ECOLOGICAL STUDY OF MICROBIAL POPULATION IN ANAEROBIC-AEROBIC ACTIVATED SLUDGE PROCESSES

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Dedication

To my parents for all their support and love…

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Summary

Effluent from wastewater treatment plants (WWTPs) is a major contributor of phosphorus (P) to receiving waters with elevated levels of P leading to environmentally detrimental eutrophication. Activated sludge systems are designed and operated globally to remove P microbiologically by a process called enhanced biological phosphorus removal (EBPR). Engineering mass-balance control strategies, empirical observations, and biochemical models have long been used to select polyphosphate accumulating organisms (PAOs) capable of EBPR metabolism (Seviour *et al.*, 2003). However, difficulties in assuring stable and reliable operation have been well recognized in both laboratory-scale reactors and full-scale EBPR plants. One suggestion is that another physiological group of bacteria broadly classified as glycogen accumulating organisms (GAOs) can compete with PAOs for anaerobic uptake of carbon source and, under certain conditions, can lead to the deterioration of EBPR activity. Basic research into community structure and function will thus provide a more complete understand and control of EBPR processes.

In this study, a laboratory-scale sequencing membrane bioreactor (MBR) fed with acetate as the sole carbon source was operated under cyclic anaerobic and aerobic conditions to mimic EBPR processes for approximately 260 days. Biomass composition [sludge total phosphorus (TP), intracellular polyhydroxyalkanoate (PHA), and carbohydrate contents] and chemical analysis data (organic substrate and soluble phosphate) suggested that a microbial consortium exhibiting the typical characteristics of GAO with no EBPR activity was enriched in the MBR. To resolve the deterioration

in rational basis, the microbial community with no EBPR activity was phylogenetically and functionally analyzed by culture-independent molecular methods.

Preliminary semi-quantitative fluorescence *in situ* hybridization (FISH) results showed that a gammaproteobacterial lineage GB (GB group) frequently observed in EBPR processes was initially the numerically dominant species (50-66% of EUBmix-stained cells) of the GAO in the MBR from day 1 to day 38. On day 85 onward, a population shift from the GB group to alphaproteobacterial tetrad-forming organisms (TFOs) (85±7% of EUBmix-stained cells) occurred. This microbial succession was suspected to be related to the applied operating conditions (long hydraulic retention time and long solids residence time) which favored the proliferation of the alphaproteobacterial TFOs rather than the GB group. Those alphaproteobacterial TFOs present in the MBR were further analyzed phylogenetically by constructing a 16S rRNA gene clone library specific for the *Alphaproteobacteria*. Results indicated that most 16S rRNA gene clones (61% of total clones) were closely affiliated with *Defluviicoccus vanus* forming a cluster within subgroup 1 of the *Alphaproteobacteria*. Combined PHA staining and FISH with specific probes designed for the member of the *Defluviicoccus* cluster suggested diversity within this TFO cluster, and that these TFOs were newly identified GAOs in EBPR systems. Subsequently, the ecological traits of the *Defluviicoccus*-TFOs present in an acetate-fed anaerobic-aerobic sequencing MBR with no EBPR activity were systemically characterized by using microautoradiography combined with FISH and PHA staining. Under anaerobic conditions, the *Defluviicoccus*-related TFOs were able to assimilate and transform volatile fatty acids, including acetate, lactate, propionate, and pyruvate into PHA under anaerobic conditions, but were not able to assimilate aspartic acid and glucose under anaerobic or aerobic conditions.

However, in alternating anaerobic-aerobic batch experiments, a previously isolated strain *D. vanus* could take up glucose under anaerobic conditions with concurrent glycogen consumption and PHA production. Overall results suggested that the *Defluviicoccus* cluster exhibited the typical phenotype described for GAOs, but differed among themselves in some of the phenotypic and morphological traits.

The occurrence of the *Defluviicoccus*-related TFOs (8 samples in Chapter 4 and 13 samples in Chapter 7) and those putative PAOs and GAOs (13 samples in Chapter 7) in 21 full-scale EBPR and non-EBPR systems was also evaluated using FISH with EBPR-related oligonucleotide probes described in the literature. The low abundance of the *Defluviicoccus*-related TFOs in the examined samples (results in Chapters 4 and 7) suggested that they were not the key populations responsible for the deterioration of full-scale EBPR processes. On the other hand, *Rhodocyclus*-related PAOs and GAOs from the GB group were the predominant populations detected, representing 4-18% and 10-31% of EUBmix-stained cells respectively, in those samples collected in Japanese WWTPs. However, a considerable proportion of *Rhodocyclus*-related PAO cells were observed with no polyphosphate (polyP) granules accumulated based on polyP staining. This was further supported by a poor correlation between *Rhodocyclus*-related PAO population and sludge TP contents.

This study revealed that the application of culture-independent molecular methods to analyze EBPR community structure has changed dramatically the understanding of the microbial populations responsible for EBPR, but many substantial gaps in the knowledge of the population dynamics of EBPR and its underlying mechanisms

remain. Cautions should be exercised to link the phylogeny of microorganism to its ecophysiology and directly apply laboratory findings on full-scale WWTPs.

Keywords: Enhanced biological phosphorus removal (EBPR); Membrane bioreactor (MBR); full-scale activated sludge plants; Polyphosphate accumulating organisms (PAOs); Glycogen accumulating organisms (GAOs); Tetrad-forming organisms (TFOs)

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Abbreviations

3HB	3-hydroxybutyrate
3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
3HV	3-hydroxyvalerate
A	Adenine (Purine)
A2O	Anaerobic-anoxic-aerobic
Ac	Acetate
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
AO	Anaerobic-aerobic
APO	Anaerobic-Pure Oxygen
ATP	Adenosine 5'-triphosphate
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical Oxygen Demand
bp	Base Pair
C	Carbon or Cytosine (Pyrimidine)
CAS	Conventional Activated Sludge
CCD	Charged-coupled Device
CFB	*Cytophaga-Flexibacteri*-group of the *Bacteroidetes*
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
Cy3	Cyanine 3 (Fluorescent Dye)

Cy5	Cyanine 5 (Fluorescent Dye)
DAPI	4',6-Diamidino-2-Phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DPAO	Denitrifying Polyphosphate Accumulating Organism
d	Day
EBPR	Enhanced Biological Phosphorus Removal
ED	Entner-Doudoroff (Pathway)
EDTA	Ethylenediaminetetraacetic Acid
EMP	Embden-Meyerhof-Parnas (Pathway) (i.e., Glycolysis)
FA	Formamide
FISH	Fluorescent *in situ* Hybridization
FITC	Fluorescein Isothiocyanate (Fluorescent Dye)
F/M Ratio	Food to Microorganism Ratio
f	Forward Primer
G	Guanine (Purine)
GALO	*Gordonia amarae*-like Organism
GAO	Glycogen Accumulating Organism
GB	Members of the lineage GB in the *Gammaproteobacteria*
G-bacteria	Glucose Bacteria?
g	Gram
HGC	Gram-positive High-G+C (i.e., *Actinobacteria*)
HRT	Hydraulic Retention Time
h	Hour
kPa	Kilopascal
kV	Kilovolt
LGC	Gram-positive Low-G+C
LPO	Lactic Acid Producing Organism
l	Liter
MAR	Microautoradiography
MBR	Membrane Bioreactor
MLSS	Mixed Liquor Suspended Solid
MLVSS	Mixed Liquor Volatile Suspended Solid

mg	Milligram
min	Minute
ml	Milliliter
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
ng	Nanogram
OD	Optical Density
OTU	Operational Taxonomic Unit
P	Phosphorus
PAO	Polyphosphate Accumulating Organism
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
Pi	Phosphate or Orthophosphate
PolyP	Polyphosphate
Pseudo-AO	Pseudo-anaerobic-aerobic
R^2	Regression Coefficient
RDP	Ribosomal Database Project
RFLP	Restrictive Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosome RNA
r	Reverse Primer
SBR	Sequencing Batch Reactor
SDS	Sodium Dodecyl Sulfate
SRT	Solids Residence Time
SRT_{min}	Minimum SRT
T	Thymine (Pyrimidine)
TAE	Tris Acetate EDTA
TCA	Tricarboylic acid (Cycle)

TCOD	Total Chemical Oxygen Demand
TFO	Tetrad-Forming Organism
TGGE	Temperature Gradient Gel Electrophoresis
T_m	Melting Temperature
TOC	Total Organic Carbon
TP	Total Phosphorus
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TSS	Total Suspended Solids
vol	Volume
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
UCT	University of Cape Town (Process)
wt	Weight
WWTP	Wastewater Treatment Plant
μ_{max}	Maximum Growth Rate of the Microorganisms
µg	Microgram
µl	Microliter
µm	Micrometer

Publications

Journal Papers

1. **Wong, M.-T.** and Liu, W.-T. (2007). Ecophysiology of *Defluviicoccus*-related tetrad-forming organisms in an anaerobic-aerobic activated sludge process. Environ. Microbiol., in press.
2. **Wong, M.-T.** and Liu, W.-T. (2006). Microbial succession of glycogen accumulating organisms in an anaerobic-aerobic membrane bioreactor with no phosphorus removal. Water Sci. Technol. 54(1), 29-37.
3. Jobbagy, A., Literathy, B., **Wong, M.-T.**, Tardy, G., and Liu, W.-T. (2006). Proliferation of glycogen accumulating organisms induced by Fe(III) dosing domestic wastewater treatment plant. Water Sci. Technol. 54(1), 101-109.
4. **Wong, M.-T.**, Mino, T., Seviour, R.J., Onuki, M., and Liu, W.-T. (2005). *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorus removal plants in Japan. Water Res. 39, 2901-2914.
5. **Wong, M.-T.**, Tan, F.M., Ng, W.J., and Liu, W.-T. (2004). Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. Microbiology 150, 3741-3748.
6. Chen, C.-L., Liu, W.-T., Chong, M.L., **Wong, M.-T.**, Ong, S.L., Seah, H., and Ng, W.J. (2004). Community structure of microbial biofilms associated with

membrane-based water purification as revealed using a polyphasic approach. Appl. Microbiol. Biotech. 63, 466-473.

Conference Papers

1. **Wong, M.-T.** and Liu, W.-T. (2005). Microbial succession of glycogen accumulating organisms in an anaerobic-aerobic membrane bioreactor with no phosphorus removal. Presented at 4th IWA Conference on Activated Sludge and Population Dynamics, July 17-20, Queensland, Australia.
2. Jobbagy, A., Literathy, B., **Wong, M.-T.**, Tardy, G., and Liu, W.-T. (2005). Proliferation of glycogen accumulating organisms induced by Fe(III) dosing domestic wastewater treatment plant. Presented at 4th IWA Conference on Activated Sludge and Population Dynamics, July 17-20, Queensland, Australia.
3. Liu, W.-T., **Wong, M.-T.**, Ong, S.L., and Ng, W.J. (2003). Why does membrane bioreactor have a low biomass yield? Presented at 1st International Tokyo Tech Bio-symposium, January 29-30, Yokohama, Japan.
4. **Wong, M.-T.**, Song, L., Ng, W.J., and Liu, W.-T. (2002). Effect of pH on the microbial population structure of submerged membrane bioreactors. Presented at 12th KIAST-KU-NTU-NUS Symposium on Environment Engineering, June 26-29, Taipei, Taiwan.

Chapter 1

Introduction

“When theory and observations come together, science often takes a great step forward.”

Stephen HAWKING, 1997

1.1 Background

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 (WHO, 2002). Although eutrophication can be part of the natural process in waterbodies associated with seasonal fluctuations, the increased frequency of such occurrences is triggered by water pollution. The primary cause of artificial eutrophication is due to an excessive discharge of nutrients [i.e., nitrogen (N) and phosphorus (P)] originating from agriculture or sewage treatment. The resultant increase in fertility in affected receiving waterbodies causes symptoms such as heavy growth of rooted aquatic plants, algal blooms, deoxygenation and, in some cases, unpleasant odor, which often affects most of the vital uses of the water such as water supply, recreation, fisheries (both commercial and recreational), and aesthetics. All these responses can further lead to deterioration of water bodies, clogging of water systems, and undesirable changes in aquatic populations. Increasing evidence has suggested that the major point sources of nutrients are the discharged effluent from wastewater treatment plants (WWTPs). Therefore, loss of recreational and economical value of a waterbody is unavoidable if controlled discharge of N and P from wastewater streams is not practiced.

Wastewater reuse and reclamation has been regarded as a viable option for potable and/or non-potable water supply. The applications of municipal wastewater reuse include agricultural irrigation, landscape irrigation, industrial recycling and reuse, groundwater recharge, recreational/environmental uses, non-potable urban uses, and potable reuse (Metcalf and Eddy, 2004). All these applications except the wastewater reuse for agricultural irrigation required P to be removed. For example, surface waters

would ultimately receive the reclaimed water for landscape irrigation and groundwater recharge. Thus, P removal from the wastewater used for these purposes should be advised to prevent the occurrence of eutrophication.

In general, P removal can be achieved using either biological treatment or chemical treatment. The chemical method employs the addition of metal salts like calcium oxide, alum, and ferric chloride to precipitate P from the wastewater, resulting in its removal with the excess sludge. Despite its simplicity and reliability for P removal, this approach is associated with problems, like high operating costs, increased sludge production, sludge with poor settling and dewatering characteristics, and depressed pH. Furthermore, the addition of chemical precipitants can introduce heavy metal contamination into the sewage and causes an increase in the salt concentration in the effluent.

Alternatively, activated sludge processes with alternating anaerobic and aerobic conditions have been successfully used for enhanced biological phosphorus removal (EBPR) from wastewater. This approach utilizes a microbiological mechanism whereby the process is conditioned to enrich polyphosphate accumulating organisms (PAOs) which accumulate P in excess of their normal metabolic requirements and store it in the form of intracellular polyphosphate (polyP). 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). PAOs utilize internally stored polyP as an energy source for the uptake and accumulation of carbon substrates as intracellular storage reserves [e.g., polyhydroxyalkanoate (PHA)]

during the anaerobic feeding stage. The required energy and reducing equivalents for this process are thought to come from the degradation of polyP and glycogen stored in the cells (Mino *et al.*, 1998). During the subsequent aerobic stage, the accumulated PHA is utilized for growth and replenishment of the polyP 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 polyP is removed from the system. EBPR processes can offer different benefits such as reduced sludge production, improved sludge settleability and dewatering characteristics, reduced oxygen requirements, and reduced process alkalinity requirements. Overall, the EBPR process is more sustainable than the chemical approach in terms of economical and environmental concerns.

EBPR processes have been widely implemented in developed countries worldwide to minimize environmental pollution and meet the stringent wastewater discharge standard. Despite their widespread application, difficulties in assuring stable and reliable operation have also been well recognized. Intermittent failures with EBPR processes in removing P have been regularly reported in both laboratory-scale reactors and full-scale WWTPs. Such failures could sometimes be attributed to external disturbances such as heavy rainfall, changes in organic loading, unbalanced nutrient condition, excessive aeration, and high nitrate loading to the anaerobic zone (Mulkerrins *et al.*, 2004). However, deteriorations in EBPR have been frequently reported for unknown reasons even in well-defined and carefully controlled laboratory-scale reactors. Thus, a standby chemical treatment is often incorporated to produce a treated effluent that meets the required discharge standards.

1.2 Problem Statements

1.2.1 Stability of Process Operation

Over the last 30 years, empirical experiences of EBPR operation have provided better guidelines for plant operators to operate EBPR processes (Mino *et al.*, 1998). 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 mechanisms of EBPR processes. Engineers have utilized the gross chemical transformations developed by these 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}. However, EBPR processes occasionally fail for no clear reason and when these happen the plant effluent contain unacceptably high levels of P. Failures often occur despite all known prerequisites for EBPR being provided and typically in periods following stable and efficient EBPR. Without any convincing supporting evidence, the success of remedial actions for such process failures is often erratic and unreliable.

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 polyP accumulation were less considered until a decade ago. As a biologically specialized reaction, the EBPR process requires the establishment of a group of pertinent and robust microorganisms that conforms to the metabolic type

targeted by the process control strategy. Improved process control and system reliability will thus certainly depend upon a better understanding of EBPR microbiology. Currently, the precise identities of those microbes involve in EBPR process have not been unambiguously revealed and the underlying mechanisms of P removal are not fully understood. In the absence of this vital information, it is anticipated that EBPR process would always operate suboptimally.

1.2.2 Limitations of Traditional Culturing Approaches

Difficulties in process optimization are primarily attributed to the inability to isolate the responsible microorganisms and verify the biochemical metabolism for the observed EBPR activity. Obtaining pure cultures of EBPR bacteria, particularly those accumulating polyP, appears to be an indispensable requirement for a description of their physiology and biochemistry. To date, there are no reports of pure cultures or fully characterized co-cultures capable of performing the anaerobic and aerobic biochemical operations conductive to EBPR. Such inability in culturing bacteria could be ascribed to their slow growth rates, their viable-but-non-culturable nature, and/or the inadequacy to mimic their native habitats. There is also a possibility that PAOs cannot grow as a single culture and interactions among different species are necessary, as is known from other microbiological systems (Seviour *et al.*, 2003). In addition, it is possible that the microorganisms may no longer exhibit their previous properties because of genetic or phylogenetic changes when they are removed from their natural environment and are grown under artificial conditions such as a laboratory setting.

While identifying and isolating the PAOs in pure culture remain a challenge, the disadvantages and inherent biases associated with the microbial community analysis in

EBPR using traditional cultivation techniques are well documented. Earlier studies using traditional culturing approaches repeatedly reported that *Acinetobacter*, a genus of the *Gammaproteobacteria*, was the potential PAO responsible for P removal in EBPR processes (Fuhs and Chen, 1975; Jenkins and Tandoi, 1991; Mino *et al.*, 1998). Although polyP accumulation in laboratory-grown *Acinetobacter* is well established, the significance of *Acinetobacter* spp. in full-scale EBPR process has been questioned. Many evidences indicated *Acinetobacter* can accumulate polyP, but fail to exhibit the described PAO metabolic characteristics and constitute only a tiny fraction of total microbial population (Cloete and Steyn, 1988; Auling *et al.*, 1991; Wagner *et al.*, 1994; Tandoi *et al.*, 1998). *Microlunatus phosphovorus* and *Lampropedia* capable of accumulating polyP have been isolated from EBPR sludge. However, they also exhibit metabolisms different from a typical PAO in an EBPR system (Nakamura *et al.*, 1995; Stante *et al.*, 1997).

Compared to cultivation-based attempts, the hunt for PAOs was more successful using culture independent molecular tools for analyses of EBPR systems. Originally, it was assumed that EBPR process was performed by a single or a few groups of microorganisms, as is common in other enrichment cultures. Despite this, it appears that EBPR activity is not carried out by a specific group of microorganisms. Instead, molecular tools showed that a consortium of phylogenetically diverse groups of microorganisms can be involved in polyP accumulation, some more numerous than others. In particular, a yet-to-be-cultured microorganism closely related to *Rhodocyclus* in the *Betaproteobacteria* subdivision has been consistently suggested to be one of the PAOs (Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000). However, Seviour *et al.* (2003) suggested that the type of bacteria responsible for EBPR may vary among

different situations. As yet, the general microbial community structure has not been well described, and therefore, the ecological mechanisms selecting for PAOs over other bacterial communities in EBPR are vaguely understood.

1.2.3 Microbial Competitors for Substrate Uptake under Anaerobic Conditions

Another difficulty in the optimization of EBPR processes is the possible involvement of microbial competitors for substrate uptake under anaerobic conditions. In EBPR processes, PAOs utilize internally stored polyP as an energy source for the uptake and accumulation of carbon substrates as intracellular PHA during the anaerobic feeding stage. This carbon sequestering mechanism enables PAOs to grow under subsequent aerobic conditions where carbon is not available to them, providing them with a competitive advantage over strictly aerobic heterotrophs. However, glycogen accumulating organisms (GAOs) share similar carbon assimilation and storage features to PAOs, and have been reported to compete with PAOs for carbon substrate by utilizing intracellular glycogen instead of polyP as internally stored energy source to synthesize PHA. GAOs were thought to achieve this by more effectively assimilating carbon substrates anaerobically, when the EBPR was operated at long solids residence time (SRT) (Wentzel *et al.*, 1989a; Smolders *et al.*, 1995; Chuang *et al.*, 1996; Brdjanovic *et al.*, 1998a; Lesjean *et al.*, 2003), high anaerobic-aerobic hydraulic retention time (HRT) ratio (Bernard *et al.*, 1985; Tammink *et al.*, 1996; Brdjanovic *et al.*, 1998a; Wang and Park, 2001), acidic pH in anaerobic phase (Smolders *et al.*, 1994; Fleit, 1995) , and high temperature (Sell, 1981; Ekama *et al.*, 1984; Barnard *et al.*, 1985; Pansward *et al.*, 2003). Presence of glucose (Cech and Hartman, 1990; 1993; Carucci *et al.*, 1994; Liu *et al.*, 1996b; Liu, 1998; Sudiana *et al.*, 1999), limited P

source (Liu *et al.*, 1994; 1997b; Wang *et al.*, 2001; Kong *et al.*, 2001; Crocetti *et al.*, 2002), and high organic loading (Liu *et al.*, 1996b; Reddy, 1998) in the influent substrate were also reported as favorable conditions for the proliferation of GAOs (Mino *et al.*, 1998; Seviour *et al.*, 2003; Mulkerrins *et al.*, 2004). When GAOs become dominant, EBPR performance is gradually deteriorated. Like PAOs, past studies have suggested GAOs consisted of several different morphotypes and phylotypes. Unfortunately, the precise identities of GAOs and their ecophysiology are still largely unknown.

1.2.4 Structure-function Relationships within EBPR Communities

With the advent of the molecular techniques for characterization of microbial communities in different EBPR systems, several phylogenetic groups of microorganisms have been suggested to be PAOs and GAOs. Previously, the bacterial diversity in a number of laboratory-scale EBPR reactors have been analyzed by 16S rRNA gene library and other molecular tools like fluorescence *in situ* hybridization (FISH). Findings from such analyses have greatly advanced the understanding of the phylogenetic diversity and accurately provided the quantitative information for the microbial community structure present in these EBPR systems. However, knowing the identity and numerical dominance of these EBPR bacteria is not sufficient to understand their ecological roles in EBPR systems. Keeping in mind that microorganisms involved in EBPR process have been found to be widespread among phylogenetically unrelated groups, there is a need to develop approaches that allow explicit links to be made between the presence of specific microorganisms and the function they exhibit. Such information not only allows a more detailed understanding of the ecology of complex microbial communities but also provides information which

can be used for the development of appropriate enrichment and isolation methods for molecularly identified but yet-to-be cultured microorganisms.

1.2.5 Applicability of Laboratory Findings to Full-scale EBPR

Studies on gross chemical changes and EBPR community structures have dramatically changed the understanding of the microbial populations responsible for EBPR. Most were carried out on biomass from laboratory-scale reactors fed with synthetic sewage and single carbon sources. These studies can also have better control over parameters like influent flows, HRTs, and SRTs. However, these laboratory-scale reactors cannot always simulate all of the important environmental conditions in full-scale WWTPs. As a result, the selected microbial populations may not always be pertinent to full-scale WWTPs. In addition, variability in real operational conditions (influent flow, load, and composition) may limit the application of these laboratory findings to full-scale WWTPs. Comparison of operational conditions and microbial populations from laboratory studies are therefore unlikely to provide definitive answers as to how a competitive advantage by an organism is gained in full-scale applications.

1.3 Research Objectives

The overall objective of the present research is to study the ecological function, interaction, and diversity of different microbial populations (e.g., PAOs and GAOs) involve in EBPR process using a laboratory-scale anaerobic-aerobic sequencing membrane bioreactor (MBR) to mimic the EBPR processes. Samples from full-scale WWTPs were also collected to verify the roles of those important bacterial groups previously found in EBPR process.

Specific objectives are:

(1) To study the microbial diversity of PAOs and GAOs using culture independent methods such as microscopy and molecular techniques;

(2) To examine the substrate assimilation and PHA synthesis abilities of GAOs under different environments, and find out their possible ecological roles in EBPR processes;

(3) To study the effect of operating conditions on the population dynamics of different GAO groups; and finally

(4) To characterize the microbial community structure of activated sludge samples taken from full-scale WWTPs with and without EBPR configuration.

1.4 Organization of Thesis

Chapter 2 presented detailed background on the research questions that are relevant to the study of EBPR process, and on the research literature reporting the findings of other investigators that shed light on these questions.

Chapter 3 described the experimental materials and methods employed.

In Chapter 4, the discussion is on tetrad-forming organisms (TFOs) dominant in an acetate-fed anaerobic-aerobic sequencing MBR showing deteriorated EBPR activity. This chapter described the *in situ* physiological traits of these TFOs, resolved their phylogenetic affiliation and diversity, and examined their occurrence in laboratory-scale and full-scale EBPR and non-EBPR systems.

The succession of two different GAO groups has been observed in the acetate-fed MBR. In Chapter 5, culture independent 16S rRNA-based methods, terminal restriction fragment polymorphism, and FISH were employed to reveal their identities. The effect of MBR operating conditions on their population dynamics was also discussed.

In Chapter 6, the operation of the acetate-fed MBR under the identical conditions as in Chapter 4 was repeated. A suite of culture-independent methods combined with microautoradiography, FISH, and PHA staining was used to understand the anaerobic and aerobic substrate assimilation and PHA accumulation abilities of yet-to-be-cultured *Defluvicoccus*-related TFOs and compared with previously examined GAOs.

In Chapter 7, the microbial community structure of thirteen activated sludge samples from nine Japanese WWTPs were characterized using the published rRNA-targeted oligonucleotide probes targeting important bacterial groups found in EBPR process and major bacterial divisions found in wastewater treatment processes. A combined approach of FISH and polyP staining was also used to identify the polyP-accumulating traits of *Rhodocyclus*-related PAO in these full-scale WWTPs.

Chapter 8 presented the overall conclusions as well as recommendations for future studies related to this research.

Chapter 2

Literature Review

2.1 Historical Development of EBPR

Biological removal of phosphorus (P) occurs in two ways. In conventional full-scale activated sludge plants, a small amount of P removal is achieved due to the growth of microorganisms. The sludge P content typically only reaches 1.5-2% of the sludge dry weight, which is the P requirement for normal growth (Schlegel, 1993). The alternative approach is achieved by encouraging the accumulation of P in bacterial cells in the form of polyphosphate (polyP) granules in excess of the levels normally required to satisfy the metabolic demand for growth. This storage process is commonly described as 'enhanced biological phosphorus removal' (EBPR). Under favorable conditions, full-scale EBPR processes can remove 80-90% of influent phosphate, achieving residual P levels in effluent of less than 1 mg l^{-1} and producing sludges with P content up to 15% of dry biomass. In contrast, the typical P removal of conventional wastewater treatment is about 20-40% (Streichan *et al.*, 1990).

2.1.1 Early Stage Observations

(A) Accidental Discovery of EBPR

Biological P removal using activated sludge processes was accidentally discovered. The first report on removal of orthophosphate (Pi) at a level more than the normal metabolic requirement in a wastewater treatment plant (WWTP) was described by Srinath *et al.* (1959) from India. They observed that the P removal was related to the aeration intensity and toxic substances. Reduction in Pi concentration to nearly zero was observed when the sludge was aerated.

Shapiro *et al.* (1967) subsequently observed that Pi taken up during aeration was rapidly released during settling or under anaerobic or anoxic conditions. Thus, they

suspected Pi uptake under aerobic conditions and release under anaerobic or low redox conditions were reversible processes. This proposition was verified by Wells (1969). Using activated sludge from the WWTP exhibiting P removal in continuous batch tests, he observed a repetitive phenomenon, in which Pi concentration decreased under aerated periods and increased under non-aerated periods. These preliminary empirical observations provided a good foundation for future studies on the design of EBPR processes.

In the late 1960s and early 1970s, many researchers tried to elucidate the observed excess P removal for full-scale treatment plants. Experimental results of Vacker *et al.* (1967) and Milbury *et al.* (1971) using plug-flow activated sludge processes implied two very important features of P removal: (1) cycling activated sludge through alternating anaerobic-aerobic conditions and (2) addition of substrate to the anaerobic zone is essential for P removal. These two features were recognized by Barnard (1974) using a 'Bardenpho' process in which anaerobic, anoxic, and aerobic zones were configured. He found that to achieve biological removal of P, 'there seemed to be a remarkable correlation between the presence of nitrates in the effluent and the inability of the plant to remove P', and 'the bacteria must pass through a short period during which fermentation takes place'. In other words, the settled activated sludge in a settling tank has to be recycled to an anaerobic basin (i.e., absence of nitrate) where the raw or settled sewage is added. The nitrate present in the returned sludge has to be denitrified to nitrogen gas, for example, by circulating the activated sludge from the aerobic basin to an anoxic basin. While cyclic anaerobic-aerobic conditions were known to be essential for excess P removal, addition of readily biodegradable substrate under anaerobic condition was recognized as a prerequisite to induce the release of Pi.

(B) Prerequisites for EBPR

From the literature review, it is not clear who first identified the addition of carbon source under anaerobic condition is a prerequisite to induce P release. Fuhs and Chen (1975) stated that the release of Pi from sludge exhibiting an excess P removal is controlled by the addition of a carbon source and unidentified diffusible substances from fermented sewage. Marais *et al.* (1983) further suggested that P release is induced by the 'readily biodegradable chemical oxygen demand (COD)', which can be rapidly metabolized in the anaerobic reactor. By adding glucose and acetate along with the unsettled municipal sewage in a laboratory-scale process, they observed an increase in P removal efficiency. It is now recognized that simple soluble organics (e.g., acetate, propionate, butyrate, lactate, succinate, valerate, and glucose) are most effective, but not equally effective, in stimulating P release under anaerobic conditions (Fukase *et al.*, 1982; Arvin and Kristensen, 1985; Comeau *et al.*, 1987; Arun *et al.*, 1989; Liu *et al.*, 1996b).

Based on these observations, the basic conditions for proper functioning of biological P removal will require: (1) repetitive anaerobic-aerobic conditions, (2) addition of influent containing readily biodegradable carbon sources in the anaerobic zone, (3) absence of nitrate in the anaerobic zone, and (4) strictly control anaerobic conditions in the anaerobic zone.

2.1.2 Verification of Biological P Removal Mechanisms

(A) Arguments against Biological P Removal

In the early development of excess P removal in activated sludge processes, there was controversy over whether the P removal mechanism is mediated biologically or

chemically. Levin and Shapiro (1965) first reported ‘luxury’ uptake of dissolved Pi was a biological reaction that was affected by the intensity of aeration. On the other hand, Menar and Jenkins (1970) credited this ‘luxury’ P removal in activated sludge systems to chemical precipitation, and although the extent of removal was a direct function of the aeration rate, the removal was oxygen-transfer independent. They stated that the aeration process stripped metabolically-produced carbon dioxide from the sludge suspension. The resultant increase in pH induced the precipitation of some forms of calcium phosphate, which was then removed with the biomass during sludge separation. The validity of these two hypotheses has been subjected to verification, and collective evidence supported the biological removal mechanism.

(B) Evidences for Biological P Removal

The first evidence of biological P removal was indicated by Levin and Shapiro (1965). They studied P removal in full-scale treatment plants and with batch experiments using sludge retrieved from these plants. Their main observations were that Pi was released under non-aerated conditions and taken up under aerobic conditions; moreover, addition of wastewater (substrate) increased P uptake. Since P was taken up under aerobic conditions, they concluded the uptake occurred via formation of ATP during oxidative phosphorylation. Uptake via substrate phosphorylation could have taken place anaerobically via the Embden-Meyerhof-Parnas (EMP) pathway (i.e., glycolysis). They showed that the process was clearly a biological one since aeration and substrate were necessary, and inhibition of oxidative phosphorylation by 2,4-dichlorophenoxyacetic acid led to inhibition of P uptake. Also, no P uptake occurred at high pH (e.g., pH 9) and thus they concluded chemical precipitation was not the

driving force for the P removal. The same result was reproduced by Fuhs and Chen (1975).

Other supportive evidence for biological P removal was also reported by Yall *et al.*, (1970) and Buchan (1981). Their X-ray diffraction and radioisotope analyses of sludge with excess P removal showed only a small portion of Pi in the sludge matrix is present as a chemical precipitate. Rensink (1981) also showed that excess P uptake was not associated with calcium. Using 10 in-series reactors (first 5 reactors non-aerated), they observed the calcium concentration did not change along the process, but the P concentration increased in the non-aerated zones, and decreased in the aerated zones. Clearly, the excess P removal was not related to dissolution and precipitation of P minerals. Miyamoto-mills (1983), Comeau *et al.* (1985), and Arvin and Kristensen (1985) also indicated that the release and uptake of Pi were closely related to the co-transport of metal ions, especially Mg and K. These two ions are known to associate with polyP formation (Dawes and Senior, 1973), but not with P precipitates. Mino *et al.* (1984, 1985), using ^{32}P-labeled Pi as a tracer, further provided evidence for intracellular polyP, especially low molecular polyP, as an energy source during the uptake of substrate under anaerobic conditions. They observed a decrease in the low molecular polyP together with an increase in the bulk Pi concentration.

Microbiological evidence from microscopic staining techniques (Fuhs and Chen, 1975) and electron microscopy (Buchan, 1981) also suggested that certain morphological types of microorganisms were able to store intracellular volutin granules. The volutin granule formed was confirmed to be inorganic polyP (Dawes and Senior, 1973). Fuhs and Chen (1975) further observed the accumulation of a carbon reserve material,

possibly polyhydroxybutyrate (PHB) after anaerobic substrate uptake. Thus, they suggested a single microorganism or closely related species with polyP accumulation was responsible for both PHB accumulation after anaerobic substrate uptake (e.g., acetate) and excess P removal.

It is worth noting that in the early days of EBPR research, attention was mainly focused on the aerobic process. A link between anaerobic process and aerobic process was rarely recognized. The aerobic P, then called ‘overplus’ or ‘luxury’, uptake (Levin and Shapiro, 1965) was supposed to result from stress conditions due to the ‘anaerobic dynamic feeding’ in activated sludge plants. It is now recognized that utilization of polyhydroxyalkanoate (PHA), replenishment of glycogen, and uptake of Pi in the aerobic processes are linked with the preceding anaerobic process.

2.2 Biochemical Models of EBPR

2.2.1 Substrate Metabolism of EBPR

Figure 2-1 shows the gross chemical changes that occur during the anaerobic-aerobic cycling of biomass in EBPR systems. Under anaerobic conditions, the extracellular carbon substrate [preferably volatile fatty acids (VFAs)] is taken up concurrently with Pi release, PHA production, and intracellular glycogen consumption. Under the subsequent aerobic conditions, the Pi released under anaerobic conditions and present in the influent is taken up and synthesized into polyP. Meanwhile, the accumulated PHA is depleted for growth and the consumed glycogen recovered to the same level as at the start of the anaerobic stage. Net P removal is achieved by wasting sludge containing a high level of polyP after the aerobic period.

Various mechanistic/biochemical models have been proposed to describe the EBPR observed in Figure 2-1 (Fuhs and Chen, 1975; Nicholls and Osborn, 1979; Rensink, 1981; Marais *et al.*, 1983). Each successive model seems to be influenced by the preceding models. They were all based on alternating anaerobic-aerobic conditions, which favor the growth of bacteria that accumulated Pi in the form of intracellular polyP granules. Under anaerobic conditions, organic substances from the sewage were taken up and stored as carbon reserves (i.e., PHA). The energy required for these processes was thought to come from the enzymatic hydrolysis of polyP. During the subsequent aerobic phase, bacterial growth and polyP accumulation occur at the expense of the stored carbon. This scheme was well accepted, since the above mentioned set of observations could explain many of the observations previously reported.

Two well-known models, the Comeau/Wentzel and the Mino models agree on many features but differed on some key features in the behavior of EBPR microorganisms (Wentzel *et al.*, 1991). Since these two models require a good understanding of the metabolism of polyP and PHA, the metabolism of these two polymers are introduced first.

