High-temperature EBPR process: The performance, analysis of PAOs and GAOs and the fine-scale population study of Candidatus “Accumulibacter phosphatis”

Ying Hui Ong a, Adeline Seak May Chua a,*, Toshikazu Fukushima b, Gek Cheng Ngoh a, Tadashi Shoji c, Atsuko Michinaka d

a Department of Chemical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, Japan
c Department of Socio-Cultural Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, S-1-5, Kashiwanoha, Kashiwa, Chiba 277-8563, Japan
d Wastewater and Sludge Management Division, Water Quality Control Department, National Institute for Land and Infrastructure Management, Asahi 1, Tsukuba, Ibaraki 305-0804, Japan

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Abstract
The applicability of the enhanced biological phosphorus removal (EBPR) process for the removal of phosphorus in warm climates is uncertain due to frequent reports of EBPR deterioration at temperature higher than 25 °C. Nevertheless, a recent report on a stable and efficient EBPR process at 28 °C has inspired the present study to examine the performance of EBPR at 24 °C–32 °C, as well as the PAOs and GAOs involved, in greater detail. Two sequencing batch reactors (SBRs) were operated for EBPR in parallel at different temperatures, i.e., SBR-1 at 28 °C and SBR-2 first at 24 °C and subsequently at 32 °C. Both SBRs exhibited high phosphorus removal efficiencies at all three temperatures and produced effluents with phosphorus concentrations less than 1.0 mg/L during the steady state of reactor operation. Real-time quantitative polymerase chain reaction (qPCR) revealed Accumulibacter-PAOs comprised 64% of the total bacterial population at 24 °C, 43% at 28 °C and 19% at 32 °C. Based on fluorescent in situ hybridisation (FISH), the abundance of Competibacter-GAOs at both 24 °C and 28 °C was rather low (<10%), while it accounted for 40% of the total bacterial population at 32 °C. However, the smaller Accumulibacter population and larger population of Competibacter at 32 °C did not deteriorate the phosphorus removal performance. A polyphosphate kinase 1 (ppk1)-based qPCR analysis on all studied EBPR processes detected only Accumulibacter clade IIF. The Accumulibacter population shown by 16S rRNA and ppk1 was not significantly different. This finding confirmed the existence of single clade IIF in the processes and the specificity of the clade IIF primer sets designed in this study. Habitat filtering related to temperature could have contributed to the presence of a unique clade. The clade IIF was hypothesised to be able to perform the EBPR activity at high temperatures. The clade’s robustness most likely helps it to fit the high-temperature EBPR sludge best and allows it...
1. Introduction

The enhanced biological phosphorus removal (EBPR) process is a modified activated sludge process for phosphorus removal in wastewater. It utilises the unique physiology of polyphosphate accumulating organisms (PAOs) to accumulate phosphorus in excess of their normal metabolic requirements and store it in the form of intracellular polyphosphate (Mino et al., 1998). To date, Candidatus “Accumulibacter phosphatis” (hereafter named Accumulibacter) are the best-known PAOs that are present in both lab-scale and full-scale EBPR processes (Oehmen et al., 2007).

While EBPR processes have been broadly reported to produce effluents with very low phosphorus concentrations, the operational unpredictability and accompanying sudden reductions in phosphorus removal have also necessitated continuous research to expanding our knowledge of the process. The effects of pertinent factors, such as the pH, temperature, nutrient limitation, etc., on the EBPR activity have been extensively studied (Seviour et al., 2003). In general, pH 7–8.5 and a temperature lower than 20 °C were reported to enhance EBPR performance (Oehmen et al., 2007). In relation to the temperature effects, the literature widely agrees that colder temperatures favour the growth of PAOs over glycogen accumulating organisms (GAOs), the competitors of PAOs for carbon substrates in the anaerobic phase. Erdal et al. (2003) observed better phosphorus removal at 5 °C than at 20 °C, linking it to the slower glycogen transformation at lower temperatures selected against GAOs. The deterioration of phosphorus removal at full-scale plants during the summer and the improved efficiencies in winter were touted as additional evidence of the competitive advantage of PAOs at colder temperatures (Gu et al., 2005). When the temperature increased from 20 °C to 30 °C, GAOs were found to gain dominance over PAOs (Panswad et al., 2003; Whang and Park, 2006; Lopez-Vazquez et al., 2007, 2008, 2009). The findings of these studies seem to indicate that the employment of the EBPR process is a challenging task for sewage treatment plants (STPs) in warm climates. A study by Cao (2011) supports this hypothesis, showing that both a lab-scale and full-scale EBPR plant operated at 30 °C in Singapore exhibited poor phosphorus removal efficiencies.

