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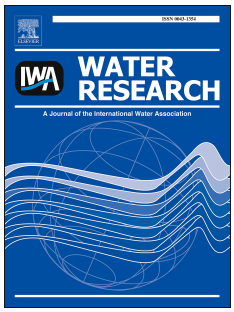

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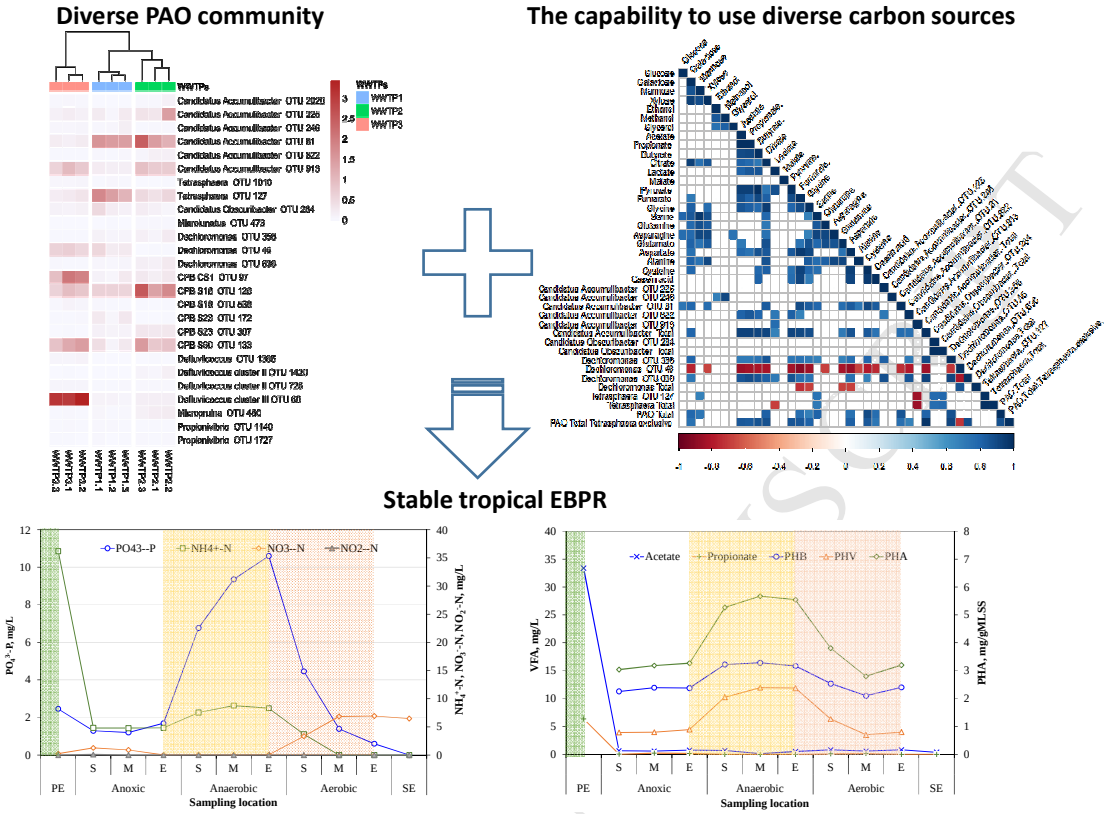
Graphical abstract



Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources

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ABSTRACT

Enhanced biological phosphorus removal (EBPR) is considered challenging in the tropics, based on a great number of laboratory-based studies showing that the polyphosphate-accumulating organism **(**PAO) *Candidatus* Accumulibacter does not compete well with glycogen accumulating organisms (GAOs) at temperatures above 25^{o}C. Yet limited information is available on the PAO community and the metabolic capabilities in full-scale EBPR systems operating at high temperature. We studied the composition of the key functional PAO communities in three full-scale wastewater treatment plants (WWTPs) with high in-situ EBPR activity in Singapore, their EBPR-associated carbon usage characteristics, and the relationship between carbon usage and community composition. Each plant had a signature community composed of diverse putative PAOs with multiple operational taxonomic units (OTUs) affiliated to *Ca.* Accumulibacter, *Tetrasphaera* spp., *Dechloromonas* and *Ca.* Obscuribacter. Despite the differences in community composition, ex-situ anaerobic phosphorus (P)-release tests with 24 organic compounds from five categories (including four sugars, three alcohols, three volatile fatty acids (VFAs), eight amino acids and six other carboxylic acids) showed that a wide range of organic compounds could potentially contribute to EBPR. VFAs induced the highest P release (12.0-18.2 mg P/g MLSS for acetate with P release-to-carbon uptake (P:C) ratios of 0.35-0.66 mol P / mol C, 9.4-18.5 mg P/g MLSS for propionate and P:C ratios of 0.38-0.60, and 9.5-17.3 mg P/g MLSS for n-butyrate), followed by some carboxylic acids (10.1-18.1 mg P/g MLSS for pyruvate, 4.5-11.7 mg P/g MLSS for lactate and 3.7-12.4 mg P/g MLSS for fumarate) and amino acids (3.66-7.33 mg P/g MLSS for glutamate with a P:C ratio of 0.16-0.43 mol P/ mol C, and 4.01-7.37 mg P/g MLSS for aspartate with a P:C ratio of 0.17-0.48 mol P/ mol C). P-release profiles (induced by different carbon sources) correlated closely with PAO community composition. High micro-diversity was observed within the *Ca.* Accumulibacter lineage, which represented the most abundant PAOs. The total population of *Ca.* Accumulibacter taxa was highly correlated with P-release induced by VFAs, highlighting the latter's importance in tropical EBPR systems. There was a

strong link between the relative abundance of individual *Ca.* Accumulibacter OTUs and the extent of P release induced by distinct carbon sources (e.g., OTU 81 and amino acids, and OTU 246 and ethanol), suggesting niche differentiation among *Ca.* Accumulibacter taxa. A diverse PAO community and the ability to use numerous organic compounds are considered key factors for stable EBPR in full-scale plants at elevated temperatures.

Keywords: Enhanced biological phosphorus removal (EBPR); high temperature; polyphosphate-accumulating organisms (PAO); *Candidatus* Accumulibacter; *Tetrasphaera*; carbon source; volatile fatty acids (VFAs); amino acids; sugars; alcohols

1 INTRODUCTION

Enhanced biological phosphorus removal (EPBR) remains one of the most cost-effective and sustainable processes and is widely employed in full-scale wastewater treatment plants (WWTPs) for the elimination from water and potential recovery of phosphorus (Oehmen et al., 2007; He and McMahon, 2011). However, a number of studies have shown that high temperature can be detrimental to EBPR, with PAOs being outcompeted by glycogen accumulating organisms (GAOs) at temperatures above 25^{o}C (Whang and Park, 2002; Panswad et al., 2003; Lopez-Vazquez et al., 2009). There have been efforts to achieve stable high-temperature EBPR in the laboratory. Freitas et al. (2009) obtained robust EBPR activity at 30^{o}C for over 100 days by applying a short cycle (consisting of a 20-min anaerobic phase and a 10-min aerobic phase), and up to 100% P removal efficiency was achieved in an aerobic granular biomass system by selectively removing sludge from the top of the sludge bed (Winkler et al. 2011). Recently, long-term EBPR stability has been

demonstrated in lab-scale reactors without applying short SRTs or selective sludge removal (Ong et al., 2014). Using acetate as a sole carbon source, the predominant PAO in the reactor was *Candidatus* Accumulibacter clade IIF. However, as the temperature increased from 24 to 32^{o}C, EBPR activities were compromised, with a significant reduction in the relative abundance of *Ca.* Accumulibacter and a concurrent increase in the GAO population. Shen et al. (2017) further showed that having multiple anaerobic/aerobic stages in one EBPR cycle helped to achieve EBPR at 30^{o}C with either acetate or propionate as carbon sources. Acetate resulted in higher process stability as compared to propionate. These laboratory-scale studies suggested the need for special operational controls to allow PAOs to outcompete GAOs at elevated temperature. In contrast, EBPR has been observed in a full-scale activated sludge plant in Singapore with year-round operation at 28-32^{o}C, although the plant was not designed for EBPR. Both GAOs and PAOs were present, but did not seem to compete with one another (Law et al., 2016). Clearly, there are remaining knowledge gaps between lab-scale studies and full-scale observations for EBPR at high temperatures. Apart from the study from Law et al (2016), very limited information is available on the key functional PAOs and their metabolic characteristics in full-scale EBPR systems under tropical conditions.

Among the many parameters governing EBPR viability and stability, the type of carbon source is key (Abu-Gharach and Randall, 1991; Shen and Zhou 2016). It is also one of the main differences between lab-scale and full-scale systems. To date, short-chain volatile fatty acids (VFAs) are the most commonly used carbon substrates in lab-scale EBPR systems. Yet PAOs consist of diverse members that can metabolise different substrates. *Ca.* Accumulibacter is commonly found in lab-scale and full-scale EBPR systems (Seviour et al., 2003; Lu et al., 2006; Fukushima et al., 2007; Oehman et al., 2007; Ong et al., 2014; Shen et al., 2017; Rubio-Rincón, et al., 2017), and primary carbon sources are restricted to low molecular weight substrates such as VFAs (Oehman et al., 2007; He and McMahon, 2011; Flowers et al., 2013). Another important group of PAOs is the actinobacterial *Tetrasphaera* spp. (Hanada et al., 2002; Kong et al., 2005). They are more abundant

than *Ca.* Accumulibacter in WWTPs in countries with lower wastewater temperatures, such as Denmark (9-18^{o}C, Saunders et al., 2016; Stokholm-Bjerregaard et al., 2017), Portugal (8-25^{o}C, Lanham et al., 2013) and Poland (5-24^{o}C, Muszyński and Załęska-Radziwiłł, 2015), as well as in some full-scale and pilot-scale MBR plants in The Netherlands, Norway, Germany and Switzerland (Silva et al., 2012), accounting for up to 40% of the biomass. In contrast to *Ca.* Accumulibacter, *Tetrasphaera* spp. are more versatile in substrate uptake capabilities, can utilise both glucose and amino acids (Nguyen et al., 2011; Nguyen et al., 2015; Marques et al., 2017), and are capable of fermenting complex organics (Nielsen et al., 2010; Kristiansen et al., 2013; Marques et al., 2017) and accumulating fermentation by-products (Nguyen et al., 2015). Some were shown to be VFA users, but in situ staining did not support that these probe-defined *Tetrasphaera* store intracellular polyhydroxyalkanoate (PHA) (Kong et al., 2005; Nguyen et al., 2011). Carbon usage characteristics are an important trait that differentiates these two groups of PAOs. Apart from *Ca.* Accumulibacter and *Tetrasphaera* spp., other putative PAOs have been identified in EBPR systems, some occurring in full-scale plants in appreciable numbers (Stokholm-Bjerregaard et al. 2017), including *Microlunatus phosphovorus* (Nakamura et al., 1995), *Ca*. Accumulimonas (Nguyen et al., 2012), *Dechloromonas* (Kong et al., 2007), *Ca.* Obscuribacter (Soo et al., 2014), *Thiothrix caldifontis* (Rubio-Rincon et al., 2017) and Comamonadaceae members (Ge et al., 2015), among others. Dissimilarity was also observed in their carbon metabolism; for example, the actinobacterium *Microlunatus phosphovorus* showed a similar metabolism to that of *Tetrasphaera*. It utilises a wide range of sugars and amino acids, but takes up acetate slowly (Nakamura et al., 1995; Ubakata and Takii, 1998). Genome analysis suggested the lack of *pha*ABC genes for PHA synthesis (Kawakoshi et al., 2012). Gammaproteobacterial *Ca.* Accumulimonas was shown to take up VFAs and store them as PHAs, similar to *Ca.* Accumulibacter (Nguyen et al., 2012).

Municipal wastewater contains a complex matrix of organic compounds. It is logical to conceive that other carbon sources, apart from VFAs, and other PAOs, apart from *Ca.* Accumulibacter, could

126 contribute to EBPR in full-scale plants. In this study, repeated field sampling was carried out in three
127 tropical WWTPs in Singapore to monitor the EBPR activity. The objectives were to (i) characterise
128 the bacterial and PAO community using 16S rRNA amplicon sequencing combined with fluorescent
129 in situ hybridisation (FISH) and poly-P chemical staining; (ii) characterise the carbon utilisation
130 profiles of these functional PAOs, by subjecting fresh activated sludge to anaerobic P-release tests
131 with 24 carbon sources from five categories (including four sugars, three alcohols, three VFAs, eight
132 amino acids and six carboxylic acids); and (iii) explore the associations between P-release induced
133 from the utilisation of different carbon sources and the bacterial community. We hypothesised that
134 differential utilisation of carbon sources is associated with a distinct PAO community. Six of the
135 carbon sources were further selected for anaerobic-aerobic cycle studies to evaluate their
136 effectiveness in supporting EBPR.

137

138 ## 2 MATERIALS AND METHODS

139 ### 2.1 Sampling of wastewater treatment plants

140 Field sampling was conducted at three domestic WWTPs with different configurations in Singapore
141 from November 2016 to February 2017 (Supplementary Table S1). WWTP1 has four identical
142 treatment trains using a 5-stage step-feeding activated sludge process, where mixed liquor passes
143 through five basins of alternating anoxic and aerobic zones with the influent equally distributed into
144 the anoxic zones of each basin. WWTP2 consists of six trains of activated sludge treatment, each one
145 featuring a modified Ludzack–Ettinger (MLE) configuration. WWTP3 has three parallel treatment
146 trains, each comprising an anoxic tank followed by an anaerobic and an aerobic tank and, finally, by
147 a membrane tank for solid-liquid separation (Table 1 and Supplementary Fig. S1). Water temperature
148 at these plants varies from 28.7 to 31.6^{o}C.

Field sampling was performed in three episodes at each plant to monitor process performance and the bacterial community composition. Liquid samples were collected from the primary effluent and at different locations of each plant (Supplementary Fig. S1). Temperature, pH and dissolved oxygen (DO) were measured with a multi-parameter portable meter (YSI Professional Plus, CA, USA) at each sampling point. The mixed liquor samples were filtered through 0.22-μm sterile filters for soluble chemical oxygen demand (SCOD), NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P and VFA analyses. Non-filtered primary effluent samples were acidified with sulphuric acid and analysed for total phosphorus (TP) and total chemical oxygen demand (TCOD). Mixed liquor samples were collected from each sampling location and fixed with 2 drops of 37% formaldehyde for PHA and glycogen analyses. For the microbial community analysis, 2 ml of mixed liquor were collected from the end of the aerobic zones, snap-frozen in liquid nitrogen and stored at -80°C before DNA extraction. For FISH imaging, activated sludge was collected from the end of the anaerobic/anoxic zones and the end of the aerobic zone, and immediately fixed using paraformaldehyde (PFA at a final concentration of 4%, for Gram-negative bacteria) and ethanol (by mixing equal volumes of 100% ethanol and mixed liquor, for Gram-positive bacteria). Fresh mixed liquor was also collected at the end of the aerobic zones for anaerobic P-release tests and anaerobic-aerobic cycle studies.

