PERFORMANCE OF HIGH-TEMPERATURE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PROCESS AND MICROBIAL STUDY OF ITS POLYPHOSPHATE ACCUMULATING ORGANISMS AND GLYCOGEN ACCUMULATING ORGANISMS

ONG YING HUI

FACULTY OF ENGINEERING
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: *Ong Ying Hui*

Registration/ Matric No.: *KHA 100053*

Name of Degree: *Doctor of Philosophy*

Title of Project/ Paper/ Research Report/ Dissertation/ Thesis ("this work"):
Performance of High-Temperature Enhanced Biological Phosphorus Removal Process and Microbial Study of Its Polyphosphate Accumulating Organisms and Glycogen Accumulating Organisms

Field of Study: *Biological Wastewater Treatment*

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ABSTRACT

Enhanced biological phosphorus removal (EBPR) process is commonly applied for the removal of phosphorus (P) from wastewater. Realizing the rapid urbanization and population growth around the world, nutrient pollution problems has gained global concerns. The increasing array of Malaysia's regulatory requirements on sewage treatment plants (STPs) is part of the abatement strategies on nutrient pollution issue. Reliably meeting low effluent limits can be difficult when EBPR process operated at temperature higher than 25^{o}C. Thus, there is an urgent need to evaluate the applicability and compliance accountability of EBPR process for STPs in tropical climates with relatively high temperatures around 25^{o}C - 32^{o}C. In this study, the EBPR performance at the temperature range of 24^{o}C - 32^{o}C and its microbiological aspects, the population abundance and dynamic of PAOs and GAOs as well as the fine scale population of *Candidatus* "Accumulibacter phosphatis" (hereafter named Accumulibacter) were investigated.

Two sequencing batch reactors (SBRs) were operated parallel for high-temperature EBPR processes. SBR-1 was continuously operated at 28^{o}C while SBR-2 first at 24^{o}C and subsequently at 32^{o}C. Both SBRs exhibited high P removal efficiencies at all three temperatures and produced effluent with P concentration less than 1.0 mg/L. Using fluorescent *in situ* hybridization (FISH), potential PAOs and GAOs were screened. It was discovered that Accumulibacter and *Candidatus* "Competibacter phosphatis" (hereafter named Competibacter) emerged as major PAOs and GAOs.

Further, the population dynamics of Accumulibacter-PAOs and the abundance of Competibacter-GAOs were monitored by real-time quantitative polymerase chain reaction (qPCR) and FISH respectively. 16S rRNA-based qPCR revealed

Accumulibacter-PAOs comprised 64% of the total bacterial population at 24^{o}C, 43% at 28^{o}C and 19% at 32^{o}C. FISH revealed the abundance of Competibacter-GAOs at both 24^{o}C and 28^{o}C was rather low (< 10%), while it accounted for 40% of total bacterial population at 32^{o}C. Obviously, smaller population of Accumulibacter-PAOs and larger population of Competibacter-GAOs did not reduce the EBPR activity in 32^{o}C. Following, polyphosphate kinase 1 gene (*ppk1*) based clone library was constructed to unveil the fine-scale population of Accumulibacter-PAOs. Clade IIF was discovered as the only Accumulibacter clade present.

More quantitatively, *ppk1* based qPCR was employed to study the dynamic patterns of clade IIF in the EBPR processes. The specific primers set for qPCR assay exclusively target on clade IIF was developed. Primers Acc-ppk1-355f and Acc-ppk1-600r were successfully designed in this study. The average abundance of Accumulibacter clade IIF assayed by qPCR was 54% at 24°C, 40% at 28°C, and 12% at 32°C. Despite lower abundance of clade IIF, the EBPR sludge of 32^{o}C possessed similar P content (7- 8%) to the other two temperatures. Good EBPR activity at 32^{o}C could be associated with the greater P accumulation capability in Accumulibacter clade IIF.

This research demonstrated good EBPR activity could be achieved at high temperatures. This is also the first report on the distribution of Accumulibacter clades in high-temperature EBPR process. Larger population in Competibacter-GAOs than Accumulibacter-PAOs did not deteriorate good EBPR performance. Accumulibacter clade IIF was robust and could tolerate high temperatures. This suggests the presence of particular Accumulibacter clade(s) and its ecophysiological role drive the EBPR activity. Also, it reflects the Accumulibacter-PAOs may have variable sensitivity to temperature among the lineages.

ABSTRAK

"Enhanced biological phosphorus removal" (EBPR) merupakan proses enap cemar yang biasanya digunakan untuk merawat air yang tercemar dengan fosforus. Kesukaran dalam mengekalkan prestasi proses ini sering berlaku apabila dioperasikan pada suhu lebih tinggi daripada 25^{o}C. Memandangkan keadaan pencemaran air yang semakin serius dan berikutan dengan penguatkuasaan undang-undang pemeliharaan alam sekitar yang semakin ketat, kesesuaian proses EBPR dalam rawatan air kumbahan di kawasan yang beriklim khatulistiwa perlu diberi pertimbangan yang sewajarnya. Oleh itu, kajian ini bermatlamat untuk mengkaji secara lebih mendalam untuk prestasi proses EBPR dalam lingkungan suhu dari 24 – 32^{o}C dan aspek mikrobiologinya dari segi organisma pengumpul fosforus (PAOs) dan organisma pengumpul glikogen (GAOs) yang terlibat.

Dua buah reaktor, SBR-1 dan SBR-2 dioperasikan secara selaras untuk process EBPR pada suhu yang berlainan. SBR-1 beroperasi secara berterusan pada suhu 28^{o}C, manakala SBR-2 beroperasi pada suhu 24^{o}C dan seterusnya tukar ke 32^{o}C. Didapati keberkesanan process EBPR pada suhu yang dikaji dalam merawat fosforus agak tinggi dan menghasilkan efluen dengan kepekatan fosforus kurang daripada 1 mg/L. Pemeriksaan PAOs dan GAOs melalui fluorescent *in situ* hybridization (FISH) menunjukkan komuniti PAOs dan GAOs didominasikan oleh *Candidatus* "Accumulibacter phosphatis" (Accumulibacter) and *Candidatus* "Competibacter phosphatis" (Competibacter).

Dinamik Accumulibacter-PAOs dan Competibacter-GAOs diprofilkan dengan menggunakan tindak balas rantai polimerase kuantifikasi (qPCR) dan FISH masing-masing. Accumulibacter-PAOs merangkumi 64% daripada jumlah populasi bacteria pada suhu 24^{o}C, 43% pada suhu 28^{o}C, dan 19% pada suhu 32^{o}C. Melalui FISH,

populasi Competibacter-GAOs didapati agak rendah (<10%) pada suhu 24^{o}C dan 28^{o}C, manakala merangkumi 40% daripada jumlah populasi bacteria pada suhu 32^{o}C. Walaupun begitu, populasi Competibacter-GAOs yang lebih besar daripada Accumulibacter-PAOs pada suhu 32^{o}C tidak mengganggu prestasi penyingkiran fosforus proses EBPR itu.

Kajian atas urutan gen polifosfat kinase 1 (*ppk1*) menunjukkan bahawa subpopulasi IIF adalah subpopulasi Accumulibacter unggul yang hadir dalam proses EBPR yang dikaji. Dengan itu, primer subpopulasi IIF, Acc-ppk1-355f and Acc-ppk1-600r, direkabentuk untuk kuantifikasi subpopulasi IIF dengan menggunakan qPCR. Subpopulasi IIF merangkumi 54% pada suhu 24^{o}C, 24% pada suhu 28^{o}C dan 12% pada suhu 32^{o}C daripada jumlah populasi bacteria. Proses EBPR pada ketiga-tiga suhu yang dikaji menunjukkan kehadiran subpopulasi IIF sebagai subpopulasi unggul. Subpopulasi ini dijangka mempunyai sifat kebolehtahanan yang lebih tinggi terhadap suhu tinggi.

Secara ringkas, proses EBPR dapat beroperasi dengan berjaya pada suhu tinggi. Kajian ini merupakan kajian pertama yang melaporkan taburan subpopulasi Accumulibacter-PAOs dalam proses EBPR yang bersuhu tinggi. Walaupun Competibacter-GAOs hadir dalam quantiti yang lebih banyak daripada Accumulibacter-PAOs, prestasi proses EBPR tidak terjejas. Kajian ini turut menunjukkan prestasi proses EBPR mungkin dipengaruhi oleh kehadiran subpopulasi Accumulibacter tertentu dan peranan ekofisiologikalnya. Subpopulasi Accumulibacter berpotensi memaparkan sensitiviti yang berlainan terhadap suhu. Keberkesanan proses EBPR pada lingkungan suhu tinggi yang ditunjukkan dalam kajian ini mencadangkan kesesuaian operasi proses EBPR di kawasan beriklim khatulistiwa.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my research supervisors, Dr. Adeline Chua Seak May and Dr. Ngoh Gek Cheng from the Department of Chemical Engineering, University of Malaya, for their invaluable support and guidance. I really appreciate their encouragement and motivation whenever I was stuck in my research.

A hearty gratitude to Prof. Takashi Mino and Dr. Hiroyasu Satoh for their incisive and thoughtful comments on my research. I also appreciate the contribution and enthusiastic support from our collaborators, Dr. Toshikazu Fukushima, Dr. Atsuko Michinaka, and Dr. Tadashi Shoji.

My sincere thanks go to all the lab assistants and technicians too in the Department of Chemical Engineering and Institute of Biological Sciences, University of Malaya, for their technical helps on my experimental works.

I would like to express my deepest gratitude to all my family members for their spiritual support and valuable time in accompanying me through all the frustration and struggles of my research.

Thanks to my past and current laboratory members for their helping hands and feedbacks on my research. I value the time and hardship we have gone through together.

Thanks to Indah Water Konsortium Sdn. Bhd. for providing the activated sludge samples.

To all my friends, thanks for your friendship, enthusiasm, practical assistance, and a ready ear. In short, thanks for your support! Valuable bonds of friendships were made and strengthened.

This research was financially supported by Fundamental Research Grant Scheme (RG152/12AET) and Postgraduate Research Grant (PV061/2011A), University of Malaya.

Last but not least, thanks to Ministry of Education Malaysia for awarding me the MyPhD scholarhip. The scholarship has lightened my financial burden which allows me to focus on my Ph.D study.

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LIST OF SYMBOLS AND ABBREVIATIONS

AO	Anaerobic-oxic
A2O	Anaerobic-anoxic-oxic
ATP	Adenosine triphosphate
ATU	Allythiourea
BOD	Biochemical oxygen demand
CAS	Conventional activated sludge
CH_3COO^-	Acetate
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EBPR	Enhanced biological phosphorus removal
FISH	Fluorescent in situ hybridization
GAOs	Glycogen accumulating organisms
GDP	Guanosine diphosphate
GLY/VFA	Glycogen to volatile fatty acid
GTP	Guanosine triphosphate
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solid
MLVSS	Mixed liquor volatile suspended solid
mRNA	messenger RNA
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide

NMR	Nuclear magnetic resonance
OTU	Operational taxonomy unit
P	Phosphorus
PAOs	Polyphosphate accumulating organisms
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PMF	Proton motive force
polyP	Polyphosphate
ppk	Polyphosphate kinase
PO_4^{3-}	Orthophosphate
P_{rel}/HAc_{up}	P release/ Acetate uptake
qPCR	Quantitative polymerase chain reaction
SBR	Sequencing batch reactor
SRT	Solid retention time
SS	Suspended solids
STPs	Sewage treatment plants
TCA	Tricarboxylic acid
TP	Total phosphorus
VFAs	Volatile fatty acids
WWTPs	Wastewater treatment plants

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CHAPTER 1

INTRODUCTION

1.1 Research Background

1.1.1 Eutrophication and Removal of Phosphorus from Sewage

Phosphorus (P) is one of the critical factors leading to the eutrophication of many surface waters. A major input of P into the water bodies is the effluent from sewage treatment plants (STPs). P removal from sewage can be achieved both using chemical or biological treatment. However, the latter is gaining the popularity due to its environmental friendly nature.

Activated sludge processes, the biological wastewater treatment which commonly employed in the conventional STPs, were initially designed to remove organic matter and suspended solids (SS) only. In the 1960s, the phenomenon of enhanced biological P removal (EBPR) was first observed when activated sludge flowed through a zone of low dissolved oxygen (DO), followed by an aeration tank (Levin and Shapiro, 1965). Certain microorganisms in activated sludge, later called polyphosphate accumulating organisms (PAOs), could luxuriously take up large amount of phosphate and store intracellularly in the form of polyphosphate (polyP) under alternating anaerobic and aerobic condition (Mino et al., 1998). P is ultimately removed from the bulk water through sludge wastage. Many activated sludge processes treating sewage have been modified to optimize conditions favorable to EBPR and can achieve effluent total phosphorus (TP) levels as low as 0.1-0.2 mg P/L (Blackall et al., 2002).

While EBPR processes have been broadly reported to produce effluents with very low P concentrations, the occurrence of operational unpredictability and accompanying sudden reductions in P removal are also being reported frequently. Factors which affecting the EBPR performance, such as the pH, temperature, nutrient limitation, etc., have been the focus of many studies. Since process performance is also likely to be strongly influenced by the microbial composition, linking the microbiology aspects with the operational factors could be highly useful to maximize the reliability and efficiency of the process. In relation to the aspect of temperature, the literatures claimed that warmer temperatures always lead to the deterioration in EBPR performance. However, detailed reasons linking the temperature effect with low EBPR activity at warm temperatures still rudimentary.

1.1.2 Problem Statements

Due to the rapid population growth and urbanization as well as the lack of appropriate water management system, water pollution has become a significant challenge in Malaysia. One of the consequences of this widespread water pollution is the occurrence of eutrophication. From a survey conducted on the status of eutrophication in Malaysia, 60% of the 90 lakes and reservoirs assessed were eutrophic and the rest mesotrophic (Anton *et al.*, 2007). The results showed the severity of eutrophication in Malaysia and the need to call for measures to prevent its proliferation.

In preventing eutrophication, P removal from sewage has been considered as a key strategy (Meganck and Faup, 1988). The need of nutrient removal from sewage has gained attention from the Malaysia's regulatory authority. In year 2009, the Malaysian government has revoked the Environment Quality (Sewage and Industrial Effluents) Regulations 1979 to the Environment Quality (Sewage) Regulations 2009 which lists

the discharge limits of phosphorus and nitrogen (N). P discharge limits imposed on effluent releasing into upstream and downstream water bodies of a water supply intake are 5 mg/L and 10 mg/respectively. Although these discharge limits are still far higher than the reported threshold limit, 0.5 mg P/L, in preventing eutrophication (Dunne and Leopold, 1978), the introduction of nutrient discharge limits in year 2009 marked the first positive move of the government in abating eutrophication.

With the nutrient discharge limits introduced, the new local STPs are facing the challenge to remove N and P from the sewage, in addition to the removal of organic matter. On P removal, the EBPR process is undoubtedly an efficient, economical and sustainable technology (Liu et al., 2007) to be considered by the local STPs operators. However, the technical and process know-how on EBPR processes were derived mainly from the laboratory reactors and real plants operated in regions with temperate climate such as North America, Europe, and Japan (Cao, 2011). There is still limited knowledge on how EBPR works in warm climates with relatively high temperature around 25 – 32^{o}C. Poor P removal efficiencies have often been experienced by the EBPR process operated at temperature higher than 20^{o}C (Panswad et al., 2003; Erdal et al., 2003; Whang et al., 2006; Cao, 2011). Rabinowitz et al. (2004) and Gu et al. (2005) also reported deterioration of EBPR during summer seasons. The findings of these studies indicate that the employment of the EBPR process to STPs in warm climates is a daunting task.

Nevertheless, there are several successful operation of high-temperature EBPR processes, i.e 28^{o}C – 30^{o}C, being reported (Freitas et al., 2009). Also, EBPR process at 28^{o}C which was inoculated by using seed sludge from a conventional activated sludge process, was successfully established in a preliminary study conducted by Ong (2010).

Following these encouraging observation, critical questions raised concerning the EBPR capacity and its microbiology as well as the involvement of *Candidatus* 'Accumulibacter phosphatis' (hereafter named Accumulibacter), the best-known PAOs, in these high-temperature EBPR processes. Thus, there is an urgent need to generate vital information pertaining to the theory and practices of EBPR process at high temperature to determine the feasibility of operating this process in warm regions.

1.2 Research Objectives

To have better understanding of EBPR at high temperature, process performance and microbiological aspect are the two research focuses of this study. The more specific objectives are outlined below:

i. **To investigate the EBPR performance and the process stability at a range of high temperatures, i.e. 28^{o}C – 32^{o}C.**

   Till date, there is still limited study which reports on the stability of EBPR process operated at high temperatures. A very crucial question in EBPR research is why EBPR activities deteriorated with temperature goes higher than 20^{o}C. To have a better insight into the process performance and sustainability of high-temperature EBPR, EBPR processes operated at 24^{o}C, 28^{o}C, and 32^{o}C were being evaluated and subjected to the subsequent microbiological study.

ii. **To identify the PAOs and glycogen accumulating organisms (GAOs) candidates involved in the high-temperature EBPR process.**

   Little is known on which PAO and GAO candidates present in high-temperature EBPR process. Therefore, screening of PAOs and GAOs population in all the studied EBPR processes is necessary. The PAOs and GAOs present in all three

temperatures will then be compared. Fluorescent in situ hybridization (FISH) approach was being used to facilitate the screening of potential PAOs and GAOs.

iii. **To examine the microbial community structure in the high-temperature EBPR process.**

There is a need to explore microbial community from the high temperature EBPR process as the identities of those microbes involved in this EBPR process have not been clearly revealed. Such information not only allows a more detailed understanding of the ecology of complex microbial communities from the successfully operated high-temperature EBPR process but also provides information which can be used for the improvement of process control and system reliability. Examination of the population diversity and phylogeny within the EBPR sludge will be done through clone library generation.

iv. **To elucidate the relationships between the process performance and the dynamics of Accumulibacter population in high-temperature EBPR processes.**

Accumulibacter is the most recognized PAOs so far in both lab-scale and full-scale EBPR processes. This study aims to assess their involvement in high-temperature EBPR. With 16S rRNA-based real-time quantitative polymerase chain reaction (qPCR), the dynamics of Accumulibacter was monitored throughout the process operation and then relate to EBPR performance.

v. **To reveal the Accumulibacter fine-scale population structure and dynamics in high-temperature EBPR processes.**

With polyphosphate kinase 1 (*ppk1*) that provides higher phylogenetic resolution, the subpopulations of Accumulibacter in high-temperature EBPR process were investigated. The abundance and dynamics of Accumulibacter fine-scale population was further estimated and profiled using *ppk1*-based qPCR method. To enhance the estimation result, qPCR assay targeted on specific Accumulibacter clade(s) which currently not available, was developed in this study.

1.3 Structure of Dissertation

This dissertation is presented in 7 chapters and the contents of each chapter are described below.

Chapter 1 presents the background and objectives of this research.

Chapter 2 presents the literature review for the overall study. The relevant background information and findings by other researchers are detailed in this chapter.

Chapter 3 details the general materials and methods used in this research.

Chapter 4 comprises the results and discussion on the performance of a long-term operated EBPR process at 28^{o}C and its microbial community.

Chapter 5 discusses the performance of EBPR processes at a range of high temperature and the dynamic profile of Accumulibacter population in it.

Chapter 6 reports the results and discussion on the fine-scale population study of Accumulibacter in high-temperature EBPR process.

Chapter 7 presents the overall conclusions of this research, the novelties and contribution of the study, as well as the recommendations for future studies in this field.