Nevertheless, successful cases of the EBPR process operated at high temperature have been reported. Freitas et al. (2009) managed to maintain a good EBPR activity at 30 °C, even in response to chemical oxygen demand (COD), nitrogen and phosphorus shock load disturbances, with a short SBR cycle that consisted of a 20-min anaerobic phase, 10-min aerobic phase, a 5-min settling and decanting phase and a 1-min idle phase. Moreover, Winkler et al. (2011) showed that the PAO-GAO competition at high temperature, i.e., 30 °C, can be controlled and further secured a 100% phosphorus removal efficiency via selective sludge removal in a segregated aerobic granular biomass system. Our previous study of the long-term operation of an EBPR reactor at 28 °C (Ong et al., 2013) showed excellent phosphorus removal. The lab-scale EBPR reactor stably showed a >95% phosphorus removal efficiency during one year of operation, resulting in an effluent phosphorus concentration of less than 1.0 mg/l. The results of these studies are exciting and call for more research efforts to further examine the feasibility of operating an EBPR process at high temperature. These findings are also promising for STP operators in warm climates because the EBPR process may still be a relevant phosphorus removal technology in these regions.

In Ong et al. (2013), Accumulibacter-PAOs were found to be responsible for the high-temperature EBPR, and specific operational control, such as short SRT (Freitas et al., 2009) and selective sludge removal (Winkler et al., 2011), was not applied to select on PAOs population. The ability of Accumulibacter-PAOs to thrive well at high temperatures (Ong et al., 2013) without being outcompeted by GAOs was unusual. This finding could be attributed to the presence of unique Accumulibacter clade(s) in Ong et al. (2013) that have better tolerance for high temperatures. Peterson et al. (2008) suggested that different subpopulations of Accumulibacter could possess different ecophysiology characteristics. With polyphosphate kinase 1 (ppk1) which providing higher phylogenetic resolution, He et al. (2007) retrieved five clades, viz I, IIA, IIB, IIC and IID emerging under the two major types of Accumulibacter and found that the clades’ population varied among the different EBPR processes as well as temporally in the system. From the study by Flowers et al. (2013) on seasonal bacteria community dynamic of an EBPR plant, it seemed that there is some correlation between temperature and certain Accumulibacter’s clades population. However, the correlation is unclear yet and further research is required.

To better understand the driving factors of EBPR at high temperatures, microbiological aspects, such as the population abundance and dynamic of PAOs and GAOs as well as the fine scale study of Accumulibacter analysis are required to be examined further. In this study, we focused on high temperatures from 24 to 32 °C, which is the temperature range of Malaysia’s sewage all year round. As for microbial aspects, the potential PAO and GAO populations were first screened via fluorescent in situ hybridisation (FISH). Following the detection of Accumulibacter and Competibacter as dominant PAOs and GAOs, respectively, their population dynamics is of great interest. Thus, the dynamics of Accumulibacter-PAOs were profiled by real-time quantitative polymerase chain reaction (qPCR) and the abundance of Competibacter-GAOs was enumerated through FISH. To provide more information and uncover the fine-scale...
population of Accumulibacter for high temperature EBPR, ppk1 gene fragments were retrieved from the EBPR processes to analyse the abundances and relative distributions of Accumulibacter clades. Overall, this work aims to elucidate the microbiology of EBPR at higher temperatures, as well as to provide additional evidence on the feasibility of operating the EBPR process in warm climates.

2. Materials and methods

2.1. SBRs operation

A sequencing batch reactor (SBR) was operated for EBPR at 28 °C for almost three years in our laboratory. The SBR, hereafter named SBR-1, showed stable and excellent EBPR performances (Ong et al., 2013). Another SBR (hereafter named SBR-2) was started up with the seed sludge collected from SBR-1. Both SBRs were operated in parallel, and their performances were evaluated for 154 days. SBR-1 was continuously operated at 28 °C, while SBR-2 was operated at 24 °C for the first 77 days, followed by 32 °C until day 154.

The configurations of SBR-1 and SBR-2 were identical except for the temperature conditions. They were double-jacketed with a working volume of 2.0 L. The reactor operations consisted of six 4-h cycles per day, with 11 min of filling, 1 h at anaerobic and 2 h aerobic conditions, followed by 40 min of settling and 9 min of decanting. The SBRs were operated with a solid retention time (SRT) of 10 days and hydraulic retention time (HRT) of 10 h.

The SBRs were fed with synthetic wastewater at 50 mg C/L, with acetate as the main carbon source. The carbon to phosphorus ratio (C:P) was 3:1. The nutrient concentrations in the feed were detailed in Ong et al. (2013).

2.2. Chemical analyses

The SBRs were monitored weekly for their EBPR performances by measuring the dissolved organic carbon (DOC), orthophosphate (PO₄³⁻), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), poly-hydroxybutyrate (PHB) and glycogen contents. The MLSS and MLVSS were measured using standard methods (APHA et al., 1998). The DOC was assayed using a TOC analyser (TOC-V CSN, Shimadzu, Japan). The orthophosphate content was analysed using ion Chromatography (861 Advanced Compact IC, Metrohm, Switzerland). The PHA content was determined via gas chromatography according to the methodology proposed by Satoh et al. (1994). The glycogen content was determined following the method described by Bond et al. (1999), which measures glucose via high performance liquid chromatography (Waters Corporation, USA) using an Agilent HPLC column.