2.2 EBPR activity tests with different carbon sources

For anaerobic P-release tests, the mixed liquor was diluted with secondary effluent from the corresponding WWTPs that was depleted of NO_2^- and NO_3^- to a final MLSS of 2.0 g/L; the solution pH was adjusted to 7.25 using 0.1M HCl or 0.1M NaOH. Fifty millilitres of diluted activated sludge were added into 50-ml culture bottles and sealed. Anaerobic conditions in each bottle were induced by N_2 gas purging for 15 min before the different carbon sources were added. Twenty-four carbon sources were tested, including four sugars: glucose, galactose, mannose, and xylose; three alcohols: ethanol, methanol and glycerol; nine amino acids: glycine, serine, glutamine, asparagine, glutamate, aspartate, alanine, cysteine and casein acid; three VFAs: acetate, propionate and n-butyrate; and five

carboxylic acids: citrate, lactate, malate, pyruvate and fumarate. Each bottle received one carbon source at a final COD concentration of 300 mg/L, and the culture bottles were placed for 3 h in a shaking incubator (Infors HT, Bottmingen, Switzerland) operated at 180 rpm and 30^{o}C. Samples were collected every hour and passed immediately through 0.45-μm sterile filters for PO_4^{3-}-P analysis. One culture bottle with activated sludge but no carbon source addition served as a control. All the experiments were done in duplicates.

Glucose, methanol, glutamate, aspartate, acetate, and propionate were further selected for anaerobic-aerobic cycle studies. Experiments were performed in 1-L reactors operated in parallel in a 30^{o}C water bath. Fresh activated sludge was diluted to a final MLSS of 2.0 g/L, followed by the addition of each carbon source to a final concentration of 30 mg C/L. Fresh primary effluent was used as one of the treatments, acting as a positive control. A reactor with activated sludge but no carbon source addition served as a negative control. The full cycle consisted of a 3-h anaerobic phase followed by a 3-h aerobic phase. N_2 gas and air were purged continuously during the anaerobic and aerobic phases, respectively, to ensure anaerobic and aerobic conditions and for mixing. The solution pH was maintained at 7.25 $\pm$ 0.05 during the experiment by addition of 0.25 M HCl or 0.1 M $NaHCO_3$. Filtered water (through 0.45μm membrane filters) and activated sludge samples were collected at different time intervals for PO_4^{3-}-P, VFAs, total organic carbon (TOC), PHA and glycogen analyses.

2.3 Analytical methods

MLSS and MLVSS were determined according to Standard Methods (APHA, 1999). COD, NH_4^{+}-N, TP, NO_3^{-}-N, NO_2^{-}-N, PO_4^{3-}-P were measured using test kits (HACH, CO, USA) following Standard Methods (APHA, 1999). TOC and TN were analysed using a TOC/TN analyser (Shimadzu, Japan), and VFAs (acetate, propionate and butyrate) were measured using a gas chromatograph (Prominence, Shimadzu, Japan) equipped with a flame ionisation detector that was fitted with a DB-FFAP column (30 × 0.25 mm) (Agilent Technology, U.S.). PHA analyses were performed according to Oehmen et

al. (2005a), using a gas chromatograph (Prominence, Shimadzu, Japan) equipped with an FID detector and fitted with a DB-5MS Ultra Inert column (30×0.25 mm) (Agilent Technology, CA, USA). Glycogen analyses were carried out by measuring glucose in the sludge after acid digestion as described by Kristiansen et al. (2013).

2.4 Fluorescence in situ hybridisation (FISH)

PFA-fixed activated sludge samples were washed with 1× phosphate-buffered saline (PBS) solution and resuspended in a mixed solution of 1×PBS and 100% ethanol (50:50). Washed PFA-fixed and ethanol-fixed samples were stored at -20°C before FISH analysis. Organisms of interest were detected using EUB probe mix, targeting most Bacteria (EUB338, EUB338II and EUB338III) (Daims et al., 1999), and PAOmix (PAO651, PAO462 and PAO846) (Crocetti et al., 2000) and Tetmix (Tet1–266, Tet2–174, Tet2–892 and Tet3–654) (Nguyen et al., 2011), targeting *Ca.* Accumulibacter- and *Tetrasphaera*- PAOs, respectively. Efforts were made to visualise the two groups of PAOs simultaneously using samples fixed with either PFA or ethanol. PFA-fixed samples allowed for good detection of both *Ca.* Accumulibacter and *Tetrasphaera*. In contrast, low detection of *Ca.* Accumulibacter was observed with ethanol-fixed samples. Thus, PFA-fixed samples were used for FISH image generation in this study.

2.5 DNA extraction, 16S rRNA gene amplicon sequencing and qPCR

Genomic DNA was extracted using the Fast DNATM 2 mL SPIN Kit for Soil samples (MP Biomedicals, CA, USA), following the optimised protocol for activated sludge (Albertsen et al. 2015). Bacterial 16S rRNA gene amplicon sequencing was performed, targeting the V1-V3 region (primer set: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG). PCR amplification was carried out in a 25-µl PCR matrix containing 10 ng of genomic DNA, 400 nM dNTPs, 1.5 mM $MgSO_4$, 2 mU Platinum R Taq DNA polymerase high fidelity, 1× Platinum R High Fidelity buffer (Thermo Fisher Scientific, MA, USA) and a pair of barcoded library adaptors (400

nM), with a thermo cycler setting of initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and final elongation at 72°C for 5 min. All PCR reactions were run in duplicate and pooled afterwards. The amplicon libraries were purified using the Agencourt R AMpure XP bead protocol (Beckmann Coulter, CA, USA) with 1.8 bead solution/PCR solution ratio. Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations. The library pool was sequenced on a MiSeq (Illumina, CA, US) using a MiSeq Reagent kit v3 (2×300 paired end). Pre-processing of all amplicon libraries was performed according to Albertsen et al. (2015). Taxonomy was assigned using MiDAS v.1.20 (McIlroy et al. 2015).

Additionally, quantitative PCR (qPCR) was used to analyse the clade level distribution of *Ca.* Accumulibacter in each plant, according to He et al. (2007).

2.6 Statistical analysis

All statistical analyses were performed using SPSS 13 (IBM, NY, USA) or R Version 3.3.33 (www.r-project.org). The heat-map of the community compositions was plotted using the R package pheatmap Version 1.0.8 (https://cran.r-project.org/web/packages/pheatmap). Complete linkage clustering analysis was based on Euclidean distances. Pearson tests were performed using the R package Hmisc Version 4.1-1 (https://cran.r-project.org/web/packages/Hmisc) to examine the correlation between the P-release values obtained with the different carbon sources and the relative abundance of putative OTUs belonging to PAOs in each WWTP. The plot of the obtained Pearson correlation coefficient matrix was conducted with the R package Corrplot Version 0.84 (https://cran.r-project.org/web/packages/corrplot), with a P-value cut-off of <0.05. Raw P-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Regularised canonical correlation analysis (CCA) was performed using the R package CCA Version 1.2 (https://cran.r-project.org/web/packages/CCA) to study the inter-relationships between the relative abundance of bacterial taxa and P-release resulted from different carbon sources. The Venn

246 diagram of the community compositions was plotted using the online tool VENNY Version 2.1
247 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

248

249 # 3 RESULTS AND DISCUSSION

250 ## 3.1 In-situ EBPR and nutrient removal activities

251 During the nine sampling events conducted at three WWTPs, high EBPR activity was observed
252 together with good nitrogen removal (Fig. 1). We recorded P-release at the end of the
253 anoxic/anaerobic zones concomitant with an increase in the intracellular PHA content of the
254 activated sludge. In the subsequent aerobic phase, P-uptake coincided with a decrease in PHA
255 content, resulting in low PO_4^{3-}-P concentrations at the end of the aerobic stages (< 2.0 mg/L). Neither
256 WWTP1 nor WWTP 2 has a defined anaerobic stage (Supplementary Fig. S1); however, P-release
257 and denitrification occurred simultaneously within the same compartment. We surmise that as long
258 as the wastewater had sufficient carbon for both denitrification and EBPR, a separation of the anoxic
259 and anaerobic zones was not necessary, as non-denitrifying PAOs were able to perform anoxic-
260 aerobic EBPR by recognising anoxic conditions as anaerobic (Cokro et al. 2017). For WWTP1, the
261 highest P-release was observed in the first anoxic stage (Fig. 1A), followed by a decrease in
262 subsequent stages due to the dilution of the primary effluent with the mixed liquor along the
263 treatment train, and carbon consumption via denitrification. This outcome agrees with results
264 obtained in a lab-scale EBPR system with multiple anaerobic/aerobic stages in one SBR cycle, where
265 decreasing P-release was observed along multi-stages (Shen et al., 2017). Additionally, a
266 significantly lower PHA content was observed at WWTP1 compared to WWTPs 2 and 3, likely
267 resulting from a faster carbon turnover in the step-feed system (Shen et al., 2017). For all plants,
268 polyhydroxybutyrate (PHB) was the major PHA polymer in the sludge, reflecting that acetate was
269 the main VFA present in the primary effluent, followed by much lower concentrations of propionate.

Low in-tank VFA concentrations (<1.0 mg/L) were observed within all three treatment trains, which is considered beneficial for the prevalence of PAOs. These organisms rely on the proton motive force generated by the hydrolysis of poly-P and the resultant efflux of proton in symport of phosphate for VFA uptake; hence they are more efficient in scavenging VFAs at low concentrations than *Defluviicoccus*-GAOs (Burow et al., 2008; Tu and Schuler, 2014).

3.2 Bacterial community composition

The bacterial communities in the three WWTPs were analysed using 16S rRNA gene amplicon sequencing. At the phylum level, all the communities were dominated by Proteobacteria (with relative abundances from 37 to 48%), primarily members of the Beta- (10-19%), Alpha- (9.5-21%) and Gamma- (3.7-7.3%) classes, and followed by Bacteroidetes (14-23%), Actinobacteria (11-19.1%), Chloroflexi (7.2-19%) and Firmicutes (2.0-3.2%) (Fig. 2A). All these phyla are common in activated sludge communities (Albertsen et al., 2015), and the relative abundance of other bacterial phyla was below 2.5%.

High community similarity was observed for samples from the same plant (>85%, Supplementary Table 1), whereas communities from different plants showed higher degrees of disparity (similarity values ranged from 29-75%). At each plant, around half (44.3-60.9%) of the OTUs with at least three reads in each library were consistently detected during the three sampling episodes. About 25% of all OTUs were detected at least once across all the plants (Fig. 2C), including almost all the putative PAO- and GAO-related OTUs. This confirms that systems performing EBPR contain a core community of functional microorganisms (Saunders et al., 2016).

A variety of sequences related to PAOs and GAOs were identified in the nine amplicon libraries (see Fig. 2B, where only taxa with a relative abundance >0.02% are listed). For PAOs, six *Ca.* Accumulibacter OTUs occurred in all three plants; WWTP2 showed the highest relative abundance (six OTUs, 2.6-3.8%), followed by WWTP1 (five OTUs, 1.70-2.04%) and WWTP3 (six OTUs,

294 0.95-1.40%). The observed relative abundance values of *Ca.* Accumulibacter fell in the lower range
295 of those found in full-scale plants in temperate countries, accounting for 2-22% as quantified by
296 FISH imaging (Kong et al., 2004; Chua et al., 2006; Gu et al., 2008; Lopez-Vazquez et al., 2008;
297 Zhang et al., 2011; Mielczarek et al., 2013), 1.2-24% by qPCR (He et al., 2007; He et al., 2008; Mao
298 et al., 2015; Zhang et al., 2016), 0.5-10% by 16S rRNA gene amplicon sequencing (Law et al., 2016;
299 Saunders et al., 2016; Stokholm-Bjerregaard et al., 2017) and 4.8% by metagenomics (Albertsen et
300 al., 2012). All the *Ca.* Accumulibacter taxa were likely type II, except for OTU 2020, which differs
301 from typical type I or type II sequences (Supplementary Table 2, Flowers et al., 2009). Based on
302 qPCR results, IIB and IIC were the predominant clades in the WWTPs (Supplementary Table 3). In
303 comparison, clades IIA, IIB and IIC were abundant in full-scale WWTPs in the U.S. (He et al. 2007),
304 and IIC and IID were the most dominant clades in 18 full-scale WWTPs from six countries (Mao et
305 al. 2015). Further, clades IA, IIB and IIC were consistently detected in eight geographically and
306 operationally distinct WWTPs (Zhang et al. 2017). Overall, it appears that clades IIB and IIC are
307 dominant in both temperate and tropical regions.

308 Two *Tetrasphaera* OTUs were detected, but only one (OTU127) was dominant in all three WWTPs.
309 Total *Tetrasphaera* OTUs were most abundant in WWTP1 (1.1-1.8%), followed by WWTP2 (0.37-
310 0.55%) and WWTP3 (0.23-0.37%) (Fig. 2B). Phylogenetic analysis of the 16S rRNA gene of the two
311 *Tetrasphaera* OTUs suggested both of them are closely related to clade 3 (Nguyen et al., 2011). Both
312 *Ca.* Accumulibacter and *Tetrasphaera* were detected by FISH in all three plants (Fig. 3). Almost all
313 the *Ca.* Accumulibacter cells and a large proportion of *Tetrasphaera* cells accumulated P as
314 indicated by FISH and poly-P dual-staining (Supplementary Fig. S2), suggesting that they are active
315 PAOs in the plants. Other putative PAOs, e.g. *Ca.* Obscuribacter (0.06-0.49%) and *Microlunatus* (1
316 OTU, 0.01-0.21%) were present in minor amount. FISH and poly-P dual-staining confirmed P
317 accumulation by other cells that were neither *Ca.* Accumulibacter nor *Tetrasphaera* (Supplementary
318 Fig. S2).

319 *Ca.* Competibacter and *Defluviicoccus* were the dominant GAOs (Fig. 2B). Six *Ca.* Competibacter-
320 related OTUs were detected across the nine samples, with the lowest relative abundance (0.87-1.42%)
321 observed in WWTP1. WWTP2 and WWTP3 showed similar relative abundance of 2.55-4.58% and
322 2.40-4.35%, respectively; however, there was a distinct community structure at the OTU level (Fig.
323 2B). Nine *Defluviicoccus*-related OTUs were detected, where none of the OTUs showed a relative
324 abundance >0.1% in any of the samples, except for *Defluviicoccus* cluster III OTU 60, which
325 consistently occurred at high relative abundance (3.04-3.43%) in WWTP3. Cluster III *Defluviicoccus*
326 members have a filamentous morphology and a GAO-phenotype (McIlroy et al., 2010). The relative
327 abundance of *Defluviicoccus* in WWTP1 (0.08-0.11%) and WWTP2 (0.27-0.33%) was much lower.
328 *Propionivibrio*- and *Micropruina*- related GAOs were detected but accounted for very minor
329 fractions of each community. *Micropruina* showed the highest relative abundance at 0.13-0.21% in
330 WWTP2.

