNA extraction process. Further, the obtained extracted DNA was detected and quantified by a DNA NanoDrop 1000 Spectrophotometer (Waltham, MA, USA), and the purified DNA was analysed in an Illumina MiSeq platform service center in the Autonomous University of Barcelona (Barcelona, Spain).

Universal primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were applied to amplify the V3–V4 regions of the small subunit (SSU) rRNA prokaryote gene (16S) [43]. The database used for the classification of organisms was based on the Greengenes database. The sequence reads were processed through Usearch software. Operational taxonomic units (OTUs) were generated with the open reference methodology. Raw sequencing data of Illumina amplicon sequencing of the 16S rRNA gene of sludge samples can be found in NCBI Sequence Read Archive with accession numbers of SUB13291801.

3. Results

3.1. Exploring the effect of influent COD limitation on the A₂O performance

Figs. 1 and 2 exhibit the removal performance of the different configurations on a load basis. Figure S3–S4 show the P fate in a concentration basis. Table S2 to Table S5 show the performance of the system regarding the different species (P, N, COD and solids, respectively) during the operational process.

After the system start-up, it took 18 days to reach a pseudo steady state with full P, ammonium and COD removal under A₂O configuration (Period Ia). Period Ib (day 18–31) had the highest influent COD concentration (585 mg/L) and P_{ANA} and P_{AER} reached 28.7 and 0.6 mg/L, respectively, with a P_{REMOVAL_ABSOLUTE} about 1.33 g/d. In terms of N, there was neither ammonium, nor nitrite detected in the effluent. NO₃-N was about 7.0 mg/L, indicating that all entering ammonium was oxidized into nitrate. Total N_{REMOVAL_ABSOLUTE} was about 4.79 g/d (Fig. 1b). Regarding organic carbon, total COD_{REMOVAL_ABSOLUTE} (76.1 g/d COD) was reached (Fig. 1c). VSS concentrations in the reactor and in the effluent were around 1.40 and 0.034 g/L (Fig. 2), which indicated a period of stability in terms of biomass concentration. In short, the A₂O plant with an excess of COD_{INF} could operate successfully.

COD_{INF} was decreased down to 500 mg/L in Period Ic (day 32–41) for 10 days. All the performance indices were still positive and COD_{INF} was successively decreased to 350 mg/L from Period Id to If (day 42–69) and, in all the cases, the high removal percentages of P, N and COD were maintained. The VSS concentration in the system showed an expected decreasing trend with the decrease of COD_{INF} (Fig. 2) (except for the Ib to Ic when undesired tap water flowrate fluctuations occurred).

The COD_{INF} was reduced to 300 mg/L on day 70 (Period Ig) and P_{ANA} declined from 26.1 to 17.6 mg/L due to the COD limitations. An increased concentration of P_{AER} (from 0.6 to 2.2 mg/L) was observed. Thus, the limit of COD_{INF} needed to maintain successful EBPR performance in the A₂O system was 350 mg/L. When COD_{INF} was increased to 500 mg/L aiming at EBPR recovery (Period Ih), the system showed bulking issues. Dispersed growth of biomass was detected probably due to the sudden and large increase of COD_{INF}. Floc-forming species may grow in a non-settleable form when exposed to a sudden high organic

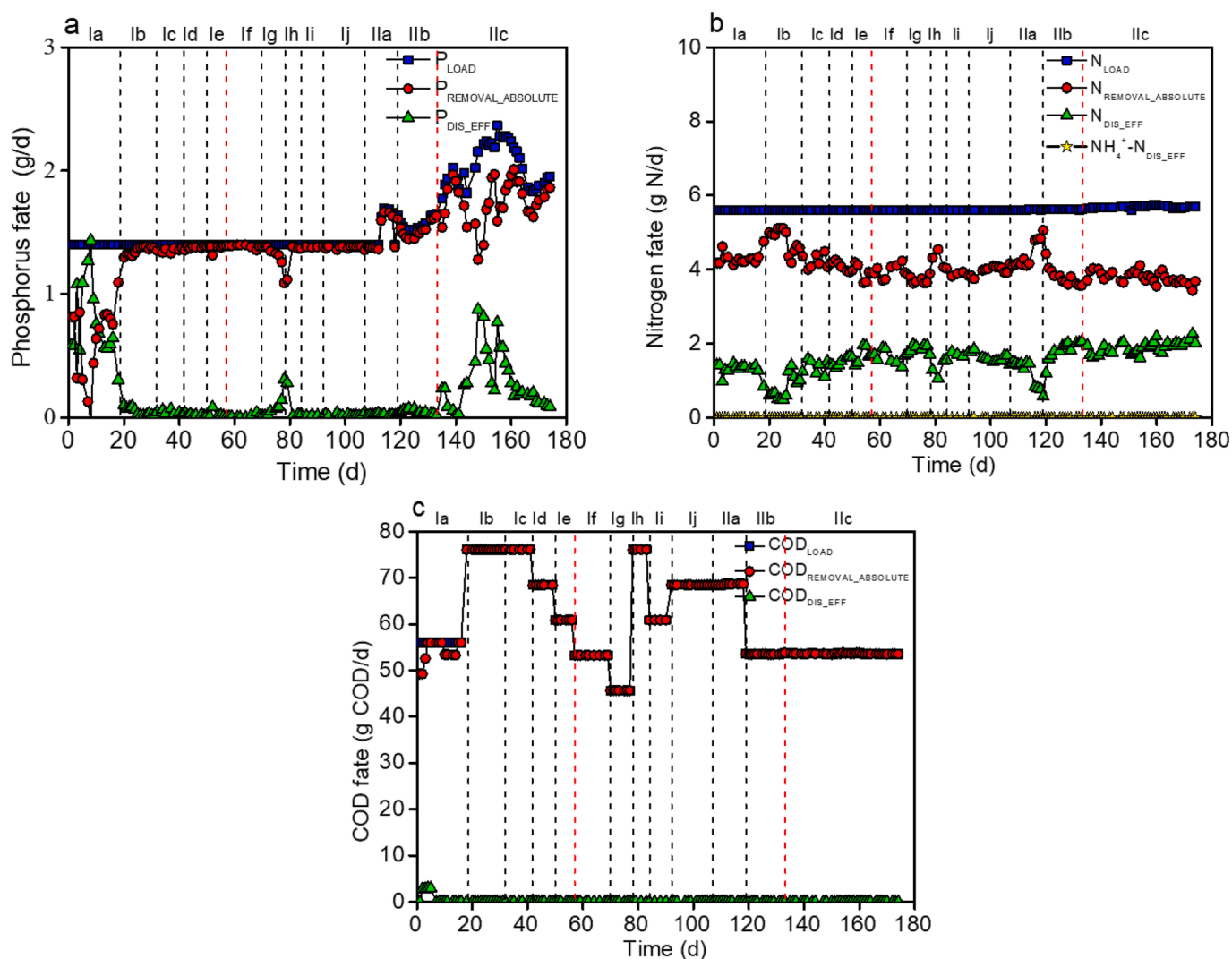


Fig. 1. The fate of P (a), N (b) and COD (c) and the removal performance of A₂O (period I) and S2EBPR (period II) configurations.

loading. The best response to this problem is a reduction ratio in the F/M of the system [14,34]. Thus, the COD_{INF} was decreased to 400 mg/L on day 84 and increased to 450 mg/L on day 92 (Periods II and Ij) to lessen the growth of dispersed biomass. P_{ANA} and P_{AER} recovered to the previous condition and the bulking problem improved. The amount of P_{REMOVAL_ABSOLUTE} and P in the effluent were similar to those of Period Id, at 1.38 g/d and 0.02 g/d, respectively.

