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The impact of pH on the anaerobic and aerobic metabolism of Tetrasphaera-enriched polyphosphate accumulating organisms

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ABSTRACT

Members of the genus *Tetrasphaera* are putative polyphosphate accumulating organisms (PAOs) that have been found in greater abundance than *Accumulibacter* in many full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plants worldwide. Nevertheless, previous studies on the effect of environmental conditions, such as pH, on the performance of EBPR have focused mainly on the response of *Accumulibacter* to pH changes. This study examines the impact of pH on a *Tetrasphaera* PAO enriched culture, over a pH range from 6.0 to 8.0 under both anaerobic and aerobic conditions, to assess its impact on the stoichiometry and kinetics of *Tetrasphaera* metabolism. It was discovered that the rates of phosphorus (P) uptake and P release increased with an increase of pH within the tested range, while PHA production, glycogen consumption and substrate uptake rate were less sensitive to pH changes. The results suggest that *Tetrasphaera* PAOs display kinetic advantages at high pH levels, which is consistent with what has been observed previously for *Accumulibacter* PAOs. The results of this study show that pH has a substantial impact on the P release and uptake kinetics of PAOs, where the P release rate was >3 times higher and the P uptake rate was >2 times higher at pH 8.0 vs pH 6.0, respectively. Process operational strategies promoting both *Tetrasphaera* and *Accumulibacter* activity at high pH do not conflict with each other, but lead to a potentially synergistic impact that can benefit EBPR performance.

1. Introduction

Enhanced biological phosphorus removal (EBPR) technology is a sustainable and cost-effective P removal process within wastewater treatment plants (WWTPs). It relies on cyclic anaerobic and aerobic conditions to select for polyphosphate accumulating organisms (PAO) that accumulate P in excess of metabolic requirements, storing it intracellularly as polyphosphate. The most well-known PAO is *Candidatus Accumumlibacter*, however, increased attention has been placed on a promising putative PAO group, *Tetrasphaera*. Many full scale WWTPs (especially those employing sidestream fermentation) have detected higher *Tetrasphaera* abundances (up to 35% in biomass) than typical *Accumulibacter* levels (Onnis-Hayden et al., 2020; Qiu et al., 2019; Stokholm-Bjerregaard et al., 2017).

Tetrasphaera is able to take up diverse organics such as amino acids and/or glucose and store these in various forms or produce other fermentation products such as volatile fatty acids (VFAs) (Kong et al., 2005; Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015, 2011). These storage products can provide carbon and energy sources for aerobic P uptake. *Tetrasphaera* can supply VFAs anaerobically for other PAOs (Herbst et al., 2019; Nguyen et al., 2015), allowing a synergistic interaction of organisms for P removal. Anaerobic PHA synthesis by *Tetrasphaera* was originally believed to be insignificant, but has since been found to be dependant on the *Tetrasphaera* clade composition (Close et al., 2021; Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015). Fernando et al. (2019) showed that while *Accumulibacter* have a higher specific P removal capacity, high *Tetrasphaera* abundances can lead to >50% of the P-removal in full-scale WWTPs. *Accumulibacter* metabolism and physiology have been well established, but *Tetrasphaera* metabolism and its response to different EBPR operational conditions are less understood (Liu et al., 2019; Rubio-Rincón et al., 2019b). Indeed, *Tetrasphaera* have a versatile physiology and a significantly different metabolism to *Accumulibacter*. Operational and environmental conditions can also affect *Tetrasphaera* abundance and

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metabolism (Barnard et al., 2017; Herbst et al., 2019; Onnis-Hayden et al., 2020; Qiu et al., 2019; Rey-Martínez et al., 2019). Improved understanding of *Tetrasphaera* is needed to better optimise EBPR, including understanding their response to operational conditions.

Domestic and industrial wastewaters contain varying chemicals and compounds that may each influence the pH. Furthermore, pH is a dynamic parameter that is impacted by multiple biological processes in WWTPs. The extracellular pH can influence the performance of EBPR systems significantly, including the anaerobic P release and aerobic P uptake rates, as well as the P release to carbon uptake ratio (Bond et al., 1999; Chen and Gu, 2006; Filipe et al., 2001a, 2001b; d; Jeon et al., 2001; Pijuan et al., 2004; Smolders et al., 1994; Liu et al., 1996). In some sequencing batch reactor (SBR) studies, P removal deterioration has been linked with low pH (Zheng et al., 2014). Several studies showed that elevated pH (> 7.25) benefits PAOs over their primary putative competitors, the glycogen accumulating organisms (GAOs), improving EBPR performance (Filipe et al., 2001a; Oehmen et al., 2005; Schuler and Jenkins, 2002).

The aforementioned EBPR studies fed VFAs as the sole carbon source, which leads to the enrichment of *Accumulibacter*, whereby the impact of pH on *Tetrasphaera* is unknown and represents a significant research gap for EBPR. Low-affinity Pit phosphate transporters that drive VFA uptake in *Accumulibacter* have also been found in *Tetrasphaera* (Herbst et al., 2019). This could suggest that extracellular pH may also affect *Tetrasphaera* carbon uptake and P release/uptake mechanisms. Considering the high abundance of *Tetrasphaera* in full-scale EBPR plants, and pH dynamics in WWTPs, it is important to understand the pH impact on *Tetrasphaera* metabolism to evaluate its impact on EBPR performance. This allows a more complete assessment of EBPR, as well as optimisation and retrofitting of WWTPs for more effective phosphorus removal (and subsequent recovery). Thus, the objective of the present study is to investigate the pH effect on *Tetrasphaera*, and its impact on the anaerobic and aerobic metabolism and kinetics in an EBPR process.

2. Results and discussion

2.1. SBR performance

The *Tetrasphaera* SBR was fed with Cas aa as the sole carbon source and was operated for about a two-month period prior to the batch tests at different pH levels. The purpose of this period was to ensure a good and stable reactor performance, with a P removal efficiency of 77.7 ± 10.1% from day 50 onwards (Fig. 1). This performance was maintained consistently in the SBR until the end of the study and could therefore be considered as steady-state operation. The typical organic carbon consumption, PHA, glycogen and phosphorus profiles of the SBR are shown in Fig. 2, while the anaerobic and aerobic activity across multiple cycles of operation is shown in Table S2, where it is compared with literature studies, in order to provide a basis for comparison with previous *Tetrasphaera* enrichments. The enriched culture displayed the traditional PAO phenotype of P release, glycogen hydrolysis, PHA synthesis and organic carbon uptake during the slow feeding period of the anaerobic phase, followed by P uptake, glycogen formation and PHA degradation in the aerobic phase. Note that the slow feeding period of the SBR led to a net increase in TOC over time in the anaerobic phase (Fig. 2).

From Table S2, the anaerobic P-release/C-uptake ratio observed in this study was consistent with Marques et al. (2017), higher than Close et al. (2021) and lower than typical *Accumulibacter* results (Table S2). A lower P-release/C-uptake ratio in these and other *Tetrasphaera* studies as compared to *Accumulibacter* studies is commonly observed (Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015; Nielsen et al., 2019; Stokholm-Bjerregaard et al., 2017), suggesting that *Tetrasphaera* may require less ATP from poly-P hydrolysis for anaerobic C-uptake. Furthermore, the higher abundance of *Accumulibacter* found in the *Tetrasphaera* enrichments of this study (Table 1, see Section 2.2 for detailed analysis of the microbial community) and Marques et al. (2017) as compared to Close et al. (2021), likely contributed to the comparatively higher P-release/C-uptake ratios. In the aerobic phase, 2.45 ± 0.57 P-mmol/L of phosphate was taken up, which is close to results from *Accumulibacter* (2.20 to 2.50 P-mmol/L) and much greater than the study of Close et al. (2021) which only reported 0.96 ± 0.39 P-mmol/L of P uptake, while Marques et al. (2017) found 1.76 ± 0.25 P-mmol/L of aerobic P uptake. It is notable that enrichments of *Tetrasphaera* that contain some *Accumulibacter* (this study; Marques et al. (2017)) have shown appreciably better P removal performance as compared to cultures that are highly enriched in *Tetrasphaera* alone, without *Accumulibacter* (Close et al., 2021). This result supports the hypothesis that enrichment of multiple PAO groups is indeed beneficial for EBPR performance. Despite the similar operational conditions applied to the enrichment SBR in each case, determining factors that cause a shift in *Tetrasphaera* clades were unclear, and were beyond the scope of the present study. Further research is warranted to better understand the cause of population shifts within this group of PAOs.