CHAPTER 2

LITERATURE REVIEW

2.1 Phosphorus and Eutrophication

Eutrophication occurs both in fresh and marine waters, where excessive development of certain types of algae and/or macrophytes can disturb the aquatic ecosystems and become a threat to animal and human health. Although eutrophication can be part of the natural process in water bodies associated with seasonal fluctuations, the increased frequency of such occurrences is triggered by water pollution. The primary cause of eutrophication is due to an excessive discharge of nutrients, i.e., nitrogen (N) and phosphorus (P) (Conley, 2000). However, P is the critical factor leading to most of the occurrence of eutrophication (Kortstee et al., 1994). The symptoms of eutrophication such as heavy growth of rooted aquatic plants, algal blooms, deoxygenation and, in some cases, unpleasant odor, often affects the vital uses of the water such as water supply, recreation, fisheries (Tchobanoglous, 2004), and aesthetics. All these responses can further lead to deterioration of water bodies, clogging of water systems, and undesirable changes in aquatic populations. Accumulative evidence has suggested that the major point sources of nutrients are the discharged effluent from wastewater treatment plants (WWTPs) (de Renzo, 1978).

Conventional WWTPs employing activated sludge processes were initially designed only to remove organic carbon, which denoted as chemical oxygen demand (COD) or biological oxygen demand (BOD), without significant reduction in the P level. Thus, the need of adopting nutrient removal technology in WWTPs is of urgency and it is evidenced from the specific legislations that have been imposed globally on the limit of

nutrient discharges from WWTPs. Within this context, in Europe, the developed regions, the effluent discharge requirements are ranged from 1-2 mg/L of total P depending on plant size, location, and potential impact on receiving waters (Lesjean et al., 2003).

In Malaysia, positive move has been taken by Malaysian government in revising the regulation imposed on sewage treatment plants (STPs). Environmental Quality (Sewage) Regulations 2009 has been put into practice since 10 December 2009, to replace the revoked Environmental Quality (Sewage and Industrial Effluents) Regulations 1979. The discharge limits for both P and N are imposed on the new sewage treatment plants. While for the existing sewage treatment plant, nutrient removal is only focused on ammoniacal nitrogen (NH_3-N). The Environmental Quality (Sewage) Regulations 2009 is presented in Table 2.1. For the effluent that is released into upstream water bodies of a water supply intake should meet Standard A, while effluent that is discharged downstream should meet Standard B.

Table 2.1: Environmental Quality (Sewage) Regulations, 2009

(i) New sewage treatment plant

Parameter	**Unit**	**Standards**	
		A	**B**
Temperature	^oC	40	40
pH Value	-	6.0-9.0	5.5-9.0
BOD_5 at 20^oC	mg/L	20	50
COD	mg/L	120	200
Suspended Solids	mg/L	50	100
Oil and Grease	mg/L	5.0	10.0
Ammoniacal Nitrogen (enclosed water body)	mg/L	5.0	5.0
Ammoniacal Nitrogen (river)	mg/L	10.0	20.0
Nitrate-Nitrogen (river)	mg/L	20.0	50.0
Nitrate-Nitrogen (enclosed water body)	mg/L	10.0	10.0
Phosphorus (enclosed water body)	mg/L	5.0	10.0

(ii) Existing sewage treatment system (approved before January 1999)

		Communal Septic Tank		Imhoff Tank		Aerated Lagoon		Oxidation Pond		Mechanical System	
Parameter	**Unit**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
BOD_5 at 20^oC	mg/L	200	200	175	175	100	100	120	120	60	60
COD	mg/L	-	-	-	-	300	300	360	360	180	240
Suspended Solids	mg/L	180	180	150	150	120	120	150	150	100	120
Oil and Grease	mg/L	-	-	-	-	-	-	-	-	20	20
Ammoniacal Nitrogen	mg/L	-	-	100	100	80	80	70	70	60	60

(iii) Existing sewage treatment system (approved after January 1999)

Parameter	**Unit**	**Standards**	
		A	**B**
BOD_5 at 20^oC	mg/L	20	50
COD	mg/L	120	200
Suspended Solids	mg/L	50	100
Oil and Grease	mg/L	20.0	20.0
Ammoniacal Nitrogen	mg/L	50.0	50.0

2.2 Enhanced Biological Phosphorus Removal (EBPR)

In general, P removal can be achieved using either biological treatment or chemical treatment in WWTPs. EBPR which is an activated sludge processes with alternating anaerobic and aerobic conditions, have been successfully used for the removal of P from wastewater without the need of chemical precipitation. This approach utilizes a microbiological mechanism whereby the process is conditioned to enrich PAOs which

accumulate P in excess of their normal metabolic requirements and store it in the form of intracellular polyphosphate. The alternating anaerobic-aerobic regime can be achieved either by spatial configuration of anaerobic and aerobic zones in series in continuous flow systems with sludge recycle or by temporal arrangement of anaerobic and aerobic in sequencing batch reactors (SBRs).

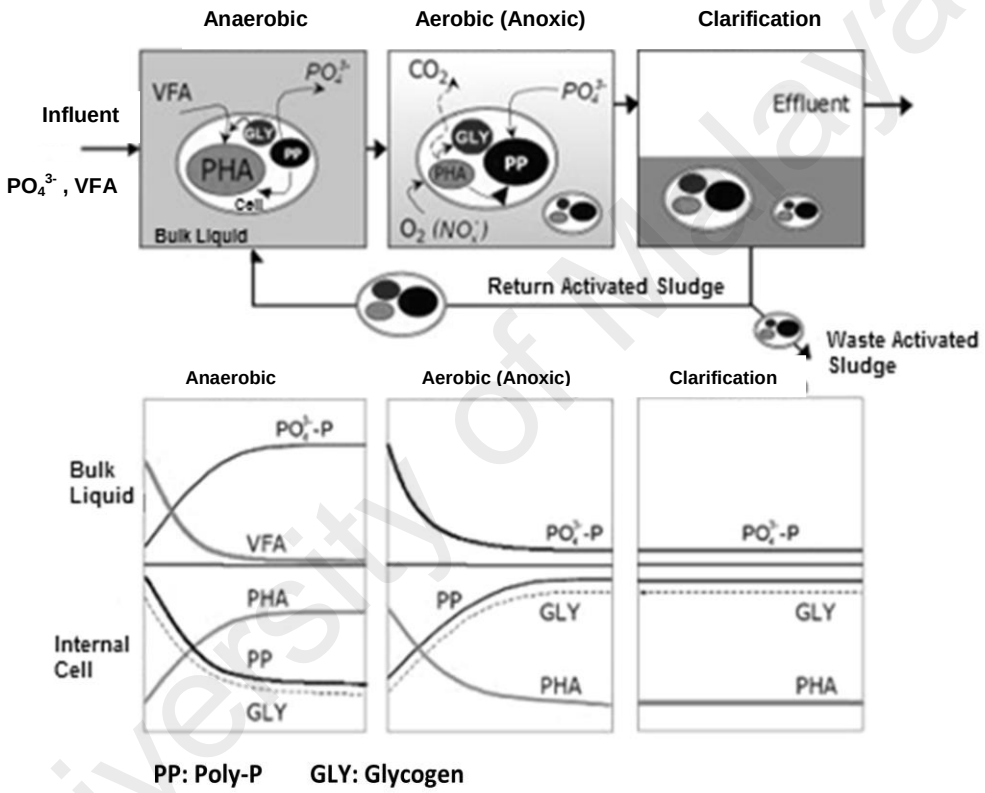


Figure 2.1: The conceptual scheme of PAOs' metabolism and typical concentration profiles of key components in EBPR (adapted from Lopez-Vazquez (2009).

PAOs utilize internally stored polyphosphate as an energy source for the uptake and accumulation of volatile fatty acids (VFAs), the carbon substrate, as polyhydroxyalkanoate (PHA) during the anaerobic feeding stage. During the subsequent aerobic stage, the accumulated PHA is utilized for growth and replenishment of the polyphosphate and glycogen pools. After a liquid/solids separation stage, a part of the

biomass is recycled to the anaerobic stage and mixed with new wastewater, whereas the excess sludge containing the intracellular polyphosphate is removed from the system. Figure 2.1 illustrates the conceptual scheme of PAOs' metabolism and typical concentration profiles of key components in EBPR.

EBPR processes offer several benefits such as reduced sludge production, generate well-settling sludge which rich in nutrient, reduced oxygen requirements, reduced process alkalinity requirements and potential P recovery. If operated successfully, the EBPR process is more sustainable approach in terms of economical and environmental concerns for P removal (Satoh et al., 1994; Oehmen et al., 2007). Thus, EBPR processes have been widely implemented in developed countries worldwide to minimize environmental pollution and meet the stringent wastewater discharge standard (Lesjean et al., 2003).

2.2.1 Biochemical Transformation and Metabolic Pathways of EBPR Process

Studies have been focused mostly on the anaerobic metabolism since this serves as a selective pressure for enrichment of PAOs. Furthermore, the anaerobic carbon uptake and storage as intracellular compound also involves complicated and unresolved biochemical dynamics.

During the early stage of EBPR-related research, polyP was considered as the only energy source for PAOs. However, glycogen was later found to provide energy too during anaerobic substrate uptake. According to Mino et al. (1998), processes occur under anaerobic condition which requires energy include: transportation of extracellular

substrates into the intracellular environment; conversion of substrates to PHA and related metabolism; and cell maintenance.

The source of reducing power generation which is utilized for anaerobic PHA formation is an essential component used in the discussion of EBPR metabolism. In the Comeau/Wentzel model (Comeau et al., 1986; Wentzel et al., 1986), the reducing equivalents required for the reduction of acetyl-CoA to polyhydroxybutyrate (PHB) were assumed to be produced by the tricarboxylic acid (TCA) cycle. As suggested, this pathway would provide sufficient Nicotinamide adenine dinucleotide (NADH) to replenish the amount which has been used up during PHB synthesis. Mino et al. (1987) later proposed that intracellular carbohydrate/glycogen utilization occurs via glycolysis which was shown by the decrease in total carbohydrate concentration under anaerobic conditions. Therefore, intracellular carbohydrate was proposed to be the source of the reducing power and supply additional acetyl-CoA for PHB synthesis. Smolders et al. (1994a) then developed the anaerobic metabolic model in accordance to the Mino model. They managed to show that the anaerobic stoichiometry could be well explained by the metabolism on the basis of intracellular glycogen. Since then, glycolysis has been widely agreed to be the main supplier of reducing power for the conversion of VFAs to PHAs by PAOs anaerobically.

Both Pereira et al. (1996) and Maurer et al. (1997) confirmed glycogen involve in EBPR via nuclear magnetic resonance (NMR) techniques. According to Perreira et al. (1996), labeled carbon dioxide ($CO_{2)}$ that derived directly from labeled acetate was detected during the anaerobic phase and the amount of reducing power supplied by glycolysis could not confidently explain the total amount required for PHA production. With these evidences, they further deduced that TCA cycle functions anaerobically.

Later, Zhou et al. (2008) also reported that Accumulibacter can use both glycogen and TCA cycle for anaerobic reducing power generation through the study of a highly Accumulibacter-enriched EBPR reactor. With comparison made between the anaerobic stoichiometry from full-scale EBPR wastewater treatment plants and the predictive models from other researchers, Pijuan et al. (2008) inferred that both glycolysis and TCA cycle for the production of reducing power in PAOs should be incorporated in EBPR metabolic models when acetate is the substrate. However, the involvement of TCA cycle could be neglected when propionate is being used as the sole carbon source.

By using metabolic inhibitors together with NMR approach, Louie et al. (2000) found out that succinate oxidation to fumarate by succinate dehydrogenase was a significant step in the PHA synthesis pathway. It requires a terminal electron acceptor with a greater redox potential than the fumarate-succinate couple and this could be the reason for complete TCA cycle not to function anaerobically (Mino et al., 1998). This problem was later addressed by Hesselmann et al. (2000) who proposed a modified succinate-propionate pathway combined with an incomplete TCA cycle where some acetyl-CoA proceeds through the oxidative pathway of the TCA cycle (forward) and later gets converted to propionyl-CoA via the methylmalonyl-CoA pathway, while a portion of pyruvate also proceeds through the reductive pathway of the TCA cycle via oxaloacetate. The involvement of partial TCA cycle was also supported by Brdjanovic et al. (1998) and Yagci et al. (2003), on top of their suggestion for the activity of the glyoxylate pathway.

PAOs used the anaerobically stored PHAs for their growth and cell maintenance under aerobic condition. Then stored PHAs are also utilized as energy source to restore the polyP and glycogen pools. As a result, the concentration of intracellular polyP and

glycogen increases with the decrease in stored PHAs and soluble orthophosphate is being taken up from the bulk liquid environment leading to the removal of P. PAOs metabolism is distinctive with the fact that apart from cell growth, noticeable parts of intracellular PHAs are being utilized in the aerobic accumulation of polyP and glycogen (Kortstee et al., 2000). All the proposed EBPR-related models agreed that catabolism proceeds via the TCA cycle. The degradation of PHB and polyhydroxyvalerate (PHV) would lead to the formation of acetyl-CoA. Both are used as the carbon and energy source for biomass growth, while a portion of adenosine triphosphate (ATP) is used for the uptake of extracellular orthophosphate and thus the replenishment of polyP. In the model proposed by Comeau et al. (1986), modification was made on Mino model (Mino et al., 1987) by including the glycogen formation and some carbon and energy were considered in the model for the replenishment of glycogen. Smolders et al. (1994b) came out with a structured metabolic model which focuses on aerobic metabolism of PAOs where linkage was made between the aerobic carbon and P transformations to the oxygen consumption rate.

2.2.2 Glycogen Accumulating Organisms (GAOs) Competing Mechanism

It had been reported that anaerobic-aerobic operation sometimes showed no EBPR activity for unknown reasons (Fukase et al., 1985; Cech and Hartman, 1993). Cech and Hartman (1993) reported the first observation of EBPR deterioration due to population shift. They observed clusters of large Gram-positive and Gram-negative cocci in distinctive tetrads, known as the ‘G-bacteria’ in a reactor fed with a mixture of glucose and acetate. Once glucose was removed from the medium, the EBPR activity slowly recovered. They elucidated that these ‘G-bacteria’ were out-competing the PAO by effectively utilizing carbon substrate during anaerobic condition for PHA production but did not contribute to aerobic P uptake. Later, Liu et al. (1994) revealed that the

intracellular carbohydrate stored in the EBPR activity deteriorated anaerobic-aerobic sludge was a polymer consisting of glycosyl units with the α-1,4- and the α-1,6-linkages, or glycogen. Thus, the 'G-bacteria' were termed glycogen accumulating organisms or GAOs (Liu et al., 1996a).

PAOs and GAOs generally fall into two different physiological groups but share some common metabolic traits. Both groups can take up extracellular carbon substrate and simultaneously convert them into PHA under anaerobic conditions. According to Satoh et al. (1994) and Liu et al. (1994), glycogen supplies ATP through glycolysis and help to maintain the intracellular redox balance through $NADH_2$ generation during anaerobic acetate uptake. ATP is essential in the transportation of acetate across the cell membrane and for its activation to acetyl-CoA (Smolders et al., 1994a). Therefore, glycolysis must proceed at a rate sufficient to satisfy the energetic requirements of acetate uptake. Part of the $NADH_2$ produced during glycolysis is consumed during the production of PHA, but excess $NADH_2$ is produced on top of the ATP requirements of other metabolic processes. As suggested by Mino et al. (1987) whereby a certain amount of the pyruvate produced through glycolysis is directed through the succinate-propionate pathway, where the excess $NADH_2$ is consumed and propionyl-CoA is produced. The total amount of pyruvate directed into the pathway is regulated to ensure the overall amount of $NADH_2$ replenished is equal to the amount consumed. In contrast to the ATP production proposed by Mino et al. (1987), Zeng et al. (2002) found that this pathway produces no ATP. They claimed that the balance of pyruvate is decarboyxlated to form acetyl-CoA which supply extra $NADH_2$ and generating CO_2. The acetyl-CoA is finally produced through glycolysis and acetate activation, while the propionyl-CoA is produced through the succinate-propionate pathway. Consequently, both acetyl-CoA

and propionyl-CoA are reduced and condensed as 3-hydroxy-valerate (3HV), a precursor of PHA.

2.3 Microbial Communities of EBPR Processes

Researchers have been trying to reveal the microorganisms primarily responsible for P removal in treatment plants and characterize their physiology to provide better operational conditions to facilitate their functions. In the early days of EBPR development, researchers hypothesized that a specific microorganism responsible for P removal would dominate EBPR sludge (Fuhs and Chen, 1975). Later, EBPR sludge is recognized comprising of a wide array of microbial species (Streichan et al., 1990; Zilles et al., 2002). Similar to conventional activated sludge, EBPR sludge harbors numerous species of viruses, bacteria, protozoa, fungi, metazoa, and algae. Molecular ecology studies show that EBPR sludge is a microbial consortia composed of phylogenetically and morphologically diverse populations with widely varying metabolic capabilities (Fukushima et al., 2007; Kawaharasaki et al., 1999). This mixed microbial population exists in a dynamic environment affected by a number of changing environmental and/or operational factors. Since very little is known regarding the microbial players in the EBPR process, organisms in EBPR sludge are classified based on their capability in accumulating P (Seviour et al., 2003).

2.3.1 Microbial Population Retrieved From Well-Performing EBPR Processes

***Acinetobacter* spp.** Through culture dependent approach, *Acinetobacter* spp. was first proposed by Fuhs and Chen (1975) as the key player responsible for P removal in EBPR process. *Acinetobacter* spp. was later reported successfully isolated from both laboratory-scale reactors and full-scale EBPR plants (Lötter, 1986; Wentzel et al.,

1986). *Acinetobacter* was also revealed by Deinema et al. (1980) for its ability in storing P in the form of polyP granule intracellularly. Hence, with all these postulations, *Acinetobacter* spp was long believed to be the only PAO present in EBPR process.

However, several studies showed that *Acinetobacter* isolates did not necessarily possess the essential characteristic which shown by EBPR sludge. Ohtake et al. (1985) found that some *Acinetobacter* isolates could not take up acetate anaerobically and could not take up P aerobically even though P release was observed (Ohtake et al., 1985). They also failed to show the phenotypic traits of EBPR sludge when subjected to repetitive anaerobic-aerobic conditions.

The use of different culture-independent measurement methods such as fluorescent antibody staining (Cloete and Steyn, 1988), quinone profile measurement (Hiraishi and Morishita, 1990) and specific FISH probe targeted *Acinetobacter* (Wagner et al., 1994; Kämpfer et al., 1996) had shown that the presence of *Acinetobacter* has little significance in laboratory-scale or full-scale EBPR processes when compared to members of other phylogenetic groups and could not account for the P removal observed in activated sludge.

Actinobacteria. *Actinobacteria* has been reported widely for their presence in EBPR processes (Kawaharasaki et al., 1999; Crocetti et al., 2000; Lee et al., 2002). They were found present in substantial numbers in full-scale EBPR plants (Eschenhagen et al., 2003; Kong et al., 2005; Nielsen et al., 2010). The isolates of the genus *Tetrasphaera* which currently available consists of *Tetrasphaera australiensis*, *Tetrasphaera japonica* (Maszenan et al., 2000a), *Tetrasphaera elongata* (Hanada et al., 2002; Onda & Takii, 2002), and the filamentous *Tetrasphaera jenkinsii*, *Tetrasphaera vanveenii*, and

Tetrasphaera veronensis (McKenzie et al., 2006). Liu et al. (2001) reported that *Tetrasphaera* spp. which dominant in the microbial population in an efficient acetate-fed laboratory-scale EBPR process were responsible for P removal. Surveys using FISH with probes targeting *Tetrasphaera, viz.* actino_101, actino_221 and actino_658, found that *Tetrasphaera* were able to take up P and stored as polyP aerobically, but did not show VFAs assimilation and PHA accumulation under anaerobic condition (Kong et al., 2005) which appears to be different from common physiological characteristic of model PAOs. The same study also revealed that *Tetrasphaera* spp existed in two different morphologies, cocci in clusters of tetrads and short rods in clumps. Nguyen et al. (2011) further shown that *Tetrasphaera* displayed in branched rod in addition to the earlier discovered morphologies.