2.3. Microbial characterisation

2.3.1. Fluorescent in situ hybridisation (FISH)

Sludge samples were collected intermittently throughout the reactor operations to screen for the potential PAOs and GA0s via FISH. During the steady state of reactors operation, sludge samples were collected weekly and subjected to FISH quantification where the relative abundance of PAOs and GA0s in the microbial community was then calculated. The mean of the relative abundance of PAOs and GA0s over steady state were also being computed and presented together with standard deviation.

The sludge samples were fixed in 4% paraformaldehyde, and FISH was performed according to Amann et al. (1995). The FISH samples were observed using a fluorescence microscope (Model DM 2500, Leica, Germany), and images were captured with a cooled charged-coupled device CCD camera (Model DFC 310 FX, Leica, Germany). The oligonucleotide probes used in this study are listed in Table 1.

A minimum of 20 microscopic fields were randomly captured for each sample. The FISH of the PAOs and GA0s was quantified using the image analysis software VideoTess-Morphology 5.1. The abundance of PAOs or GA0s was defined as the mean image area with a positive signal for PAOs or GA0s probes relative to the area with a positive signal for EUBMix.

2.3.2. Genomic DNA extraction

The sludge samples were collected weekly, and their total genomic DNA was extracted using Nucleospin® Soil (Macherey–Nagel, Germany) according to the manufacturer's instructions. The DNA concentration was determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3.3. ppk1 clone library construction and phylogenetic analysis

The Accumulibacter ppk1 fragments were amplified on genomic DNA extracted from EBPR sludge taken from SBR-1 when its EBPR activity reached steady state. The DNA template was subjected to PCR by using Accumulibacter ppk1-specific Acc-ppk1-254f (5'-TCACCCACCGACCAGCAGAAGC-3') and Acc-ppk1-1376r (5'-AGATCATCAGCATCTTGGC-3') primers (McMahon et al., 2007). The PCR mixture contained 4.0 μL of DNA template, 3 μL of each primer (10 μM), 37.5 μL GoTaq® Green Master Mix 2X (Promega), and 27.5 μL of sterile ultrapure water. PCR amplification was carried out in a thermocycler (MyCycler, Biorad) with an initial denaturation step at 95 °C for 10 min, followed by 25 cycles of denaturation at 95 °C for 45 s, annealing at 68 °C for 1 min, and elongation at 72 °C for 2 min, followed by an final extension at 72 °C for 5 min (He et al., 2007). The PCR products were then purified using a Wizard® SV Gel and PCR Clean-Up System (Promega) gels and cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer’s instructions. After blue white screening, a total of 135 colonies were retrieved and sequenced. The clones were sequenced using an ABI 3730xl DNA analyser (Applied Biosystems). The Accumulibacter ppk1 fragments sequences found in the sludge were compared with sequences in the GenBank database using the BLAST software (www.ncbi.nlm.nih.gov) (Altschul et al., 1990). The closest sequences were aligned, and a phylogenetic tree was constructed with the neighbour-joining method using MEGA 5 (Tamura et al., 2011).

The GenBank accession number for the nucleotide sequence determined in this study is KF985964.
2.3.4. Primer design

A primer set was designed to exclusively target the ppk1 gene of Accumulibacter clade IIF. The specificity was compared to the sequences obtained from the clone library mentioned in section 2.3.3 as well as the sequences available in GenBank. The qPCR primers were designed to avoid long amplicon lengths and degenerate bases to ensure accurate quantification. Specificity was ensured for both forward and reverse primers to avoid amplification in other clades. Qualitative PCR was performed on all samples using the clade IIF primer sets designed in this study, and the details are listed in Table 2. Single bands on the agarose gel at the expected amplicon size were obtained for all the positive results (data not shown), indicating that the ppk1 genes were amplified.

2.3.5. Real-time quantitative PCR

Real-time quantitative PCR was conducted with the real time thermal cycler CFX 96 Real Time PCR Detection System (BioRad, USA) using IQ™SYBR® Green Supermix (BioRad, USA) and a total reaction volume of 20 µl. The DNA extract (5 ng) from sludge samples or quantitative standard DNA was used as the template DNA. The specific primers used in this study are listed in Table 2. To amplify the Accumulibacter 16S rRNA and ppk1 genes, the qPCR programmes consisted of an initial 3-min denaturation at 95 °C, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing for 45 s and extension at 72 °C for 30 s. The annealing temperatures for each primer set are listed in Table 2.

