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RESEARCH ARTICLE

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ENVIRONMENT RESEARCH

Assessing the performance of polyphosphate accumulating organisms in a full-scale side-stream enhanced biological phosphorous removal

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Abstract

Phosphorous (P) removal in wastewater treatment is essential to prevent eutrophication in water bodies. Side-stream enhanced biological phosphorous removal (S2EBPR) is utilized to improve biological P removal by recirculating internal streams within a side-stream reactor to generate biodegradable carbon (C) for polyphosphate accumulating organisms (PAOs). In this study, a full-scale S2EBPR system in a water resource recovery facility (WRRF) was evaluated for 5 months. Batch experiments revealed a strong positive correlation ($r = 0.91$) between temperature and C consumption rate (3.56–8.18 mg-COD/g-VSS/h) in the system, with temperature ranging from 14°C to 18°C. The anaerobic P-release to COD-uptake ratio decreased from 0.93 to 0.25 mg-P/mg-COD as the temperature increased, suggesting competition between PAOs and other C-consumers, such as heterotrophic microorganisms, to uptake bioavailable C. Microbial community analysis did not show a strong relationship between abundance and activity of PAO in the tested WRRF. An assessment of the economic feasibility was performed to compare the costs and benefits of a full scale WRRF with and without implementation of the S2EBPR technology. The results showed the higher capital costs required for S2EBPR were estimated to be compensated after 5 and 11 years of operation, respectively, compared to chemical precipitation and conventional EBPR. The results from this study can assist in the decision-making process for upgrading a conventional EBPR or chemical P removal process to S2EBPR.

Practitioner Points

- Implementation of S2EBPR presents adaptable configurations, exhibiting advantages over conventional setups in addressing prevalent challenges associated with phosphorous removal.

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- A full-scale S2EBPR WRRF was monitored over 5 months, and activity tests were used to measure the kinetic parameters.
- The seasonal changes impact the kinetic parameters of PAOs in the S2EBPR process, with elevated temperatures raising the carbon demand.
- PAOs abundance showed no strong correlation with their activity in the full-scale S2EBPR process in the tested WRRF.
- Feasibility assessment shows that the benefits from S2EBPR operation can offset upgrading costs from conventional BPR or chemical precipitation.

KEYWORDS

Economic feasibility, Nutrient recovery, Phosphorous, Polyphosphate accumulating organisms (PAOs), S2EBPR, Wastewater, WRRF

INTRODUCTION

The removal of phosphorus (P) from wastewater through chemical precipitation using agents such as lime, aluminum sulfate (alum), and iron salts is a common approach (Tomei et al., 2020). However, chemical P removal poses a current challenge for sustainable P recovery thus necessitating the minimization of chemical dosing (Strickland, 1999). Furthermore, the addition of chemical agents increases the operational cost, which limits sustainable and economical applications for recovering P from wastewater (Parsons & Smith, 2008). Enhanced biological phosphorous removal (EBPR) is an alternative technology to improve P removal by achieving reduced concentrations of P in effluent in water resource recovery facilities (WRRF) (Fernando et al., 2019; Melia et al., 2017). P removal in an EBPR system is achieved by polyphosphate (Poly-P) accumulating organisms (PAOs) that are capable of storing poly-P during the alternation of anaerobic and aerobic phases (Serralta et al., 2004). Under anaerobic conditions, PAOs can take up organic matter and store it as poly-hydroxy-alkanoates (PHAs) using the energy from poly-P degradation that is associated with orthophosphate (OP) release to the wastewater (Wang et al., 2019). During the aerobic phase, PAOs can oxidize the stored PHAs and use the released energy to accumulate P from the wastewater. The P removal can be completed by withdrawing the excess P-rich activated sludge in clarifiers (Izadi et al., 2021; Shen et al., 2017). However, practical challenges remain for applying EBPR at large scale and many WRRFs are still adding precipitation agents to ensure the effluent meets the permitting requirements with an increase in the total cost of treatment as a result (Mulkerrins et al., 2004). Unstable or incomplete P removal in EBPR may be due to operational upsets (e.g., low bioavailable C in the influent) and subsequently growth of competitive microorganisms (e.g., glycogen accumulating organisms [GAOs]) (Yu et al., 2021). To supply sufficient C for PAOs, side-stream EBPR (S2EBPR) recirculates a portion of organic carbon-rich streams (e.g., returned activated sludge [RAS]) to a side-stream anaerobic reactor. Here, hydrolysis and fermentation can generate a readily biodegradable C source mostly in the form of volatile fatty acids (VFAs), which can be used as organic substrate by PAOs (Zhao et al., 2022).

Activated sludge contains other microorganisms that potentially compete with PAOs for organic substrate such as denitrifying polyphosphate accumulating organisms (DPAOs) and GAOs. DPAOs can utilize nitrite (NO_2^-) and nitrate (NO_3^-) as electron acceptor in addition to oxygen (Massoompour et al., 2022). GAOs are able to store C source anaerobically, but not contribute to aerobic P removal (Emel et al., 2011). To assess the competitive dynamics between PAO and GAO, operational and environmental factors, such as pH and temperature, were identified as determinants (Carvalheira et al., 2014). Jeon et al. (2001) evaluated conditions impacting PAOs activity and growth and showed that GAOs can outcompete PAOs at pH of 7 and subsequently EBPR failed, while higher pH values (~8.4) revealed a complete P removal (Jeon et al., 2001). In another study, Lopez-Vazquez operated GAO-enriched lab-scale SBRs and acclimated the sludge at 20°C for 8 days. Then they altered the reactors' temperature to various values within the range of 10–40° C and examined the impact on GAOs activity. Their results suggested that GAOs cannot compete with PAOs at temperatures <20°C due to lower acetate uptake and biomass growth rates. However, temperatures >20°C favored GAOs activity as they can effectively compete with PAOs as the acetate uptake rate will be higher (Lopez-Vazquez et al., 2009). Therefore, the role of GAOs in full-scale plants implementing S2EBPR and the relation between GAOs abundance, PAOs activity, and operational parameters need further examination.

The stability and reliability of S2EBPR depend on PAO activity, which can be measured via kinetic parameters such as anaerobic P release rate and chemical oxygen demand (COD) uptake rate, aerobic P uptake rate, and operational factors such as the temperature of the sludge and raw influent characteristics (C/P ratio, ammonium [NH_4^+], total Kjeldahl nitrogen [TKN], total suspended solid [TSS], volatile suspended solid [VSS], etc.) (Jeon et al., 2001; Onnis-Hayden et al., 2020). Schauer et al. (2019) monitored the performance of a full-scale WRRF in which PAO-enrichment was confirmed during their summer operation. The results showed that as the temperature increased from 15°C to 23°C, the abundance of PAOs increased from 6.5% to 11% concurrently with increasing P-uptake rates from 7.5 to 12 mg-P/g-VSS/h. The authors explained that the competition between PAOs and GAOs was not significant in the full-scale WRRF due to the stable P release to acetate uptake ratios observed in their work (Schauer et al., 2019). The varied observations reported by Schauer et al. (2019) and Lopez-Vazquez et al. (2009) concerning PAOs activity and their competitive potential with other microorganisms suggest that temperature's influence on the PAO process may be also affected by other factors, such as system scales, acclimation, and the composition of the original microbial community. The application of S2EBPR can compensate for the limited bioavailable carbon in the influent and is expected to enhance the activity and growth of PAOs, leading to improved process stability. Lanham et al. (2013) reported a more stable biological P removal (94%–96%) and higher abundance of PAOs (3.5%–6.5%) in full-scale S2EBPRs compared to conventional EBPRs with removal efficiency of 43%–92% and PAOs' abundance of 3%–4% (Lanham et al., 2013). However, the contribution of PAOs to biological P removal and their activity under different operational/seasonal conditions in a full-scale S2EBPR is not well-understood.