As shown in Table S1, higher average glycogen consumption/C-

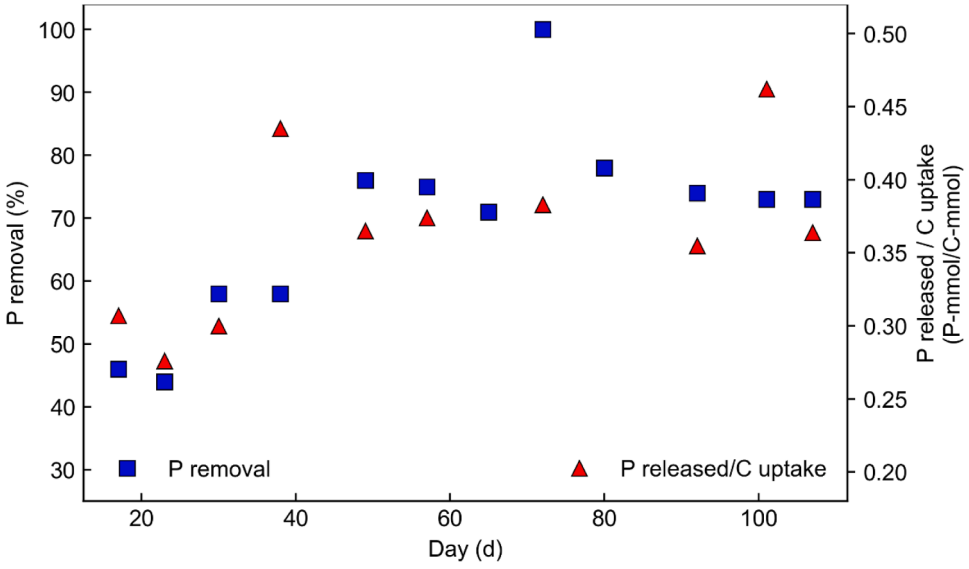

Fig. 1. Phosphorus removal (%) and anaerobic P-release/C-uptake ratio over time in the enriched SBR.

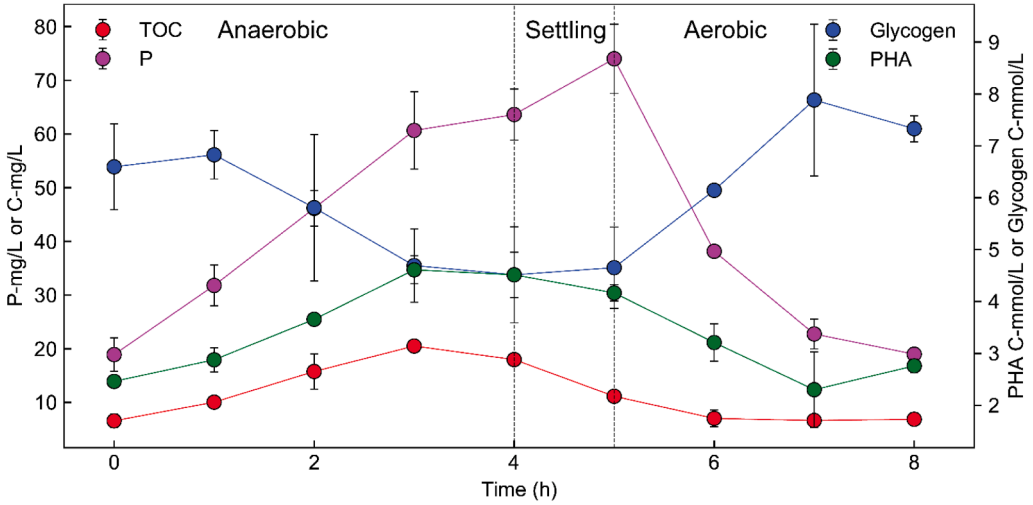

Fig. 2. Typical profiles of extracellular P-PO_4 and TOC, and intracellular Glycogen and PHA of the enriched SBR during the batch test period. The values are averages of cycle studies in day 49 and day 101. Between the dashed lines, the settling and decant phases occurred.

Table 1
Semi-quantitative FISH results obtained after visual analysis and classification of the communities.

Sample	PAOMIX	Tet1–266	Tet2–892	Tet2–174	Tet3–654
SBR	(++)	(+)(-)	(-)	(++)	(+++)
pH=6	(++)	(+) or (+)(-)	(-)	(++)	(++)
pH=7.5	(++)	(+) or (+)(-)	(-)	(++)	(++)
pH=8	(++)	(+) or (+)(-)	(-)	(++)	(+)
pH=6.5	(++)	(+)(-)	(-)	(++)	(+)
pH=7	(++)	(+)	(-)	(++)	(++)

(-) Not detected (0%);.
(+)(-) Very low abundance (1–5%);.
(+) Present (5–25%);.
(++) Abundant (25–50%);.
(+++) Dominant (>50%).

uptake (0.51 ± 0.17 C-mmol/C-mmol) and PHA production/C uptake (0.52 ± 0.13 C-mmol/C-mmol) were observed as compared to those obtained in the study of Close et al. (2021) and Marques et al. (2017). A similar level of glycogen production hydrolysis, but a lower level of PHA accumulation per carbon mole uptake were observed as compared with previous *Accumulibacter* studies (Table S1). The higher PHA storage by the present enriched culture could be due to either a higher fraction of PHA-storing *Tetrasphaera,* a higher *Accumulibacter* fraction, or both (see Section 2.2 for analysis of the microbial community). Through FISH analysis, Tet2–174 (25–50%) and Tet3–654 (>50%) were the two most abundant *Tetrasphaera* clades in this study, while Close et al. (2021) mainly found Tet2–892 (59.5 ± 10.7%) and Tet3–654 (24.1 ± 4.9%) and Marques et al. (2017) found Tet1–266 (38.5 ± 1.4%) and Tet3–654 (31.0 ± 4.3%). Close et al. (2021) proposed that PHA-storing *Tetrasphaera* may be more prominent within clade 2, which is consistent with the results of this study. Also, *Accumulibacter* has a higher PHA production /substrate uptake ratio (Table S2) than *Tetrasphaera. Accumulibacter* abundance is 25–45% in this study, which is higher than the 22% observed by Marques et al. (2017) and the <0.5% observed by Close et al. (2021). The high fraction of *Accumulibacter* in this study is also likely to have contributed to the higher PHA storage.

The nature of the carbon source (casein hydrolysate in this study) fed to the SBR is known to have a significant influence on PHA storage by PAOs, where a much higher PHA storage is observed through the uptake of VFAs, such as acetate and propionate (Marques et al., 2017). As also shown in Table S1, PHV was the most produced fraction of PHA, accounting for 74.7 ± 6.3%, whereas the amount of PHB formed was only 25.3 ± 6.3%. A similar PHA fractionation was reported in Marques et al. (2017), which also showed the presence of some *Accumulibacter* in their *Tetrasphaera* enrichment. *Accumulibacter* are known to produce mainly PHB from fermentation products such as acetate (Smolders et al., 1994). However, PHV accounted for 100% of the produced PHA in Close et al. (2021), which had a very high *Tetrasphaera* enrichment without *Accumulibacter*. This study supports the hypothesis that PHV is the major PHA fraction that is produced within *Tetrasphaera*, where the observed PHB production could be formed by the *Accumulibacter* fraction of the sludge.