The quantification results obtained from qPCR were further used to estimate the percentage of the total bacterial population comprised by Accumulibacter as well as the total Accumulibacter lineage relative to the total bacteria population. The abundance of the ppk1 gene determined based on the qPCR using ppk1-specific primers can represent the abundance of Accumulibacter cells because it is a single copy gene. However, the number of rrn operons must be taken into consideration to determining the cell number based on 16S rRNA primers. The Accumulibacter genome has previously been assumed to have 2 copies of the rrn operon, and the other bacterial genomes in the activated sludge had an average of 4.1 copies of the rrn operon (Garcia et al., 2006; He et al., 2007).

### Table 1 – 16S rRNA oligonucleotide probes for the identification of potential PAOs and GAOs.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence 5′ − 3′</th>
<th>Specificity</th>
<th>% FA Conc.</th>
<th>Fluorophores</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338³</td>
<td>GCTGCCCTCCGGCTAGGTTG</td>
<td>Eubacteria</td>
<td>35</td>
<td>FITC</td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>EUB 338-II</td>
<td>GAGCCCAACCGCAGTTG</td>
<td>Eubacteria</td>
<td>35</td>
<td>FITC</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB 338-III</td>
<td>GTCGCCACCACCGAGTTG</td>
<td>Eubacteria</td>
<td>35</td>
<td>FITC</td>
<td>Daims et al. (1999)</td>
</tr>
</tbody>
</table>

**Probes for potential PAOs**

| PAO 462²   | CGGCTATCAACWCAAGGATTAAAC | Most Accumulibacter              | 35         | Cy3          | Crocetti et al. (2000) |
| PAO 651²   | GCTCTGGCAAACTCCAGG       | Most Accumulibacter              | 35         | Cy3          | Crocetti et al. (2000) |
| PAO 846²   | GTTAGCTAGGCACCTAAAGG     | Most Accumulibacter              | 35         | Cy3          | Crocetti et al. (2000) |
| HGC69⁵     | TATAGTATACCGCAGGCGGT     | Actinobacteria – high G + C      | 25         | Cy3          | Roller et al. (1994)  |
| Actino 1011 | TGCGGGCGACCCATCTAC      | Tetrasphaera-related Actinobacteria | 30        | Cy3          | Liu et al. (2001)    |

**Probes for potential GAOs**

| GB          | CGATCTCCTACGGCCTACT      | Competibacter (GB group)         | 35         | Cy3          | Kong et al. (2002)   |
| TFO_DF218   | GAAGCCCTTTGCCCTCAG       | Defluvicoccus-related organisms  | 35         | Cy3          | Wong et al. (2004)   |
| TFO_DF618   | GCTACTCTTTGCTAACCAGG     | Defluvicoccus-related organisms  | 35         | Cy3          | Wong et al. (2004)   |
| DF988       | GATGCCACCGGATCTGAGAGG    | Defluvicoccus-related organisms  | 35         | Cy3          | Meyer et al. (2006)  |
| DF1020      | CGGGCGGAGCGGACTCCTCC     | Defluvicoccus-related organisms  | 35         | Cy3          | Meyer et al. (2006)  |

### Table 2 – qPCR Primers information. The section highlighted in bold signifies the details of primers set developed in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ − 3′)</th>
<th>Target</th>
<th>Annealing temperature (Tₐ)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>518f</td>
<td>CCAGGAGCAGCGGCTAAT</td>
<td>Acc 16S rRNA genes</td>
<td>65</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>PAO846</td>
<td>GTAGCTAGCGAGCTAAAAG</td>
<td>Bacterial 16S rRNA genes</td>
<td>60</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>341f</td>
<td>CCTACGGCGGAGCGAGAG</td>
<td>Bacterial 16S rRNA genes</td>
<td>60</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>534r</td>
<td>ATTACGGCGGCTGCTG</td>
<td>Bacterial 16S rRNA genes</td>
<td>60</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-763f</td>
<td>GAGCGAGAAGCGGCTCAAG</td>
<td>Acc-1 ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-1170r</td>
<td>AAGGCTACCTGTTAGAGAC</td>
<td>Acc-IIA ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-893f</td>
<td>AGTCTAATCTACACGGAGAC</td>
<td>Acc-IIA ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-997r</td>
<td>CAGCGAAGACCTGATTGC</td>
<td>Acc-IIB ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-870f</td>
<td>GTAGACCCGAGATCTGCTG</td>
<td>Acc-IIB ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>954r</td>
<td>ACCGCTGACGGCTG</td>
<td>Acc-IIC ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-1002r</td>
<td>TCACCCAGACGCTCAGAGC</td>
<td>Acc-IIIC ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc-ppk1-254f</td>
<td>CGGGCTGAGCTGGGAGGAG</td>
<td>Acc-IIIC ppk 1</td>
<td>61</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>530f</td>
<td>ATGGACCCAGTGCTG</td>
<td>Acc-III ppk 1</td>
<td>63</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>534r</td>
<td>CATAGGCTCGGCTG</td>
<td>Acc-III ppk 1</td>
<td>63</td>
<td>He et al. (2007)</td>
</tr>
<tr>
<td>Acc–ppk1-355f</td>
<td>CGAACCCTCGGAAAGCGGAT</td>
<td>-- IIIF ppk 1</td>
<td>70</td>
<td>This study</td>
</tr>
</tbody>
</table>
Kaetzke et al., 2005; He et al., 2007; Flowers et al., 2013). qPCR was performed with Accumulibacter- and general bacterial-targeted 16S rRNA primers to determine the percentage of the total bacterial population comprised by Accumulibacter; qPCR was performed with ppk1-specific primers and general bacterial-targeted 16S rRNA primers to determine the percentage of the total bacterial population comprised by the total Accumulibacter lineage.