Four batch tests were performed to assess PAO activity (Table S6). High anaerobic release and aerobic uptake rates of P were obtained with the system operation at 500 mg/L COD_{INF} (0.37 and 0.18 mgP/gVSS min respectively). The lowest aerobic and anoxic P uptake rates were observed for the biomass withdrawn under the lowest COD_{INF} condition (300 mg/L). Complete P removal was observed under A₂O configuration except for the period with limited COD_{INF} (300 mg/L).

3.2. Performance of the S2EBPR configuration

On day 107, the SSSF was installed and fed with a biomass-enriched stream from the settler (8.4 L/d, i.e. 6% of the RAS flowrate). The SSSF effluent was connected to the anaerobic reactor (Period IIa). The initial integration of the SSSF with a high daily WAS volume (10 L) and 450 mg/L of COD_{INF} exhibited bulking issues (VSS in the reactor decreasing from 0.98 to 0.75 g/L and in the effluent increasing from 0.023 to 0.095 g/L). Period IIb (day 119–132) was designed as a VSS upturn period with

lower COD_{INF} and lower WAS flow. The system recovered subsequently without affecting the P performance. Then, the WAS was increased again in day 133 (Period IIc) to reach the targeted SRT (11.7 ± 0.1 d) and the same COD_{INF} of 350 mg/L as Period If to allow a thorough comparison of S2EBPR vs A₂O (sections 3.3 and 3.4 and the Discussion section are based on Period If and Period IIc). The SSSF performance for the different periods is shown in Tables S2 to S5 (the concentrations of P, N, COD and solids in the SSSF were indicated as P_{SSSF}, NH₄⁺-N_{SSSF}, COD_{SSSF}, VSS_{SSSF} and TSS_{SSSF}) and Figure S3.

Significant P release was observed in the SSSF due to VFA production and its simultaneous *in-situ* consumption by PAO, as reported in previous works [31,46,48]. The SSSF showed an absolute P release of about 88.7 mg/L and a concentration of COD around 40 mg/L. Thus, as a result of SSSF integration, the system received higher P_{LOAD} compared to A₂O in period If (2.07 vs 1.40 g/d), and showed higher P_{ANA} (37.5 vs 26.1 mg/L), and P_{REMOVAL_ABSOLUTE} (1.76 vs 1.39 g/d). Therefore, considering the amount of P removed, EBPR performance with SSSF integration improved by around 27%. However, the high increase in P_{LOAD} led to an undesired and unstable effluent quality, with P_{AER} about 2.0 ± 1.6 mg/L.

In terms of N, the SSSF effluent provided around 11.0 mg NH₄⁺-N/L (without nitrate and nitrite) due to the fermentation/hydrolysis of the biomass and cell lysis or decay in SSSF. Thus, the S2EBPR showed almost no change in the N_{LOAD} (5.69 vs 5.60 g/d, i.e. + 1.6%) and a slightly

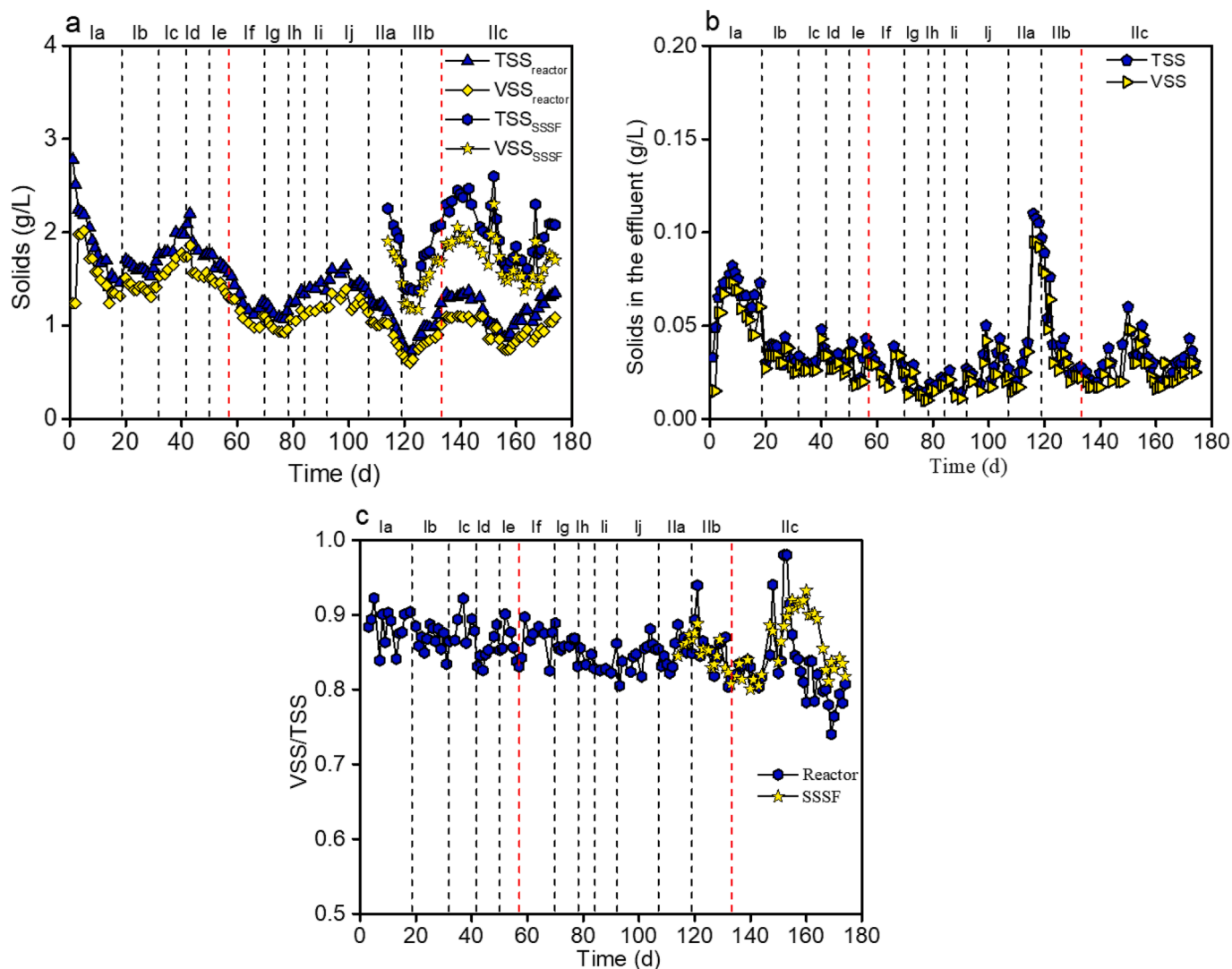


Fig. 2. Evolution of solids during the A₂O (Period I) and S2EBPR (Period II) operation. (a) Solids concentration in the reactor and the SSSF, (b) Solids concentration in the effluent, and (c) Ratio of volatile suspended solids (VSS) to total suspended solids (TSS).

higher value of N_{EFF} (1.91 vs 1.64 g/d) without any observed influence on nitrification. Mass balances showed that the percentage of influent N incorporated into the biomass ($N_{BIOMASS}$) decreased from 33% in A₂O to 19% in S2EBPR, and it was reasonable due to the lower WAS discharged. Accordingly, the percentage of $N_{DENITRIFIED}$ improved from 35% (A₂O) to 46% (S2EBPR), which showed that the integration of SSSF improved the denitrification extent.