Overall, the *Tetrasphaera*-enriched SBR in this study displayed similar P release and uptake behaviour as compared to previous studies (Close et al., 2021; Marques et al., 2017), with slightly higher PHA and glycogen storage, perhaps related to the higher *Accumulibacter* fraction of the present study. The consistency of this reactor performance as compared to literature suggests a successfully enriched *Tetrasphaera* reactor culture that was suitable for subsequent analysis of the impact of pH on their anaerobic and aerobic stoichiometry and kinetics.

2.2. Microbial analysis

Semi-quantitative FISH analysis indicated the presence of both PAO groups, *Tetrasphaera* and *Accumulibacter*, in the enriched culture as shown in Table 1. FISH samples were analysed from the enrichment reactor during the batch experiment period (day 65 and day 101) as well as from samples taken during the batch tests (at the end of the batch tests). Figure S1 shows representative FISH images of the most dominant PAOs observed. From FISH analysis, *Tetrasphaera* comprises a volume fraction of >50% of the total bacterial biovolume (Table 1 and Figure S1), which was slightly higher than the values observed with high throughput sequencing (37 – 43.5% of the total 16S rRNA gene reads analysed corresponded to *Tetrasphaera* – see Table 2). Within all organisms observed through 16S rRNA gene sequencing, *Tetrasphaera* represented the largest group. Thus, both FISH and 16S rRNA analysis indicate that *Tetrasphaera* dominated the SBR culture and batch tests. The dynamics of *Tetrasphaera* abundance was relatively low in this work as compared to the dynamics observed in most biological wastewater treatment studies, with only some fluctuation observed between the batch tests using probe Tet3–654 according to FISH.

The average volume fraction of *Accumulibacter* was 25% to 45% biovolume from FISH analysis. Marques et al. (2017) had previously detected 22% *Accumulibacter* in an enriched *Tetrasphaera* culture also fed with amino acids as the carbon source. It is likely that *Accumulibacter* can survive on fermentation products, such as VFAs that have been found to be produced by *Tetrasphaera* under anaerobic conditions (Close et al., 2021; Herbst et al., 2019; Kong et al., 2008; Nguyen et al., 2015; Nielsen

Table 2
The 15 most abundant bacteria identified by 16S rRNA gene amplicon sequencing of the cultures enriched in the SBR on days 65 and day 92 and in the batch test at pH 7. The seeding sludge was also analysed and is presented for comparison.

	Enriched SBR (day 92_05/12)		Enriched SBR (day 65_08/11)		Seeding culture			Batch test, pH=7		
	Phylum	**%**	**Phylum**	**Genus**	**%**	**Phylum**	**Genus**	**%**	**Phylum**	**Genus**	**%**
1	Actinobacteria	**42.2**	Actinobacteria	***Tetrasphaera***	**38.8**	Actinobacteria	***Tetrasphaera***	**41.2**	Actinobacteria	***Tetrasphaera***	**36**
	Actinobacteria	6.4	Saccharibacteria	*Candidatus Saccharimonas*	12.1	Actinobacteria	*Leucobacter*	10.5	Saccharibacteria	*Candidatus Saccharimonas*	13.9
2	Bacteroidetes	5.1	Actinobacteria	*Leucobacter*	8.8	Saccharibacteria	*Candidatus Saccharimonas*	7.2	Actinobacteria	*Leucobacter*	8.9
3	Saccharibacteria	3.7	Bacteroidetes	*Flavobacterium*	4.1	SHA−109	*p_SHA−109_OTU_33*	2.7	SHA−109	*p_SHA−109_OTU_33*	3.2
4	Actinobacteria	3.5	Chloroflexi	*Chloronema*	2.5	Bacteriodetes	*Terrimonas*	2.2	Betaproteobacteria	*Propionivibrio*	2.4
5	Gammaproteobacteria	3	Betaproteobacteria	*Propionivibrio*	2.3	Chloroflexi	*o_C0119_OTU_82*	2.2	Actinobacteria	*c_Actinobacteria_OTU_43*	2.2
6	Chloroflexi	2.4	Betaproteobacteria	*Ottowia*	2.3	Betaproteobacteria	***Candidatus Accumulibacter***	**2**	Bacteriodetes	*Terrimonas*	2.1
7	Betaproteobacteria	2.2	Actinobacteria	*f_Dermatophilaceae_OTU_105*	1.8	Alphaproteobacteria	*Ochrobactrum*	2	Firmicutes	*Fusibacter*	2
8	Betaproteobacteria	2.1	Gammaproteobacteria	*CPB_C22&F32*	1.6	Betaproteobacteria	*Propionivibrio*	2.1	Alphaproteobacteria	*Ochrobactrum*	2
9	Actinobacteria	1.5	Bacteriodetes	*Terrimonas*	1.5	Actinobacteria	*c_Actinobacteria_OTU_43*	2.1	Bacteroidetes	*Flavobacterium*	1.9
10	Alphaproteobacteria	1.5	Chloroflexi	*Candidatus Defluviflum*	1.4	Bacteroidetes	*Flavobacterium*	1.5	Firmicutes	*Acidaminobacter*	1.6
11	Alphaproteobacteria	1.5	Chloroflexi;	*Candidatus Chloroploca*	1.4	Firmicutes	*Acidaminobacter*	1.3	Actinobacteria	*Propioniciclava*	1.2
12	Deltaproteobacteria	1.4	Actinobacteria	*c_Actinobacteria_OTU_43*	1.3	Chloroflexi	*Candidatus Defluviflum*	1.3	Alphaproteobacteria;	*Chelatococcus*	1.2
13	Betaproteobacteria	**1.3**	Alphaproteobacteria	*Sphingomonas*	1.2	Alphaproteobacteria;	*Chelatococcus*	1.2	Betaproteobacteria	***Candidatus Accumulibacter***	**1.1**
14	Alphaproteobacteria	1.3	Deltaproteobacteria	*Desulfovibrio*	1.1	Alphaproteobacteria	*Bosea*	1.2	Chloroflexi	*Candidatus Defluviflum*	1.1
15	Chloroflexi;	1.2	Alphaproteobacteria	*Chelatococcus*	1	Gammaproteobacteria	*Candidatus Competibacter*	1	Alphaproteobacteria	*Bosea*	1
	Total abundance of OTUs reported as PAOs (in bold)	**43.5**	**Total abundance of OTUs reported as PAOs (in bold)**		**38.8**	**Total abundance of OTUs reported as PAOs (in bold)**		**43.2**	**Total abundance of OTUs reported as PAOs (in bold)**		**37.1**

et al., 2012).

From high throughput sequencing results, GAOs from the genus *Propionivibrio* were detected at 2.1–2.4% of the microbial community (Table 2). Semi-quantitative FISH analysis results showed a similar estimate, constituting <5% of the biovolume (data not shown). *Propionivibrio* is commonly observed in full-scale EBPR plants at a similar level, constituting up to 3% of the microbial community (Albertsen et al., 2016).