Six-point calibration curves for qPCR were produced using a tenfold serial dilution in triplicate for each assay, from $10^0$ to $10^4$ target copies per reaction. Controls for ppk1- or 16S rRNA-targeted PCR were generated from appropriate clones from ppk1 or 16S clone libraries. For all unknown samples, 5 ng of community-derived genomic DNA was added as the template. In each assay, a no-template control was included to check for contamination and primer-dimer formation.

### 3. Results and discussion

#### 3.1. SBRs performance

Throughout the operation of the SBRs, selected weekly cycles of SBR-1 and SBR-2 were monitored for the EBPR performances. Fig. 1 shows the PO$_4$-P concentrations at the beginning of the anaerobic phase, as well as at the beginning and the end of the aerobic phase in each monitored cycle.

Similar to the EBPR performance reported in Ong et al. (2013), SBR-1 continued to exhibit a good phosphorus removal at 28 °C throughout the 154-day reactor operation. Consistent with the observed anaerobic phosphorus release and aerobic phosphorus uptake, the effluent phosphorus concentrations remained below 0.5 mg P/L. This finding further supports that stable EBPR activity can be maintained at a higher temperature, such as 28 °C. However, the EBPR activity in SBR-2 was disturbed immediately after reactor start-up due to the adaptation of seed sludge to the temperature switch from 28 °C to 24 °C. Drastic decreases in the anaerobic phosphorus release and aerobic phosphorus uptake were observed on day 7 of SBR-2 operation, resulting in an effluent phosphorus concentration of 7.3 mg/l. Subsequently, the EBPR activity gradually recovered, and by day 35, the EBPR performance of SBR-2 was as good as that of SBR-1, producing effluent with less than 1 mg P/L and reaching its steady state.

On day 77 (Fig. 1(b)), the temperature of SBR-2 changed from 24 °C to 32 °C, an adaptation pattern similar to that during the start-up of SBR-2. A substantial decrease in the anaerobic phosphorus release and aerobic phosphorus uptake took place until day 112. Thereafter, the EBPR activity showed signs of recovery and reached steady state after day 119; the effluent phosphorus concentration stabilised at less than 1 mg/l. These results indicate that the EBPR biomass cultivated in this study was robust to the higher temperatures applied and capable of restoring its EBPR activity upon temperature changes. Moreover, the EBPR performances at all the temperatures tested in this study showed high phosphorus removal efficiencies ranging from 95 to 99% with phosphorus removal rates of 1.9–2.5 mgP gVSS$^{-1}$ h$^{-1}$ during steady state.

Reactor cycle studies demonstrated that the EBPR processes operated at 24 °C, 28 °C, and 32 °C exhibited metabolic transformations typical of EBPR (Fig. 2). During the steady state operation of SBRs, we observed little difference in the MLVSS of 24 °C and 28 °C, with 4.3 and 4.6 g/L respectively. However, these MLVSS values were higher than that observed at 32 °C, i.e., 3.3 g/L. The amount of biomass produced seems to negatively correlate with the temperature higher than 28 °C which could be due to bacteria having fewer endogenous needs at lower temperatures (24 °C–28 °C), which allows more substrate to be available for cell production. Based on the cyclic profiles, anaerobic phosphorus release rate, aerobic phosphorus uptake rate, phosphorus release/acetate uptake ratio, biomass phosphorus, and glycogen content at all three temperatures were calculated and are summarised in Table 3.

Table 3 shows that the biomass glycogen content was notably higher at 32 °C, i.e., 13% w/w, than at 24 °C and 28 °C, which indicates the higher abundance of GAOs at 32 °C. Based on our current understanding of the biochemical pathways of the EBPR process, the theoretical $P_{re}/HAc_{up}$ ratio is proposed to be 0.5–0.7 P-mole/C-mole for PAOs (Schuler and Jenkins, 2003). The $P_{re}/HAc_{up}$ ratio obtained in this study ranged from 0.4 to 0.6 P-mole/C-mole. A $P_{re}/HAc_{up}$ ratio of 0.4, which is lower than the range of the theoretical value, indicates a noticeable GAO population at 32 °C. This finding agrees with the earlier hypothesis based on the biomass glycogen content at 32 °C. Lower anaerobic phosphorus release observed in 32 °C also hinted the increase of GAOs population in the process. Most of the EBPR biochemical models (Comeau et al., 1986; Wentzel et al., 1986; Mino et al., 1998) agree that
Polyphosphate is hydrolysed to generate energy for carbon uptake. As the GAOs population increase, there is less carbon source available for PAOs and thus, less polyphosphate is hydrolysed for the carbon uptake.