331 The genus *Dechloromonas* was moderately abundant in all plants (0.30-0.68%, 0.20-0.48% and 0.54-
332 0.68% for WWTPs 1, 2 and 3, respectively). OTU 46 predominated in WWTPs 1 (0.2-0.53%) and 3
333 (0.51-0.63%), and OTU 636 (0.11-0.23%) and OTU356 (0.08-0.22%) dominated in WWTP2. The
334 *Dechloromonas* genus is closely related to *Ca.* Accumulibacter in the Rhodocyclaceae family. Some
335 taxa may be PAOs, as an in-situ study showed that they behave similarly to *Ca.* Accumulibacter in
336 terms of substrate uptake and storage of PHAs (Kong et al., 2007). They may assimilate both acetate
337 and amino acids anaerobically (McIlroy et al. 2016) or assimilate acetate and store PHA, but without
338 poly-P cycling (Ahn et al., 2007; Günther et al., 2009). Therefore, the *Dechloromonas* OTUs
339 observed in this study may represent PAOs, GAOs or neither.

340 Overall, PAOs were more abundant (3.23-4.33%) than GAOs (1.02-1.74%) in WWTP1, while in
341 WWTP2, the abundances of PAOs (3.19-4.51%) and GAOs (3.04-5.05%) were similar. GAOs (4.26-
342 4.70%) were much more abundant than PAOs (1.30-1.78%) in WWTP3. All three plants contained a
343 diverse EBPR community composed of a rich collection of PAOs and GAOs with many OTUs. Most

of the OTUs were detected across plants but with distinct relative abundances, suggesting that each plant had its own signature EBPR community (Fig. 2B).

3.3 Anaerobic P-release profiles with different carbon sources

Systematic anaerobic P-release tests were performed on the activated sludge samples obtained from the three WWTPs, using 24 carbon sources from five categories representing the most commonly found hydrolysates of the three major groups of organic matter: carbohydrates, lipids, and proteins (Nielsen et al., 2010). All samples had PAOs capable of using a wide range of compounds and despite the disparity in PAO community structures, similar P-release patterns were observed across all plants (Fig. 4). VFAs resulted in the highest P-release of 24.0-39.4, 18.8-37.0 and 17.0-34.6 mg/L for acetate, propionate and butyrate, respectively, with an MLSS of 2 g/L. The values were comparable to those obtained with acetate in temperate EBPR plants, such as 8-15 mg P/g SS in 28 Danish WWTPs (Mielczarek et al., 2013), 5.1-24.3 mg P/g VSS in six WWTPs in the United States (Gu et al., 2008), and up to 15.8 mg P/g SS in ten EBPR plants in China (Zhang et al., 2011). Substantial P release was observed for some carboxylic acids (i.e., 20.2-36.2 mg/L for pyruvate, 9.0-23.4 mg/L for lactate and 7.4-24.8 mg/L for fumarate), which constituted the second most efficient group of carbon sources, following VFAs. This result was not unexpected since pyruvate and fumarate are important intermediates in the anaerobic carbon metabolism of *Ca.* Accumulibacter and *Tetrasphaera* (He and McMahon, 2011; Kristiansen et al., 2013). All nine amino acids resulted in substantial P-release (up to 23.2 mg/L); values were comparable to those in Danish WWTPs (where high P-release of up to 12.0 mg P/g VSS was obtained with glycine), despite the much lower relative abundances of *Tetrasphaera* and *Ca.* Accumulibacter in our study. Relatively low P-release was observed for sugars, alcohols and other carboxylic acids (e.g. malate). These findings highlight the importance of VFAs in tropical EBPR systems, as generally understood for temperate EBPR (Oehmen et al., 2007), but at the same time underscores the potential roles of other compounds (e.g.,

pyruvate, fumarate, lactate, amino acids and in some cases, glucose), which could serve as complementary carbon sources.

The P-release values induced by each carbon source were normalised against the P-release from the acetate treatment and from the same activated sludge source, and a clustering analysis was performed (Fig. 5). Interestingly, the P-release profiles corresponding to the utilisation of different carbon sources for activated sludge from each plant clustered together, suggesting a connection between carbon usage characteristics and PAO community composition.

3.4 Relating carbon source usage to bacterial/PAO community composition

If certain taxa in the activated sludge preferentially do utilise specific carbon sources for P-release, then these associations may be detectable by examining correlation statistics between P-release and the relative abundance of OTUs across samples, under the assumption that a greater abundance of the relevant genes will be associated with an increase in P-release magnitude. Among the top 89 most abundant bacterial OTUs that had no missing values, PAOs tended to strongly correlate with P-release associated with different carbon sources (Supplementary Fig. S3). We note that other non-PAO taxa showed a comparable degree of correlation, which could result from similar mechanistic association with carbon utilisation in a non-EBPR context, mechanistic associations with PAOs unrelated to carbon source utilization, OTUs that represent cryptic PAO species or false positive correlations associated with the sample sizes employed here. Interestingly, GAOs showed a tendency to be anti-correlated with the P-release profiles (induced from the utilisation of different carbon sources), consistent with an underlying competition with PAOs (Oehmen et al., 2007). Overall, there was a positive correlation between P-release and the PAO community.

For different carbon sources, strong positive correlations were observed for compounds within the same category (Fig. 6A), suggesting that similar compounds were processed via similar metabolic pathways and/or by specific groups of bacteria. Among categories, positive correlations were

observed between sugars and amino acids, which suggests that bacteria that can use amino acids may also be able to use sugars (Fig. 6A). *Tetrasphaera* is known to have the ability to metabolise sugars and amino acids (Kong et al., 2005; Nguyen et al., 2011; Kristiansen et al., 2013; Nguyen et al., 2015; Marques et al., 2017). Additionally, positive correlations were observed between some VFAs and amino acids, particularly, between aspartate and all the VFAs and between acetate and glutamate.

Strong positive correlations (Pearson correlations >0.91 with $P<0.001$) were observed between the total population of *Ca.* Accumulibacter and all VFAs, pyruvate and fumarate (Fig. 6A), underscoring the role of VFAs as primary carbon sources utilised by *Ca.* Accumulibacter (Oehmen et al., 2007; Flowers et al., 2013). Interestingly, strong positive correlations were also observed between the total population of *Ca.* Accumulibacter and some amino acids (aspartate, glutamate, glycine and casein acid). As also suggested by the CCA analysis (Fig. 6B), this correlation was most prominent for *Ca.* Accumulibacter OTU 81 (the most abundant *Ca.* Accumulibacter OTU in WWTP1 and the second most abundant *Ca.* Accumulibacter OTU in WWTP2), indicating a significant role in amino acid-induced P-release. Via MAR-FISH analysis, Kong et al. (2004) showed that PAO651-defined *Ca.* Accumulibacter from three full-scale WWTPs in Denmark could assimilate glutamate, but not other amino acids tested. None of the *Ca.* Accumulibacter cells from these three full-scale WWTPs in Denmark that were targeted by the PAOmix probe could use glycine (Nguyen et al. 2015). Most of the *Ca.* Accumulibacter cells from four full-scale WWTPs in Japan that hybridised with the probe could take up glutamate and aspartate anaerobically (Chua et al. 2006). Metatranscriptomic characterisation of an enrichment culture of *Ca.* Accumulibacter clade IIC strain UW-1 showed the expression of genes involved in anaerobic glycine metabolism, and P-release associated with anaerobic glycine was further demonstrated in a batch test (Oyserman et al., 2016). Using FISH and poly-P dual staining, we observed that a greater number of *Ca.* Accumulibacter cells released their poly-P after anaerobic incubation with glutamate or aspartate, suggesting they are capable of using these carbon sources anaerobically. At the same time, a number of *Ca.* Accumulibacter cells retained

417 their poly-P even after anaerobic incubation with glutamate or aspartate, indicating they are not
418 efficient glutamate/aspartate users (Supplementary Fig. S2). These observations highlight that *Ca.*
419 Accumulibacter taxa have different affinities toward amino acids and might explain the
420 differentiation of the amino acid-using *Ca.* Accumulibacter (OTU 81) from other taxa in the
421 correlation test (Fig. 6).

422 The relative abundance of *Ca.* Accumulibacter OTU 246 was closely related to alcohol-induced P-
423 release. By comparison, a previous metagenomic study revealed two *Ca.* Accumulibacter taxa from
424 clade IIF with a set of genes encoding the necessary enzymes to convert ethanol into acetate, which
425 might enable them to use ethanol for EBPR (Skennerton et al. 2013). Based on a comparison with
426 type I and type II FISH probes (Flowers et al., 2009), the less abundant *Ca.* Accumulibacter OTU
427 246 (0.01-0.16%) in the plants sampled in this study can most likely be assigned to type II
428 (Supplementary Table 2). Given that it was the only OTU related to alcohols among all the putative
429 PAOs, its ability to use alcohols should be further studied.

430 Through CCA analysis (Fig. 6B), we observed that the predominant *Tetrasphaera* OTU 127 was
431 positively related to the P-release induced by amino acids (asparagine, serine), sugars (glucose,
432 mannose), malate and all the alcohols. However, except for serine and mannose, none of these
433 correlations were significant ($P<0.05$) in the Pearson correlation test (Fig. 6A). It is possible that the
434 capability of some *Ca.* Accumulibacter taxa like OTU81 to use amino acids weakened the statistical
435 relationship between *Tetrasphaera* and amino acids. In addition, a study suggested that some
436 *Tetrasphaera* members might be able to achieve anaerobic P-uptake with sugars and/or amino acids,
437 due to energy generation through fermentation of the carbon sources (Marques et al., 2017). This
438 ability would mask the relationship between P-release and their population.

439 *Ca.* Obscuribacter (0.05-0.49%) showed no correlation between P-release and any of the carbon
440 sources tested (Fig. 6A), and was only weakly connected to the same group of carbon sources that

correlated with *Tetrasphaera* (Fig.6B). *Ca.* Obscuribacter is a putative PAO predicted to be able to utilise VFAs, glucose and amino acids (Soo et al., 2014), although these features need to be further confirmed. Among the three *Dechloromonas* OTUs, OTU 46 showed significant anti-correlation with a wide range of carbon sources, including most VFAs, sugars and amino acids. Meanwhile, the other 2 OTUs (OTU 356 and 636) correlated with these compounds, but it is possible that correlations were the result of the positive correlation between total Ca. Accumulibacter and OTU_356 and OTU_636. Hence we are unable to unambiguously assign *Dechloromonas* OTUs to the P-release stimulated by any of the carbon sources. *Dechloromonas* was the third most abundant group of putative PAOs in the three plants, so it is necessary to further investigate their putative roles in EBPR systems. However, amplicon analysis may overestimate the relative abundance of some *Dechloromonas* OTUs as much as 10-fold compared to FISH-based quantification due to a high copy number of the 16S rRNA gene (McIlroy et al., 2016). None of the available FISH probes hybridised with the few *Dechloromonas* OTUs in our sludge samples.

3.5 Performance of selected carbon sources for EBPR

Anaerobic-aerobic cycle studies were performed with selected carbon sources (glucose, methanol, glutamate, aspartate, acetate and propionate) at a concentration of 30 mg C/L (Fig. 7). Acetate, propionate and wastewater (i.e., primary effluent) showed similar P-release profiles, resulting in high P-release at the end of the anaerobic stage. The greatest P-release was observed for WWTP2 (Fig. 7B) and corresponded with the highest relative abundance of PAOs (especially, *Ca.* Accumulibacter) (Fig. 2B). Substantial P-release was observed for glutamate and aspartate, with very similar profiles that were almost constant throughout the anaerobic operation, but at much lower rates when compared to wastewater and VFAs. Interestingly, an initial adaptation period seemed to be necessary for glucose or methanol to stimulate a substantial P-release. It is possible that these compounds were first fermented by *Tetrasphaera* or other bacteria, and that some fermentation products induced P-release by PAOs (Nielsen et al., 2010; Kristiansen et al., 2013; Marques et al., 2017). However, during the

466 anaerobic phase, no VFA formation was observed for any of the carbon sources and neither was
467 significant PHA formation detected in the sludge (Supplementary Fig. S3). The lack of observable
468 VFA formation is reasonable given that the uptake rate of VFAs is higher than that of other carbon
469 sources (Supplementary Fig. S3). No PHA detection suggests that these carbon sources may have
470 been converted into other intracellular storage compounds.

471 In the subsequent aerobic stage, substantial P-uptake was observed for all carbon sources. Some
472 carbon sources (glutamate and methanol) allowed aerobic P-uptake without the necessity for
473 anaerobic conversion of these carbon sources to known intracellular storage compounds (e.g., PHAs,
474 Fig. S4). Acetate and wastewater had the highest P-uptake rates. Significantly lower P-uptake was
475 observed for propionate. This lower P-uptake is likely due to the need for PAOs to acclimate to the
476 different intracellular storage polymer content (higher in PHV and PH2MV). Since acetate was the
477 major VFA in these plants, the lower P-uptake observed for propionate was not unexpected. A
478 similar explanation might apply to other carbon sources. Despite the distinct PAO community
479 composition among plants, glutamate always showed lower P-uptake compared to aspartate. In lab-
480 scale reactors, aspartate and glutamate were suggested to be favoured by *Ca.* Accumulibacter and
481 *Tetrasphaera*, respectively (Fukushima et al., 2007; Zengin et al., 2011). However, the consistent
482 behaviour of glutamate and aspartate in the present work, across three plants, suggests that glutamate
483 and aspartate were probably used by the same group of PAOs.

484 Stoichiometric values were calculated for P and carbon transformations and compared to published
485 model values (Table 2) for acetate, propionate and wastewater; model values for other carbon
486 sources were not available. Our stoichiometric values differ somewhat from the published ones for
487 *Ca.* Accumulibacter (or a mixed PAO-GAO community), but generally fall within the range obtained
488 from full-scale sludge studies in temperate regions. Aerobic stoichiometry is expected to show a
489 higher degree of variability, since the production of biomass, polyphosphate, and glycogen from
490 PHA can proceed independently (Smolder et al., 1995). In general, higher aerobic P-uptake/PHA-

consumption (P/PHA) values were observed in all three plants, which is in line with the observation of Lanham et al. (2013).

Among plants, the highest anaerobic P-release/VFA-uptake (P/VFA) ratios and aerobic P/PHA ratios were observed in WWTP2 samples, corresponding to the high relative abundance of *Ca.* Accumulibacter. WWTP1 had similar ratios to WWTP3, but also the lowest number of GAOs (*Ca.* Competibacter, 0.87-1.42%; *Defluviicoccus*, 0.38-0.69%). Apart from the fact that GAOs take up VFAs, which results in lower P/VFA values, different groups of PAOs have been found to show distinct stoichiometric ratios. Welles et al. (2015) suggested that, when poly-P is not limiting, type II *Ca.* Accumulibacter performed a PAO metabolism with a P/VFA ratio of 0.64 P-mol/C-mol; in contrast, type I members displayed a mixed PAO-GAO metabolism with a P/VFA ratio of 0.22 P-mol/C-mol and a correspondingly high PHV content in the PHA. Although a majority of the detectable *Ca.* Accumulibacter OTUs in the three plants seemed to be type II members, the predominance of different species in different plants might also have contributed to differences in the stoichiometric values. Additionally, the conversion of VFAs into PHA requires reducing equivalents, where for *Ca.* Accumulibacter, these reducing equivalents can be obtained from glycolysis and/or the anaerobic operation of the reductive branch of the TCA cycle (Comeau et al., 1986; Mino et al., 1987; Schuler and Jenkins, 2003; Hesselmann et al., 2000; Pijuan et al., 2008; Zhou et al., 2010). The involvement of the TCA cycle results in a higher PHV content. The contribution of each pathway varies among different *Ca.* Accumulibacter taxa (Majed et al., 2012), and depends on the availability of glycogen in the cells (Zhou et al., 2009). PAOs in full-scale temperate EBPR plants often employ the anaerobic TCA cycle in addition to, or instead of, the glycolysis pathway (Lanham et al., 2013). Law et al. (2016) also suggested that glycolysis and the TCA cycle were of equal importance in supplying reducing power in a full-scale tropical EBPR system, based on both stoichiometric and metatranscriptomic results, where genes in both pathways were highly expressed at approximately the same levels. The relatively lower anaerobic Gly/VFA and aerobic Gly/PHA values observed in

WWTP1, together with PHV/PHB values comparable to those found in WWTP3 (with a higher relative abundance of GAOs), would suggest a greater use of the TCA cycle in WWTP1.