***Rhodocyclus*-related organisms**. The presence of the *Rhodocyclus*-related organisms in EBPR processes was first reported by Bond et al. (1995) using 16S rRNA gene clone library. *Rhodocyclus*-related bacteria in EBPR garnered more attention after being confirmed by several other studies on their involvement in EBPR (Hesselmann et al., 1999; Crocetti et al., 2000; Liu et al., 2001; Kawaharasaki et al., 2002; Onuki et al., 2002; Jeon et al., 2003). Hesselmann et al. (1999) and Kong et al. (2004) reported that *Rhodocyclus* group was responsible for the key metabolism of PAO as predicted in EBPR model. Also, Hesselmann et al. (1999) named the Rhodocyclus-related PAO as Candidatus 'Accumulibacter phosphatis'.

Microlunatus phosphovorus. *Microlunatus phosphovorus* was another microorganism isolated from a laboratory scale EBPR process (Nakamura et al., 1995) and hypothesized responsible for EBPR. This gram-positive high G+C coccus bacteria was reported showed carbon assimilation and P release during anaerobic condition while

accumulated large amounts of polyP under aerobic condition. However, through FISH probe targeted on *Microlunatus phosphovorus* in EBPR sludge, Kawaharasaki et al. (1998) revealed that *Microlunatus phosphovorus* insignificantly contributed to the bacterial cells which stained positive for polyP. Santos et al. (1999) further studied the role of *Microlunatus phosphovorus* to EBPR using ^{31}P and ^{13}C NMR. It was found in this study that *Microlunatus phosphovorus* could assimilate and transform glucose to acetate and poly-glucose under anaerobic conditions and later utilized the stored poly-glucose for growth under aerobic condition. But, *Microlunatus phosphovorus* showed neither PHA nor glycogen involved in their metabolism. Moreover, *Microlunatus phosphovorus* is not commonly present or significantly contributes to the total bacterial population in EBPR process (Seviour et al., 2003). *Microlunatus phosphovorus* found constituted 3% and 7% of the total bacterial population in EBPR sludge investigated by Kawaharasaki et al. (1999) and Lee et al. (2002). Thus, *Microlunatus phosphovorus* was not considered major PAOs.

Paracoccus denitrificans. *Paracoccus denitrificans* was a denitrifying isolate first isolated from fluidized-bed reactor operated for nitrate removal (Barak and van Rijn, 2000). *Paracoccus denitrificans* which found exhibited P removing capability was then studied for polyP accumulation under aerobic, anoxic, and anaerobic conditions. *Paracoccus denitrificans* utilized PHAs only with the presence of external carbon supplement and polyP synthesis took place with the presence of oxygen or nitrate as the electron acceptor In short, *Paracoccus denitrificans* could remove P without the need for alternating anaerobic/aerobic conditions.

Malikia spp. *Malikia granosa* and *Malikia spinosa* are gram-negative, motile, rod-shape *β-Proteobacteria* isolates which retrieved from a municipal WWTP (Spring et al.,

2005). They phylogenetically close with the family *Comamonadaceae*. Both *Malikia* spp. possess ability in accumulating PHAs in carbon-rich medium. Under carbon-limited conditions, PHAs were degraded and polyP were stored intracellularly. Nevertheless, their existence and ability to transform PHAs and polyP in the EBPR process is yet to be investigated hitherto.

Quadricoccus australiensis. *Quadricoccus australiensis* which related to family of *Rhodocyclaceae* was recovered from an Australian activated sludge process showing foaming and bulking problems (Maszenan et al., 2002). *Quadricoccus australiensis* possessed tetrad morphotype and stained positively for intracellular polyP and PHA in pure culture. But, their EBPR behavior under anaerobic/aerobic conditions has not been determined.

Filamentous foaming bacteria. *Candidatus Microthrix parvicella* and *Nostocodia limicola* II, are some of the candidates in filamentous foaming bacteria group that sometimes detected in EBPR system and showing polyP accumulation (Seviour et al., 1990). Their ability to store phosphorus in the form of polyp granule intracellularly was supported by the positive response showed to Neisser staining. these filamentous bacteria stain positively for Neisser stain in pure culture and *in situ* (Blackall et al., 2000; Liu et al., 2001). However, there is still lack of detailed information on their involvement in P removal of EBPR processes.

2.3.2 Microbial Population Retrieved From Deteriorated EBPR Processes

Defluviicoccus spp. *Defluviicoccus spp.* which reported by Wong et al. (2004) and Meyer et al. (2006) is phylogenetically related to *Alphaproteobacteria* and displaying

tetrad forming organism (TFO) morphotype. *Defluviicoccus spp*. could be the potential competitor for PAOs as it was revealed through FISH-MAR their ability in taking up carbon sources, such as acetate and propionate. It was found abundant in a deteriorated acetate-fed anaerobic/aerobic reactor but not in different full-scale EBPR or non-EBPR plants (Wong et al., 2004). Meyer et al. (2006) found *Defluviicoccus vanus* highly enriched in a propionate-fed anaerobic/aerobic reactor with poor P removal performance. Phylogenetic analysis showed that this *Defluviicoccus vanus* formed in two clusters, namely Cluster 1 and Cluster 2. Cluster 1 comprised sequences from Wong et al. (2004) while Cluster 2 also found existed in two full-scale EBPR plants in Australia.

GB group. The GB group, cluster forming coccobacilli which related to *γ-proteobacteria* is often observed in sludge samples of non-functioning laboratory-scale and full-scale EBPR systems (Nielsen et al., 1999; Liu et al., 2000; Dabert et al., 2001, Kong et al., 2002a; Saunders et al., 2003; Pijuan et al., 2004). The phenotype of GB group was later identified by Crocetti et al. (2002) and named *Candidatus* "Competibacter phosphatis" (Competibacter). Kong et al. (2002b) extensively examined the diversity and distributions of the GB group in both poorly and well performed EBPR process. Phylogenetic analysis of 16S rRNA sequences further classified the *γ-proteobacteria* into seven subgroups (Kong et al., 2002b). FISH probes targeted these seven subgroups, namely GAO989 (identical with GB_1) and GB_1 up to GB_2) were designed in the same study. Either the mixture of probes GB or mixture of probes GB_2 and GAO989 could target on Competibacter (Kong et al., 2002b; Zeng et al., 2003; Oehmen et al., 2004). Competibacter have frequently observed in abundance in laboratory scale acetate-fed EBPR process and full-scale plants (Crocetti et al., 2002; Kong et al., 2002b; Saunders, 2005; Kong et al., 2006). The established correlations of

high abundance of Competibacter with the VFAs uptake and poor EBPR activity have caused long-believed of Competibacter as an active competitor to PAOs in competing carbon substrate under anaerobic condition but did not contribute to P removal under aerobic condition.

Amaricoccus spp. Cech and Hartman (1993) retrieved isolates with TFO morphology from a laboratory-scale anaerobic and aerobic alternating reactor that exhibited poor EBPR performance. These isolates were later identified and named by Maszenan et al. (1997) as *Amaricoccus* spp. Falvo et al. (2001) reported that *Amaricoccus* spp. could generate glycogen aerobically but did not compete with PAOs for acetate or glucose anaerobically; and PHA was synthesized aerobically but not anaerobically. Liu et al. (1996) further claim that these physiological properties are different from the behaviors of proposed GAO. A probe designed specifically to target for *Amaricoccus* spp. was tested against various activated sludge samples from EBPR systems (Maszenan et al., 2000b) and found that *Amaricoccus* commonly exist as tetrads in flocs or dispersed cell in both conventional activated sludge and EBPR treatment plants.

Micropruina glycogenica. *Micropruina glycogenica* was first isolated from an EBPR SBR fed with both acetate and peptone (Liu et al., 1997). It was later proposed as candidate of GAOs as they could assimilate either acetate or glucose and synthesize PHA under anaerobic condition, but could not store polyP aerobically (Jeon and Park, 2000; Shintani et al., 2000; Kong et al., 2001)

Kineosphaera limosa. *Kineosphaera limosa* were another isolates recovered from deteriorated laboratory-scale EBPR reactor (Liu et al., 2002). It is high G+C gram

positive and non-spire forming coccus which capable in storing intracellular PHA. There is limited information available about their existence in full-scale EBPR systems.

2.4 Candidatus 'Accumulibacter phosphatis' (Accumulibacter)

Accumulibacter, the *Rhodocyclus*-related PAO, is widely recognized as an important bacterial population that responsible for EBPR in lab-scale acetate-feed EBPR systems (Dabert et al., 2001; Zeng et al., 2003; Carvalho et al., 2007). Their involvement in P removal in full scale EBPR systems is also undeniable as Zilles et al. (2002) found that Accumulibacter cells represented approximately 20% of the total bacterial population in sludge from EBPR plants in the United States, and took part in over 70% of the P-accumulating activity. Other studies reported that, compared with the total bacterial community, Accumulibacter constituted 7 to 12% in four Australian plants (Saunders et al., 2003), 5 to 22% in three plants in Denmark (Kong et al., 2004), 7 to 17% in four Japanese plants (Chua et al., 2006), and 6 to 16% in seven plants in the Netherlands (Lopez-Vazquez et al., 2008). A study by He et al. (2008) also revealed that Accumulibacter represented 40 to 70% of the total PAO population in sludge samples from five plants in the United States.

2.4.1 Accumulibacter's Polyphosphate Metabolism and Polyphosphate Kinase (*ppk*)

The biochemistry of polyP is relatively well studied in model organisms because of its multiple and important biological functions. Its metabolism is of particular interest in EBPR systems since its synthesis is the direct mechanism associated with P removed from wastewater. In EBPR metabolic, polyphosphate serves as an energy source for the

synthesis of acetate-acetyl-CoA (AcCoA) and in re-establishing the proton motive force (PMF) consumed by substrate transport during the anaerobic phase.

Polyphosphate kinase 1 (*ppk1)* catalyzes the reversible reaction of polyP formation from ATP, as shown in eq. 2.1, with the forward reaction to synthesize polyphosphate is preferred.

$$polyP_n + ATP \leftrightarrow polyP_{n+1} + ADP \qquad \text{(eq. 2.1)}$$

Conversely, polyphosphate kinase 2 (*ppk2),* a smaller enzyme than *ppk1*, operates in reverse manner to generate guanosine triphosphate (GTP) from guanosine diphosphate (GDP) by the degradation of polyphosphate.

Although other bacteria were also identified as potential PAOs in certain EBPR systems, focus is given to Accumulibacter in this research as this lineage has been widely studied till date *ppk1* appears to be a single-copy gene in Accumulibacter which can evolve four times faster than 16S rRNA genes (Kunin et al., 2008). These characteristics also have made *ppk1* a good phylogenetic marker to show fine-scale differences.

The first study on *ppk1* in EBPR activated sludge was performed by McMahon et al. (2002). Exploiting the high level of conservation of the *ppk1* amino acid sequence among bacteria, they designed degenerate primers to retrieve fragments of putative *ppk1* gene from activated sludge samples from acetate-fed laboratory-scale EBPR SBRs, where Accumulibacter comprised approximately 80% of the total bacteria in the sludge. Two genotypes of *ppk1* were retrieved from Accumulibacter, designated Type I and

Type II. The Type I *ppk1* was shown to be expressed during EBPR by messenger RNA (mRNA) dot blot analysis. The metagenomic analysis of Accumulibacter-enriched lab scale reactors revealed that Accumulibacter possessed genes encoding for *ppk1* and *ppk2* and both genes were present as a single copy (Garcia et al., 2006). From an extensive census of bacterial *ppk1* genes from a number of full scale EBPR and non-EBPR sludges, McMahon and colleagues (2007) observed two major Types of Accumulibacter (I and II respectively), and designed a *ppk1* primer set targeting total Accumulibacter. Using this primer set, He et al. (2007) retrieved fragments of Accumulibacter *ppk1* genes from a few more EBPR facilities, and found phylogenies reconstructed using 16S rRNA and *ppk1* genes were largely congruent. Moreover, the *ppk1* phylogeny exhibited a higher resolution, with at least five subgroups (clades), viz I, IIA, IIB, IIC and IID, emerging under the major Types, suggesting the Accumulibacter lineage was more diverse than previously realized. The distribution patterns of these clades suggested that Accumulibacter population structure varied among different EBPR systems and also changed temporarily within a system. They further postulated that different clades within the lineage are ecologically distinct. Peterson et al. (2008) have shown that other than activated sludge EBPR systems, Accumulibacter also inhabit environment, such as freshwater and estuarine sediments. They recover several new clades of Accumulibacter not previously detected in activated sludge, to give a total of 12 clades, which including two major groups, Type I and Type II. They also found that habitat characteristics were key factors in determining Accumulibacter distribution in the local environment, providing evidence of ecological differences among the different subpopulations.

Using the 16S rRNA FISH probes designed by Flowers and colleagues (2009) that differentiate Types I and II, Oehmen and colleagues (2010) were able to relate the

nitrate reducing and non nitrate reducing Accumulibacter subgroups observed by Carvalho and colleagues (2007) to Type I and Type II. Further incorporating this metabolic difference into modeling enabled a successful prediction of the abundances of these Accumulibacter subgroups in EBPR sludges. As lab-scale reactors only enrich for a subset of the Accumulibacter lineage (He et al., 2007), the denitrification difference which can be generalized to all clades within each Type needs further investigation. It should be noted that the Type I/II probes designed by Flowers and colleagues (2009) cannot inclusively target all members within each Type, and in particularly they missed some sequences that were exclusively present in full-scale treatment plants. Therefore, when using these two probes, FISH with the PAOmix probes should be applied to check if the sum from Type I/ II probes can account for the total Accumulibacter, especially for full scale sludges. By operating two lab scale EBPR reactors, He et al. (2010) found that the distribution of the two major clades within the total Accumulibacter population was quite stable in one reactor but comparatively dynamic in the other reactor under undisturbed operation. However, the variance in the clade distribution did not appear to affect the reactor performance. Instead, good EBPR activity was positively associated with the abundance of total Accumulibacter. They further concluded that the different clades in the system provided functional redundancy.

Kim and colleagues (2010) have differentiated Accumulibacter into four clades, Acc-SG1, Acc-SG2, Acc-SG3, and Acc-SG4, based on fragments of Accumulibacter 16S rRNA genes retrieved from EBPR sludge of their SBR. By using FISH and flow cytometric cell sorting, they tried to link the 16S rRNA gene sequences of these four Accumulibacter's clades to their *ppk1* genes in Accumulibacter lineages. The phylogenetic analysis of the *ppk1* gene homologs was overall congruent with that of the 16S rRNA genes, showing affiliation to clades IA, IB, IIA, IIC, IIF. Members from

Acc-SG3 with a distinct morphology comprised two different *ppk1* genes and affiliated to both clade IB and IIC. With this, they further suggest that Accumulibacter strains may be diverse physiologically and ecologically. Later, Kim et al. (2013) further characterize the denitrifying P uptake properties of Accumulibacter. A SBR was gradually acclimated from anaerobic-oxic (AO) to anaerobic-anoxic-oxic (A2O) conditions by stepwise increases of nitrate concentration and the anoxic time. The acclimation process led to a clear shift in the relative abundances of recognized Accumulibacter subpopulations from clades IIA > IA > IIF to clades IIC > IA > IIF, while the overall Accumulibacter abundance decreased (from 55.1% to 29.2%).

Similarly, Mielczarek et al. (2013) used qualitative *ppk1*-PCR on a single time point to better resolve the Accumulibacter community detected using FISH in the same 28 Danish WWTPs discussed above. Accumulibacter Clade IA and IIC were found in only a few plants. In both studies, however, the phylogenetically resolved analysis was conducted on samples collected only on a single day, and no information was available regarding the variability in Accumulibacter clade abundances over time. Thus, it is not currently possible to draw conclusions about representative Accumulibacter clade composition in any particular WWTP.

2.5 Process Stability and Factors Influencing the EBPR Process Performance

Over the last 40 years, empirical experiences of EBPR operation have provided better guidelines for operating EBPR processes. The metabolic behaviors of PAOs have been described by different biochemical models based on the experimental results from extensive mixed culture studies. These studies have provided insights into the biological mechanism of EBPR processes. Engineers have utilized the gross chemical transformations developed by the biochemical models to design and optimize EBPR

processes. It has been demonstrated in many full scale WWTPs that EBPR processes are able to reduce P concentrations to less than 0.5 mg l^{-1} (Blackall et al. 2002).

Despite their widespread application, difficulties in assuring stable and reliable operation have also been well recognized (Seviour et al., 2003; Thomas et al., 2003; Oehmen et al., 2007). Intermittent failure with EBPR processes in removing P has been regularly reported in both laboratory-scale reactors and full scale WWTPs. Failures often occur despite all known prerequisites for EBPR being provided and typically in periods following stable and efficient EBPR.

The possible reasons of these perturbations could be ascribed to the traditional engineering conceptualization of EBPR processes. Engineering mass balance control strategies, empirical observations, and biochemical models have long been used to select microbial communities that carry out EBPR metabolism. However, proper operational conditions used to promote and maintain the growth of appropriate microbial communities for polyphosphate accumulation were hardly considered until two decades ago.

2.5.1 Effect of Temperature/ Climatic

Temperatures or climate in particular, is a significant consideration in the design and operation of EBPR processes. It is also one of the most widely studied environmental factors influencing EBPR (McClintock et al., 1993; Brdjanovic et al., 1998; Panswad et al., 2003; Whang and Park, 2006; Lopez-Vazquez et al., 2007, 2008). However, consensus is lacking on the effect of temperature on P removal efficiency and stability. Low temperatures (lower than 15^{o}C) has been observed to decrease the rates of

biochemical transformations (e.g. P release/uptake, acetate uptake, PHA oxidation, growth), as in most of biological reactions (McClintock et al., 1993; Brdjanovic et al., 1998). However, these findings have been contradicted by studies that showed successful EBPR operation at colder temperature (Barnard et al., 1985). Considering the effect of temperature as a selective pressure on competition between PAOs and GAOs, the EBPR literature widely agrees that colder temperatures favor PAOs over GAOs. A number of studies found that GAOs appeared to gain dominance over PAOs with increasing temperatures over the range from 20 to 36^{o}C (Panswad et al., 2003; Whang and Park, 2006). Erdal et al. (2003) reported better P removal at 5^{o}C than at 20^{o}C, because slower glycogen transformation at lower temperatures selected against GAOs. Deterioration of P removal at full-scale plants during summer at higher temperatures and its improved efficiency in winters at colder temperatures have been touted as additional proof of PAO's competitive advantage at colder temperatures. The application of FISH to laboratory EBPR systems always found that GAOs outcompeted PAOs at temperature higher than 20^{o}C (Lopez-Vazquez et al., 2007, 2008). The researchers also reported that anaerobic carbon uptake rate was higher for GAOs versus PAOs at temperature higher than 20^{o}C, thereby giving GAOs a competitive advantage at higher temperatures. In general, PAOs can compete successfully against GAOs, at temperatures between 20^{o}C and 25^{o}C. It is hypothesized that the deterioration of P removal in EBPR processes at temperatures higher than 25^{o}C is caused partly by thermally induced reduction of polyphosphate-accumulating capacity in PAOs.