Nevertheless, the possible higher abundance of GAOs at 32°C did not deteriorate the phosphorus removal performance. The EBPR sludge at all three temperatures possesses similar phosphorus content, and the aerobic phosphorus uptake rate was higher at 32°C. The anaerobic phosphorus release rate was slightly higher at 32°C than at 28°C and 32°C. Our observations contradicted those reported in Panswad et al. (2003), who showed that the phosphorus release rate increased with temperature from 20°C to 35°C, while the phosphorus uptake rate decreased. Thus, the microbiology of these EBPR processes should be elucidated to understand the characteristics of high-temperature EBPR sludge and better link them to the process performance.

### 3.2. Population dynamics and abundance of PAOs and GAOs

The screening of potential PAOs and GAOs via FISH revealed that the PAO communities at all three temperatures were dominated by Accumulibacter-PAOs. The samples showed negative responses to high G+C Actinobacteria- and Tetrasphaera-related Actinobacteria probes. The presence of Competibacter-GAOs in the sludge samples was found to be significant, but the alphaproteobacterial GAOs related to the

### Table 3 – Summary of the anaerobic phosphorus release rate, aerobic phosphorus uptake rate, phosphorus release/acetate uptake ratio, biomass phosphorus and glycogen content in the EBPR processes operated at 24°C, 28°C and 32°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Anaerobic phosphorus release rate (mgP gVSS⁻¹ h⁻¹)</th>
<th>Aerobic phosphorus uptake rate (mgP gVSS⁻¹ h⁻¹)</th>
<th>Phosphorus release/acetate uptake ratio (P mol/HAc mol⁻¹)</th>
<th>Biomass phosphorus content (% w/w)</th>
<th>Biomass glycogen content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>7.6</td>
<td>1.9</td>
<td>0.60</td>
<td>7.6</td>
<td>5.4</td>
</tr>
<tr>
<td>28</td>
<td>6.2</td>
<td>1.5</td>
<td>0.57</td>
<td>7.4</td>
<td>7.5</td>
</tr>
<tr>
<td>32</td>
<td>6.1</td>
<td>2.5</td>
<td>0.38</td>
<td>7.7</td>
<td>13.8</td>
</tr>
</tbody>
</table>
members of the genus Defluviicoccus were absent or present at low, statistically insignificant abundance.

Because Accumulibacter was identified as the major PAO at all three temperatures, qPCR was employed to profile the dynamics and abundance of Accumulibacter. This method has been reported as a rapid and accurate approach for routine monitoring throughout the operation of the process (Fukushima et al., 2007; He et al., 2007). Fig. 3(a) shows the Accumulibacter percentage estimated according to the Accumulibacter 16S rRNA gene and bacterial 16S rRNA genes via qPCR analyses. Because SBR-1 had been operating at 28 °C for a long term prior to this study, a relatively stable Accumulibacter population was observed throughout the reactor operation that only fluctuated within 40–46% of total bacterial population. This finding agreed with the stable EBPR activities observed in SBR-1 (Fig. 1). Although EBPR activity in SBR-2 was disturbed when the sludge experienced a temperature change from 28 °C to 24 °C, the Accumulibacter population increased steadily from approximately 40% on day 0–65% on day 42 and beyond. When changing the temperature from 24 °C to 32 °C in SBR-2, the Accumulibacter abundance dropped drastically from approximately 64% on day 77 to approximately 19% on day 119 and beyond. The average abundances of Accumulibacter during the steady state operation were 64%, 43%, and 19% at 24 °C, 28 °C, and 32 °C, respectively. The FISH quantification data shows a similar trend as that given by qPCR: the increase in the temperature negatively correlates with the proliferation of PAOs. The abundances of Accumulibacter revealed by FISH during the steady state operation of reactors operation were 51% at 24 °C, 33% at 28 °C and 12% at 32 °C (Table 4). Lopez-Vazquez et al. (2008) observed a similar phenomenon, and they claimed that Accumulibacter possess a clear advantage in growth at lower temperatures, which encourages the cell lineages. This hypothesis is also supported by the claim that different clades within the Accumulibacter lineage possess unique ecological differences or habitat characteristics (McMahon et al., 2007; Peterson et al., 2008). Thus, in order to better explain the current observation, ppk1 is of great interest, as it could provide greater phylogenetic resolution in revealing finer scale differences within Accumulibacter.