In general, based on the PHA/VFA ratios, most of the PHA was composed of PHB when acetate was supplied. Anaerobic Gly/VFA and aerobic Gly/PHA ratios were also at a low level. Additionally, the P/VFA ratios were close to the model values of *Ca.* Accumulibacter, suggesting that *Ca.* Accumulibacter (and probably other PAOs) could effectively acquire organic carbon in the presence of high numbers of GAOs. This was particularly evident in WWTP3, where the relative abundance of *Ca.* Competibacter (2.40-4.35%) and *Defluviicoccus* (3.04-3.43%) was 5.4 to 6.7 times higher than that of *Ca.* Accumulibacter (0.95-1.40%) (Fig. 2B). The stoichiometric values were not different from those observed at other plants, implying that these GAOs were not significantly affecting carbon uptake and P removal by PAOs.

When primary effluent was used, the observed P/VFA values were much higher compared to acetate or propionate, suggesting that other organic compounds present in the wastewater were utilised by PAOs and contributed to additional P-release (Supplementary Fig. S3C). Higher Gly/VFA ratios (compared to those when acetate or propionate were supplied) were also observed, together with elevated PHA/VFA values as well as a high PHV content of the PHA, suggesting that both PAOs and GAOs were more active. Overall it appears, in view of the high observed P/VFA ratios, that both PAOs and GAOs could access other organic compounds from the wastewater. More work is needed to understand the roles of carbon sources in mediating the interactions between PAOs and GAOs in full-scale EBPR systems.

Conclusions

- Three WWTPs in tropical Singapore showed high in-situ EBPR activities. Each plant possessed a characteristic microbial community composed of diverse putative PAOs with multiple OTUs (affiliated to *Ca.* Accumulibacter, *Tetrasphaera*, *Dechloromonas* and *Ca.* Obscuribacter), highlighting that PAOs commonly found in temperate EBPR systems also thrive at higher temperatures.
- Despite differences in composition, all microbial communities were capable of P-release by using a wide range of carbohydrates, lipids, and protein hydrolysates. This flexibility is important in dynamic environments and with complex organic-carbon mixtures, as found in full-scale WWTPs.
- The PAO community composition was highly correlated with the EBPR-associated carbon usage profiles, attributable in part to the different capabilities of specific taxa in using specific carbon sources.
- *Ca.* Accumulibacter was the most dominant group of PAOs in the WWTPs, and VFAs were the most effective carbon sources for release and uptake of P. These findings highlight the important role of *Ca.* Accumulibacter and VFAs, similar to temperate EBPR systems.
- Even within the lineage of *Ca.* Accumulibacter, different taxa were associated with the P-release induced by distinct carbon sources, implying that *Ca.* Accumulibacter contains diverse members with versatile carbon metabolisms. In complex and variable environments, these differences may allow them to occupy different ecological niches, where they can co-exist and contribute to a robust EBPR.
- Apart from *Ca.* Accumulibacter and *Tetrasphaera*, other PAOs contributed to the EBPR, However, currently, very limited information is available on those PAOs and future work should investigate their roles and metabolisms in full-scale plants.

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1 **Table 1** Operating conditions, primary effluent and secondary effluent characteristics and EBPR activity at the three WWTPs in Singapore

Characteristic	Parameter, unit	WWTP1	WWTP2	WWTP3
Operating conditions	Process configuration	5-stage step feeding	Modified Ludzack–Ettinger (MLE)	Anoxic/Anaerobic/Aerobic-MBR
	Treatment capacity, m^3/day	361,000	205,000	68,000
	SRT, day	5.0	5.0	15 - 20
	HRT, h	5.6	6.8	10 - 12
	T, ^oC	28.7 - 31.2	29.8 - 31.1	29.3 - 31.6
	pH	6.32 - 7.49	6.82 - 7.67	6.62 - 8.70
	MLSS[a], g/L	2.30 - 2.76	2.18 - 2.45	3.33 - 3.80
	MLVSS[a], g/L	1.70 - 1.87	1.84 - 1.95	2.27 - 2.56
Primary effluent	TCOD, mg/L	237 - 324	337 - 381	348 - 394
	PO_4^{3-}P, mg/L	4.44 - 5.31	5.14 - 7.38	2.46 - 5.39
	TP, mg/L	5.53 - 6.52	6.02 - 8.19	3.48 - 6.37
	NH_4^+-N, mg/L	38.7 - 42.8	37.7 - 43.8	36.2 - 43.9
	TN, mg/L	42.6 - 50.1	40.9 - 44.7	38.1 - 45.6

	TOC, mg/L	37.8 - 62.2	63.8 - 69.9	68.6 - 87.1
	Acetate, mg/L	26.9 - 31.5	50.3 - 62.5	32.7 - 44.5
	Propionate, mg/L	3.60 - 4.41	4.90 - 8.37	5.41 - 7.20
Secondary effluent	TCOD, mg/L	10 - 12	15 - 20	13 - 21
	PO_4^{3-}P, mg/L	0.51 - 1.61	0	0 - 2.38
	TP, mg/L	0.62 - 1.70	0	0 - 2.29
	NH_4^+-N, mg/L	0.57 - 3.93	0 - 12.8	0-0.56
	NO_3^--N, mg/L	2.11 - 3.19	6.18-9.61	6.47 - 9.28
	NO_2^--N, mg/L	0.24 - 0.34	0-1.78	0
	TN, mg/L	3.69 - 8.76	10.6 - 21.7	8.89 - 11.4
	TOC, mg/L	5.17 - 6.31	6.75 - 9.91	8.38 - 10.6
In situ EBPR activity	P-release [b], mgP/mgVSS	5.16 - 5.58	6.79 - 16.7	3.36 - 5.26
	P-uptake [c], mgP/mgVSS	6.21 - 6.50	9.19 - 18.2	3.63 - 6.35

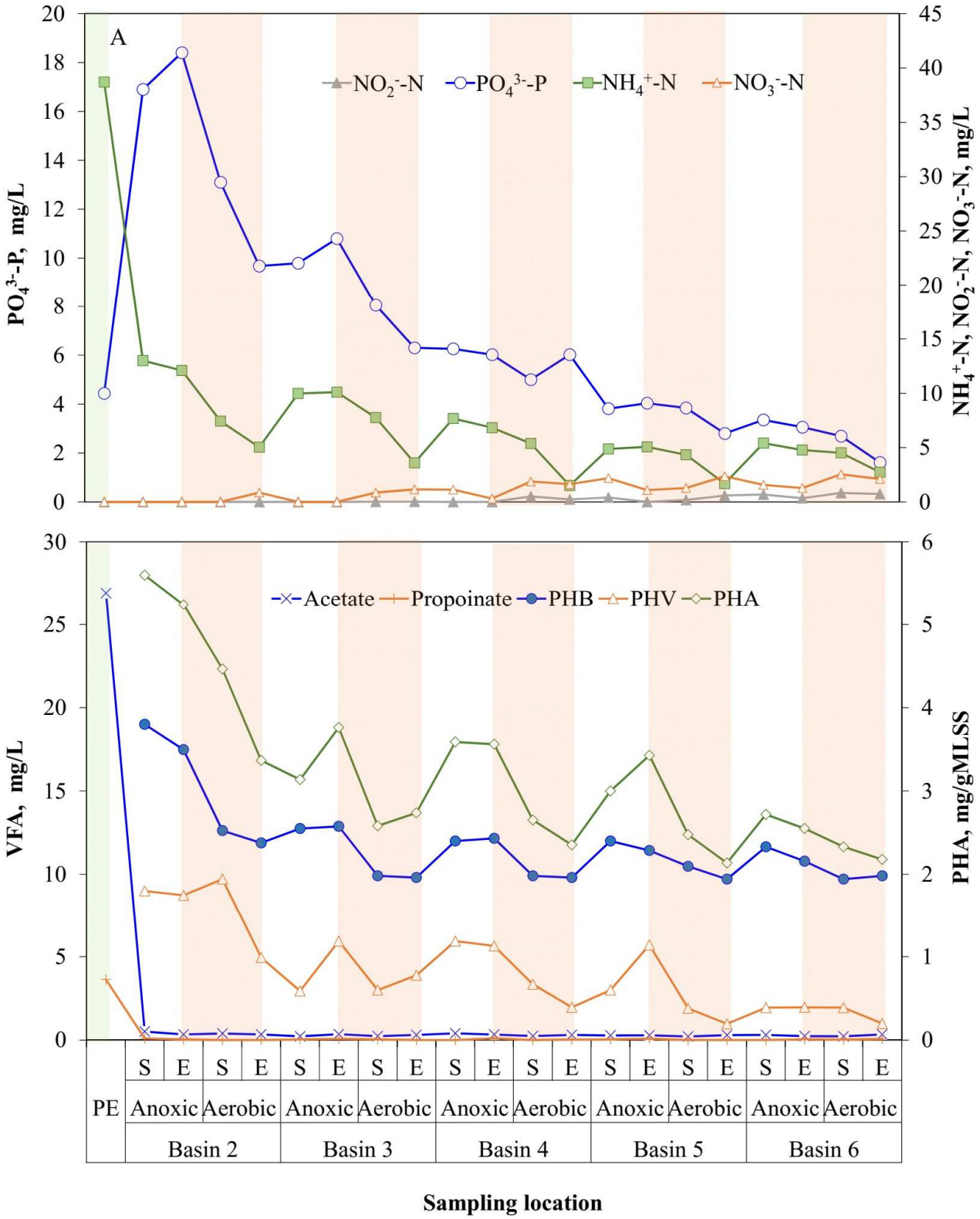
[a] The values show the concentrations at the end of the aerobic stage; [b] Calculated by subtracting the primary effluent PO_4^{3-}-P concentration from the PO_4^{3-}-P concentration measured at the end of the anaerobic\anoxic stage; [c] Calculated as difference between the PO_4^{3-}-P concentration at the end of the anaerobic\anoxic stage and that at the end of the aerobic stage.

Table 2 Stoichiometric ratios of phosphorus and carbon transformations during the anaerobic-aerobic cycle tests. Values represent the mean with standard deviation in parentheses (n = 3).

Carbon source	Origin	Anaerobic stoichiometry						Aerobic stoichiometry		Reference
		P/VFA[a]	PHA/VFA[b]	PHB/VFA[c]	Gly/VFA[f]	PHV/VFA[d]	PHV/PHB[e]	P/PHA[h]	Gly/PHA[i]	
		P-mol/C-mol	C-mol/C-mol					P-mol/C-mol	C-mol/C-mol	
Acetate	WWTP1	0.35 (0.01)	0.63 (0.06)	0.52 (0.09)	0.58 (0.10)	0.11 (0.02)	0.20 (0.08)	0.65 (0.22)	0.22 (0.02)	This study
	WWTP2	0.66 (0.06)	0.78 (0.24)	0.69 (0.18)	0.61 (0.26)	0.09 (0.07)	0.11 (0.07)	1.21 (0.46)	0.17 (0.05)	This study
	WWTP3	0.41 (0.08)	0.78 (0.10)	0.65 (0.04)	0.64 (0.15)	0.13 (0.07)	0.20 (0.10)	0.57 (0.08)	0.26 (0.01)	This study
	PAO TCA model	0.50	0.90	0.90	0.00	0.00	0.00	-	-	Comeau et al., 1986
	PAO Gly model	0.50	1.33	1.33	0.50	0.00	0.00	0.41	0.42	Smolder et al., 1995
	PAO TCA/Gly model	0.37	1.40	1.11	0.60	0.29	0.26	-	-	Hesselmann et al., 2000
	Ca. Accumulibacter Type I	0.64	1.36	1.27	0.29	0.09	0.07	-	-	Welles et al., 2015
	Ca. Accumulibacter Type II	0.22	1.47	1.24	0.96	0.23	0.19	-	-	Welles et al., 2015
	Full-scale sludge	0.30 - 1.30	0.67 - 1.74	-[g]	0.04 - 0.82	-	-	0.3-1.8	0.2-1.1	Lanham et al., 2013
	Full-scale sludge	0.33 - 0.45	-	-	-	-	-	-	-	Lopez-Vazquez et al., 2008
	Full-scale sludge	0.29 - 0.75	-	-	-	-	-	-	-	Gu et al., 2008
	Full-scale sludge	0.42 - 0.59	1.20 - 1.35	1.05 - 1.08	0.34 - 0.40	0.15 - 0.27	0.14 - 0.25	-	-	Pijuan et al., 2008

	GAO Model	0.00	1.86	1.36	1.12	0.46	0.34	0.00	0.65	Zeng et al., 2003
Propionate	WWTP1	0.38 (0.04)	0.56 (0.10)	0.04 (0.02)	0.69 (0.25)	0.53 (0.07)	-	0.49 (0.37)	0.35 (0.14)	This study
	WWTP2	0.60 (0.07)	0.60 (0.13)	0.05 (0.04)	0.68 (0.19)	0.48 (0.08)	-	1.06 (0.31)	0.52 (0.15)	This study
	WWTP3	0.46 (0.16)	0.61 (0.16)	0.06 (0.04)	0.71 (0.22)	0.55 (0.12)	-	0.31 (0.12)	0.38 (0.16)	This study
	PAO model	0.42	1.22	0.00	0.33	0.56	-	-	-	Oehmen et al., 2005b
	GAO model (*Defluviicoccus*)	0.00	1.50	0.00	0.67	0.83	-	-	-	Oehmen et al., 2006
Primary effluent	WWTP1	0.63 (0.03)	1.39 (0.00)	0.72 (0.06)	1.15 (0.05)	0.59 (0.08)	0.83 (0.19)	0.90 (0.28)	0.38 (0.13)	This study
	WWTP2	1.00 (0.05)	1.20 (0.16)	0.64 (0.03)	1.74 (0.42)	0.55 (0.13)	0.86 (0.11)	0.78 (0.28)	0.18 (0.11)	This study
	WWTP3	0.94 (0.00)	1.51 (0.08)	0.90 (0.07)	1.49 (0.16)	0.57 (0.05)	0.63 (0.14)	0.71 (0.07)	0.30 (0.18)	This study

[a]P-release to VFA-uptake molar ratio; [b]PHA-formation to VFA-uptake molar ratio; [c]PHB-formation to VFA-uptake molar ratio; [d]PHV-formation to VFA-uptake molar ratio; [e]PHB to PHV molar ratio in the PHA; [f]Glycogen-consumption to VFA-uptake molar ratio; [g]values not available; [h]P-uptake to PHA-consumption molar ratio; [i]Glycogen-formation to PHA-consumption molar ratio.