In this study, the P removal performance of a full-scale WRRF was evaluated to assess the potential for S2EBPR implementation. Samples from the S2EBPR process were tested for kinetic parameters of PAOs, including rates of anaerobic P release, anaerobic COD uptake, and aerobic P uptake, using laboratory-scale batch experiments. Operational data, such as temperature, influent composition, and alum consumption, were statistically analyzed to elucidate the relationships between operational and seasonal factors and PAOs kinetic parameters. Furthermore, the abundance of PAOs and GAOs in S2EBPR was determined to assess the conventional PAO contribution in biological P removal and assess the competition between PAOs and GAOs for organic substrate in this WRRF. It should be noted that this study did not study the role of DPAOs within the system. Additionally, an economic analysis was performed to evaluate the feasibility of upgrading from the conventional chemical precipitation process to the S2EBPR process.

MATERIALS AND METHODS

Water resource recovery facility (WRRF)

Operation

The full-scale WRRF (The Parkway WRRF), which is located in Laurel, MD, USA, with a capacity of 2.8×10^7 L/day (7.5 MGD), was selected for study in this work. This facility uses a four-stage Bardenpho process for nutrient removal (Curtin et al., 2011). To further enhance the P removal efficiency, an unused secondary clarifier was rehabilitated as a fermenter (side-stream anaerobic zone), upgrading the process to S2EBPR starting in Sep. 2020. The 1.1×10^6 L (0.3 MG) fermenter was intermittently mixed, and HRT was about 12 hours. The mixer was on for about 20 min every 4–6 h to target an oxidation reduction potential (ORP) of −360 mV in the fermenter. Figure 1 provides a schematic diagram of the current WRRF integrated with S2EBPR. Details about the volume and HRT are provided in Table S1. The facility uses two reactor basins (RB-1 and RB-2) that are operating in parallel, where the effluent of each is combined before entering the secondary clarifiers. The supplementary C source, MicroC® 3000 (characteristics provided in Supporting Information S1 and Table S2), is added in the post-anoxic phase for enhancing nitrate removal via denitrification. It should be noted that the MicroC® 3000 is predominantly methanol (72.1%), and PAOs are not capable of utilizing it; however, it could be beneficial for EBPR by depleting the available nitrate through denitrification (Shen et al., 2017).

Characterization of influent and activated sludge

Both influent and sludge samples were collected from February through June 2022 for characterization. Table 1 summarizes the influent characteristics using the data collected from The Parkway. During the study period, a total of 19 sludge sample events were conducted in the WRRF for performing batch activity tests. These events included eight samples collected on weekends and 11 samples collected on weekdays. Out of those samples, seven were collected from RB-1, and 12 from RB-2. The collected sludge samples were characterized, and the results show that the TSS and VSS were an average of

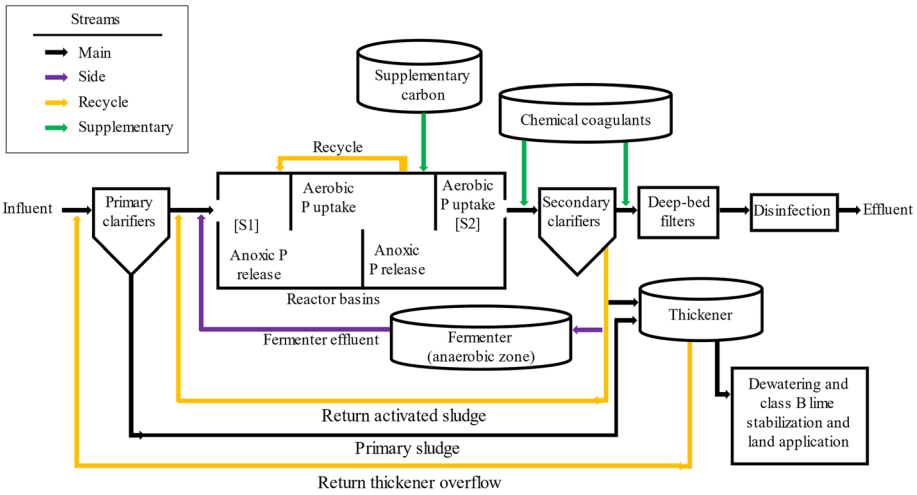

FIGURE 1 Overview of schematic diagram in the Parkway water resource recovery facility (WRRF).

TABLE 1 Characteristics of the influent wastewater to the WRRF (data was collected from 02/01/2022 to 06/05/2022).

		Range	
Parameters (mg/L)	**Average values**	**Min**	**Max**
TSS	126 ± 180	28	918
BOD_5	204 ± 109	80	648
TKN	39 ± 11	24	96
NH_4-N	27 ± 4	16	34
TP	5 ± 3	2	22
T (°C)	17 ± 2	14	23

3.93 ± 0.40 and 3.12 ± 0.29 g/L in the RB-1, and 4.05 ± 0.40 and 3.23 ± 0.31 g/L in the RB-2, respectively. The sludge volume index (SVI) was also measured on-site to assess the settleability of the sludge. SVIs were ranging from 99 to 221 mL/g for RB-1 and 95 to 206 mL/g for RB-2, which indicated good settleability (Liu et al., 2011). Filamentous index (FI) was also estimated using the light microscope according to the method suggested by Eikelboom (2000) and all samples revealed a FI of ~0 (Eikelboom, 2000).

Laboratory activity tests

Before performing the activity test, a 30-min aeration was applied to deplete the sludge from remaining organics to obtain a soluble COD <20 mg/L (Wang et al., 2018). This pre-aeration step ensures the designed initial COD concentration is achieved in the batches. After this pre-aeration, the anaerobic phase was initiated by sparging the reactor with nitrogen gas (>99% purity, Robert Oxygen Company, Inc.) to achieve a dissolved oxygen (DO) level <0.2 mg/L. Acetate was then added to an initial concentration of 100 mg/L of COD (time: 0 min). The anaerobic phase was maintained for 150 min (time: 0 to 150 min). The P uptake test was conducted for 5 h in the same reactor (150 to 450 min). During this stage, aeration was maintained with DO concentration >2 mg/L to avoid oxygen limitation in the system. During both phases, pH was maintained at 7.0 ± 0.2 using 0.05 M HCl/NaOH. Samples were collected at (min): 0, 30, 60, 90, 120, 150, 180, 210, 270, 360, and 450 for analysis of orthophosphate (OP) and COD (Oehmen, Yuan, et al., 2005). A reactor without acetate (COD) addition was used to assess the endogenic P release and the resulting P uptake. The results showed (Figure S1) that the contribution of endogenic activity to the calculation of kinetic parameters was insignificant (detailed results are provided in Supporting Information S2 and Table S3). Also, nitrate concentration was detected as negligible in batch bioassays. Therefore, it is anticipated that the P uptake and other kinetic parameters calculated from the batch bioassays did not include DPAOs' activity. Activity tests were performed in duplicate at 20 ± 1°C.

Chemical analyses

Activated sludge samples were filtered through a 0.45-μm filter prior to soluble COD (sCOD) and OP measurements. sCOD concentration was determined using a HACH colorimeter DR900 with COD premade digestion vials for the range of 20 to 1500 mg/L (HACH, CO, USA). OP was measured using a UV–VIS spectrophotometer (Agilent, CA, USA) at 880 nm following the ascorbic acid method (Nivens et al., 1999). pH was measured using a digital pH probe using a 3-point calibration and ±0.01 sensitivity (9156BNWP, Thermo Fisher, MA, USA). Other parameters, including TSS and VSS, were measured according to standard methods (APHA, 2017).