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), N and P shock load disturbances, with a short SBR cycle that consisted of a 20-minute anaerobic phase, 10-minute

aerobic phase, a 5-minute settling and decanting phase and a 1-minute idle phase. Furthermore, Winkler et al. (2011) showed that the PAO-GAO competition at high temperature, *i.e.*, 30°C, can be controlled and a 100% P removal efficiency was secured via selective sludge removal in a segregated aerobic granular biomass system. Previous study on the establishment of EBPR reactor at 28°C (Ong, 2010) showed excellent P removal. The lab-scale EBPR reactor stably showed more than 95% P removal efficiency during one year operation period, resulting in an effluent P concentration of less than 1.0 mg/l. The results of these studies are encouraging and call for more research efforts to further examine the feasibility of operating an EBPR process at high temperature.

2.5.2 Effect of pH

The pH of the anaerobic zone has been long recognized as an important operational condition that affects the ability of PAOs and GAOs to take up VFAs. The pH value of 5 was found to cause complete deterioration in EBPR performance due to the collapse of pH gradient across the cell membrane of PAOs (Smolders et al., 1994a; Fleit, 1995). Using a PAO-enriched culture, Smolders et al. (1994a) found that the amount of P released in the anaerobic zone during acetate uptake increased significantly with an increase in pH. High pH usually causes more P release because more energy is required to maintain a constant pH gradient across the cell membrane. At higher pH, more energy is required to transport acetate across the membrane; therefore, more polyP is cleaved to produce the energy, releasing more P. Subsequently, the amount of PHA used for uptake of P in the aerobic phase will be increased.

A series of batch tests has shown that aerobically P uptake, PHA utilisation and biomass growth were inhibited by a low pH (6.5), suggesting that a higher aerobic pH (7–7.5)

would be more beneficial for PAOs (Filipe et al., 2001a). On the contrary, the acetate uptake rate of GAOs has been reported in short-term tests to decrease with increasing pH (Filipe et al., 2001b). This suggests that a higher pH not only results in a higher energy demand for acetate uptake, but also negatively affects the ability of GAOs to take up acetate. For GAOs, the energy for acetate transport is obtained by the degradation of internally stored glycogen. An increase in the pH of the anaerobic zone causes an increase in the amount of glycogen consumed under anaerobic conditions. Thus, more glycogen is required to be produced in the aerobic phase. A larger percentage of the PHA accumulated in the anaerobic phase would be used, leaving less PHA for the growth of GAOs. This would lead to a decrease in the yield of GAOs as the pH of the anaerobic zone increased. Since the amount of PHA utilized for the production of glycogen for GAOs is much higher than the amount used for the replenishment of polyP for PAOs, an increase in pH in the anaerobic zone makes the growth of GAOs less favorable. It has been postulated that an anaerobic pH of 7.25 is a critical point, whereby GAOs are able to anaerobically take up VFAs faster than PAOs below pH 7.25, and above this pH value, PAOs take up acetate faster (Filipe et al., 2001b). An improved level of P removal has been observed when the anaerobic pH setpoint was increased from 6.8 to 7.25 (Filipe et al., 2001b). The reason for the improved performance was hypothesized to be from a shift in the microbial competition from GAOs to PAOs. This hypothesis has been supported through assessing population changes in the microbial community. Zhang et al. (2005) found that the deterioration of P removal accompanied by a change in pH from 7.0 to 6.5, while a clear shift in the microbial community structure was simultaneously observed through examination of 16S rRNA clone libraries developed at each pH.

2.5.3 Effect of carbon availability and sources

The availability of carbon is a primary control in EBPR process. The EBPR system tends to use substantially more influent COD than conventional activated sludge, likely because EBPR organisms accumulate storage products that require carbon alone for synthesis. The form of the COD is also a crucial factor controlling EBPR. The COD must have a sufficient portion of VFAs, or readily biodegradable COD that could be fermented into VFAs in sewer lines or in fermentation units, to achieve EBPR (Randall et al., 1992). A readily biodegradable COD to P ratio of 16 in influent has been recommended to achieve low P levels in effluent (Barnard and Abraham, 2006). However, some researchers have hypothesized that high COD/P ratios may result in P-limited conditions that favor the proliferation of GAOs, thereby leading to EBPR failure. To balance these constraints, Schuler and Jenkins (2003) suggested acetate-COD to P ratios of approximately 10 to achieve high P removal, while avoiding carbon- and P-limiting conditions.

Limited P loadings were demonstrated to suppress the development of PAO leading to the establishment of GAO (Liu et al., 1994). Liu et al. (1997) proposed that the PAO could assimilate acetate anaerobically at a higher rate than the GAO. However, under limited P loading conditions, insufficient intracellular polyP was available to generate enough energy for anaerobic acetate assimilation and hence the PHA synthesis. Thus, no PHA was available to cells for subsequent aerobic growth. Alternatively, the GAO could use their stored glycogen reserves to provide energy for anaerobic acetate transport and PHA synthesis. Subsequently, PHA could be utilized aerobically for growth and glycogen synthesis. This P-limited strategy was further used to obtain stable GAO enriched cultures for research purpose (Wang et al., 2001; Kong et al., 2001; Crocetti et al., 2002).

Since acetate and propionate are the dominant carbon sources present in the influent of full-scale treatment plants, most of the research has focused on the effect of VFA on the PAO-GAO competition and the EBPR stability. Both stable and unstable EBPR processes have been reported when acetate or propionate was used as sole carbon source. However, when studying the VFA effects on the metabolisms of the EBPR microbial communities, the use of either acetate or propionate as sole carbon source does not seem to ensure the dominance of PAO. According to Oehmen et al. (2005), Accumulibacter PAOs are able to take up acetate and propionate with the same efficiency and at a similar kinetic rate.

CHAPTER 3

MATERIALS AND METHODS

3.1 Operation of Sequencing Batch Reactors (SBRs)

Two SBRs with working volume of 2.0 L were operated on six 4-hour cycles per day, with 11 minutes filling, 1 hour anaerobic and 2 hours aerobic conditions, followed by 40 minutes settling and 9 minutes decanting. The SBRs' cycle is illustrated in Figure 3.1.

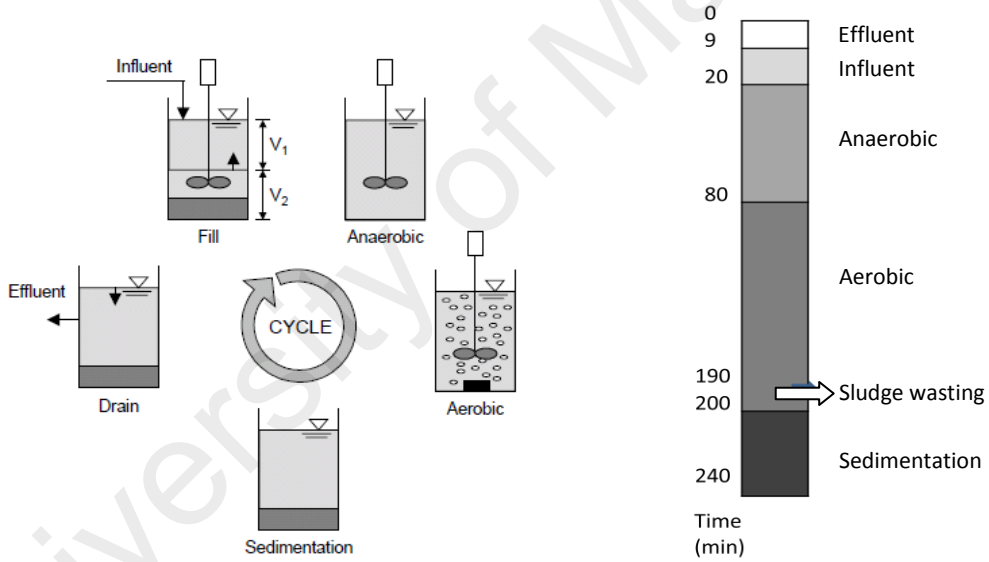


Figure 3.1: Operating cycle of SBR.

The SBRs were constantly mixed with 6-blades Rushton turbine except during settling and decanting periods. Aerobic condition was maintained by delivering air from air pump to the mixed liquor. At the end of each aerobic period and before mixing was stopped, mixed liquor was wasted from the system to keep the sludge retention time (SRT) at approximately 10 days. In the last 40-minute of the cycle, all the pumps and the mixer were turned off to allow sludge sedimentation. After the settling period, 800

ml of supernatant was discharged, resulting in a hydraulic retention time (HRT) of 10 hours and the SBR was then replaced with fresh synthetic wastewater during the filling stage. Either 0.5 M HCl or 0.5 M NaOH was added to adjust the operational pH of the process to 7.50 ± 0.10.

The SBRs were fed with synthetic wastewater which prepared from concentrated feed solutions and "P-water". The compositions of the concentrated feed solutions are listed in Table 3.1. Both SBRs were operated at 50 mg C/L with carbon-to-phosphorus (C:P) ratio at approximately 3.3 or COD:P ratio of 10:1. Concentrated feed and "P-water" was diluted according to the C:P ratio before feeding into SBRs.

Table 3.1: Composition of concentrated feed solution and concentrated "P-water"

Concentrated feed solution (g/L)		Concentrated phosphate solution (g/L)	
Sodium acetate	2.79	KH_2PO_4	0.39
Peptone	0.93	K_2HPO_4	0.34
Yeast extract	0.15		
NH_4Cl	0.32		
$MgSO_4•7H_2O$	0.68		
$MgCl_2•6H_2O$	1.21		
$CaCl_2•H_2O$	0.32		
ATU	0.02		

3.1.1 Continuous Operation of the Parent SBR (SBR-1)

SBR-1 was initially set up to provide fundamental knowledge on the establishment of EBPR process in Malaysia. Since there is no EBPR process for sewage treatment available in Malaysia, SBR-1 was seeded with sludge collected from the conventional activated sludge process. The sludge was later successfully cultivated to exhibit the

characteristics of EBPR sludge. SBR-1 was confirmed enriched with PAO and possess good EBPR capacity at 28^{o}C (Ong, 2010).

SBR-1 was then served as the parent reactor for the EBPR related study of this research. During the phase I of the study, SBR-1 was evaluated for its long-term process stability and the microbial community structure. It was then involved in temperature effect study on high-temperature EBPR in phase II and III of this study. The SBRs operating strategy is shown in Table 3.2.

3.1.2 Operation of the Parallel SBR (SBR-2)

In order to evaluate the effect of high temperatures on the EBPR performance and the dynamics of PAOs and GAOs population, a separate SBR, hereafter named SBR-2 was operated. SBR-2 was physically identical to SBR-1 and also operated under similar conditions. For the execution of high-temperature study, the temperature of SBR-2 was set according to the designed temperature, firstly at 24^{o}C and subsequently at 32^{o}C. SBR-2 was operated parallel with SBR-1 during phase II and III (Table 3.2) of this study.

Table 3.2: SBRs operating strategy

Phase	Operation duration (days)	Operating temperature (°C)	
		SBR 1*	SBR 2**
I	728	28	-
II	77	28	24
III	77	28	32

*SBR-1 was operated continuously from Phase I, followed by Phase II and then to Phase III.
**SBR-2 was operated continuously from Phase II to Phase III.

3.2 Monitoring of EBPR Performance

The SBRs were monitored weekly for their EBPR performances by measuring the dissolved organic carbon (DOC), orthophosphate (PO_4^{3-}), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), PHB and glycogen contents.

3.2.1 MLSS and MLVSS

Both MLSS and MLVSS measurement were adopted from the Standard Methods for Water and Wastewater Analysis (APHA, 1998). For MLSS measurement, 20 ml mixed liquor sample was withdrawn from the SBRs and filtered through a weighed standard glass-fiber filter. The residue remained on the filter was dried in oven for 1 hour to a constant weight at 105^{o}C. MLSS is calculated using eq. 3.1:

$$\text{MLSS, mg/L} = \frac{(\text{A-B}) \times 1000}{\text{Sample volume, mL}} = \frac{(\text{A-B}) \times 1000}{20 \text{ mL}} \quad \text{(eq. 3.1)}$$

where:

A = (weight of filter + weight of dried residue) after dried at 105^{o}C for 1 hour, mg

B = weight of filter, mg

For MLVSS measurement, dried residue from MLSS measurement was ignited in a muffle furnace (ThermConcept KL 15/11, German) at 550^{o}C for 20 minutes. It is calculated as in eq. 3.2:

$$\text{MLVSS, mg/L} = \frac{(\text{A-C}) \times 1000}{\text{Sample volume, mL}} = \frac{(\text{A-C}) \times 1000}{20 \text{ mL}} \quad \text{(eq. 3.2)}$$

Where:

A = (weight of filter + weight of dried residue) after drying at 105^{o}C for 1 hour, mg

C = (weight of filter +weight of ignited residue) after ignition at 550^{o}C, mg

3.2.2 Dissolved Organic Carbon (DOC)

Mixed liquor samples were withdrawn from SBRs. The samples were then centrifuged (Sigma 3-16 P, United Kingdom) for 5 minutes at 3500 rpm. Supernatant of the samples was immediately filtered through 0.45 µm cellulose acetate syringe filter and kept in glass vials. DOC was assayed by Total organic carbon analyzer (TOC-V CSN, Shimadzu, Japan). The method used is in accordance with Standard Methods (APHA, 1998).

3.2.3 Phosphorus (PO_4-P) and Acetate (CH_3COO^-)

PO_4^{3-} and acetate was analysed by Ion Chromatography (861 Advanced Compact IC, Metrohm, Switzerland). Column Methrom A supp 5 150/4.0 mm was used. Reagents used were ultra pure water with conductance < 0.1 µS/cm, eluent solution which consisted of 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$, and 0.01 M H_2SO_4 as regenerant solution. Concentrations of PO_4^{3-} were calculated by comparing the chromatogram areas of samples to standard solutions.

3.2.4 Polyhydroxyalkanoate (PHA)

The method used in PHA analysis was proposed by Satoh et al. (1994). PHA was analyzed by gas chromatography after methanolytic derivatization. 10 ml sludge mixed

liquor was taken and put in a 10-ml glass centrifuge tube. The sludge mixed liquor was centrifuged at 3500 rpm for 5 minutes. Once the supernatant was decanted, the sludge pellet was frozen at -20 ^{o}C and lyophilized using freeze dryer, (Eyela FDU-1100, Japan). 2 ml of chloroform and 2 ml of acidified methanol (H_2SO_4, 10 v/v %) were added to the lyophilized pellet. The tube was then closed tightly with a Teflon lined screw cap and mixed vigorously to homogenize the mixture of reagent and sample. After heated for 24 hours in oven at 99^{o}C, the mixture was cooled to room temperature, 1 ml of 14% aqueous ammonia solution was then added gently into the tube. It was again closed with the cap and mixed vigorously for about 1 minute. After centrifuged at 3500 rpm for 5 minutes, about 1.5 ml of the chloroform phase was collected in a 2-ml GC vial and 0.5 ml pure water was added into the vial. It was then mixed vigorously and centrifuged. After centrifugation, the chloroform phase was again transferred into another 2-ml GC vial before injected into a gas chromatography.

The gas chromatographic analyses were conducted using gas chromatography (Shimadzu GC-2010, Japan) (Figure 3.6(ii)), equipped with a capillary column (J&W, DB-Wax; film thickness: 1µm ; length: 30m; I.D: 0.53mm) and flame ionization detector. Helium gas was used as the carrier gas (3 ml/min) and nitrogen as the make up gas (20 ml/min). Injection mode used is split. The initial column temperature was retained at 90^{o}C for 5 minutes. Then, the column temperature was increased to 160^{o}C at 3^{o}C/min, and further incresed to 180^{o}C and retained for another 2 minutes. Injector temperature was set at 230^{o}C and the detector temperature at 250^{o}C.

3.2.5 Glycogen

Glycogen was determined following the method described by Bond et al. (1999) which was measured as total cellular carbohydrate with high performance liquid chromatography. 5-ml samples were acidified to a final concentration of 0.6 M hydrochloric acid in duplicate. The samples were digested in oven at 100^{o}C for 1 h. After cooling and centrifugation of the samples, glucose in the supernatant was quantified in high-performance liquid (Waters Corporation, USA) using Agilent Hi-Plex H column. Sulfuric acid (0.008 M) was the mobile phase, with a flow rate of 0.6 ml/min, and the volume of sample injected was 30 µl. The column temperature and the detector temperature were set at 65°C and 35°C respectively.

3.3 Screening of PAOs and GAOs Population Through FISH

Sludge samples were collected weekly during reactor operations for FISH analyses. The samples were fixed in 4% paraformaldehyde. FISH was performed according to Amann et al. (1995) to study relative abundance of PAOs and GAOs in the microbial community. FISH samples were observed using a fluorescence microscope (Model DM 2500, Leica, German) and images were captured with a cooled charged-coupled device CCD) camera (Model DFC 310 FX, Leica, German).

The oligonucleotide probes used in this study are listed in Table 3.3. The probes include 5' FITC labeled EUBmix (i.e., EUB338, EUB338-II, EUB338-III) that targets most of the bacteria (Daims et al., 1999); 5' Cy3 labeled probes for the identification of potential PAOs: PAOmix probes (i.e., PAO462, PAO651, PAO846) that targets most of the PAOs members in Accumulibacter (Crocetti et al., 2000), HGC69a probe targets on Actinobacteria – high G+C Gram positive bacteria (Roller et al., 1994) and Actino 1011

that targets *Tetrasphaera*-related Actinobacteria (Liu et al., 2001); 5' Cy3 labeled probes for the identification of potential GAOs: GB probe that targets most of the GAO members in Competibacter (Crocetti et al., 2002; Kong et al., 2002b), TFO_DF218 and TFO_DF618 that targets *Defluviicoccus*-related organisms (cluster 1) (Wong et al., 2004), DF988 and DF1020 targets *Deflluviicoccuse* related organisms (cluster 2) (Meyer et al., 2006).A minimum of 20 microscopic fields was captured randomly for each sample. FISH quantification of the PAOs and GAOs was done by image analysis software VideoTesT- Morphology 5.1. The abundance of PAOs or GAOs was determined as the mean image area with a positive signal for PAOmix or GB relative to the area with a positive signal for EUBmix.

3.4 The Microbial Community Structure of the Long-Term Operated 28^{o}C EBPR Process (SBR-1) Through Clone Library Generation

3.4.1 DNA Extraction and PCR Amplification

The sludge collected from the SBR-1 on day 700 of reactor operation was extracted for total genomic DNA using ZR Soil Microbe DNA Micro Preps (Zymo Research, USA) according to the manufacturer's instructions. The DNA template was subjected to polymerase chain reaction (PCR) by using bacterium specific 11f (5'-GTTTGATCCTGGCTCAG-3') and 1512r (5'-GGYTACCTTGTTACGACTT- 3') primers.

3.4.2 16S rRNA Gene Clone Library Construction

Cloning of the purified PCR product was conducted using pGEM-T easy vector system (Promega, USA) and JM 109 Competent Cells (Promega, USA). After blue white screening, a total of 80 colonies were retrieved and sequenced.

3.4.3 DNA Sequencing and Phylogenetic Analysis

Sequencing of the 16S rRNA genes of the clones was carried out by Protech Technology Enterprise Co., Ltd. (Taiwan) using ABI 3730DNA analyzer (USA). The 16S rRNA sequences found in the sludge were compared with sequences in GenBank database using the BLAST software (www.ncbi.nih.nlm.gov). The closest sequences were aligned with and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5 (Tamura et al. 2011).

3.5 Reveal of Accumulibacter Fine-Scale Population in High-Temperature EBPR Process

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 *ppk*1 specific Acc-ppk1-254f (5'-TCACCACCGACGGCAAGAC-3') and Acc-ppk1-1376r (5'-ACGATCATCAGCATCTTGGC-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 ultra pure water. PCR amplification was carried out in a thermocycler (MyCycler, Biorad) with an initial denaturation step at 95 ^{o}C for 10 minutes, followed by 25 cycles of denaturation at 95 ^{o}C for 45 sec, annealing at 68 ^{o}C for 1 minutes, and elongation at 72 ^{o}C for 2 minutes, followed by a final extension at 72 ^{o}C for 5 minutes (He et al., 2007). The PCR products were then purified by 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. Sequencing of the clones was carried out using ABI 3730xl DNA analyzer (Applied Biosystems). The Accumulibacter *ppk1* fragments sequences found in the sludge were compared with sequences in GenBank database using BLAST software (www.ncbi.nih.nlm.gov) (Altschul et al., 1990). The closest sequences were aligned with and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5 (Tamura et al., 2011).