A
NO_2^--N
PO_4^{3-}-P
NH_4^+-N
NO_3^--N
PO_4^{3-}-P, mg/L
NH_4^+-N, NO_2^--N, NO_3^--N, mg/L
Acetate
Propoinate
PHB
PHV
PHA
VFA, mg/L
PHA, mg/gMLSS
S E S E S E S E S E S E S E S E S E S E S E
PE
Anoxic Aerobic Anoxic Aerobic Anoxic Aerobic Anoxic Aerobic Anoxic Aerobic
Basin 2 Basin 3 Basin 4 Basin 5 Basin 6
Sampling location

1

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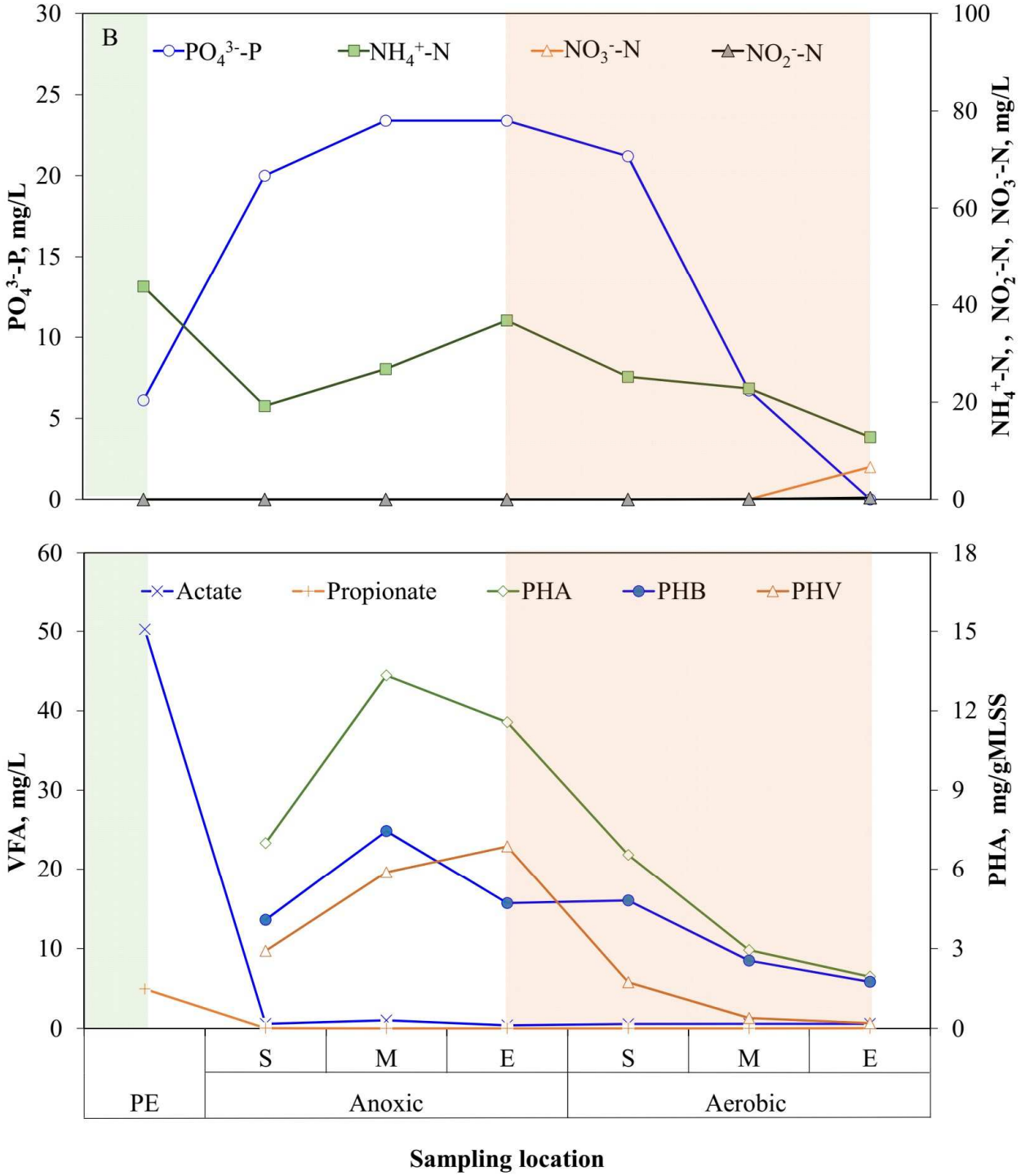
3

4


B
PO4 3--P
NH4 +-N
NO3 -N
NO2 -N
PO4 3-P, mg/L
NH4 +-N, , NO2 -N, NO3 -N, mg/L
Actate
Propionate
PHA
PHB
PHV
VFA, mg/L
PHA, mg/gMLSS
S
M
E
S
M
E
PE
Anoxic
Aerobic
Sampling location

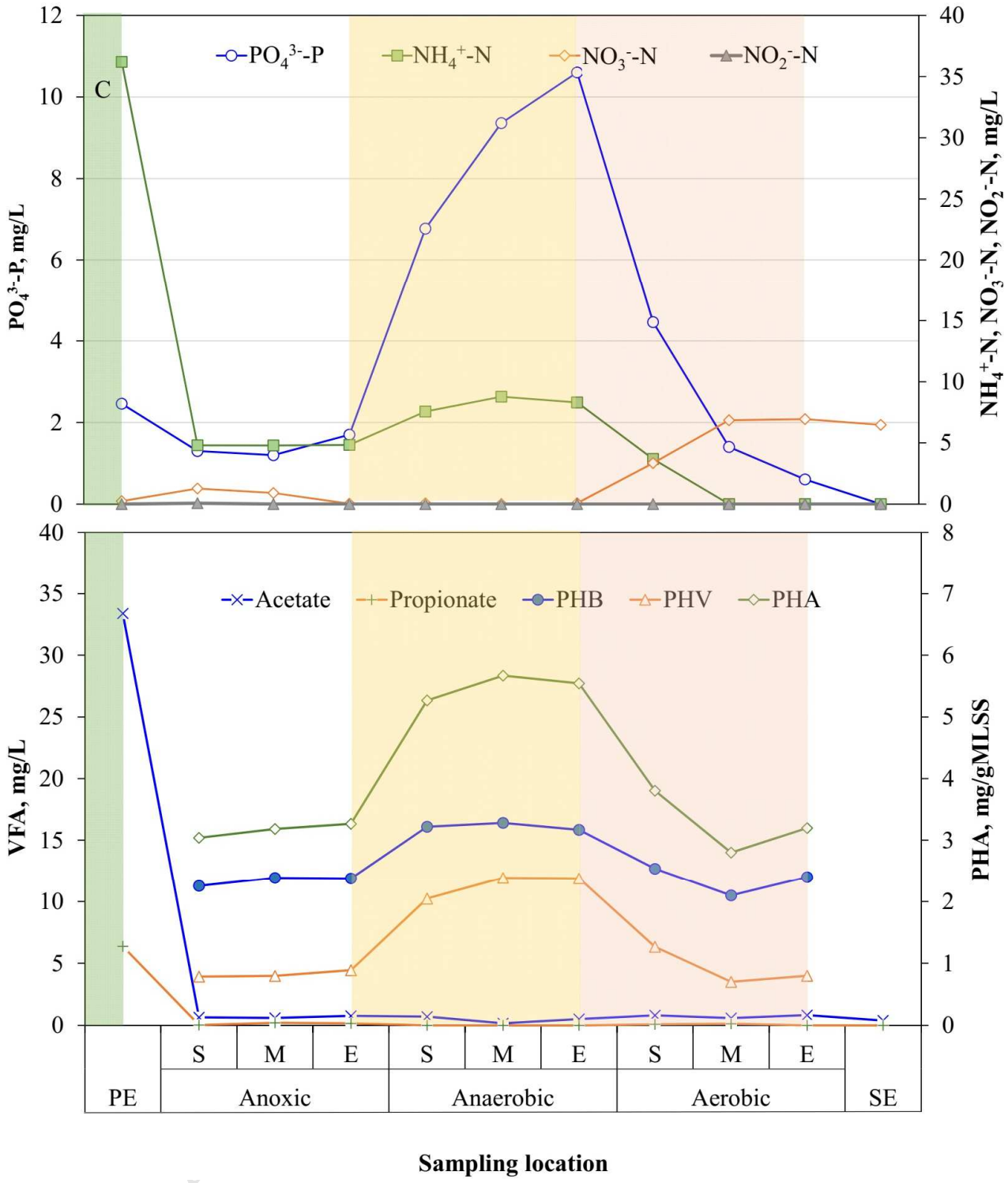
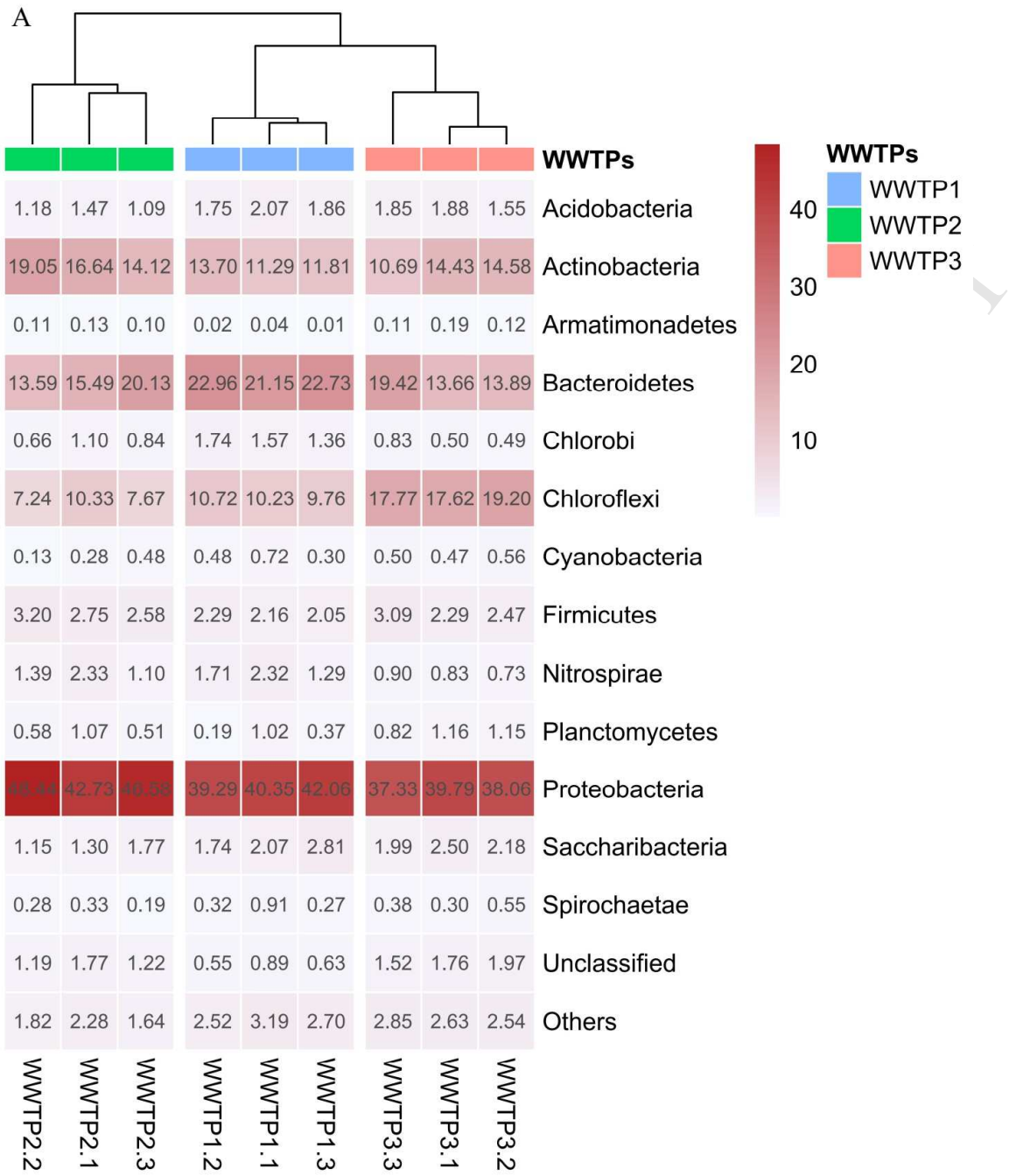
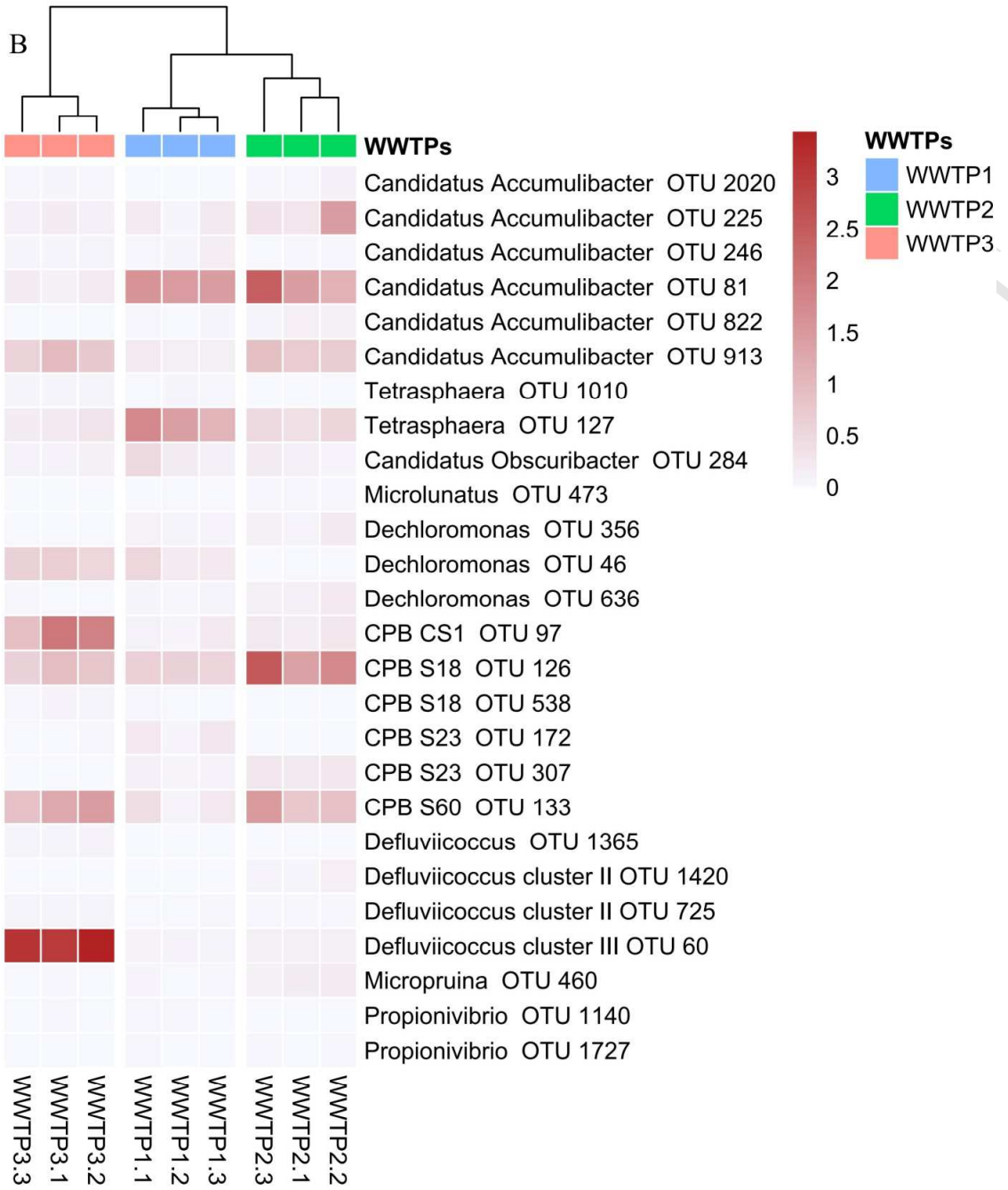


Figure 1 - Nutrient concentrations at the three WWTPs plants showing EBPR activity. (**A**), WWTP1; (**B**), WWTP2; (**C**), WWTP3. PE, primary effluent; S, start; M, mid; E, end; SE, secondary effluent. Green shading, PE; yellow shading, anaerobic stage; orange shading, aerobic stage.