TABLE 2 qPCR primer sequences for total bacteria (Huse et al., 2008), PAOs (Crocetti et al., 2002), and GAOs (Winkler et al., 2011).

Specificity	Primer	Sequence (5′–3′)	qPCR performance		Calibration (ng/μL)
			Standard curve (R^2)	PCR efficiency (%)	
Bacteria	338f	ACTCCTACGGGAGGCAGCAG	0.999	93.46	From 10.8
	533r	TTACCGCGGCTGCTGGCAC			To 1.1×10^{-9}
PAOs	518f	CCAGCAGCCGCGGTAAT	0.994	106.06	From 11.8
	846r	GTTAGCTACGGCACTAAAAGG			To 1.2×10^{-11}
GAOs	GAOQ 413f	AAGCCCTTTAGGCGGGGA	1.000	95.83	From 12.7
	GAOQ 989f	TTCCCCGGATGTCAAGGC			To 1.3×10^{-11}

Microbial community analyses

Microbial community changes were assessed in activated sludge samples from the reactor basins. After centrifuging 50 mL of samples at 3500 rpm for 10 min, 250 mg of the resulting sludge pellets was used for DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen, CA, USA). Genomic DNA yield and purity were quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, CA, USA). To assess the abundance of PAOs and GAOs, primers targeting the Ca. *Accumulibacter* (the model PAO) and the Ca. *Competibacter phosphatis* (the model GAO) were used (Table 2). To prepare the calibration curves for the qPCR runs, activated sludge collected from the WRRF was used (as positive control) to run a PCR with each primer set. PCR thermocycler protocols were adopted from Winkler et al. (2011): starting with 3 min at 95°C, and then 45 cycles of (i) denaturation: 30 s at 95°C, (ii) annealing: 45 s at 60°C, and (iii) elongation: 30 s at 72°C (Winkler et al., 2011). PCR reaction wells contained 2-μL DNA template, 0.4 μL of each primer (reverse and forward), 9.7 μL RT-PCR grade water (Invitrogen, CA, USA), and 12.5 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, CA, USA). The resulting products were run through 1.5% agarose gel (at 100 V), where the amplicon size was confirmed as well as the absence of non-specific binding. The PCR product was then cleaned up using a Wizard® SV Gel and PCR clean-up system (Promega, WI, USA). A NanoDrop spectrophotometer was used to measure the quality and concentration of amplified DNA. Table 2 shows both the primer sets for the PCR amplification and relative abundance measurements, and the calibration curve ranges for each qPCR run. Calibration curve for the qPCR using serial dilution of these cleaned up PCR products. The abundance was quantified using a CFX Opus 96 Real-Time PCR detection system (Bio-Rad, CA, USA). qPCR thermocycler protocols were the same as the PCR thermocycler protocols (Winkler et al., 2011). qPCR melting curves were evaluated to confirm a single melting peak and ensure the quality of runs. The 2 μL of RT-PCR grade water (Invitrogen, CA, USA) was used as a negative control for both PCR and the qPCR runs. All of the samples were amplified in triplicate for the quality assessment purposes.

Data processing and analysis

P release and COD uptake rate were calculated by the amount of the OP released to the system and the amount of the COD consumed in the linear range, respectively, in the anaerobic phase. The total P released to COD uptake ratio was calculated based on the difference between concentrations of OP and COD at $t = 0$ min and $t = 150$ min (anaerobic phase). P uptake rate was calculated based on the OP concentration during the aerobic phase (150–450 min). The values were normalized by the mass of the volatile suspended solid (g-VSS) and time (h) (Drewnowski & Makinia, 2011; Larriba et al., 2020; Mulkerrins et al., 2004). The results were statistically analyzed using the Student's paired *t*-test with a *p-value* of 0.05, corresponding to a 95% confidence interval. Pearson correlation analysis was used to investigate the correlation between kinetic parameters and the operational and seasonal factors (Qiu et al., 2019). The 7-day averages were used to account for variability. The correlation output ranges from +1 to −1, with the symbols (+ or −) indicating a positive or negative linear relationship, respectively. A strong relationship is concluded when the value falls within the range of +1 to +0.7 (positive correlation) or −1 to −0.7 (negative correlation). Values between −0.7 and +0.7 indicate a weak relationship.

Feasibility study

Before the startup of the fermenter in the tested WRRF (Sep. 2020), chemical P precipitation was applied by

TABLE 3 Base value (2021) and input parameters for the economic analysis.

Parameter	Purpose	Unit	Base value
Discount rate	Determining the present value of future cash flow	Annual rate, %	4%
Inflation rate	Represent increase in price levels	Annual rate, %	3%
Treatment goal	Final concentration of the TP at the effluent	mg/L	0.3[a]
Capital investment	The costs at the beginning of the project	$	400,000[b]
Sludge handling	The costs for handling/discharging each lb of sludge	$/kg	0.08
Electricity costs	The electricity costs for the facility	$/kwh	0.11
Alum costs	The costs for chemical precipitation	$/L	0.20
Sludge selling[f]	Benefits from selling the sludge to other facilities	$/kg	0.0046
P removal%	Represent the efficiency of biological P removal	%	Chemical: 0%[c] Biological: 47%[d] S2EBPR: 81%[e]

[a]Effluent TP required by the tested water resource recovery facility (WRRF).
[b]Based on the actual cost that the tested WRRF spent for reconstructing an abandoned primary clarifier to fermenter.
[c]Assuming that in the chemical precipitation technology, no P will be removed biologically.
[d]The efficiency of the conventional EBPR to remove 50% of the P.
[e]Efficiency of biological P removal in the tested WRRF.
[f]https://bloomsoil.com/.

dosing alum to polish the effluent quality and remain below the effluent standard of 0.3 mg/L of TP. An Excel-based economic analysis was performed to evaluate the economical outcomes of P removal with S2EBPR in comparison with (1) a fully chemical precipitation without any biological P removal and (2) a hybrid P removal based on the equal contribution of biological P removal (conventional EBPR) and chemical precipitation. The assessment considered the capital costs and operational costs for a period of 15 years (starting 2021). Capital costs include the costs of installing the new anaerobic reactor and new pipeline systems. Operational costs include the costs for chemical agents, electricity for the additional pumps and mixers, and sludge handling. Table 3 summarizes the main parameters considered in this assessment (detailed calculations provided in Supporting Information S3). In addition, sensitivity analysis was performed to address concerns regarding the changes over time in three directions: (i) if the performance of the S2EBPR in this facility changed due to the process upsets/optimization, (ii) if the P-rich sludge selling can benefit the facility, (iii) if the goal of the treatment decrease as the standards may improve. It should be noted that the sensitivity analysis was conducted without including the operational cost associated with the actions of the WRRFs for changing P removal efficiency and/or target effluent P concentration, and this factor should be counted when applying the results from this work for practical implementation.

RESULTS AND DISCUSSION

Full scale WRRF P removal performance

The Parkway WRRF revealed a total P (TP) removal efficiency of 96.8 ± 2.3% through a combined treatment of biological P removal and chemical precipitation, while biological P removal contributed to 81 ± 9.1% (based on the samples from reactor basins) of TP removal. The effluent's TP concentration was measured at 0.17 ± 0.12 mg/L, meeting the discharge limit of 0.3 mg/L for TP according to the national pollution discharge elimination system (NPDES) permit. Our monitoring data showed that the temperatures of raw influent and the aeration basin were similar (Figure S2). Therefore, the temperature of the raw influent can be used as the temperature of the biological P removal process in the studied full-scale WRRF.