The Gen Bank accession number for the nucleotide sequence determined in this study is KF985964.

3.6 Development of Accumulibacter's Clade IIF Targeted qPCR Primers

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 3.5 as well as the sequences available in GenBank. For accurate quantification using qPCR, primers were designed to avoid long amplicon length and degenerate bases. Specificity was ensured for both forward and reverse primers, to avoid amplification occurs on other clades. Qualitative PCR was performed on all samples, using the clade IIF primer sets designed in this study.

3.7 Dynamics of 16S rRNA and *ppk1* Gene Based Accumulibacter Population at Different Operational Temperatures

3.7.1 Genomic DNA Extraction

The mixed liquor from SBR-1 and SBR 2 was collected weekly for chemical profile analyses and microbial analyses. For the quantification of Accumulibacter population and total bacterial population, the total genomic DNA samples were extracted using NucleoSpin®Soil (Macherey-Nagel, Germany) according to the manufacturer's instructions. DNA concentration was determined using NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.7.2 Real-time Quantitative PCR

Real time quantitative PCR was conducted with real time thermal cycler CFX 96 Real Time PCR Detection System (BioRad, USA) using iQ™ SYBR® Green Supermix (BioRad, USA) with a total reaction volume of 20 µl. The DNA extract (5ng) from sludge samples or quantitative standard DNA was used as the template DNA. The specific primers used in this study are listed in Table 3.4. Specificity of the primers was

evaluated based on melting curve analysis of PCR products. To determine the relative abundance of Accumulibacter in the total bacterial population of EBPR sludge, the 16S rRNA gene abundance levels from total Accumulibacter, Accumulibacter *ppk1* genes and total bacteria were measured by qPCR with the specific primer sets (Table 3.4). For amplification of Accumulibacter 16S rRNA and *ppk1* genes, qPCR programmes consisted of an initial 3-min denaturation at 95^{o}C, followed by 35 cycles of denaturing at 94^{o}C for 30 sec, annealing for 45 sec and extension at 72^{o}C for 30 sec. Annealing temperature for each primer sets are listed in Table 3.4.

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; 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 Acumulibacter; 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 by tenfold serial dilution with triplicate within each assay, from $10^3 – 10^8$ target copies per reaction. Controls for *ppk1* or 16S rRNA-targetted 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.

Table 3.4: qPCR Primers information

Primer	Sequence (5' – 3')	Target	Annealing Temperature (T_a)	Reference
518f	CCAGCAGCCGCGGTAAT	Acc 16S rRNA genes	65	He et al. (2007)
PAO846 r	GTTAGCTACGGCACTAAAAGG			
341f	CCTACGGGAGGCAGCAG	Bacterial 16S rRNA genes	60	He et al. (2007)
534r	ATTACCGCGGCTGCTGG			
Acc-ppk1-763f	GACGAAGAAGCGGTCAAG	Acc-I *ppk 1*	61	He et al. (2007)
Acc-ppk1-1170r	AACGGTCATCTTGATGGC			
Acc-ppk1-893f	AGTTCAATCTCACCGAGAGC	Acc-IIA *ppk 1*	61	He et al. (2007)
Acc-ppk1-997r	GGAACTTCAGGTCGTTGC			
Acc-ppk1-870f	GATGACCCAGTTCCTGCTCG	Acc-IIB *ppk1*	61	He et al. (2007)
Acc-ppk1-1002r	CGGCACGAACTTCAGATCG			
Acc-ppk1-254f	TCACCACCGACGGCAAGAC	Acc-IIC *ppk1*	66	He et al. (2007)
Acc-ppk1-460r	CCGGCATGACTTCGCGGAAG			
Acc-ppk1-375f	GGGTATCCGTTTCCTCAAGCG	Acc-IID *ppk1*	63	He et al. (2007)
Acc-ppk1-522r	GAGGCTCTTGTTGAGTACACGC			
Acc-ppk1-355f	**CGAACTCGGCGAAAGCGAGTA**	**Acc-IIF *ppk1***	**70**	**This study**
Acc-ppk1-600r	**ATCGCCTCCGAGCAACTGTTC**			

Table 3.3: Oligonucleotide probes used in FISH experiments

Probe	Sequence 5' – 3'	Specificity	% FA Conc.	Fluorophores	Reference
EUB 338_a	GCTGCCTCCCGTAGGAGT	Eubacteria	35	FITC	Amann et al. (1995)
EUB 338- II_a	GCAGCCACCCGTAGGTGT	Eubacteria	35	FITC	Daims et al. (1999)
EUB 338- III_a	GCTGCCACCCGTAGGTGT	Eubacteria	35	FITC	Daims et al. (1999)
Probes for potential PAOs					
PAO 462_b	CCGTCATCTACWCAGGGTATTAAC	Most Accumulibacter	35	Cy3	Crocetti et al. (2000)
PAO 651_b	CCCTCTGCCAAACTCCAG	Most Accumulibacter	35	Cy3	Crocetti et al. (2000)
PAO 846_b	GTTAGCTACGGCACTAAAAGG	Most Accumulibacter	35	Cy3	Crocetti et al. (2000)
HGC69a	TATAGTTACCACCGCCGT	Actinobacteria – high G+C	25	Cy3	(Roller et al., 1994)
Actino 1011	TTGCGGGGCACCCATCTC T	Tetrasphaera-related Actinobacteria	30	Cy3	Liu et al. (2001)
Probes for potential GAOs					
GB	CGATCCTCTAGCCCACT	Competibacter (GB group)	35	Cy3	Kong et al. (2002)
TFO_DF218	GAAGCCTTTGCCCCTCAG	Defluviicoccus-related organisms	35	Cy3	Wong et al. (2004)
TFO_DF618	GCCTCACTTGTCTAACCG	Defluviicoccus-related organisms	35	Cy3	Wong et al. (2004)
DF988	GATACGACGCCCATGTCAAGGG	Defluviicoccus-related organisms	35	Cy3	Meyer et al. (2006)
DF1020	CCGGCCGAACCGACTCCC	Defluviicoccus-related organisms	35	Cy3	Meyer et al. (2006)

a EUB338, EUB338-II, & EUB338-III were labelled with FITC and prepared in an equimolar mixture, known as EUBmix.
b PAO462,PAO651, & PAO846 were labelled with Cy3 and prepared in an equimolar mixture, known as PAOmix

CHAPTER 4

PERFORMANCE OF A LONG-TERM OPERATED EBPR PROCESS AT 28°C AND ITS MICROBIAL COMMUNITY

In a previous study on the establishment of 28°C EBPR process (Ong, 2010), SBR-1 was inoculated with seed sludge collected from a conventional activated sludge (CAS) process. It was then successfully acclimatized to EBPR behavior at 28°C, a relatively high-temperature for EBPR operation. This EBPR process had its effluent P concentrations constantly maintained at around 1.0 mg/L, which is about 96% P removal efficiency. The excellence P removal achieved, has served as the motivation to prolong the reactor operation in order to further investigate its long-term process stability and insights into its microbial community in current study.

4.1 EBPR Reactor Performance

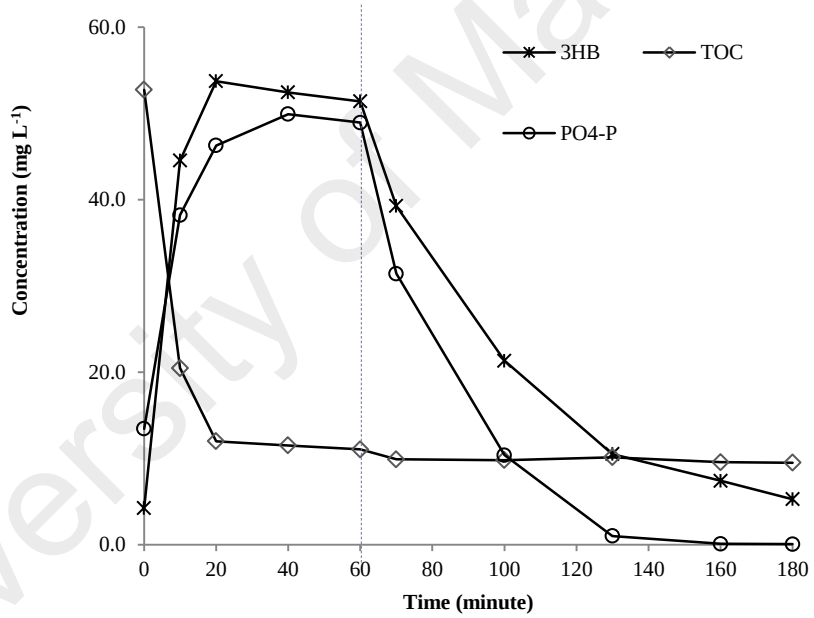
SBR-1 which set up for preliminary study (Ong, 2010) was continuously operated in order to examine the long-term stability of a high-temperature EBPR process. Figure 4.1(a) illustrates typical concentration profile of mixed liquor P and TOC and intracellular PHB of a monitored SBR cycle during Phase I of this study (or on week 72 of reactor operation). Figure 4.1(a) shows that more than 75% of carbon sources were readily sequestered during the anaerobic stage. This reduction of carbon substrate in the bulk liquid environment corresponded to the increase of PHB concentration from 3 mg C /L to 50 mg C /L in the EBPR sludge.

Since hydrolysis of intracellular polyphosphate in PAOs provides the energy for anaerobic substrate uptake (Mino et al., 1985), the increment of P concentration in line with the reduction of DOC concentration in the wastewater during anaerobic phase was thus observed. In the subsequent aeration stage of this work, phosphate decrease rapidly from 50 mg P/L to 10 mg P/L in 40 minutes, and continuously decrease to below 1.0 mg P/L in one hours. As for PHB, it decreased to 4 mg C /L at the end of aeration stage. These observations were concorded with the typical EBPR characteristics where PHA was utilized for cell growth, cell maintenance and also energy generation for the recovery of intracellular polyP by PAOs. P in the wastewater was removed and aerobic P uptake always surpassed the anaerobic P release. The continuous operation of EBPR process (Phase I) also showed stable MLVSS concentration of 4100 – 4500 mg/L. The EBPR capacity continued to increase with P concentration in effluent stably and was reliably maintained below 1.0 mg/L and resulted a P removal efficiency of 99%, or the removal rate of 0.02 g-P/ g-MLVSS•day as shown in Figure 4.1(b).

The stable and good EBPR activity showed during Phase I of this study was different from the poor EBPR performance that always reported by researchers who studied the effect of higher temperature (> 20 ^{o}C) on EBPR process (Panswad et al., 2003; Erdal et al., 2003; Whang and Park, 2006; Lopez-Vazquez et al., 2008; Cao, 2011). The deterioration of EBPR process during summer seasons (> 25 ^{o}C) was also observed (Gu et al., 2005). In Cao's (2011) study, a tropical based EBPR process operated at 30 ^{o}C , the effluent PO_4^{3-}-P concentration of the process was between 3.0 – 6.5 mg/L. Furthermore, Cao (2011) claimed that EBPR alone is not sufficient for P removal operated in tropical climate, but together with chemical precipitation, following the operation of EBPR process at 30 ^{o}C which showed effluent P concentration at between 3.0 – 6.5 mg/L.

The positive results shown in this work, encourage the retrofitting of existing activated sludge sewage treatment plants in Malaysia into EBPR process in order to comply with the revised regulations on nutrient removal. This study also provides the first report on a successful long term operation of EBPR process at a relatively high temperature. It certainly contribute useful information for the operation of EBPR in the tropics which is scarce till date. Research on the microbiology of this high temperature EBPR process will help in elucidating the microbial community involved.

(a)



(b)

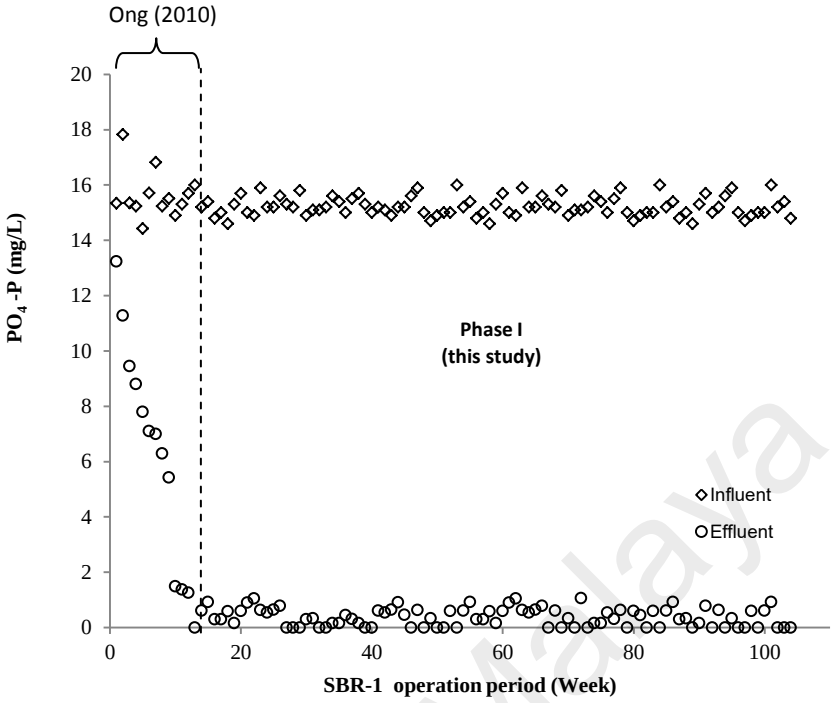


Figure 4.1: (a) Typical concentration profiles of TOC, PO_4-P and 3HB (mg C/L) in one SBR cycle in week 72 of SBR-1 operation; (b) Concentration profiles of PO_4-P in the monitored cycles along a period of two years operation of SBR-1.

4.2 PAO and GAO Candidates in the EBPR Process

Apart from chemical analysis, microbial study is encouraged following the good EBPR showed in 28^{o}C, the high-temperature EBPR process. Since PAOs and GAOs are the two main groups of bacteria that widely recognized in determining the EBPR performance, the potential PAO and GAO populations were first screened via FISH. The Accumulibacter-PAOs and Competibacter-GAOs appeared to be dominated the PAOs and GAOs population. The samples were also screened for high G + C Actinobacteria and Tetrasphaera-related Actinobacteria PAOs as well as alphaproteobacterial GAOs. It was showed that their abundances were statistically insignificant. Based on the FISH results, during Phase I of SBR-1 operation, the number

of Accumulibacter-PAOs contributed to 32 ± 4% of the total bacterial population while Competibacter-GAOs contributed 7 ± 5%, as shown in Fig. 4.2(a) and (b). Throughout the two-year reactor operation, the ratio of Accumulibacter-PAOs population to Competibacter-GAOs population was relatively constant at around five (5).

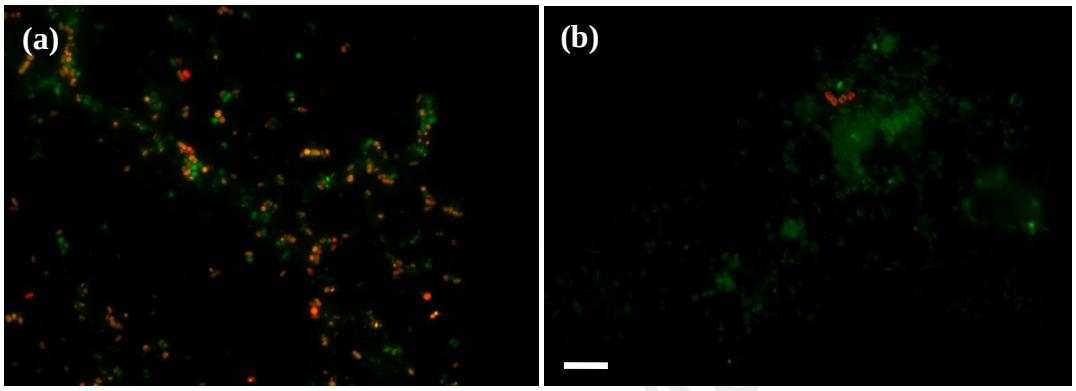


Figure 4.2: Overlay FISH images of sludge sample from day 700 of reactor operation showing (a) the PAOs (orange cell clusters) hybridized with both FITC-labelled EUBmix probe (green) and Cy3-labelled PAOmix probe (red); (b) the GAOs (orange cell clusters) hybridized with both FITC-labelled EUBmix probe (green) and Cy3-labelled GB probe (red) (Scale bar = 10μm).

The results of the FISH technique were cross-checked by noting that a main metabolic difference between PAOs and GAOs is in their primary energy source for anaerobic carbon uptake. While PAOs obtain energy from the hydrolysis of polyphosphate, GAOs use glycogen as their sole energy source. Thus, in a GAOs-enriched system, larger amount of glycogen is consumed anaerobically. By calculating the ratio of glycogen degradation to VFA uptake during the anaerobic phase (hereafter abbreviated as Gly/VFA), the relative activity of PAOs and GAOs can be estimated. In this study, the Gly/VFA ratio was 0.69, close to the Gly/VFA ratio reported in a few efficient EBPR systems (Table 4.1). This Gly/VFA ratio was also much lower than 1.12 reported in a GAOs-enriched system (Zeng et al., 2003). Together with the higher abundance of

Accumulibacter-PAOs shown by FISH, the low Gly/VFA ratio further supports that the EBPR reactor of this study is a PAOs enriched system.

Table 4.1: The ratio of anaerobic glycogen degradation to VFA (Gly/VFA) uptake of a few EBPR systems

Studies	Carbon source	Gly/VFA	Temperature
This study	Acetate (80% of the total carbon source)	0.69	28^{o}C
Smolders et al. (1994b)	Acetate model	0.50	20^{o}C
Hesselmann et al. (2000)	Acetate	0.60	20^{o}C
Filipe et al. (2001c)	Acetate	0.53	22^{o}C
Lu et al. (2006)	Acetate	0.46	20 -24^{o}C

*Gly/VFA expressed in C-mol/ C-mol.

The success of maintaining a stable population of Accumulibacter-PAOs in a relatively high-temperature EBPR process for a long-term indicates the feasibility of the biological P removal technology in warm temperature regions. Freitas et al. (2009) and Winkler et al. (2011) also shed some light on high temperature EBPR. Freitas et al. (2009) found that through a relatively short 36-minute SBR cycle, with 20 minutes of anaerobic phase and 10 minutes of aerobic phase, the sludge became more robust and ready to adjust to typical disturbances, such as shock load, and PAOs-GAOs competition. As for Winkler et al. (2011), selective removal of GAOs dominated at the top of the sludge bed had proven to be influential to the PAOs-GAOs competition in forming a desired microbial population for EBPR. Their approaches offer possibility to engineer the competition between PAOs and GAOs. The encouraging EBPR performances obtained by Freitas et al. (2009), Winkler et al. (2011) and in this study,

strongly suggest that the enrichment of PAOs at high temperature is possible with certain operating strategy and conditions.

On the other hand, a few other studies reported that EBPR performance deteriorates at temperatures above 20^{o}C, hypothesized to result from a shift in the community from PAOs to GAOs (Erdal et al., 2003; Panswad et al., 2003; Whang and Park, 2006). The reasons for the different EBPR performance in the past studies are not clear yet. The high efficiency of EBPR observed in this work could be attributed to the variant of metabolism behavior exhibited by PAOs in this high temperature EBPR sludge rather than population shift. Due to the information available for the factors controlling the structure and function of the related microbial communities is still rudimentary; hence microbial study is needed to address this inadequacy.