Regarding the COD, the SSSF effluent contained around 40 mg/L COD, which only represented a 0.5% increase of the COD_{LOAD} in S2EBPR (from 53.3 to 53.6 g/d). Full COD removal efficiency indicated that the SSSF integration did not affect the COD removal since most of the COD produced was used *in-situ* in the SSSF. The COD mass balance showed a higher percentage of $COD_{MINERALIZED}$ in S2EBPR (77%) vs A₂O (60%), which agreed with part of the theoretical WAS being degraded in the SSSF. The solids in the reactors in both scenarios (S2EBPR and A₂O) were around 1 g/L. However, the solids in the SSSF were always higher (around 2 g/L) due to the use of the concentrated biomass stream from the settler. The ratio of VSS/TSS in the SSSF tended to be higher than that of A₂O (Fig. 2c), which indicated that the biomass in SSSF was releasing P and that the polyphosphate (PolyP) levels were much lower. In addition, the sludge production decreased from 15.1 to 8.5 g/d because the WAS in S2EBPR was half of that of A₂O to maintain a similar SRT (see below in the discussion section).

Period III (day 206–283) aimed at comparing the different combination of locations for the effluent of the SSSF (Figures S5 and S6): anaerobic, anoxic and aerobic reactors. More detailed information can be found in the [Supplementary Information](#). According to the results obtained, the optimum integration position of SSSF to A₂O for EBPR performance under low COD_{INF} conditions is the anaerobic reactor. The worst EBPR performance was obtained with the connection of the SSSF to the anoxic reactor, but the reason for this needs further study. Connection to the aerobic reactor could enhance P-removal activity, but at the expense of a detrimental effect on nitrification.

3.3. Energy recovery based on BMP in S2EBPR

Recovering part of the chemical energy contained in the wastewater is a hot trend in current water resource recovery facilities. The integration of the SSSF into the A₂O process decreased the amount of WAS (due to the hydrolysis and fermentation of solids in the SSSF) and, thus, biogas production would be minimized. The sludge coming from an EBPR system has different BMP depending on the WAS location because of the different polyhydroxyalkanoate (PHA) content [8,20]. Fig. 3 and Table 2 compare the BMP of different sludge samples from the S2EBPR system: sludge from anaerobic, aerobic reactors and SSSF in Period II c. The highest BMP was obtained from the anaerobic sludge with about

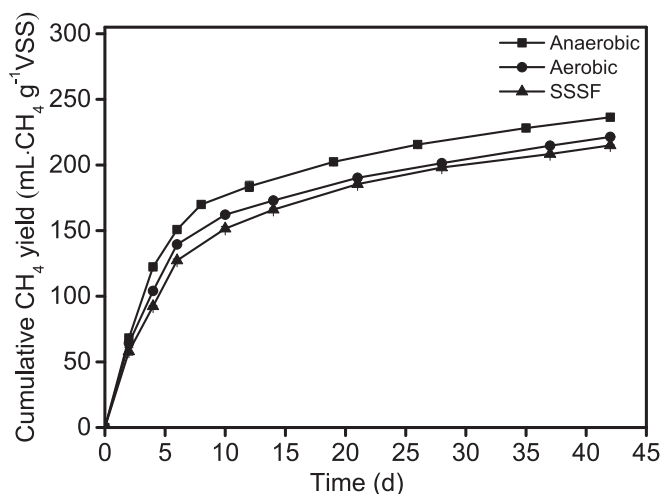


Fig. 3. Anaerobic biochemical methane potential tests for biomass samples obtained from anaerobic, aerobic and SSSF reactors under S2EBPR operation in period IIc.

Table 2

Biochemical methane potential (BMP) tests with biomass from anaerobic, aerobic reactor and SSSF.

Sludge sample ^a	BMP ^b (mL CH ₄ /gVSS)	Initial methane production rate ^c (mL CH ₄ /(gVSS·d))
Anaerobic reactor	250 ± 10	27.3 ± 0.8
Aerobic reactor	221 ± 3	24.7 ± 0.6
SSSF	215 ± 11	22.3 ± 1.7

^a The sludge samples were taken from anaerobic, aerobic and SSSF reactors in Period IIc during stable state.

^b The final cumulative CH₄ yield obtained from different sludge samples in 42 days.

^c The rates for the first 10 days of the BMP test.

250 mL CH₄/gVSS, followed by aerobic and SSSF, with 221 and 215 mL CH₄/gVSS, respectively. Anaerobic biomass showed a 16% higher BMP than aerobic sludge. Unexpectedly, the BMP of SSSF was closer to that in the aerobic reactor rather than being similar to the anaerobic BMP. It could be speculated that the internal levels of PHA in the biomass from the SSSF were lower to those from the anaerobic reactor, however PHA was not measured. The initial methane production rates in the anaerobic sludge were the highest: 27.3 mL CH₄/(gVSS·d), whereas 24.7 and 22.3 mL CH₄/(gVSS·d) were observed for aerobic and SSSF sludge, respectively.

3.4. The microbiological community in A₂O and S2EBPR

The variations and relative abundances of the bacteria selected in A₂O (period I) and S2EBPR (period II and III) were analyzed by 16S rRNA gene sequencing. Fig. 4 compares the microbiological community observed at the genus level in the operation of periods I and II (identity derived at an OTU threshold of 96.5% similarity). The number of OTUs was similar in each operational period: about 1414 in the A₂O system in period I (Table S7) and in periods II and III from S2EBPR, the OTUs were 1430 in the reactor and 1480 in the SSSF.

Among the observed OTUs, the clusters *Desulfovibrio*, *Anaerostipes*, *Insolitospirillum* and *Dechloromonas* were found to be dominant along the whole operational process. Thereinto, *Desulfovibrio* was considered as heterotrophic denitrifying bacteria [9], and it has been observed in a system with the simultaneous removal of N and P [41]. *Desulfovibrio* was generally the most abundant organism during the whole study, no matter if the system was A₂O (4%-10% when the COD_{INF} was 500, 400, 350, 300 and 450 mg/L respectively), or S2EBPR (about 6.3% and 4.2%

when COD_{INF} was 350 mg/L in the aerobic reactor and SSSF respectively). *Dechloromonas*, which can use oxygen or NO_x-N as electron acceptors, have been reported to exist frequently in full-scale WWTPs and have been considered as functional PAOs [32]. The relative abundance of *Dechloromonas* in the A₂O system was in the range of 1.22–5.15%, and in the S2EBPR system was found to be 5.38% in the aerobic reactor and 5.78% in the SSSF.

Rhodobacter [19] and *Thauera* [52] have been implicated as potential PAOs in EBPR systems. Interestingly, these organisms were found to be of higher abundance during the A₂O operation rather than S2EBPR. The most common PAO, *Ca. Accumulibacter*, was not detected in the system. However, *Thiothrix* was much higher in abundance in the S2EBPR process (more than 6.5%) as compared to A₂O (from 0.01% to 2.7%). *Thiothrix* has been recognized as candidate PAO in a broad range of reports [27,33,37] and grew in a low COD condition [37]. This correlated well with the results of this study, since the ratio of C/P reached the lowest level under the operation of the S2EBPR system. *Thiothrix* was also considered as a typical filamentous bacteria which could lead to settling problems of activated sludge [45]. Notably, the percentage of *Thiothrix* began to increase in the A₂O process when the COD_{INF} was 450 mg/L and sludge bulking was observed at that time (SVI around 571 mL/g). However, in S2EBPR there was no sludge bulking, with the SVI about 200 mL/g, which indicated that the presence of *Thiothrix* in S2EBPR system doesn't seem to affect the stability of the sludge despite of their filamentous cell morphology.

In addition, fermenters *Dysgonomonas* and *Propionispora* were reported to ferment organics to VFA [53], which accounted for significant abundances with 1.5%-3.4% and 0.8%-2.2%, respectively, during the whole operation. In terms of *Propionivibrio* and *Deftuviococcus*, which are groups of known GAO [1,35], they were less abundant in the A₂O system with less than 0.3% and 0.8% observed during period I, respectively, which corresponded with successful EBPR performance. However, the presence of GAO is not a necessary indicator of the EBPR deterioration if PAOs are favoured kinetically [24,30]. It is interesting that in the S2EBPR system, both organisms showed higher proportions. Especially, *Propionivibrio* accounted for 1.5% in the aerobic reactor and 5.5% in the SSSF. These results were contrary to that of Wang et al., [49], since they found *Propionivibrio* showed an inferior population in conventional EBPR than S2EBPR. Nevertheless, in our S2EBPR system, PAOs still held a competitive advantage over GAOs, both in terms of total population abundance (Table 4) Table S8 and in terms of P removal performance.