Based on the biomass glycogen content presented earlier (Table 3), the abundance of GAOs was expected to increase as the temperature increased from 24 °C to 32 °C. This expectation was confirmed by FISH, as shown in Table 4. The significant growth of Competibacter at 32 °C clearly showed that they are the major competitors of Accumulibacter for the carbon substrate available in the EBPR process. However, the GAOs did not seem to totally outcompete Accumulibacter and also did not deteriorate the EBPR activity. Appendix A shows the FISH images of sludge samples taken from the EBPR processes operated at 24 °C and 32 °C.

In agreement with previous studies (Whang and Park, 2006; Erdal et al., 2003; Panswad et al., 2003), we observed an increase in the GAO population as the temperature increased. However, despite the smaller population of Accumulibacter and larger population of Competibacter at 32 °C, the EBPR performances at 32 °C were similar to those at 24 °C and 28 °C. The good EBPR capacity at high temperatures and the coexistence of Accumulibacter-PAOs and Competibacter-GAOs shown in this study are very interesting. The reason for this rare observation is uncertain. The Accumulibacter-PAO present at these high-temperature EBPR reactors may be a unique clade(s) within the lineage that is more tolerant to higher temperatures. This hypothesis is also supported by the claim that different clades within the Accumulibacter lineage possess unique ecological differences or habitat characteristics (McMahon et al., 2007; Peterson et al., 2008). Thus, in order to better explain the current observation, ppk1 is of great interest, as it could provide greater phylogenetic resolution in revealing finer scale differences within Accumulibacter.

### 3.3 Fine-scale population of *Accumulibacter* in high-temperature EBPR processes

#### 3.3.1. ppk1 based phylogeny of high-temperature EBPR process

To empirically observe the Accumulibacter clades present in the high-temperature EBPR biomass, a ppk1 gene clone

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Accumulibacter (%)</th>
<th>Competibacter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>51 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>28</td>
<td>33 ± 5</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>32</td>
<td>12 ± 1</td>
<td>40 ± 4</td>
</tr>
</tbody>
</table>

*Errors indicate the standard deviation of mean (n = 6); n represents the frequency of sludge sampling during steady state.

Fig. 3 – Percentage of Accumulibacter according to (a) 16S rRNA; and (b) ppk1 in the total bacterial population of EBPR processes operated in SBR-1 and SBR-2 throughout the reactor’s operation; (×) 28 °C-SBR 1; (○) 24 °C-SBR 2; (●) 32 °C-SBR 2.
library was constructed from SBR-1 (28 °C). The phylogram indicating the inferred relationships between ppk1 gene homologs from the cloned Accumulibacter clades is shown in Fig. 4. All clones obtained are highly affiliated with the reported clone Acc-SG2 ppk1, whose accession number is HM046427 (Kim et al., 2010). This clone is related to clade IIF of Accumulibacter. Peterson et al. (2008) and Shoji et al. (2011) also reported other clade IIF-related strains.

3.3.2. Development of the clade IIF primers set for qPCR

Because all of the ppk1 sequences recovered from SBR-1 (28 °C) in our study are closely related to clade IIF, a qPCR assay with specific primers targeted to this clade was necessary in the subsequent quantification study. However, the current available specific ppk1 primers are only limited to clades I, IIA, IIB, IIC, and IID, which were developed by He et al. (2007). Thus, the clade IIF primers set for qPCR was developed in this study. FastPCR 6.3 (Kalendar et al., 2011) was used to design the primers. The length of the sequence was shortened to adjust for an appropriate Tm (near 60 °C) and GC content (20–80%). Gradient PCR was carried out to check the potential annealing temperatures. The optimum annealing temperature was further validated and identified via qPCR runs to avoid mis-amplification on other non-targeted clades as well as mis-annealing on non-target positions of clade IIF. Negative controls, which consisted of the clones with the non-targeted Accumulibacter clades, were negative.

**Fig. 4** – Phylogram indicating inferred relatedness of ppk1 gene homologs from the cloned ‘Accumulibacter’ clades. Sequences found in this study were contrasted with those from reference sequences in the GenBank database, and the ppk1 genes were classified based on the clade names assigned previously (He et al., 2007). Bootstrap values are shown in percentages of 1000 replicates. Numbers in parentheses indicate frequencies of colonies exhibiting the same restriction patterns of ppk1 gene homologs in libraries constructed from the respective clones of ‘Accumulibacter’ clades.
employed to check the non-specific cross-detection of ppk1 fragments from other clades. The accession numbers of the clones used for negative controls were AB830356 (clade I), AB830378 (clade IIA), AB830355 (clade IIC), and AB830336 (clade IID). Amplification was not detected in these negative controls, which indicated that the primer set was specific to clade IIF. Specificity of the clade IIF primer set was also further examined based on the melting curve. The melting peaks of the PCR product from the activated sludge, the no template control and nuclease free water (Promega) are shown in Fig. 5(a). Only one melting peak was observed for the sludge sample. This melting temperature of this peak was the same (near 89 °C) as that of the PCR product from cloned ppk1 gene fragments of Accumulibacter’s clade IIF. The melting curve analysis did not show primer-dimers. In the no template control, peak was not seen due to no amplification within 35 PCR cycles.