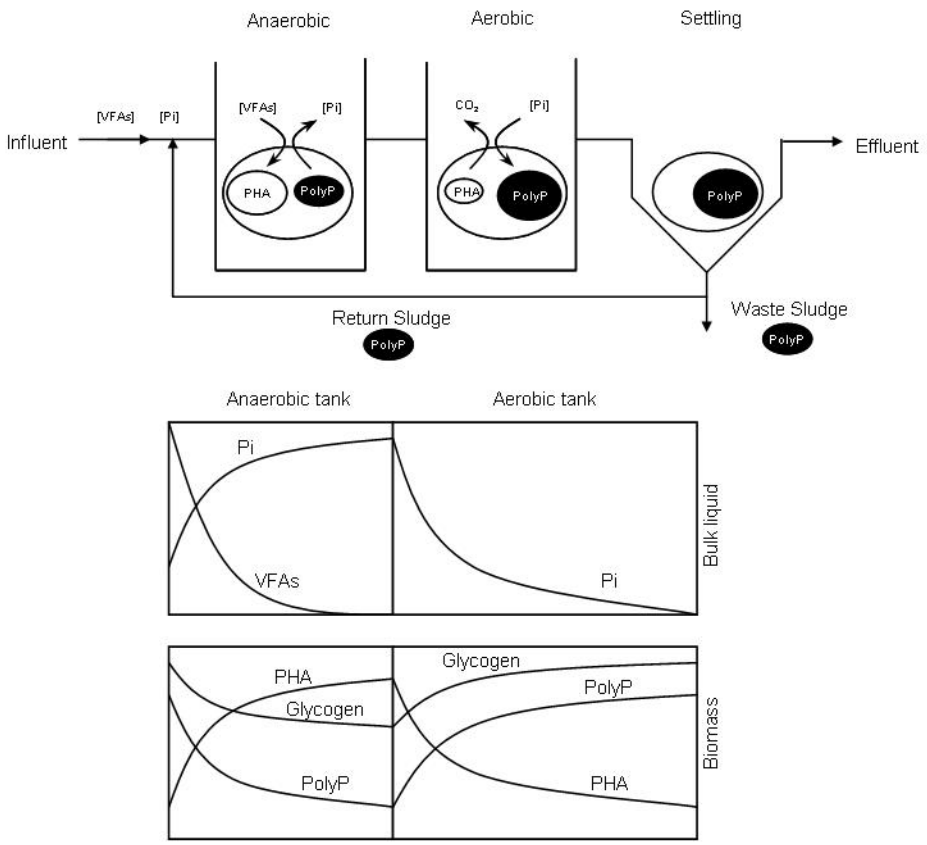


Figure 2-1 Gross chemical changes observed during the anaerobic/aerobic cycling of biomass in an EBPR process (van Loosdrecht *et al.*, 1997).

2.2.2 PolyP Metabolism

(A) Nature of PolyP

PolyP ($M_{(n+2)}P_nO_{(3n+1)}$) is a polyanionic linear inorganic polymer consisting of many Pi monomers linked by high-energy phosphoanhydride bonds (Figure 2-2) to produce linear or circular chains (Schlegel, 1993). The terminal hydroxyl groups of the polyP are mildly acidic while the intermediate hydroxyl groups are strongly acidic (van Wazer and Holst, 1950). The level of polymerization is known to vary between different organisms from three to a thousand monomers, and for activated sludge, the acid-soluble fraction contains between 3 and 20 monomer units while the acid-insoluble fraction contains a higher number of monomers (Kulaev, 1979). After

staining metachromatically with basic dyes such as methylene blue and toluene blue, it can be readily detected as intracellular granules (Serafim *et al.*, 2002; Jenkins *et al.*, 2004). 4'6-diamidino-2-phenylindole (DAPI), which imparts a yellow fluorescence to polyP granule, has frequently been used to detect polyP in EBPR studies (Kawaharasaki *et al.*, 1999; Liu *et al.*, 2001; Onuki *et al.*, 2002; Tsai and Liu, 2002; Lee *et al.*, 2003). Thermodynamically the standard free energy of hydrolysis of the anhydride linkage yields about 38 kJ per phosphate bond at pH 5. The energy storage function of polyP depends on the ability of the bond cleavage reaction to affect phosphorylation and thereby conserve the energy associated with the hydrolytic action (Dawes, 1992). In the absence of divalent cations, linear polyP is stable under alkaline conditions. It is completely hydrolyzed by acid to Pi within 15 min at 100ºC.

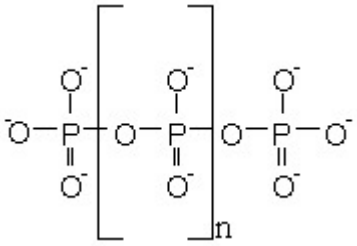


Figure 2-2 Structure of linear inorganic polyP. The n may vary from 3 to 1000 (Kortstee *et al.*, 2000).

(B) Synthesis of PolyP

Table 2-1 summarizes all enzymatic reactions involved in polyP synthesis and degradation. PolyP-ADP phosphotransferase (polyP kinase) from *Escherichia coli* can reversibly transfer the phosphate residue from a high energy donor to the polyP chain (Equation 2.1). Toerien *et al.* (1990) suggested that polyP kinase appears to be the key enzyme in polyP metabolism of some prokaryotic organisms. The second enzyme

catalyzing the synthesis of polyP is 1,3-diphosphoglycerate-polyphosphate phosphotransferase (Equation 2.2).

(C) Degradation of PolyP

The degradation of polyP is not a readily reversible reaction of synthesis catalyzed by polyP kinase. Enzymes involved in the polyP degradation were reported by Dawes and Senior (1973). These included polyP-AMP phosphotransferase, polyP-dependent NAD + kinase, polyP-glucokinase, polyP-fructokinase, polyP depolymerase, and polyphosphatases (Toerien *et al.*, 1990).

(D) Presence of PolyP in Microorganisms

PolyPs stored by PAOs are also known as 'volutin granules', because they were first found in *Spirillum volutans* (Schlegel, 1993). Accumulation of polyP is widely observed among bacteria, blue-green algae, fungi, protozoa, and algae (Dawes and Senior, 1973). Regulation of polyP metabolism and hence the polyP content in these microorganisms differs considerably and depends strongly on their growth conditions. Synthesis of polyP requires counter ions like Mg^{2+}, Ca^{2+}, and K^{+}.

Kulaev (1985) pointed out that polyP is important in several cellular functions. For example, (1) accumulating energy-rich ATP phosphorylic residues in an osmotically inert reserve material containing 'activated phosphate', (2) making cells more independent of environmental conditions through the accumulation of polyP reserves, (3) regulating ATP and other nucleotide levels in cells, (4) fulfilling the function of ATP in some cases by direct participation in phosphorylation reactions, (5) linking cations (e.g., K^{+}, Mg^{2+}, and Mn^{2+}) metabolism with that of polyP, (6) excreting heavy

metals (e.g., cadmium) via a metal phosphate transport system, and (7) contributing to cellular homeostasis and osmotic regulation. Whether these functions apply to PAOs in EBPR systems is not clear. However, polyP is assumed to act as an energy source for anaerobic substrate assimilation and PHA synthesis. Also, polyP may buffer EBPR biomass under alkaline conditions, regulating the intracellular pH of PAO by its degradation (Andreeva *et al.*, 1993). Studies further indicate a potential application of polyP metabolism in heavy metal bioremediation (Keasling and Hupf, 1996; Keyhani *et al.,* 1996). The ability of polyP to alter the behavior of cells during periods of starvation for inorganic Pi or energy can be further exploited to improve production of heterologous proteins in bacteria.

Table 2-1 Summary of all enzymatic reactions for polyP synthesis and degradation (McGrath and Quinn, 2003)

Enzyme	**Reaction**	**Equation No.**
PolyP Synthesis		
PolyP-ADP phosphotransferase (PolyP kinase)	$(polyP)_n + ATP \leftrightarrow (polyP)_{n+1} + ADP$	2.1
1,3-diphosphoglycerate-polyP phosphotransferase	$(polyP)_n$ + 1,3-diphosphoglycerate $\rightarrow$ 3-phosphoglycerate + $(polyP)_{n+1}$	2.2
PolyP Degradation		
PolyP-AMP phosphotransferase	$(polyP)_n + AMP \leftrightarrow (polyP)_{n-1} + ADP$	2.3
PolyP-dependent NAD + kinase	$(polyP)_n + NAD \leftrightarrow (polyP)_{n-1} + NADP$	2.4
PolyP glucokinase	$(polyP)_n$ + Glucose $\leftrightarrow (polyP)_{n-1}$ + Glucose 6-phosphate	2.5
PolyP fructokinase	$(polyP)_n$ + Fructose $\leftrightarrow (polyP)_{n-1}$ + Fructose 6-phosphate	2.6
PolyP depolymerase	$(polyP)_n + H_2O \rightarrow (polyP)_{n-x} + (polyP)_x$	2.7
Polyphosphatase	$(polyP)_n + H_2O \rightarrow (polyP)_{n-1} + (polyP)_1$	2.8

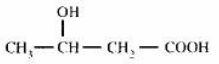
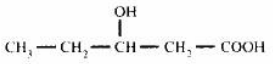
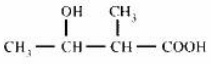
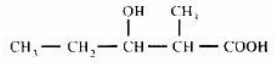
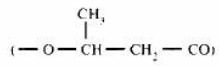
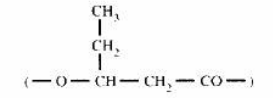
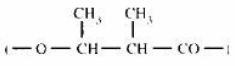
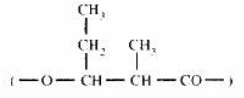
2.2.3 PHA Metabolism

(A) PHA

PHAs are biodegradable polyesters of various hydroxyalkanoates. They are accumulated as carbon/energy and/or reducing power storage material in various microorganisms usually under limiting conditions of nutritional elements (e.g., N, P, S, O or Mg) in the presence of excess carbon (Anderson and Dawes, 1990; Brandl *et al.*, 1990; Lee, 1996) or during adverse growth conditions (Reyes *et al.*, 1997). PHA normally occurs in granules with intensely staining response to Sudan black B (Serafim *et al.*, 2002; Jenkins *et al.*, 2004). Another specific and sensitive staining of PHA granules can be obtained with Nile blue A stain (Ostle and Holt, 1982).

To date, approximately 120 different constituents of PHAs have been identified (Steinbüchel and Valentin, 1995). Of these, 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 3-hydroxy-2-methylbutyrate (3H2MB), and 3-hydroxy-2-methylvalerate (3H2MV) are the common monomers of PHA found in the EBPR sludge (Table 2-2). The monomer composition of the PHA produced is variable, and dependent on the substrate(s) used and the environmental conditions (Lemos *et al.*, 1998). When acetate is the sole carbon source available in the anaerobic phase, the 3HB unit is usually the major unit in the PHA formed (Satoh *et al.*, 1992; Smolders *et al.*, 1994).

Table 2-2 Monometric units of PHA found in the EBPR sludge (Mino *et al.*, 1998)

	3-hydroxybutyrate (3HB)	3-hydroxyvalerate (3HV)	3-hydroxy-2-methylbutyrate (3H2MB)	3-hydroxy-2-methylvalerate (3H2MV)
Free Acid				
In PHA				
Precursors	2 acetyl-CoA	1 acetyl-CoA 1 propionyl-CoA	1 acetyl-CoA 1 propionyl-CoA	2 propionyl--CoA

(B) Biosynthesis and Degradation of PHB

The biosynthesis and degradation of PHB, which is very important to the understanding of substrate metabolism in EBPR process, has been well illustrated using *Azotobacter beijerinckii* (Toerien *et al.*, 1990) (Figure 2-3). The enzyme, β-ketothiolase catalyzes the condensation of two moles of acetyl-CoA to produce one mole of acetoacetyl-CoA. Acetoacetyl-CoA reductase further catalyzes the formation of β-hydroxybutyryl-CoA from acetoacetyl-CoA. $NADPH_2$ is the preferred coenzyme, while $NADH_2$ gives one-fifth of the rate. The final reaction is catalyzed by PHB granule-bound β-hydroxybutyryl CoA polymerase, which uses β-hydroxybutyryl-CoA as a substrate and releases CoASH.

Degradation of PHB to acetyl-CoA is not a reversible process of synthesis. The initial hydrolysis of PHB involves the production of β-hydroxybutyric acid, catalyzed by a PHB depolymerase (Toerien *et al.*, 1990). This intermediate is further oxidized to acetoacetic acid by a constitutive NAD-specific β-hydroxybutyrate dehydrogenase found in bacteria accumulating and degrading PHB. Acetoacetate degradation further proceeds to acetoacetyl-CoA, and acetoacetate succinyl-CoA: CoA-transferase is the

responding enzyme. The last degradation step of PHB to acetyl-CoA is catalyzed by ketothiolase, the same enzyme catalyzing the first reaction of biosynthesis.

(C) Presence of PHA in Microorganisms

The role of PHA, especially PHB, in microorganisms was reviewed and summarized by Dawes and Senior (1973) and Anderson and Dawes (1990). More than 300 different microorganisms are known to synthesize and accumulate PHAs intracellularly (Lee, 1996). Physiologically, this polymer can function as a carbon and/or energy source during starvation and in survival; a reserve of carbon and energy for the endergenic reactions involved in sporulation and encystment; an energy source of nitrogen fixation process between the bacteria and leguminous plants; or an electron sink during oxygen limiting conditions. The ability to accumulate and degrade intracellular storage PHA helps microorganisms to survive and compete in natural microbial communities. This polymer can be used as taxonomic characteristics for identifying the subspecies of the genus *Pseudomonas*. In addition, the level of PHA in a community can change rapidly due to variations in nutritional status (Elhottová *et al.*, 1997) and the ratio of PHA concentration to the total concentration of microbial biomass can thus serve as an important marker for the growth and nutritional status of microbial communities (Tunlid and White, 1992).

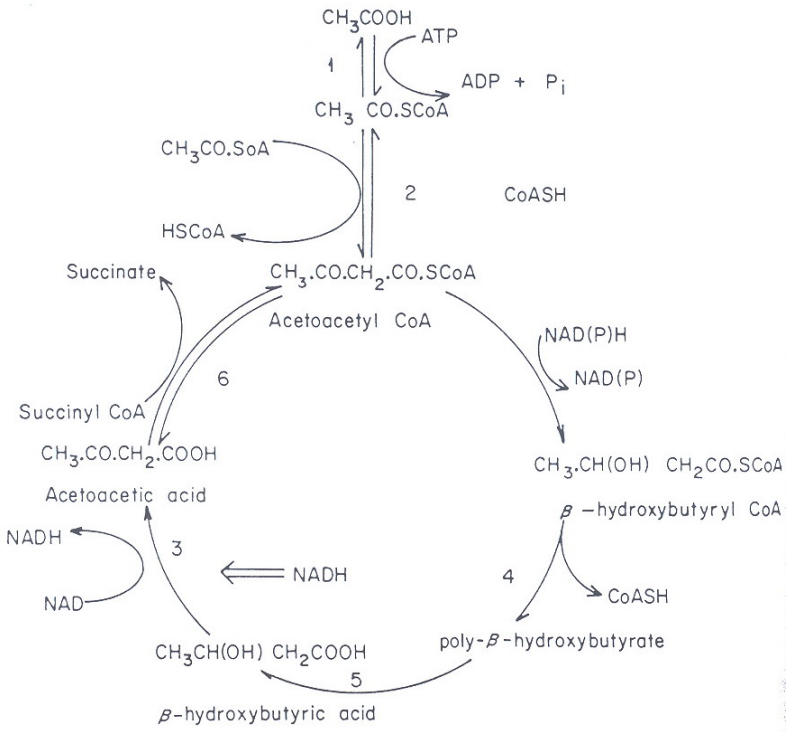


Figure 2-3 Biosynthesis and degradation of PHB. Key to enzyme: 1, thiokinase; 2, β-ketothiolase; 3, β-hydroxybutyrate dehydrogenase; 4, β-hydroxybutyryl CoA polymerase; 5, poly-β-hydroxybutyrae depolymerase; 6, acetoacetate succinyl CoA: CoA transferase. ⇒, inhibition. (Dawes and Senior, 1973).

(D) Factors Influencing PHB Biosynthesis and Degradation

Dawes and Senior (1973) indicated that oxygen limitation is the initiating factor for PHB accumulation by cells of *A. berijerinckii* in the presence of excess glucose. They found that accumulation of $NADPH_2$ can inhibit glucose metabolism via the Entner-Doudoroff (ED) pathway. As a result, citrate synthase and isocitrate dehydrogenase is inhibited, and acetyl-CoA, which is no longer able to be oxidized at a rapid rate via the tricarboxylic acid (TCA) cycle, is accumulated and channeled into PHB biosynthesis. It is because the cell can adjust to the new growth condition by re-oxidizing the excess reducing power in the PHB synthesis process. Thus, PHB would be functioning as an 'electron sink' in a quasi-fermentation process. Degradation of PHB as a result of

relaxation of oxygen limitation and exhaustion of external substrate proceeds when intracellular concentration of $NADP^+$ is increased and acetyl-CoA is decreased. Dawes and Senior (1973) further indicated that the regulation of PHB degradation and, specifically the PHB depolymerase, is controlled through the inhibition of β-hydroxybutyrate dehydrogenase by $NADH_2$, pyruvate, 2-oxoglutarate, or oxaloacetate.

However, very few studies have investigated the degradation and synthesis of PHB using bacterial strains isolated from EBPR processes. Lötter and Dubery (1989) studied the production of PHB in a polyP accumulating *Acinetobacter* strain. β-hydroxybutyrate dehydrogenase, an enzyme involved in the degradation of PHB, was extracted and detected, but was inhibited by high substrate concentrations, NADH, and acetyl-CoA.

2.2.4 The Comeau/Wentzel Model and the Mino Model

(A) Anaerobic Conversion of Acetate

The Comeau/Wentzel model uses acetate as the typical substrate and assumes *Acinetobacter* spp. as the bacteria mediating EBPR (Comeau *et al.*, 1986; Wentzel *et al.*, 1986). Under anaerobic conditions, polyP is postulated to serve as energy reserve both for the transport of carbon substrate (i.e., acetate) into the cell and for the synthesis of PHB. Release of Pi in the bulk solution is observed. This model further assumed that acetate diffuses into the cell in a protonated form and then dissociates in the cytoplasm at the expense of the pH gradient across the cell membrane. To maintain the cytoplasmic pH and the proton motive force, protons are translocated across the membrane at the expense of polyP energy bonds. As a result of the polyP breakdown, Pi is accumulated inside the cell, and released across the membrane into the bulk

solution. Acetate after transporting into the cell is converted to acetyl-CoA or via the formation of acetyl phosphate. In both cases, energy such as ATP is required. Finally, the model suggested that the acetyl-CoA is synthesized into PHB, and the required reducing equivalent can be supplied by assimilating part of the acetate taken up through the TCA cycle under anaerobic conditions. Since the synthesis of PHB from 2 acetyl-CoA can accept 1 reducing equivalent (i.e., $NADH_2$), the polymer can function as an electron sink when an electron acceptor is not available. The mass balance of the anaerobic substrate metabolism can be described by Equation 2.9.

$$9\text{Acetate} + (\text{polyP})_n + (\text{PHB})_n \rightarrow (\text{PHB})_{n+4} + (\text{polyP})_{n-9} + 9\text{Pi} + 2CO_2 \qquad 2.9$$

Based on the above equation, the expected theoretical molar ratio of P released to acetate take up (P/Ac) is 1. However, as reported by Comeau *et al.* (1987) the energy required for acetate transport inside the cell must be provided by the polyP hydrolysis. Thus, the P/Ac molar ratio increases to 1.5.

Mino *et al.* (1987) developed a similar biochemical pathway to explain the mechanisms of acetate uptake and P release under anaerobic conditions (Figure 2-4a). They proposed glycogen as another source of reducing equivalents for the conversion of substrates into PHA. This concept was developed based on a significant decrease in intracellular carbohydrate, possibly glycogen, during anaerobic acetate uptake. The Mino model suggested that the required reducing equivalent is supplied from the degradation of glycogen through the EMP pathway. Thus, the acetyl-CoA is produced and further converted into PHB. The overall mass balance for the model can be expressed by Equation 2.10.

$$6\text{Acetate} + (\text{polyP})_n + (\text{PHB})_n + (\text{glycogen})_n \rightarrow (\text{PHB})_{n+4} + (\text{polyP})_{n-3} + 3\text{Pi} + 2CO_2 + (\text{glycogen})_{n-1} \qquad 2.10$$

Based on this model, the P/Ac molar ratio is 0.5, lower than that suggested by the Comeau/Wentzel model.

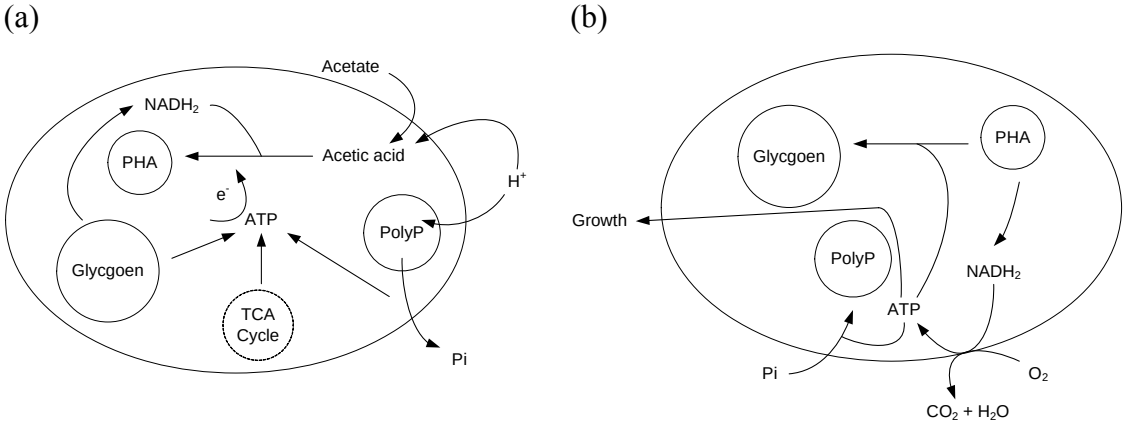


Figure 2-4 A summary of the major features of the biochemical models for EBPR. (a) Substrate uptake under anaerobic condition with acetate as the sole carbon source. PAOs obtain energy from the hydrolysis of polyP and release of Pi. Reducing equivalents come from the degradation of glycogen. (b) Growth under aerobic condition with no external carbon source present. The accumulated PHA is utilized for growth and for restoration of the pools of glycogen and polyP (modified from van Loosdrecht *et al.*, 1997 and Seviour *et al.*, 2003).

It is generally believed that the major part of the required reducing power is generated through the glycogen metabolism as suggested in the Mino model rather than through the TCA cycle as predicted by the Comeau-Wentzel model (Smolder *et al.*, 1994; Pereira *et al.*, 1996). By using P-removing sludge from a well-controlled SBR, it was shown that under absolute anaerobic conditions (1) extracellular acetate was completely converted to PHA, (2) the molar 3HB:Acetate ratio was 1.2:1.3, (3) cellular glycogen was consumed during the conversion of acetate into PHB, and (4) all cells contained glycogen at the start of the experiment. These data also show that glycogen not only served as a source of reducing equivalents but also as an energy source in addition to polyP. Furthermore, a ^{13}C tracer experiment using nuclear magnetic

resonance (NMR) (Satoh *et al.*, 1992) indicated that the acetate taken up by the sludge anaerobically was not oxidized to CO_2, suggesting TCA cycle was not involving in the substrate uptake.

Wentzel *et al.* (1991) evaluated the existing biochemical models and suggested a new one called the 'Adapted Mino Model'. They proposed that the glucose degradation following glycogen breakdown proceeded through the ED pathway, rather than the EMP pathway as originally suggested by Mino and his coworkers. This modification was based on a report that the EMP pathway did not operate in *Acinetobacter* spp (Juni, 1978).

On the other hand, evidences indicate the possibility of partial involvement of the TCA cycle in the generation of reducing power by PAO in the anaerobic stage of the EBPR process (Pereira *et al.*, 1996; Hesselmann *et al.*, 2000; Louie *et al.*, 2000; Yagci *et al.*, 2003). After feeding PAO-enriched sludge with ^{13}C-labeled acetate, CO_2 release was observed under absolute anaerobic conditions (Pereira *et al.*, 1996). So far, this is the only experimental result indicating the possible function of the TCA cycle in the anaerobic phase of the EBPR process. The function of the TCA cycle in the anaerobic uptake of carbon sources by PAO as well as contribution to the microbial selection in the EBPR process remains to be further investigated.

(B) Anaerobic Conversion of Lactate

When lactate was used anaerobically by a PAO-enriched sludge, a significant amount of 3HV was detected in the PHA produced (Satoh *et al.*, 1992). A 3HV unit of PHA is made from an acetyl-CoA molecule and a propionyl-CoA molecule. This experimental

result strongly implied that there should be a pathway to convert lactate to propionyl-CoA. To explain this result, Satoh *et al.*, (1992) assumed partial conversion of pyruvate to propionyl-CoA through the succinate-propionate pathway. The succinate-propionate pathway is usually observed in certain anaerobic bacteria during the metabolism of pyruvate to propionate (Gottschalk, 1986). This pathway consumes reducing power (NADH), which is provided from the glycolysis of glycogen to acetyl-CoA. Based on such an assumption, a comprehensive concept (Figure 2-5) is proposed to describe the metabolic strategy of PAO to grow in EBPR processes (Mino, 2000). According to this concept, the glycogen storage functions as a regulator of the redox balance in the cell. Conversion of glycogen to acetyl-CoA and CO_2 generates reducing power, whereas conversion to propionyl-CoA via the succinate-propionate pathway consumes reducing power. It is essential for PAO to take up carbon sources faster than other bacteria in the anaerobic stage regardless of the types of substrates present in the influent wastewater. The above mechanism can provide PAO with a capability to take up various kinds of reduced or oxidized organic substrates in the anaerobic phase without disturbing the redox balance in the cell.

(C) Aerobic Condition

Under aerobic conditions, both the Wentzel/Comeau and Mino models assumed that the responding bacteria can utilize the accumulated PHB as a reserve of carbon and energy both for polyP accumulation from Pi uptake and for cell synthesis and growth, in the absence of external carbon sources (Figure 2-4b). The Mino model further suggested that part of the accumulated PHB can be used for the re-synthesis of glycogen.

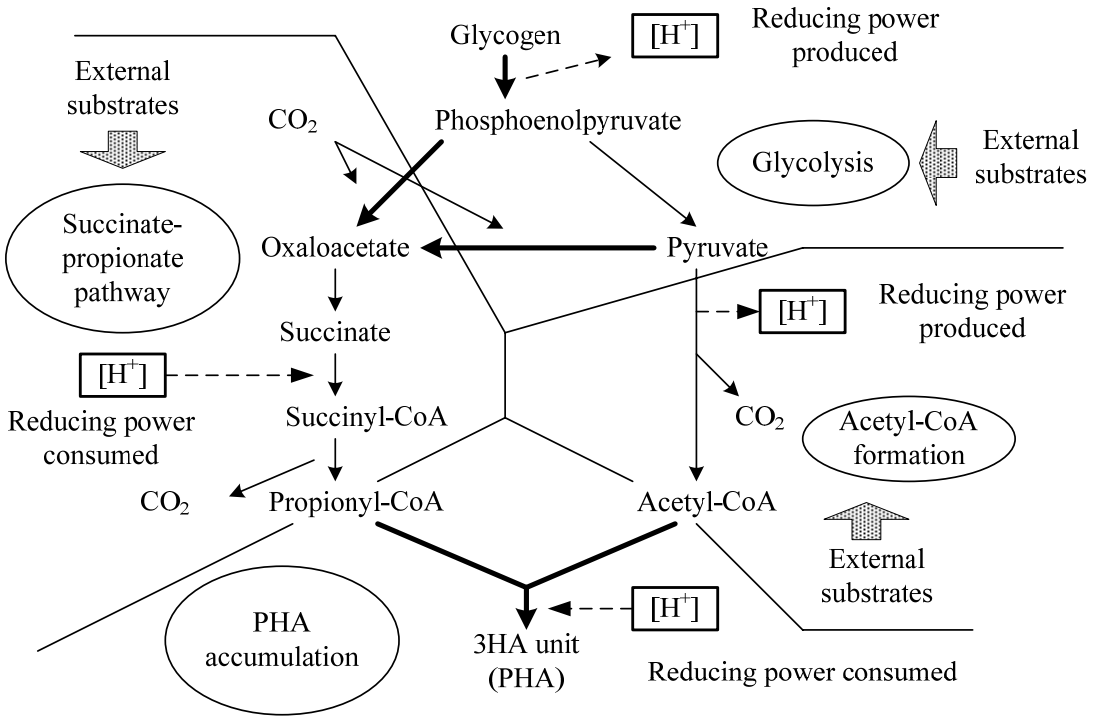


Figure 2-5 A conceptual model for anaerobic carbon metabolism in an EBPR process (Mino, 2000).

2.2.5 GAOs Competing Mechanism

(A) Presence of GAOs

It was reported that anaerobic-aerobic operation sometimes does not exhibit EBPR activity for unknown reasons (Matsuo *et al.*, 1982; Fukase *et al.*, 1985; Cech and Hartman, 1990; 1993). The first observation of EBPR deterioration due to population change was reported by Cech and Hartman (1993). They observed clusters of large Gram-positive and Gram-negative cocci in distinctive tetrads, called 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 substrates in the anaerobic zone for PHA production with no concomitant Pi release. This stored PHA could support the growth of these populations in the aerobic zone and for intracellular carbohydrate accumulation instead of polyP storage. Subsequently, Liu *et al.* (1994)

showed that the intracellular carbohydrate stored in the anaerobic-aerobic sludge with a minimized polyP content was a polymer consisting of glycosyl units with the α-1,4- and the α-1,6-linkages, or glycogen. Thus, these populations were named as the glycogen accumulating organism (GAO) (Liu *et al.*, 1996).

(B) Anaerobic Conditions

PAOs and GAOs apparently fall into two different physiological groups but share some common metabolic traits (Table 2-3). Both groups can take up exogenous carbon substrate and simultaneously transform them into PHA under anaerobic conditions. According to Satoh *et al.* (1994) and Liu *et al.* (1994), glycogen serves as a source of ATP through glycolysis and as a means of maintaining the redox balance inside the cell through production of $NADH_2$ during acetate uptake under anaerobic conditions. ATP is used for transport 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. Some of the $NADH_2$ produced during glycolysis is utilized during the production of PHA, but excess $NADH_2$ is produced because of the ATP requirements. Therefore, to maintain the redox balance, other metabolic processes were involved in the consumption of $NADH_2$. Mino *et al.* (1987) suggested that 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 amount of pyruvate directed to this pathway is regulated so that the overall amount of $NADH_2$ produced is equal to the amount consumed during the process. Therefore, the succinate-propionate pathway is used to balance the redox potential inside the cell. In contrast to the ATP production initially proposed by Mino *et al.* (1987), Zeng *et al.* (2002) indicated that

this pathway produces no ATP. The remaining pyruvate is decarboyxlated to form acetyl-CoA, providing additional $NADH_2$ as well as generating CO_2. Finally, the acetyl-CoA is produced through glycolysis and acetate activation, and the propionyl-CoA is produced through the succinate-propionate pathway. Both acetyl-CoA and propionyl-CoA are then reduced and condensed as 3HV, a precursor of PHA.

Table 2-3 Comparison of metabolisms of PAOs and GAOs (Mino *et al.*, 1998)

Metabolism	**PAOs**	**GAOs**
In the anaerobic phase	+	+
Uptake of external organic substrates	+	+
Consumption of intracellular glycogen	+	+
Accumulation of intracellular PHA	+	+
Consumption of intracellular polyP and consequent release of Pi	+	-
In the aerobic phase	+	+
Recovery of intracellular glycogen	+	+
Consumption of stored PHA	+	+
Growth	+	+
Recovery of intracellular polyP	+	-

'+': involve; '-ve': not involve.

2.3 Factor Influencing the EBPR Process

2.3.1 Operational Conditions

(A) Solids Residence Time

In general, the EBPR process is not very sensitive to the solids residence time (SRT) and it has been shown in practice good P removal is possible at SRTs ranging from 3 to 68 days (Reddy, 1998). Smolders *et al.* (1995) observed that the biomass yield did not depend on the SRT, because of relative low maintenance requirement for PAOs in comparison with normal heterotrophs. A low decay rate observed with the PAOs

suggested that a proportionally larger part of the active biomass would consist of PAOs at longer SRTs. Consequently, the total phosphorus (TP) content of the biomass also increased with an increase in SRT (Wentzel *et al.*, 1989a; Chuang *et al.*, 1996; Lesjean *et al.*, 2003).

For non-PAOs, an increased SRT was linked only with an increased biomass concentration, i.e., the minimum SRT was directly linked with the maximum growth rate of the microorganisms ($SRT_{min} = 1/\mu_{max}$). However, Smolders *et al.* (1995) and Brdjanovic *et al.* (1998a) reported experimental evidences for a more complicated linkage between SRT and EBPR operation since storage polymers were involved in PAOs. Changing SRT resulted in variation in internal storage composition and possibly deterioration of the EBPR process. Smolders *et al.* (1995) indicated that at an SRT < 3 days, the polyP content was too low to allow complete uptake of acetate at anaerobic condition. As a result, EBPR efficiency was deteriorated under sufficient external acetate conditions. Wentzel *et al.* (1989a) also observed an unstable process and poor quality effluent when the EBPR process was operated at an SRT < 3 days.

(B) Anaerobic and Aerobic Hydraulic Retention Times

Anaerobic and aerobic hydraulic retention times (HRTs) are among the most important design and operation parameters affecting EBPR efficiency (Brdjanovic *et al.*, 1998a; Wang and Park, 2001). Long anaerobic and aerobic HRTs will result in a negative effect on EBPR processes (Tammink *et al.*, 1996; Brdjanovic *et al.*, 1998a). Bernard *et al.* (1985) termed the system with an excessive anaerobic HRT with no uptake of acetate as a secondary P release. Because the secondary P release is not associated with the storage of PHA, PAOs will have lower cellular energy for subsequent P uptake in

the aerobic zone. Consequently, they may lose their competitive edge against GAOs in EBPR systems. Therefore, an optimal anaerobic HRT should be based on the VFA uptake rate. Like anaerobic HRT, an extensive aerobic HRT, could cause the depletion of cellular PHA, and should be avoided (Bedjanovic *et al.*, 1998a). An optimal aerobic HRT should be based on the amount of PHA synthesized (or the influent organic concentration) during the anaerobic stage and the target effluent Pi concentration.

In contrast, Filipe *et al.* (2001) showed that the energetics of GAOs are less favorable under anaerobic starvation conditions than the energetics of PAOs. Thus, a way to promote the growth of PAOs over GAOs is to hold the community under anaerobic conditions for a longer period than is needed for uptake of VFAs, thereby forcing the bacteria to use internal storage products to meet maintenance energy needs. PAOs can generate ATP for maintenance energy from polyP degradation, whereas GAOs must provide ATP through glycolysis of stored glycogen. Because recovery of polyP is easier than recovery of glycogen, increasing the time under anaerobic conditions could have a larger effect on GAOs than PAOs, giving the PAOs a competitive advantage. In support of this concept, Matsuo (1994) observed that when the anaerobic HRT was increased in a deteriorated EBPR system, effective P removal was recovered.

(C) pH in Anaerobic Phase

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.*, 1994; Fleit, 1995). Using a PAO-enriched culture, Smolders *et al.* (1994) 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 Pi release because more energy is required to maintain a constant pH gradient through 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 Pi. Subsequently, the amount of PHA used for uptake of Pi in the aerobic phase will be increased.

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 will cause an increase in the amount of glycogen consumed under anaerobic conditions. Thus, more glycogen will need to be produced in the aerobic phase. Thus, a larger percentage of the PHA accumulated in the anaerobic phase will 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 is 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 will make the growth of GAOs less favorable.