Notably, high throughput sequencing data revealed a much lower abundance of *Accumulibacter* than was indicated by FISH analysis (Tables 1 and 2). Discrepancies between results from the amplicon-based method and FISH analysis have previously been attributed to (i) inefficient DNA extraction prior to high throughput sequencing, (ii) specificity of the FISH probes or incomplete probe coverage, or (iii) inconsistencies between biovolume measurements versus relative DNA abundance (Albertsen et al., 2012). In particular, this tendency has been frequently observed for *Accumulibacter*, where the abundance of *Accumulibacter* has been routinely estimated to be lower by sequencing as compared to FISH in other EBPR studies (Carvalho et al., 2021; Rubio-Rincón et al., 2019b; Valverde-Pérez et al., 2016). Quantitative links between *Accumulibacter* abundance as assessed by FISH and their metabolic activity have been well established in engineering-related EBPR studies (Acevedo et al., 2012; Kolakovic et al., 2021; Oehmen et al., 2010a, 2007; Rubio-Rincón et al., 2019a; Tu and Schuler, 2013; Zhang et al., 2021), whereas similar quantitative correlations have so far been less established when applying other microbial techniques (Oehmen et al., 2010b).

Although different in absolute numbers, both the FISH analysis and the 16S rRNA gene-based amplicon sequencing analysis showed *Tetrasphaera* were dominant in the enriched SBR around the time of the batch test period. Moreover, the dynamics of the microbial community over the operational period was not substantial, suggesting that the microbial community of the enriched SBR was in pseudo-steady-state.

2.3. Batch test results at different pH levels

Anaerobic–aerobic batch assays using casein hydrolysate as the sole C-source were performed at different controlled pH values, ranging from 6.0 to 8.0, using the sludge from the enriched SBR to investigate the impact of pH on *Tetrasphaera* and their metabolism. From Figures S2-S6, during the batch tests, Cas aa was taken up (reflected by the decrease of TOC), which was accompanied by phosphorus release and a small level of glycogen hydrolysis. PHA was oxidised in the subsequent aerobic phase, supplying the C and energy for biomass growth and replenishing the glycogen and polyphosphate pools.

In the pH range 6.0–8.0, the P release rate increased linearly from 2.56 ± 0.39 to 8.63 ± 0.79 mgP/gVSS/h (Table 3 and Fig. 3f). These values were significantly lower than the rates observed with *Accumulibacter* (61.5 to 88.8 mgP/gVSS/h in pH range from 6.5 to 8.5) (Oehmen et al., 2005). *Accumulibacter* also take up organic carbon much quicker than *Tetrasphaera* (Table 3). This higher rate of anaerobic activity is consistent with previous suggestions that *Tetrasphaera* possess a lower relative EBPR activity than *Accumulibacter* on a per-cell basis (Fernando et al., 2019).

The substrate uptake, PHA production and glycogen degradation rates were independent of pH over the range of 6.0 to 8.0 (Fig. 3d, 3b and 3c, respectively), which agreed with studies on PAOs fed with acetate (Filipe et al., 2001a; Smolders et al., 1994; Liu et al., 1996). The resulting P release/C-uptake ratio has a positive correlation with pH (Fig. 3a), which was likely because of additional energy requirements for taking up the negatively charged amino acid ions at higher pH against the negative electrical potential of the cells. *Tetrasphaera* are also capable of generating energy from the fermentation of amino acids, leading to the lower P release to substrate uptake ratios as compared to *Accumulibacter* metabolism that were observed in this study. This lower anaerobic P release also leads to a lower demand for storage compound

Table 3
Stoichiometric and kinetic metabolism comparison between an enriched *Tetrasphaera* + *Accumulibacter* reactor and *Accumulibacter* reactor during the batch tests at different pH values.

	Tetrasphaera + *Accumulibacter* (fed with Casein hydrolysate)					*Accumulibacter* (fed with propionate) (Oehmen 2005)				
pH controlled	6.0	6.5	7.0	7.5	8.0	6.5	7.0	7.5	8.0	8.5
Anaerobic										
P-release (P-mmol/L)	0.42 ± 0.01	0.91 ± 0.03	0.94 ± 0.09	1.03 ± 0.32	1.00 ± 0.16					
PHA production (C-mmol/L)	1.91 ± 0.79	1.91 ± 1.11	1.68 ± 0.47	2.17 ± 0.09	1.73 ± 0.64					
Glycogen consumption (C-mmol/L)	1.18 ± 0.01	1.35 ± 0.52	1.10 ± 0.15	1.29 ± 0.29	1.46 ± 0.07					
Substrate uptake rate (C-mg/gVSS/h)	11.07 ± 2.39	10.80 ± 2.61	12.18 ± 0.30	10.73 ± 0.13	12.29 ± 3.78	61.39	56.25	88.71	79.71	74.89
P-release rate (P-mg/gVSS/h)	2.56 ± 0.39	5.15 ± 0.09	6.36 ± 1.55	7.94 ± 1.15	8.63 ± 0.79	61.5	56.3	93.2	103.8	88.8
Aerobic										
PHA consumption (C-mmol/L)	1.3 ± 0.44	2.19 ± 0.68	2.07 ± 0.73	0.98 ± 0.79	1.11 ± 0.24					
P-uptake (P-mmol/L)	1.7 ± 0.05	2.58 ± 0.12	2.62 ± 0.14	2.57 ± 1.21	3.08 ± 0.04					
Glycogen production (C-mmol/L)	0.90 ± 0.14	1.05 ± 0.16	0.80 ± 0.43	1.29 ± 0.25	0.54 ± 0.07					
P uptake rate (P-mg/gVSS/h)	5.98 ± 0.89	8.27 ± 0.97	10.10 ± 0.71	10.82 ± 4.42	13.18 ± 0.29	23.9	25.6	37.7	36.2	27.3
Stoichiometry										
PHA production/substrate uptake (C-mmol/ C-mmol)	0.47 ± 0.19	0.41 ± 0.19	0.42 ± 0.13	0.59 ± 0.03	0.45 ± 0.16	1.24	1.23	1.10	0.95	1.01
PHA degradation rate (C-mmol/mg active biomass)	0.53 ± 0.06	0.75 ± 0.12	0.92 ± 0.28	0.59 ± 0.26	0.65 ± 0.13					
P release / substrate uptake (P-mmol/C-mmol)	0.10 ± 0.00	0.20 ± 0.01	0.23 ± 0.02	0.28 ± 0.08	0.26 ± 0.04	0.39	0.39	0.41	0.51	0.46
Glycogen degradation/substrate uptake (C-mol/ C-mol)	0.29 ± 0.01	0.29 ± 0.08	0.27 ± 0.05	0.35 ± 0.08	0.38 ± 0.01	0.36	0.32	0.25	0.12	0.15
P-uptake/PHA utilisation (P-mmol/C-mmol)	0.71 ± 0.32	0.5 ± 0.15	0.51 ± 0.2	1.5 ± 1.19	0.95 ± 0.20					
% P in TSS (%)	8 – 10	8 – 10	8 – 10	8 – 10	5 – 10					
PHB:PHV	31:69	31:69	32:68	26:74	37:63					