4. Discussion

4.1. Integrating SSSF in an A₂O process

The main objective of integrating a SSSF into an A₂O system is to increase the stability of biological P removal under potentially detrimental situations (i.e. low influent rbCOD or excessive nitrate entering the anaerobic reactor). The SSSF should provide an extra source of VFAs that would come from the degradation of part of the WAS. When a SSSF is integrated into a non-EBPR system, the SSSF effluent contains a high amount of VFA that could be used, for example, to enhance denitrification. In this work, the effluent of the SSSF was not VFA-rich but it contained a significant amount of P [21,48,49]. PAO had consumed the VFA produced *in-situ* in the SSSF linked to P release, with an average of P concentration about 88.7 mg/L in SSSF. Thus, the SSSF integration increased the average P_{LOAD} (47.9%) and concentration of P_{ANA} (43.7%) in the S2EBPR system (2.07 g/d and 37.5 mgP/L) when compared to A₂O (1.40 g/d and 26.1 mgP/L) under the same COD_{INF} conditions. The increase of P_{LOAD} was partially mitigated by the better P performance of the S2EBPR configuration: the P removal capacity in S2EBPR increased by 26.6 % (1.76 vs 1.39 g P/d). Wang et al. [49] reported a 24.5 % higher P release (132 kg P/d vs 106 kg P/d) and a 80% higher P removal efficiency when comparing S2EBPR with A₂O. Onnis-Hayden et al. [31] also reported higher P removal performance with S2EBPR vs A₂O (90%

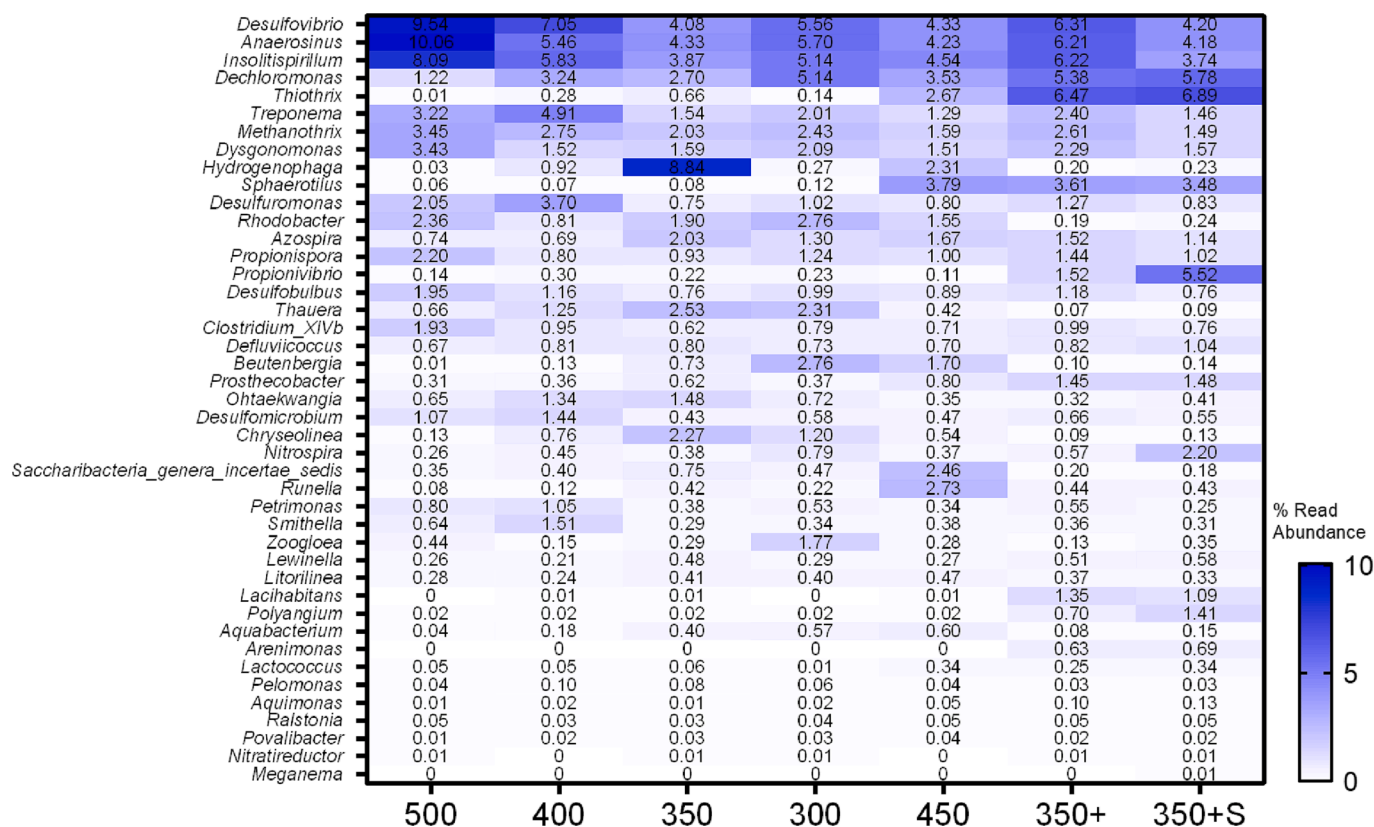


Fig. 4. Microbial communities at the genus level observed during the different operational periods: 500, 400, 350, 300, 450 mg/L of COD_{inf} in the A_2O system (period I); 350 + and 350 + S are samples from the aerobic reactor and SSSF of the S2EBPR system (period IIc) obtained operating with COD_{inf} of 350 mg/L. The order shown is based on the total abundance of that genus calculated by adding up its abundance across all analyzed samples.

vs 82%).

This extra P_{LOAD} can challenge the plant performance if the ratio of RAS diverted to the SSSF is too high. In our case, P_{AER} was about 2.0 ± 1.6 mgP/L since not all the entering P could be removed despite the observed increase in P removal performance. Vale et al. [46] showed 2.8 mg/L of P in the effluent in a full-scale S2EBPR with 6% of RAS to SSSF (without reporting the specific extra P_{LOAD}). A potential solution would be to integrate P removal/recovery strategies at this point to avoid a drastic increase of the P entering the plant.

The produced VFA in the SSSF was up-taken *in-situ* by PAO and others leading to a low COD SSSF effluent (around 40 mg/L). Vollertsen et al. [48] estimated that about half of the rbCOD was stored by PAO in the SSSF with HRT about 30 to 35 h, which left half of the COD in the SSSF effluent (50–90 mg rbCOD/L). Andreasen et al. [2] and Wang et al. [49] reported that an increase of 14% and 16% of influent COD could be due to the implementation of a SSSF in full-scale S2EBPR. The extent of the COD_{LOAD} increase depends on both the HRT of SSSF and the proportion of PAO in the sludge. Too low HRTs (less than 1 day) would lead to low VFA production and combined with a high amount of PAO would lead to a low COD increase since all the potential COD released would be directly used in the SSSF.