(D) Temperature

The effects of temperature on the efficiency and kinetics of EBPR systems have been studied in the past two decades, but the results were inconsistent. Early studies (Sell, 1981; Ekama *et al.*, 1984; Barnard *et al.*, 1985) reported that EBPR efficiency at low temperatures (5 to 15ºC) was higher than at high temperatures (15 to 24ºC). Although lower temperatures appear to reduce metabolic activities of the microbial population, EBPR systems could perform more efficiently at low temperatures. Panswad *et al.* (2003) suggested that PAOs could be either mesophilies or psychrophiles, and

predominated when temperature was at 20ºC or lower, and GAOs could be mesophilies with optimum temperatures between 25 and 32.5ºC.

Contradictory findings (McClintock *et al.*, 1992; Jones and Stephenson, 1996; Brdjanovic *et al.*, 1997, 1998b; Baetens *et al.*, 1999) indicated a complete or partial loss of EBPR performance at cold temperatures. Such deterioration of EBPR performance might be caused by a population shift from PAOs to GAOs or to other heterotrophs, but no investigation on PAO-GAO population shifts has been performed. The impact of long-term (6-7 weeks) changes in temperature on kinetics of the anaerobic and aerobic phases of EBPR showed that temperature had only a moderate impact on the aerobic P-uptake, but a strong effect on PHA consumption, oxygen uptake, and bacterial growth (Brdjanovic *et al.*, 1998b).

2.3.2 Influent Substrate Composition

(A) Presence of Glucose

Like PAOs, GAOs can assimilate VFAs and glucose under anaerobic conditions, but without concurrent P release (Cech and Hartman, 1993; Carucci *et al.*, 1994; Liu *et al.*, 1996b; Liu, 1998; Sudiana *et al.*, 1999). This suggests a potential competition in substrate between PAOs and GAOs. Liu *et al.* (1994) indicated that GAO could use external glucose instead of intracellular glycogen as the reducing power and energy source for anaerobic PHA formation. Compared to anaerobic glucose assimilation, Maurer (1997) concluded from their NMR studies that anaerobic acetate assimilation has higher energy demand and cells need to store higher levels of polyP to achieve it. This could be a reason for the fact that presence of glucose in the influent often induces proliferation of GAOs (Cech and Hartman, 1990; 1993).

In contrast, good EBPR has been occasionally achieved with glucose as a major source (Jeon *et al.*, 2000; Jeon and Park, 2000; Wang *et al.*, 2002). Wang *et al.* (2002) suggested that a stable EBPR process fed with glucose can be maintained in a SBR under longer anaerobic reaction time, higher glucose content in the feed, and shorter aerobic reaction time with lower DO level. Comparing to an acetate-fed EBPR reactor, less Pi release into the medium was observed with PHV as the major PHA component accumulated under anaerobic conditions, suggesting different metabolic pathways for anaerobic acetate and glucose assimilations, respectively.

(B) Limited P Source

Limited Pi loadings were demonstrated to suppress the development of PAO leading to the establishment of GAO (Liu *et al.*, 1994). Liu *et al.* (1997b) proposed that the PAO could assimilate acetate anaerobically at a higher rate than the GAO. However, under limited Pi loading conditions, insufficient intracellular polyP was available to generate enough energy for anaerobic acetate assimilation and, hence, 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 and, subsequently, utilize the PHA 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).

(C) High Organic Loading

In EBPR processes, increases in the organic matter will generally increase the P release and the PHA storage in the anaerobic zone under an optimum VFA versus P

ratio (Reddy, 1998). When VFAs were present in the anaerobic phase in excess of the requirements for PAOs, GAOs could possibly function as a scavenger for organic substrates, and become the dominated population in the deteriorated EBPR processes (Liu *et al.*, 1996b). In addition to the proliferation of GAOs, if the organic substrate is not totally sequestered in the anaerobic zone, residual substrate could support the growth of filamentous bacteria in the aerobic zone (Chang *et al.*, 1996).

(D) Deficiency of Amino Acids

Although amino acids are present in considerable amounts in the wastewater influent at EBPR plants, their roles in related to the EBPR activity and the microbial competition between PAOs and GAOs have attracted surprisingly little interest. Both glutamate and aspartate could be used as sole carbon sources to support EBPR in laboratory-scale reactors (Satoh *et al.*, 1990; 1998). Satoh *et al.* (1994) postulated that if the influent contains a low content of amino acids or proteins, the growth rate of PAOs could be reduced, leading to the proliferation of GAOs. Furthermore, it was found that the stored polymers composed of intracellular PHA and polypeptide (not yet chemically characterized and identified) when glutamate was used as substrate (Satoh *et al.*, 1990; 1998).

2.3.3 Other Mechanisms of EBPR Deterioration

Although the dominance of GAOs were usually assumed to cause the deterioration of EBPR, Fang *et al.* (2002) and Okunuki *et al.* (2004) recently observed that no substrate uptake was observed under anaerobic periods and suggested that the GAOs competing mechanism could not apply to their reactors. Okunuki *et al.* (2004) suggested the excess P release in the anaerobic period could lead to deterioration, while Fang *et al.*

(2002) suspected that GAO might not be the sole group of bacteria responsible for the deterioration of P removal efficiency in an EBPR reactor.

2.4 Microbial Communities Observed in Effective EBPR Processes

2.4.1 Isolation of PAO from EBPR Systems

Traditional microbiological techniques of enrichment and isolation have isolated a few bacterial species may participate in EBPR (Table 2-4).

(A) *Acinetobacter* spp.

Acinetobacter spp. were first isolated by Fuhs and Chen (1975) from biomass with high P removal capacity. Subsequently many researchers also isolated *Acinetobacter* spp. from laboratory-scale reactors and full-scale EBPR plants (Buchan, 1983; Lötter, 1985; Wentzel *et al.*, 1988). Some of these *Acinetobacter* spp. were reported to store Pi as intracellular polyP granules, up to 10-13.6% P of biomass dry weight, under aerobic conditions (Deinema *et al.*, 1980), and hence were suspected as the organisms responsible for EBPR in treatment systems.

However, the phenotypic traits of these *Acinetobacter* isolates do not necessarily match with that of PAO-enriched sludge. These isolates could not take up acetate anaerobically even though Pi release was observed (Ohtake *et al.*, 1985), and could not take up Pi in the absence of carbon sources under aerobic conditions. When subjected to repetitive anaerobic-aerobic conditions (Tandoi *et al.*, 1987), they also failed to show the essential characteristics that observed in EBPR processes.

Different culture-independent methods like fluorescent antibody staining (Cloete and Steyn, 1988), quinone profile measurement (Hiraishi *et al.*, 1989; Hiraishi and Morishita, 1990) and fluorescence *in situ* hybridization (FISH) with an oligonucleotide probe specific for *Acinetobacter* (Wagner *et al.*, 1994; Kämpfer *et al.*, 1996) have been used to investigate the role of *Acinetobacter* in laboratory-scale or full-scale EBPR processes. In all cases, *Acinetobacter* spp. were found to represent only a small proportion of the total EBPR microbial population and could not account for the P removal observed in activated sludge. Thus, it was suggested that the classical culture-dependent methods were strongly biased to the isolation of *Acinetobacter* spp.

Considerable effort was also made to resolve the taxonomy of *Acinetobacter* spp. isolated from EBPR processes. Most of them were successfully identified as *A. junii*, *A. lwoffii*, and *A. johnsonii*, but other could not be identified (Seviour *et al.*, 2003). Of these, low levels of anaerobic P-release were detectable in *A. johnsonii* strain 210A but it could not assimilate acetate and synthesize PHA under anaerobic conditions (inconsistent with EBPR biochemical model). There are several explanations for such unusual results (Tandoi *et al.*, 1998). These include the possibility that the biochemical models are incomplete or incorrect, that *Acinetobacter* in pure culture behaves differently than that in EBPR systems, or that *Acinetobacter* is not the major PAO responsible for carrying out the chemical changes so characteristics of EBPR sludge.

(B) *Microlunatus phosphovorus*

M. phosphovorus, a gram-positive high G+C coccus, was isolated from a laboratory-scale EBPR process (Nakamura *et al.*, 1991; 1995). This isolate exhibited some metabolic traits described for the EBPR model. These included the accumulation of

large amounts of polyP under aerobic conditions, and uptake of carbon sources like glucose and casamino acids with simultaneous release of Pi and degradation of polyP under aerobic conditions. This isolate could not take up acetate or accumulate PHA under anaerobic conditions. Results of FISH using a 16S rRNA-targeted oligonucleotide probe for *M. phosphovorus* in EBPR sludge indicated that *M. phosphovorus* constituted less than 3% of total bacterial cells showing positive polyP stain (Kawaharasaki *et al.*, 1998). The role of *M. phosphovorus* to EBPR was further studied using ^{31}P and ^{13}C NMR (Santos *et al.*, 1999). *M. phosphovorus* could assimilate and transform glucose to acetate and poly-glucose under anaerobic conditions, and could respire the stored poly-glucose aerobically for growth. Ubukata and Takii (1994) also isolated a bacterium that resembled *M. phosphovorus* morphologically and physiologically. They demonstrated that the bacterium exhibited the anaerobic utilization and aerobic accumulation of polyP only after cultivation under alternating anaerobic and aerobic conditions. This result implied that the enzyme system for the polyP metabolism is not constitutive but inductive.

(C) *Lampropedia* spp.

Lampropedia spp., a gram-negative coccus, was isolated from an SBR system designed for EBPR (Stante *et al.*, 1997). This isolate could take up acetate and store it as PHA with concomitant polyP degradation and Pi release under anaerobic conditions. However, the Pi release to acetate uptake ratio (i.e., Pi/Ac ratio) was higher than that predicted by the Mino model. Despite exhibiting the key metabolic characteristics of PAO, it has a very unique sheet-like cell morphology that is not commonly observed in EBPR processes. In general, the role of *Lampropedia* spp. in EBPR still remains unclear.

(D) *Tetrasphaera* spp.

The genus *Tetrasphaera* in the family *Intrasporangiaceae* of the class *Actinobacteria* currently consists of three main species, *T. australiensis* (cocci growing in tetrads), *T. japonica* (cocci growing in tetrads), and *T. elongate* (short rods). These isolates were all isolated from samples of activated sludge biomass by micromanipulation (Maszenan *et al.*, 2000a; Hanada *et al.*, 2002). In the study by Liu *et al.* (2001) with an efficient laboratory-scale EBPR process, *Tetrasphaera* spp. were found as one of the dominant microbial populations. Using FISH with probe actino_1011 targeting a rod-shaped bacterial morphotype related to *Tetrasphaera*, probe action_1011 hybridized to cells showing positive polyP staining but did not accumulate PHA anaerobically. In a full-scale EBPR plant, Kong *et al.* (2005) further designed two oligonucleotide probes to clarify the phylogenies of different morphotypes hybridizing by probe actino_1011. Two morphotypes, cocci in clusters of tetrads and short rods in clumps, could be hybridized with probes actino_221 and actino_658 respectively, and responded positively with the Neisser stain.

(E) *Tessaracoccus bendigoensis*

T. bendigoensis, a high-G+C gram-positive coccus, was isolated using micromanipulation from a laboratory-scale anaerobic-aerobic SBR fed with a mixture of acetate and glucose (Maszenan *et al.*, 1999). It appeared as cocci or clusters of cocci in regular tetrads, and was morphologically similar to the dominant organism observed in the biomass. *T. bendigoensis* can accumulate polyP aerobically, with no PHA accumulation. The environmental factors affecting the accumulation of these polymers as well as its physiological traits remain unknown.

(F) *Paracoccus denitrificans*

P. denitrificans is a gram-negative cocci from the *Alphaproteobacteria*. PolyP accumulation by *P. denitrificans* was examined under aerobic, anoxic, and anaerobic conditions (Barak and van Rijn, 2000). PolyP synthesis took place with either oxygen or nitrate as the electron acceptor and in the presence of an external carbon source. Cells contained PHB granule, but no polyP could be produced when PHB-rich cells were incubated under anoxic conditions in the absence of an external carbon source. In contrast to PAOs, *P. denitrificans* was able to remove P without the need for alternating anaerobic/aerobic conditions.

(G) *Burkholderia cepacia*

B. cepacia isolated from a municipal WWTP was observed to contain detectable intracellular polyP inclusions under acidic conditions as revealed by Neisser stain (Mullan *et al.*, 2002). At pH 5.5, a maximum level of intracellular polyP that comprised 13.6% of cellular dry weight was accumulated and high polyphosphate kinase activity was detected in actively growing cells.

(H) *Malikia* spp.

M. granosa is a gram-negative motile, rod-shape bacterium isolated from a municipal WWTP (Spring *et al.*, 2005). It is phylogenetically affiliated with the family *Comamonadaceae*. One of its closest relatives is *Pseudomonas spinosa*. As *P. spinosa* was misclassified, and it was renamed as *M. spinosa*. Cells of the genus *Malikia* were able to accumulate large amounts of PHA and polyP in the form of large intracellular granules. However, their occurrence in full-scale EBPR process has not been investigated yet.

2.4.2 Culture-independent Approach

Culture-independent technologies for direct measures of abundance, diversity, and phylogeny of individual members of complex microbial communities have provided new insights to EBPR processes (Tables 2-5 and 2-6). These technologies applied different biomarker-based methods like polyamines, respiratory quinones, fatty acid profiles, FISH, construction of 16S rRNA clone libraries, and the electrophoretic separation and analysis of PCR-amplified 16S rRNA fragments have contributed a radical revision of the understanding of the microbial ecology of the process. Using these methods, at least 30 different phylotypes from major phyla of the domain *Bacteria* have been described (Liu *et al.*, 2000).

(A) *Rhodocyclus*-related PAO

The presence of the *Rhodocyclus* group in EBPR processes was first reported by Bond *et al.* (1995) using 16S rRNA gene clone library, and later confirmed by other studies (Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000; Liu *et al.*, 2001). FISH with oligonucleotide probes targeted for the *Rhodocyclus*-related clones showed that an acetate-fed EBPR reactor was dominated by the *Rhodocyclus* group up to 81% of the DAPI-stained cells. Staining of intracellular polyP and PHA confirmed that the dominant populations in the *Rhodocyclus* group were responsible for the key metabolism of PAO, namely acetate uptake, PHA accumulation, P-release, and polyP degradation under anaerobic conditions. This result strongly suggested that members of the *Rhodocyclus* group were responsible for EBPR observed in treatment systems.

(B) Yeast Spores

It was reported that in addition to bacteria, eukaryotic yeast spores in distinctively large clustered cells seen in a full-scale EBPR plant community could involve in EBPR (Melasniemi *et al.* 2000). These yeast cells showed positive response on polyP staining. An unusual response to the Gram stain was detected which characteristically stained in orange brown color. Cells with similar characteristics have also been reported elsewhere (Crocetti *et al.*, 2000) but were hybridized by the FISH probe designed against the *Rhodocyclus*-related PAO.

Chua *et al.* (2004) revealed the identity and the *in situ* physiology of large clustered cells in the biomass from a conventional plant at Rosebud, Victoria, Australia. FISH analysis showed that these cells were likely prokaryotic and related to the *Betaproteobacteria*, but were not related to *Rhodocyclus*-related PAO, since they did not respond to the PAOmix FISH probes.

(C) Filamentous Bacteria

PolyP accumulation was occasionally observed with some filamentous foaming bacteria like *Microthrix parvicella* and *Nostocodia limicola* II in EBPR system (Seviour *et al.*, 1990). Both these filamentous bacteria stain positively for Neisser stain in pure culture and *in situ* (Erhart *et al.*, 1997; Blackall *et al.*, 2000; Liu *et al.*, 2001). However, it is still not clear if they can participate in EBPR activity.

Table 2-4 Bacterial species and genera that might be involved in induction of or participating in EBPR (modified from de-Bashan and Bashan, 2004)

Bacterial genera or species	Evidence for involvement in EBPR	Evidence against involvement in EBPR	Reference
Acinetobacter spp.	1. Dominant populations based on culturing method 2. Accumulating polyP and PHA under aerobic conditions	1. Selective culturing method for *Acinetobacter* spp. 2. Not primarily responsible for EBPR and detecting as a small percentage in the sludge (< 10% of total bacteria) using fluorescent antibody staining, quinine profile or FISH with oligonucleotide probe 3. No strain possesses the typical metabolic pathways of EBPR (e.g., acetate uptake, PHA accumulation, polyP hydrolysis, and Pi release under anaerobic conditions 4. Using quinine Q-9 as dominant respiratory mechanism, when PAO possesses Q-8 or MK8(H_4). 5. Containing polyamine diaminopropane, which was not detected in EBPR processes	Fuhs and Chen (1975), Cloete and Steyn, (1988), Hiraishi *et al.* (1989), Hiraishi and Morishita, (1990), Wagner *et al.* (1994), Kämpfer *et al.* (1996), Christensson *et al.* (1998), Mino *et al.* (1998)
Microlunatus phosphovorous	1. Accumulating large amounts of polyP under aerobic conditions 2. Anaerobic uptake of glucose	1. Not taking up acetate and not accumulating PHA under anaerobic conditions 2. Detecting only <3% of total bacteria in the sludge using FISH with oligonucleotide probe, when PAO are about 9% of the total population 3. Containing Q-9 as a major quinine but not the common Q-8 and MK-8(H_4) of PAO-enriched sludge	Nakamura *et al.* (1995), Kawaharasaki *et al.* (1998), Santos *et al.* (1999), Eschenhagen *et al.* (2003)
Lampropedia sp.	1. Possessing key metabolic characteristics of PAO. Accumulating polyP and PHB 2. Taking up acetate and storing it as PHA with polyP degradation and releasing of Pi	A unique sheet-like organization, which is uncommon in EBPR processes	Stante *et al.* (1997)
Rhodocyclus sp.	1. Possessing key metabolism of PAO 2. FISH show that the group dominates the EBPR process (> 81% of the population) 3. The behavior of these bacteria generally was consistent with the biochemical models proposed for	No available data	Bond *et al.* (1999), Hesselmann *et al.* (1999), Crocetti *et al.*, (2002), Ahn *et al.* (2002), Eschenhagen *et al.* (2003), Lee *et al.*

	PAO		(2003), Kong *et al.* (2004)
Tetrasphaera japonica, *Tetrasphaera australiensis*	PolyP accumulating cocci	Microscopically similar to GAO from activated sludge	Maszenan *et al.* (2000), Eschenhagen *et al.* (2003)
Tessaracoccus bendiogoensis	1. Containing intracellular polyP granules 2. Morphologically similar to dominant microorganisms in activated sludge	Resembles GAO	Maszenan *et al.* (1999)
Paracoccus denitrificans	PolyP accumulating bacterium	1. Does not need the alternating anaerobic-aerobic cycle for polyP accumulation 2. Accumulating PHB, but not polyP when cells are rich in PHB	Barak and van Rijn (2000)
Burkholderia cepacia	Containing intracellular polyP granules	No available data	Mullan *et al.* (2002)
Malikia spp.	Containing intracellular polyP granules	No available data	Spring *et al.* (2005)
Agrobacterium sp. *Aquaspirillum* sp., *Micrococcus* sp., *Staphylococcus* sp., *Acidovorax* sp., *Microsphaera multipartite*, *Dechlorimonas* spp., Unidentified yeast, CFB group	Found in large and dominate numbers in EBPR processes	No available data	Melasniemi *et al.* (1998), Merzouki *et al.* (1999), van Ommen Kloeke and Geesey (1999), Melasniemi and Hernesmaa (2000), Ahn *et al.* (2002)

Table 2-5 A summary of laboratory-scale studies on the population composition of EBPR communities using culture independent molecular techniques (modified from Seviour *et al.*, 2003)

Reactor configuration	**Reactor feed**	**Method of community analyses**	**Major populations detected (> 10% of total)**	**PAO identified**	**References**
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	16S rRNA clone library	*Alphaproteobacteria* (13%) *Betaproteobacteria* (33%) *Planctomycetes* (13%)	n.d	Bond *et al.* (1995)
Continuous anaerobic-aerobic reactor	Synthetic sewage with acetate as carbon source	16S rRNA clone library	*Alphaproteobacteria* (16%) *Actinobacteria* (37%)	n.d.	Christensson *et al.* (1998)
Anaerobic-aerobic SBR	Synthetic sewage with glucose as carbon source	Quinone profiles, FISH	*Alphaproteobacteria* (14%) *Betaproteobacteria* (30%) *Gammaproteobacteria* (12%) *Actinobacteria* (14%)	n.d.	Sudiana *et al.* (1998)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	Quinone profiles, FISH	*Alphaproteobacteria* (15%) *Betaproteobacteria* (34%) *Gammaproteobacteria* (12%) *Actinobacteria* (16%)	n.d.	Sudiana *et al.* (1998)
Anaerobic-aerobic SBR	Synthetic Sewage with acetate and peptone as carbon sources	FISH	*Betaproteobacteria* (45%) *Beta-2-proteobacteria* (55%) *Actinobacteria* (35%)	n.d.	Bond *et al.* (1999)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	FISH	*Betaproteobacteria* (64%) *Actinobacteria* (10%)	n.d.	Kawaharasaki *et al.* (1999)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	16S rRNA clone library, FISH	*Betaproteobacteria* (89%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Hesselmann *et al.* (1999)
Anaerobic-aerobic SBR	Complex synthetic sewage	16S rRNA clone library, FISH	*Betaproteobacteria* (34%) *Actinobacteria* (20%) CFB Group (16%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Hesselmann *et al.* (1999)
Anaerobic-aerobic SBR	Synthetic sewage with acetate and peptone as carbon sources	Quinone profiles, DGGE and 16S rRNA clone library	*Alphaproteobacteria* (n.d.) *Betaproteobacteria* (n.d.) *Actinobacteria* (n.d.) (with quinone profiling)	n.d.	Liu *et al.* (2000)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	16S rRNA clone library, FISH	*Alphaproteobacteria* (12%) *Beta-2-proteobacteria* (80%) *Actinobacteria* (28%) CFB Group (14%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Crocetti *et al.* (2000)
Anaerobic-aerobic SBR	Synthetic sewage with	16S rRNA clone	CFB Group (39%)	*Rhodocyclus*-related organisms	Dabert *et al.*

	acetate as carbon source	library, SSCP profiles	*Betaproteobacteria* (17%)	(*Betaproteobacteria*)	(2001)
Continuous anaerobic-aerobic reactor	Synthetic sewage with acetate, propionate and peptone as carbon sources	16S rRNA clone library, DGGE, FISH	*Betaproteobacteria* (41%) *Actinobacteria* (18%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Liu *et al.* (2001)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	DGGE and FISH	*Betaproteobacteria* (n.d.)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Ahn *et al.* (2002)
Anaerobic-aerobic SBR	Synthetic sewage with mixture of volatile fatty acids as carbon sources	FISH	*Alphaproteobacteria* (25%) *Betaproteobacteria* (54%) *Gammaproteobacteria* (12%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Levantesi *et al.* (2002)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	FISH	*Alphaproteobacteria* (14%) *Betaproteobacteria* (77%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Kong *et al.* (2002a)
Continuous anaerobic-aerobic reactor (EBPR, no nitrification)	Complex municipal sewage	16S rRNA clone library, T-RFLP, FISH	*Alphaproteobacteria* (15 with FISH; 22% with 16S rRNA) *Betaproteobacteria* (20% with FISH) *Gammaproteobacteria* (21% with 16S rRNA) CFB Group (13% with FISH) *Actinobacteria* (18% with FISH; 31% with 16S rRNA)	*T. australiensi* and *T. Japonica* (*Actinobacteria*)	Eschenhagen *et al.* (2003)
Continuous Phoredox-system (EBPR, nitrification and denitrification)	Complex municipal sewage	16S rRNA clone library, T-RFLP, FISH	*Alphaproteobacteria* (15% with FISH; 25% with 16S rRNA) *Betaproteobacteria* (17% with FISH) CFB Group (12% with FISH) *Actinobacteria* (14 with FISH; 23% with 16S rRNA)	*T. australiensi* and *T. Japonica* (*Actinobacteria*)	Eschenhagen *et al.* (2003)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	Quinone profiles and 16S rRNA clone library	*Betaproteobacteria* (n.d.) (with quinone profiling)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Jeon *et al.* (2003)
Anaerobic-aerobic SBR	Synthetic sewage with propionate as carbon source (varied pH)	FISH	'*Candidatus* Accumulibacter phosphatis' (55%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Pijuan *et al.* (2004)

n.d.: no data

Table 2-6 A summary of full- and pilot-scale studies on the population composition of EBPR communities using culture independent molecular techniques (modified from Seviour *et al.*, 2003)

Reactor configuration	**Reactor feed**	**Method of community analyses**	**Major populations detected (> 10% of total)**	**PAO identified**	**References**
Full-scale (Phoredox) in Hirblingen, Germany	Complex municipal sewage	FISH	*Betaproteobacteria*	n.d.	Wagner *et al.* (1994)
Pilot Plant (3-stage Phoredox) at Darvill WWTP in Pietermaritzburg, KwaZulu-Natal, South Africa	Complex municipal sewage with substrate supplementation	FISH	*Alphaproteobacteria* (19%) *Betaproteobacteria* (22%) *Gammaproteobacteria* (17%) *Actinobacteria* (11%)	n.d.	Mudaly *et al.* (2000)
Pilot Plant (anaerobic-aerobic), No N Removal at Malmö WWTP in Sweden	Complex municipal sewage with substrate supplementation	FISH/MAR	*Betaproteobacteria* *Gammaproteobacteria* Varied over time	*Rhodocyclus*-related organisms (*Betaproteobacteria*) But others suspected	Lee *et al.* (2002)
Pilot Plant (UCT) with N Removal at Malmö WWTP in Sweden	Complex municipal sewage with substrate supplementation	FISH/MAR	*Betaproteobacteria* *Gammaproteobacteria*	*Rhodocyclus*-related organisms (*Betaproteobacteria*) But others suspected	Lee *et al.* (2002)
Full-scale (UCT) at Nine Springs WWTP in Madison, Wisconsin, USA	Complex municipal sewage	FISH	*Alphaproteobacteria* (10%) *Betaproteobacteria* (25%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Zilles *et al.* (2002)
Full-scale Aerobic-anoxic (Orbal Process with N Removal) at Dane-Iowa WWTP in Mazomanie, Wisconsin, USA	Complex municipal sewage	FISH	*Betaproteobacteria* (12%) *Actinobacteria* (11%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Zilles *et al.* (2002)
Full-scale (Biodenipho) at Aalborg East WWTP in Denmark	Complex municipal sewage	FISH/MAR	*Rhodocyclus*-related organisms (5-10%) Rod-shaped *Tetrasphaera*-	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Kong *et al.* (2004, 2005)

			related organisms (3%) Coccus-shaped *Tetrasphaera*-related organisms (<1%)		
Full-scale (Biodenipho) at Egaa WWTP in Denmark	Complex municipal sewage	FISH/MAR	*Rhodocyclus*-related organisms (10-15%) Rod-shaped *Tetrasphaera*-related organisms (6%) Coccus-shaped *Tetrasphaera*-related organisms (10%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*)	Kong *et al.* (2004, 2005)
Full-scale (Biodenipho) at Skagen WWTP in Denmark	Industrial wastewater from the fishing industry	FISH/MAR	*Rhodocyclus*-related organisms (17-22%) Rod-shaped *Tetrasphaera*-related organisms (7%) Coccus-shaped *Tetrasphaera*-related organisms (12%)	*Rhodocyclus*-related organisms (*Betaproteobacteria*), *Tetrasphaera*-related organisms (*Actinobacteria*)	Kong *et al.* (2004, 2005)

n.d.: no data

2.5 Microbial Communities Observed in Deteriorated EBPR Processes

Past studies have suggested that GAOs consist of several different morphotypes and phylotypes. The TFOs, with a distinctive morphology of cocci arranged in tetrads or clusters, were the first GAO reported by Cech and Harman (1993) in a deteriorated EBPR reactor fed with glucose. Culture-dependent studies have obtained several bacterial isolates with morphological traits resembling TFO observed in EBPR systems. These isolates were related to the *Alphaproteobacteria* (*Amaricoccus* spp. and *D. vanus*), *Betaproteobacteria* (*Quadricoccus* sp.), *Gammaproteobacteria*, and *Actinobacteria* (*Tetrasphaera* spp., *M. glycogenica*, and *K. limosa*) (Maszenan *et al.*, 1997; Kong *et al.*, 2001; Shintani *et al.*, 2000; Hanada *et al.*, 2002; Liu *et al.*, 2002a) (Table 2-7). However, except for *M. glycogenica*, none of those isolates were reported to be in abundance in full-scale or laboratory-scale EBPR processes (Kong *et al.*, 2001; Seviour *et al.*, 2003). While the absence of these TFO isolates in EBPR systems remains unexplained (Seviour *et al.*, 2003), molecular approaches have revealed several yet-to-be-cultured TFOs from different major phylogenetic lineages dominated in laboratory-scale EBPR systems (Kong *et al.*, 2001, 2002a; Levantesi *et al.*, 2002; Tsai and Liu, 2002) (Table 2-8). TFOs were often observed in large numbers in many anaerobic-aerobic EBPR plants (Seviour *et al.*, 2000). They are sometimes present in processes with good EBPR activities (Christensson *et al.*, 1998; Hesselmann *et al.*, 1999) or in processes with deteriorated EBPR activities and the absence of glucose in the feed (Nielsen *et al.*, 1999; Dabert *et al.*, 2001; Lee *et al.*, 2002; Crocetti *et al.*, 2002).

Another GAO morphotype that has been described was the coccobacillus-shaped organism observed in an acetate-fed deteriorated EBPR reactor (Liu *et al.*, 1996). It formed a deeply branched lineage GB in the *Gammaproteobacteria* (referred to GB group henceforth) and consisted of at least seven phylogenetic subgroups (Nielsen *et al.*, 1999; Kong *et al.*, 2002) including '*Candidatus* Competibacter phosphatis' (Crocetti *et al.*, 2002).

2.5.1 Isolation of GAO from EBPR Systems

(A) *Amaricoccus* spp.

Isolates of TFO from a laboratory-scale reactor showing poor EBPR capacity by Cech and Hartman (1993) were later characterized and named as *Amaricoccus* spp. in the *Alphaproteobacteria* (Maszenan *et al.*, 1997). Four species were described, *A. veronensis*, *A. kaplicensis*, *A. macauensis*, and A. *tamworthensis*. They could not synthesize polyP aerobically, could synthesize glycogen aerobically, and could not assimilate acetate or glucose anaerobically (Falvo *et al.*, 2001). PHA synthesis occurred aerobically but not anaerobically. These physiological properties are different from those described for GAO (Liu *et al.*, 1996b). It is possible that *Amaricoccus* spp. were not the organisms dominating in the reactor operated by Cech and Hartman (1993).

A probe specific for *Amaricoccus* spp. was designed and tested against various activated sludge samples from EBPR systems (Maszenan *et al.*, 2002b). The results showed that *Amaricoccus* is a commonly occurring organism in both conventional and EBPR treatment plants treating different wastes. They could appear as tetrads in flocs, or as single, or as dispersed cells in the bulk liquid.

(B) *Defluviicoccus vanus*

D. vanus, a gram-negative coccus/coccobacillus, was isolated from a sample of activated sludge biomass from a full-scale EBPR process in the Czech Republic (Maszenan *et al.*, 2005). It often occurs as tetrads, clusters or aggregates. Phylogenetic analysis revealed that it belonged to the *Alphaproteobacteria*. This strain is a very slow-growing organism, and responds negatively to many biochemical tests even after prolonged incubation. No polyP was detected by staining, but accumulation of intracellular PHA granules was apparent after staining with Nile blue A, after culturing aerobically in media containing acetate, propionate, or glucose.

(C) *Quadricoccus australiensis*

Q. australiensis, a gram-negative coccus, was isolated using micromanipulation from an Australian activated sludge process showing foaming and bulking problems. This isolate was a strict aerobe, appearing in biomass samples as clusters or cocci in tetrads. These morphological traits resembled 'G-bacteria' commonly observed in activated sludge samples. It stained positively for intracellular polyP and PHA.

(D) *Micropruina glycogenica*

M. glycogenica is a slow growing organism, originally isolated from an EBPR SBR fed with a mixture of acetate and peptone (Liu *et al.*, 1997). Its ability to anaerobically assimilate glucose and accumulate glycogen has been demonstrated in pure culture (Shintani *et al.*, 2000). Kong *et al.* (2001) analyzed the bacterial community in an anaerobic-aerobic non-P removing SBR biomass fed with a mixture of acetate and glucose. Clone library analysis and DGGE profiling of this community revealed that *M. glycogenica* and a closely related clone sbr-gs28 (Jeon and Park, 2000) were detected

among the *Actinobacteria* from a glucose-fed SBR. FISH using probe MIC184 designed against these populations showed that they accounted for almost all of the *Actinobacteria* in this community and the fluorescing cells appeared as small cocci in clusters and sheets. FISH in combination with microautoradiography (MAR) revealed that *M. glycogenica* and the sbr-gs28 clone could assimilate both acetate and glucose anaerobically, synthesized PHA anaerobically, and could not store polyP aerobically. These observations supported the possibility that *M. glycogenica* is a member of GAO in EBPR processes (Shintani *et al.*, 2000).

(E) *Kineosphaera limosa*

K. limosa is a high-G+C gram-positive, motile, non-spore-forming coccus capable of accumulating significant amounts of PHA. It was isolated from an inefficient laboratory-scale EBPR reactor (Liu *et al.*, 2002). However, no report on its occurrence in full-scale EBPR process is available.

2.5.2 Culture-independent Approach

(A) *Sphingomonas* spp.

Using FISH and DGGE to analyze the communities from their laboratory-scale anaerobic-aerobic SBRs, Beer *et al.* (2004) reported that members of the genus *Sphingomonas* in the *Alphaproteobacteria* were present in large numbers in sludge samples with poor P removal capacity and high glycogen content. This group could store PHA anaerobically but not aerobically, and could not accumulate polyP aerobically. These metabolic traits were similar as those reported for GAO. However, this population was seldom observed in biomass samples taken from 10 full-scale EBPR plants from eastern states of Australia using FISH with probe SBR9-1a. Beer *et*

al. (2004) observed that this population accounted for 5±3% of the total cell area responding to the EUBmix probe targeted cells in plants with high EBPR capacity (e.g., the Bendigo plant, Victoria). The cells appeared as typical TFO but scattered throughout the flocs. None were stained DAPI positive for polyP.

(B) GB Group

The GB group consists of large coccoid cells (2-4 μm) to occasionally long rod (up to 20 μm) and are often observed in laboratory-scale acetate-fed SBRs with low EBPR capacity (Liu *et al.*, 1996b; Bond *et al.*, 1999; Nielsen *et al.*, 1999). Although the presence of this group in laboratory-scale anaerobic-aerobic reactors (Nielsen *et al.*, 1999; Dabert *et al.*, 2001; Liu *et al.*, 2001; Kong *et al.*, 2002b; Levantesi *et al.*, 2002; Pijuan *et al.*, 2004) and full-scale EBPR plants (Lee *et al.*, 2003; Saunders *et al.*, 2003) was well documented, the ecological functions of this group in EBPR processes require further studies. Although cells of the GB group were present in high number (12% of total cells), no evidence was found to suggest that these might impact on EBPR performance of systems fed with glucose and acetate (Lee *et al.*, 2003). Nielsen *et al.* (1999) and Liu *et al.* (2001) observed this novel group in various EBPR systems and observed the accumulation of cellular inclusions such as PHA and occasionally polyP. In contrast, Dabert *et al.* (2001) observed a marked increase in the GB group in an SBR during the transition from good to poor EBPR capacity. Blackall *et al.* (2002) proposed that members of the subgroup GB_6 had a phenotype consistent with those of GAOs. Kong *et al.* (2002b) extensively examined the diversity and distributions of the GB group in 13 different laboratory-scale and full-scale systems with or without EBPR activity, they found that it was 1-4 times more abundant in most EBPR systems investigated (contributing 10-50% of total cells) than in the CAS systems (< 10%).

Phylogenetic analysis based on 16S rRNA sequences further classified this GB group into seven subgroups (Kong *et al.*, 2002b) encompassing '*Candidatus* Competibacter phosphatis'. Cells of the subgroups GB_4 and GB_6 exhibited typical GAO phenotypes, (e.g., storing PHA anaerobically and utilizing it aerobically), and are frequently detected by FISH in full-scale EBPR processes (Crocetti *et al.*, 2002; Kong *et al.*, 2002b).