4.3 The EBPR Microbial Population from 16S rRNA Gene Retrieval and Phylogenetic Analysis

To acquire more detailed taxonomic information on the microbial community involved in this stable high-temperature EBPR process, a clone library of the EBPR sludge was constructed. It was earlier proposed that a community rich in diversity contributed to a stable ecosystem, particularly in activated sludge systems (Siripong and Rittman, 2007). A total of 80 clones were selected. The partial 16S rRNA sequence of 600-800 bps was identified for each clone. These partial 16S rRNA sequences were grouped into 26 OTUs (operational taxonomy units). The phylogenetic analysis categorized the sequenced clones into 7 groups as shown in Figure 4.3.

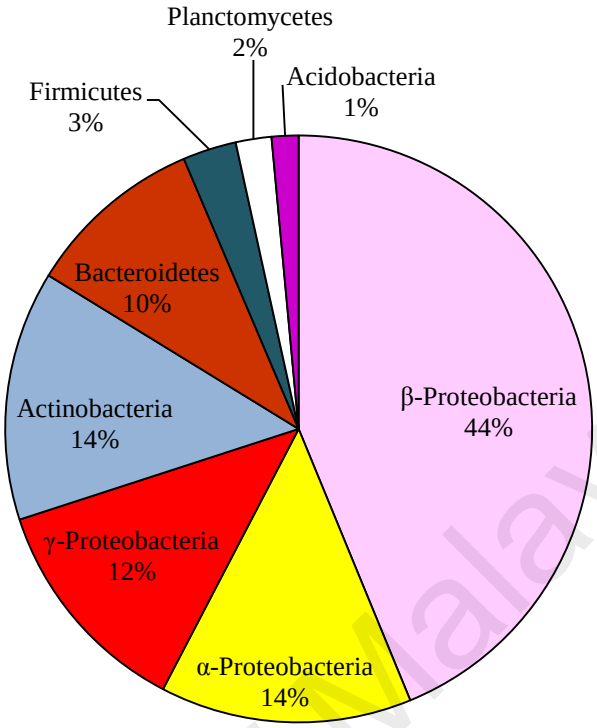


Figure 4.3: Composition of microbial community through clone library generation.

The numerically largest bacterial group is the Proteobacteria which accounting for 69.6%, including *α-proteobacteria* (13.8%), *β-proteobacteria* (43.6%), *γ-proteobacteria* (12.3%), followed by *Actinobacteria* (13.7%), *Bacteroidetes* (9.8%), *Firmicutes* (2.94%), *Planctomycetes* (1.96%), *Acidobacteria* (1.47%). At the level of class, *β-proteobacteria* was the dominant group in the EBPR sludge, which was in accordance with previous studies (Rani et al. 2008). Table 4.2 summarizes the clone number of each OTU, the closest sequence found in the NCBI database, and their similarity. The phylogenetic affiliation of the sequences was also analyzed using the neighbor-joining method. Fig. 4.4 illustrates the phylogenetic tree of the 26 OTUs.

Table 4.2: Affiliations of OTUs in the EBPR sludge

Affiliation	Closest Sequences in GenBank	Similarity (%)	OTU	No. of Clones
α-Proteobacteria	*Rhodobacter* sp.	98	SBR1-2_9	3
	Methylocystis sp.	100	SBR1-2_40	2
	Brevundimonas diminuta	97	SBR1-2_86	4
	Mesorhizobium plurifarium	98	SBR1-2_74	2
β-Proteobacteria	*Thauera* sp.	99	SBR1-2_13	2
	Nitrosomonas sp.	98	SBR1-2_34	4
	Comamonadaceae sp.	98	SBR1-2_47	3
	Uncultured *Canditatus* Accumulibater phosphatis SBRA220	99	SBR1-2_27	8
	Uncultured Accumulibacter clone LPU28	98	SBR1-2_110	7
	Uncultured bacterium clone LBP60	95	SBR1-2_1	2
	Uncultured bacterium PHOS-HE 23	94	SBR1-2_91	3
	Uncultured bacterium MO 111_27	99	SBR1-2_22	5
γ-Proteobacteria	Uncultured gammaproteobacterium AY172151	97	SBR1-2_11	2
	Uncultured gammaproteobacterium AY172170	99	SBR1-2_76	2
	Uncultured bacterium PHOS-HE54	99	SBR1-2_61	2
	Uncultured bacterium clone A_SBR_64	94	SBR1-2_6	3
Actinobacteria	Uncultured Actinobacteria bacterium	99	SBR1-2_15	5
	Uncultured *Candidatus* Microthrix calida strain TNO2-4	94	SBR1-2_105	6
Bacteroidetes	*Runellazeae sp.*	99	SBR1-2_10	3
	Uncultured Flavobacterium clone HP1A39	95	SBR1-2_25	1
	Uncultured Flexibacteriaceae bacterium	98	SBR1-2_71	2
	Uncultured Bacteroidaceae bacterium	97	SBR1-2_83	2
	Uncultured Sphingobacteriaceae bacteriu,	96	SBR1-2_53	2
Firmicutes	*Bacillus* sp.	98	SBR1-2_5	1
Planctomycetes	*Planctomyces* sp. Schlesner 664	99	SBR1-2_52	1
Acidobacteria	Uncultured Acidobacteria bacterium	96	SBR1-2_90	1

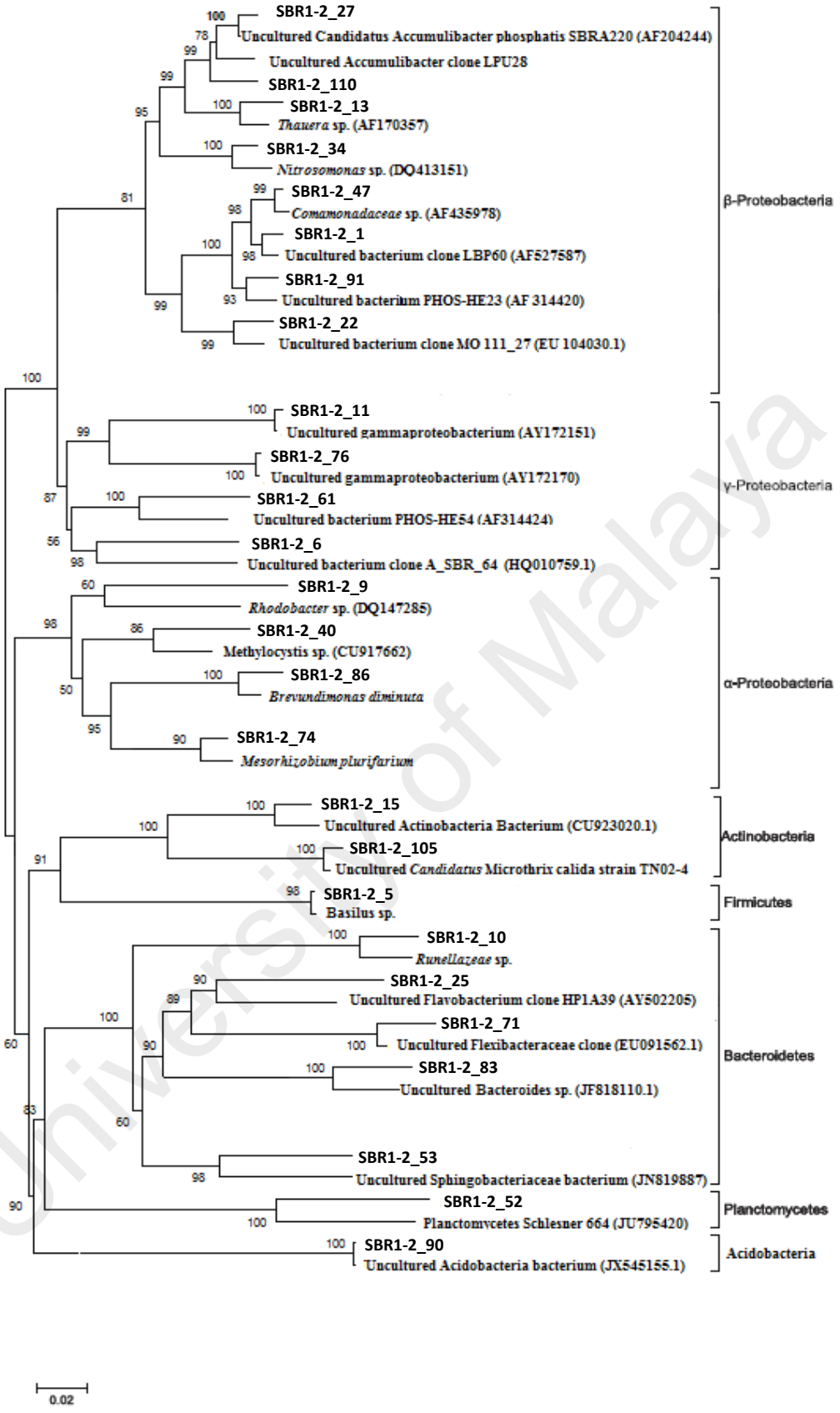


Figure 4.4: Neighbor- Joining trees deduced from partial sequences of 16S rRNA genes of clones from EBPR sludge sample. Bootstrap confidence values obtained with 1000 replicates are given at the branch plant.

The phylum *Proteobacteria* encompassed 56 clones forming 18 OTUs. Among the 8 OTUs in the *β-proteobacteria*, clones SBR1-2_13 and SBR1-2_34 had cultured species *Thauera* sp. and *Nitrosomonas* sp., respectively. Clones SBR1-2_47 and SBR1-2_94 had *Comamonadaceae* sp. as the closest relatives, which is strictly aerobic, non fermentative, capable of accumulating PHB (Holt et al., 1994) and commonly found in activated sludge and biofilm reactors. The remaining *β-proteobacteria* affiliated clones were related to sequences of uncultured bacteria represented by SBR1-2_27 and SBR1-2_110, showing greater than 97.0% similarity with Accumulibacter. A lower abundance of Accumulibacter of 18.7% was obserbed by 16S rRNA analysis compared with the 32% determined by FISH. This observation reflects the known quantitative bias of clone library as reported by other studies (Liu et al., 2001; Crocetti et al., 2000).

Four OTUs were found in *Gammaproteobacteria*. Two of them, *viz.* SBR1-2_11 and SBR1-2_76, were related to Competibacter-GAOs from the GB lineage. The presence of Competibacter-GAOs in the reactor was also detected through FISH analaysis. This correlates well with the earlier claim that the existence of Competibacter is common in lab-scale EBPR process fed with acetate (Crocetti et al., 2002; Zeng et al., 2003; Oehmen et al., 2004). Meanwhile, the SBR1-2_61 is closely affiliated with an uncultured bacterium PHOS-HE54 from an aerobic phosphate removal ecosystem (Dabert et al., 2001) and with several uncultured species in the EBPR systems (Nielsen et al., 1999; Crocetti et al., 2002). Among the *Alphaproteobacteria*, four OTUs were identified. Two had *Rhodobacter* sp. and *Methylocystis* sp. (86.4% and 94.5% similarity, respectively) as their closest relatives. As for the remaining two *α-proteobacteria* affiliated clones, SBR1-2_86 is closely related to *Brevundimonas diminuta* of little know characteristics, whereas, SBR1-2_74 is closely related to

Mesorhizobium plurifarium, a common soil bacterium capable of forming nodules at the root and stem of plants.

Several reports claiming that members of *Actinobacteria* accounted for a large proportion of the microbial population in the EBPR process. In this study, although clones of the *Actinobacteria* were detected, they made up only about 14% of the total clones. Both the OTUs identified do not belong to the putative actinobacterial PAO commonly reported (Kong et al., 2005). In the phylum *Bacteroidetes*, one of the OTUs has the cultured bacterium *Runellazeae* as its closest relative with 91.6% similarity; others were related to uncultured environmental clones. Other OTUs clustered to sequences in the families *Flavobacteriaceae, Flexibacteriaceae*, *Bacteroidaceae* and *Sphingobacteriaceae.* The three phyla *Firmicutes*, *Planctomycetes*, and Acidobacteria were represented respectively by only one clone namely SBR1-2_5, SBR1-2_52, and SBR1-2_90.

From the clone library, it appears that the only recognized and relatively abundant PAOs existed in the 28^{o}C EBPR process is Accumulibacter-related. Accumulibacter-PAOs also observed contributing about 36% of total bacteria population according to FISH. Thus, the presence and contribution of Accumulibacter towards the P removal in the reactor is undeniable. Since genotypic differences within the Accumulibacter lineage are always reported in studies using the gene encoding polyphosphate formation, *ppk1* (McMahon et al., 2007; He et al., 2007; Kim et al., 2010), it is of great interest to further investigate the subpopulation (or clade) of Accumulibacter which contributed to this high-temperature EBPR. Also, it is also necessary to monitor Accumulibacter clade dynamics over time in order to infer and elucidate their effects on the EBPR performance.

4.4 Conclusions

An EBPR process operated at a relatively high temperature, 28^{o}C, was stably removing 85% carbon and 99% phosphorus from wastewater over a period of two years. Through FISH, considerably more Accumulibacter-PAOs than Competibacter-GAOs were detected in the reactor, at 36% and 7% of total bacterial population respectively. A low ratio of Gly/VFA of 0.69 further indicated the dominance of PAOs in the reactor. From th clone library generated, 26 OTUs were retrieved from the the sludge and a diverse population was shown, comprising *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Planctomycetes*, and *Acidobacteria*. Accumulibacter are the only recognized PAOs revealed by the clone library. Both the clone library and FISH results strongly suggest that Accumulibacter are the major PAOs responsible for the P removal in this long-term 28^{o}C EBPR.

CHAPTER 5

PERFORMANCE OF EBPR PROCESSES AT 24 - 32^{o}C AND THE PROFILES OF ACCUMULIBACTER POPULATION DYNAMICS

Following the stable and efficient P removal shown by the 28^{o}C EBPR process in SBR-1 (detailed in chapter 4), SBR-2 was set-up and operated at 24^{o}C and 32^{o}C for the execution of high-temperature study. As Accumulibacter was the most relevant PAOs identified in the sludge of SBR-1, and as it had been used to seed the SBR-2, the goal of the subsequent study was to investigate the dynamic profiles of Accumulibacter-PAOs population at different temperatures.

5.1 EBPR Performance at 24^{o}C, 28^{o}C and 32^{o}C

Throughout the reactor operation, SBR-1 and SBR-2 were monitored weekly for their EBPR performances. Figure 5.1 shows the PO_4-P concentrations at the beginning of the anaerobic phase, as well as the beginning and the end of the aerobic phase in each monitored cycle during Phase II and Phase III of this study.

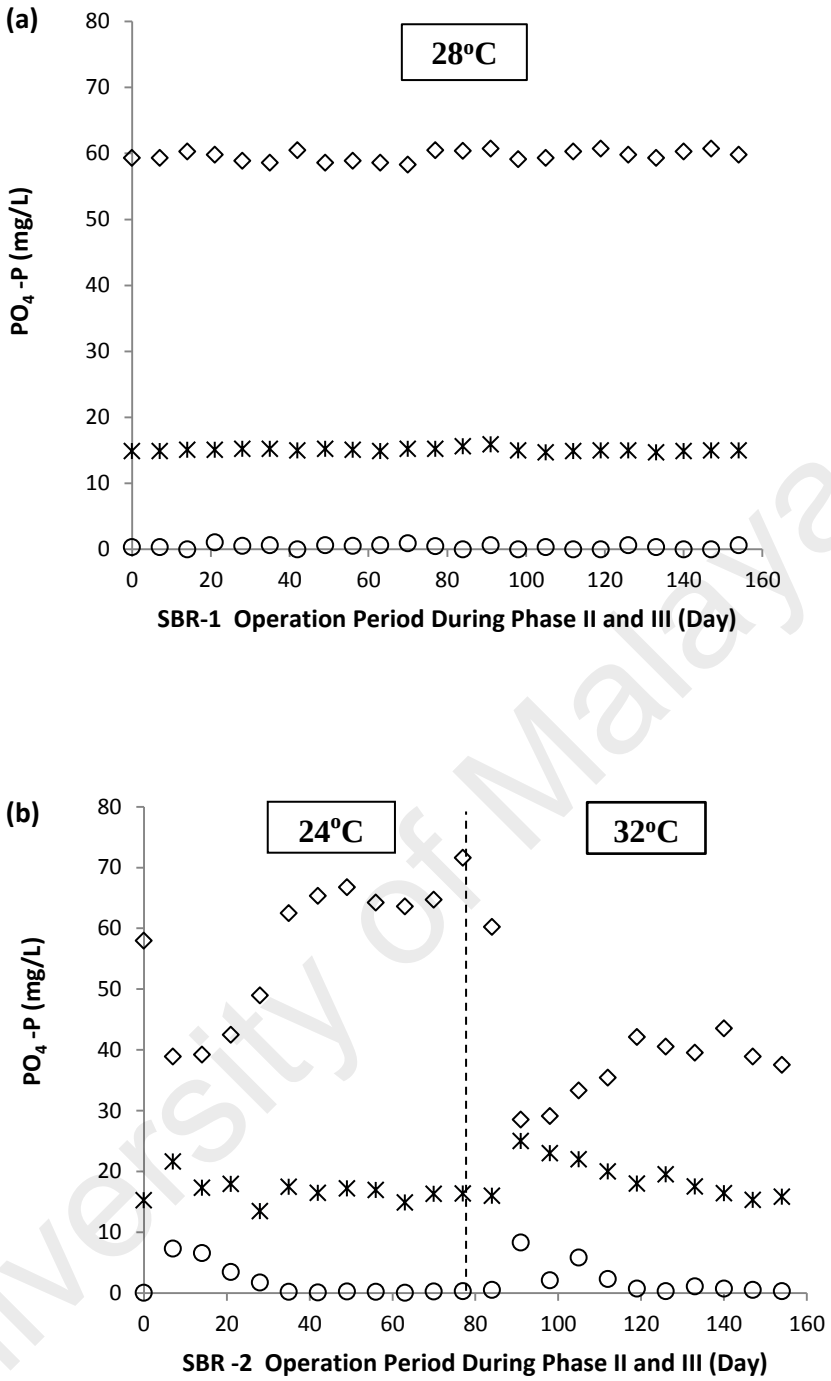


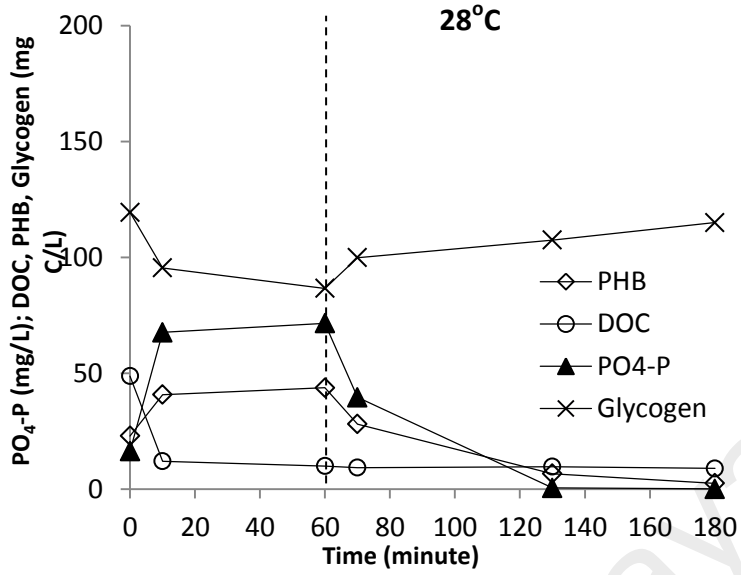
Figure 5.1: Concentration profiles of PO_4-P in the monitored cycles throughout the 154-day operation of **(a)** SBR-1 at 28°C and **(b)** SBR-2 at 24°C and 32°C; (✳) Beginning of the anaerobic phase; (◇) Beginning of the aerobic phase; (○) End of the aerobic phase. The vertical dashed line denotes the changing of temperature from 24°C (Phase II) to 32°C (Phase III) in SBR-2.