In addition, the biomass fermentation also led to ammonium release and about 11 mgN/L was detected in the SSSF effluent, which resulted in a slight increase of the total N_{LOAD} to the system of 1.6%. The VFA released by biomass fermentation was not only used by PAO, but also acted as an electron donor for denitrification, since the nitrate in the ER recycled to the SSSF was fully denitrified. The concept is similar to that of the so-called Johannesburg WWTP configuration that aims at avoiding nitrate entering to the anaerobic reactor by providing an external carbon source as electron donor. Thus, the percentage of $N_{DENITRIFIED}$ increased from 35% to 46%. However, the $N_{REMOVAL_ABSOLUTE}$ decreased

from 3.96 to 3.79 g/d with the N removal efficiency decreasing from 69% to 63% due to the lower amount of WAS from the S2EBPR (5L/d) compared with A_2O (10L/d) to keep the same SRT (see equations (1) and (2)).

Equation (1) shows the typical SRT calculation, while equation (2) accounts for the amount of biomass degraded in the SSSF. It is important to include this biomass degraded in the SSSF, as it has a comparable impact to an additional purge. This means that less WAS is required in S2EBPR compared to A_2O to maintain the SRT. The SRT concept is only related to the fate of the solids: part of the COD as biomass was degraded in the SSSF, however, P and N were not degraded but released to the medium because of the biomass fermentation. As mentioned above, the SSSF integration decreased the amount of WAS and increased the nutrient load to the plant, and these factors pose complex interactions to the system and may compromise the effluent quality.

4.2. Influent C/P ratio and the COD requirement

In our case, different influent COD/P ratios were obtained by the integration of SSSF despite the plant influent was not changed. Considering the increase of P_{LOAD} from the SSSF and the low increase of COD, the COD/P (g/g) was only 26.3 in the S2EBPR, being 31% lower than that of the A_2O system under the same COD_{INF} (COD/P ratio about 38). This COD/P ratio was even lower than that of the A_2O system under the $COD_{INF} = 300$ mg/L condition (period I; COD/P ratio about 32.6), which resulted in EBPR deterioration. Thus, the S2EBPR system showed a higher $P_{REMOVAL_ABSOLUTE}$ at a much lower COD/P ratio (26.3), which indicated the promising advantage on P removal under a low COD/P ratio scenario of S2EBPR vs A_2O .

The influent COD/P ratio plays an important role in EBPR performance [17,38]. However, the carbon biodegradability/fractionation is

as important as the amount. PAO need VFA-like organic matter for their anaerobic metabolism. Calculating the minimum COD required for a successful N and P removal is not a straightforward issue and depends not only on the N and P loads but also on the COD biodegradability. Metcalf and Eddy [42] suggest for simultaneous C, P and N removal in an A₂O configuration, the following values of readily biodegradable COD (rbCOD): 10 g rbCOD/gP and 6.6 g rbCOD/gNO₃-N. Then, 334 mg/L rbCOD would be required to remove the influent concentrations of 9 mgP/L and 37 mgN/L. This theoretical value agrees with the required COD_{INF} experimentally observed during the A₂O evaluation (350 mg/L). In the case of the S2EBPR, P_{LOAD} and N_{LOAD} increased to 2.07 gP/d and 5.69 gN/d with the input from the SSSF, which would correspond to an influent concentration of 13.3 mgP/L and 37.6 mgN/L. Considering the effluent P concentration during period IIc (2.0 mgP/L), 11.3 mgP/L were removed in the S2EBPR system with the same COD_{INF} = 350 mg/L instead of only 9 mgP/L of A₂O. This would imply a decrease in the COD requirements down to 9 g rbCOD/g P, i.e. 10% lower than for the A₂O configuration. Since the generated organic matter is mostly used by PAO in the anaerobic reactor and not for denitrification, it is reasonable to assume organic matter requirements of 9 g rbCOD/g P and 6.6 g rbCOD/g NO₃-N for simultaneous C, P and N removal in a S2EBPR system.

4.3. Energy recovery indices

Table 3 shows the energy recovery indices of A₂O and S2EBPR at the same COD_{INF} conditions. One should previously think whether low sludge production is beneficial or detrimental for the plant performance. When considering energy recovery, the integration of the SSSF should decrease the potential energy recovery of the plant since not all the WAS is derived to biogas production but part of it is degraded in the SSSF. However, lower sludge production would as well lead to lower sludge handling costs. Operational costs of a WWTP are typically about 40–50% due to sludge production, with only 10% related to energy [16,50], so less sludge produced with less energy produced could still be an economic win from an operational costs standpoint. Besides that, less WAS would also mean a smaller digester too (and, thus, lower capital costs and operation costs).

The WAS in the S2EBPR was lower than in the A₂O for the same targeted SRT. We can assume similar BMP values from these two systems since the BMP value is mostly dependent on the internal PHA content and, thus, on the SRT [8,51]. We adopted 221 mL CH₄/gVSS from aerobic sludge under the condition of S2EBPR for the calculation process (see section 3.3). Thus, both energy recovery indices for the S2EBPR system were around 45 % lower than those from the A₂O for the period with the same SRT, since half volume of wasted sludge was discharged in S2EBPR with similar amount of COD_{LOAD}. MRI and ERI are about 0.253 gCOD_{CH4}/gCOD_{REM} and 0.166 kJ_{CH4}/kJ_{INF} for the A₂O case and 0.139 gCOD_{CH4}/gCOD_{REM} and 0.093 kJ_{CH4}/kJ_{INF} for the S2EBPR. MRI is also an indicator for mineralization degree, which was in accordance with the COD mineralization degree in these two systems (60% in A₂O and 77% in S2EBPR), and higher mineralization results in higher cost in S2EBPR. Literature reports show that the conversion efficiency from influent COD to methane is in the range of 15–35% and this energy could be recovered as a form of electricity by combined heat and power technologies. Assuming 35% of the transformation efficiency from methane to electricity, only 5% (S2EBPR) or 12% (A₂O) of the COD_{INF} could be recovered in the form of electricity [26].

As a consequence, the S2EBPR would still be an economic win with half sludge produced though both energy recovery indices for the

S2EBPR system were around 45 % lower than those from the A₂O.

4.4. Key functionally populations- relevant PAO and GAO

Reactor operation with a S2EBPR process showed a higher percentage of known candidate PAOs than the A₂O system (Table 4), which was in accordance with the higher P removal capability of the S2EBPR. Specifically, under the same COD_{INF} condition of 350 mg/L, more PAO clades (e.g. *Thiothrix* and *Dechloromonas*) could be enriched in S2EBPR. GAO seemed to hold higher percentages in S2EBPR (2.34% in the aerobic reactor and 6.55% in the SSSF) than in A₂O (1.02%), in contrast to previous findings that S2EBPR showed lower GAO abundance than conventional EBPR [31,49]. Different from other investigations [29,31,49], *Tetrasphaera* was not observed in this study, despite of its reported low decay rate and fermenting ability. Dold and Conidi [13] also pointed out some potential conflicts for *Tetrasphaera*, such as the conflict in its abundance due to different quantification procedures, the implied importance in RAS fermentation processes and its importance in its ability to ferment in EBPR systems. Apart from that, it should be noted that in S2EBPR the microbial communities showed a relatively lower percentage of PAO and higher percentage of GAO in the SSSF compared with the aerobic reactor. SSSF was speculated to induce more decay of GAO and other ordinary heterotrophic organisms due to the extended anaerobic condition, thus giving PAO a competitive advantage [13,18,49], which was not consistent with the observed community dynamics of this study. It suggests the existence of some unknown PAO in S2EBPR. For example, Ca. *Accumulibacter* PAO were not detected in this study, but it should be considered that its quantification by 16S rRNA techniques has usually led to lower values than other techniques as fluorescent in-situ hybridization [36,47]. As a result, future work should also focus on identifying other potential groups of PAOs that may exist in S2EBPR processes. In any case, the S2EBPR sustained the coexistence of an abundant PAO community that was appropriate for EBPR, despite the higher P effluent observed.