A
WWTPs
1.18 1.47 1.09 1.75 2.07 1.86 1.85 1.88 1.55 Acidobacteria
19.05 16.64 14.12 13.70 11.29 11.81 10.69 14.43 14.58 Actinobacteria
0.11 0.13 0.10 0.02 0.04 0.01 0.11 0.19 0.12 Armatimonadetes
13.59 15.49 20.13 22.96 21.15 22.73 19.42 13.66 13.89 Bacteroidetes
0.66 1.10 0.84 1.74 1.57 1.36 0.83 0.50 0.49 Chlorobi
7.24 10.33 7.67 10.72 10.23 9.76 17.77 17.62 19.20 Chloroflexi
0.13 0.28 0.48 0.48 0.72 0.30 0.50 0.47 0.56 Cyanobacteria
3.20 2.75 2.58 2.29 2.16 2.05 3.09 2.29 2.47 Firmicutes
1.39 2.33 1.10 1.71 2.32 1.29 0.90 0.83 0.73 Nitrospirae
0.58 1.07 0.51 0.19 1.02 0.37 0.82 1.16 1.15 Planctomycetes
42.73 39.29 40.35 42.06 37.33 39.79 38.06 Proteobacteria
1.15 1.30 1.77 1.74 2.07 2.81 1.99 2.50 2.18 Saccharibacteria
0.28 0.33 0.19 0.32 0.91 0.27 0.38 0.30 0.55 Spirochaetae
1.19 1.77 1.22 0.55 0.89 0.63 1.52 1.76 1.97 Unclassified
1.82 2.28 1.64 2.52 3.19 2.70 2.85 2.63 2.54 Others
WWTP2.2
WWTP2.1
WWTP2.3
WWTP1.2
WWTP1.1
WWTP1.3
WWTP3.3
WWTP3.1
WWTP3.2
40
30
20
10
WWTPs
WWTP1
WWTP2
WWTP3

1


B
WWTPs
Candidatus Accumulibacter OTU 2020
Candidatus Accumulibacter OTU 225
Candidatus Accumulibacter OTU 246
Candidatus Accumulibacter OTU 81
Candidatus Accumulibacter OTU 822
Candidatus Accumulibacter OTU 913
Tetrasphaera OTU 1010
Tetrasphaera OTU 127
Candidatus Obscuribacter OTU 284
Microlunatus OTU 473
Dechloromonas OTU 356
Dechloromonas OTU 46
Dechloromonas OTU 636
CPB CS1 OTU 97
CPB S18 OTU 126
CPB S18 OTU 538
CPB S23 OTU 172
CPB S23 OTU 307
CPB S60 OTU 133
Defluviicoccus OTU 1365
Defluviicoccus cluster II OTU 1420
Defluviicoccus cluster II OTU 725
Defluviicoccus cluster III OTU 60
Micropruina OTU 460
Propionivibrio OTU 1140
Propionivibrio OTU 1727
WWTP3.3
WWTP3.1
WWTP3.2
WWTP1.1
WWTP1.2
WWTP1.3
WWTP2.3
WWTP2.1
WWTP2.2
3
2.5
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1.5
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WWTPs
WWTP1
WWTP2
WWTP3

2

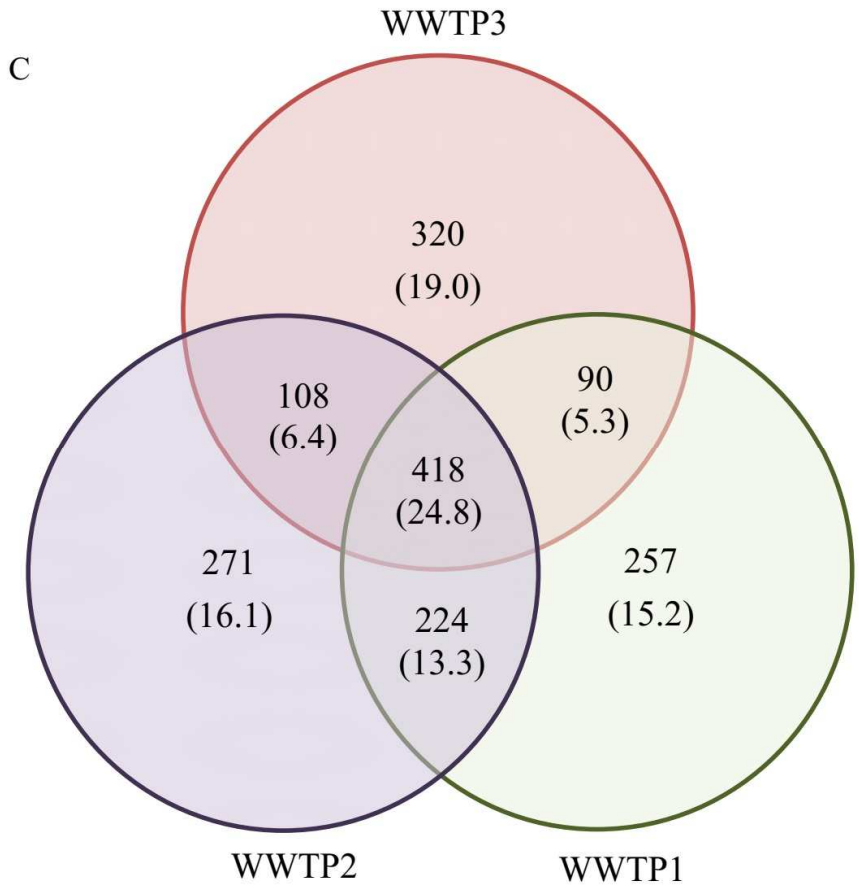


Figure 2 - Bacterial community composition at three WWTPs in Singapore. (**A**), communities at phylum level, **(B),** putative PAO and GAO community communities (only OTUs with relative abundance >0.02% are included); and **(C)** Venn diagram of the shared OTUs (with no less than 3 reads) among different WWTPs as suggested by 16S rRNA gene amplicon analysis. Parentheses show percentages.

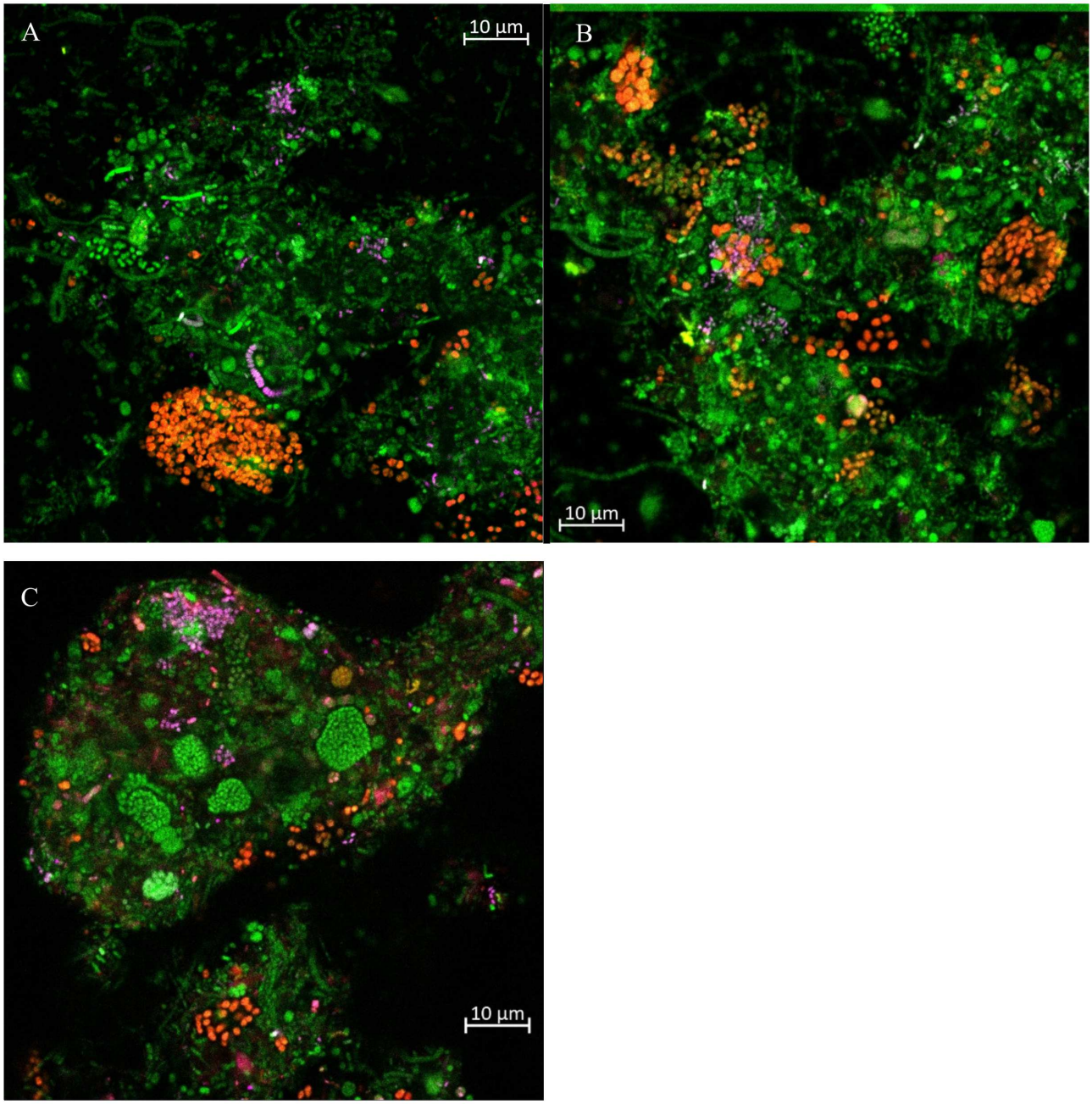
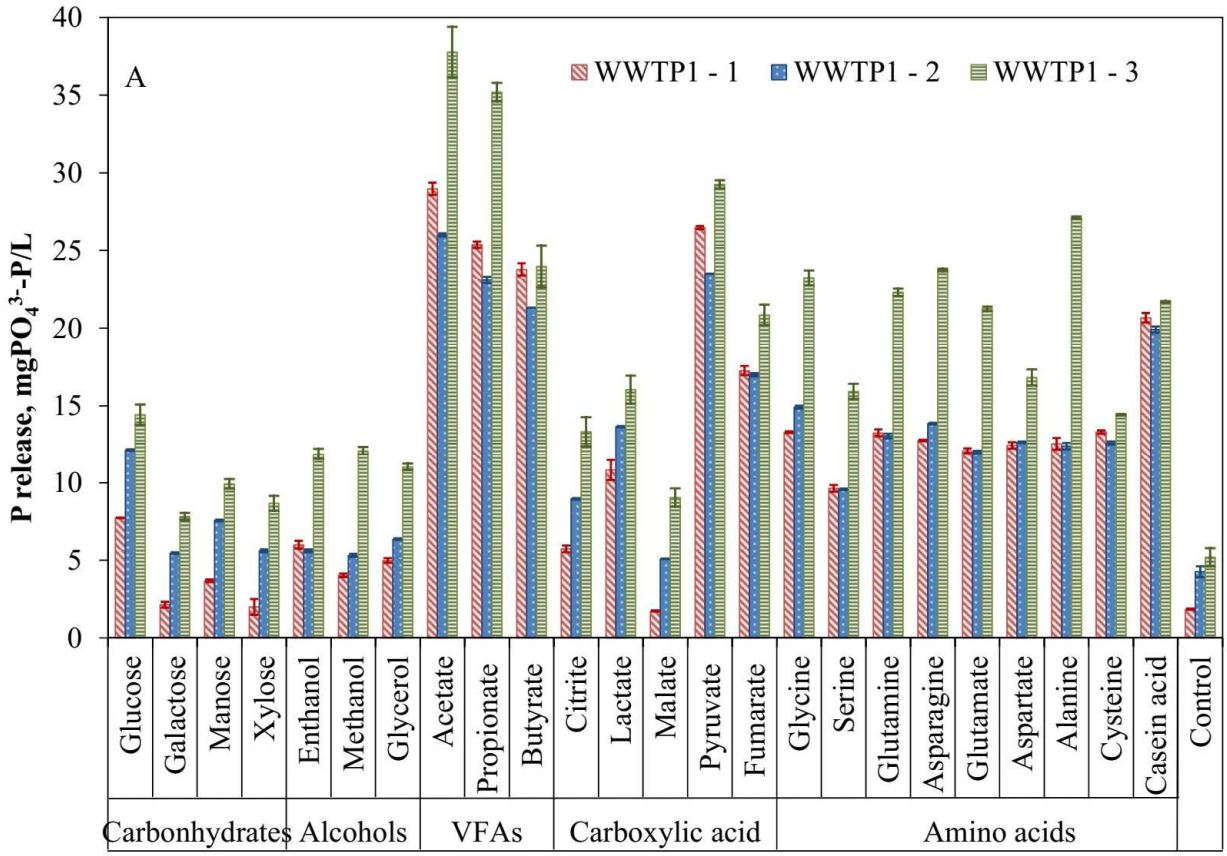


Figure 3 – Whole cell fluorescence in-situ hybridisation imaging of activated sludge from three plants. (**A**), WWTP1; **(B),** WWTP2; and **(C)** WWTP3. Bacteria hybridised with EUBmix (green), *Ca.* Accumulibacter–PAOs with PAOmix (red), and *Tetrasphaera*–PAOs with Tetmix (magenta).