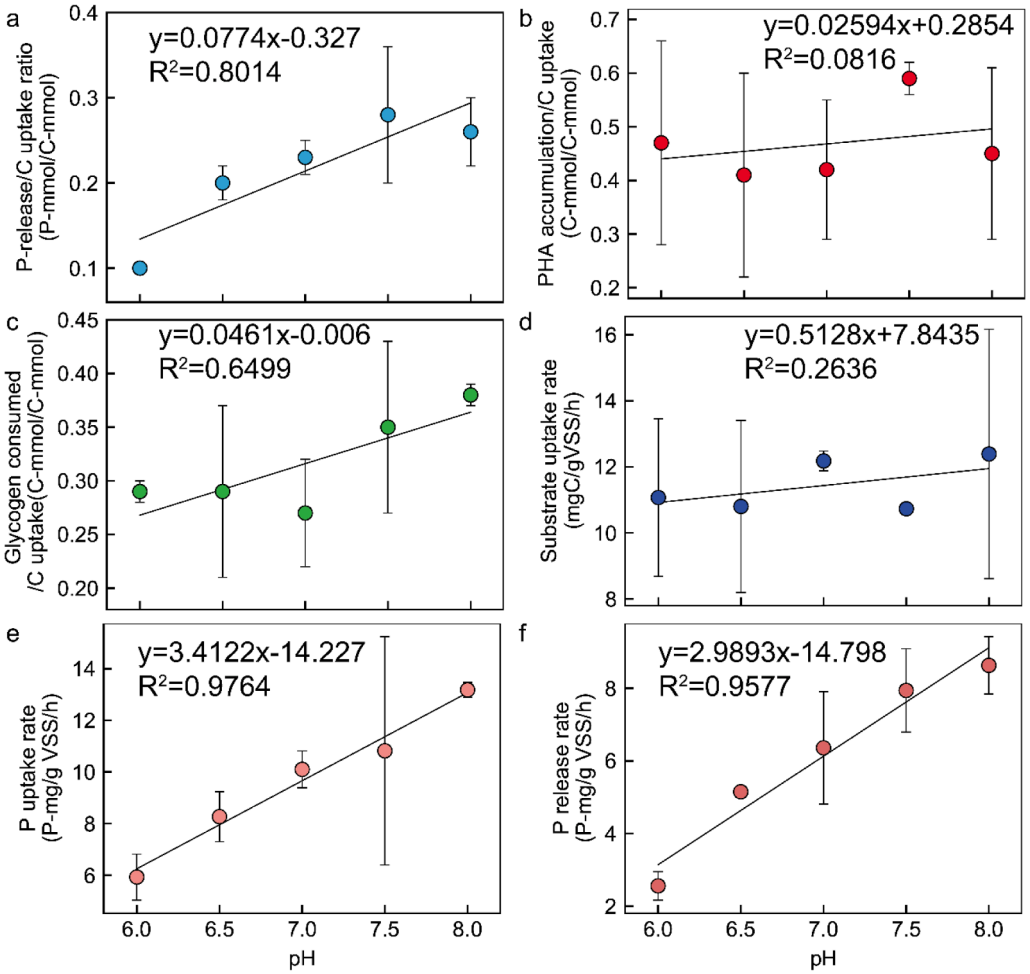

Fig. 3. Batch test results as a function of pH, showing (a) P-release/Carbon uptake ratio, (b) PHA synthesis/Carbon uptake ratio, (c) Glycogen consumption/Carbon uptake ratio, (d) Substrate uptake rate, (e) Phosphate uptake rate, (f) Phosphate release rate.

consumption in the aerobic phase to drive P uptake and achieve successful EBPR.

The linear increase in P release rate as a function of pH, coupled with the slight increase in the ratio of glycogen degradation per substrate uptake as a function of pH, shows that the energy for amino acid uptake by *Tetrasphaera* relies more on polyphosphate hydrolysis than glycogen degradation as pH increases. The main role of glycogen in *Tetrasphaera* metabolism is likely for generating reducing equivalents in order to close the redox balance of the cell (Close et al., 2021; Marques et al., 2017). The PHA/substrate uptake ratio was higher in this work as compared to previous studies on *Tetrasphaera* (Close et al., 2021; Marques et al., 2017), though lower than that typically observed for *Accumulibacter* (Table 3). The reasons could be attributed to the higher abundance of *Accumulibacter* in this study (according to FISH) as compared to previous studies (Close et al., 2021; Marques et al., 2017). The low PHA production/substrate uptake ratio as compared to *Accumulibacter* suggests that PHA is not the sole storage compound within *Tetrasphaera*. Close et al. (2021) found that glutamate and aspartate were important storage polymers during amino acid uptake by *Tetrasphaera*.

Similarly to what was observed in anaerobic conditions, in aerobic conditions, no clear correlation was observed between PHA utilization or glycogen production as a function of pH for *Tetrasphaera* (Table 3). However, the results in Fig. 3e show a clear linear increase in the P uptake rate as pH increases from 6.0 to 8.0 for the *Tetrasphaera*-enriched culture. An increase in P uptake at high pH was also observed in acetate and propionate-fed *Accumulibacter* PAOs between pH 6.5 and 7.5 (Filipe et al., 2001c; Oehmen et al., 2005). van Veen et al. (1993) have previously proposed that the rate of P release or P uptake in *Acinetobacter johnsonii* was limited by the rate of deprotonation of a protein carrier located on the outside or inside of the cell membrane, respectively, during the transport of P across the cell membrane. The rate of deprotonation of this carrier was proposed to be directly related to pH, causing higher P release and uptake rates at higher pH levels in *E. coli* and *Acinetobacter johnsonii* (van Veen et al., 1993; 1994). van Veen et al. (1994) suggested that this pH mechanism impacting P release and uptake may be generally applicable to all types of bacteria, giving examples of not only gram-negative bacteria such as *E. coli* and *Acinetobacter johnsonii*, but also gram-positive bacteria such as *Micrococcus lysodeikticus* and *Bacillus cereus*. The increased P release and uptake kinetics as a function of pH found in this study, enriched in gram-positive *Tetrasphaera*, also observed in previous studies focussed on gram-negative *Accumulibacter* (Filipe et al., 2001c; Oehmen et al., 2005), is consistent with the hypothesis of van Veen et al. (1994), and suggests that a similar pH impact on P release/uptake could be expected for all PAOs.

The net P removal from the batch tests was calculated as the difference between P uptake and P release, which revealed a significant increase from pH = 6 to pH = 8, where 2.08 ± 0.12 P mmol/L of net P removal was observed at pH = 8 and 1.28 ± 0.04 P mmol/L of net P removal was observed at pH = 6. For comparison, the pH=7 tests showed a net P removal of 1.68 ± 0.05 P mmol/L, where the tests at pH=6.5 and pH=7.5 showed <10% difference as compared to pH=7. As a result, reduced net P removal at low pH levels will also reduce the ability of PAOs to regenerate their polyphosphate pool for substrate uptake under anaerobic conditions, and could lead to out-competition by other organisms in the system, e.g. GAOs.

2.4. Implications of the study

This study demonstrated how the pH affects *Tetrasphaera* metabolism in an enriched culture. The P release and uptake kinetics showed a strong dependence on the pH, while intracellular PHA and glycogen production and carbon source consumption rates were largely independent of pH changes. At high pH levels, chemical precipitation can potentially be a competing mechanism for P removal. However, chemical precipitation was considered unlikely to be a significant mechanism of P uptake in this study for two reasons: 1) iron and aluminium salts were not present in appreciable quantities within the reactor media (iron was only added at micronutrient levels) and 2) the P release rate was observed to increase as a function of pH (Fig. 3f), as well as the P uptake rate (Fig. 3e), whereby a decrease in P release rate at the higher pH level would be expected if chemical precipitation had increased appreciably.

It is noteworthy that high pH conditions lead to higher rates of P release and uptake in both *Tetrasphaera* and *Accumulibacter*. This suggests that high pH can improve the synergistic interaction of *Tetrasphaera* and *Accumulibacter*, and enhance EBPR performance. While *Tetrasphaera* is capable of contributing to P removal through the anaerobic uptake of sugars and amino acids, simultaneously producing fermentation products such as VFA (Nielsen et al., 2019; Zhao et al., 2022), *Accumulibacter* can survive on these fermentation productions while also contributing to further P removal (Close et al., 2021; Marques et al., 2017). This promotes functional redundancy, decreasing the reliance on only one type of PAO to facilitate EBPR, which can benefit EBPR process robustness. Functional redundancy has been previously shown to result in process performance benefits in wastewater treatment in other situations (Paulo et al., 2020; Wang et al., 2021). High pH has been previously found to benefit PAOs over GAOs (Filipe et al., 2001d; Oehmen et al., 2005), thus a high pH (~8) can also lead to multiple benefits to the microbial community composition. Moreover, considering the typical pH profile observed in uncontrolled EBPR processes at lab-scale or full-scale (Serafim et al., 2002; Serralta et al., 2004; Flores-Alsina et al., 2015), the pH can increase substantially from ~7.0–8.0 between the anaerobic and aerobic phases. According to the results of this study, such a pH profile would result in a nearly optimal aerobic P uptake rate for both PAO groups, coupled with an anaerobic P release rate that is ~30–40% lower than what would have been observed if the pH were maintained at 8.0 anaerobically. The typical pH profile in EBPR systems therefore leads to an increased net P removal capacity of the WWTP for both groups of PAOs, further underlying the potential process benefits that can be achieved through selecting both *Tetrasphaera* and *Accumulibacter*.