4.5. Practical implications

This comprehensive work systematically evaluated the effect of introducing a SSSF in an A₂O plant (S2EBPR configuration) under a wide range of influent COD scenarios. The S2EBPR system showed improvement of EBPR and denitrification even at lower COD/P ratios than that being minimum threshold in an A₂O configuration. Besides the conventional performance, we assessed the possibility of improving energy recovery and the connection of a SSSF to different locations of the A₂O. Implementing a SSSF into a conventional A₂O system can have the following implications:

- 1) The connection of a SSSF to the anaerobic reactor can allow higher SRT (i.e. lower WAS) for fermentation processes and thus lead to lower COD/P requirements for EBPR. The connection of a SSSF to the anaerobic reactor of A₂O showed the optimum EBPR performance compared with other locations (i.e. to the anoxic or aerobic reactor). If an energy recovery process is implemented (i.e. anaerobic digestion), the energy recovery indices would decrease due to the lower volume of WAS. Despite the energy recovery decline, it would still be advantageous from the economic point of view due to the savings in terms of the sludge disposal and the need of a smaller digester.
- 2) Combining the S2EBPR with P recovery provides a novel opportunity for P recovery by chemical precipitation due to the higher P

Table 3

Comparison of energy recovery indices of A₂O and S2EBPR under the same COD_{INF} conditions.

Systems	Period	VSS (g/L)	Y _{OBS} (gCODx/gCODs)	Influent load (gCOD/d)	CH ₄ production (gCOD/d)	MRI (gCOD _{CH4})/(gCOD _{REM})	ERI (kJ _{CH4})/(kJ _{INF})
A ₂ O	If	1.13 ± 0.13	0.40 ± 0.04	53.3	9.544	0.253	0.166
S2EBPR	IIc	0.95 ± 0.12	0.22 ± 0.03	53.6	5.373	0.139	0.093

Table 4

The read abundance of relative PAOs and GAOs at the genus level observed during different operational periods: 500, 400, 350, 300, 450 mg/L of COD_{INF} in the A₂O system (period I); 350 + and 350 + S are samples from the aerobic reactor and SSSF of the S2EBPR system (period IIc) obtained operating with COD_{INF} of 350 mg/L.

COD _{INF} (mg/L)	500	400	350	300	450	350+	350 + S
Genus	%	%	%	%	%	%	%
<i>Thiothrix</i>	0.01 ± 0.00	0.28 ± 0.31	0.66 ± 0.04	0.14 ± 0.02	2.67 ± 1.31	6.47 ± 1.40	6.89 ± 0.39
<i>Dechloromonas</i>	1.22 ± 1.20	3.24 ± 3.18	2.70 ± 0.01	5.14 ± 1.54	3.53 ± 0.70	5.38 ± 1.35	5.78 ± 1.47
<i>Desulfuromonas</i>	2.04 ± 0.57	3.70 ± 3.72	0.75 ± 0.02	1.02 ± 0.06	0.80 ± 0.17	1.27 ± 0.31	0.83 ± 0.00
<i>Desulfovibrio</i>	9.54 ± 2.80	7.05 ± 2.53	4.08 ± 0.36	5.56 ± 0.22	4.33 ± 1.50	6.31 ± 1.23	4.20 ± 0.64
<i>Rhodobacter</i>	2.36 ± 1.67	0.81 ± 0.90	1.90 ± 0.08	2.76 ± 0.34	1.55 ± 0.56	0.19 ± 0.06	0.24 ± 0.03
<i>Desulfobulbus</i>	1.95 ± 0.82	1.16 ± 0.29	0.76 ± 0.09	0.99 ± 0.07	0.89 ± 0.33	1.18 ± 0.26	0.76 ± 0.13
<i>Thauera</i>	0.66 ± 0.43	1.25 ± 1.11	2.53 ± 0.10	2.31 ± 0.87	0.42 ± 0.41	0.07 ± 0.01	0.09 ± 0.02
<i>Desulfomicrobium</i>	1.07 ± 0.25	1.44 ± 1.16	0.43 ± 0.04	0.58 ± 0.13	0.47 ± 0.15	0.66 ± 0.09	0.55 ± 0.00
Total PAO	18.84 ± 7.84	18.91 ± 13.20	13.81 ± 0.76	18.50 ± 3.25	14.66 ± 5.13	21.54 ± 4.72	19.35 ± 2.67
<i>Propionivibrio</i>	0.14 ± 0.08	0.30 ± 0.06	0.22 ± 0.01	0.23 ± 0.20	0.11 ± 0.04	1.52 ± 0.38	5.52 ± 0.67
<i>Deftuviococcus</i>	0.67 ± 0.60	0.81 ± 0.92	0.80 ± 0.15	0.73 ± 0.28	0.70 ± 0.31	0.82 ± 0.14	1.03 ± 0.01
Total GAO	0.81 ± 0.69	1.11 ± 0.98	1.02 ± 0.16	0.96 ± 0.48	0.81 ± 0.35	2.34 ± 0.52	6.55 ± 0.69

concentrations attained: on one hand, it would allow removing part of the P accumulated in the system due to the lower WAS volume required to maintain biomass concentration in this configuration, on the other hand, it would decrease the potential of additional P load from SSSF to threaten the quality of mainstream effluent.

- 3) In this work, we have not considered another potential P input to the plant: the reject wastewater or the effluent from the waste sludge treatment. We only considered the P-load increase due to part of the RAS being diverted to the SSSF. In real plants, the whole reject water may be recycled to the plant. This effluent would increase the P-load to the plant without providing any extra COD and, thus, would even make more evident the need of novel configurations such as the SSSF.

5. Conclusions

The S2EBPR configuration with the connection of the SSSF to the anaerobic reactor was studied by introducing 6% of the RAS with HRT = 2.4 d and COD_{INF} of 350 mg/L (minimum value for successful P/N removal in the A₂O configuration).

The main benefits with S2EBPR compared with A₂O were:

- Higher P and N removal capacity (26.6% and 11%) without compromising full COD and ammonium oxidation.
- EBPR could be sustained with a low influent COD/P of only 26.3, while EBPR deterioration in A₂O was observed with a ratio about 32.6. Organic matter needs decreased from 10 g rbCOD/gP to 9 g rbCOD/gP.
- A higher abundance of functional PAO were observed in S2EBPR, which gave PAO the advantage for EBPR performance.

However, some underlying drawbacks were:

- Lower effluent quality (with 2.0 ± 1.6 mgP/L) was observed due to the additional P_{LOAD}, accompanied with a relatively insignificant increase of residual COD (0.5%) and N (1.6%).
- The energy recovery indices were around 45% lower than those of A₂O (with the same SRT), and less of the input COD could be recovered as electricity in S2EBPR (5%) compared with A₂O (12%), which may not a real drawback from an economic standpoint due to the sludge reduction.

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

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2023.143700>.