PAOs activity in the full-scale S2EBPR

As two reactor basins (RB-1 and RB-2) are operated at a parallel mode in the WRRF, it is expected that similar results will be observed from the two basins. As shown in Figure 2a, calculated kinetic parameters of PAOs activity in RB-1 and RB-2 showed no significant difference. Therefore, the data from activity tests in two basins were combined for further investigation, while the results of

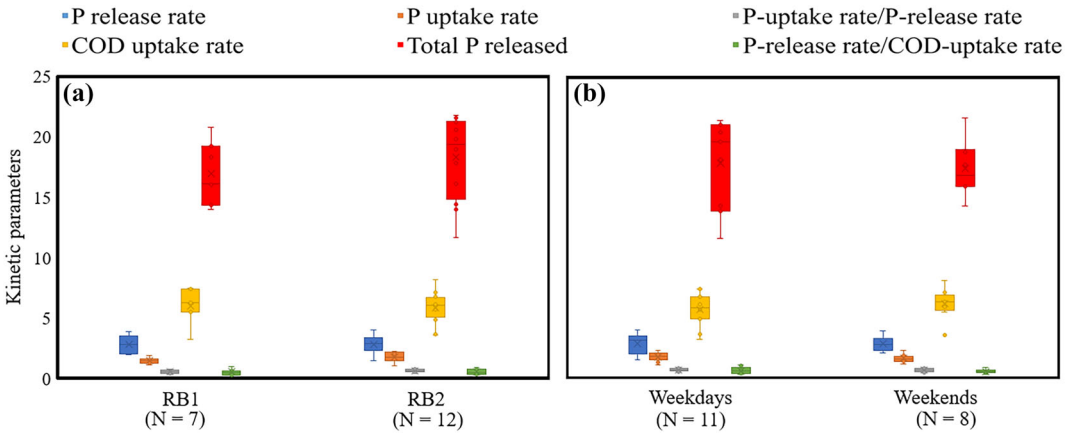

FIGURE 2 Calculated kinetic parameters of polyphosphate accumulating organisms (PAOs) (a) in reactor basins RB-1 and RB-2 and (b) during weekdays and weekends. P release rate and P uptake rate are expressed as mg-P/g-VSS/h; P-uptake/P-release is a dimensionless ratio; COD uptake rate is expressed as mg-COD/g-VSS/h; Total P released is expressed as mg/L of P released during anaerobic phase; P-release rate/COD-uptake rate is expressed as mol-P/mol-C. The number of samples (*N*) used for the test and calculation is shown.

activity tests for each RB are provided in Figures S3 and S4.

In the laboratory experiments, PAOs' activity was assessed by providing acetate (100 mg/L) as a readily biodegradable COD (rbCOD) source. The kinetic parameters determined from these activity tests reflect the potential of microorganisms to remove P in the system. As shown in Table S4, anaerobic P release and COD uptake rates ranged from 1.4–4.0 mg-P/g-VSS/h and 3.2–8.2 mg-COD/g-VSS/h, respectively. In comparison, anaerobic P release rate of 5.6–31.9 mg-P/g-VSS/h and COD uptake rate of 16.1–42.5 mg-COD/g-VSS/h were reported from other full-scale EBPR systems (Gu et al., 2008), while laboratory-scale EBPRs showed a higher anaerobic P release rate ranging from 31.2–62.4 mg-P/g-VSS/h (Gao et al., 2011). These results suggested that the WRRF studied in this work had a lower PAO activity potential (or higher abundance of GAOs) in the full-scale EBPR process than other WRRFs with EBPR implementation. Lower PAOs activity could be a result of the high C/P ratio in the raw influent. In this work, a C/P ratio of 90 ± 34 mg-COD/mg-P in the influent was found, compared with a C/P ratio of 5–38 mg-COD/mg-P in other full-scale EBPR systems with a higher anaerobic P release rate (5.6–31.9 mg-P/g-VSS/h) (Gu et al., 2008). Ma et al. (2005) showed that a C/P ratio of >32 can decrease the PAOs activity due to the potential competition and proliferation of GAOs as the excessive amount of carbon will be delivered to them (Ma et al., 2005).

The ratio of the P release rate to the COD uptake rate in the anaerobic phase can indicate the dominant metabolic pathway of the PAOs and provide insight into the relative abundance of PAOs/GAOs within the system (Schuler & Jenkins, 2003). Previous studies have shown that a P-release/COD-uptake ratio of 0.5 or higher can indicate a polyphosphate accumulating metabolism (PAM), while values <0.5 suggest a glycogen accumulating metabolism (GAM) pathway (Zhang et al., 2022). The results (Figure 3) suggest an alteration of the dominant metabolic pathway of PAOs through the sampling period. In February (T = 15.2 ± 0.6°C), the calculated P-release/COD-uptake ratio was 0.81 ± 0.16 mol-P/mol-C, which is higher than 0.5. This suggests that the PAM pathway was the dominant mechanism for carbon uptake. As temperature increased from 15.9 ± 0.8°C (March), to 17.1 ± 1.0°C (April), and further to 19.5 ± 1.1°C (May), the calculated ratio of P release to COD uptake in the anaerobic phase dropped below 0.5, suggesting that GAM became the primary pathway for C uptake in the PAOs. In June, when T increased to 21.4 ± 0.9°C, a P release to COD uptake ratio of 0.72 ± 0.07 was observed, indicating that PAM was again the primary mechanism responsible for the C uptake. A similar alteration of the PAOs' metabolic pathway was observed by Lanham et al. (2013). In their work, they compared microbial community and EBPR activity in two full-scale WRRFs with anaerobic/anoxic/aerobic configurations (located in Portugal) through winter (five samplings) and summer (two samplings). They conducted P-release/COD-uptake measurements and the results showed that the dominant pathway in both WRRFs changed from PAM to GAM when temperature increased from 15°C to 22°C. This result was similar to what we observed in our study from Feb to May (temperature increased from 15.2°C to 19.5°C). However, our results showed that when the temperature is higher than 18°C (June), the P release to C uptake ratio tends to remain consistent or slightly increase (Figure 4b), indicating a metabolic pathway change from

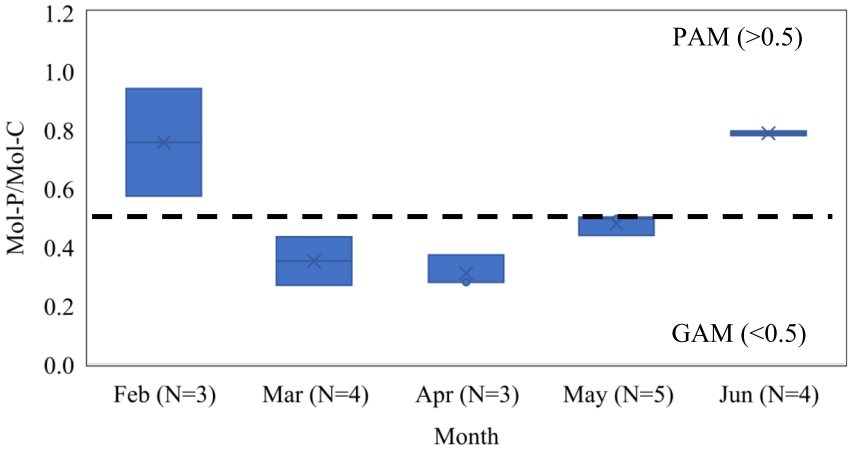

FIGURE 3 Anaerobic P-release/COD-uptake ratio over sampling period (02/01/2022 to 06/05/2022). N represents the number of samples used for the calculation.