Similar to the EBPR performance shown during Phase I, SBR-1 continued to exhibit a good phosphorus removal at 28°C throughout the 154-day (Phase II and Phase III) reactor operation, as shown in Figure 5.1(a). 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.

The EBPR activity in SBR-2 was disturbed immediately after reactor start-up due to the adaptation of seed sludge (from SBR-1) to temperature switching 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. Nevertheless, the EBPR activity gradually recovered after day 14. 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 till the end of Phase II.

On day 77 (Phase III), 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 (Figure 5.1(b)). A substantial decrease in the anaerobic phosphorus release and aerobic phosphorus uptake continued until day 112. Thereafter, the EBPR activity showed sign of recovery and reached the steady state on 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 - 99 % with phosphorus removal rates of 1.9 - 2.5 mgP $gVSS^{-1}$ h^{-1} during steady state.

(a)



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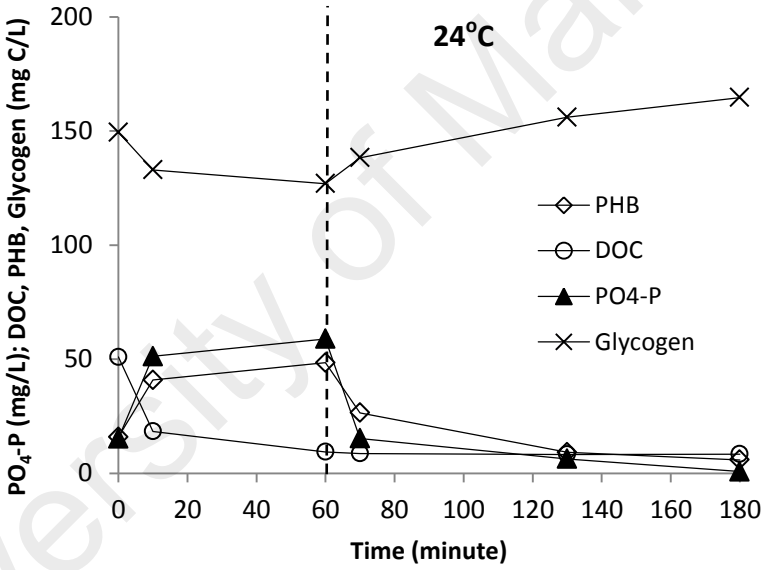
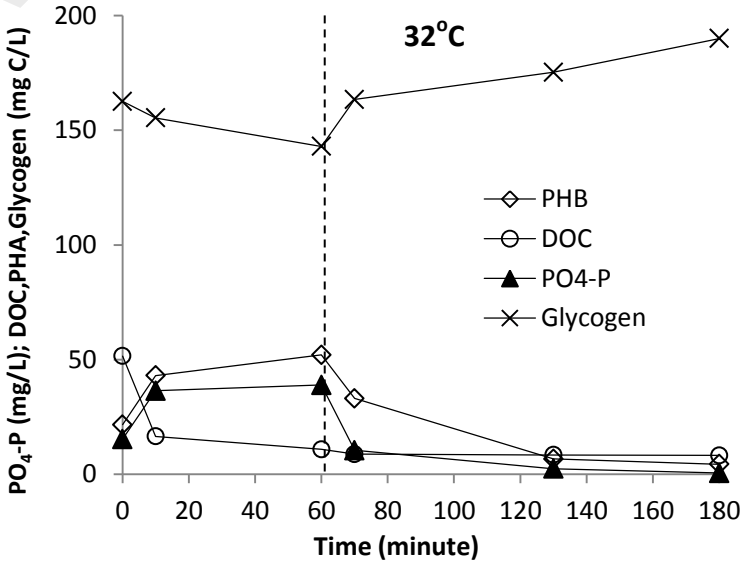


(c)



Reactor cycle studies demonstrated that the EBPR processes operated at all 24°C, 28°C, and 32°C exhibited metabolic transformations typical of EBPR (Figure 5.2). Since acetate was used as the main carbon source, PHB was the main type of PHA produced. Though PHV also detected, the concentration is rather low compared to PHB and statistically insignificant. During the steady state operation of SBRs, little difference in the MLVSS was observed for 24°C and 28°C, at 4.3 and 4.6 g/L respectively, as shown in Figure 5.3. 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 decrease with the temperature higher than 28^{o}C which could be due to the bacteria having fewer endogenous needs at lower temperatures (24^{o}C and 28^{o}C) and allowing more substrate available for cell production.

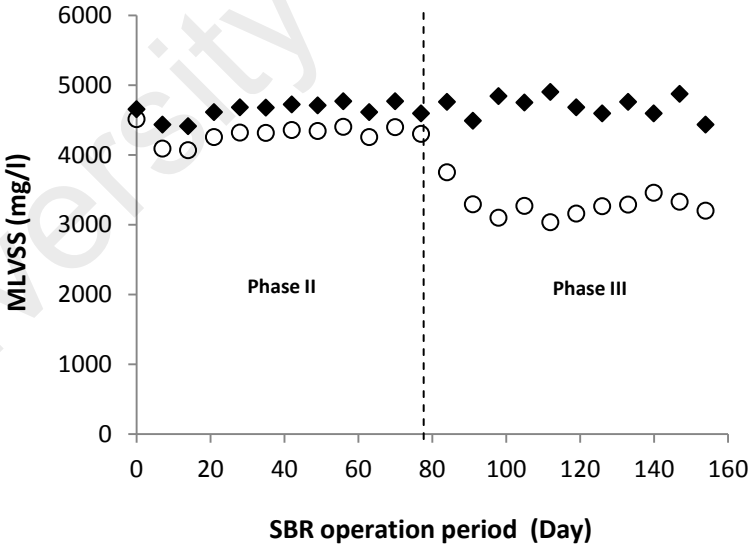


Figure 5.3: MLVSS of EBPR processes operated in SBR 1 and SBR 2 throughout Phase II and Phase III; (◆) SBR 1; (○) SBR 2.

Based on the cyclic profiles throughout the reactors operation, 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 summarised in Table 5.1.

Table 5.1: Summary of the anaerobic P release rate, aerobic P uptake rate, P release/acetate uptake ratio, biomass P and glycogen content in the EBPR processes operated at 24°C, 28°C and 32°C

T (^{o}C)	Anaerobic P release rate (mgP gVSS^{-1} h^{-1})	Aerobic P uptake rate (mgP gVSS^{-1} h^{-1})	P release/ Acetate uptake (P_{rel}/HAc_{up}) ratio (P-mole /C-mole)	Biomass P content (% w/w)	Biomass glycogen content (% w/w)
24	7.6	1.9	0.60	7.6	5.4
28	6.2	1.5	0.57	7.4	7.5
32	6.1	2.5	0.38	7.7	13.8

Table 5.1 shows that the biomass glycogen content was notably higher at 32°C, i.e., 13 % w/w, than those at 24°C and 28°C, indicating there is higher abundance of GAOs at 32°C. Based on our current understanding of the biochemical pathways of the EBPR process, the theoretical P_{rel}/HAc_{up} ratio was proposed to be 0.5-0.7 P-mole/ C-mole for PAOs (Schuler and Jenkins, 2003). The P_{rel}/HAc_{up} ratio obtained in this study ranged from 0.4 to 0.6 P-mole/ C-mole. A P_{rel}/HAc_{up} ratio of 0.4, which is lower than the range of the theoretical value, signifies 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 at the temperature also implied 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 hydrolyzed to generate energy for carbon uptake. As the GAOs population increase, there is less

carbon source available for PAOs and thus, less polyphosphate is hydrolyzed 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 possessed similar phosphorus content, and the aerobic phosphorus uptake rate was higher at 32 °C. The anaerobic phosphorus release rate was slightly higher at 24°C than the other two temperatures. These findings contradicted with those reported by Panswad et al. (2003), which suggesting that the phosphorus release rate increased with temperature from 20°C to 35°C, while the phosphorus uptake rate decreased. Based on the discrepancy between the two studies, it suggests that the P metabolism in PAO might be an inducible trait in which the environmental factors control the induction is yet to be identified. To delve further, the microbiology of these high-temperature EBPR processes should be elucidated to and better linked with the process performance.

5.2 Population Dynamics and Abundance of PAOs and GAOs

Similar to the observation made on SBR-1 during Phase I, the screening of potential PAOs and GAOs via FISH revealed that the PAO communities at all three temperatures during Phase II and Phase III 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 members of the genus Defluviicoccus were absent or present in low and statistically insignificant abundance.

Accumulibacter was identified as the major PAO at all three temperatures and thus, the qPCR method which has been reported as a rapid and accurate approach for routine monitoring throughout the operation of the process was employed to profile the dynamics and abundance of Accumulibacter (Fukushima et al., 2007; He et al., 2007). Figure 5.4 shows the Accumulibacter percentage estimated according to the Accumulibacter 16S rRNA gene and bacterial 16S rRNA genes via qPCR analyses. Due to SBR-1 had been operating at 28°C for a long term during Phase I, a relatively stable Accumulibacter population was observed during Phase II and Phase III, which only fluctuated within 40 - 46 % of total bacterial population. This finding agreed with the stable EBPR activities observed in SBR-1 (Figure 5.1).

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 to 65 % on day 42 and beyond. When the temperature was changed 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.

Through FISH, the abundances of Accumulibacter revealed during the steady state operation of reactors operation were 51 % at 24°C, 33 % at 28°C and 12 % at 32°C (Table 5.2). It was found that the % of Accumulibacter quantified through qPCR was slightly higher than through FISH. The reason is, only active cells are quantified by FISH, while both active and dead cells are positively quantified by the quantitative PCR method (Fukushima et al., 2007). However, both qPCR and FISH quantification data shows a similar trend: the increase of temperature inversely proportional to the

proliferation of PAOs. Lopez-Vazquez et al. (2008) observed a similar phenomenon too, and they claimed that Accumulibacter possess a clear advantage in anaerobic carbon uptake and lower required cell maintenance at lower temperatures, which encourages the cell growth of Accumulibacter.

Reduction in the Accumulibacter-PAOs population without sacrificing the P removal performance is an interesting finding of this study. Many studies have reported involvement of the Accumulibacter-PAOs population for EBPR, but most studies were based on a handful of samples collected at single time and rarely consider the dynamics over a period of time. In those EBPR-related studies which reported low effluent P concentration, the percentage of Accumulibacter-PAOs population can vary from as low as 4% to 81% (Oehmen et al., 2006; Hesselmann et al, 1999). The average Accumulibacter-PAOs population in the bacterial community of EBPR sludge for a success EBPR operation is hardly concluded. The size of Accumulibacter-PAO may not be the critical factor in determining good P removal but probably the induction of P metabolism.

Table 5.2: Abundance of Accumulibacter and Competibacter over the steady state as enumerated by FISH

Temperature (^{o}C)	**Accumulibacter (%)**	**Competibacter (%)**
24	51 ± 3	4 ± 1
28	33 ± 5	7 ± 2
32	12 ± 1	40 ± 4

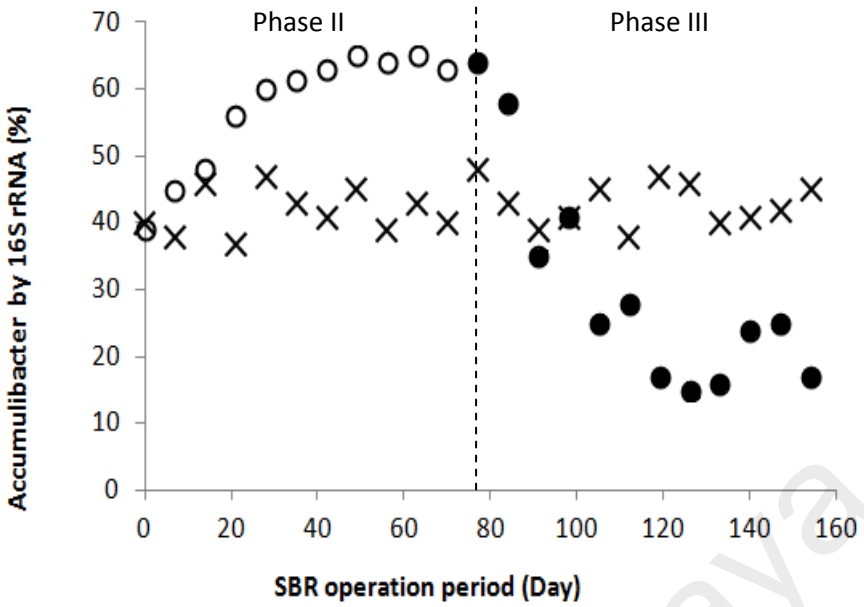


Figure 5.4: Percentage of Accumulibacter according to 16S rRNA in the total bacterial population of EBPR processes operated in SBR-1 and SBR-2 during Phase II and Phase III; (x) 28°C-SBR 1; (○) 24°C-SBR 2; (●) 32°C-SBR2.

Based on the biomass glycogen content presented earlier in Table 5.1, 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 5.2. 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 Accumulibcater and also did not deteriorate the EBPR activity. Figure 5.5 shows the FISH images of sludge samples taken from the EBPR processes operated at 24°C and 32°C.

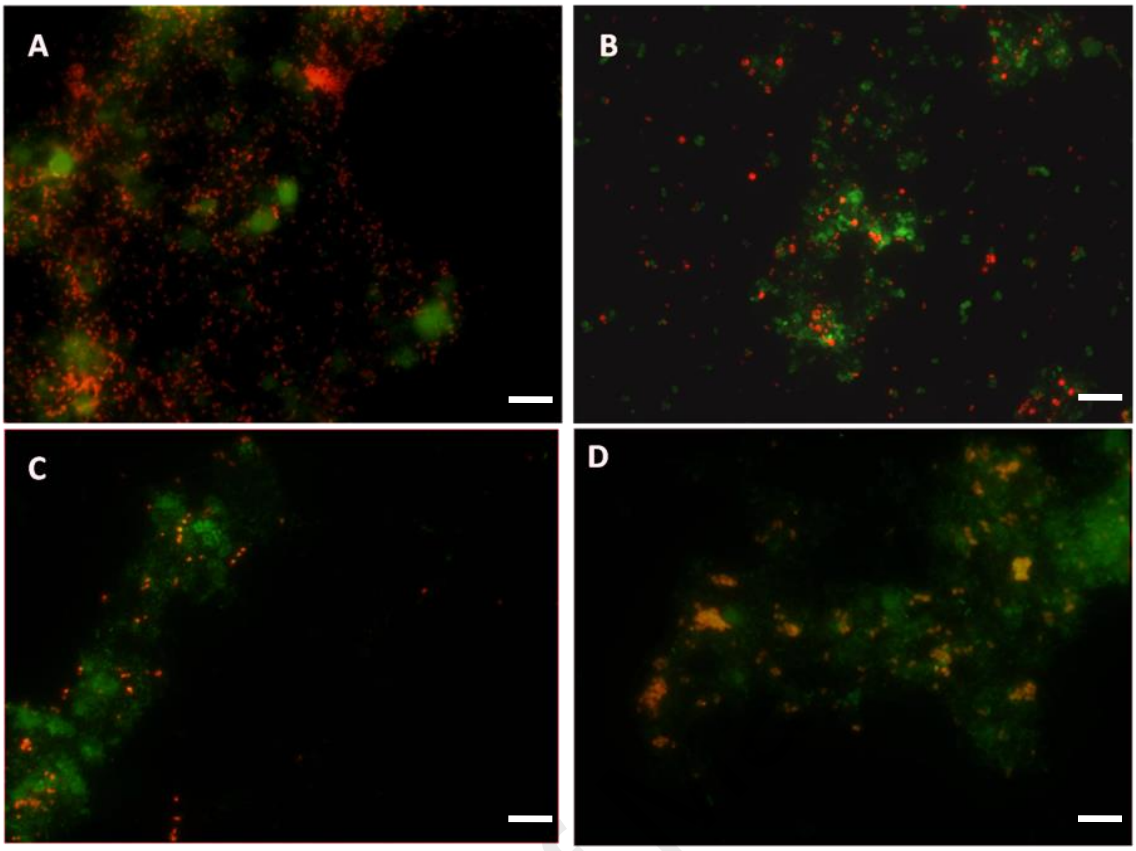


Figure 5.5: Overlay FISH images of EBPR sludge samples collected at 24°C and 32°C on day 77 and day 154, respectively, of SBR-2 operation. Panel A and Panel B show Accumulibacter (orange cell clusters) hybridised with both FITC-labelled EUBmix probe (green) and Cy3-labelled PAOmix probe (red), from EBPR 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 = 10μm).

In agreement with previous studies (Whang and Park, 2006; Erdal et al., 2003; Panswad et al., 2003), an increase in the GAO population as the temperature increased was observed in this study. 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 most interesting. Though the reason for this rare observation is yet to be confirmed. The Accumulibater-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). *ppk1* is probably best to explain the current observation, as it could provide greater phylogenetic resolution in revealing finer scale differences within Accumulibacter.

5.3 Conclusions

This study demonstrated that EBPR can be achieved at 24^{o}C, 28^{o}C and 32^{o}C, typical range of sewage's temperature in warm climate. High phosphorus removal efficiencies, ranging from 96% to 99% achieved at these temperatures. Relatively higher Accumulibacter-PAOs abundance observed at 24^{o}C and 28^{o}C compared to 32^{o}C. The observation of good phosphorus removal in 32^{o}C system with predominance of Compectibacter also challenges the traditional concept in maximizing PAOs and minimizing GAOs to enhance the EBPR activity.

CHAPTER 6

FINE-SCALE POPULATION STUDY OF ACCUMULIBACTER IN HIGH-TEMPERATURE EBPR PROCESSES

ppk1 appears to be evolving faster than 16S rRNA genes in Accumulibacter (Kunin et al., 2008) and it may provide enough resolution to observe fine-scale differences within Accumulibacter's lineage. Thus, one of the objectives of this study was to demonstrate the use of *ppk1* as a genetic marker to study the more resolved population structure of Accmulibacter-PAOs in the high-temperature EBPR processes. In addition, this provide insightful information for EBPR operation at high temperatures. To facilitate this purpose, Accumulibacter *ppk1* gene fragments were retrieved from EBPR sludge of SBR-1, which also served as seed sludge, to construct Accumulibacter phylogenies. qPCR methods were then employed to measure the abundance and relative distribution of Accumulibacter clades of both SBR-1 and SBR-2.