Overall, this study reveals the short-term impacts of pH on *Tetrasphaera* kinetics and metabolism. High pH can enhance *Tetrasphaera* net P removal and thereby improve EBPR performance. Maintaining a relatively high pH, often achieved without a dedicated pH controller, could be a route to ensure efficient P removal. However, it is still unknown how pH impacts the long-term performance and microbial selection of *Tetrasphaera* communities, which warrants further study.

3. conclusions

In this study, the effect of pH on anaerobic and aerobic metabolism of an enriched culture of *Tetrasphaera*-PAOs was studied, for the first time. The P-uptake and release rates by the enriched *Tetrasphaera* sludge increased linearly with an increase of pH from 6 to 8. *Tetrasphaera* are capable of taking up different organic carbon sources over a wide range of pH conditions, producing fermentation products that can be consumed by *Accumulibacter*. High pH improves EBPR effectiveness, as *Tetrasphaera* display kinetic advantages at high pH levels, similar to *Accumulibacter*. The typical pH variations observed in EBPR plants can promote higher net P removal by both groups. Optimisation of EBPR in WWTPs may benefit from strategies that support a robust community of both *Accumulibacter* and *Tetrasphaera*, experiencing a positive synergy between the two PAO groups.

4. Materials and methods

4.1. Sequencing batch reactor (SBR) for tetrasphaera enrichment

A *Tetrasphaera* PAO culture was enriched in a laboratory-scale, anaerobic-aerobic sequencing batch reactor (SBR) with a working

volume of 2 L. The SBR was inoculated with biomass that was already enriched in *Tetrasphaera* PAOs (Close et al., 2021). The SBR was operated with an 8-h cycle, which consisted of an initial anaerobic period of 4 h, followed by a settling/decanting period of 1 hour and an aerobic period of 3 h. At the end of the decant phase, 1 L of the supernatant was withdrawn from the SBR within 10 mins.

400 mL of synthetic medium, named solution A, was fed continuously into the SBR during the first 3 h of the anaerobic phase, resulting in an organic carbon concentration of approximately 5 C-mmol/L in the reactor. The SBR was fed with sodium casein hydrolysate (a mixture composed mainly of amino acids and peptides, and designated as Cas aa) (Fluka, USA) as the sole C-source. Solution A contained per litre: 0.771 g Cas aa, 0.3698 g NH_4Cl, 0.5938 g $MgSO_4{\cdot}7H_2O$, 0.276 g $CaCl_2{\cdot}2H_2O$, 7.3 mg allyl-N thiourea (ATU, a nitrification inhibitor), 19.8 mg ethylene-diaminetetraacetic (EDTA) and 1.9792 mL of a micro-nutrient solution. The micronutrient solution (based on Smolders et al. (1994)) contained per litre: 1.5 g $FeCl_3{\cdot}6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4{\cdot}5H_2O$, 0.18 g KI, 0.12 g $MnCl_2{\cdot}4H_2O$, 0.06 g $Na_2MoO{\cdot}2H_2O$, 0.12 g $ZnSO_4{\cdot}7H_2O$, 0.15 g $CoCl_2{\cdot}6H_2O$. Solution B was composed of: K_2HPO_4 (84.2 mgP/L) and KH_2PO_4 (65.8 mgP/L), where 600 mL of solution B (150 mgP/L) was fed into the SBR during the first 2 min of the aerobic phase. The reactor had a hydraulic retention time (HRT) of 16 h and 100 mL sludge was removed from the reactor during 1 min of each day, resulting in a sludge retention time (SRT) of 20 d Around 0.2 mL/min argon was bubbled into the SBR to maintain anaerobic conditions, while 0.5 L/min air was bubbled during the aerobic phase. The reactor temperature was maintained at 20 ± 1 °C and the pH was at 7.1 ± 0.1 by dosing 0.1 M HCl. The sludge was mixed at 300 rpm stirring during the anaerobic and aerobic phases.

The reactor performance was assessed biologically and chemically (see Section 4.3). SBR dynamics were analysed by cycle studies, where samples were taken every hour throughout a cycle and analysed for total organic carbon (TOC), PHA, glycogen, ammonia and phosphate, via chemical analytical methods. Cell concentration in the reactor was tracked by analysing total suspended solids (TSS) and volatile suspended solids (VSS). Fluorescence in situ hybridisation (FISH) and high throughput sequencing analyses were also performed to assess the microbial community dynamics.

4.2. SBR batch experiments

To study the impact of pH on *Tetrasphaera* PAO metabolism and kinetics under anaerobic and aerobic conditions, batch experiments were carried out in duplicate on the sludge enriched in the SBR. Duplicated batch tests were performed for each pH (ranging between 6 and 8), following experimental conditions similar to those applied for the SBR enrichment, but at different controlled pH setpoints. Sludge for the batch experiments was taken from the enriched SBR during a period when high and stable P removal occurred (from day 59 to day 93). The sludge was not returned to the parent SBR after finishing each experiment, to avoid influencing the parent SBR. Due to the limitation of the sludge volume of the parent SBR, the batch tests were performed sequentially on a weekly basis. During this period, the average P-removal was 80 ± 12%.

The batch tests were performed with 500 mL of working volume, at 20 °C. For each batch test, a 400 mL sample of mixed liquor was taken from the enriched SBR at the end of the aerobic stage, when the cells have accumulated most of the phosphate. The sludge was centrifuged for 10 min at 6000 rpm, and the pellet was resuspended in 250 mL of the aforementioned solution A (without Cas aa). Argon was bubbled to ensure anaerobic conditions prior to feeding of the carbon source. The pellet was inoculated into the reactor followed by additions of 150 mL of phosphorus (solution B) and 100 mL of Cas aa solution, resulting in an initial concentration of 45 ppm of P-PO_4^{3-} and 5 C-mmol/L, respectively. The temperature was maintained by a water bath at 20 ± 1 °C with the stirring rate at 300 rpm. The pH of the reactor was controlled to the respective fixed setpoint (± 0.1 pH units) by addition of 0.1 M HCl and 0.1 M NaOH, when the pH values were above/below the targeted pH values, respectively. The batch reactors were operated with a cycle of 8 h, consisting of an anaerobic (4 h) followed by a settling/decant period (1 h) and an aerobic phase (3 h), similarly to the parent SBR.

To prevent any organic carbon entering the aerobic phase, the reactor content was centrifuged at 4000 rpm for 10 min at the end of the anaerobic phases. Then the pellet was resuspended again in mineral medium (i.e. Solution A, but without carbon source), water, and the phosphorus media of Solution B, with a volume proportion of 2.5:1:1.5, respectively, to mimic the media proportions of the anaerobic phase.

Samples were taken at various points throughout the cycle and analysed by the chemical methods described in Section 4.3, including FISH sampling performed at the end of the anaerobic phases.