Table 2-7 A summary of the known anaerobic substrate assimilation patterns and intracellular storage polymer production by the GAO in anaerobic-aerobic activated sludge systems (modified from Seviour *et al.*, 2003)

Organisms	Phylogenetic affiliation	Cell morphology	Anaerobic substrate assimilation	Anaerobic polymer			Aerobic polymer			References
				PHA	Glycogen	PolyP	PHA	Glycogen	PolyP	
Amaricoccus spp.[a,b]	*Alphaproteobacteria*	Cocci in tetrads	None in pure culture or *in situ*	-ve	-ve	-ve	+ve	+ve	-ve	Maszenan *et al.* (1997), Falvo *et al.* (2001)
D. vanus	*Alphaproteobacteria*	Cocci/cocco bacilli in tetrads and clusters	Acetate, propionate, and glucose in pure culture	n.d.	n.d.	n.d.	+ve	n.d.	-ve	Maszenan *et al.* (2005)
Tetrasphaera spp.[a]	*Actinobacteria*	Cocci in tetrads	None in pure culture; n.d. *in situ*	n.d.	n.d.	n.d.	-ve	n.d.	+ve	Liu *et al.* (2001)
M. phosphovorus [a,b]	*Actinobacteria*	Cocci in clusters	Glucose, not acetate in pure culture; n.d. *in situ*	-ve	n.d.	-ve	-ve	n.d.	+ve	Santos *et al.* (1999)
M. glycogenica [b,c]	*Actinobacteria*	Cocci in clusters	Acetate and glucose in pure culture and *in situ*	n.d.	+ve	-ve	n.d.	+ve	-ve	Shintani *et al.* (2000), Kong *et al.* (2001)
Unnamed[a]	*Gammaproteobacteria*	Large coccoid rods	Acetate *in situ*	+ve	n.d.	-ve	-ve	n.d.	-ve	Hesselmann *et al.* (1999)
Unnamed[a]	*Gammaproteobacteria*	Large coccoid rods	Acetate *in situ*	n.d.	n.d.	n.d.	+ve	n.d.	+ve	Liu *et al.* (2001)
β-TFO1[a]	*Betaproteobacteria*	Large coccoid rods	Glucose *in situ*	n.d.	n.d.	n.d.	+ve	n.d.	-ve	Tsai and Liu (2002)
γ-TFO1[a]	*Gammaproteobacteria*	Large coccoid rods	Glucose *in situ*	n.d.	n.d.	n.d.	-ve	n.d.	-ve	Tsai and Liu (2002)
HGC-TFO1[a]	*Actinobacteria*	Large coccoid cells in sheets	Glucose *in situ*	n.d.	n.d.	n.d.	±	n.d.	±	Tsai and Liu (2002)
Clone sbr-gs28[c]	*Actinobacteria*	Cocci in tetrads	Acetate and glucose *in situ*	+ve	n.d.	-ve	n.d.	n.d.	-ve	Jeon and Park (2000), Jeon *et al.* (2000)
'*Candidatus* Competibacter phosphatis' [b,c]	*Gammaproteobacteria*	Large coccoid rods	Acetate *in situ*	+ve	n.d.	-ve	-ve	n.d.	-ve	Blackall *et al.* (2002), Crocetti *et al.* (2002), Levantesi *et al.* (2002)

[a]Determined using staining; [b]determined by direct chemical analysis; [c]determined by MAR; '+ve': contain; '-ve': not contain; '±': variable; 'n.d.': no data

Table 2-8 A summary of laboratory studies on the population composition of deteriorated EBPR communities using culture independent molecular techniques

Reactor configuration	**Reactor feed**	**Method of community analyses**	**Major populations detected (> 10% of total)**	**GAO identified**	**References**
Anaerobic-aerobic SBR	Synthetic sewage with glucose as carbon source	Quinone profiles, FISH	*Alphaproteobacteria* (17%) *Betaproteobacteria* (39%) *Gammaproteobacteria* (11%) *Actinobacteria* (12%)	n.d.	Sudiana *et al.* (1998)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	Quinone profiles, FISH	*Alphaproteobacteria* (19%) *Betaproteobacteria* (33%) *Gammaproteobacteria* (11%) *Actinobacteria* (11%)	n.d.	Sudiana *et al.* (1998)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	DGGE, FISH	Novel *Gammaproteobacteria* (35% with FISH; 75% with DGGE)	Novel *Gammaproteobacteria*	Nielsen *et al.* (1999)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	FISH	*Betaproteobacteria* (58%)	*Betaproteobacteria* from groups other than β-1 or β-2-*Proteobacteria*	Bond *et al.* (1999)
Anaerobic-aerobic SBR	Synthetic sewage with acetate and glucose as carbon sources	16S rRNA clone library, DGGE, FISH/MAR	*Alphaproteobacteria* (26%) *Gammaproteobacteria* (14%) *Actinobacteria* (29%) LGC bacteria (22%)	*M. glycogenica* (*Actinobacteria*), *Sphingomonas* spp. (*Alphaproteobacteria*)	Kong *et al.* (2001), Beer *et al.* (2004)
Anaerobic-aerobic SBR	Synthetic sewage with acetate as carbon source	FISH/MAR	*Alphaproteobacteria* (49%) *Betaproteobacteria* (38%)	*Sphingomonas* spp. (*Alphaproteobacteria*)	Kong *et al.* (2002a), Beer *et al.* (2004)

n.d.: no data

2.6 Ecological Functions of EBPR Communities

The ecophysiological traits, which are important to interpret the role of EBPR communities, were commonly investigated using FISH combined with histochemical staining or MAR. Intracellular polymer storage plays an important role in PAO's metabolism. The storage capacity of PAOs gives them a competitive advantage over other microorganisms present that are not able to accumulate internal reserves. Histochemical staining procedures for qualitative visualization of polymers (e.g., polyP and PHA) by optical microscopy and combinations of these procedures with FISH have been used to reveal the ability of PAO to accumulate polymer (Kawaharasaki *et al.*, 1999; Liu *et al.*, 2001; Levantesi *et al.*, 2002; Onuki *et al.*, 2002; Tsai and Liu, 2002).

MAR is an elegant tool to evaluate substrate utilization capability of individual cells *in situ* in complex ecosystems (Andreasen and Nielsen, 1997). It involves first the incubation of microbial cells with radiolabeled substrates. The uptake of radiolabeled substrates by individual cells is confirmed based on the presence of silver granules surrounding those cells that are immobilized on a glass slide and subjected to prior exposure under dark for an optimized period of time. By combining the MAR technique with FISH, *in situ* substrate utilization patterns of individual populations can be resolved, allowing their ecophysiology in EBPR processes to be examined (Lee *et al.*, 1999 and Ouverney and Fuhrman, 1999). In several EBPR systems, the MAR-FISH approach has been applied to analyze specific activities and functions in a few phylogenetic groups of PAO and GAO (Lee *et al.*, 1999; 2003; Kong *et al.*, 2002a; 2004; 2005; 2006; Chua *et al.*, 2004).

2.6.1 *Rhodocyclus*-related PAO

(A) Polymer Accumulation Ability

The polyP- and PHA-accumulating traits of the *Rhodocyclus*-related PAO in acetate-fed laboratory EBPR reactors have been well studied. Onuki *et al.* (2002) showed that sequences of the dominant bands in the DGGE profile of sludge biomass with EBPR capacity were closely related to the *Rhodocyclus*-related clones. After simultaneous polyP and FISH staining targeting the *Rhodocyclus*-related PAOs, Onuki *et al.* (2002) further indicated the presence of polyP in these *Rhodocyclus*-related cells. By combining FISH with polyP and PHA staining, Liu *et al.* (2001) and Levantesi *et al.* (2002) revealed that the *Rhodocyclus*-related organisms present in efficient EBPR systems behaved according to the biochemical models (i.e., synthesizing PHA anaerobically and polyP aerobically).

On the other hand, using the dual staining of polyP and FISH targeting the *Rhodocyclus*-related PAOs in full-scale EBPR systems, a considerable proportion of *Rhodocyclus*-related PAOs appeared to contain little or no polyP granules (Zilles *et al.*, 2002; Lee *et al.*, 2003; Kong *et al.*, 2004). The role of *Rhodocyclus*-related PAOs in full-scale EBPR processes was found to be less important than in laboratory-scale EBPR systems fed with acetate as sole carbon source.

(B) Substrate Assimilation Ability

Kong *et al.* (2002a) employed MAR-FISH on biomass from an SBR operating under conditions with high and low P removal. The FISH analysis revealed that the numbers of *Betaproteobacteria* and *Rhodocyclus*-related organisms in the community corresponded directly to the level of P removal. Furthermore, most but not all the

Rhodocyclus-related organisms, which appeared as pleiomorphic cells mainly in clusters, assimilated acetate and synthesized PHA anaerobically. Under aerobic conditions, most of the cells responding to the *Rhodocyclus* probes assimilated ^{33}Pi the same way as the biochemical models predicted.

Lee *et al.* (2003) obtained very similar results using MAR-FISH in two activated sludge pilot systems. The *Rhodocyclus*-related organisms were observed to accumulate P aerobically. They further noted uneven acetate and ^{33}Pi assimilation with their *Rhodocyclus*-related PAO, and proposed that either some of these cells are physiologically inactive *in situ* or there is considerable physiological variation among the populations detected by the probes used. Furthermore, because the changes in population levels of *Rhodocyclus*-related PAO did not correspond to changes in the P removal capability of their sludge, it was concluded that other bacteria are likely to be involved in EBPR.

The ecophysiological traits of *Rhodocyclus*-related PAOs present in full-scale EBPR plants was studied by using MAR-FISH (Kong *et al.*, 2004). These traits were found to be consistent with the biochemical models proposed for PAO. The *Rhodocyclus*-related PAOs were able to accumulate short-chain substrates including acetate, propionate, and pyruvate under anaerobic conditions, and could not assimilate many other low-molecular-weight compounds such as ethanol and butyrate. Furthermore, they were able to simultaneously assimilate two substrates (e.g., acetate and propionate). Leucine and thymidine could not be assimilated as sole substrates and could only be assimilated as cosubstrates with acetate perhaps as N sources. Glucose could not be assimilated by the *Rhodocyclus*-related PAOs, but was easily fermented

by other microbial populations in the sludge. Glycolysis instead of the TCA cycle was likely the source providing the reducing power needed by the *Rhodocyclus*-related PAOs to form the intracellular PHA storage compounds during anaerobic substrate assimilation (Kong *et al.*, 2004). The *Rhodocyclus*-related PAOs were able to take up Pi and accumulate polyP when oxygen, nitrate, or nitrite was present as an electron acceptor. In the presence of acetate, growth was sustained by using oxygen as well as nitrate or nitrite as an electron acceptor. This strongly indicated that *Rhodocyclus*-related PAOs were able to denitrify and thus played a role in the denitrification occurring in full-scale EBPR plants.

2.6.2 *Tetrasphaera*-related PAO

The ecophysiological traits of *Tetrasphaera*-related PAOs in a full-scale EBPR plant was studied by using MAR-FISH (Kong *et al.*, 2005). These PAOs took up Pi aerobically only if, in a previous anaerobic phase, they had taken up organic matter from wastewater or a mixture of amino acids. They could not take up VFAs (e.g., acetate), glucose, or ethanol under anaerobic or aerobic conditions. The storage compound produced during the anaerobic period was not PHA, as for *Rhodocyclus*-related PAOs, and its identity is still unknown. Growth and uptake of Pi took place in the presence of oxygen and nitrate but not nitrite, indicating the inability of denitrification. In an efficient EBPR system containing *T. elongata* in large number, Hanada *et al.* (2002) revealed that *T. elongata* can accumulate polyP but not PHA *in situ* under aerobic conditions, suggesting its contribution to P removal.

2.6.3 Yeast-like Betaproteobacterial Cell

The *in situ* physiology of large yeast-like betaproteobacterial cells was studied with MAR-FISH in order to understand their substrate assimilation abilities under different conditions as well as their Pi uptake ability (Chua *et al.*, 2004). These cells were able to take up acetate, glutamate, and aspartate, but not glucose under both aerobic and anaerobic conditions. PHA staining in combination with MAR showing cells incubated under anaerobic conditions contained PHA granules. In addition, MAR showed aerobic ^{33}Pi assimilation with all these substrates supporting the EBPR capacity in these large cells.

2.7 Ecological Functions of Deteriorated EBPR Communities

Like PAOs, knowing the identity of these GAOs is not sufficient to understand their role in anaerobic-aerobic EBPR systems. Being able to compete with PAO for substrate uptake under anaerobic conditions, a clearer picture of which organic substrates these GAOs are able to assimilate anaerobically, and what intracellular storage compounds are synthesized *in situ* need to be revealed.

Generally, many GAOs are known to synthesize storage polymers (Table 2-7). Combinations of *in situ* identification by FISH and stains for polyP and PHA have been used to indicate GAO identity (Hesselmann *et al.*, 1999; Nielsen *et al.*, 1999; Liu *et al.*, 2001; Tsai and Liu, 2001). Results indicated that GAOs that are phylogenetically related and indistinguishable by FISH may have different physiological behavior in activated sludge.

2.7.1 Large Gammaproteobacterial Coccoid

(A) Polymer Accumulation Ability

Hesselmann *et al.* (1999) found that large gammaproteobacterial coccoid rods produced PHA anaerobically but not aerobically, and no polyP was detected in cells exposed to aerobic conditions. They suggested that these characteristics might mean that such bacteria could compete anaerobically with the PAO for acetate. These large gammaproteobacterial coccoid rods behaved in a similar way to those revealed by Nielsen *et al.* (1999) and the GB group revealed by Kong *et al.* (2006). However, the large cocci reported by Liu *et al.* (2001) were observed to accumulate both polyP and PHA.

(B) Substrate Assimilation Ability

The ecophysiological traits of GB group present in twelve full-scale EBPR plant was studied by using MAR-FISH (Kong *et al.*, 2006). Under aerobic or anaerobic conditions, all members of the group GB could take up acetate, pyruvate, propionate, and some amino acids, while some subgroups in addition could take up formate and thymidine. Glucose, ethanol, butyrate, and several other organic substrates were not taken up. Glycolysis was found to be essential for the anaerobic uptake of organic substrates. Members of the subgroup GB_6 was possibly able to denitrify, and members of the subgroup GB_1, GB_4, and GB_5 were able to reduce nitrate to nitrite.

2.7.2 TFOs

(A) Polymer Accumulation Ability

Using a combined approach of FISH and polyP staining together with a successive staining of PHA granules, Tsai and Liu (2002) characterized the betaproteobacterial,

gammaproteobacterial, and actinobacterial TFOs that dominated glucose-fed SBRs without EBPR activity. In addition to phylogenetic diversity, Tsai and Liu (2002) also revealed considerable *in situ* physiological variations in this morphotype. The betaproteobacterial TFOs stained polyP negative and PHA positive, indicating that they could utilize compounds other than polyP (i.e., glycogen) as reducing power for PHA synthesis from glucose. In contrast, two types of actinobacterial TFO stained polyP positive and PHA negative, indicating their capacity to accumulate polyP without the synthesis and degradation of PHA. This metabolic trait was different from the widely accepted biochemical model of EBPR and non-EBPR metabolisms. Other actinobacterial and gammaproteobacterial TFOs showed negative responses to both polyP and PHA stains, and their function in the deteriorated EBPR system need to be further clarified.

(B) Substrate Assimilation Ability

Kong *et al.* (2001) applied MAR-FISH to look at the ecophysiological traits of various TFOs in laboratory-scale anaerobic-aerobic SBR. No clear distinction could be seen there in the substrate uptake patterns of TFOs from the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Micropruina glycogenica*, a member of the *Actinobacteria*. All these populations assimilated acetate but not glucose anaerobically, and this finding questioned the original suggestion (Cech and Hartman, 1993) that GAO became dominant over PAOs because of their preferential anaerobic assimilation of glucose. However, it should be noted in the study of Cech and Hartman (1993) that no *in situ* physiological experiments were carried out to confirm the observation that the TFOs were in fact GAO. Although dominant bacterial populations from this reactor were successfully isolated, characterized, and named as *Amaricoccus* spp. in the

Alphaproteobacteria (Masznan *et al.*, 1997), pure culture studies indicated that these *Amaricoccus* spp. were not able to assimilate either acetate or glucose anaerobically (Falvo *et al.*, 2001). Thus, the question whether *Amaricoccus* spp. were actually the dominant populations observed by Cech and Hartman (1993) or not remained unresolved.

Chapter 3

Materials and Methods

3.1 Laboratory-scale Reactor

3.1.1 Seed Sludge

Original seed sludge for activated sludge cultivation in this study was obtained from a conventional activated sludge process treating mainly municipal sewage at a water reclamination plant (capacity = 1,400,000 population equivalent), (Bedok, Singapore). It was obtained from a sludge wasting line and stored for 3 days at 4°C prior to use. The initial total phosphorus content (TP) of the sludge was about 3% of total suspended solids (TSS).

3.1.2 Feed Composition

The concentrated synthetic wastewater consisted of solutions A and B. Solution A contained (per liter) $CH_3COONa \cdot 3H_2O$, 86.89 g; and $CaCl_2 \cdot 2H_2O$, 3.08 g. Solution B contained (per liter) $MgSO_4 \cdot 7H_2O$, 19.82 g; $(NH_4)_2SO_4$, 2.64 g; KH_2PO_4, 6.75 g; and 140 ml trace elements solution. The trace elements solution contained (per liter) citric acid, 2.73 g; $Na_3EDTA \cdot 4H_2O$, 0.36 g; $FeCl_3 \cdot 6H_2O$, 0.15 g; H_3BO_3, 1.50 g; $ZnSO_4$, 0.25 g; $MnCl_4 \cdot H_2O$, 0.15 g; $CuSO_4 \cdot 5H_2O$, 0.12 g; KI, 0.06 g; $Na_2MoO_4 \cdot 2H_2O$, 0.03 g; and $NiCl \cdot 6H_2O$, 0.03 g. The total organic carbon (TOC) and phosphorus (P) concentrations in the bulk solution after feeding were 200 mg l^{-1} and 20 mg l^{-1}, respectively (P:TOC ratio = 1:10).

3.1.3 Reactor Setup and Operation

A 30-l rectangular, polyacrylamide vessel (22.5×22.5×40 cm, L×W×H) with a working volume of 20 l was used as a sequencing membrane bioreactor (MBR) (Figure 3-1). The sequencing MBR was operated under cyclic anaerobic and aerobic conditions to mimic enhanced biological phosphorus removal (EBPR) processes. It was operated with four 6 h cycles per day for 260 days. Each cycle consisted of a feeding phase (10 min), an anaerobic phase (140 min), and an aerobic phase [210 min (inclusive of 120 min decanting at the end of aerobic phase)]. During the feeding phase, a synthetic

medium as indicated above with acetate as the sole carbon source was delivered into the sequencing MBR at an organic loading rate of 0.8-0.9 g l^{-1} d^{-1}. Mechanical mixing was used during each cycle. Aeration was carried out during the aerobic phase, to maintain a minimum dissolved oxygen of 1 mg l^{-1}. In the final 60 min of the aerobic phase, clarified liquor was separated from the mixed liquor using a submerged membrane (effective filtration area, 0.1 m^2; pore size 0.4 μm) purchased from Kubota. The suction pressure was maintained at 50 kPa with a peristaltic pump. The solids residence time (SRT) and equivalent hydraulic retention time (HRT) were maintained at 15 d and 5 d, respectively, and pH was controlled at 8.0±0.1 using a pH controller through acid and base addition (Chemitreat pH/Redox 800). The operating conditions were selected based on the previously described (Liu *et al.*, 1994; Kong *et al.*, 2002a) and primarily aimed to promote good EBPR activity in the sequencing MBR. Certain modifications and improvements have been made in consideration of the difficulties associated with laboratory operation of MBR (e.g., membrane fouling and feed degradation).

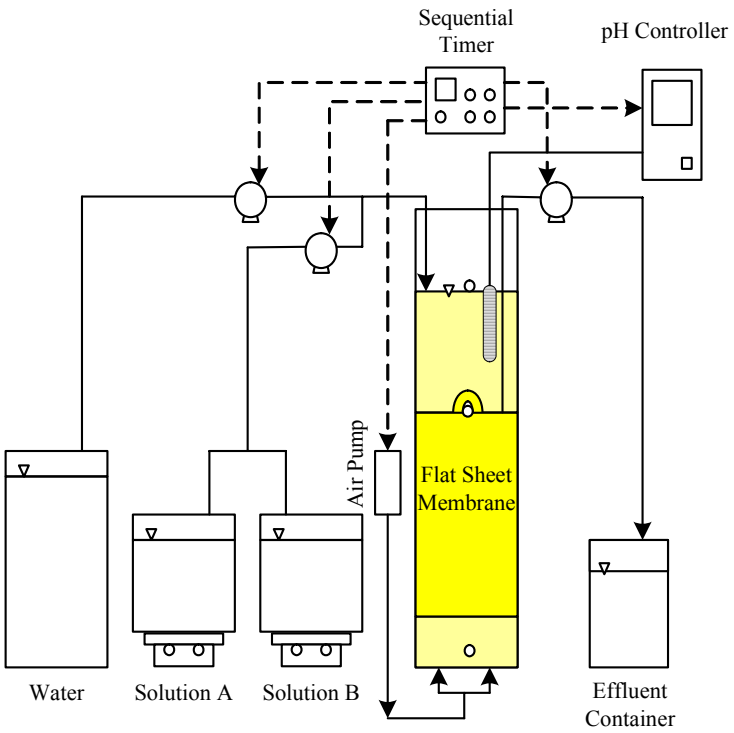


Figure 3-1 Schematic layout of the sequencing MBR.

3.2 Full-scale Wastewater Treatment Plant Samples

Table 3-1 lists the locations, treatment capacities, wastewater sources, process configurations and TP contents of the nine Japanese wastewater treatment plants from where samples were taken. These plants were configured as aerobic (CAS), anaerobic-aerobic (AO), pseudo-anaerobic-aerobic (Pseudo-AO), and anaerobic-anoxic-aerobic (A2O) modes. Samples Nakagawa_CAS, Shin-Gashi_CAS, and Sunamachi_CAS were collected from these systems comprising a single aeration tank and showing no orthophosphate (Pi) removal. Samples Nakagawa_AO, Kosuge_AO, Mikawasima_AO, and Nakano_AO were obtained from systems comprising a mechanically-mixed anaerobic tank followed by an aerobic tank. Sample Todoroki_APO was obtained from a treatment system where pure oxygen was used for aeration, while samples Shibaura_Pseudo-AO, Kosuge_Pseudo-AO, and Mikawasima_Pseudo-AO were collected from systems comprising an air-mixed anaerobic tank followed by an aerobic tank. Samples Ariake_A2O and Nakagawa_A2O were collected from systems comprising an additional anoxic tank for denitrification between the mechanically-mix anaerobic and aerobic tanks. The sludge samples used in this study were all collected at the end of the aeration tanks, and were kept on ice during transportation back to the laboratory for further analyses.

3.3 Anaerobic and Aerobic Batch Experiments for Substrate Uptake

Batch tests for determining substrate uptake of the enriched sludge under anaerobic and aerobic conditions, respectively, were performed as described previously (Liu *et al.*, 1996). Briefly, 200 ml of sludge was collected from the reactor at the end of an aerobic phase, and washed twice with filtered reactor effluent before suspended in a

250 ml sealed serum bottle. For anaerobic tests, each bottle was gassed with oxygen-free nitrogen gas for 15 min to remove any residue oxygen and then capped with a butyl rubber stopper before substrate addition. Aerobic tests were conducted by continuously aerating the liquid. To start a batch test, 1 ml of concentrated substrate solution at a final concentration of 30 mg C per g of TSS was added into the serum bottle. The bottles were then incubated at 20±1°C with shaking for 4 h. The tested substrates included acetate, aspartic acid, glucose, lactate, propionate, and pyruvate.

Table 3-1 Summary of the 13 activated sludge samples

Plant	**Treatment capacity (m^3d^{-1})**	**Wastewater source**	**Sample name**	**TP content (mg P g^{-1} SS)**
Ariake, Tokyo	11,000	Domestic	Ariake_A2O	32.3
Kosuge, Tokyo	180,000	Domestic	Kosuge_Pseudo-AO	33.1
			Kosuge_AO	32.0
Mikawashima, Tokyo	750,000	Domestic	Mikawashima_Pseudo-AO	28.8
			Mikawashima_AO	31.3
Nakagawa, Tokyo	188,590	Domestic	Nakagawa_A2O	30.5
			Nakagawa_AO	28.9
			Nakagawa_CAS	20.4
Nakano, Tokyo	N/A[1]	Domestic	Nakano_AO	30.8
Shibaura, Tokyo	70,000	Domestic and industrial	Shibaura_Pseudo-AO	38.8
Shin-Gashi, Tokyo	N/A	Domestic	Shin-Gashi_CAS	18.5
Sunamachi, Tokyo	N/A	Domestic and industrial	Sunamachi_CAS	19.7
Todoroki, Kawasaki	N/A	Domestic	Todoroki_APO	30.8

[1]N/A: not available.

3.4 Analytical Methods

3.4.1 Microscopy

Epifluorescence microscopy and confocal laser scanning microscopy (CLSM) were both used for microscopic observations and staining methods. The epifluorescence microscope (model BX51, Olympus) was equipped with a cooled CCD camera SPOT-RT Slider (Diagnostic Instruments, USA), a 100 W HBO bulb, and three different fluorescence filter sets (U-MWU2, U-MWB2 and U-MF2). The CLSM model LSM 5 Pascal (Carl Zeiss) was equipped with an inverted microscope, an argon-ion laser (458 to 514 nm), two helium/neon lasers (543 nm and 633 nm), three Zeiss filter sets (01, 09 and 15), and different objective lenses (×20, ×40, ×63 and ×100 oil-immersion). Image processing and analysis were performed with the software packages provided by Zeiss, Metamorph (Universal Imagine) and Adobe Photoshop software (Adobe).

3.4.2 Chemical Staining

For preliminary microscopic characterization, cells were stained by the Gram, Neisser, and Sudan black B stains (Jenkins *et al.*, 2004). Neisser and Sudan black B staining procedures were used to confirm the presence of intracellular polyphosphate (polyP) and polyhydroxalkanoate (PHA) granules, respectively. The frequencies of occurrence of the morphotypes of polyP-stained cells were assessed using a subjective rating system developed by Jenkins *et al.* (2004), with zero being the lowest score and 6 reflecting excessive polyP containing cells numbers present. Due to equipment setup, Nile Blue A staining (Ostle and Holt, 1982) combined with FISH were used in CLSM to detect accumulated intracellular PHA in microbial cells of interest.

3.4.3 PHA Analysis

PHA of lyophilized sludge samples was digested, methylated, and chloroform extracted according to the method of Brandl *et al.* (1988) and Comeau *et al.* (1988). The composition of the extracted methyl esters was assayed using a gas chromatography-mass spectroscopy system (Hewlett Packard HP 6890 series) equipped with a DB 5.625 column. 5 µl of the extracted solution was injected and analyzed before split injection (split ratio, 1/30). The temperatures of the injector and detector, and the temperature program used for separation of the esters were the same as described by Brandl *et al.* (1988).

3.4.4 Other Analytical Methods

Soluble TOC was measured with a TOC analyzer (Shimazu). The sludge carbohydrate content and dissolved sugars concentration were determined using the phenol method (Herbert *et al.*, 1971). Intracellular glycogen content was first separated according to the method of Yamamoto and Matsui (1989), and then determined using the phenol method. Sludge TP contents after presulfate digestion and dissolved Pi concentrations were determined using the ascorbic acid method (APHA, 1998). Unless otherwise stated, other analyses used in this study were performed in accordance with Standard Methods (APHA, 1998).

3.5 Clone Library and Terminal Restriction Fragment Polymorphism

3.5.1 DNA Extraction

DNA from biomass samples was obtained using the protocol described by Liu *et al.* (1997) with minor modifications. Approximately 10 mg (dry weight) of biomass

suspended in 600 µl of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), and 0.75 M sucrose]. Lysozyme (900 mg l^{-1}) and achromopeptidase (10 mg l^{-1}) were added, and the suspension was incubated at 37°C for 30 min. After the addition of proteinase K (100 mg l^{-1}) and sodium dodecyl sulfate (SDS) (1%, wt/vol), the samples were incubated at 37°C for 2 h. During that period, each tube was gently inverted several times every 30 min. The mixtures were then subjected to three freeze-thaw cycles. Hexadecyltrimethyl ammonium bromide and sodium chloride were added to final concentrations of 0.7% (wt/vol) and 0.5 M, respectively, and the mixtures were incubated for 30 min at 60°C to precipitate polysaccharides and residual proteins. Each precipitate was removed by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by extraction with chloroform-isoamyl alcohol (24:1). DNA was precipitated by the addition of 1 volume of isopropanol and incubation at -20ºC overnight. The precipitated DNA was centrifuged at 13,000×g for 30 min. The pellet was washed with 70% ethanol twice, dried, and resuspended in 50 to 100 µl of distilled water.

3.5.2 PCR Conditions

Reaction mixtures contained 1× PCR buffer, each deoxynucleoside triphosphate at a concentration of 200 µM, 2.5 mM $MgCl_2$, each primer at a concentration of 0.2 µM, and 2.5 U of Taq DNA polymerase (Promega) in a final volume of 100 µl. Table 3-2 lists the primers and their specificities used for PCR reactions in this study. PCR was performed with a Hybaid thermal cycler (Hybaid) by using the following program: an initial denaturation at 94°C for 5 min; 30 cycles consisting of denaturation (45 s at 94°C), annealing (45 s at 55°C), and extension (1 min at 72°C); and a final extension

at 72°C for 5 min. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (3 µl) in 1.0% agarose in 1× TAE buffer.

Table 3-2 Primers used for PCR reactions

Primer	**Specificity**	**rRNA target site**[a]	**Sequence (5' to 3')**	**Usage**	**Reference**
11f	*Bacteria* (forward)	16S, 11-27	GTTTGATCCTGGC TCAG	Clone library, sequencing	Amann *et al.* (1995)
47f[b]	*Bacteria* (forward)	16S, 47-66	Cy5-C(C/T)TAACACAC ATGCAAGTCG	T-RFLP	Chen *et al.* (2004)
519f	*Bacteria* (forward)	16S, 519-536	CAGC(C/A)GCCGC GGTAAT(A/T)C	Sequencing	Amann *et al.* (1995)
519r	*Bacteria* (reverse)	16S, 519-536	G(A/T)ATTACCGC GGC(G/T)GCTG	Sequencing	Amann *et al.* (1995)
968fGC[c]	*Bacteria* (forward)	16S, 968-984	AACGCGAAGAAC CTTAC	DGGE	Heuer *et al.* (1997)
1392r	*Bacteria* (reverse)	16S, 1392-1406	ACGGGCGGTGTG T(A/G)C	DGGE	Amann *et al.* (1995)
1055r	*Bacteria* (reverse)	16S, 1055-1069	CGGCCATGCACC ACC	T-RFLP	Chen *et al.* (2004)
EUB1512r	*Bacteria* (reverse)	16S, 1512-1527	TACCTTGTTACGA CTT	Clone library, sequencing	Kane *et al.* (1993)
T7	TOPO vector (forward)	n.a.[e]	TAATACGACTCA CTATAGGG	Sequencing	Sambrook and Russell (2001)
SP6	TOPO vector (reverse)	n.a.[e]	ATTTAGGTGACA CTATAG	Sequencing	Sambrook and Russell (2001)
ALF1b	*Alphaproteobacteria* (forward)	16S, 19-35	CGTTCG(C/T)TCT GAGCCAG	Clone library, sequencing	Manz *et al.* (1992)
ALF968	*Alphaproteobacteria* (reverse)	16S, 968-986	GGTAAGGTTCTG CGCGTT	Clone library, sequencing	Neef *et al.* (1999)
GB	GAO - group GB (reverse)	16S, 612-628	CGATCCTCTAGCC CACT	T-RFLP	Kong *et al.* (2002)

GB_G1[d]	Subgroup G1 in group GB (reverse)	16S, 989-1006	TTCCCCGGATGTC AAGGC	T-RFLP	Kong *et al.* (2002)
GB_G2[d]	Subgroup G2 in group GB (reverse)	16S, 989-1006	TTCCCCAGATGTC AAGGC	T-RFLP	Kong *et al.* (2002)
M13f	TOPO vector (forward)	n.a.[e]	GTAAAACGACGG CCAG	Clone library	Sambrook and Russell (2001)
M13r	TOPO vector (reverse)	n.a.[e]	CAGGAACACGCT ATGAC	Clone library	Sambrook and Russell (2001)

[a]*Escherichia coli* rRNA numbering.

[b]A Cy5 fluorescent dye was labeled at the 5' end.

[c]GB_G1 and GB_G2 were used in the mixture called G1G2.

[d]A 40 bp GC clamp (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3') was included at the 5' end.

[e]n.a, not applicable.

3.5.3 Construction of 16S rRNA Gene Clone Library

Primer pair ALF1b-EUB1512r or 11f-ALF968r was used to selectively amplify the 16S rRNA gene of the *Alphaproteobacteria* in the extracted community DNA. PCR products were cloned using a TA cloning kit (Invitrogen) according to the manufacturer's instruction. The clone library was screened for redundant clones. Cells of white *E. coli* colonies that contained correct DNA insertion were directly used as DNA template for PCR with a vector-specific primer set (i.e., M13f and M13r) to confirm the presence or absence of DNA fragment In the second step of the screening, clones with different 16S rRNA sequence insertions were identified using denaturing gradient gel electrophoresis polymorphism (DGGE) or restriction fragment length polymorphism (RFLP) techniques. The DGGE screening was performed as reported previously (Liu *et al.*, 2002b). Briefly, the M13f-M13r amplified PCR products of colonies with correct DNA insertion were diluted 20 to 50 times, and subsequently used as the DNA templates in a PCR reaction with a DGGE primer set, 968fGC and 1392r. After the confirmation of PCR products by electrophoresis, multiple DGGE-

PCR products were loaded into a well in a DGGE gel instead of one product per well. A 6% DGGE gel with an increasing gradient of denaturants (i.e., urea and formamide) from 40 to 60% was prepared. DGGE was performed with a DCode system (Bio-Rad) at 200 V, 60ºC and 3 h. After DGGE electrophoresis, the total number of clones with different rDNA insertions was estimated from the total number of DGGE bands with different electrophoretic positions. A clone with a different electrophoresis position on a DGGE gel was defined as an operational taxonomic unit (OTU). For RFLP screening, inserts from individual clones were amplified using the vector-specific primer set, digested with restriction enzymes *Hha*I and *Msp*I, and grouped into OTUs based on the RFLP pattern. 16S rRNA gene sequences for the ALF1b-1512r clone library were analyzed using an ABI model 377 automated sequencer (Applied Biosynthesis), a *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems), and the following primers: ALF1b, 1512r, T7, and SP6. 16S rRNA gene sequences for the 11f-ALF968r clone library were analyzed using a CEQ 8000 genetic analysis system (Beckman Coulter), a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter), and the following primers: 11f, ALF968, 519f, and 519r.

3.5.4 Phylogenetic Analysis of 16S rRNA Gene Clones

The sequences of representative clones from individual OTUs were fully sequenced. The 16S rRNA gene sequences obtained were checked for chimeric artifacts with the CHECK_CHIMERA tool in the Ribosomal Database Project (RDP) (Maidak *et al.*, 2001), and then compared with sequences in the GenBank using the BLAST algorithm (Altschul *et al.*, 1997). For phylogenetic analysis, those retrieved sequences and their related bacterial sequences were aligned using the integrated aligners in the ARB software package (Ludwig *et al.*, 2004). Resulting alignments were manually verified

against known 16S rRNA secondary structure regions. The aligned sequences were then imported into the BioEdit software package (Hall, 1999) to remove any surplus nucleotides and unnecessary gaps. A neighbor-joining tree (Saitou and Nei, 1987) with the Juke–Cantor method (1,000 replicate bootstraps) and a maximum parsimony tree under heuristic searches (Nei and Kumar, 2000) were constructed using the MEGA3 program (Kumar *et al.*, 2004).