References

- M. Albertsen, S.J. McIlroy, M. Stokholm-Bjerregaard, S.M. Karst, P.H. Nielsen, "Candidatus Propionivibrio aalborgensis": A Novel Glycogen Accumulating Organism Abundant in Full-Scale Enhanced Biological Phosphorus Removal Plants, *Front. Microbiol.* 7 (2016) 1–17, <https://doi.org/10.3389/fmicb.2016.01033>.
- K. Andreasen, G. Petersen, H. Thomsen, R. Strube, Reduction of nutrient emission by sludge hydrolysis, *Water Sci. Technol.* 35 (1997) 79–85, [https://doi.org/10.1016/S0273-1223\(97\)00215-1](https://doi.org/10.1016/S0273-1223(97)00215-1).
- I. Angelidakis, M. Alves, D. Bolzonella, L. Borzacconi, J.L. Campos, A.J. Guwy, S. Kalyuzhnyi, P. Jenicek, J.B. van Lier, Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays, *Water Sci. Technol.* 59 (2009) 927–934, <https://doi.org/10.2166/wst.2009.040>.
- APHA. *Stand. methods exam. water wastewater*, 20th ed., 1995.
- J.A. Baeza, *Advanced Direct Digital Control (AddControl): lessons learned from 20 years of adding control to lab and pilot scale treatment systems*, in: *13th IWA Conference on Instrumentation, Control and Automation. ICA 2022, Tsinghua University, Beijing (China), 2022*, pp. 13–15.
- J.L. Barnard, K. Abraham, Key features of successful BNR operation, *Water Sci. Technol.* 53 (2006) 1–9, <https://doi.org/10.2166/wst.2006.400>.
- J.L. Barnard, P. Dunlap, M. Steichen, Rethinking the Mechanisms of Biological Phosphorus Removal, *Water Environ. Res.* 89 (2017) 2043–2054, <https://doi.org/10.2175/106143017X15051465919010>.
- C. Chan, A. Guisasaola, J.A. Baeza, Correlating the biochemical methane potential of bio-P sludge with its polyhydroxyalkanoate content, *J. Clean. Prod.* 242 (2020), 118495, <https://doi.org/10.1016/j.jclepro.2019.118495>.
- L. Chu, J. Wang, Denitrification performance and biofilm characteristics using biodegradable polymers PCL as carriers and carbon source, *Chemosphere* 91 (2013) 1310–1316, <https://doi.org/10.1016/j.chemosphere.2013.02.064>.
- E.R. Coats, K. Eyre, C. Bryant, T. Woodland, C.K. Brinkman, Assessing the Effects of RAS Fermentation on EBPR Performance and Associated Microbial Ecology, *Water Environ. Res.* 90 (2018) 659–671, <https://doi.org/10.2175/106143017X15131012153130>.
- Comeau, Y., Oldham, W.K., Hall, K.J., 1987. Dynamics of Carbon Reserves in Biological Dephosphatation of Wastewater, *Biological Phosphate Removal from Wastewaters*. International Association on Water Pollution Research and Control. <https://doi.org/10.1016/b978-0-08-035592-4.50010-9>.
- Copp, J.B., Belk, I., Vale, P., 2012. Operational Control of a RAS Fermentation Process for Enhancing Biological Phosphorus Removal.