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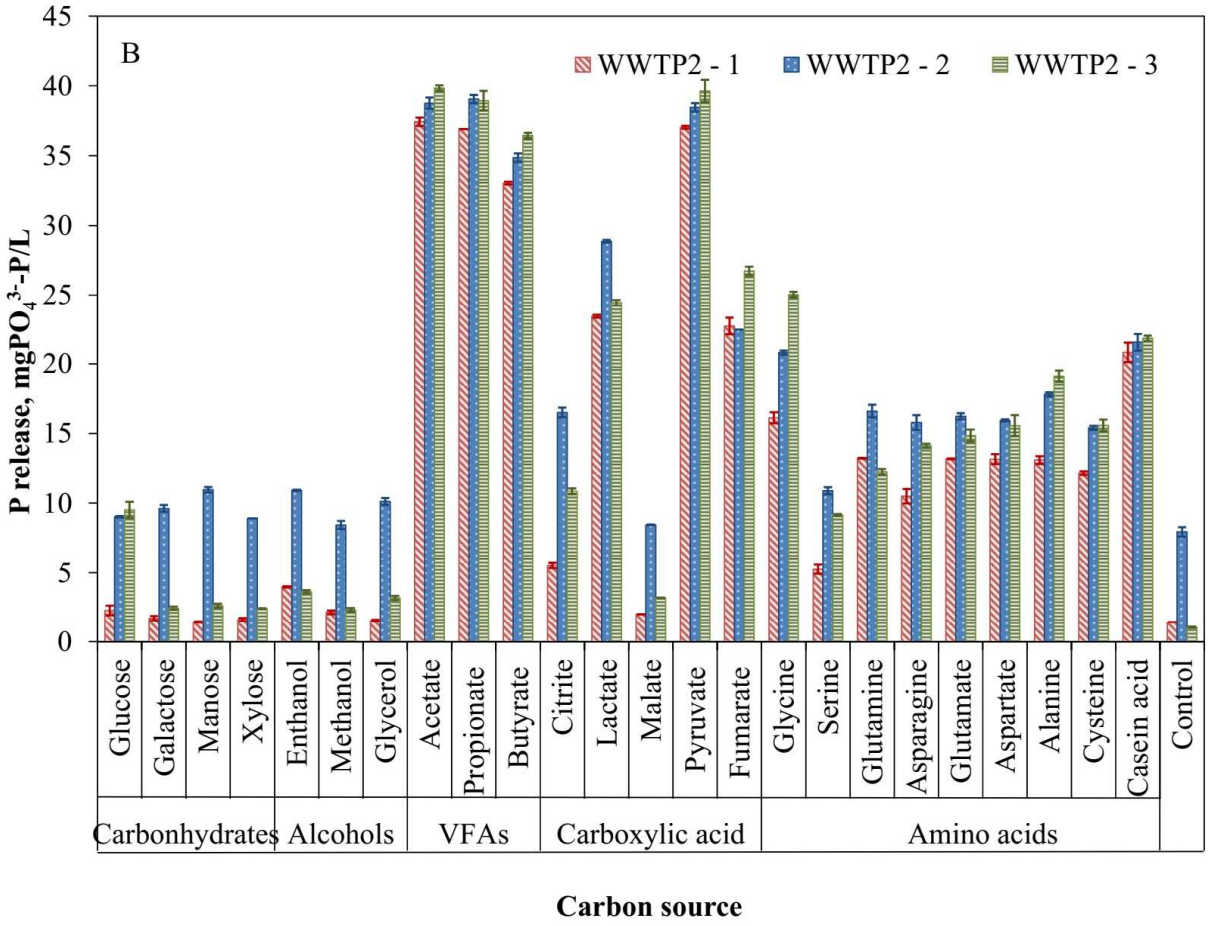

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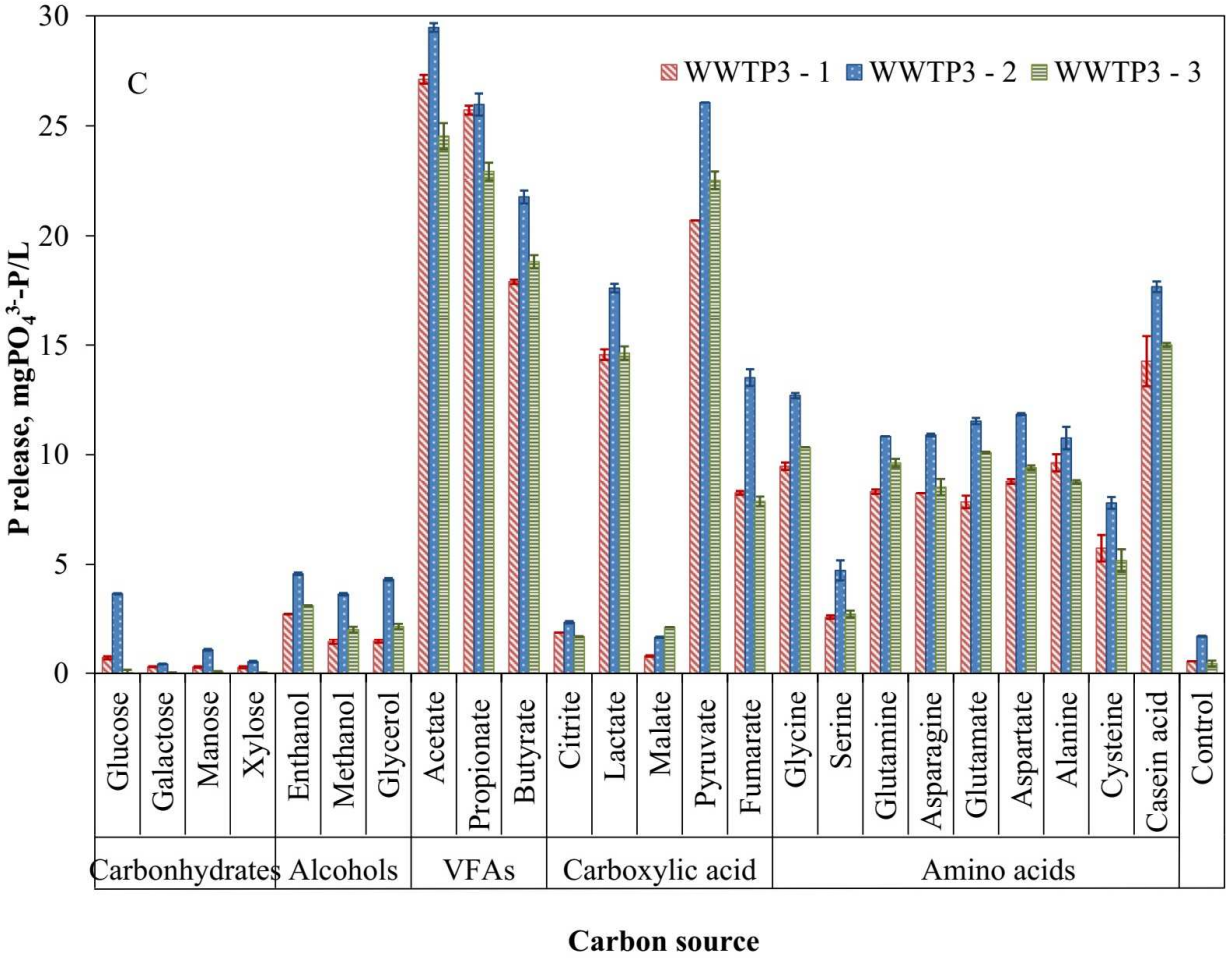


Figure 4 – Phosphorus levels after release from activated sludge from (**A**), WWTP1; (**B**), WWTP2; and (**C**), WWTP3 after a 3-h anaerobic incubation with different carbon sources. Initial COD = 300 mg/L, MLSS = 2.0 g/L. No carbon source was added for the control. Error bars show the range of duplicated tests.

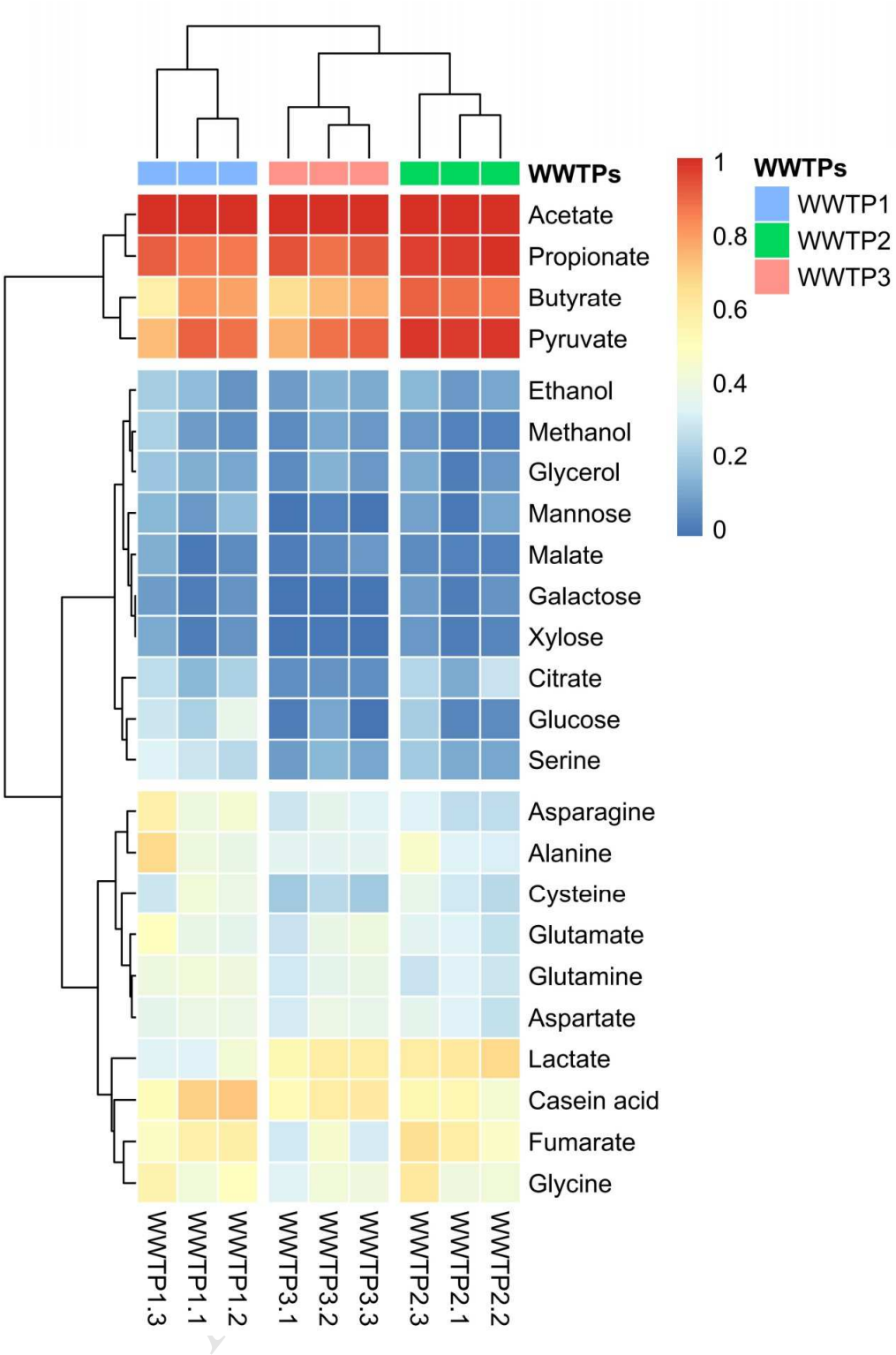
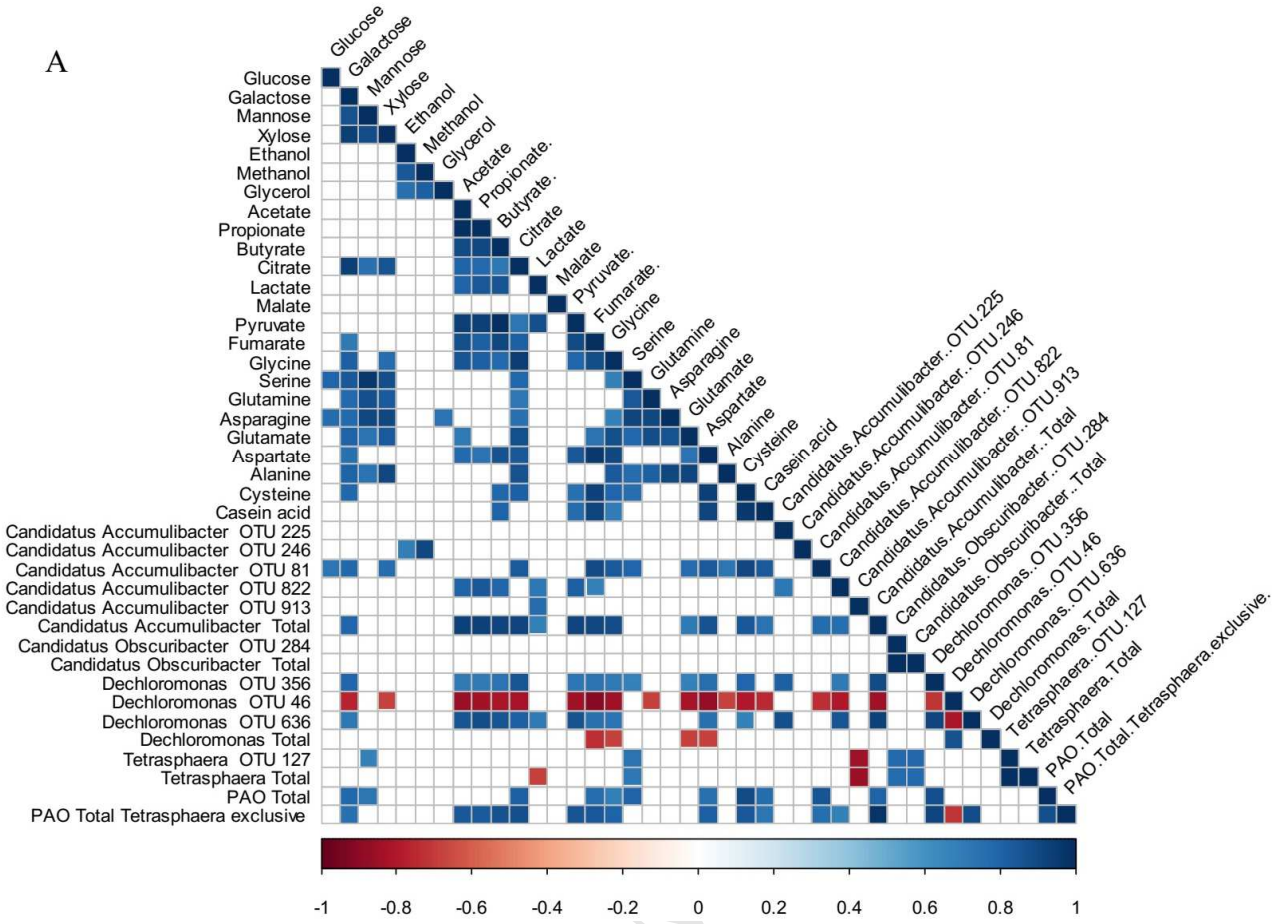


Figure 5 - Heat-map with cluster analysis of the normalised P-release activities of activated sludge from three plants with different carbon sources. Values were normalised to the corresponding P-release obtained with acetate for each sampling episode.