3.5.5 Terminal Restriction Fragment Length Polymorphism Analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes was performed according to a previously described protocol (Liu *et al.*, 1997). The extracted DNA was used as the template in the PCR amplification using either the domain *Bacteria*-specific primers 47f and 1055r or group GB-specific primers GB and G1G2. The amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen), and digested with four different tetrameric restriction nucleases (*Alu*I, *Hha*I, *Hae*III, *Msp*I or *Rsa*I). The digested products were denatured at 95ºC for 2 min, immediately chilled on ice, and analyzed using a model CEQ 8000 automated sequencer (Beckman Coulter) at 55ºC and 4.8 kV for 2 h. The lengths of fluorescently labeled fragments were determined by comparison with internal standards using CEQ 8000-genetic analysis system software (Beckman Coulter). Only the T-RFs with abundance greater than 1% of total intensity were considered. Cluster analysis (Minitab 14) was employed to statistically discern patterns in the T-RFLP data. Dendrograms were constructed by using the single linkage and Euclidean distance rules in the Minitab program.

3.5.6 Design and Optimization of Oligonucleotide Probes

16S rRNA-targeted oligonucleotide probes were designed using the probe design function in ARB (Ludwig *et al.*, 2004). The specificity of these probes was confirmed against the Check Probe program in the Ribosomal Database Project (Maidak *et al.*, 2001), and experimentally optimized for FISH by determining a proper dissociation temperature or formamide concentration against a reference strain (Manz *et al.*, 1992).

3.5.7 Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences obtained have been deposited at the GenBank, EMBL and DDBJ nucleotide sequence database under the accession numbers AY351635 to AY351641, AY351643 and DQ250532 to DQ250540.

3.6 Fluorescence *in situ* Hybridization

3.6.1 Cell Fixation and Oligonucleotide Probes

Fresh sludge samples were fixed in both paraformaldehye and ethanol solutions for Gram-negative bacteria and Gram-positive bacteria respectively, then washed with phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2), and stored in PBS/ethanol solution at -20ºC prior to further analysis (Manz *et al.*, 1992; Roller *et al.*, 1994). When necessary, the sludge flocs were briefly dispersed by sonicating for 60 s with an ultrasonic processor (Vibracell, Sonics) at a pulse of 5 s and an output power of 4 W. Table 3-3 lists the oligonucleotide probes, their specificities, and conditions of stringency used for FISH. These probes were commercially synthesized and 5'-labelled with fluorescein isothiocyanate (FITC), cyanine 3 (Cy3), or cyanine 5 (Cy5) (MWG Biotech or Proligo).

3.6.2 Hybridization and Washing

Fixed cells were spotted on precleaned epoxide-coated microscopic slides (Paul Marienfeld GmbH), dried, and dehydrated in 50%, 80%, and then 96% (vol/vol) ethanol (3 min each). Hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris/HCl pH 7.4, 0.01% SDS and varied concentrations of formamide. Ten microliters of hybridization buffer containing 50 ng of each probe were added into individual wells on the slide and dissipated carefully. The slide was positioned horizontally in a 50-ml falcon tube containing a tissue paper soaked with hybridization buffer and then incubated for 3 h at 46ºC. Post-hybridization washing was performed at slightly elevated stringency (48ºC). Wash buffer consisted of 20 mM Tris/HCl pH 7.4, 0.01% SDS, 5 mM EDTA, and a reduced amount of NaCl. The slide was quickly rinsed with 1 ml pre-warmed buffer, and then immersed in buffer for 20 min. After rinsing with distilled water and air-dried, the slide was mounted with a SlowFade light antifade kit (Molecular Probes) or VectaShield (Vecta) before microscopic observation. Sludge samples were initially hybridized with the probe NON338 labeled with Cy3 to exclude nonspecific-probe-binding (Wallner *et al.*, 1993), and then analyzed with the domain- and group-specific oligonucleotide probes listed in Table 3-3 to provide information on microbial community structure.

3.6.3 Semi-quantitative FISH

Semi-quantitative FISH analysis was performed on the CLSM-captured or epifluorescence microscope-captured images according to protocols described previously (Bouchez *et al.*, 2000; Schmid *et al.*, 2000; Liu *et al.*, 2001). At least 20 microscopic fields selected randomly in the X, Y, and Z planes taken from the hybridization of individual probes were analyzed. Within each field, cells hybridized

to a given probe were statistically expressed as a percentage of the total area of bacteria hybridizing with the EUBmix probe, 4'6-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1} for 10 min) or SYTO9 (Molecular Probes) (3 μM for 15 min) using the function provided in MetaMorph (Universal Imagine) or Leica QWin. The numbers obtained from individual fields were averaged and standard deviations of the mean were calculated. Results were corrected for nonspecific binding with the results of the negative control using probe NON338 (Gieseke *et al.*, 2001).

3.6.4 Combined FISH and PolyP Staining Methods

Cells were fixed onto a glass slide, stained with a freshly prepared DAPI solution (50 μg ml^{-1}) at room temperature for 10 min, rinsed thoroughly with water, and air dried. Microphotograph images of the DAPI positive cells were taken with the epifluorescence microscope equipped with the cooled CCD camera in true color using suitable filter set and optimized exposure times. Under these settings, the polyP granules appeared as bright yellow while the other regions of the cells remained pale blue (Kawaharasaki *et al.*, 1999). The immersion oil on the glass slide was then washed off carefully with ethanol and the sample was then subjected to FISH as described above. The locations where the DAPI images were taken were relocated, and the corresponding FISH images captured.

3.6.5 Floc Characteristics of Probe PAOmix Targeted *Rhodocyclus*-related PAO Cells

The floc characteristics of probe PAOmix targeted *Rhodocyclus*-related polyphosphate accumulating organism (PAO) cells were analyzed based on the numbers of *Rhodocyclus*-related PAO cells present in the microcolonies, and the diameters and

areal cell densities of these microcolonies. At least 20 different microphotographic fields (×100 oil immersion) containing more than 40 microcolonies or 1000 cells in total were randomly chosen for each sample. The numbers of *Rhodocyclus*-related PAO cells present in a microcolony were counted manually. The diameters and areal cell densities of these microcolonies were measured using the package provided in Metamorph (Universal Imagine).

3.6.6 Statistical Analysis

Linear regression analyses were conducted to determine the correlations between the *Rhodocyclus*-related PAO abundances and the sludge TP contents, or between the polyP-stained cells abundance and the sludge TP contents using Microsoft Excel. Regression coefficient (R^2) was used to assess the linear correlations between dependent and independent variables. The linear relationships were also checked graphically to eliminate any outlier.

Table 3-3 Oligonucleotide probes used in FISH analysis

Probe	**Specificity**	**rRNA target site**[a]	**Sequence (5' to 3')**	**FA (%)**[b]	**Reference**
NON338	Nonspecific binding	16S, 338-335	ACTCCTACGGGAGGCAGC	20	Wallner *et al.* (1993)
ARCH915	*Archaea*	16S, 915-934	GTGCTCCCCCGCCAATTCCT	35	Amann *et al.* (1995)
EUB338[c]	Most *Bacteria*	16S, 338-355	GCTGCCTCCCGTAGGAGT	35	Amann *et al.* (1990)
EUB338-II[c]	*Planctomycetales* & other *Bacteria* not detected by EUB338	16S, 338-355	GCAGCCACCCGTAGGTGT	35	Daims *et al.* (1999)
EUB338-III[c]	*Verrucomicrobiales* & other *Bacteria* not	16S, 338-355	GCTGCCACCCGTAGGTGT	35	Daims *et al.* (1999)

	detected by EUB338				
ALF968	*Alphaproteobacteria*, except of *Rickettsiales*	16S, 968-986	GGTAAGGTTCTGCGCGTT	35	Neef *et al.* (1999)
BET42a[d]	*Betaproteobacteria*	23S, 1027-1043	GCCTTCCCACTTCGTTT	35	Manz *et al.* (1992)
GAM42a[d]	*Gammaproteobacteria*	23S, 1027-1043	GCCTTCCCACATCGTTT	35	Manz *et al.* (1992)
HGC69a	*Actinobacteria*	23S, 1901-1918	TATAGTTACCACCGCCGT	25	Roller *et al.* (1994)
CF319a	*Cytophaga-Flexibacter*-group of the *Bacteroides*	16S, 319-336	TGGTCCGTGTCTCAGTAC	35	Manz *et al.* (1996)
LGC354A[e]	*Firmicutes*	16S, 354-371	TGGAAGATTCCCTACTGC	35	Meier *et al.* (1999)
LGC354B[e]	*Firmicutes*	16S, 354-371	CGGAAGATTCCCTACTGC	35	Meier *et al.* (1999)
LGC354C[e]	*Firmicutes*	16S, 354-371	CCGAAGATTCCCTACTGC	35	Meier *et al.* (1999)
PAR651	Genus *Paracoccus* in *Alphaproteobacteria*	16S, 651-668	ACCTCTCTCGAACTCCAG	40	Neef *et al.* (1996)
GB	GAO - group GB	16S, 612-628	CGATCCTCTAGCCCACT	35	Kong *et al.* (2002)
GB_G1 (GAOQ989)[d,f]	Subgroup G1 in group GB	16S, 989-1006	TTCCCCGGATGTCAAGGC	35	Kong *et al.* (2002)
GB_G2[d]	Subgroup G2 in group GB	16S, 989-1006	TTCCCCAGATGTCAAGGC	35	Kong *et al.* (2002)
GB_1 and 2	Subgroups 1 and 2 in group GB	16S, 587-604	GGCTGACTGACCCATCC	20	Kong *et al.* (2002)
GB_2	Subgroup 2 in group GB	16S, 64-81	GGCATCGCTGCCCTCGTT	35	Kong *et al.* (2002)
GB_3	Subgroup 3 in group GB	16S, 642-659	CCACTCAAGTCCAGCCGT	35	Kong *et al.* (2002)
GB_4 [c]	Subgroup 4 in group GB	16S, 1020-1037	GGCTCCTTGCGGCACCGT	35	Kong *et al.* (2002)

GB_5	Subgroup 5 in group GB	16S, 69-86	CTAGGCGCCGAAGCGCCC	35	Kong *et al.* (2002)
GB_6 (GAM1019)[f]	Subgroup 6 in group GB	16S, 1054-1071	GGTTCCTTGCGGCACCTC	35	Kong *et al.* (2002)
GB_7[d]	Subgroup 7 in group GB	16S, 1000-1017	CATCTCTGGACATTCCCC	35	Kong *et al.* (2002)
PAO462[g]	*Rhodocyclus*-related PAO in *Betaproteobacteria*	16S, 462-485	CCGTCATCTACWCAGGGTATTAAC	35	Crocetti *et al.* (2000)
PAO651[g]	*Rhodocyclus*-related PAO in *Betaproteobacteria*	16S, 651-668	CCCTCTGCCAAACTCCAG	35	Crocetti *et al.* (2000)
PAO846[g]	*Rhodocyclus*-related PAO in *Betaproteobacteria*	16S, 846-866	GTTAGCTACGGCACTAAAAGG	35	Crocetti *et al.* (2000)
actino_1011	*Tetrasphaera japonica*, EBPR clone Ebpr19, Ebpr20 in *Actinobacteria*	16S, 1011-1029	TTGCGGGGCACCCATCTCT	30	Liu *et al.* (2001)
AMR839	*Amaricoccus* spp. in *Alphaproteobacteria*	16S, 839-860	CTGCGACACCGAACGGCAAGCC	20	Maszenan *et al.* (2000)
MIC184	*Micropruina glycogenica* in *Actinobacteria*	16S, 438-456	CATTCCTCAAGTCTGCC	20	Kong *et al.* (2001)
TET63	*Tetrasphaera* spp. in *Actinobacteria*	16S, 63-80	GCTCCAGGGTCACCGTTC	20	Kong *et al.* (2001)
TFO_DF218	*Defluviicoccus*-related TFO in *Alphaproteobacteria*	16S, 218-235	GAAGCCTTTGCCCCTCAG	25-35	This study
TFO_DF618	*Defluviicoccus*-related TFO in *Alphaproteobacteria*	16S, 618-635	GCCTCACTTGTCTAACCG	25-35	This study
TFO_DF629	*Defluviicoccus*-related TFO in *Alphaproteobacteria*	16S, 629-646	AGGACTTTCACGCCTCAC	n.d.[h]	This study

TFO_DF776	*Defluviicoccus*-related TFO in *Alphaproteobacteria*	16S, 776-794	GCTATAGCGTCAGTTACGG	25-35	This study
TFO_DF862	*D. vanus*	16S, 862-880	AGCTAAGCTCCCCGACAT	35	This study

[a]*Escherichia coli* rRNA numbering.

[b]FA concentration in the hybridization buffer.

[c]EUB338, EUB338-II, and EUB-III were used in the mixture called EUBmix.

[d]BET42a, GAM42a, GB_G1, GB_G2, GB_4, and GB_7 were used in combination with their corresponding unlabeled competitor probes.

[e]LGC354A, LGC354B, and LGC354C were used in the mixture called LGCmix.

[f]Probes GAOQ989 and GB_6 were reported by Crocetti *et al.* (2002) and Nielsen *et al.* (1999) respectively.

[g]PAO462, PAO651, and PAO846 were used in the mixture called PAOmix.

[h]Not determined.

3.7 Microautoradiography

3.7.1 Incubation with Radioactive Substrates

Microautoradiography (MAR) experiments were carried out under 12 different treatments (Table 3-4) according to protocols previously described (Lee *et al.*, 1999) with a slight modification. Fresh activated sludge samples taken from the end of the aerobic phase were diluted with filtered reactor effluent to a biomass concentration of approximately 1 g of mixed liquor suspended solids per liter. For each experimental system, 1.9 ml of diluted activated sludge was added to a 9-ml serum bottle. For anaerobic incubation, the serum bottles were sealed with gas-tight rubber stoppers, flushed with oxygen-free nitrogen gas for 10 min to remove any residual oxygen, and preincubated for 1 h to ensure that oxygen was completely removed. Subsequently, 0.1 ml of oxygen-free substrate solution containing sterile radioactive and non-radioactive substrates in analytical grade was injected with syringes through the rubber stoppers into individual bottles. For aerobic incubation, substrates and/or other chemicals were

added after the activated sludge was vigorously shaken for 20 min to ensure aerobic conditions. During all preincubations and incubations, the serum bottles were shaken (250 rpm) at 20±1ºC. Radioactive and non-radioactive substrates were obtained from Amersham or Sigma-Aldrich. Those radioactive substrates included: D-[6-^{3}H]glucose (specific activity, 33000 mCi mmol^{-1}), [2-^{14}C]acetate (specific activity, 59 mCi mmol^{-1}), L-[U-^{14}C]lactate (specific activity, 161 mCi mmol^{-1}), L-[U-^{14}C]aspartic acid (specific activity, 207 mCi mmol^{-1}), [1-^{14}C]propionate (specific activity, 6.5 mCi mmol^{-1}), and [1-^{14}C]pyruvate (specific activity, 27 mCi mmol^{-1}).

To exclude the possibility of substrate adsorption onto sludge biomass, parallel control treatments with different radioactive and non-radioactive substrates were carried out with sludge samples pasteurized at 70ºC for 10 min. Negative control treatments (i.e., no addition of radioactive substrate) were also performed to check for chemography. At the end of each treatment, samples were fixed with 6 ml of 4% freshly prepared paraformaldehyde in PBS for 3 h at 4ºC. The samples were washed three times with 5 ml of PBS to remove the excess soluble radioactive substrate and fixative.

3.7.2 MAR/FISH and MAR/PHA Analyses

The microscopic slides (or coverslips) after FISH or PHA staining were coated with a preheated (43°C) LM-1 autoradiography emulsion (Amersham), exposed at 4ºC for 3-5 d, and developed in 4% D19 developer (Kodak) for 0.5-3 min, selected to give optimal balance between the extent of the silver grain deposition and the fluorescent signal from the FISH probed cells. To test for the optimal exposure time, a slide was developed every day, and the increase in the number of silver grains surrounding cells was determined manually. A MAR-positive cell was defined as an assembly of more

than 6 silver grains covering an area of 2-3 μm^2, while the background grain density was less than 0.01 silver grain per μm^2. The background grain density was counted in areas >10 μm away from any MAR-positive bacteria. To further confirm that no substrate assimilation was taking place, the emulsion exposure and development times were extended up to 10 days and 10 min, respectively.

3.8 Pure Cultures

3.8.1 *Defluviicoccus vanus*

D. vanus (NCIMB 13612) was obtained from National Collections of Industrial Food and Marine Bacteria (Scotland, UK). It was grown on glucose sulfide (GS) agar medium containing (per liter) glucose, 0.15 g; $(NH_4)_2SO_4$, 0.5 g; $CaCO_3$, 0.1 g; $Ca(NO_3)_2$, 0.1 g; KCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $Na_2S \cdot 9H_2O$, 0.187 g; K_2HPO_4, 0.05 g; vitamin solution, 0.01 ml; agar, 15 g (final pH 7.3±0.2). The vitamin solution contained (per liter) biotin, 0.5 mg; calcium pantothenate, 40 mg; cocarboxylase, 40 mg; vitamin B12, 0.5 mg; folic acid, 0.5 mg; inositol, 0.5 mg; p-aminobenzoic acid, 40 mg; pyridoxine, 40 mg; riboflavin, 40 mg; thiamine, 40 mg. Liquid cultures were grown in the same medium without agar at 30 ºC with gentle shaking.

3.8.2 Anaerobic-aerobic Batch Incubation of *D. vanus*

Batch tests with alternating anaerobic-aerobic regimes were performed in 100-ml serum bottles. *D. vanus* cells were harvested from the late log-phase by centrifugation, and resuspended in saline to a final OD_{600} of 0.5. To ensure that the cell did not accumulate PHA, the bottle was mixed by shaking under aerobic conditions till the PHA granules were consumed as determined by microscopic observation. For anaerobic incubation, cultures were flushed with pure nitrogen gas for 5 min to remove

the oxygen and then preincubated for 25 min to ensure that oxygen was completely removed. Subsequently, cultures were supplemented with sterile concentrated GS medium without $NaNO_3$ and $Na_2S•9H_2O$. After incubation at 30ºC for 12 h, the rubber stopper of the bottle was opened, and the serum bottle was thoroughly mixed for 11.5 h by shaking. This anaerobic and aerobic incubation was repeated 5 times. At the end of the anaerobic and aerobic incubations, samples were taken and analyzed as described previously (Liu *et al.*, 1996).

Table 3-4 Incubation conditions used for MAR experiments[a]

Expt.	Non-radioactive substrate	Radioactive tracer Compound	Conc (µCi mg^{-1} SS)	Incubation conditions	Incubation time (h)	Substrate assimilation[c]	PHA accumulation[d]
1	Acetate (1 mM)[b]	[2-^{14}C]acetate (Na)	10	Anaerobic	2	++	++
2	Acetate (3 mM)	[2-^{14}C]acetate (Na)	20	Anaerobic	4	++	+++
3	Acetate (3 + 1 mM)	[2-^{14}C]acetate (Na)	10	Anaerobic preincubation	4 + 2[e]	++	+++
4	Acetate (1 mM)	[2-^{14}C]acetate (Na)	10	Aerobic	2	++	+++
5	Aspartic acid (1 mM)	L-[U-^{14}C]aspartic acid	10	Anaerobic	4	-	-
6	Aspartic acid (1 mM)	L-[U-^{14}C]aspartic acid	10	Aerobic	4	-	-
7	Glucose (1 mM)	D-[6-^{3}H]glucose	20	Anaerobic	4	-	-
8	Glucose (1 mM)	D-[6-^{3}H]glucose	20	Aerobic	4	-	-
9	Lactate (1 mM)	L-[U-^{14}C]lactate	10	Anaerobic	4	++	++
10	Propionate (1.5 mM)	[1-^{14}C]propionate	10	Anaerobic	3	++	++
11	Propionate (4.5 + 1.5 mM)	[1-^{14}C]propionate	10	Anaerobic preincubation	4 + 2[e]	++	+++
12	Pyruvate (1 mM)	[1-^{14}C]pyruvate	10	Anaerobic	2	++	+++

[a]All experiments were performed at room temperature at pH 7 to 7.5 when substrates were added.
[b]The concentrations in parentheses are the absolute amounts added.
[c]"-": no labeling; "+": weaker labeling, but clearly positive; "++"; strong labeling.
[d]"-": not detected; "+/-": very few; "+": few; "++"; some; "+++": common.
[e]Preincubation with non-radioactive substrate + incubation with radioactive substrate.

Chapter 4

Identification and Occurrence of Tetrad-Forming *Alphaproteobacteria* in Anaerobic-aerobic Activated Sludge Process

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4.1 Abstract

In an acetate-fed anaerobic-aerobic membrane bioreactor, a deteriorated enhanced biological phosphorus removal (EBPR) community was developed [as determined based on the chemical profiles of organic substrate, soluble phosphate, and intracellular carbohydrate and polyhydroxyalkanoate (PHA) concentrations]. Microscopic observations revealed the dominance of tetrad-forming organisms (TFO), of which the majority stained positively for PHA under anaerobic conditions. Fluorescence *in situ* hybridization (FISH) confirmed that the *Alphaproteobacteria* (85.0±7.0% of total cells) were the most dominant group. A 16S rRNA gene clone library specific for the *Alphaproteobacteria* indicated that most 16S rRNA gene clones (61% of total clones) were closely affiliated with *Defluviicoccus vanus* forming a cluster within subgroup 1 of the *Alphaproteobacteria*. Combined PHA staining and FISH with specific probes designed for the member of the *Defluviicoccus* cluster suggested diversity within this TFO cluster, and that these TFOs were newly identified new glycogen-accumulating organisms in EBPR systems. However, these *Defluviicoccus*-related TFO were only seen in low abundance in 12 different EBPR and non-EBPR systems, suggesting that they were not the key populations responsible for the deterioration of full-scale EBPR processes.

4.2 Introduction

Enhanced biological phosphorus removal (EBPR) processes employ a cyclic anaerobic and aerobic configuration to enrich microbial populations capable of removing phosphate from the bulk solution by intracellular polyphosphate accumulation (Seviour *et al*., 2003). These polyphosphate-accumulating organisms (PAOs) utilize internally stored polyphosphate as an energy source for the uptake and accumulation of carbon

substrates as intracellular storage reserves, for example polyhydroxyalkanoate (PHA), during the anaerobic feeding stage. This carbon sequestering mechanism enables PAO to grow under subsequent aerobic conditions where a carbon source is not available to them, providing them with a competitive advantage over strictly aerobic heterotrophs (Seviour *et al.*, 2003). Glycogen accumulating organisms (GAOs) share similar carbon assimilation and storage features to PAOs under anaerobic conditions, and have been reported to compete with PAOs for carbon substrate by utilizing glycogen as an internally stored energy source (Liu *et al.*, 1996). Though GAOs are often more dominant than PAOs in deteriorated EBPR systems (Liu *et al.*, 1996), the mechanism of EBPR deterioration is still not well understood. One possible reason for this is the poor understanding of the microbial diversity and physiological function of GAOs in various EBPR systems.

Culture-dependent and culture independent molecular techniques have been used to identify GAO. One postulated GAO that has been described is the coccobacillus shaped organisms seen in an acetate-fed deteriorated EBPR reactor (Liu *et al.*, 1996), and phylogenetically placed in the bacterial lineage GB consisting of at least seven phylogenetic subgroups (Nielsen *et al.*, 1999; Kong *et al.*, 2002b), or '*Candidates* Competibacter phosphorus' (Crocetti *et al.*, 2002) in the *Gammaproteobacteria*. This group exhibits several metabolic traits similar to those proposed for GAOs (Nielsen *et al.*, 1999; Crocetti *et al.*, 2002), and appears to be widely distributed in laboratory- and full-scale EBPR processes (Crocetti *et al.*, 2002; Kong *et al.*, 2002b).

The tetrad-forming organisms (TFOs), which occur as clusters of four or more cells, are another hypothesized GAO (Cech & Hartman, 1993; Liu *et al.*, 1996; Tsai & Liu,

2002). So far, TFOs dominate mainly in laboratory-scale EBPR systems fed with synthetic carbon sources. Culture-dependent studies have obtained several bacterial isolates with morphological traits resembling TFOs observed in EBPR systems. These isolates have been shown phylogenetically to be members of the *Alphaproteobacteria* (*Amaricoccus* spp., and *Defluviicoccus vanus*), *Betaproteobacteria* (*Quadricoccus* sp.), *Gammaproteobacteria*, and *Actinobacteria* (*Tetrasphaera* spp., *Micropruina glycogenica*, and *Kineosphaera limosa*) (Maszenan *et al.*, 1997; Kong *et al.*, 2001; Shintani *et al.*, 2000; Hanada *et al.*, 2002; Liu *et al.*, 2002a). However, except for *M. glycogenica*, none of those isolates have been seen in abundance in either full- or laboratory-scale EBPR processes (Kong *et al.*, 2001; Seviour *et al.*, 2003). While the absence of these TFO isolates in EBPR systems remains unexplained (Seviour *et al.*, 2003), molecular approaches have detected several as-yet-unidentified TFOs from different major phylogenetic lineages dominating in laboratory-scale EBPR systems (Kong *et al.*, 2001, 2002a; Levantesi *et al.*, 2002; Tsai & Liu, 2002). These findings suggest that using morphological traits to identify members of a physiologically functional group like the GAO is inappropriate. There needs to be an improvement in the current understanding of the microbial diversity and metabolic functions of TFOs, and their distribution and possible role as GAOs in systems designed to perform EBPR.

Membrane bioreactors (MBRs) have emerged as important wastewater treatment technologies because of their small footprint, high mass/liquid separation efficiencies and low sludge production and operational costs (i.e., high biomass concentration) (Stephenson *et al.*, 2000). Applying MBRs to biological phosphorus removal is also promising (Adam *et al.*, 2002). Here, a sequencing MBR with anaerobic, aerobic and liquid-solid separation stages was established, but it failed to perform EBPR over an operation period of 260 days. Microscopic observation revealed that the microbial

community was dominated by cells with a TFO morphology. This paper describes the *in situ* physiological traits of these TFO, resolves their phylogenetic affiliation and diversity, and examines their occurrence in laboratory- and full-scale EBPR and non-EBPR systems.

4.3 Results and Discussion

4.3.1 Performance of an Acetate-fed MBR Exhibiting Deteriorated EBPR Activity

The bioreactor was operated continuously for approximately 260 days. During this period, the MLSS concentration gradually increased from 3000 mg l^{-1} at day 1, and levelling at around 12860±2524 mg l^{-1} from day 41 onwards. The carbon removal efficiencies at the end of the anaerobic phase after day 41 were 85±6.4% of the feed concentration. The sludge P content at the end of the aerobic phase ranged from 1 to 2% of biomass dry weight during bioreactor operation. Figure 4-1 shows the typical chemical changes that occurred during sequencing batch reactor cycles which were measured on three occasions between days 55 and 260. The carbon source (acetate) expressed as TOC was rapidly consumed; it concentration decreased from 190±36 mg l^{-1} initially to less than 23±2.7mg l^{-1} within the first hour of the anaerobic stage. The remaining TOC was possibly soluble microbial products, which are often reported in MBR systems (Urbain *et al.*, 1998). The decrease in carbon concentration was accompanied by a decrease in biomass glycogen content from 253±35 to 154±14 mg (g VSS)$^{-1}$ and an increase in biomass PHA content from 13±8 to 57±8 mg (g VSS)$^{-1}$. The PHA produced consisted of mainly 3-hydroxybutyrate (~ 80% of total PHA detected) and 3-hydroxyvalerate (~ 20%). No obvious Pi release and Pi uptake were observed during the anaerobic phase and the aerobic phase, respectively. At the

aerobic stage, the biomass carbohydrate level increased gradually while the PHA level decreased to almost zero. This metabolic behaviour suggested a deteriorated EBPR activity occurring in the reactor.

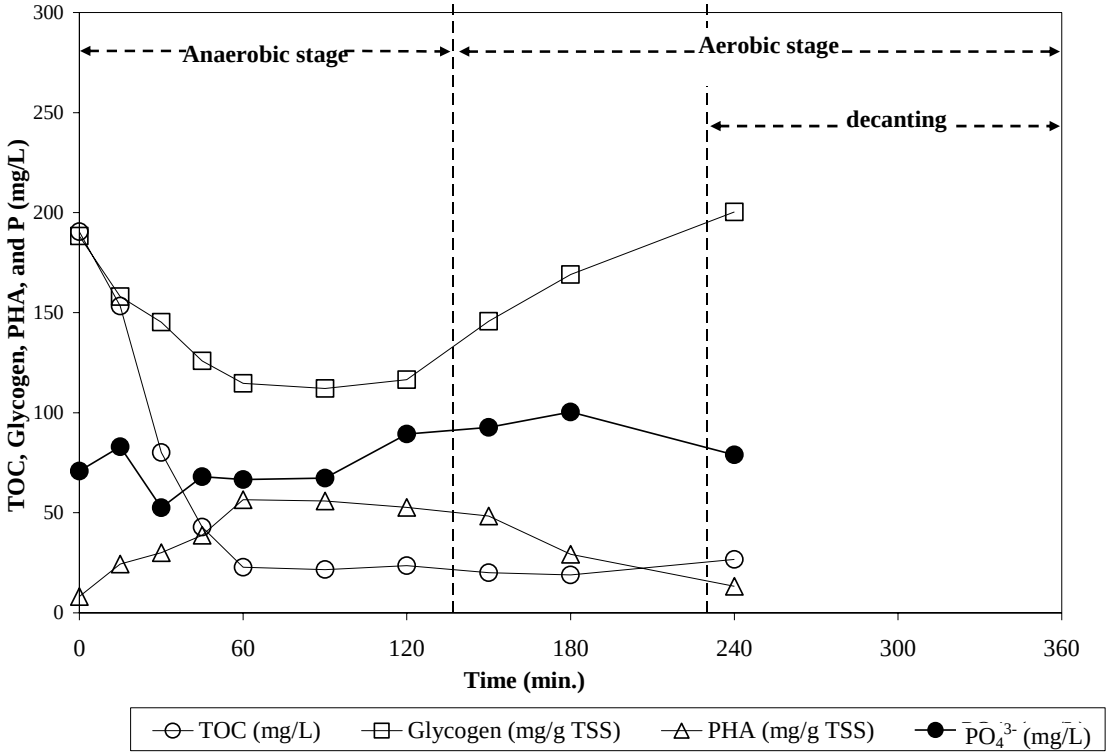

Figure 4-1 Chemical changes that occurred during an anaerobic-aerobic cycle in the acetate-fed MBR. A 10-min feeding phase at the beginning of anaerobic stage is not shown.

4.3.2 Microscopic Observations of the Community

Activated sludge samples taken at the end of anaerobic phase were frequently observed by light microscopy. After the enrichment period (day 55 and onwards) the predominant morphotype was bacterial cells occurring in clusters of four or more cells (the so-called TFO) (Figure 4-2a). Different morphotypes of TFO, based on size and cluster formation patterns, were observed and recognized. For example, one (Figure 4-2a, circle 4) was a large cluster which typically contained at least 16 cells (resembling sarcina-type). Another image of this pattern from another sludge sample is shown in

the inset of Figure 4-2(a). In addition, most TFOs stained positively with Sudan black B, with granule inclusions or whole cells stained at different intensities (Figure 4-2b), which suggested the accumulation of biomass PHA. This was further confirmed using Nile blue A staining, which is reported to be a more specific and sensitive PHA staining method than Sudan black B staining (Ostle & Holt, 1982). As shown in Figure 4-2(c), more cells stained positively using Nile blue A stain than Sudan black B stain, and different degrees of fluorescence intensity were observed among different cell morphotypes, and among cells from a single cluster.

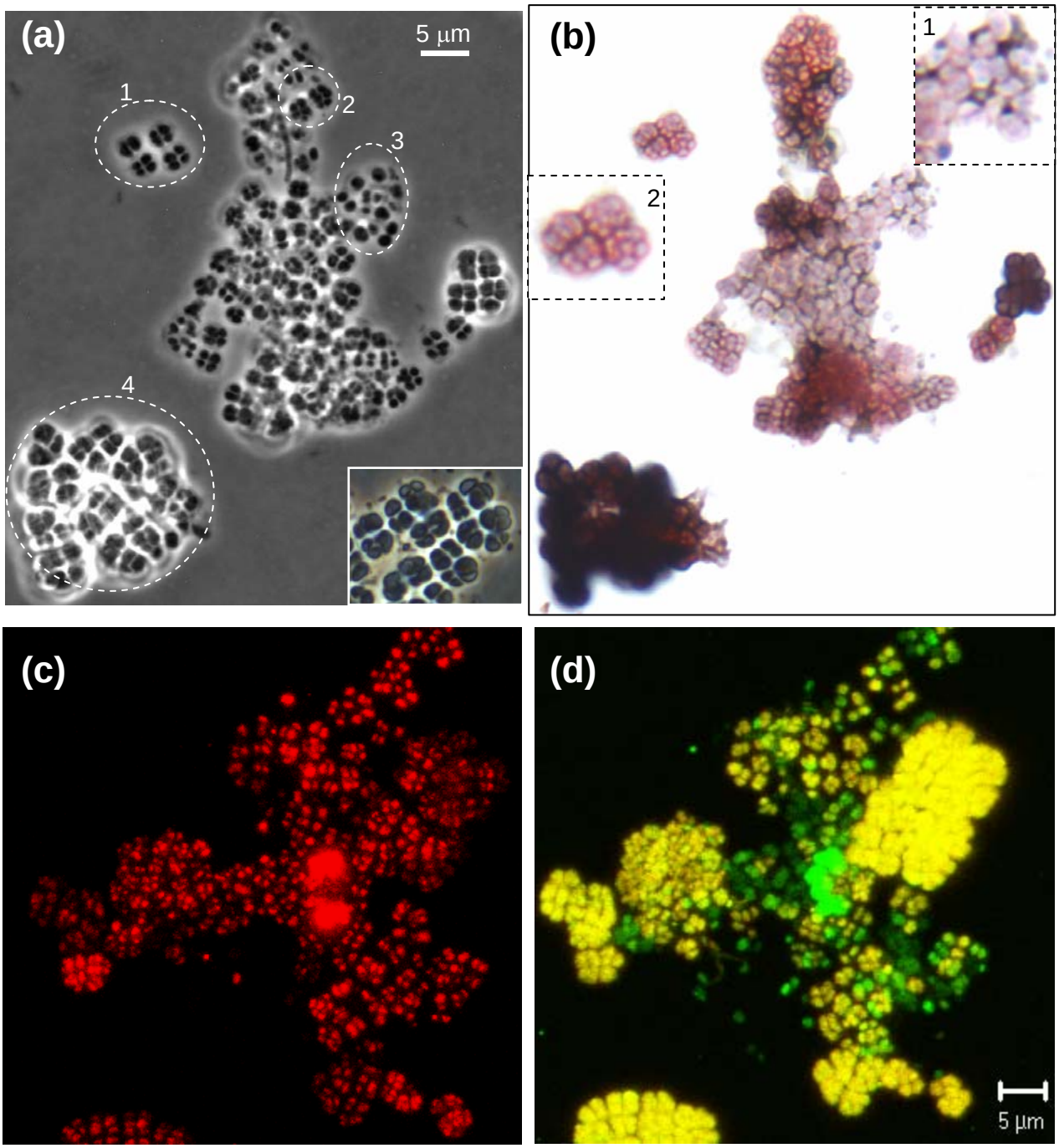

Figure 4-2 Micrographs of activated sludge samples from the acetate-fed MBR. (a) Phase-contrast image of different morphotypes of TFO. (b) Sudan black B-stained image of the same field as in (a) with two insets providing close-up views on the PHA accumulations in two different types of TFO. (c) Nile blue A-stained image. (d) CLSM micrograph of FISH of the same field as in (c) showing the TFOs in yellow, which were simultaneously hybridized by probe ALF968 (Cy3 labeled, red)

targeting the alphaproteobacterial group and probe EUBmix (FITC labeled, green) targeting total bacterial cells.