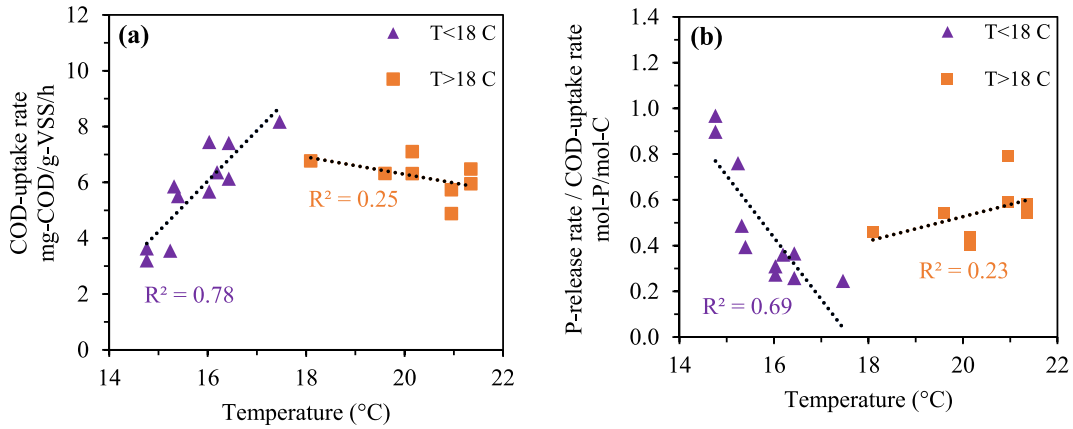

FIGURE 4 Correlation analysis between temperature and (a) COD (acetate) uptake rate (mg-COD/g-VSS/h) and (b) P-release rate/COD-uptake rate (mol-P/mol-C).

GAM to PAM. This result is different from that reported by Lanham et al. (2013). It should be noted that Lanham et al. (2013) collected samples in separate time periods and used the average temperature of each sampling period for correlation analysis, which might overlook some details. In comparison, in our study, we adopted a continuous sampling approach from Feb to June and utilized daily temperature recordings for the correlation analysis in this study, offering a more comprehensive and detailed assessment. Our results suggest that temperature could play a critical role in changing the microbial community and subsequently altering the C uptake pathway within a specific temperature range (i.e., 14–18°C) in the tested S2EBPR system of this study. Nevertheless, the impact of temperature on the process might be diminished when it falls outside of that range or when other critical factors, such as the carbon source, undergo significant variation.

Aerobic P uptake can be used to assess the abundance and activity of PAOs in the system (Izadi et al., 2020). P uptake rate of 2.4–9.7 mg-P/g-VSS/h and around 4.4 mg-P/g-VSS/h was reported for the full-scale and laboratory-scale EBPRs with enriched PAOs, respectively (Gu et al., 2008; Puig et al., 2008). However, a lower P uptake rate, ranged from 0.8 to 2.2 mg-P/g-VSS/h, was measured in the WRRF studied in this work. This could be attributed to the lower abundance of PAOs and reduced activity. Gu et al. (2008) reported that the disturbance of the balance between intracellular energy and PHA synthesis in PAOs due to the P release during sampling transfer can result in lower P uptake (Gu et al., 2008). Understanding the PAOs mechanisms in the P uptake and the factors affecting P uptake rate could clarify the reason behind the lower P uptake and prevent the EBPR from failure.

The operational conditions at the tested WRRF vary between weekdays and weekends. During the weekend, there is a higher influent flow rate occurs, which could potentially decrease the hydraulic residence time (HRT) in the reactor basins, from 16.1 hours (at 6.3 MGD) on the weekdays to 14.4 hours (7.5 MGD) on weekends due to higher flow rates. In addition, although the gravity

thickeners continuously receive primary sludge and WAS 7 days per week, the following unit, the dewatering facility, only operates during the weekdays. Therefore, when the dewatering facility is not in operation on the weekend, the thickener overflow is pumped back to the head of the plant, and the thickened solids are stored in the solid blend tank. Although the TP concentration in the influent did not show a significant change from weekday (5.39 ± 1.08 mg/L) to weekend (5.73 ± 1.19 mg/L), a higher OP loading of 33.9 ± 5.3 kg-P/day (caused by overflow from thickener unit) was experienced on the weekend in comparison to 14.0 ± 2.3 kg/day during the weekdays. Therefore, it is crucial to examine whether the thickener overflow that is routed to the head of the WRRF on the weekends can have an impact on the PAOs within the reactor basins. Eleven and eight samples were taken on weekdays and weekends, respectively, and the results (shown in Figure 2b) were compared to investigate the changes of the PAOs activity. The P release rate did not change significantly from weekdays to weekends (*p-value* > 0.05), while the plant data showed a stable P removal efficiency over weekends (96.4 ± 2.0%) and weekdays (96.9 ± 2.4%). These results suggest that the biological P removal process is resistant against changes in HRT and loading variations caused by alterations in the dewatering process and increased TP concentration from the thickener overflow. Additionally, there was no change in the dosage of the MicroC® 3000 in the plant over weekends compared to weekdays, which also indicates the resistance of the S2EBPR against the temporal changes. Deterioration of the EBPR process was reported by Brdjanovic et al. (1998) after heavy rainfall or higher hydraulic loading during weekends. These types of events can cause dilution and result in lower available C sources and thus starvation of PAOs (Brdjanovic et al., 1998). In another study, Henze et al. (1995) highlighted the negative impact of prolonged exposure to stormwater on the efficiency of the EBPR process. The reason was either due to the lower activity of PAOs or the COD in the influent after dilution with stormwater (Henze et al., 1995).

Correlation analysis

To investigate the change of COD uptake rate and P-release/COD-uptake over different temperatures, correlation analysis was performed (Figure 4a,b). The results showed a strong positive correlation ($r = 0.88$) between temperature (14 to 18°C) and COD uptake rate (3.2–8.2 mg-COD/g-VSS/h). However, when the temperature is >18°C, the COD uptake rate seemed to level off was maintained in the range of 4.9–7.1 mg-COD/g-VSS/h. This result is consistent with previous work in which a higher temperature caused an alteration in the dominant groups of microorganisms within the microbial community in bench-scale SBRs (Panswad et al., 2003). Panswad et al. (2003) found that raising the temperature from 20°C to 30–35°C changed the dominant groups of microorganisms in the system from PAOs (20°C) to GAOs (30°C) and to ordinary heterotrophs organisms (OHOs) (35°C), respectively (Panswad et al., 2003). Higher COD uptake rates that are associated with temperature increases due to seasonal variation could highlight the contribution of co-existing heterotrophs to COD consumption within the microbial community and potential competition for organic substrate. The correlation between temperature and P-release rate/COD-uptake rate showed a strong negative correlation ($r = -0.83$) between temperature (14 to 18° C) and P-release rate/COD-uptake rate (0.58–0.28 mol-P/mol-C) (Figure 4b). Previous results from Oehmen, Zeng, et al. (2005) indicated the ratio of the P-release rate/COD-uptake rate of 0.5 as the PAO metabolism model in acetate-fed systems (Oehmen, Zeng, et al., 2005). According to Yu et al. (2021) results, the ratio of the P-release rate/COD-uptake rate, when <0.5 mol-P/mol-C, can be attributed to the PAO-GAO mixed metabolism. This may lead to a change in the balance of the dominant species within the microbial community or potential proliferation of denitrifying bacteria that can compete with PAOs for acetate (Yu et al., 2021). In this study, the P-release rate/COD-uptake rate of PAOs decreased as the temperature of the reactor basins increased, indicating a decline in PAM dominance within the system. However, the effects of temperature on the dominant metabolic pathway at higher temperatures (>18°C) were diminished, and the calculated P-release rate/COD-uptake rate was in the range of 0.40–0.79 mol-P/mol-C. A gradual increase in the P-release rate/COD-uptake rate was observed at temperature >18°C, indicating potential adaptation of the PAO to sustain PAM in the system. This was consistent with the decreasing COD uptake rate at temperature >18°C (Figure 4a**)**. These findings imply that the uptake and degradation of organic substrates during the anaerobic phase may be regulated by different microbial groups under varying temperature conditions. Temperature fluctuations or shocks caused by seasonal changes and/or weather anomalies can potentially lead to the dominance of heterotrophic microorganisms over PAOs, consequently reducing the abundance of PAOs in the biological P removal process. Unfortunately, controlling the temperature to maintain the abundance of PAOs dominant is not applicable in full-scale WRRFs. Further investigation of the microbial community in the biological P removal process may provide insights into the competitiveness of various carbon-consumers at different