Standard curves were constructed from 1.0 × 10³ to 1.0 × 10⁸ copies of the DNA quantification standard per reaction. A linear regression line of the Ct values for the DNA standards versus the logarithm of their starting copy number was constructed. A typical linear regression line is shown in Fig. 5(b). The averages of R² value and the slope of the calibration line were 0.999 and 3.552, respectively (n = 3). The average amplification efficiency (E) based on the slope was 91.2%. These data indicate that the primers set was successfully developed for the quantification of Accumulibacter clade IIF using a qPCR assay.

3.3.3. The presence and dynamic of accumulibacter’s clades revealed by the ppk1 gene
The sludge samples from SBR-1 and SBR-2 throughout the operation of the reactors were subjected to agarose gel electrophoresis and qPCR analysis to detect the presence and abundance of clade I, IIA, IIB, IIC, IID and IIF. The qPCR results were used to estimate the Accumulibacter percentage based on the total Accumulibacter ppk1 genes and bacterial 16S rRNA genes. However, except for the positive result for clade IIF, the amplification of fragments from the other clades were below the detection limit of both the qPCR assay and the visualisation by agarose gel electrophoresis for all three temperatures. As shown in Fig. 3(b), the average abundances of Accumulibacter during the steady state operation were 54% at 24 °C, 40% at 28 °C, and 12% at 32 °C. The qPCR-based estimation of the total Accumulibacter abundance using the ppk1 primer sets did not significantly differ from that derived by qPCR using the 16S rRNA gene-targeted primer. This observation again proves that clade IIF was the only Accumulibacter clade present in the sludge, which also represents the total Accumulibacter population.

The three EBPR processes operated at 24 °C, 28 °C, and 32 °C harboured similar Accumulibacter clades and showed good phosphorus removal efficiencies exceeding 95%. Without employing specific operational control to select on Accumulibacter clades population, the presence of clade IIF in all 24 °C, 28 °C, and 32 °C strongly suggests their tolerance and participation in high-temperature EBPR. Although a lower abundance of clade IIF was observed at 32 °C than at 24 °C and 28 °C, the phosphorus contents (%) in the EBPR sludge at all three temperatures were similar at approximately 7–8% (Table 3), indicating Accumulibacter clade IIF accumulated more phosphorus at 32 °C. These interesting results lend support to the claim that temperature appears to shape some of the Accumulibacter clade populations (Flowers et al., 2013). Flowers et al. (2013) found that certain Accumulibacter clades population showed seasonal dynamic in patterns with different cycles of growth and decline. Their findings support the previous hypothesis on differentiation in physiologies among Accumulibacter clades (He et al., 2007). The capability of clade IIF to sustain good EBPR at high temperatures seems to suggest that it could potentially carry out EBPR in warm climate. As other Accumulibacter’s clades are absent in our EBPR processes, their capability to survive in high temperatures is yet to confirm.

This study is the first to report the distribution of Accumulibacter clades in high-temperature EBPR processes. However, further research efforts are needed to elucidate the relationship of Accumulibacter population on an individual clade basis with the temperature effects. Data on the dynamics of the fine-scale structure of Accumulibacter in the EBPR processes operated in different seasons as well as in warm climates could help in facilitate this elucidation.

Fig. 5 – (a) Melting peak of DNA extracted from activated sludge and negative control; (b) Standard curve for the primer sets Acc-ppk1-355f and Acc-ppk1-600r constructed using a series of tenfold dilution.
4. Conclusions

- Efficient EBPR was achieved at high temperatures, which suggested the feasibility of the EBPR process for phosphorus removal in warm climates.
- Good EBPR activity was maintained with the coexistence of Accumulibacter-PAOs and Competibacter-GAOs in the 32 °C system.
- The presence of the single clade IIF in 24 °C, 28 °C, and 32 °C EBPR processes seems to reflect the robustness and tolerance of clade IIF at high temperatures.
- The phosphorus removal during the high-temperature EBPR process may not solely depend on the size of the Accumulibacter population in the EBPR process but rather on the presence of a particular Accumulibacter clade(s) and its ecophysiological role.

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Appendix A

Overlay FISH images of EBPR sludge samples collected at 24 °C and 32 °C on day 77 and day 154, respectively, of SBR-2 operation. Panel A and Panel B show Accumulibacter (orange cell clusters) hybridised with both FITC-labelled EUBmix probe (green) and Cy3-labelled PAOmix probe (red), from EBPR processes operated at 24 °C and 32 °C, respectively; Panel C and Panel D show Compectibacter (orange cell clusters) hybridised with both FITC-labelled EUBmix probe (green) and Cy3-labelled GB probe (red), from EBPR processes operated at 24 °C and 32 °C, respectively (Scale bar = 10 μm).

References