4.3.3 Preliminary Phylogenetic Profiling of the Microbial Community Structure

FISH with oligonucleotide probes targeting different phylogenetic groups was performed to provide information on the microbial community structure in the reactor. Over 80% of DAPI-stained or SYTO 9-stained cells were detected with the domain *Bacteria* probes (EUBmix). Based on data from epifluorescence microscopy and CLSM, the biomass sample taken at day 55 was dominated by *Alphaproteobacteria* (82.7±3.0/85.0±7.0% of EUBmix-stained cells, epifluorescence microscope/CLSM), followed by *Gammaproteobacteria* (10.1±4.4/4.5±1.8%), *Betaproteobacteria* (5.3±1.7/4.8±1.5%), *Cytophaga-Flexibacterium-Bacteroides* (3.8±3.3/8.1±4.5%), and *Actinobacteria* (1.9±1.2/<1%). Differences between the data obtained by the two methods were not statistically significant.

Figure 4-2(d) shows the CLSM-FISH image of the bacterial cells simultaneously hybridized by probes EUBmix (FITC-labelled, green) and ALF968 (Cy3-labelled, red). The bacterial cells that appear yellow (due to superimposition of red and green) represented members of the *Alphaproteobacteria*. From the *Alphaproteobacteria* at least two morphotypes of TFO by size were observed. The first, which resembled the one in Figure 4-2(a) (circle 4), contained large coccoid cells in clusters of 16 or more. The second resembled the TFO observed in Figure 4-2(b) (circle 2), appeared after FISH as ‘flower buds’, and differed from the first in the cell size and in FISH staining responses. Since the *Alphaproteobacteria* appeared to dominate the community, a 16S

rRNA gene clone library was constructed for this subdivision to phylogenetically place the TFOs.

4.3.4 Phylogenetic Diversity of TFOs in the *Alphaproteobacteria*

A total of 51 clones were selected from the clone library, screened, and classified into 11 different operational taxonomic units (OTUs) after fully sequencing (~1400 bp). Phylogeny analysis (Figure 4-3) shows that the majority of these clones (eight OTUs, 67% of the total clones) were related to the *Alphaproteobacteria*. Five of those eight OTUs (61% of the total clones) formed a lineage within the family *Rhdospirillales* of the *Alphaproteobacteria*. Of these five OTUs, four formed a tight cluster with *Defluviicoccus vanus* isolated from a full-scale EBPR process (Kong *et al.*, 2002a, b) with a sequence similarity of between 92.6-97.2%. Within this *Defluviicoccus* cluster, OTU TFOa28 (47% of the total clones) represented the most dominant OTU or population in the reactor. The remaining fifth OTU TFOa27, with low clone abundance in the lineage, was related to three environmental clones previously retrieved from a laboratory-scale EBPR reactor (McMaholm *et al.*, 2002). The other three *Alphaproteobacteria*-related OTUs not within this lineage were related to *Paracoccus* and *Rhodobacter* species, and *Hyphomonas* species (Figure 4-3). Finally, of the non-*Alphaproteobacteria*-related OTUs, one was identified as a chimeric sequence and the other two were related to the genus *Prosthecobacter* from the phylum *Verrucomicrobia*. It should be noted that the primer ALF1b used in clone construction is not very specific for *Alphaproteobacteria*, and the successful construction of the clones was attributed to the high abundance of the *Alphaproteobacteria* organisms as revealed through FISH analysis.

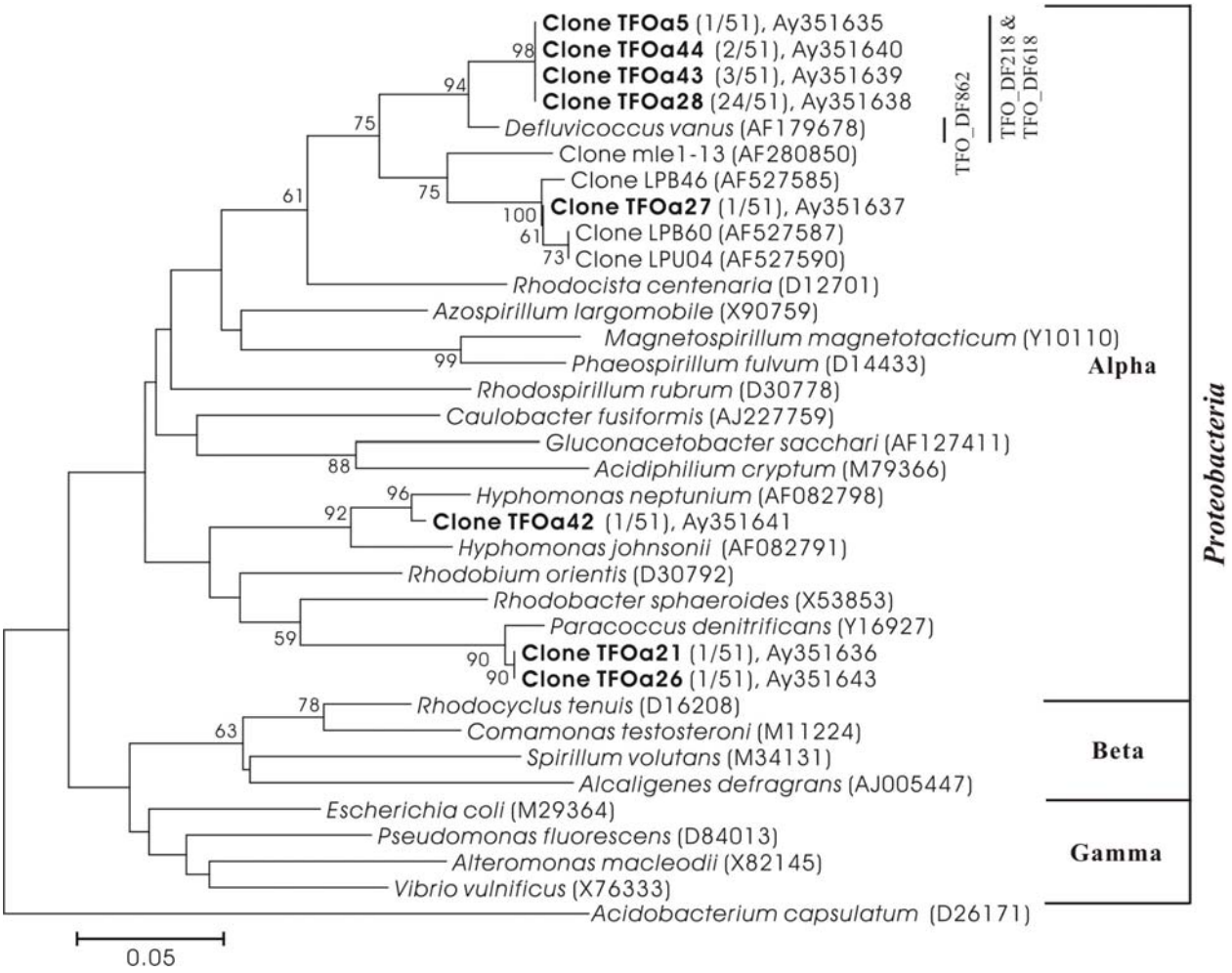

Figure 4-3 Distance matrix-based phylogenetic tree for the novel TFOs found in the *Alphaproteobacteria*. Calculation was based on the neighbor-joining algorithm with 1000 bootstrap sampling. All the sequences found, along with representative sequences from different subdivisions of the *Proteobacteria*, were used in the construction of phylogeny tree. The tree was rooted with the 16S rRNA gene sequences of *Acidobacterium capsulatum* (D26171). The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

4.3.5 Design and Optimization of Probes for the *Defluviicoccus*-related TFO

Kong *et al.* (2001) designed a probe DEF438 targeting *D. vanus*. However, sequence comparison revealed that the probe sequence was identical instead of complementary to the *Defluviicoccus*-related OTUs (Figure 4-3), and had one mismatched nucleotide to four environmental clone sequences (i.e., LPB46, TFOa27, LPB60 and LPU04) closely related to *D. vanus*. These two issues could possibly lead to a failure in detecting the *Defluviicoccus*-related cells or reduce the probe specificity for detecting target cells. Therefore, three further oligonucleotide probes (TFO_DF218, TFO_DF618 and TFO_DF862) that targeted the OTUs found in the *Defluviicoccus* were designed, and were used in FISH analysis to clarify whether they represented the different morphotypes of TFOs (Figure 4-2a) observed in the bioreactor. These probes (listed in Table 4-1) had at least two mismatched sequences to any non-target sequences. The multiple probe strategy could also be used to avoid any failure of probe design associated with poor accessibility of the 16S rRNA sequence (Fuchs *et al.*, 1998), and to determine if inter-group diversity existed within the *Defluviicoccus* cluster. Based on the probe-target dissociation curves (data not shown) obtained at different formamide concentrations with *D. vanus* as a perfect-matched reference strain, the optimized formamide concentrations for probes TFO_DF218, TFO_DF618, and TFO_DF862 were determined to be 25-35%, 25-35% and 35%, respectively.

4.3.6 Phylogenetic Confirmation and PHA-accumulating Traits of the *Defluviicoccus*-related TFO

The CLSM-FISH results indicated that probes TFO_DF218 and TFO_618 could bind to approximately 86.8-93.2% and 64.1-78.6%, respectively of all alphaproteobacterial cells in three biomass samples taken every 2-3 weeks over a period of two months.

This suggests that the probe TFO_DF218 has a broader specificity than probe TFO_DF618 toward these TFOs. Probe TFO_DF862 gave no hybridization signals to any cells in these biomass samples, suggesting that *D. vanus* was not present in the reactor.

Figure 4-4(a) shows a FISH image of a biomass sample taken at the end of anaerobic phase hybridized simultaneously with probes TFO_DF218 and TFO_DF618. At least two different FISH-positive TFO were observed. One, in clusters of 4-6 cells, fluoresced with both probe TFO_DF618 (red) and probe TFO_DF218 (green) as yellow-color cells. The other type fluoresced only with probe TFO_DF218 (green) and usually formed clusters of more than 16 cells, represented about 8-20% of the bacterial cells in the reactor community. These differences in probe responses suggested the existence of other as-yet-uncultured populations within this *Defluviicoccus* cluster. Figure 4-4(b) shows the corresponding Nile blue A-stained image of the FISH-positive cells as shown in Figure 4-4(a). All the *Defluviicoccus*-related TFOs reacted positively to Nile blue A stain but with different levels of fluorescence intensity, for example, among the TFO_DF218-positive, TFO_DF618-negative cells (indicated by arrows in Figure 4-4b). These observations suggested variation among the TFOs in their ability to accumulate PHA.

Currently, no methods are available to directly link glycogen consumption with PHA production for microbial populations of interest following anaerobic substrate uptake. Thus, the involvement of glycogen in the metabolism of substrates and production of PHA in microbial cells was indirectly suggested by the concurrent anaerobic consumption and aerobic production of cellular glycogen (Crocetti *et al.*, 2002;

Levantesi *et al.*, 2002). Using this approach, probable glycogen-accumulation-ability of cells hybridized by probes TFO_DF218 and TFO_DF618 could be indirectly supported by combining the metabolic profiles in Figure 4-1 and the FISH results in Figure 4-4. In biomass samples dominated by cells positive for the probes TFO_DF218 and TFO_DF618 (>70% of the total cells), the PHA content increased from 0% to ~5% of sludge dry weight after anaerobic uptake of acetate. These levels subsequently decreased to almost 0% by the end of the aerobic phase. Thus, these *Defluviicoccus*-related TFOs may be tentatively described as putative GAOs, since their key phenotypic traits are similar to those proposed for other GAOs in EBPR processes (Nielsen *et al.*, 1999; Crocetti *et al.*, 2002; Kong *et al.*, 2002b).

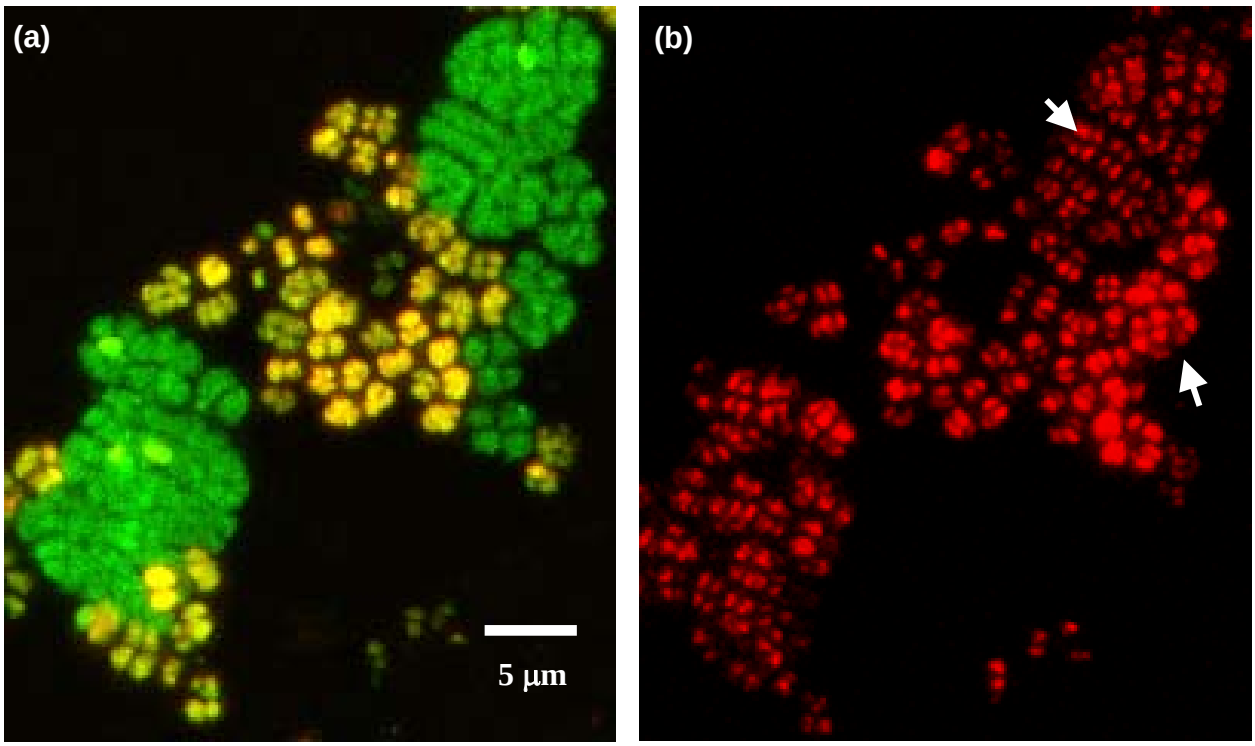

Figure 4-4 (a) CLSM micrographs of FISH showing different types of TFO hybridized by probe TFO_DF618 (Cy5 labeled, red) and/or probe TFO_DF218 (Cy3 labeled, green) targeting the *Defluviicoccus* cluster at different levels of specificity. (b) Nile blue A-stained image of the same field as in (a) showing the accumulation of PHA inside some of the TFO cells.

4.3.7 Occurrence of *Defluviicoccus*-related TFOs in Activated Sludge Treatment Processes

The occurrence of these *Defluviicoccus*-related TFOs in biomass samples from the laboratory-scale and full-scale systems with or without EBPR activity studied in Kong *et al.* (2002b) was examined using FISH and the probes designed here. With the exception of the biomass sample taken from the bioreactor operated in this study, none of the other biomass samples had a high percentage of their total cells as *Defluviicoccus*-related TFO. Unlike the gammaproteobacterial lineage GB (Kong *et al.*, 2002b), this survey suggested that *Defluviicoccus*-related TFO were not the dominant populations in laboratory- and full-scale EBPR systems or in conventional activated sludge processes. This difference could be due to the MBR system used here, where the biomass concentration and organic loading were much higher and lower, respectively, than other conventional gravity settling systems.

In fact, dominance of TFO in EBPR processes has been reported mainly in laboratory-scale systems. Using light microscopy, Cech and Hartman (1993) and Liu *et al.* (1994) observed the proliferation of TFOs in deteriorated lab-scale EBPR processes. Kong *et al.* (2001, 2002a) reported that diverse unidentified alphaproteobacterial TFOs represented more than 50% of the total bacterial cells in a deteriorated EBPR reactor fed with acetate or a mixture of acetate and glucose. They did not detect any TFOs related to the genera *Defluviicoccus* and *Amaricoccus* in their systems, and suspected the existence of other novel TFOs in the *Alphaproteobacteria*. Likewise, Levantesi *et al.* (2002) reported that TFOs other than *Amaricoccus* spp. from the *Alphaproteobacteria* were dominating (25% of total bacterial cells) in an acetate-fed EBPR reactor, but whether the reported TFO were related to the *Defluviicoccus* cluster

revealed here remains to be validated. Thus, the role of the *Defluviicoccus*-related TFOs in the deterioration of full-scale EBPR processes was less clear than the role for members of the gammaproteobacterial lineage GB reported previously (Kong *et al.*, 2002b).

4.4 Conclusions

In summary, this chapter has clearly identified the phylogenetic diversity, phenotypic traits and distribution of three different types of TFO from the *Defluviicoccus* cluster in various laboratory-scale and full-scale activated sludge systems. It is important to further understand the preferred growth environments of the *Defluviicoccus*-related TFO through long-term monitoring of this population in a system under different operating conditions, and through pure culture studies for example using *D. vanus*. This information can provide further understanding on the interactions between/among different GAO and PAO, and improve the process stability of EBPR processes.

Chapter 5

Microbial Succession of Glycogen Accumulating Organisms in an Anaerobic-aerobic Membrane Bioreactor with no Phosphorus Removal

The content of this chapter has been accepted for publication as:

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5.1 Abstract

The succession of glycogen accumulating organisms (GAOs) has been observed in an acetate-fed, anaerobic-aerobic sequencing membrane bioreactor (MBR) operated for 260 days without enhanced biological phosphorus removal (EBPR) activity. Semi-quantitative fluorescence *in situ* hybridization results showed that a gammaproteobacterial lineage GB frequently observed in EBPR processes was initially the numerically dominant species (50-66% of total cells) of the GAO in the MBR from day 1 to day 38. During this period, succession of two different subgroups of group GB was also observed. On day 85 onward, a population shift from GB group to *Defluviicoccus*-related tetrad-forming organisms (TFO) occurred. This microbial succession was suspected to be related to the applied operating conditions (long hydraulic retention time and long solids residence time) which favored the proliferation of *Defluviicoccus*-related TFO rather than the GB group. Application of terminal restriction fragment length polymorphism on selected samples further revealed that the initial sludge inoculum had a high level of phylogenetic diversity of bacterial communities, but the microbial assemblage entered a relative stable population state after day 85.

5.2 Introduction

The deterioration of phosphorus (P) removal in enhanced biological phosphorus removal (EBPR) systems has been commonly ascribed to the proliferation of a physiological group of bacteria broadly classified as glycogen accumulating organisms (GAOs) (Seviour *et al.*, 2003). It is generally accepted that GAOs outcompete polyphosphate accumulating organisms (PAOs) for carbon substrates by utilizing intracellular glycogen instead of polyphosphate as an internally stored energy source to

synthesize polyhydroxyalkanoate (PHA) during the anaerobic feeding stage (Liu *et al.*, 1996).

Past studies have suggested that GAOs consist of several different morphotypes and phylotypes. The tetrad-forming organisms (TFOs), with a distinctive morphology of cocci arranged in tetrads or clusters, were first reported as GAO by Cech and Hartman (1993) in their deteriorated EBPR reactor fed with glucose. Using culture-dependent methods and molecular techniques, microorganisms with the TFO morphotype have been found to be widespread among phylogenetically unrelated groups, including different subdivisions of the *Proteobacteria* and the *Actinobacteria* division (Seviour *et al.*, 2003). Another GAO morphotype that has been described was the coccobacillus-shaped organism observed in an acetate-fed deteriorated EBPR reactor (Liu *et al.*, 1996). It was phylogenetically placed in the *Gammaproteobacteria* (referred to GB group henceforth) and consisted of at least seven phylogenetic subgroups (Nielsen *et al.*, 1999; Kong *et al.*, 2002) including '*Candidatus* Competibacter phosphatis' (Crocetti *et al.*, 2002). Given this complicated phylogenetic picture, it has become apparent that GAOs can be enormously complex assemblages of populations with diverse physiologies. Therefore, a single general ecophysiological model does not appear to be suitable in explaining the proliferation of different GAOs that lead to the failure of EBPR.

Factors influencing the dominance of different GAOs were also important for optimizing EBPR operation, but were so far poorly addressed. Many studies dealt with how certain operational conditions (e.g. long solids residence time (SRT), high anaerobic-aerobic hydraulic retention time (HRT) ratio, acidic pH in anaerobic phase,

and high temperature) and influent substrate composition (e.g. presence of glucose, limited P source, and low P:TOC ratio) may affect EBPR performance (Mino *et al.*, 1998; Seviour *et al.*, 2003). Unfortunately, most of these studies were carried out without structure-function analyses of the changes in the microbial community, making uncertain their general applicability to EBPR operation.

In this study, the succession of two different GAO groups has been observed in an acetate-fed anaerobic-aerobic sequencing membrane bioreactor (MBR) without EBPR activity over an operation of 260 days. Culture independent 16S rRNA-based methods, terminal restriction fragment polymorphism (T-RFLP) and fluorescence *in situ* hybridization (FISH) were employed to reveal their identities. The effect of MBR operating conditions on their population dynamics was also discussed. Such information will allow a better basis for understanding the niches for different GAO groups in EBPR processes and provide a stronger basis for analyses of factors influencing the stability of P removal in these systems.

5.3 Results and Discussion

5.3.1 Reactor Performance

Biomass composition and chemical analysis data suggested that a microbial consortium exhibiting the typical characteristics of GAO was enriched in the MBR over the period of 260 days. The carbon removal efficiencies at the end of the anaerobic phase were consistently 85±6.4% and the accompanying biomass PHA content was steady at 57±8% from day 41 onwards. At the end of the aerobic phase, intracellular glycogen level increased from 6% to 20% of biomass dry weight and P

content in the sludge decreased from 2.5% to 1-2% of biomass dry weight during the first 20 and 5 days of operation respectively.

5.3.2 Microscopic Observations of the Community

A large fraction of total bacterial cells in a sludge sample from day 20 was observed to be Sudan black B-positive and accumulated several small intracellular granules (Figure 5-1a). Most of these Sudan black B-stained cells were randomly scattered in the sludge. They were either large coccobacilli or irregular long rods up to 10-15 μm in length and resembled one of the putative GAO (GB) (Figure 5-1b). A significant morphological change was observed from day 55 onwards. The predominant morphotype became bacterial cells occurring in clusters of four or more cells (the so-called TFOs) (Figure 5-1e). Based on size and cluster formation patterns, various morphotypes of TFO were observed and recognized. Most TFOs showed positive response to Sudan black B staining, but to different extents (Figure 5-1d). No Neisser-positive cells could be detected after day 5, further suggesting that a microbial consortium with no EBPR activity was enriched in the MBR.

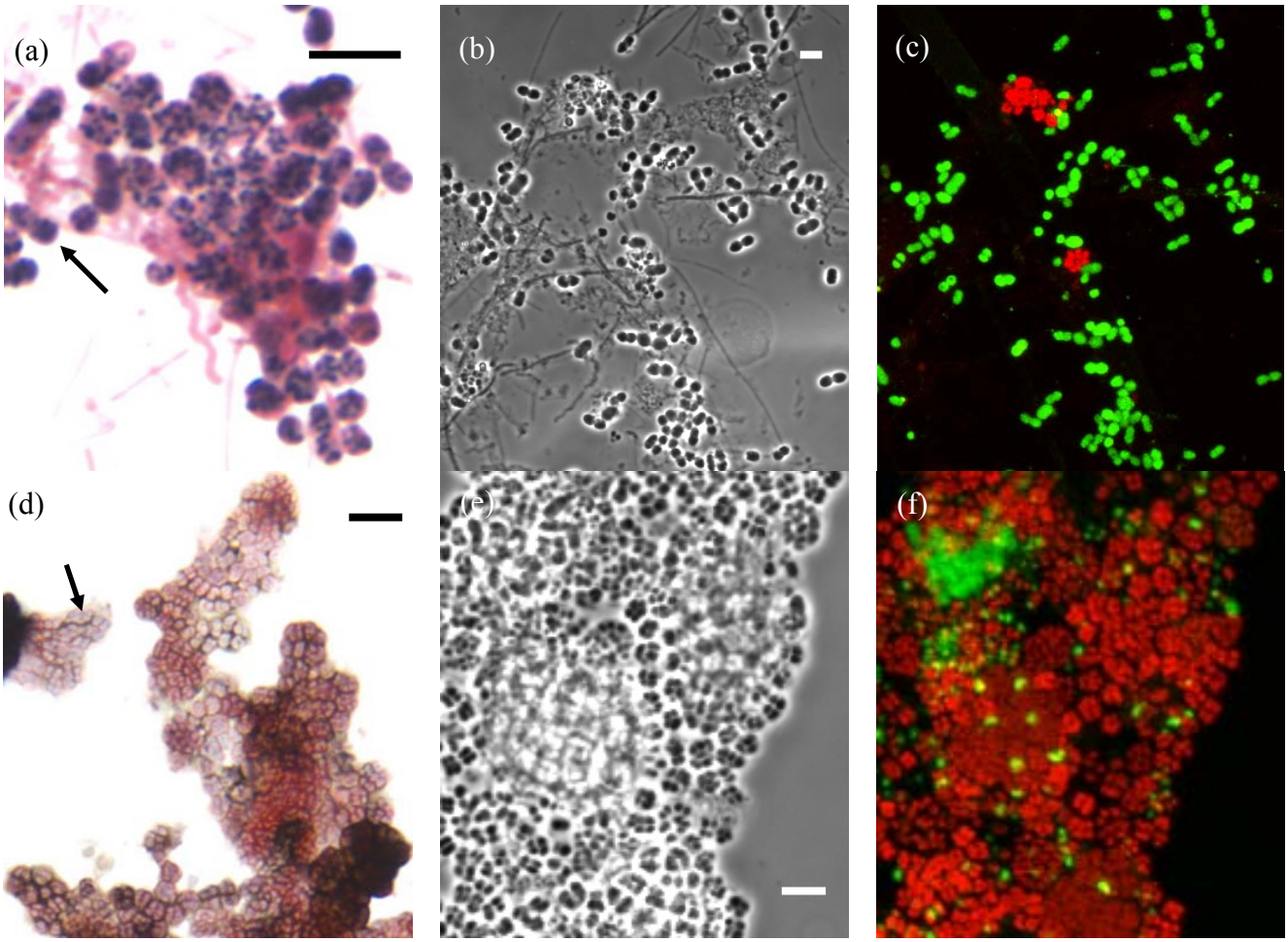

Figure 5-1 Micrographs of activated sludge samples from the acetate-fed MBR. (a and d) Sudan black B-stained images on days 21 and 117 respectively showing PHA accumulation of biomass. Cells marked with an arrow indicate the presence of intracellular PHA granules. (b and e) Phase-contrast images on days 21 and 117 respectively showing different predominant morphotypes of GAO. (c and f) CLSM micrographs of FISH of the same field as in (b) and (e) with probes TFO_DF218 (Cy3-labeled, red) and GB (Cy5-labeled, green). Scale bar = 5 μm. The scale bars in (b) and (e) apply to (c) and (f) respectively.

5.3.3 Microbial Population Dynamics in the MBR as Revealed by FISH

The microbial population dynamics was determined by semi-quantitative FISH analysis with oligonucleotide probes targeting five major phylogenetic groups commonly found in activated sludge systems. Figure 5-2 shows that the *Alpha*-, *Beta*-, *Gammaproteobacteria*, *Actinobacteria*, and CFB group accounted for 18±4, 39±10,

22±5, 7±3, and 17±4% of total bacterial cells respectively in the sludge inoculum at day 1. During the next 38 days of operation, the *Betaproteobacteria* population changed significantly. Starting as the most predominant group, it decreased to 27±7% of total bacterial cells by day 10, then increased rapidly to 60±8% by day 21 and subsequently gradually decreased to 19±8% by day 38. The changes of *Alpha*- and *Gammaproteobacteria*, *Actinobacteria*, and CFB group were not as explicit as the *Betaproteobacteria* during this period. However, a significant shift in composition and abundance of the predominant microbial populations was recorded between days 38 and 85. *Alphaproteobacteria*, which accounted for over 80% of the bacterial cells, became the numerically abundant group and remained as the predominant group until the end of operation. This apparent shift in community structure was consistent to the change in morphologies of the predominant populations noted in the previous section. In some samples, the summations of the percentage of cells detected with group-specific probes were slightly greater than 100%. During detection with the bacterial probe EUBmix, the signal from small and weakly fluorescing cells may be obscured by adjacent cells that are larger and brighter (Bond *et al.*, 1999). An overestimation of cells binding group-specific probes in a bacterial community could thus result.

Subsequent semi-quantitative FISH analysis using the group and subgroup-specific probes targeting GB, *Defluviicoccus*-related TFO, and *Rhodocyclus*-related PAO were carried out to reveal their abundance during MBR operation (Figure 5-3). Probes GB_G1 and GB_G2 separately hybridized with different subgroups of GB cell in the sludge, together constituting 4±2% of total bacterial cells in the inoculum. Their total counts increased rapidly to 51±10% at day 10 and to approximately 60% between days 15 and 21, before decreasing to 20±7% at day 38, leveling off at 2-10% from day 85.

Among all GB subgroups, GB_4 was found to be the most predominant one from day 15 onwards (data not shown). However, the remaining fraction of probe GB hybridized cells could not be further defined by any one of the GB subgroup probes designed by Kong *et al.* (2002), suggesting the presence of a new GB subgroup in the samples. It was also noted that the inconsistencies of probes GAM42a and GB counts were attributed to the specificity of probes BET42a and GAM42a which differed from each other by a central nucleotide and originally designed based on a limited number of 23S rRNA sequences available (Yeates *et al.*, 2003). Simultaneous FISH analysis with probes BET42a and GB_4 has performed to correlate the abundances between *Betaproteobacteria* and GB subgroup 4 (data not shown).

On the other hand, the *Defluviicoccus*-related TFOs, targeted by probes TFO_DF218 and TFO_DF618, started to become significant at 9±3% on day 34, and overwhelmingly dominated the microbial assemblage (> 50% of total bacterial cells) after day 85. Between days 85 and 117, most of the *Defluviicoccus*-related TFOs were equally targeted by both probes TFO_DF218 and TFO_DF618. After day 117, the *Defluviicoccus*-related TFOs targeted by the probe TFO_DF218 alone replaced and became the numerically abundant group. Figures 5-1 (c and f) show the CSLM-FISH images of the GAO cells simultaneously hybridized by probes TFO_DF218 (Cy3-labeled, red) and GB (Cy5-labeled, green) on days 21 and 117 respectively, clearly demonstrating the succession of predominant population.

Rhodocyclus-related PAO consisted of less than 5% of total bacterial cells at day 1, and decreased to undetectable levels after day 10. These FISH findings agreed with the observations that the sludge was low in P contents and had undetectable amounts of

Neisser-positive cells. This suggested that the applied operating environment in the MBR favored the proliferation of GAOs but not PAOs.

5.3.4 Analysis of Microbial Community Structure Shift by T-RFLP

Microbial community shift was also monitored using T-RFLP fingerprinting methods with three different restriction endonucleases (*Hha*I, *Msp*I, and *Rsa*I) on selected samples. The discernible T-RF patterns revealed the microbial assemblage had a high level of phylogenetic diversity, but entered a relatively stable population state, characterized by a reduction in the overall species richness (T-RFs) after day 85. Clustering analysis in Figure 5-4 indicates the samples from days 10 to 38 formed one large cluster with high similarity (> 70%) while those from day 85 onwards formed a distinctive group (Figure 5-4).

In a computer simulation, GB sequences from 28 activated sludge clones obtained in previous studies (Dabert *et al.*, 2001; Crocetti *et al.*, 2002; Kong *et al.*, 2002) were cleaved with four restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, and *Msp*I). The lengths of these theoretical T-RFs were calculated, and assigned to peaks found in the chromatograms from the samples collected in days 10, 15, 21, 29, and 34. Results indicated that PCR products amplified with primer pairs 47f-GB or 47f-G1G2 followed by separate restriction digestion with these four restriction endonucleases reproducibly yielded a single sharp peak for the selected samples. These peaks accounted for 78±16% of total T-RF abundances and corresponded to the predicted cleavage positions (±3 mismatched nucleotides) of clone SBRC_10 (Kong *et al.*, 2002). The clone SBRC_10 was originally assigned to be GB subgroup 7 but detailed sequence comparison revealed that it had 2-5 mismatched nucleotides to all the GB

subgroup probes designed by Kong *et al.* (2002). This finding further supported the presence of a new GB subgroup in the samples. Further clone library analysis followed by probe design for this MBR sludge is necessary to elucidate the identity of this new GB subgroup.

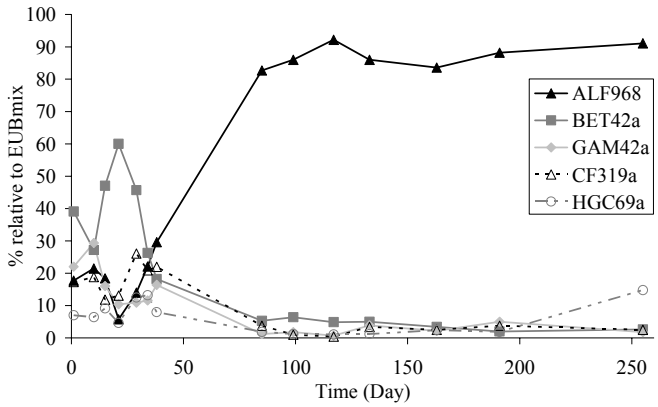

Figure 5-2 Microbial population change in the biomass as determined by semi-quantitative FISH analysis.

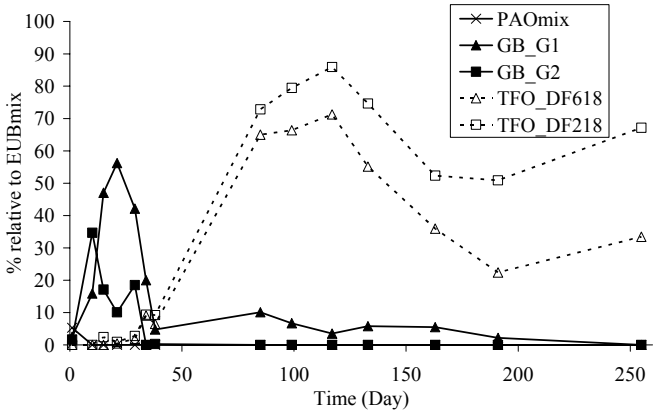

Figure 5-3 Population dynamics of different members of GB and *Defluviicoccus*-related TFO in the biomass during the entire operation.

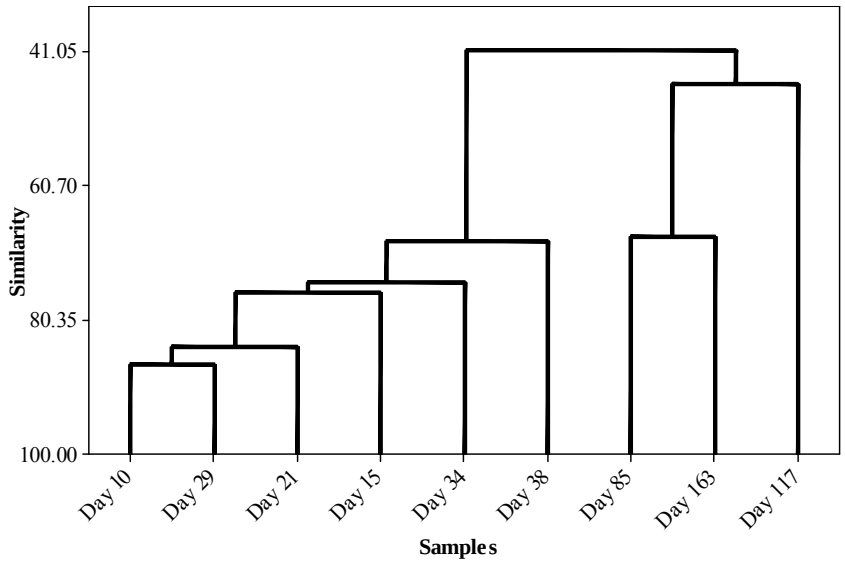

Figure 5-4 Dendrogram of the cluster analysis on the T-RFLP profile after *Msp*I digestion of PCR amplifying of the 16S rRNA gene for the domain *Bacteria* for selected samples. The dendrogram was constructed by selecting the single linkage and Euclidean distance rules in the Minitab 14 program.