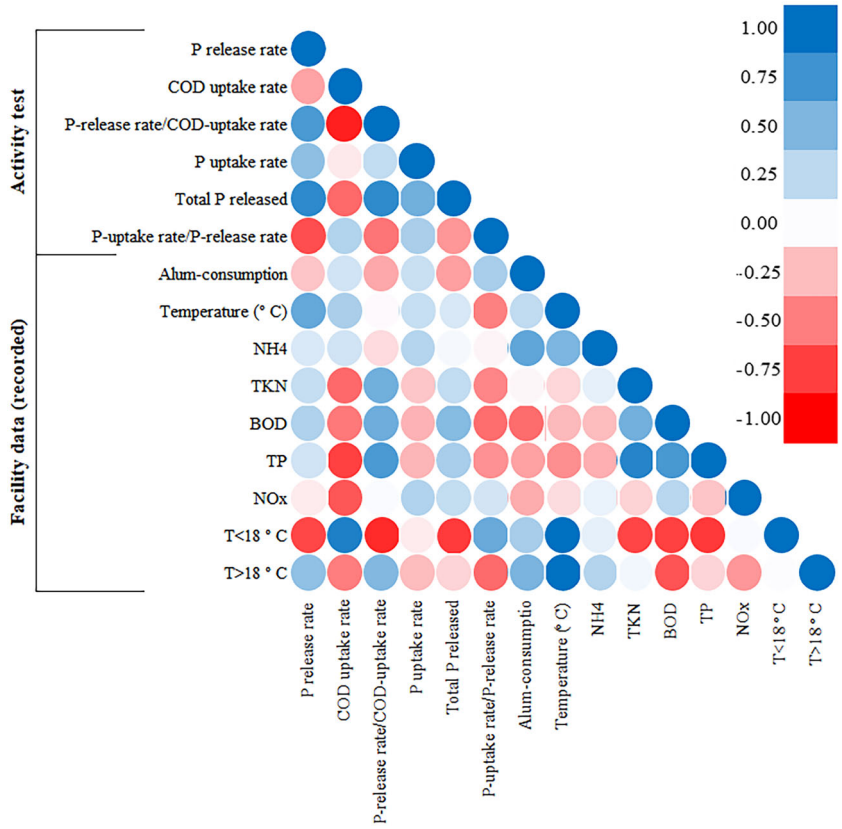

FIGURE 5 Pearson correlation analysis between the calculated kinetic parameters and the operational parameters. The colors represent the correlation coefficient, increasing from blue (positive) to red (negative). A strong relationship is concluded if the value is +1 to +0.7 (positive correlation) or −1 to −0.7 (negative correlation). Other values between −0.7 and +0.7 indicate a weak relationship.

operational conditions. This understanding can facilitate the optimization of the carbon supplementation strategy in each phase to prevent the growth of competitive organisms for carbon.

To elucidate the correlation between the calculated kinetic parameters and the operational parameters that may affect the EBPR performance, Pearson correlation analysis was conducted (Figure 5). The assessed operational data included alum dosing, temperature, influent characteristics (e.g., NH_4^+, TKN. TP, biological oxygen demand [BOD], NO_2^-, and NO_3^-), and rainfall. Results showed that the activity of PAOs is decreasing as the total P released and COD consumption are negatively correlated (lower total P released while higher COD consumed). This was shown by the lower P release rate concurrent with less total P released to the system, and higher COD consumption rate in the anaerobic phase. Studies by Wang et al. (2016) and Pijuan et al. (2008) have indicated that the higher P release rate and the total amount of the P released during the anaerobic phase could be an indicator for higher PAO activity (Pijuan et al., 2008; Schauer et al., 2019; Wang et al., 2016). For example, Schauer et al. (2019) concluded that in a well-functioning EBPR (without VFA limitation), the initial P release rate was positively correlated to the abundance of PAOs in the system (Schauer et al., 2019). They also reported that a total P release/COD uptake ratio between 0.5 and 0.75 mol-P/mol-C could indicate a PAO dominant microbial community, whereas lower values indicated lower abundance.

Seasonal changes were shown to affect the pathway to consume COD (Figure 3), therefore showing the potential to impact the performance and stability of the biological P removal process. This is supported by the Pearson correlation analysis. As shown in Figure 5, the total amount of the P released to the system was negatively correlated ($r = -0.76$) with temperatures <18°C. Previous studies reported that temperatures >20°C can create unfavorable conditions for an efficient EBPR as higher temperatures may benefit the activity and growth of other C-consumers that can compete with PAOs for limited resources and consequently reduce the abundance of PAOs within the system (Liau et al., 2015; Panswad et al., 2003). Our results confirmed the negative effects of the temperature on PAOs activity between 14°C and 18°C (Feb to May) while the P release rate decreased, COD uptake increased, and the total amount of P decreased. However, a slightly moderate positive correlation between P release and temperature >18°C can be potentially due to the diminished impacts of temperature on the activity of PAOs outside of the 14–18°C temperature range. Consistent with previous results, the results from this study suggest that the competition between other heterotrophic bacteria with PAOs for C