4.3. Analytical methods

4.3.1. Chemical analyses

Colorimetric methods were implemented in a segmented flow analyser (Skalar 5100, Skalar Analytical, The Netherlands) to determine inorganic phosphate, P/TSS and ammonia. For P/TSS, the sample from the end of the aerobic period was digested with 0.3 M H_2SO_4 and 73 mg/L of $K_2S_2O_8$, then analysed by the segmented flow analyser as described by Carvalheira et al. (2014). After sample digestion, the supernatant phosphate concentration was subtracted from the obtained total phosphate concentration to determine the P/TSS.

Glycogen was extracted by digestion (2–4 mg biomass, HCl 0.9 M and 3 h of digestion time), and quantified by HPLC using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. 10 µL sodium hydroxide (NaOH 18 mM) was injected at a flow rate of 1 mL/min as eluent (25 °C) (Lanham et al., 2012).

The casein hydrolysate uptake was indicated by total organic carbon (TOC) with a Shimadzu TOC-VCSH (Shimadzu, Japan).

PHAs were determined by Bruker 430-GC gas chromatograph (GC) equipped with a FID detector and a BR-SWax column (60 m, 0.53 mm internal diameter, 1 µm film thickness, Bruker, USA) (Lanham et al., 2013).

For dry weight determination (TSS and VSS), standard methods were applied (APHA et al., 2005).

4.3.2. FISH analysis

Semi-quantitative fluorescence in situ hybridisation (FISH) was used to assess the SBR and batch test microbial composition, using the following specific fluorescently labelled oligonucleotide probes: EUBMIX (equimolar concentrations of EUB338, EUB338II, and EUB338III (Amann et al., 1990; Daims et al., 1999; Nielsen and Daims, 2009), which targets all Bacteria; PAOMIX (PAO651, PAO461 and PAO846 Crocetti et al. (2000)), which targets most members of the Ca. *Accumulibacter* cluster (with 89% target). *Tetrasphaera*-related PAOs were identified with the Tet1–266, Tet2–892, Tet2–174 and Tet3–654 probes (Nguyen et al., 2011) (see Table S2, Supplementary Information (SI)). Hybridised samples were observed using an epifluorescence microscope, Zeiss Imager D2, with a magnification of 1000x. The% biovolume of *Accumuibacter* or *Tetrasphaera* is indicated by the ratio between the area of each organism population over the FITC-labelled EUBmix population. Semi-quantification of each of the specific probes against EUBmix was performed by visual inspection of a minimum 10 independent fields by an expert operator. A summary of results and microphotographs for the most abundant populations are shown in Table 1 and Figure S1, respectively.

4.3.3. High throughput sequencing

To complete the microbiological analysis, samples from the enriched SBR were analysed through sequencing of the 16S rRNA gene. Biomass from the enriched SBR within the batch test period were analysed (Day 65, Day 92) to characterise the microbial composition and relative

abundance of different bacteria by high throughput DNA sequencing (performed by DNASense Aps, Denmark), through methods that have been previously described by Wang et al. (2017).

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2023.100177.

References

Acevedo, B., Oehmen, A., Carvalho, G., Seco, A., Borrás, L., Barat, R., 2012. Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage. Water Res. 46 (6), 1889–1900.

Albertsen, M., Hansen, L.B.S., Saunders, A.M., Nielsen, P.H., Nielsen, K.L., 2012. A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal. ISME J. 6 (6), 1094–1106.

Albertsen, M., McIlroy, S.J., Stokholm-Bjerregaard, M., Karst, S.M., Nielsen, P.H., 2016. Candidatus propionivibrio aalborgensis”: a novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants. Front Microbiol 7.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172 (2), 762–770.

APHA, AWWA and WEF (2005) Standard Methods For the Examination of Water and Wastewater, 21st edition, Washington, D.C.

Barnard, J.L., Dunlap, P., Steichen, M., 2017. Rethinking the mechanisms of biological phosphorus removal. Water Environ. Res. 89 (11), 2043–2054.

Bond, P.L., Keller, J., Blackall, L.L., 1999. Anaerobic phosphate release from activated sludge with enhanced biological phosphorus removal. A possible mechanism of intracellular pH control. Biotechnol. Bioeng. 63 (5), 507–515.

Carvalheira, M., Oehmen, A., Carvalho, G., Eusébio, M., Reis, M.A.M., 2014. The impact of aeration on the competition between polyphosphate accumulating organisms and glycogen accumulating organisms. Water Res. 66, 296–307.

Carvalho, V.C.F., Kessler, M., Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2021. Achieving nitrogen and phosphorus removal at low C/N ratios without aeration through a novel phototrophic process. Sci. Total Environ. 793, 148501.

Chen, Y., Gu, G., 2006. Effect of changes of pH on the anaerobic/aerobic transformations of biological phosphorus removal in wastewater fed with a mixture of propionic and acetic acids. J. Chem. Technol. Biotechnol. 81 (6), 1021–1028.

Close, K., Marques, R., Carvalho, V.C.F., Freitas, E.B., Reis, M.A.M., Carvalho, G., Oehmen, A., 2021. The storage compounds associated with Tetrasphaera PAO metabolism and the relationship between diversity and P removal. Water Res. 204, 117621.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L. L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. 66 (3), 1175–1182.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22 (3), 434–444.

Fernando, E.Y., McIlroy, S.J., Nierychlo, M., Herbst, F.-A., Petriglieri, F., Schmid, M.C., Wagner, M., Nielsen, J.L., Nielsen, P.H., 2019. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman–FISH. ISME J. 13 (8), 1933–1946.

Filipe, C.D.M., Daigger, G.T., Grady Jr., C.P.L., 2001a. Effects of pH on the rates of aerobic metabolism of phosphate-accumulating and glycogen-accumulating organisms. Water Environ. Res. 73 (2), 213–222.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001b. A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH. Biotechnol. Bioeng. 76 (1), 17–31.

Filipe, C.D.M., Daigger, G.T., Grady Jr., C.P.L., 2001c. pH as a Key Factor in the Competition Between Glycogen-Accumulating Organisms and Phosphorus-Accumulating Organisms. Water Environ. Res. 73 (2), 223–232.

Filipe, C.D.M., Daigger, G.T., Grady Jr., C.P.L., 2001d. Stoichiometry and kinetics of acetate uptake under anaerobic conditions by an enriched culture of phosphorus-accumulating organisms at different pHs. Biotechnol. Bioeng. 76 (1), 32–43.

Flores-Alsina, X., Mbamba, C.K., Solon, K., Vrecko, D., Tait, S., Batstone, D.J., Jeppsson, U., Gernaey, K.V., 2015. A plant-wide aqueous phase chemistry module describing pH variations and ion speciation/pairing in wastewater treatment process models. Water Res. 85, 255–265.

Herbst, F.-A., Dueholm, M.S., Wimmer, R., Nielsen, P.H., 2019. The proteome of tetrasphaera elongata is adapted to changing conditions in wastewater treatment plants. Proteomes 7 (2), 16.

Jeon, C.O., Lee, D.S., Lee, M.W., Park, J.M., 2001. Enhanced biological phosphorus removal in an anaerobic–aerobic sequencing batch reactor: effect of pH. Water Environ. Res. 73 (3), 301–306.

Kolakovic, S., Freitas, E.B., Reis, M.A.M., Carvalho, G., Oehmen, A., 2021. Accumulibacter diversity at the sub-clade level impacts enhanced biological phosphorus removal performance. Water Res. 199, 117210.

Kong, Y., Nielsen, J.L., Nielsen, P.H., 2005. Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. Appl. Environ. Microbiol. 71 (7), 4076–4085.

Kong, Y., Xia, Y., Nielsen, P.H., 2008. Activity and identity of fermenting microorganisms in full-scale biological nutrient removing wastewater treatment plants. Environ. Microbiol. 10 (8), 2008–2019.