5.3.5 Competitive Advantage of TFO over GB in Anaerobic-aerobic MBR

The complex interactions that lead to the selection of different microorganisms in an activated sludge system are not well understood. Using the applied conditions that should select for PAOs (high P:TOC ratio), GAOs predominated instead. Furthermore, the succession of predominance from the GB group to *Defluviicoccus*-related TFOs indicated a competitive interaction between these two GAO groups. GAO-GAO competition was less documented than PAO-GAO competition. This study suggested that the MBR operating conditions applied a selective pressure on the dominant organisms, resulting in the competition between the GB group and *Defluviicoccus*-related TFOs. The sludge inoculum was initially collected from a local conventional wastewater treatment plant which comprised a considerable fraction of GB group (5±3%). The applied cyclic anaerobic-aerobic phase in the MBR which was the preferred growth environment for the GB group provided a selective pressure for them to outcompete other strictly aerobic heterotrophs (Kong *et al.*, 2002). This may explain the initial dominance of the GB group in MBR sludge. However, after a few SRTs of operation, the *Defluviicoccus*-related TFO gradually became the major population and outgrew the GB group as the predominant population. This may be attributed to the applied long HRT and SRT operation that favored the proliferation of the *Defluviicoccus*-related TFO rather than the GB group. As opposed to the different TFO phylogenetic groups, the GB group has only been reported to be more prevalent in laboratory- and full-scale EBPR systems operating at relatively short HRTs (< 12 h) and SRTs (7-8 d) (Nielsen *et al.*, 1999; Liu *et al.*, 2001; Crocetti *et al.*, 2002; Kong *et al.*, 2002). In addition, microbial succession was also perceived within the GB group and *Defluviicoccus*-related TFOs as revealed by the semi-quantitative FISH analysis

using their subgroup-specific probes. No clear explanation could be provided on this interspecies competition until the isolation of their pure cultures. Further useful information on their nutritional requirements could be obtained using FISH with the microautoradiography technique by allowing insight into their physiology *in situ*.

5.4 Conclusions

The biomass of a sequencing acetate-fed MBR operating with anaerobic-aerobic cycling produced a biomass dominated by GAOs which exhibited glycogen and PHA accumulation traits commonly observed in deteriorated EBPR systems. Microbial succession occurred between the GB group and *Defluviicoccus*-related TFOs. A long SRT (> 10 d) and a long HRT (> 12 h) were considered to be the important factors for the *Defluviicoccus*-related TFOs to outcompete the GB group.

Chapter 6

Ecophysiology of *Defluviicoccus*-related Tetrad-forming Organisms in an Anaerobic-aerobic Activated Sludge Process

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6.1 Abstract

A group of uncultured tetrad-forming organisms (TFOs) was enriched and dominating in an acetate-fed anaerobic-aerobic sequencing membrane bioreactor showing deteriorated enhanced biological phosphorus removal capacity. Results of 16S rRNA gene clone library and fluorescence *in situ* hybridization (FISH) showed that these TFOs were novel members of the *Defluviicoccus* cluster in the *Alphaproteobacteria*, accounting for 90±5% of the EUBmix FISH-detectable bacterial cells in the reactor biomass. Microautoradiography in combination with FISH and polyhydroxyalkanoate (PHA) staining revealed that these *Defluviicoccus*-related TFOs could take up and transform acetate, lactate, propionate, and pyruvate, but not aspartic acid and glucose, as PHA under anaerobic conditions. In contrast, under continuous anaerobic-aerobic cultivation, *Defluviicoccus vanus* NCIMB 13612, the only isolate from the cluster, was able to take up glucose with concurrent glycogen consumption and PHA production under anaerobic conditions. Under subsequent aerobic conditions, the accumulated PHA was utilized and the biomass glycogen levels restored. These findings not only re-confirm that these *Defluviicoccus*-related TFOs behave as glycogen accumulating organisms, but also reveal unexpected levels of physiological, phylogenetic and morphological diversity within members of the *Defluviicoccus* cluster.

6.2 Introduction

In order to minimize the risks of eutrophication in receiving bodies of water, enhanced biological phosphorus removal (EBPR) processes are widely used to remove soluble orthophosphate (Pi) in domestic wastewater. Engineering strategies implemented through mass-balance control and empirical practices have made important contributions in enabling the selection of the microbial communities capable of

performing EBPR. However, achieving stable and reliable operation of these systems at both laboratory- and full-scales has been shown to be problematic. This is in part related to our poor understanding of the phylogenetic and functional complexity of the microbial populations in these systems (Seviour *et al.*, 2003).

Both rRNA gene sequence analysis and fluorescence *in situ* hybridization (FISH) with targeted rRNA-targeted oligonucleotide probes has greatly advanced our understanding of the microbial complexity in laboratory-scale EBPR processes. So far, any different bacterial populations have been phylogenetically identified as having the phenotype expected of either polyphosphate accumulating organisms (PAOs) that promote EBPR, or glycogen accumulating organisms (GAOs) that compete with PAOs anaerobically for substrates and possibly lead to deterioration of EBPR capacity. These populations are affiliated with several subdivisions of the *Proteobacteria*, the *Actinobacteria*, and the *Bacteroidetes* group (Seviour *et al.*, 2003). For example, one putative PAO is closely related to members of the genus *Rhodocyclus* (Crocetti *et al.*, 2000) while one putative GAO is the coccobacillus-shaped organism deeply branched in the *Gammaproteobacteria* (GB group) (Kong *et al.*, 2002). To effectively monitor these populations in EBPR systems, oligonucleotide probes have been developed, validated and used in FISH analysis (Seviour *et al.*, 2003).

However, our recent survey on the bacterial community composition in nine different full-scale EBPR plants in Japan revealed that only 24-48% of the total bacterial cells in these plants could be identified by FISH using the available oligonucleotide probes targeting the populations reportedly found in EBPR processes (Wong *et al.*, 2005). As with other similar studies (Zilles *et al.*, 2002; Lee *et al.*, 2003; Kong *et al.*, 2004;

Wong *et al.*, 2005), the role of *Rhodocyclus*-related PAOs in full-scale EBPR processes may be less important than in laboratory-scale EBPR systems fed with acetate as sole carbon source. Thus to further elucidate the microbial community structure in EBPR processes, experimental approaches that can explicitly link the presence of an individual population to its involvement in mediating the chemical transformations of importance are needed (Seviour *et al.*, 2003).

Microautoradiography (MAR) is an elegant tool to evaluate substrate assimilation capability of individual cells in situ in complex ecosystems (Andreasen and Nielsen, 1997). By combining MAR with FISH, *in situ* substrate utilization patterns of individual populations can be resolved, allowing their ecophysiology in EBPR processes to be examined (Lee *et al.*, 1999; Ouverney and Fuhrman, 1999). Findings suggest that both the *Rhodocyclus*-related PAO in the *Betaproteobacteria* and the *Tetrasphaera*-related PAO in the *Actinobacteria* division are actively involved in the aerobic assimilation of Pi in full scale plants (Lee *et al.*, 2003; Kong *et al.*, 2004, 2005b). The observed metabolic behavior of the *Rhodocyclus*-related PAOs was generally consistent with the biochemical models proposed for EBPR (Mino *et al.*, 1998). However, the diversity of organic substrates taken up anaerobically by the *Rhodocyclus*-related PAOs and the *Tetrasphaera*-related PAOs differed markedly (Kong *et al.*, 2004, 2005b). For example, the *Tetrasphaera*-related PAOs utilized only amino acids (i.e., casamino acids) while the *Rhodocyclus*-related PAOs consumed a number of substrate including acetate, propionate, and glutamic acid. Furthermore PHA synthesis was detected only with the *Rhodocyclus*-related PAOs.

Only one study has applied MAR-FISH to look at the ecophysiological traits of GAOs in laboratory-scale anaerobic-aerobic sequencing batch reactor (SBR) fed with a mixture of acetate and glucose (Kong *et al.*, 2001). No clear distinction could be seen there in the substrate uptake patterns of tetrad-forming organisms (TFOs) from the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Micropruina glycogenica*, a member of the *Actinobacteria*. All these populations assimilated acetate but not glucose anaerobically, and this finding questioned the original suggestion (Cech and Hartman, 1993) that GAO became dominant over PAOs because of their preferential anaerobic assimilation of glucose. However, it should be noted in the study of Cech and Hartman (1993) that no *in situ* physiological experiments were carried out to confirm the observation that the TFOs were in fact GAO. Although dominant bacterial populations from this reactor were successfully isolated, characterized, and named as *Amaricoccus* spp. in the *Alphaproteobacteria* (Masznan *et al.*, 1997), pure culture studies indicated that these *Amaricoccus* spp. were not able to assimilate either acetate or glucose anaerobically (Falvo *et al.*, 2001), and the question whether *Amaricoccus* spp. were actually the dominant populations observed by Cech and Hartman (1993) or not remained unresolved.

We recently reported the dominance of TFOs in an acetate-fed, anaerobic-aerobic sequencing membrane bioreactor (MBR) showing deteriorated EBPR capacity (Wong *et al.*, 2004). These TFOs were identified as members of the *Defluviicoccus* cluster in the *Alphaproteobacteria*. A combination of biomass composition data, metabolic profiling and FISH, together with polyhydroxyalkanoate (PHA) staining suggested these possessed the phenotype expected of GAOs in EBPR systems. However, little else is known about their ecophysiology in EBPR processes. To resolve this question,

this study was carried out to characterize the TFO community in this MBR run under identical conditions as before. Several culture-independent methods combined with MAR, FISH, and PHA staining was applied to this community to elucidate their ecophysiology, and the data compared with those from similar studies with other putative GAOs. In addition, alternating anaerobic-aerobic batch experiments were conducted to examine the metabolic attributes of *Defluviicoccus vanus* NCIMB 13612 to assess the possible levels of metabolic variation existing within the *Defluviicoccus* cluster.

6.3 Results

6.3.1 Performance of an Acetate-fed MBR

The MBR was operated under cyclic anaerobic and aerobic conditions with acetate as the sole carbon source for 474 days. During this period, the total suspended solids (TSS) concentration gradually increased from 3000 mg l^{-1} at day 1, and leveled off at around 12500±2500 mg l^{-1} from day 50 onwards. On days 135, a failure with the pH controller occurred. Although this problem was remedied immediately, a marked drop in the mixed liquor pH to 2 was observed. Overall, the average carbon removal efficiency at the end of the anaerobic phase and the Pi removal efficiency at the end of the aerobic phase were 85±5% and 10±4% of the levels originally added, respectively.

Figure 6-1 illustrates a typical MBR chemical profile, which were measured on four different occasions between days 200 and 420. The carbon source (acetate) expressed as TOC was rapidly consumed from 200±45 mg l^{-1} to less than 20±5 mg l^{-1} within the first hour of the anaerobic stage. The remaining TOC was possibly soluble microbial products, often reported in MBR systems (Urbain *et al.*, 1998). Decrease in medium

carbon levels was accompanied by a decrease in the biomass intracellular glycogen content from 158±8 to 105±27 mg (g TSS)$^{-1}$, and a corresponding increase in intracellular PHA content from 4±2 to 32±9 mg (g TSS)$^{-1}$. No obvious anaerobic Pi release or aerobic Pi uptake were observed. During the subsequent aerobic stage, the intracellular glycogen level increased gradually while the PHA level decreased to almost zero. Overall reactor performance suggested a deteriorated EBPR capacity even though the operational conditions (high P:TOC ratio) were designed to promote EBPR activity in the system.

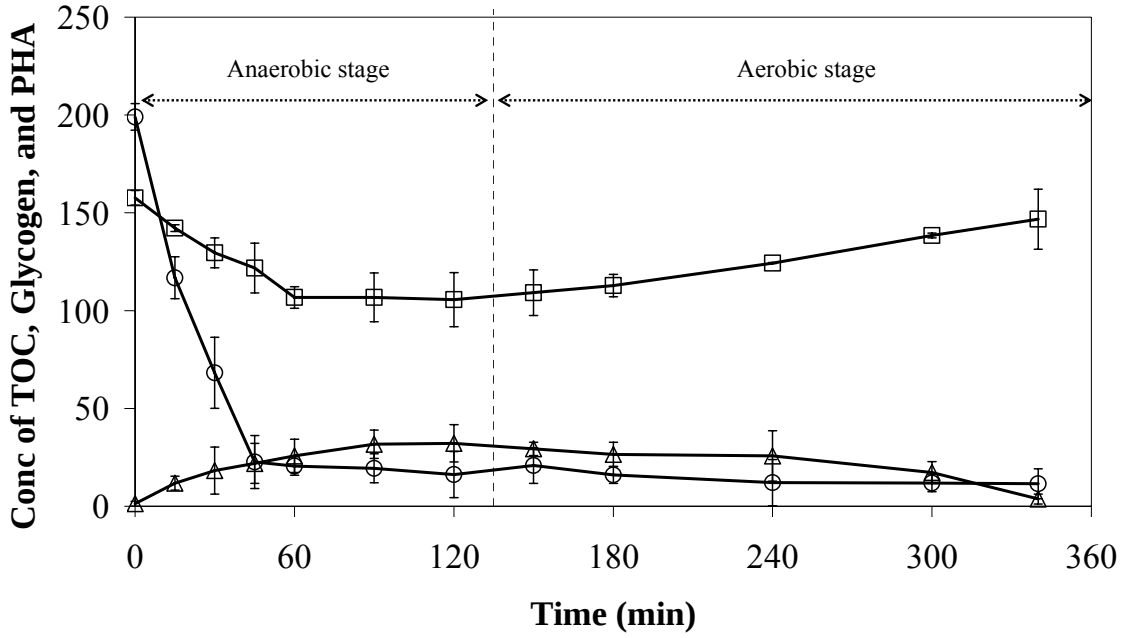


Figure 6-1 Chemical changes that occurred during an anaerobic-aerobic cycle in the acetate-fed MBR. Standard derivations of the mean were also included. ○, TOC [mg l^{-1}]; △, PHA [mg (g TSS)$^{-1}$]; □, glycogen [mg (g TSS)$^{-1}$]. A 10-min feeding phase at the beginning of anaerobic stage is not shown.

6.3.2 Anaerobic and Aerobic Batch Experiments for Substrate Uptake

Substrate assimilation patterns of the biomass in the MBR were further investigated (see Figure 6-2). Among the six carbon substrates examined, acetate, lactate,

propionate, and pyruvate were all completely taken up under both anaerobic and aerobic conditions. Concurrently, a decrease in intracellular glycogen and increase in PHA biomass levels were detected under anaerobic conditions. Compared to anaerobic conditions, the uptake rate of a same substrate was always faster, and the amounts of PHA synthesized and glycogen consumed were higher under anaerobic conditions.

Under anaerobic conditions, the amount of glycogen consumed varied with the substrate used, from 30 to 100 mg (g TSS)$^{-1}$. The highest amount of glycogen was consumed during the uptake of acetate, followed by propionate, lactate, and then pyruvate uptake. Acetate and propionate were the most effective substrates supporting the anaerobic synthesis of PHA [~ 80 mg (g TSS)$^{-1}$], followed by lactate and pyruvate [~ 50 mg (g TSS)$^{-1}$]. The PHA produced from acetate, propionate, pyruvate, and lactate consisted of a mixture of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). However, the molar percentage of 3HB and 3HV varied with the substrate assimilated and the incubation conditions used. 3HB (~ 71% of total PHA detected) was the major component produced after acetate uptake, 3HV (~ 80%) from propionate and lactate uptake, and equal amounts of 3HB and 3HV from pyruvate uptake. Although glucose and aspartic acid were hardly consumed by the enriched sludge under either anaerobic or aerobic conditions, slight degradation of intracellular glycogen and PHA was still observed, and possibly used for cell maintenance.

Under aerobic conditions with the addition of acetate and propionate, their uptake was accompanied by an accumulation of PHA and consumption of glycogen. Compared with anaerobic PHA synthesis, more amounts of 3HB were produced from the uptake of acetate (~ 98%), pyruvate (~ 100%), and lactate (~ 86%). Only minor changes in

intracellular glycogen levels were observed when either pyruvate or lactate was used as the substrate.

6.3.3 Microscopic Observations of the Community

During the reactor operation, activated sludge samples were regularly taken at the end of the anaerobic phase, and examined under a light microscope. Small cocci/coccobacilli (1.5-2 μm) occurring mostly in pairs, tetrads, or aggregates (the so-called TFOs) were the most dominant populations observed in samples taken at day 65 and onwards. However, these TFOs were morphologically different from those previously described, in terms of both their cell size and cluster formation patterns (Figure 6-3a) (Wong *et al.*, 2004). Most stained positively with Sudan black B, and their cells contained several small granular inclusions (Figure 6-3b). Nile blue A staining confirmed these granules were PHA (Figure 6-3c). More PHA positively-stained cells were observed using Nile blue A stain than with Sudan black B staining, and Nile blue A stain was reported to be more specific and sensitive for PHA (Ostle and Holt, 1982).

6.3.4 Confirmation of the TFO as *Defluviicoccus*-related Organisms Using FISH

Using FISH technique, the most numerically abundant group in samples taken from day 65 was the *Alphaproteobacteria*, accounting for 95±5% of EUBmix-stained cells. The majority of these alphaproteobacterial cells were further identified to be *Defluviicoccus*-related TFO using three existing oligonucleotide probes targeting this cluster (Figure 6-4) (Wong *et al.*, 2004). Approximately 90±5% of EUBmix-stained cells fluoresced with probe TFO_DF218 in four different biomass samples taken every 30-50 days over a 200 day period (Figure 6-3d), but no hybridization signal was

observed with the TFO_DF618 probe or TFO_DF862 probe. These observation suggested that these *Defluviicoccus*-related TFOs were phylogenetically different from the two phylotypes described previously i.e., one targeted by probes TFO_DF218+TFO_DF618 and the other by probe TFO_DF218 only (Wong *et al.*, 2004).

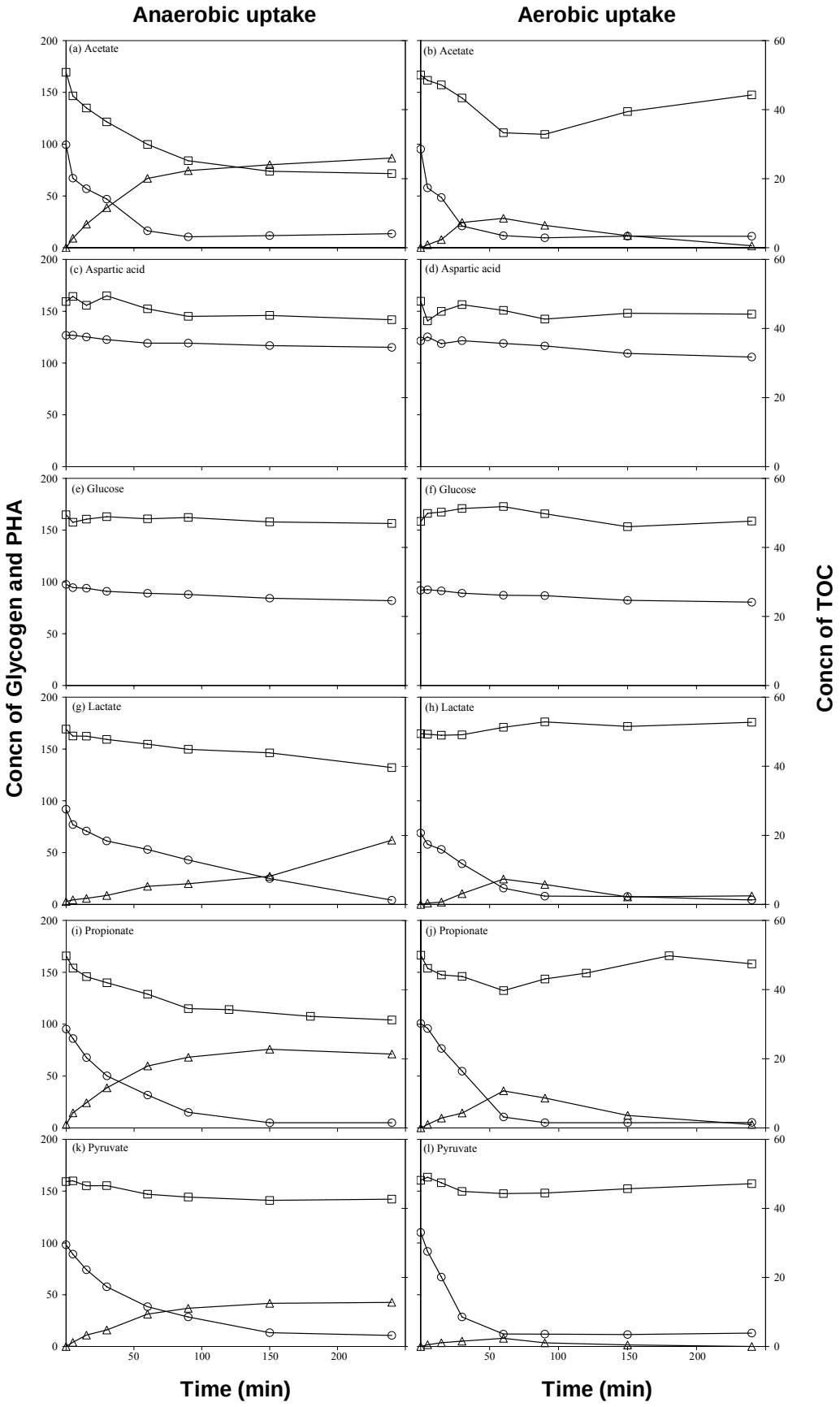


Figure 6-2 Chemical changes that occurred during batch experiments with (a) anaerobic acetate uptake, (b) aerobic acetate uptake, (c) anaerobic

aspartic acid uptake, (d) aerobic aspartic acid uptake, (e) anaerobic glucose uptake, (f) aerobic glucose uptake, (g) anaerobic lactate uptake, (h) aerobic lactate uptake, (i) anaerobic propionate uptake, (j) aerobic propionate uptake, (k) anaerobic pyruvate uptake, and (l) aerobic pyruvate uptake. ○, TOC [mg (g TSS)$^{-1}$]; △, PHA [mg (g TSS)$^{-1}$]; □, glycogen [mg (g TSS)$^{-1}$].

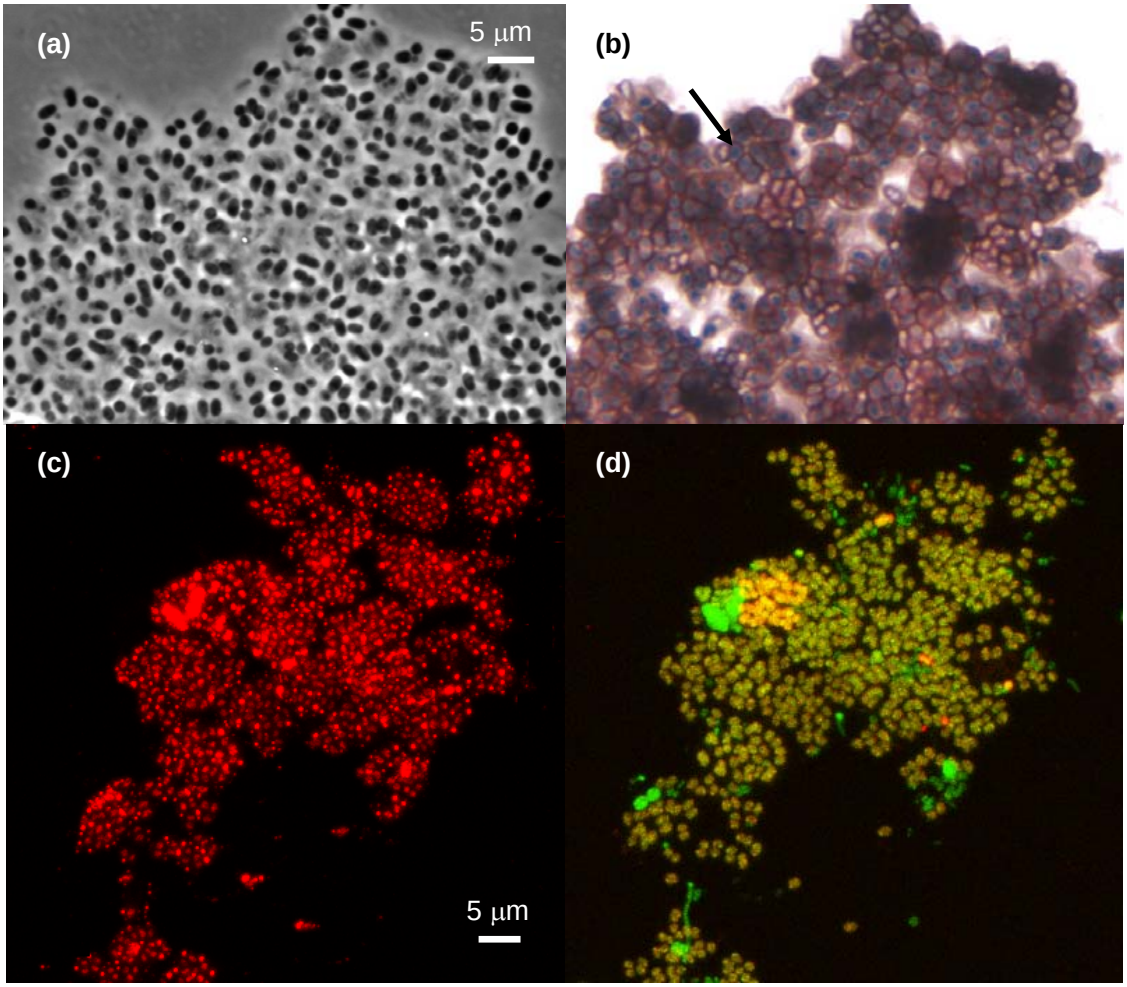


Figure 6-3 (a) Phase-contrast image. (b) Sudan black-B stained image of the same field as in (a). Black arrow indicated PHA accumulating cells. (c) Nile blue A-stained image. (d) CLSM micrograph of FISH of the same field as in (c) showing the *Defluviicoccus* -related TFOs in yellow, which were simultaneously hybridized by probe TFO_DF218 (Cy3-labeled, red) targeting the *Defluviicoccus* -related TFOs and probe EUBmix (Cy5-labeled, green) targeting total bacterial cells.

6.3.5 Phylogenetic Analysis of the *Defluviicoccus*-related TFOs

To clarify the level of diversity within members of this *Defluviicoccus* cluster, two 16S rRNA gene clone libraries targeting the *Alphaproteobacteria* were constructed from sludge samples taken at days 187 and 338. In sample day-187, 12 OTUs (designated TFOb clones) were obtained from the 52 clones screened. Nine OTUs (representing 83% of the total clones) were related to the *Alphaproteobacteria*, and the remaining OTUs detected in this clone library were identified as chimeric sequences or sequences related to the genus *Providencia* from the *Gammaproteobacteria* and members of the *Planctomycetes*. Among the *Alphaproteobacteria*-related OTUs, four were closely related to the *Defluviicoccus*-related TFO clones described previously (sequence similarity > 97%) (Wong *et al.*, 2004). The remaining five OTUs were related to environmental clones previously retrieved from the paralytic shellfish poison-related dinoflagellagte *Alexandrium tamarense* (Groben *et al.*, 2000). In sample day-338, 13 OTUs (designated TFOc clones) were obtained (48 clones). Ten OTUs (representing 69% of the total clones) were related to the *Alphaproteobacteria*, and the remaining three OTUs were affiliated with the genus *Pseudoxanthomonas* from the *Gammaproteobacteria*, the *Actinobacteria*, and the *Verrucomicrobia*. Among the *Alphaproteobacteria*-related OTUs, two were closely related to the *Defluviicoccus*-related TFO clones, while the remaining eight were related to *Porphyrobacter* spp., *Hyphomonas* spp., and *Paracoccus* spp..

A phylogenetic tree (Figure 6-4) was constructed for all known 16S rRNA sequences of *Defluviicoccus*-related organisms. These sequences formed a single clade (100% bootstrap value) that did not relate closely to any currently recognized species within the family *Rhodospirillaceae* (67% bootstrap value). This cluster could be further

divided into three distinct monophyletic clusters supported by bootstrap values. Cluster I comprised sequences of the type strain of *D. vanus* NCIMB 13612 (Maszenan *et al.*, 2005), isolated from a full-scale EBPR process in the Czech Republic, and environmental clones from laboratory-scale EBPR reactors fed with short chain fatty acids (SCFAs) (Wong *et al.*, 2004; Meyer *et al.*, 2006; this study). Clusters II was comprised solely of sequences of environmental clones from laboratory-scale EBPR reactors fed with SCFAs and/or amino acids (McMaholm *et al.*, 2002; Wong *et al.*, 2004; Meyer *et al.*, 2006). Cluster III comprised a single environmental clone sequence (clone mle1-13) retrieved from a non-EBPR bioreactor treating pharmaceutical wastewater (LaPara *et al.*, 2000). The phylogenetic tree produced with maximum parsimony exhibited an identical topology to the neighbor-joining tree (data not shown).

6.3.6 Probe Design and Optimization

An hierarchical probe strategy was used in FISH to minimize any possible problems in probe design associated with poor accessibility to their targeted 16S rRNA sequences (Fuchs *et al.*, 1998), and to reveal if any inter-group population structure existed within members of this *Defluviicoccus*-related cluster. Besides the existing probes for these populations, two additional oligonucleotide probes named TFO_DF629 and TFO_DF776 were designed (Figure 6-4). Probe TFO_DF629 targeted cluster II. It had one internal C-A mismatch with the target sequences of members of the cluster I and at least two mismatches with all other known 16S rRNA sequences. The TFO-DF776 probe targeted all members of the cluster I, and seemed to have a specificity similar the TFO_DF218 probe described previously (Wong *et al.*, 2004). Based on the probe-target dissociation curve obtained at different formamide concentrations with the

sludge samples, the optimized formamide concentrations for the TFO_DF776 probe was determined to be 25-35% (data not shown). We also noted that it was not possible to design an oligonucleotide probe targeting exclusively the dominant clones (i.e., TFOb5 and TFOc18) within cluster I.

FISH results indicated that the TFO_DF776 probe could bind to approximately 95±3% of all the TFO_DF218-stained cells in four biomass samples taken every 30-50 days over a period of 200 days (Figure 6-5d). This suggests that probe TFO_DF218 has a broader specificity than the TFO_DF776 probe towards to these populations. Probe TFO_DF629 gave no hybridization signals with any cells in these biomass samples, suggesting either an absence of the targeted subgroup in the reactor, the inaccessibility of probe to the targeted sequences, or the heterogeneity among different 16S rRNA copies within a single bacterial chromosome (Head *et al.*, 1998). Overall FISH results suggested certain degree of phylogenetical structure within cluster I. Similar results were reported for cluster II (Meyer *et al.*, 2006). It is still not clear to what extent the ecological properties (tolerances to biotic or abiotic conditions, substrate affinities etc) of members of each of these clusters might vary.

The abundance of *Paracoccus*-related populations in these biomass samples was also examined with probe PAR651, as a considerable proportion of clones from the sample taken at day 338 (21% of the total clones) was affiliated with this genus. However, only less than 1% of EUBmix-stained cells were targeted by probe PAR651 in all the samples examined.

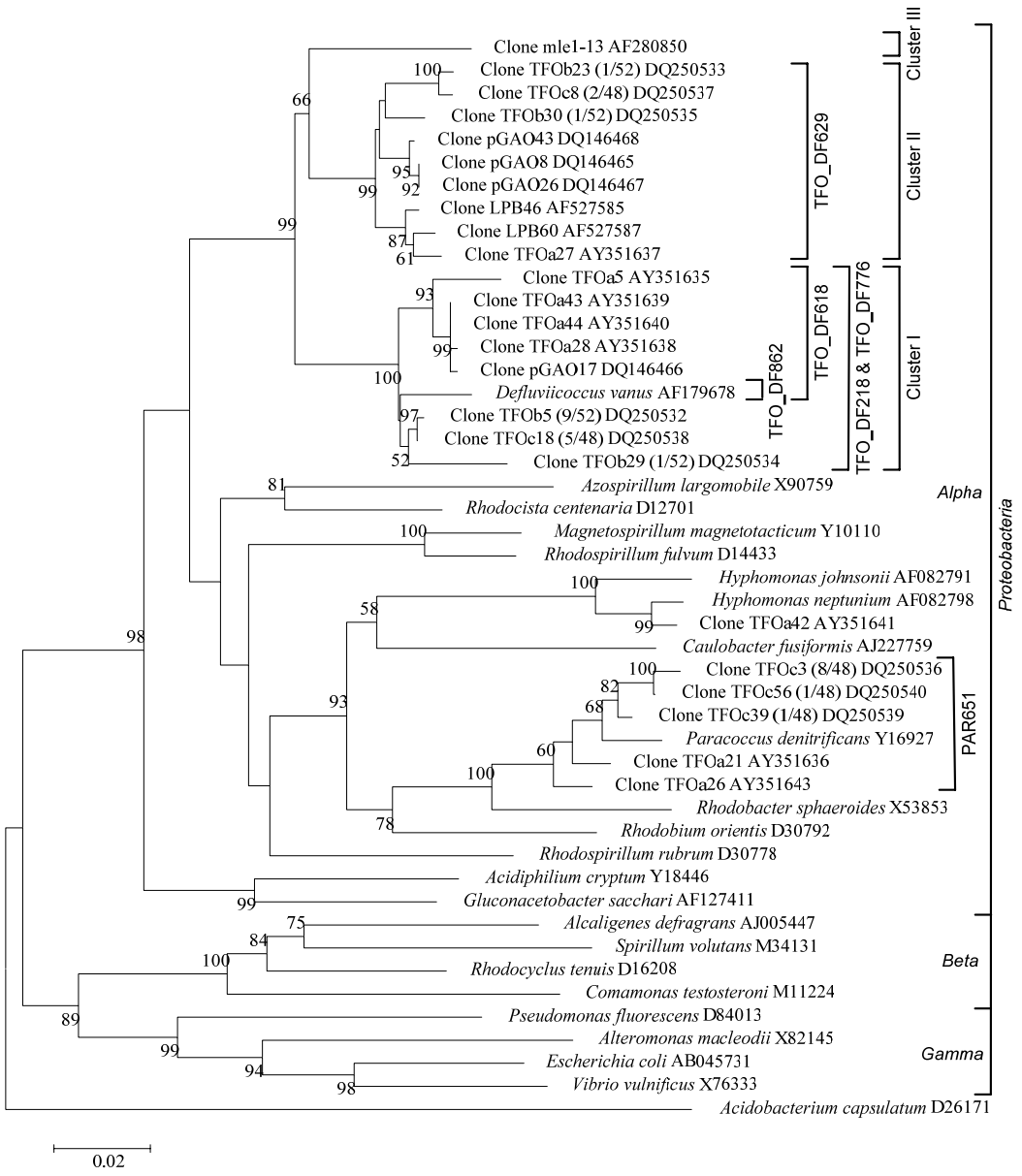


Figure 6-4 Distance matrix-based phylogenetic tree for the *Defluviicoccus* -related TFOs present in the MBR. Samples were collected at days 187 and 338 and approximately 50 clones were screened for each sample (TFOb clones from the sample at day 187 while TFOc clones from the sample at day 338). Calculation was based on the neighbor-joining algorithm with 1000 bootstrap sampling. All the sequences found, along with representative sequences from different subdivisions of the *Proteobacteria* subdivision, were used in the construction of phylogeny tree. The tree was rooted with the 16S rRNA gene sequences of *Acidobacterium capsulatum* (D26171). The scale bar corresponds to 2 substitutions per 100 nucleotide positions.