A



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2

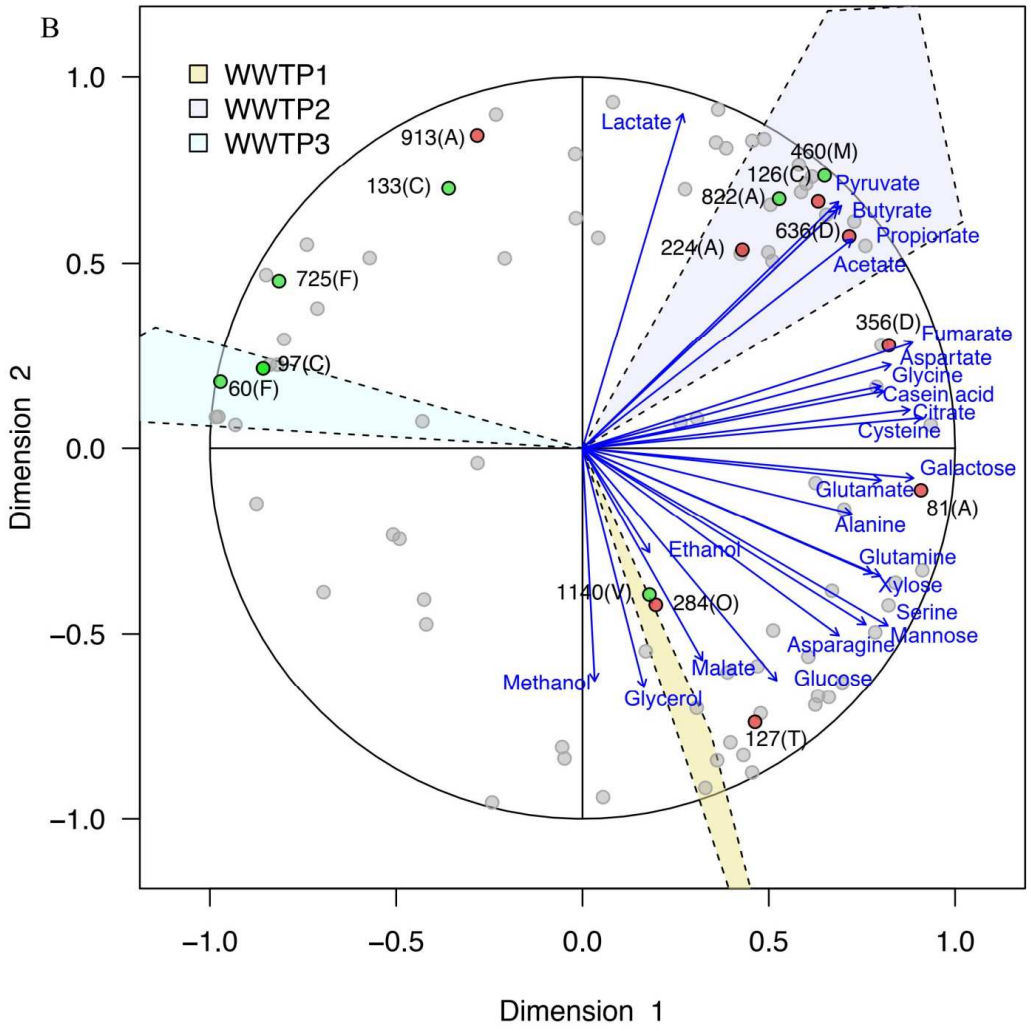
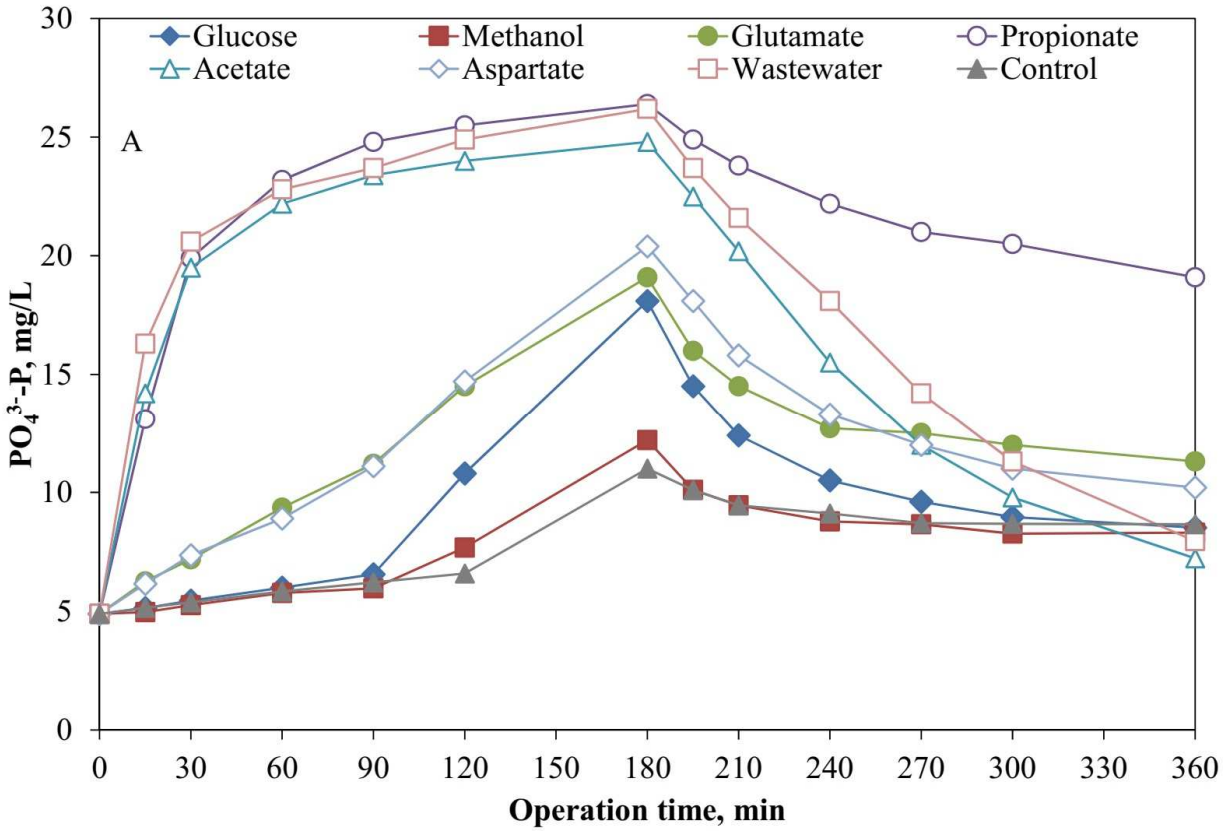
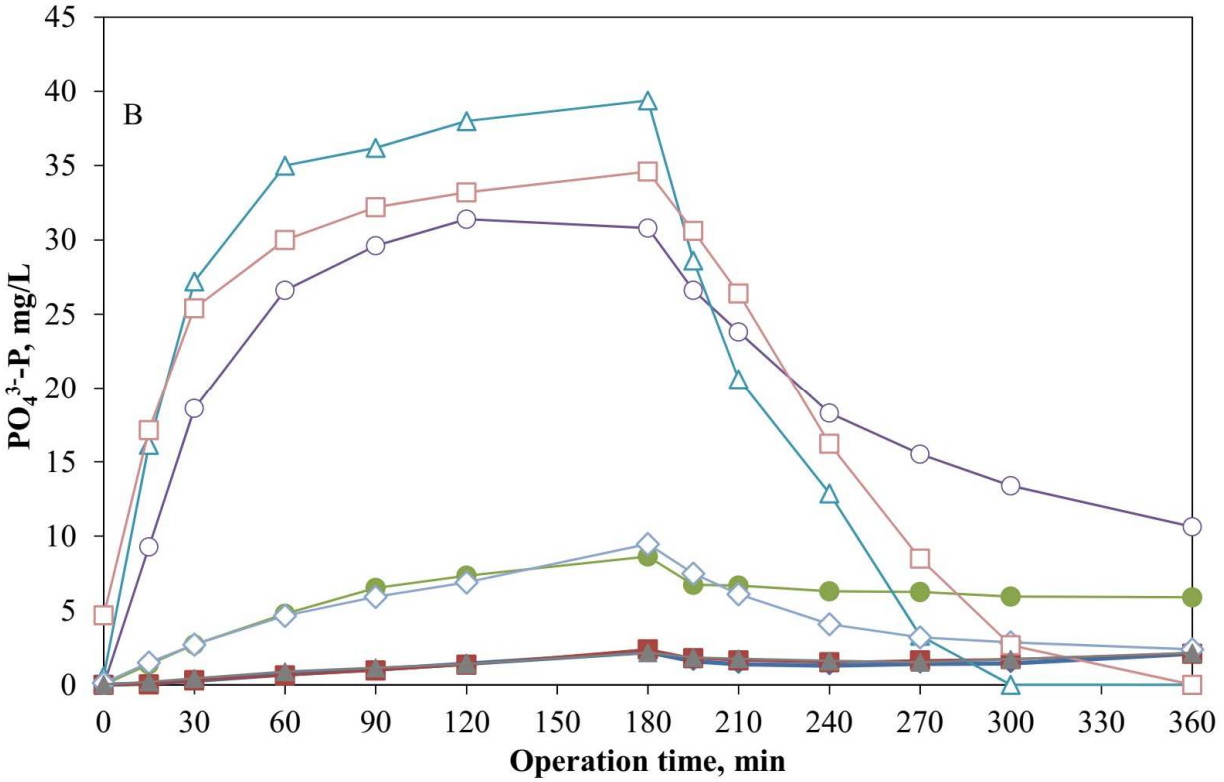


Figure 6 – Relationship between carbon utilization patterns leading to P release and abundance of specific PAOs. (**A**), Heatmap representation of Pearson's correlation matrix for P-release obtained with different carbon sources and the relative abundance of each group of putative PAOs (with a P-value cut-off of 0.05 and the calculated false discovery rate of 14.5%) and (**B**), canonical biplot of correlations between P-release profiles obtained from different carbon sources, relative abundance profiles of member taxa and sample identity based on regularised canonical correlation analysis (CCA). Correlations shown are between the 89 bacterial taxa that contain no missing values and P-release values obtained with different carbon sources. Data are projected onto the first two canonical variables. P-release variables are plotted as blue lines from the origin terminating in an arrow, and labelled with the name of the carbon source. OTU profile data are plotted with grey circles, with the

exception of those annotated to selected PAOs or GAOs (red and green, respectively), and which are tagged with their OTU number and abbreviation ((A) *Ca.* Accumulibacter; (T) *Tetrasphaera*; (O) *Ca.* Obscuribacter; (D) *Dechloromonas*; (F) *Defluviicoccus*; (C) *Ca.* Competibacter; (M) *Micropruina*; (V) *Propionivibrio*. The colored convex hulls show the projections of samples from each WWTP.


Glucose
Methanol
Glutamate
Propionate
Acetate
Aspartate
Wastewater
Control
A
PO_4^{3-}-P, mg/L
Operation time, min

1


B
PO_4^{3-}-P, mg/L
Operation time, min

2

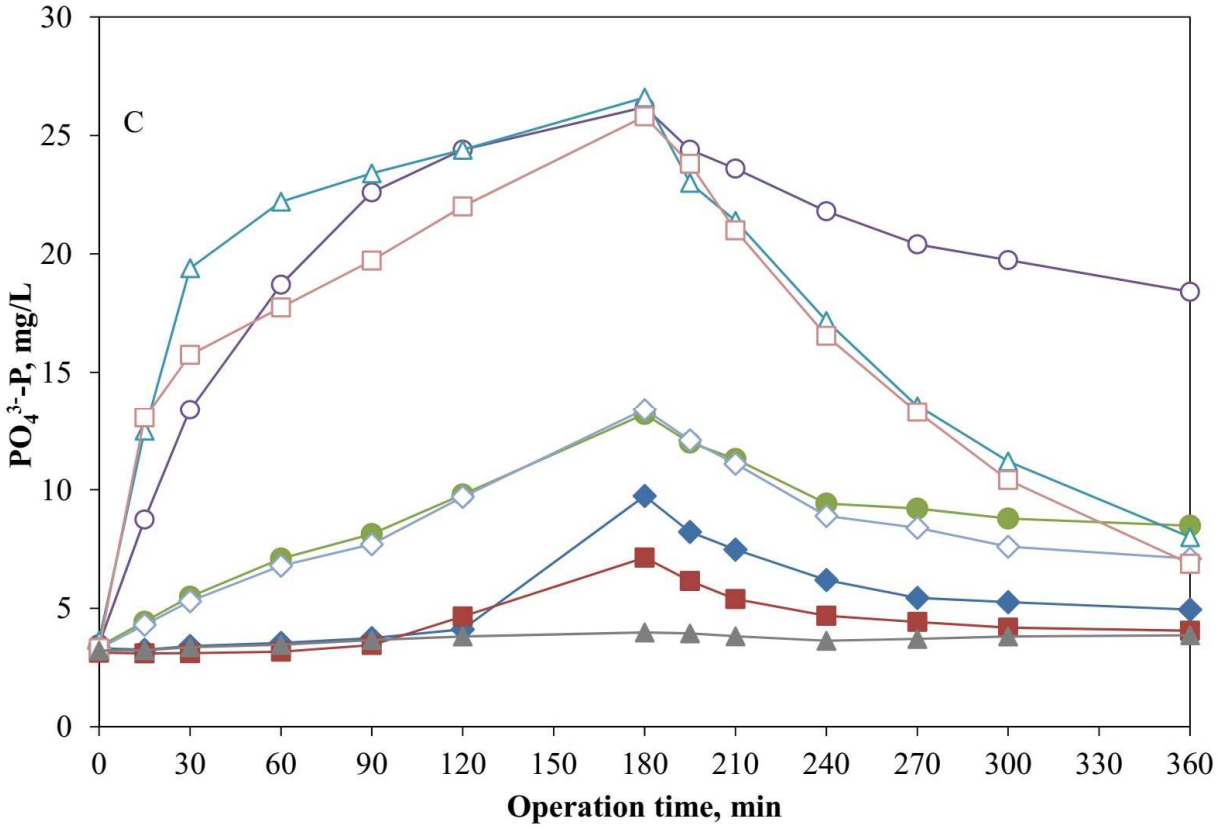


Figure 7 - P-release and -uptake profiles obtained with selected carbon sources in anaerobic-aerobic cycle studies (**A**), WWTP1; (**B**), WWTP2; and (**C**), WWTP3. MLSS = 2.0 g/L.

Highlights

- Three full-scale tropical WWTPs in Singapore showed high in-situ EBPR activity;
- Each plant was occupied by a diverse PAO community using various carbon sources;
- *Ca.* Accumulibacter was the main active PAO at temperatures above 28^{o}C;
- Acetate remains the most important carbon source for EBPR in tropical WWTPs;
- Carbon usage profiles were highly correlated with PAO community composition;