Kristiansen, R., Nguyen, H.T.T., Saunders, A.M., Nielsen, J.L., Wimmer, R., Le, V.Q., McIlroy, S.J., Petrovski, S., Seviour, R.J., Calteau, A., Nielsen, K.L., Nielsen, P.H., 2013. A metabolic model for members of the genus Tetrasphaera involved in enhanced biological phosphorus removal. ISME J. 7 (3), 543–554.

Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. Process Biochem. 48 (11), 1626–1634.

Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2012. Optimisation of glycogen quantification in mixed microbial cultures. Bioresour. Technol. 118, 518–525.

Liu, R., Hao, X., Chen, Q., Li, J., 2019. Research advances of Tetrasphaera in enhanced biological phosphorus removal: a review. Water Res. 166, 115003.

Liu, W.T., Mino, T., Matsuo, T., Nakamura, K., 1996. Biological phosphorus removal processes - effect of pH on anaerobic substrate metabolism. Water Sci. Technol. 34 (1–2), 25–32.

Marques, R., Santos, J., Nguyen, H., Carvalho, G., Noronha, J.P., Nielsen, P.H., Reis, M.A. M., Oehmen, A., 2017. Metabolism and ecological niche of Tetrasphaera and Ca. Accumulibacter in enhanced biological phosphorus removal. Water Res. 122, 159–171.

Nguyen, H.T.T., Kristiansen, R., Vestergaard, M., Wimmer, R., Nielsen, P.H., 2015. Intracellular accumulation of glycine in polyphosphate-accumulating organisms in activated sludge, a novel storage mechanism under dynamic anaerobic-aerobic conditions. Appl. Environ. Microbiol. 81 (14), 4809–4818.

Nguyen, H.T.T., Le, V.Q., Hansen, A.A., Nielsen, J.L., Nielsen, P.H., 2011. High diversity and abundance of putative polyphosphate-accumulating Tetrasphaera-related bacteria in activated sludge systems. FEMS Microbiol. Ecol. 76 (2), 256–267.

Nielsen, J.L., Nguyen, H., Meyer, R.L., Nielsen, P.H., 2012. Identification of glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal plant by stable isotope probing. Microbiology 158 (7), 1818–1825.

Nielsen, P., Daims, H., 2009. FISH Handbook For Biological Wastewater Treatment. Iwa publishing.

Nielsen, P.H., McIlroy, S.J., Albertsen, M., Nierychlo, M., 2019. *Re*-evaluating the microbiology of the enhanced biological phosphorus removal process. Curr. Opin. Biotechnol. 57, 111–118.

Oehmen, A., Carvalho, G., Lopez-Vazquez, C.M., van Loosdrecht, M.C.M., Reis, M.A.M., 2010a. Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Res. 44 (17), 4992–5004.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Res. 41 (11), 2271–2300.

Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010b. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res. 44 (15), 4473–4486.

Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J., 2005. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Res. 39 (15), 3727–3737.

Onnis-Hayden, A., Srinivasan, V., Tooker, N.B., Li, G., Wang, D., Barnard, J.L., Bott, C., Dombrowski, P., Schauer, P., Menniti, A., Shaw, A., Stinson, B., Stevens, G., Dunlap, P., Takács, I., McQuarrie, J., Phillips, H., Lambrecht, A., Analla, H., Russell, A., Gu, A.Z., 2020. 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 (3), 403–417.
Paulo, L.M., Castilla-Archilla, J., Ramiro-Garcia, J., Escamez-Picón, J.A., Hughes, D., Mahony, T., Murray, M., Wilmes, P., O'Flaherty, V, 2020. Microbial community redundancy and resilience underpins high-rate anaerobic treatment of dairy-processing wastewater at ambient temperatures. Front. Bioeng. Biotechnol. 8.
Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnol. Bioeng. 85 (1), 56–67.
Qiu, G., Zuniga-Montanez, R., Law, Y., Thi, S.S., Nguyen, T.Q.N., Eganathan, K., Liu, X., Nielsen, P.H., Williams, R.B.H., Wuertz, S., 2019. Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources. Water Res. 149, 496–510.
Rey-Martínez, N., Badia-Fabregat, M., Guisasola, A., Baeza, J.A., 2019. Glutamate as sole carbon source for enhanced biological phosphorus removal. Sci. Total Environ. 657, 1398–1408.
and Rubio-Rincón, F.J., Weissbrodt, D.G., Lopez-Vazquez, C.M., Welles, L., Abbas, B., Albertsen, M., Nielsen, P.H., van Loosdrecht, M.C.M., Brdjanovic, D., 2019a. Candidatus Accumulibacter delftensis": a clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res. 161, 136–151.
and Rubio-Rincón, F.J., Welles, L., Lopez-Vazquez, C.M., Abbas, B., van Loosdrecht, M.C. M., Brdjanovic, D., 2019b. Effect of lactate on the microbial community and process performance of an EBPR system. Front. Microbiol. 10.
and Schuler, A.J., Jenkins, D., 2002. Effects of pH on enhanced biological phosphorus removal metabolisms. Water Sci. Technol. 46 (4–5), 171–178.
Serafim, L.S., Lemos, P.C., Reis, M.A.M., 2002. Effect of pH control on EBPR stability and efficiency. Water Sci. Technol. 46 (4–5), 179–184.
Serralta, J., Borrás, L., Blanco, C., Barat, R., Seco, A., 2004. Monitoring pH and electric conductivity in an EBPR sequencing batch reactor. Water Sci. Technol. 50 (10), 145–152.
Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.
Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Front. Microbiol. 8.
Tu, Y., Schuler, A.J., 2013. Low acetate concentrations favor polyphosphate-accumulating organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal from wastewater. Environ. Sci. Technol. 47 (8), 3816–3824.
Valverde-Pérez, B., Wágner, D.S., Lóránt, B., Gülay, A., Smets, B.F., Plósz, B.G., 2016. Short-sludge age EBPR process – Microbial and biochemical process characterisation during reactor start-up and operation. Water Res. 104, 320–329.
Van Veen, H.W., Abee, T., Kortstee, G.J., Konings, W.N., Zehnder, A.J., 1993. Mechanism and energetics of the secondary phosphate transport system of Acinetobacter johnsonii 210A. J. Biol. Chem. 268 (26), 19377–19383.
Van Veen, H.W., Abee, T., Kortstee, G.J., Konings, W.N., Zehnder, A.J., 1994. Translocation of metal phosphate via the phosphate inorganic transport system of Escherichia coli. Biochemistry 33 (7), 1766–1770.
Wang, X., Oehmen, A., Freitas, E.B., Carvalho, G., Reis, M.A.M., 2017. The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production. Water Res. 112, 269–278.
Wang, Y., Ye, J., Ju, F., Liu, L., Boyd, J.A., Deng, Y., Parks, D.H., Jiang, X., Yin, X., Woodcroft, B.J., Tyson, G.W., Hugenholtz, P., Polz, M.F., Zhang, T., 2021. Successional dynamics and alternative stable states in a saline activated sludge microbial community over 9 years. Microbiome 9 (1), 199.
Zhang, C., Guisasola, A., Baeza, J.A., 2021. Achieving simultaneous biological COD and phosphorus removal in a continuous anaerobic/aerobic A-stage system. Water Res. 190, 116703.
Zhao, W., Bi, X., Peng, Y., Bai, M., 2022. Research advances of the phosphorus-accumulating organisms of Candidatus Accumulibacter, Dechloromonas and Tetrasphaera: metabolic mechanisms, applications and influencing factors. Chemosphere 307, 135675.
Zheng, X., Sun, P., Han, J., Song, Y., Hu, Z., Fan, H., Lv, S., 2014. Inhibitory factors affecting the process of enhanced biological phosphorus removal (EBPR) – A mini-review. Process Biochem. 49 (12), 2207–2213.