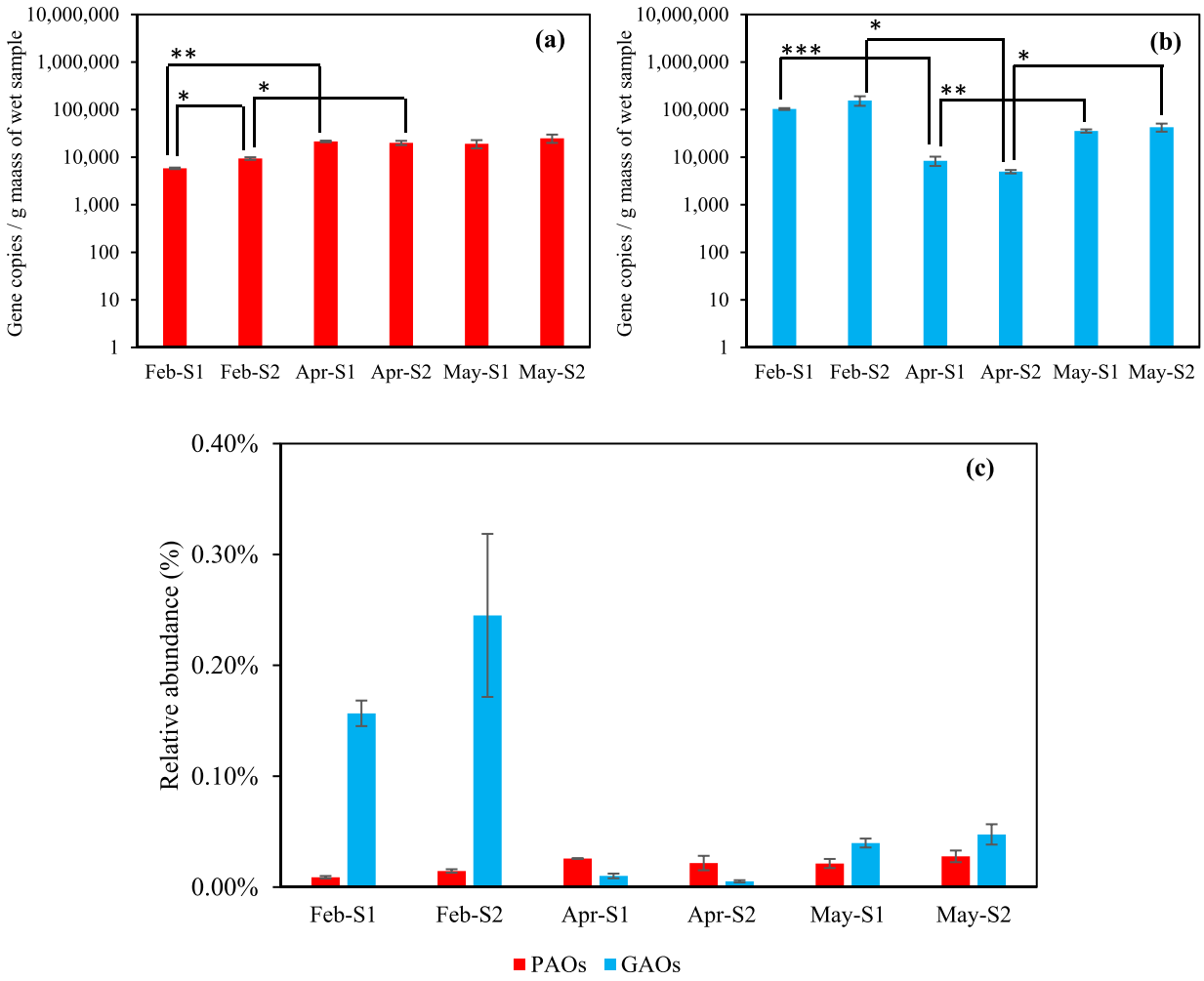

FIGURE 6 Abundance of (a) polyphosphate accumulating organisms (PAOs) and (b) glycogen accumulating organisms (GAOs) at two locations in the reactor basin (S1 and S2). (c) Relative abundance of PAOs and GAOs per total 16S gene copies. Significant differences showed with * (*p-value* < 0.05), ** (*p-value* < 0.01), and *** (*p-value* < 0.001).

consumption will increase as temperature increases. Meanwhile, the effects of system scale, acclimation period and composition of the original microbial community should be accounted into the comparison as they might affect the temperature range that PAOs might perform their highest potential activity.

Microbial community analysis

Sludge samples from RB-2 were analyzed to determine the abundance of PAO and GAO and monitor their activity (Figure 6), while the abundance qPCR results showed total number of bacteria was similar in all samples (Figure S5). A significant increase of PAOs' abundance was observed as the temperature increased from 15.2°C to 17.1°C. When comparing the abundance of PAOs in April and May, non-significant change was observed. Considering the reduced P-release rate (from 3.4 to 2.0 mg-P/g-VSS/h) as temperature increased (from 14 to 18°C) concurrent with higher abundance of PAOs, it can be concluded that the presence of PAOs does not guarantee a high activity. Our activity tests showed an increased COD consumption rate (from 3.2–8.2 mg-COD/g-VSS/h) at higher temperatures (from 14 to 18°C). However, the results, including lower PAOs activity (as illustrated by the lower P-release rate in Figure 5) and a diminished GAOs population (as shown by lower GAOs abundance in Figure 6b,c), suggest that neither PAOs nor GAOs had the potential to be responsible for higher COD demand. This implies the possible involvement of other heterotroph microorganisms in COD consumption at higher temperatures. However, it should be also noted that this study exclusively focused on Ca. *Accumulibacter* for the examination of PAOs and Ca. *Competibacter* for the investigation of GAOs and may have overlooked the contribution of other PAOs and GAOs species within the system. Additionally, it should be noted the relative

abundance of these species within the system (Figure 6c) was lower than the literature. For example, Winkler et al. (2011) estimated the relative abundance of PAOs as 0.9%–24.5% and GAOs as 0.2%–11.5% in their PAO-enriched lab-scale SBRs. In a study by Gu et al. (2008), microbial community of six full-scale WRRFs was analyzed and three of them showed the abundance of PAOs (Ca. *Accumulibacter*) as 5 ± 5%, one was 10 ± 5%, and two with 15 ± 5%. Additionally, they measured the abundance of Ca. C*ompetibacter* as <1% in three facilities, 5 ± 5% in two facilities and 15 ± 5% in one facility. These results indicate that although the batch bioassays conducted in this study showed the presence of active PAO, the tested WRRF has a limited enrichment of PAOs in the S2EBPR process. This suggests that the operational conditions of the S2EBPR need enhancement to bolster the competition of PAOs against other competitors, aiming for a more stable and efficient biological P removal. Recently, Farmer et al. (2023) reported that the relative abundance of *Tetrasphaera* and *Dechloromonas* was as high as Ca. *Accumulibacter* in their monitoring of the microbial community of a full-scale S2EBPR system (Farmer et al., 2023). However, their study was published after our microbial community analysis was concluded. In another full-scale WRRF, *Defluviicoccus* and Ca. *Competibacter* were found to be the dominant types of GAO (Qiu et al., 2019), while Qiu et al. (2022) showed that *Defluviicoccus* and Ca. *Competibacter* were present at all sampling points from a laboratory-scale SBR during 315 days of operation (Qiu et al., 2022). In a laboratory-scale simultaneous EBPR and semi-nitritation process, the presence of other bacterial groups such as *Nitrosomonas* (0.02%), *Bacillus* (0.46%), *Azoarcus* (0.11%), were observed together with Ca. *Accumulibacter* and Ca. *Competibacter* (0.19%) (Yuan et al., 2020). These results document that the newly identified group of GAO *Defluviicoccus* and Ca. *Competibacter* are important for biological P removal despite their low (<1%) relative abundance in the microbial community. Additional microbial community analysis including other types of PAOs, GAOs and OHOs will be helpful in clarifying the relationship between COD uptake and microorganisms' abundancies.

Feasibility assessment

The feasibility assessment was performed to provide insights into the costs and benefits of a potential transition to S2EBPR at the WRRF in this study. Three treatment scenarios were selected for this assessment: (1) chemical precipitation (with 0% biological P removal), (2) conventional EBPR (with equal parts of biological P removal and chemical P precipitation), and (3) S2EBPR (with 81% biological P removal). The assessment compared the cumulative costs, including the operation and maintenance costs, and capital costs associated with each technology's application in the WRRF. The results showed that chemical precipitation was the most economically infeasible P removal technology due to the high costs of chemical agents. Compared to the conventional EBPR, the implementation of the S2EBPR technology requires a higher initial investment, primarily for installation of the new fermenter and the new piping system. The installation of a fermenter for the Parkway WRRF in 2020 incurred a cost of $400 K, including expenses for retrofitting the fermenter, as well as adding piping, mixers, pumps, and other miscellaneous items. Based on the economic analysis (Figure 7), the costs of the construction (capital cost) of the fermenter can be compensated after five and 11 years of operation compared to chemical precipitation and conventional EBPR, respectively. An example of the calculation is provided in Supporting Information S3.