6.1 *ppk1*-Based Phylogeny of High-Temperature EBPR Process

To empirically examine the Accumulibacter clades present in the high-temperature EBPR processes, a *ppk1* gene clone library was constructed. The phylogram indicating the inferred relationships between *ppk1* gene homologs from the cloned Accumulibacter clade and relevant sequences from other EBPR studies are shown in Figure 6.1. All the 135 sequences retrieved were identical and thus only one representative sequence being deposited into the GenBank database and the *ppk1* genes were classified based on the clade names assigned previously in He et al. (2007). In order to show these retrieved sequenced were identical, a closeup phylogenetic tree exclusively for clade IIF (all the

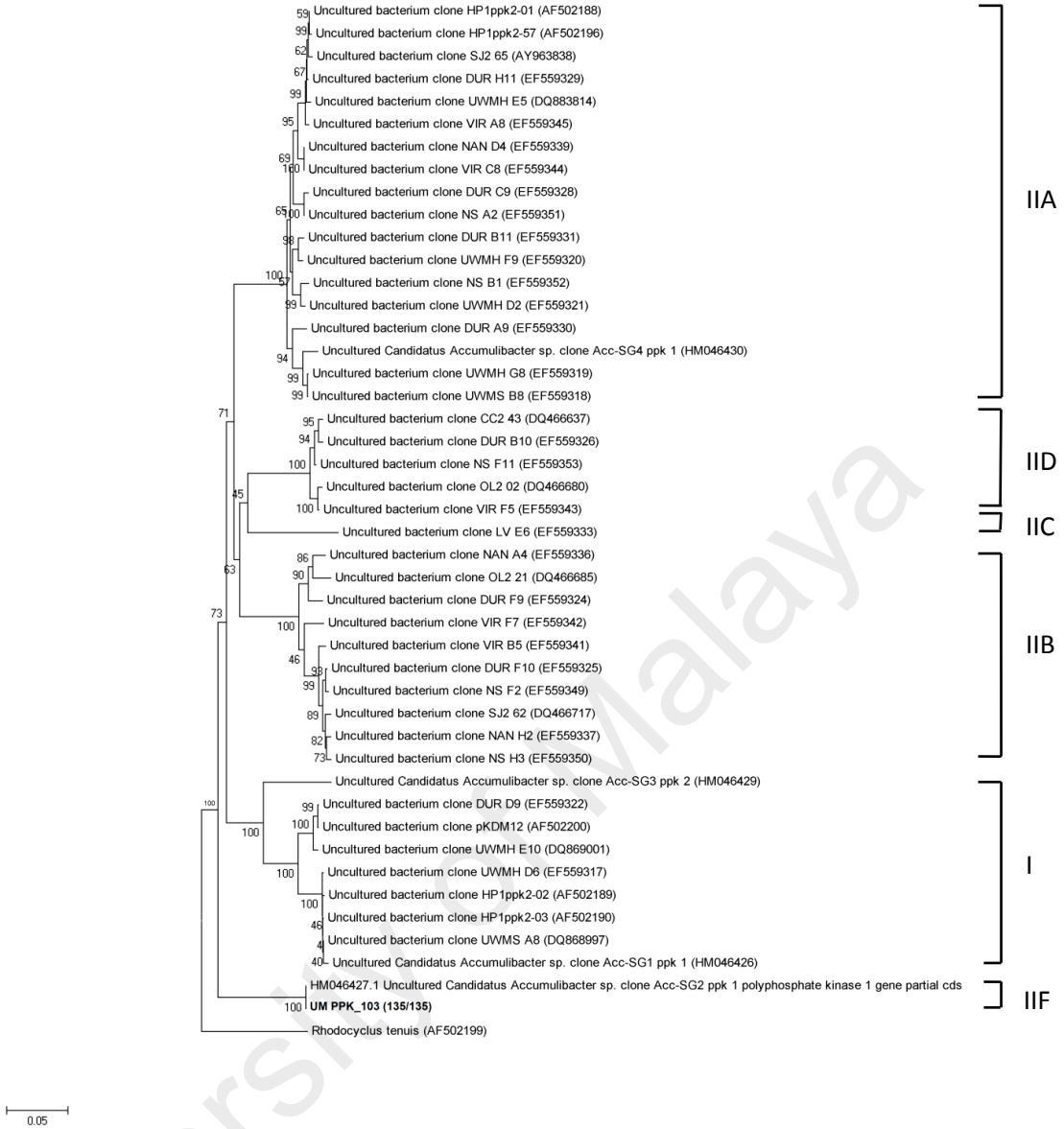


Figure 6.1: Phylogram indicating inferred relatedness of *ppk1* gene homologs from the cloned Accumulibacter clades. Sequences found in this study were contrasted with those from reference sequences in the GenBank database, Bootstrap values are shown in percentages of 1,000 replicates. Numbers in parentheses indicate frequencies of colonies exhibiting the same restriction patterns of *ppk1* gene homologs in libraries constructed from the respective clones of Accumulibacter clades.

sequences from our study and the available sequences in database) is attached in Appendix C.

It is noteworthy that all the clones obtained are highly affiliated with the reported clone Acc-SG2 ppk_1, with accession number is HM 046427 (Kim et al., 2010). This clone is related to clade IIF of Accumulibacter. Besides that, Peterson et al. (2008) and Shoji et al. (2011) also reported other clade IIF-related strains. The Gen Bank accession number for the nucleotide sequence determined in this study is KF985964.

6. 2 Development of the Clade IIF Primers Set for qPCR

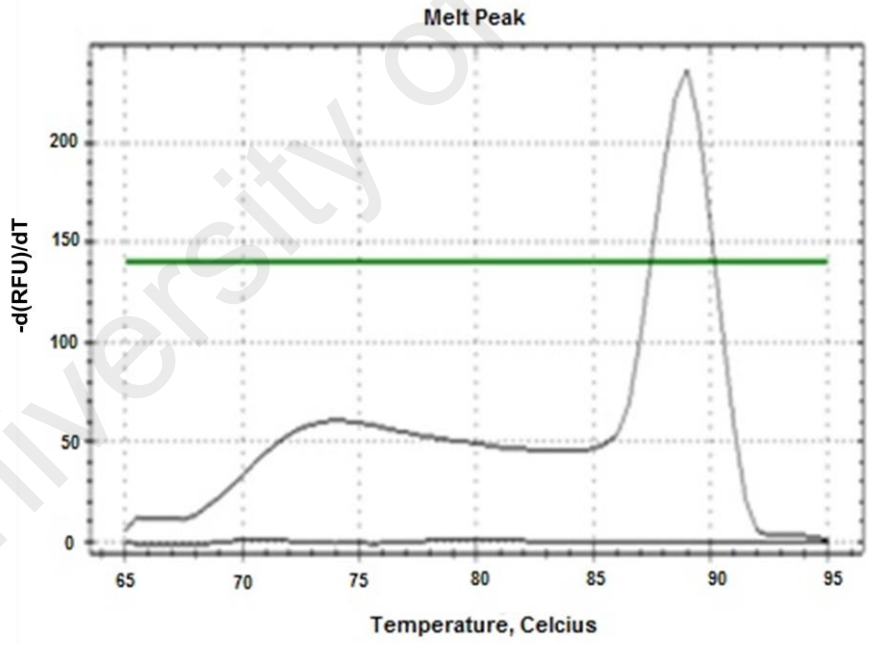
Because all of the *ppk1* sequences recovered from SBR-1 (28°C) in our study are closely related to clade IIF, a qPCR assay with specific primers targeted to this clade was necessary in the subsequent quantification study. However, the current available specific *ppk1* primers are only limited to clades 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 (Kalendra et al., 2011) was used to design the primers. The length of the sequence was shortened to adjust for an appropriate T_m (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 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 Figure 6.2(a). Only one melting peak was observed for the sludge sample. The 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 detected due to no amplification within 35 PCR cycles.

(a)



(b)

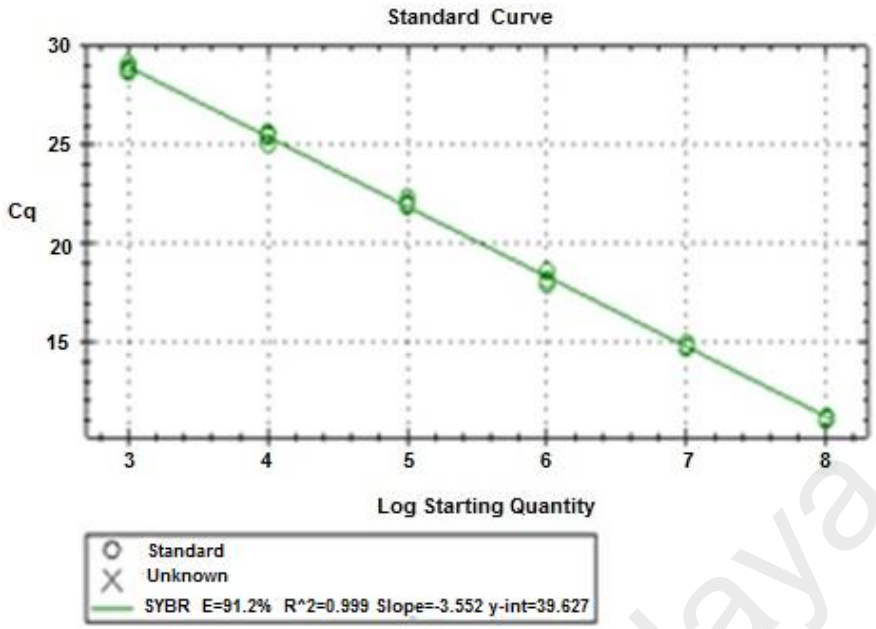


Figure 6.2: (a) Melting peak of DNA extracted from activated sludge and negative control; (b) Standard curve for the primer sets Acc-ppk1-355f and Acc-ppk1-600r constructed using a series of tenfold dilution.

Standard curves were constructed from 1.0×10^3 to 1.0×10^8 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 Figure 6.2(b). The averages of R^2 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.

6. 3 Presence and Dynamic of Accumulibacter's Clades Revealed by *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. With the exception of 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 Figure 6.3, 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 result lends support to the suggestion made by He et al (2007) that *ppk1* provides as adequate measurement as 16S rRNA. Furthermore, this observation again proves that clade IIF was the only Accumulibacter clade present in the sludge, which also represents the total Accumulibacter population.

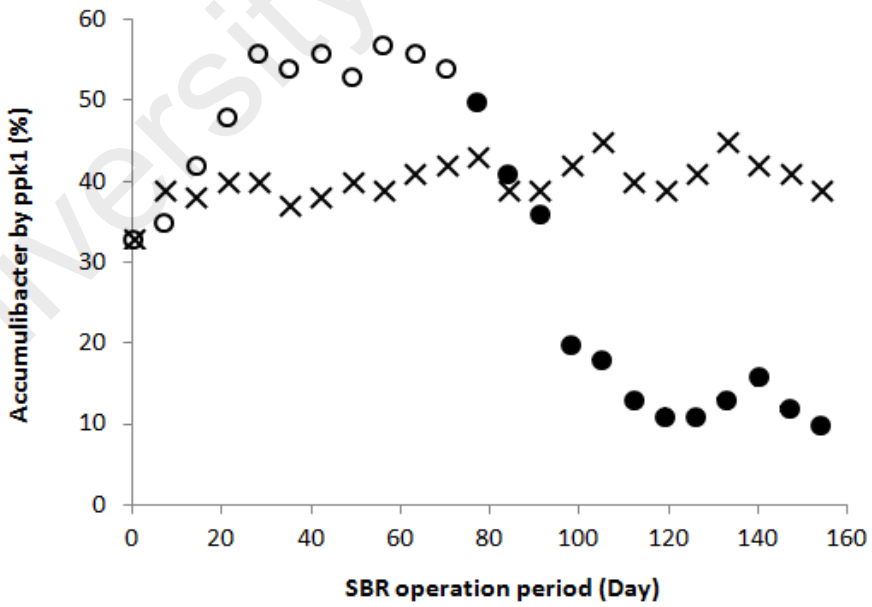


Figure 6.3: Percentage of Accumulibacter according to *ppk1* in the total bacterial population of EBPR processes operated in SBR-1 and SBR-2 throughout the reactor's operation; (x) 28°C-SBR 1; (○) 24°C-SBR 2; (●) 32°C-SBR2.

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 three temperatures 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 P contents (%) in the EBPR sludge at all three temperatures were similar at approximately 7 – 8 % (Table 5.1), indicating Accumulibacter clade IIF accumulated more phosphorus at 32°C.

Accumulibacter subpopulations structure was stable throughout the reactors operation as clade shift did not observed. The similarities in the Accumulibacter assemblages in all three high-temperature range EBPR process substantiated the claim that temperature appears to shape some of the Accumulibacter clade populations (Flowers et al., 2013). Flower 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 climates. Since there is no significant presence of other Accumulibacter's clades in our EBPR processes, their capability to survive in high temperatures could not be ruled out definitively at this stage. This is the first *ppk1*-based study that reveals the existence of Accumulibacter clade IIF with respect to high-temperature EBPR. Though seasonal dynamics on the abundance of Accumulibacter clades over a period of two-year was shown in Flowers et al. (2013), the wastewater temperature of the studied WWTP ranging from 10 to 22^{o}C which was

much lower than 28 to 32^{o}C. In addition, the presence of clade IIF was not detected in their study. A relationship between temperature and the selection of Accumulibacter subpopulation cannot be drawn conclusively at this point. More research on the distribution of Accumulibacter fine-scale population from cold to warm temperature are awaited.

6.4 Conclusions

Accumulibacter *ppk1*-based clone library showed the retrieved clones were closely related to Accumulibacter clade IIF. qPCR assay targeted on Accumulibacter clade IIF was successfully developed. The *ppk1*-based qPCR analysis detected only Accumulibacter clade IIF in all the studied EBPR processes. The estimation of Accumulibacter population measured using *ppk1* was not significantly different from the estimation measured using 16S rRNA (as presented in Chapter 5). The abundance of Accumulibacter clade IIF showed a decrease with the increase of temperature from 24 to 32^{o}C. The clade's robustness most likely helps it to fit the high-temperature EBPR sludge best and able to maintain its EBPR activity even at lower abundance in 32^{o}C.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Although EBPR has been considered a well-established wastewater treatment technology, the applicability of it in warm climates is uncertain due to frequent reports of EBPR deterioration at temperature higher than 20°C. However, in a preliminary study, EBPR sludge was successfully cultivated from non-EBPR sludge and the EBPR process was subsequently maintained at 28^{o}C. Thus, this research is initiated to further investigate the EBPR performance at a range of high temperatures. Along with this, the microbiology aspect of the high-temperature EBPR process also being evaluated. The conclusions from the research finding are as follow:

i. The studies collectively presented that good EBPR activity could be achieved at high temperatures, between 24^{o}C - 32^{o}C. Robustness of the high-temperature EBPR sludge was shown with rapid recovery from P and DOC removal deterioration when the process experienced a change in operational temperature. Overall, these high-temperature EBPR systems demonstrated stable EBPR performance without obvious fluctuation after reaching steady state of reactor operations.

ii. Screening of potential PAOs and GAOs candidates were carried out via FISH for all 24^{o}C, 28^{o}C and 32^{o}C. From the screening, Accumulibacter-PAOs and Competibacter-GAOs were found to be emerged as the major PAOs and GAOs

population in all three temperatures. Their abundance in the sludge community is of statistical significance.

iii. With the clone library generated, 26 OTUs were retrieved from the sludge sample of 28^{o}C EBPR process which operated for more than 2 years. Its microbial community structure comprised of Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, and Acidobacteria. Again, 16S rRNA clone library evidenced that Accumulibacter were the major PAOs responsible for the P removal in this high-temperature EBPR process. This work contributes valuable reference to the bacteria community structure of high-temperature EBPR process.

iv. Although the abundance of Accumulibacter-PAOs decreased with increasing temperature, still stable and high P removal efficiencies achieved in all 24^{o}C, 28^{o}C and 32^{o}C. The EBPR activities were not disrupted by the smaller Accumulibacter-PAOs population and a larger population of Competibacter-GAOs in 32^{o}C system. The high resilience and stable P removal function were always ensured in these high-temperature EBPR process regardless the size of Accumulibater-PAOs' population.

v. qPCR assay targeted on Accumulibacter clade IIF was successfully developed. Accumulibacter's clade IIF was the unique clade recovered by *ppk1*-targeted PCR from the studied high-temperature EBPR processes. The qPCR estimation of the total Accumulibacter fraction as a proportion of the bacterial community based on 16S rRNA genes was similar to the *ppk1*-based estimation. 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 allow it coexist with GAOs without compromising EBPR activity.

7.2 Novelties and Implications of Study

Some significant novelties and implications of this research are highlighted below:

i. This is the first study reports on the excellence and stable EBPR performance exhibited by a 28^{o}C high-temperature EBPR process which operated for a course of more than two years.

ii. This study presents the microbial community structure of a high-temperature EBPR process with stable and high P removal efficiency. The information obtained serves as a good reference for future works dealing with high-temperature EBPR.

iii. This study has comprehensively examined the feasibility of operating EBPR process across a range of high temperatures, 28^{o}C - 32^{o}C. The involvement of Accumulibacter-PAOs and their population size which range between 19 – 64 % of the total bacterial population could be a benchmark for good P removal performance in high-temperature EBPR process.

iv. The Accumulibacter clade IIF-specific *ppk1*-based qPCR primers set were successfully designed and validated in this study. With this, Accumulibacter clade IIF can now be quantified in addition to the current available clade I, IIA, IIB, IIC and IID specific primers sets developed by He et al. (2007).

v. Reduction in the PAO population and predominance of GAOs population without sacrificing the P removal performance is an interesting finding from this study. This finding challenges the earlier claims that relate deteriorated EBPR performance at warm temperatures with the decreasing of PAOs.

vi. A noteworthy finding from this research includes the ability of Accumulibacter clade IIF in tolerating high temperatures and continued showing high EBPR activities, especially exhibiting greater capability in P accumulation at 32^{o}C. This discovery is certain to revolutionize the understanding of ecophysiological traits of each Accumulibacter clade.

vii. Municipal regulations are increasingly mandating more stringent effluent quality standards globally. This research further addresses the applicability of EBPR process in tropical climates. It also provides useful process operating guidelines from both operational and microbiological aspects, in order to achieve a more consistent and higher level of nutrient removal.

7.3 Recommendations

All the scopes outlined in the study have been accomplished. Some further research efforts are needed to address issues and unresolved shortcomings encountered in this research. The possible future studies are recommended as below:

i. Most of the EBPR-related microbial studies currently available were based on the samples originated from temperate regions. Microbial data for high-temperature EBPR process is still limted. Further research is needed in the exploration of microbial diversity from a variety of high-temperature EBPR systems. There is pragmatic interest in charting microbial community structure and function in high-temperatrue EBPR ecosystems in relation to process stability and treatment performance.

ii. Further research efforts are needed to elucidate the relationship of Accumulibacter subpopulations on an individual clade basis with the temperature effects. Data on the dynamics and distribution pattern of the fine-scale structure of Accumulibacter in the EBPR processes operated across seasons as well as in warm climates could help in facilitate this purpose.

iii. It is utmost important to revisit the claim of population shift of PAOs induced by high temperatures. Based on the biochemical characteristics of PAOs and GAOs, PAOs could be more robust to the environmental change due to their dependence on two energy reserves (i.e poly-P and glycogen) whereas GAOs only possess glycogen reserve. The possibility of alternative energy pathway in PAOs being triggered to secure their EBPR capability under stress conditions (high temperatures) requires further research.

iv. While it is feasible to operate high-temperature EBPR process in lab scale, testing on the operation of full scale EBPR process in local STPs which subjected to high-temperature influent all year round has its constraints. Due to the distinct and inherent differences between lab scale and full scale operation, the transferability between them is challenging. Furthermore, the sludge history, complexities in microbial diversity, variations of physiological behaviors of microorganisms involved added further complication into the transferability. Configure a pilot plant treating the real municipal wastewater with the operating conditions mimic real plants would serve as the foundation for the later full scale operation. The experience and information obtained from the pilot run can be used as an appropriate guideline for researchers and EBPR operators to troubleshoot process upsets in real.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of Publications

- **Ong, Y. H.,** Chua, A. S. M., Ngoh, G. C., Fukushima, T., Shoji, T., and Michinaka, A. (2014). High-temperature EBPR process: The performance, analysis of PAOs and GAOs and the fine-scale population study of *Candidatus* 'Accumulibacter phosphatis'. *Water Res*., 64, 102-112.
  [ISI-Cited Publication. Q1 in the JCR® Category of Engineering, Environmental. Impact Factor: 5.323]

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