- [13] P. Dold, D. Conidi, Achieving enhanced biological P removal: Have we forgotten how to design a bioP plant? WEFTEC 2019–92nd Annu. Water Environ. Fed. Tech. Exhib. Conf. (2019) 1452–1466.
- [14] W. Eckenfelder, J. Muserman, Activated sludge: treatment of industrial wastewater, CRC Press, 1998.
- [15] E.Y. Fernando, S.J. McIlroy, M. Nierychlo, F.A. Herbst, F. Petriglieri, M.C. Schmid, M. Wagner, J.L. Nielsen, P.H. Nielsen, Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman–FISH, ISME J. 13 (2019), <https://doi.org/10.1038/s41396-019-0399-7>.
- [16] R. Ferrentino, M. Langone, F. Merzari, L. Tramonte, G. Andreottola, A review of anaerobic side-stream reactor for excess sludge reduction: Configurations, mechanisms, and efficiency, Crit. Rev. Environ. Sci. Technol. 46 (2016) 382–405, <https://doi.org/10.1080/10643389.2015.1096879>.
- [17] A.Z. Gu, A. Saunders, J.B. Neethling, H.D. Stensel, L.L. Blackall, Functionally Relevant Microorganisms to Enhanced Biological Phosphorus Removal Performance at Full-Scale Wastewater Treatment Plants in the United States, Water Environ. Res. 80 (2008) 688–698, <https://doi.org/10.2175/106143008x276741>.
- [18] D. Herbst, N. Wimmer, The Proteome of *Tetrasphaera elongata* is adapted to Changing Conditions in Wastewater Treatment Plants, Proteomes 7 (2019) 16, <https://doi.org/10.3390/proteomes7020016>.
- [19] A. Hiraishi, A. Yanase, H. Kitamura, Polyphosphate accumulation by Rhodospirillum rubrum grown under different environmental conditions with special emphasis on the effect of external phosphate concentrations, Bull. Japanese Soc. Microb. Ecol. 6 (1991) 25–32, <https://doi.org/10.1264/microbes1986.6.25>.
- [20] S.M.S. Huda, H. Satoh, T. Mino, Anaerobic degradation of polyhydroxyalkanoate accumulated in activated sludge in the absence of anaerobic digested sludge, J. Water Environ. Technol. 14 (2016) 236–246, <https://doi.org/10.2965/jwet.15-037>.
- [21] K. Jönsson, J. la C. Jansen, Hydrolysis of return sludge for production of easily biodegradable carbon: effect of pre-treatment, sludge age and temperature, Water Sci. Technol. 53 (2006) 47–54, <https://doi.org/10.2166/wst.2006.405>.
- [22] A.B. Lanham, A. Oehmen, A.M. Saunders, G. Carvalho, P.H. Nielsen, M.A. M. Reis, Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal, Water Res. 47 (2013) 7032–7041, <https://doi.org/10.1016/j.watres.2013.08.042>.
- [23] APHA. Method 4500-P C. Stand. methods exam. water wastewater, 20th ed., 1995.
- [24] Li, G., Tooker, N.B., Wang, D., Srinivasan, V., Barnard, J.L., Russell, A., Takacs, I., Bott, C., Dobrowski, P., Onnis-Hayden, A., Gu, A.Z., 2020. Modeling side-stream enhanced biological phosphorus removal (S2EBPR) system using agent-based model with adaptive maintenance, decay and TCA metabolism. bioRxiv. <https://doi.org/10.1101/2020.11.18.387589>.
- [25] APHA. Method 4500-NH3 D. Stand. methods exam. water wastewater, 20th ed., 1995.
- [26] P.L. McCarty, J. Bae, J. Kim, Domestic Wastewater Treatment as a Net Energy Producer—Can This be Achieved? Environ. Sci. Technol. 45 (2011) 7100–7106, <https://doi.org/10.1021/es2014264>.
- [27] Q. Meng, W. Zeng, B. Wang, Z. Fan, Y. Peng, New insights in the competition of polyphosphate-accumulating organisms and glycogen-accumulating organisms under glycogen accumulating metabolism with trace Poly-P using flow cytometry, Chem. Eng. J. 385 (2020), 123915, <https://doi.org/10.1016/j.cej.2019.123915>.
- [28] J.B. Neethling, Factors Influencing the Reliability of Enhanced Biological Phosphorus Removal, Water Intell. Online 5 (2006), <https://doi.org/10.2166/9781780404479>.
- [29] H.T.T. Nguyen, V.Q. Le, A.A. Hansen, J.L. Nielsen, P.H. Nielsen, High diversity and abundance of putative polyphosphate-accumulating *Tetrasphaera*-related bacteria in activated sludge systems, FEMS Microbiol. Ecol. 76 (2011) 256–267, <https://doi.org/10.1111/j.1574-6941.2011.01049.x>.
- [30] P.H. Nielsen, S.J. McIlroy, M. Albertsen, M. Nierychlo, Re-evaluating the microbiology of the enhanced biological phosphorus removal process, Curr. Opin. Biotechnol. 57 (2019) 111–118, <https://doi.org/10.1016/j.copbio.2019.03.008>.
- [31] A. Onnis-Hayden, V. Srinivasan, N.B. Tooker, G. Li, D. Wang, J.L. Barnard, C. Bott, P. Dombrowski, P. Schauer, A. Menniti, A. Shaw, B. Stinson, G. Stevens, P. Dunlap, I. Takács, J. McQuarrie, H. Phillips, A. Lambrecht, H. Analla, A. Russell, A.Z. Gu, Survey of full-scale sidestream enhanced biological phosphorus removal (S2EBPR) systems and comparison with conventional EBPRs in North America: Process stability, kinetics, and microbial populations, Water Environ. Res. 92 (2020) 403–417, <https://doi.org/10.1002/wer.1198>.
- [32] F. Petriglieri, C. Singleton, M. Peces, J.F. Petersen, M. Nierychlo, P.H. Nielsen, “*Candidatus* Dechloromonas phosphoritropha” and “*Ca. D. phosphorivorans*”, novel polyphosphate accumulating organisms abundant in wastewater treatment systems, ISME J. 15 (2021) 3605–3614, <https://doi.org/10.1038/s41396-021-01029-2>.
- [33] N. Rey-Martínez, M. Badia-Fabregat, A. Guisasola, J.A. Baeza, Glutamate as sole carbon source for enhanced biological phosphorus removal, Sci. Total Environ. 657 (2019) 1398–1408, <https://doi.org/10.1016/j.scitotenv.2018.12.064>.
- [34] Richard, M., 2003. Michael Richard, Ph.D. Sear-Brown Fort Collins, CO. 20th Annu. USEPA Natl. Oper. Trainers Conf. 1–21.
- [35] S. Roy, Q. Guanglei, R. Zuniga-Montanez, R.B. Williams, S. Wuertz, Recent advances in understanding the ecophysiology of enhanced biological phosphorus removal, Curr. Opin. Biotechnol. 67 (2021) 166–174, <https://doi.org/10.1016/j.copbio.2021.01.011>.
- [36] F.J. Rubio-Rincón, D.G. Weissbrodt, C.M. Lopez-Vazquez, L. Welles, B. Abbas, M. Albertsen, P.H. Nielsen, M.C.M. van Loosdrecht, D. Brđjanovic, “*Candidatus* Accumulibacter delftensis”: A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate, Water Res. 161 (2019) 136–151, <https://doi.org/10.1016/j.watres.2019.03.053>.
- [37] F.J. Rubio-Rincón, L. Welles, C.M. Lopez-Vazquez, M. Nierychlo, B. Abbas, M. Geleijnse, P.H. Nielsen, M.C.M. van Loosdrecht, D. Brđjanovic, Long-term effects of sulphide on the enhanced biological removal of phosphorus: The symbiotic role of *Thiothrix caldifontis*, Water Res. 116 (2017) 53–64, <https://doi.org/10.1016/j.watres.2017.03.017>.
- [38] F. Sabba, M. Farmer, Z. Jia, F. Di Capua, P. Dunlap, J. Barnard, C.D. Qin, J. A. Kozak, G. Wells, L. Downing, Impact of operational strategies on a sidestream enhanced biological phosphorus removal (S2EBPR) reactor in a carbon limited wastewater plant, Sci. Total Environ. 857 (2023), 159280, <https://doi.org/10.1016/j.scitotenv.2022.159280>.
- [39] N. Shen, Y. Zhou, Enhanced biological phosphorus removal with different carbon sources, Appl. Microbiol. Biotechnol. 100 (2016) 4735–4745, <https://doi.org/10.1007/s00253-016-7518-4>.
- [40] G.J.F. Smolders, J. van der Meij, M.C.M. van Loosdrecht, J.J. Heijnen, Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence, Biotechnol. Bioeng. 43 (1994) 461–470, <https://doi.org/10.1002/bit.260430605>.
- [41] H. Sun, Q. Zhou, L. Zhao, W. Wu, Enhanced simultaneous removal of nitrate and phosphate using novel solid carbon source/zero-valent iron composite, J. Clean. Prod. 289 (2021), <https://doi.org/10.1016/j.jclepro.2020.125757>.
- [42] G. Tchobanoglous, F.L. Burton, H.D. Stensel, Wastewater Engineering: Treatment and Reuse, Metcalf & Eddy Inc, Fourth. ed., McGraw-Hill, New York, 2013.
- [43] H. Tian, X. Xu, J. Qu, H. Li, Y. Hu, L. Huang, W. He, B. Li, Biodegradation of phenolic compounds in high saline wastewater by biofilms adhering on aerated membranes, J. Hazard. Mater. 392 (2020), 122463, <https://doi.org/10.1016/j.jhazmat.2020.122463>.
- [44] N.B. Tooker, G. Li, C. Bott, P. Dombrowski, P. Schauer, A. Menniti, A. Shaw, J. L. Barnard, B. Stinson, G. Stevens, P. Dunlap, I. Takacs, H. Phillips, H. Analla, A. Russell, A. Ellsworth, J. McQuarrie, K. Carson, A. Onnis-Hayden, A.Z. Gu, Rethinking and Reforming Enhanced Biological Phosphorus Removal (EBPR) strategy - Concepts and mechanisms of side-stream EBPR, in: Water Environment Federation Technical Exhibition and Conference 2017, WEFTEC 2017, Water Environment Federation, 2017, pp. 4387–4404. <https://doi.org/10.2175/193864717822153076>.
- [45] E. Vaiopoulou, P. Melidis, A. Aivasidis, Growth of filamentous bacteria in an enhanced biological phosphorus removal system, Desalination 213 (2007) 288–296, <https://doi.org/10.1016/j.desal.2006.02.101>.
- [46] P. Vale, J. Barnard, D. Thomas, P. Dold, RAS Fermentation to Enhance Biological Phosphorus Removal, Proc. Water Environ. Fed. 2008 (2008) 1775–1789, <https://doi.org/10.2175/193864708788733981>.
- [47] B. Valverde-Pérez, D.S. Wágner, B. Lórant, A. Gülay, B.F. Smets, B.G. Plósz, Short-sludge age EBPR process - microbial and biochemical process characterisation during reactor start-up and operation, Water Res. 104 (2016) 320–329, <https://doi.org/10.1016/j.watres.2016.08.026>.
- [48] J. Vollertsen, G. Petersen, V.R. Borregaard, Hydrolysis and fermentation of activated sludge to enhance biological phosphorus removal, Water Sci. Technol. 53 (2006) 55–64, <https://doi.org/10.2166/wst.2006.406>.
- [49] D. Wang, N.B. Tooker, V. Srinivasan, G. Li, L.A. Fernandez, P. Schauer, A. Menniti, C. Maher, C.B. Bott, P. Dombrowski, J.L. Barnard, A. Onnis-Hayden, A.Z. Gu, Side-stream enhanced biological phosphorus removal (S2EBPR) process improves system performance - A full-scale comparative study, Water Res. 167 (2019), 115109, <https://doi.org/10.1016/j.watres.2019.115109>.
- [50] S.-S. Yang, W.-Q. Guo, X.-J. Zhou, Z.-H. Meng, B. Liu, N.-Q. Ren, Optimization of operating parameters for sludge process reduction under alternating aerobic/oxygen-limited conditions by response surface methodology, Bioresour. Technol. 102 (2011) 9843–9851, <https://doi.org/10.1016/j.biortech.2011.07.079>.
- [51] C. Zhang, A. Guisasola, J.A. Baeza, Achieving simultaneous biological COD and phosphorus removal in a continuous anaerobic/aerobic A-stage system, Water Res. 190 (2021), 116703, <https://doi.org/10.1016/j.watres.2020.116703>.
- [52] Y. Zhang, Z. Hua, H. Lu, A. Oehmen, J. Guo, Elucidating functional microorganisms and metabolic mechanisms in a novel engineered ecosystem integrating C, N, P and S biotransformation by metagenomics, Water Res. 148 (2019) 219–230, <https://doi.org/10.1016/j.watres.2018.10.061>.
- [53] L. Zhao, H. Chen, Z. Yuan, J. Guo, Interactions of functional microorganisms and their contributions to methane bioconversion to short-chain fatty acids, Water Res. 199 (2021), 117184, <https://doi.org/10.1016/j.watres.2021.117184>.