A sensitivity analysis was also performed to assess the effect of the P removal efficiency, potential benefits from selling the P-rich sludge in the future, as well as P discharge permit on the process cost, with the results provided in Figure 8a,b, and Supporting Information S6, respectively. The P removal efficiency of 81% detected in the reactor basins of the Parkway WRRF in this study was used as the biological P removal efficiency of S2EBPR. A 10% increase in P removal efficiency using optimization of the process and without additional costs can lead to a 58% reduction in cumulative costs over a 15-year operational period. Conversely, a decrease in efficiency to 71% would result in a 31% increase in costs. This highlights the potential benefits of S2EBPR if optimal conditions for improved P removal are ensured, while also emphasizing the technology's resilience in preventing significant cost increases during periods of reduced performance or operational disruptions such as the temperature effect that is discussed in previous sections. As P is a non-renewable resource, there is potential for benefits from selling P-rich sludge. In such case, the revenue generated from sludge selling can reduce the total operational costs of the S2EBPR. As an example, if 100% of the generated sludge in this full-scale S2EBPR is sold, it can lead to a 26% decrease in operational costs compared to the current technology that does not sell P-rich sludge. These findings also underscore the cost-benefits analysis of S2EBPR over a 15-year period, especially in response to the requirements of next generation WRRF for resource recovery and stricter regulation on P effluent limits (Figure S6). However, it is worth mentioning that the sensitivity

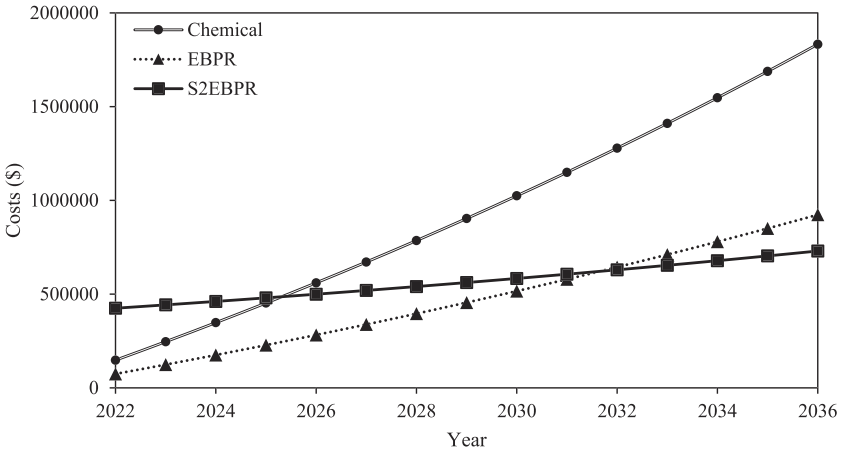

FIGURE 7 Accumulative costs of three P removal technologies in the Parkway water resource recovery facility (WRRF): (1) chemical precipitation, (2) enhanced biological phosphorous removal (EBPR), and (3) side-stream enhanced biological phosphorous removal (S2EBPR).

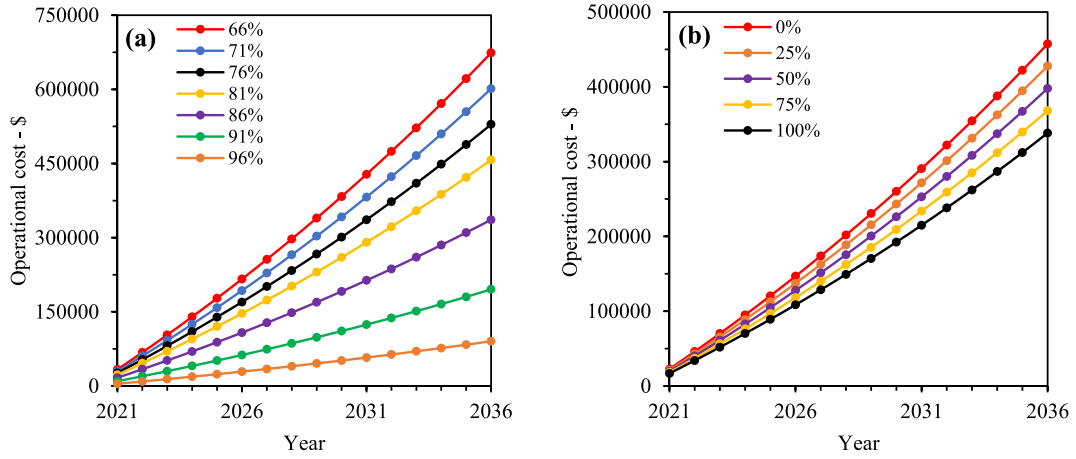

FIGURE 8 Sensitivity analysis of operational costs for (a) different biological P removal efficiencies and (b) different percentage of P-rich sludge selling within the side-stream enhanced biological phosphorous removal (S2EBPR) technology.

analysis was conducted without factoring in the operational costs associated with the actions taken by the WRRFs to modify phosphorus removal efficiency and/or attain the target effluent phosphorus concentration. Depending on the system scale, process adaptability, and strategy selection, these costs can indeed be substantial, especially when striving for an exceptionally high level of P removal or low level of P concentration required for discharging. Therefore, it may be necessary for WRRFs to conduct additional cost analysis to quantify this marginal operational cost and incorporate it with the results from this work.

CONCLUSION

A WRRF implementing the S2EBPR configuration was assessed for PAO activity, abundance, and relationship with seasonal/operational parameters over a five-month sampling period. The results indicated an insignificant difference in PAO activity among reactor basins operated in parallel mode and between weekends/weekdays when different HRTs and P loadings were applied. The study also showed that observed PAO activity was found to be higher at lower temperatures, but lower compared to other EBPR processes reported in literature. Furthermore, PAOs activity, and microbial community analysis did not show a strong relationship between the abundance and activity of PAO in the tested WRRF. The Pearson correlation analysis revealed a positive correlation between COD uptake and bioreactor basin temperature within the range of 14–18°C. However, no dominant GAO metabolism in C consumption pathways was found, and microbial community analysis did not show an increased GAOs abundance, suggesting the potential involvement of other heterotrophic microorganisms at higher temperatures. The cost and benefit assessment of the S2EBPR

in comparison to other technologies can provide insights for facilities currently employing conventional EBPR, chemical precipitation, or a combination. These findings may encourage exploration of potentially retrofitting existing systems and transition toward a more environmentally sustainable approach for P removal, where the recovered P can be utilized on for instance farmland. However, it should be noted that this research was mainly focused on the activity of conventional PAOs and their contribution to the P removal in the full-scale S2EBPR, further efforts should be conducted to investigate the presence of DPAOs and their role in the system. The outcomes of this study are informative for decision-makers and policy developers by providing information about the critical role of seasonal and operational parameters in PAO activity and P removal. Furthermore, these findings contribute to enhancing the current understanding of applying S2EBPR in full-scale WRRFs to address current challenges such as insufficient C source and promote an efficient, sustainable, and cost-effective technology.

AUTHOR CONTRIBUTIONS

Khashayar Aghilinasrollahabadi: Investigation; methodology; writing; visualization; and conceptualization. **Shahrzad Saffari Ghandehari:** Investigation; methodology; and writing. **Birthe Veno Kjellerup:** Conceptualization; methodology; writing; validation; and supervision. **Caroline Nguyen:** Conceptualization; methodology; and writing. **Yerman Saavedra:** Conceptualization. **Guangbin Li:** Conceptualization; methodology; writing; visualization; validation; and supervision.

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CONFLICT OF INTEREST STATEMENT

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 STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information data associated with this article can be found in the online version.

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