6.3.7 Substrate Assimilation Patterns of the *Defluviicoccus*-related TFOs

In this study, 12 different MAR treatments were conducted before applying the FISH and PHA staining techniques (Table 3-4). Results of MAR-FISH and MAR-PHA analyses reveal the physiological attributes of the *Defluviicoccus*-related cells from cluster I *in situ*. Under anaerobic conditions, these populations assimilated acetate, lactate, propionate, and pyruvate, and used them to synthesize intracellular PHA. In each case, more than 95% of the FISH-positive *Defluviicoccus* -related TFO cells were MAR positive for each (Figure 6-5a-b, d). We further noted a higher fraction of cells containing PHA granules under a higher substrate dosage or a longer incubation time. Under the aerobic uptake of acetate, strong MAR signals were also observed with the *Defluviicoccus*-related cells. Furthermore, these *Defluviicoccus*-related cells could not take up aspartic acid or glucose after 4-h incubation under either anaerobic or aerobic conditions, and did not show PHA accumulation with them. Instead, a minor unidentified bacterial population in these communities assimilated glucose under aerobic conditions (Figure 6-5c). When pasteurized sludge samples were used as controls, none of the radioactive substrates was taken up, since no silver grain-coated cells were observed after MAR (results not shown).

To determine whether the assimilated acetate and propionate were used by the *Defluviicoccus*-related TFO cells for storage and/or growth under anaerobic conditions, cells were preincubated with unlabeled with each for 4 h before any labeled acetate or propionate was added (Table 3-4). Only a few *Defluviicoccus*-related TFO cells were observed under these conditions to assimilate radiolabeled acetate and propionate. Figure 6-5e revealed that positive PHA-staining response was observed with almost all the *Defluviicoccus*-like organisms. However, MAR analysis suggested

that very little radio-labeled substrate was taken up by these cells after the preincubation with the non-radioactive substrate (Figure 6-5f). It is likely that the storage capacity of the *Defluviicoccus*-related cells was probably saturated after the preincubation treatment.

6.3.8 Anaerobic-aerobic incubation of *D. vanus* NCIMB 13612

The ability of *D. vanus* NCIMB 13612 to take up substrates under anaerobic conditions was investigated by subjecting it to five consecutive anaerobic-aerobic cycles (Figure 6-6). Under these conditions, *D. vanus* NCIMB 13612 took up glucose rapidly during the anaerobic phase. Glucose concentrations decreased from 152±29 mg l^{-1} to less than 22±4 mg l^{-1} by the end of the anaerobic phase. This was accompanied by a decrease in intracellular glycogen levels from 31±4 to 18±2 mg (g TSS)$^{-1}$ and an accumulation of intracellular PHA inclusions, as determined by Nile blue A staining. During the aerobic phase, intracellular glycogen levels were restored to their initial levels presumably from PHA degradation, since its level fell. No detectable Pi release and Pi uptake were observed during the anaerobic phase and the aerobic phase, respectively. These metabolic traits are similar to those proposed for the GAO phenotype (Liu *et al.*, 1996).

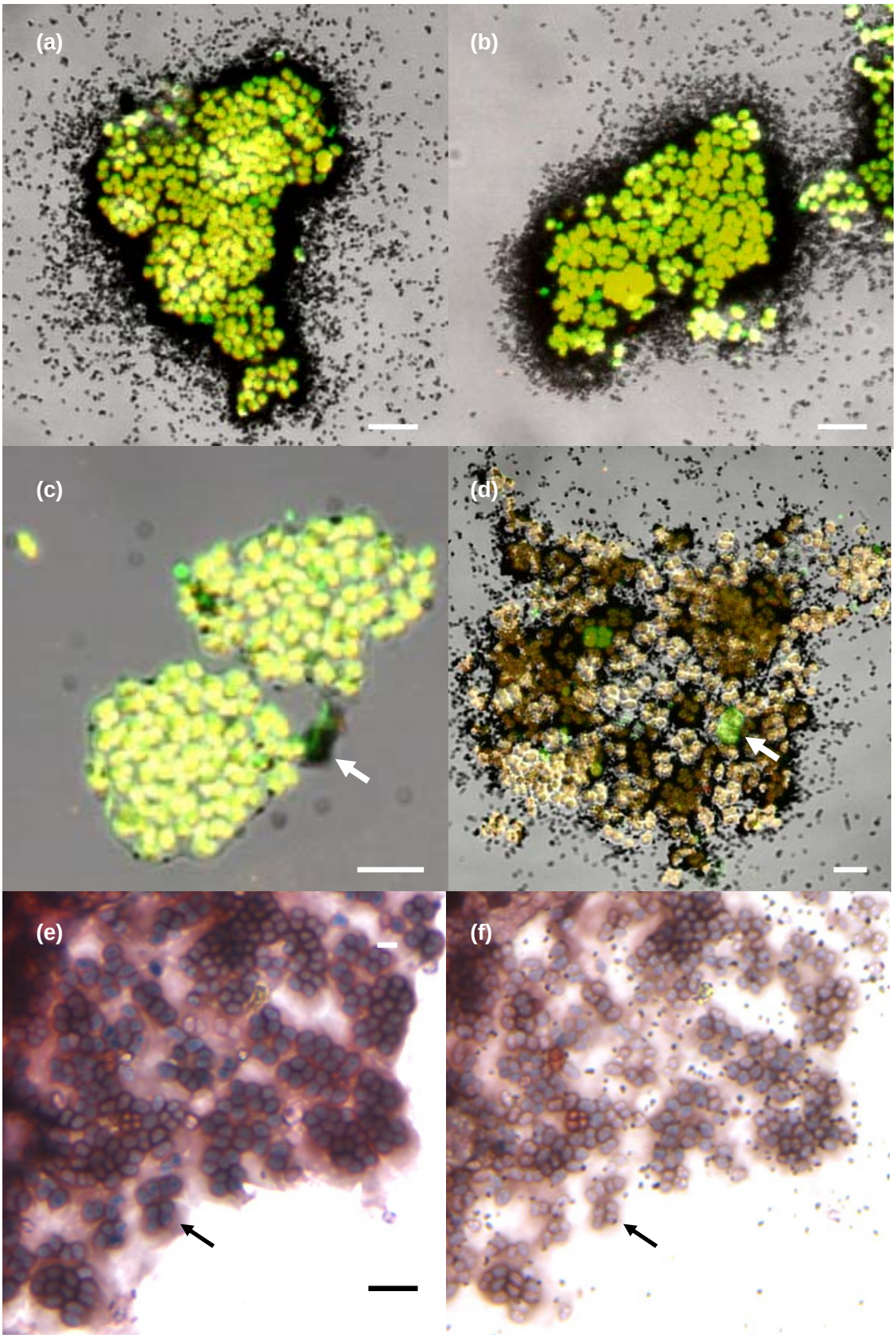


Figure 6-5 (a-d) Combined MAR-FISH images of the *Defluviicoccus* -related TFO in a laboratory-scale sequencing MBR. (a) Anaerobic uptake of

[^{14}C]acetate, (b) anaerobic uptake of L-[U-^{14}C]lactate, (c) aerobic uptake of [^{3}H]glucose, and (d) anaerobic uptake of [1-^{14}C]propionate. (a-c) In situ hybridizations were performed with probe EUBmix (FITC-labeled, green) and probe TFO_DF218 (Cy3-labeled, red). (d) In situ hybridization was performed with probe TFO_DF218 (Cy5-labeled, green) and probe TFO_DF776 (Cy3-labeled, red). White arrows in (c) indicted aerobic uptake of glucose by cells other than *Defluviicoccus* -related TFO. White arrows in (d) showed the co-existence of two subpopulations of *Defluviicoccus* -related TFO. (e and f) Anaerobic uptake of [^{14}-C]acetate with 3 h anaerobic preincubation. (e) Sudan black-B stained image. (f) MAR image of the same field as in (e). Black arrow indicated that less MAR-positive cells resulted due to the saturation of PHA accumulating ability. The scale bars represent 5 μm.

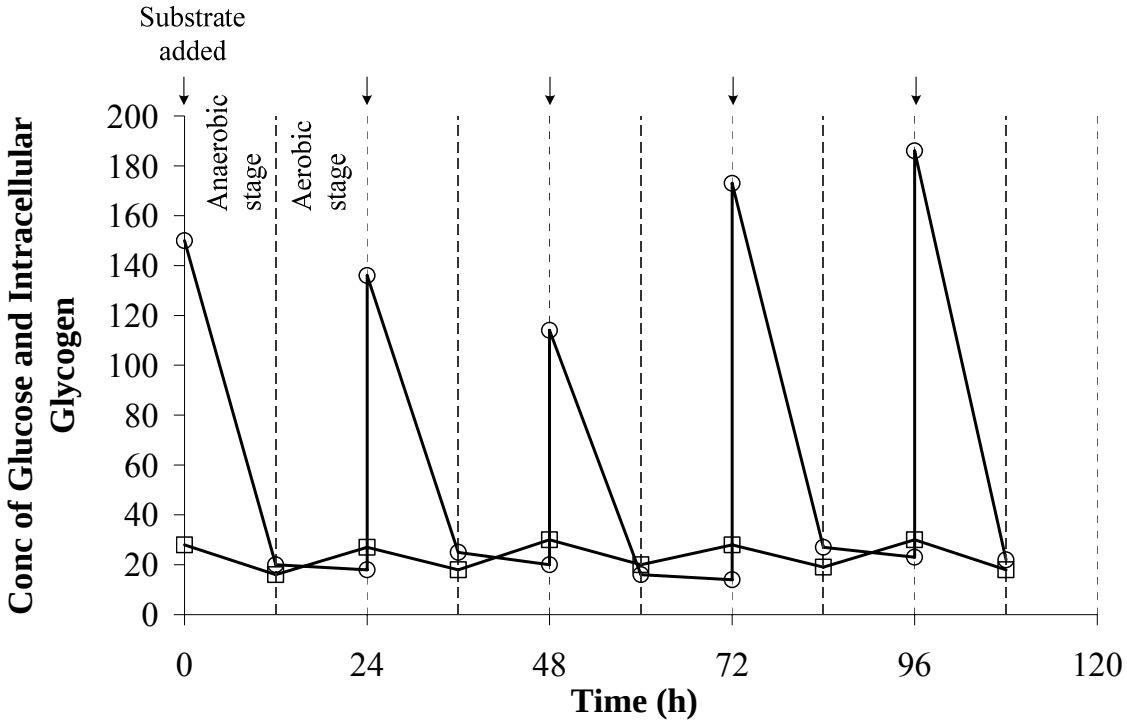


Figure 6-6 Chemical changes that occurred during five cycles of anaerobic-aerobic batch incubation with *D. vanus*. ○, Glucose [mg l^{-1}]; □, intracellular glycogen [mg (g TSS)$^{-1}$]. Substrate was added at the beginning of each anaerobic phase.

6.4 Discussion

6.4.1 "Glycogen-accumulating Organisms"

The early definition of "glycogen-accumulating organisms (GAOs)" is based on the observations made by Cech and Hartman (1993). They observed that the EBPR capacity was deteriorated when glucose was added into an acetate-fed laboratory-scale reactor. At the same time, a microbial population without polyphosphate accumulation was observed to dominate, and hence was named as "G-bacteria" (G stands for glucose). Upon the removal of glucose from the influent feed, the EBPR capacity slowly recovered. Later on, based on thorough chemical profile measurements, enzyme inhibitory experiments and microscopic observations, a biochemical model was proposed for GAO (Liu *et al.*, 1994; Satoh *et al.*, 1994). This model defined GAO as organisms that can accumulate intracellular glycogen and utilize it as an energy source to take up substrates and transform them into carbon storage (i.e., PHA) under the anaerobic conditions of EBPR processes. GAOs were also observed to use a wide range of substrates from SCFAs, mono-, di, and tri-saccharides, to complex substrates like peptone and yeast extracts, and consist of populations with different morphotypes including small and large cocci in a cluster of four or more (i.e., TFOs), and large oval rods (Liu *et al.*, 1996).

Summaries of a recent review (Seviour *et al.*, 2003) further implied that the definition of GAOs may be broadened to include microorganisms that can take up one or more types of substrates and transform them into storage compounds other than PHA, and can proliferate in the EBPR processes. For examples, several studies have reported the dominance of TFOs in glucose-fed laboratory-scale anaerobic-aerobic systems with and without EBPR capacities (Carucci *et al.*, 1994; Jeon and Park, 2000; Tsai and Liu,

2002). A bacterial isolate, *M. glycogenica*, was also demonstrated to take up glucose not acetate anaerobically and accumulate it as glycogen (Shintani *et al.*, 2000). *D. vanus* observed in this study also exhibited similar physiological traits as *M. glycogenicus*.

At present, two major groups, the GB group from the *Gammaproteobacteria* and *Defluviicoccus*-related organisms from the *Alphaproteobacteria*, were demonstrated as the putative GAOs using molecular approaches, and representing the major morphotypes observed (Liu *et al.*, 1996). Tsai and Liu (2002) further reported considerable morphological variation among those TFO detected from phylogenetically unrelated groups, suggesting that the phylogenetic diversity of GAOs was apparently greater than what has been reported (Seviour *et al.*, 2003). However, it should be noted that not all TFOs (e.g., *Amaricoccus* spp. and *Tetrasphaera* spp.) are GAO though they represent a major morphotype of GAOs.

6.4.2 Physiological Traits and Diversity of the *Defluviicoccus* Cluster

The *Defluviicoccus*-related populations seen in the community examined here were confirmed to exhibit the physiological traits considered necessary for the GAOs in EBPR processes (Liu *et al.*, 1996; Wong *et al.*, 2004). Acetate, propionate, lactate, and pyruvate were all taken up by these cells but glucose and aspartic acid were not. While these substrates were assimilated, PHA synthesis of varying composition was observed. Though we did not determine the Pi assimilation using MAR, chemical profile from sludge biomass suggested no release of Pi anaerobically.

Comparing the substrate uptake patterns for the dominant *Defluviicoccus* populations observed in this study and those reported previously (Wong *et al.*, 2004) suggested that all these populations behave similarly, even though unexpected differences in FISH response were observed. However, these populations differed from *D. vanus* in their inability to take up glucose anaerobically. We could not conclude whether the substrate utilization patterns between the *Defluviicoccus* related organisms in clusters I and II are different or not, since only a limited range of substrates was examined for cluster I *in situ* and no populations from cluster II were found here. Thus, further studies using MAR-FISH and MAR-PHA as demonstrated in this study and elsewhere (Kong *et al.*, 2005a) are clearly needed, and may help to clarify whether if such differences do exist. One group might be preferentially selected by the substrate composition of the influent, and hence become dominant.

In fact, a recent study using MAR-FISH has clearly revealed the difference and similarity in the substrate uptake patterns among different subgroups of the GB bacteria (Kong *et al.*, 2005a). Among those six subgroups examined, all could assimilate acetate, propionate, pyruvate, and glucose anaerobically, but only some subgroups (1 and 6) could further assimilate formate and/or thymidine. Additional differences were observed under different electron acceptors and pre-treatment conditions. Between *Defluviicoccus*-related organisms in cluster I and GB bacteria, they shared several common substrates but differed mainly in the glucose uptake (Kong *et al.*, 2005a).

6.4.3 Microbial Interaction of *Defluviicoccus* Organisms and Other GAOs

As most of the TFOs in the *Defluviicoccus* cluster and other GAOs can all utilize acetate anaerobically (Wong *et al.*, 2004; Kong *et al.*, 2005a), competition among them is anticipated to take place. In fact, a microbial succession involving different GAO phylotypes was observed in the community of a laboratory-scale deteriorated EBPR system (Wong and Liu, 2006). From reactor startup to day 38, the GB bacteria group dominated numerically (50-66% of total cells). However, a shift in the dominant populations from subgroup GB_7 to subgroup GB_4 was observed. Then from day 85, these GB populations were replaced by the *Defluviicoccus* -related TFO organisms. Between days 85 and 117, most of these were targeted equally by both the TFO_DF218 and TFO_DF618 probes, but after day 117, those cells responding to the TFO_DF218 probe became the numerically abundant group. The reasons for this are not clear because MAR-FISH studies were not carried out on these changing communities, but the long hydraulic retention times and long solids residence times were suspected to cause the proliferation of the *Defluviicoccus*-related organisms over the GB group. This suggested that *Defluviicoccus* related organisms in cluster I could have relatively lower substrate affinity and growth rate than the GB bacteria. In this present study, identical operational conditions were used, but the *Defluviicoccus*-related organisms now selected were markedly different phylogenetically to those observed earlier. And so the precise determinants responsible for these shifts in phylotype of GAO remain unclear.

Competitive interaction between *Defluviicoccus* and GB in full-scale EBPR processes was not clear, but recent surveys suggested that *Defluviicoccus* related populations from cluster I was not found in full-scale EBPR plants in Japan (Wong *et al.*, 2005),

and populations from cluster II were present in Australian plants (Meyer *et al.*, 2006). In contrast, the GB bacteria were often observed in full-scale EBPR plants in Japan, Australia, Hungary and Denmark (Nielsen *et al.*, 1999; Kong *et al.*, 2002; Kong *et al.*, 2005a; Wong *et al.*, 2004). Subgroups 2, 4 and 6 of the GB bacteria were the dominant types found in 13 different Japanese plants (Wong *et al.*, 2005), and all subgroups except 2 were observed to present at different frequency in 12 different Danish EBPR plants (Kong *et al.*, 2005a). These observations further confirm that the growth conditions in the full-scale EBPR plants were not favorable for the growth of *Defluviicoccus* related populations from cluster I.

6.4.4 Microbial Interaction between *Defluviicoccus*-related Organisms and PAO

At present, the key determinants of the outcomes in any PAO-GAO competition remain unclear. One possible basis for competition between PAO and GAO was described as an "internal energy-based" type competition in EBPR bioreactors fed mainly acetate (Liu *et al.*, 1997b). This concept indicated that the amount of acetate taken up anaerobically reflects the amount of intracellular stored energy by PAOs (i.e., polyphosphate) or by GAOs (i.e., glycogen). However, unlike the glycogen pool size in GAOs, the polyphosphate pool size is often affected by the Pi concentration present in the feed (Liu *et al.*, 1997b). A low feed Pi will lead to a low polyphosphate content and less substrate taken up by PAOs than GAOs. When internally stored energy is not limited, the PAO-GAO competition is determined by the rate of substrate uptake under anaerobic conditions (Liu *et al.*, 1997b). It has also been reported that the type of SCFA can affect competition between the PAO and GAO (Oehmen *et al.*, 2005), since propionate instead of acetate appeared to selectively promote the growth of PAOs at

the expense of the GAOs, and thus serve as a better substrate for EBPR. However, in full-scale plant operation, it is rather difficult and costly to manipulate community composition in this way. Likewise, several other operational parameters (e.g., long solids residence time, high anaerobic-aerobic hydraulic retention time ratio, acidic pH in anaerobic phase, and high temperature) and influent substrate composition (e.g., limited P source and low P:TOC ratio) have all been reported to affect the competition between GAOs and PAOs (Seviour *et al.*, 2003), although in most of these, the GAOs were not identified.

MAR-FISH results here and from a recent study with full-scale EBPR systems (Kong *et al.*, 2005b) would suggest that the *Defluviicoccus*-related organisms can potentially compete for acetate with known PAOs especially the *Rhodocyclus*-related PAOs anaerobically. However, those *Defluviicoccus* populations from cluster I may not be able to compete with PAOs in full-scale EBPR plants, as the growth conditions as aforementioned was not favorable to them. Instead, they may outcompete PAOs in systems with long SRT (solids residence time) and HRT (hydraulic retention time).

Many PAOs have been reported to assimilate amino acids anaerobically. These PAOs included *Microlunatus phosphovorus* (Nakamura *et al.*, 1995), the *Rhodocyclus*-related PAO (Kong *et al.*, 2004), the *Tetrasphaera*-related PAOs (Kong *et al.*, 2005b), and unknown PAOs observed in either laboratory-scale or full-scale EBPR systems (Liu *et al.*, 1996; Satoh *et al.*, 1998). Although amino acids are present in considerable amounts in the wastewater influent at EBPR plants, their roles in related to the EBPR activity and the microbial competition between PAOs and GAOs have attracted surprisingly little interest. The possible competition between *Defluviicoccus* and PAOs

that assimilate amino acids is not clear, as only one amino acid was tested here for the substrate uptake by *Defluviicoccus* related populations, and some amino-utilizing PAOs may also utilize acetate (Kong *et al.*, 2005b). Thus, further studies using MAR-FISH to examine the PAO-GAO competition under presence of either amino acids or a mixture of amino acids and SCFAs are critical.

6.5 Conclusions

In summary, this study has provided important insights into the ecophysiological traits of *Defluviicoccus*-related organisms found in laboratory-scale anaerobic-aerobic reactors. Based on biomass composition data, metabolic profiling and a suite of culture-independent methods and staining techniques, these populations appear to behave there as GAOs, and could dominate in systems with long SRT and HRT. But development of novel molecular techniques is further required to directly link the substrate uptake and storage with glycogen consumption under anaerobic conditions. Their *in situ* substrate utilization patterns differed from those reported for pure cultures of *D. vanus* and other GAOs for glucose uptake (Kong *et al.*, 2005b). The phylogenetic and phenotypic diversity among these *Defluviicoccus*-related TFOs, like the GB bacteria (Kong *et al.*, 2005a), are likely to be complex, and should be further investigated using *in-situ* characterization approaches like MAR-FISH and MAR-PHA. For this reason, it should be careful in the future to cast a name for yet-to-be-cultured populations that are phylogenetically closely related. We believed that *Candidatus* Competibacter phosphatis was a subgroup of the GB bacteria, and suspected that differences in substrate utilization may also exist among different subgroups of the *Rhodocyclus*-related PAOs, or *Candidatus* Accumulibacter phosphatis. More efforts using *in-situ* physiological techniques are needed to further address the

ecophysiological and phylogenetical diversity of PAOs and GAO, and to elucidate the microbial interactions between these GAOs and PAOs, and within different members of GAOs or PAOs in laboratory-scale and full-scale EBPR systems.

Chapter 7

In Situ Identification and Characterization of the Microbial Community Structure of Full-scale Enhanced Biological Phosphorus Removal Plants in Japan

The content of this chapter has been published as:

Wong, M.-T., Mino, T., Seviour, R.J., Onuki, M., and Liu, W.-T. (2005). *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorus removal plants in Japan. Water Res. 39, 2901-2914.

7.1 Abstract

Fluorescent *in situ* hybridization (FISH) and polyphosphate (polyP) staining methods were used to characterize the microbial community structure of 13 activated sludge samples taken from nine different Japanese wastewater treatment plants with and without enhanced biological phosphorus removal (EBPR) activities. FISH with published rRNA-targeted oligonucleotide probes for important bacterial groups involving in the EBPR process revealed that *Rhodocyclus*-related polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) from a gammaproteobacterial lineage GB were the predominant populations detected, representing 4-18% and 10-31% of EUBmix-stained cells respectively. However, a considerable proportion of *Rhodocyclus*-related PAO cells were observed with no polyP granules accumulated based on polyP staining. This was further supported by a poor correlation between *Rhodocyclus*-related PAO population and sludge total phosphorus (TP) contents. In contrast, high correlations between polyP-stained cells and sludge TP contents were observed. In particular, among those polyP-stained cells in samples Ariake_A2O and Nakano_AO, more than 85% of them could not be targeted by probe PAOmix. These non-*Rhodocyclus*-related PAOs included populations from other bacterial divisions and members of the *Betaproteobacteria* other than those in the *Rhodocyclus*-related group.

7.2 Introduction

Enhanced biological phosphorus removal (EBPR) processes are widely used for phosphate (Pi) removal from wastewater in addition to or instead of chemical precipitation methods. In EBPR processes, microorganisms include mainly those favorable for EBPR [i.e., polyphosphate accumulating organisms (PAOs)] and those

competing with PAOs [i.e., glycogen accumulating organisms (GAOs)] (Seviour *et al.*, 2003). Successful operation of EBPR processes is thought to require the selection of PAOs over GAOs under optimal growth conditions (Seviour *et al.*, 2003), and thus it is important to identify which of these physiological groups are present in such communities.

Culture-independent molecular methods have been extensively used to characterize microbial populations involved in EBPR processes (Hesselmann *et al.*, 1999; Kawaharasaki *et al.*, 1999; Nielsen *et al.*, 1999; Crocetti *et al.*, 2000 and 2002; Kong *et al.*, 2001, 2002a, and 2002b; Liu *et al.*, 2001; Zilles *et al.*, 2002; Wong *et al.*, 2004). These methods include clone library construction, DNA fingerprinting, and fluorescent *in situ* hybridization (FISH). For PAOs, the proposed candidates include *Microlunatus phosphovorus*, *Rhodocyclus*-related species, *Tetrasphaera*-related species, and *Malikia* spp. (Nakamura *et al.*, 1995; Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000; Liu *et al.*, 2001; Spring *et al.*, 2005). For GAOs, putative candidates can be grouped into two morphotypes: tetrad-forming organisms (TFOs) and large coccobacillus-shaped organisms. The TFOs are known to include *Amaricoccus* spp., *Micropruina glycogenica*, *Tetrasphaera* spp., and *Defluvicoccus vanus* and related species (Maszenan *et al.*, 2000; Kong *et al.*, 2001; Wong *et al.*, 2004). The large coccobacillus-shaped organisms are members of the lineage GB in the *Gammaproteobacteria* (referred to subsequently as the GB group) or '*Candidatus* Competibacter phosphatis' (Crocetti *et al.*, 2002; Kong *et al.*, 2002b). A comprehensive set of rRNA-targeted oligonucleotide probes has been developed and successfully applied for the *in situ* detection of these organisms but applied mainly to

communities in laboratory-scale EBPRs fed with acetate as sole carbon source or other chemically defined media.

Only a few studies have attempted to verify the roles of those PAOs and GAOs in different full-scale EBPR processes (Crocetti *et al.*, 2000; Kong *et al.*, 2002b and 2004; Zilles *et al.*, 2002; Lee *et al.*, 2003; Wong *et al.*, 2004). With application of selected probes in FISH analysis, these studies have suggested that the predominance and involvement of the *Rhodocyclus*-related PAO (probe PAOmix) in full-scale EBPRs was dependent on the plant configuration, electron acceptor condition, and spatial distribution of the cells (Crocetti *et al.*, 2000; Zilles *et al.*, 2002; Lee *et al.*, 2003; Kong *et al.*, 2004). For GAOs, cells of the GB group exhibited a typical GAO phenotype, storing PHA anaerobically and utilizing it aerobically but not accumulating polyphosphate (polyP) aerobically, have been frequently detected by FISH in full-scale EBPR processes (Crocetti *et al.*, 2002; Kong *et al.*, 2002b). Unlike the GB group, dominance of TFOs in EBPR processes has been reported mainly in communities developed in laboratory-scale systems. Thus, any possible role for the various TFOs in the deterioration of full-scale EBPR processes is less clear than for the GB group (Wong *et al.*, 2004). It is apparent that more effort is required to better understand the occurrence and role of these PAOs and GAOs in full-scale EBPR processes.

In this study, the microbial community structures of 13 activated sludge samples from nine Japanese wastewater treatment plants (WWTPs) were characterized using the published rRNA-targeted oligonucleotide probes targeting important bacterial groups found in EBPR process and major bacterial divisions found in wastewater treatment processes. A combined approach of FISH and polyP staining was also used to identify

the polyP-accumulating traits of *Rhodocyclus*-related PAO in these full-scale WWTPs. Such information will allow a better basis for understanding the microbial ecology of EBPR communities in full-scale plants and provide a stronger basis for analyses of factors influencing the stability of P removal in these systems.

7.3 Results

7.3.1 Chemical Analysis of Sludge Samples

The influent and effluent Pi concentrations in the domestic and industrial wastewater samples varied from 6 to 10 mg-P l^{-1}, and less than 2 mg-P l^{-1}, respectively. Table 3-1 shows the TP contents of the sludge samples. The TP contents from the EBPR plants, which ranged from 28 to 39 mg-P g^{-1}-SS, and were generally higher than those observed in CAS sludge samples (~20 mg-P g^{-1}-SS). Among all the samples, Shibaura_Pseudo-AO and Todoroki_APO had the highest sludge TP contents, at around 39 mg-P g^{-1}-SS and 38 mg-P g^{-1}-SS, respectively. No correlation was observed for samples taken from a same location with different process configurations.

7.3.2 Microscopic Analysis of Sludge Samples

Microscopic examinations for polyP containing cells were conducted with both Neisser and DAPI staining. A morphological diversity was observed among those cells containing polyP granules in the 13 sludge samples. These polyP-containing cells included coccobacilli in different spatial organizations and filaments of different sizes (Figure 7-1).

Table 7-1 summarizes the types and abundance of the polyP-containing morphotypes in each of the sludge samples. In general, polyP-containing cells were coccobacilli and

were packed into dense clusters (Figure 7-1a). Each cluster contained several hundred polyP-containing coccobacilli, resembling the putative PAO, '*Candidatus* Accumulibacter phosphatis' or the *Rhodocyclus*-related cells. Cells of this kind dominated the communities in samples Nakagawa_A2O, Nakagawa_AO, Todoroki_APO, and Shibaura_Pseudo-AO. Another polyP-containing morphotype was a filamentous bacterium resembling the *Nostocoidia limicola*-II like cell, and was occasionally found in samples Mikawasima_AO, Nakano_AO, and Shibaura_Pseudo-AO (Figure 7-1c). *In situ* hybridization of samples with probe HGC69a suggested that this filament was the actinobacterial *N. limicola*-II (Blackall *et al.*, 2000). Furthermore, a polyP-containing filament with irregular true branching was observed either inside the floc or in the bulk solution, and was especially common in sample Todoroki_APO (Figure 7-1d). This filament was the Gordonia amarae-like organism (GALO) morphotype, some of which are known to accumulate polyP and often associated with foaming (Soddell, 1999).

Table 7-1 Subjective scoring of polyP-stained cells in each sample (0: none; 1: few; 2: some; 3: common; 4: very common; 5: abundant; 6: excessive)

	Major morphotype of polyP-stained cell			
	Coccobacilli		Filament	
Sample name	Cluster	Single cells	Short and thin	Long and thick
Ariake_A2O	1	1	1	0
Nakagawa_A2O	3	1	1	0
Nakagawa_AO	3	1	0	1
Kosuge_AO	2	0	1	0
Mikawasima_AO	2	1	0	3
Nakano_AO	1	1	0	3
Todoroki_APO	3	1	4	0
Shibaura_Pseudo-AO	3	1	0	3
Kosuge_Pseudo-AO	2	0	1	1
Mikawasima_Pseudo-AO	2	1	0	2
Nakagawa_CAS	1	2	0	1
Shin-Gashi_CAS	0	1	0	0
Sunamachi_CAS	1	1	0	0

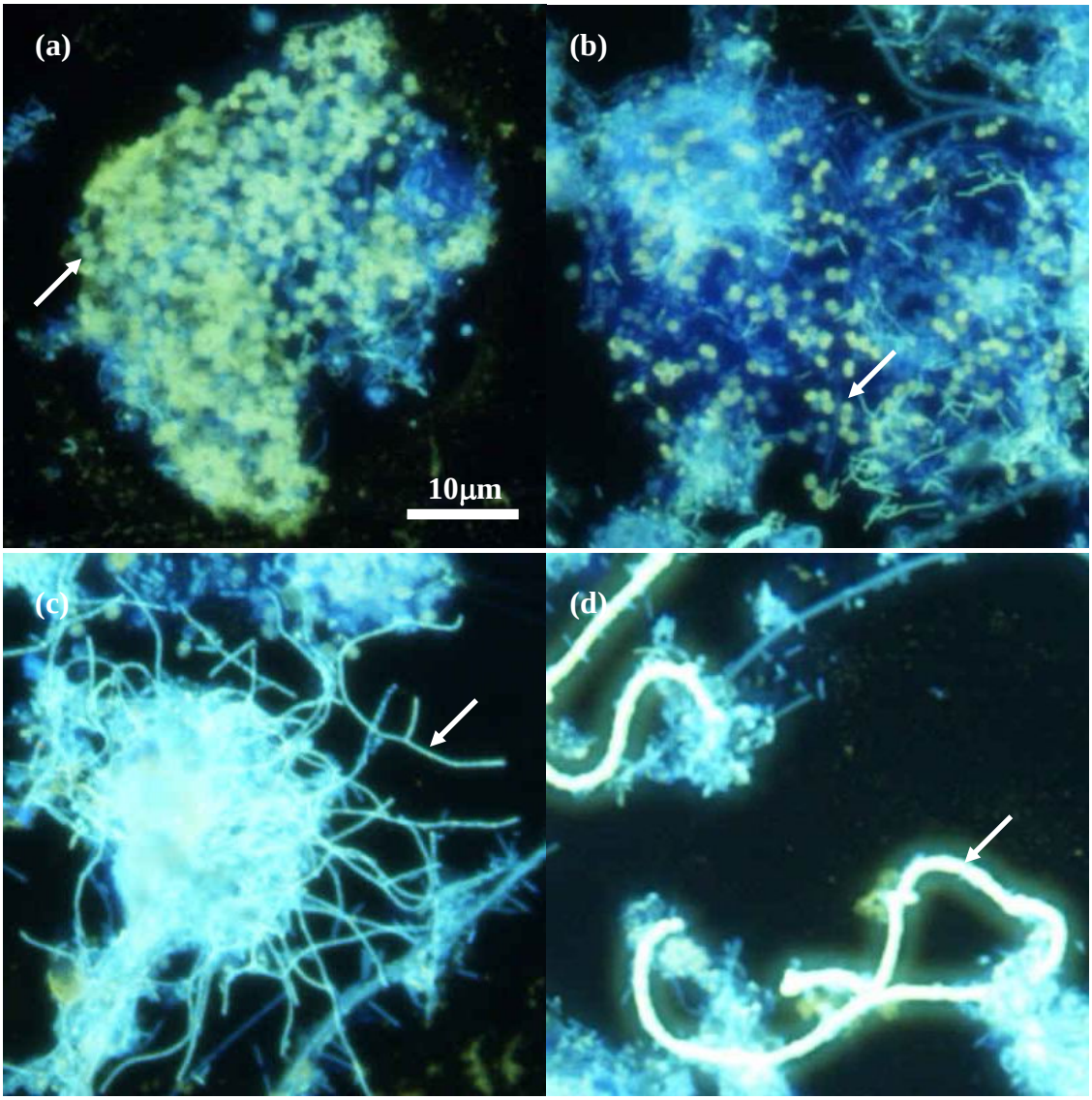


Figure 7-1 Polyphosphate (DAPI) staining images of 13 activated sludge samples showing the morphology of different PAO cells. (a) coccobacilli in cluster; (b) coccobacilli; (c) irregularly shaped true-branching filaments; (d) thick filamentous. The scale bar on panel a (10 μm) also applies to all other panels.

7.3.3 Microbial Community Profiles as Revealed by FISH Analyses

The activated sludge samples were first analyzed by DAPI staining and FISH analyses with the domains *Bacteria* and *Archaea*-targeted probes. At least 80±12% of DAPI-stained cells in all the 13 activated sludge samples were detected with the domain *Bacteria* probes (EUBmix). None of the sludge samples contained any detectable archaeal cells, although the occurrence and activity of *Archaea* in aerated activated sludge has been reported previously (Gray *et al.*, 2002).

The presence of '*Candidatus* Accumulibacter phosphatis' cells was then examined using FISH with probe PAOmix. Figure 7-2a shows that the PAOmix-probe-fluorescing cells contributed more than 9% of EUBmix-stained cells for 11 of those 13 samples, the exceptions being samples Ariake_A2O and Nakano_AO. Among those EBPR samples, the highest abundance of PAOmix-probe-binding cells was observed in samples Nakagawa_A2O, Nakagawa_AO, Kosuge_AO, Todoroki_APO, and Kosuge_Pseudo-AO (15-18%). Samples Mikawasima_AO, Shibaura_Pseudo-AO, and Mikawasima_Pseudo-AO contained slightly smaller fractions of PAOmix-probe-binding cells (10-12%). The three CAS samples from Nakagawa, Shin-Gashi, and Sunamanchi plants contained about 10% of probe PAOmix hybridized cells. This abundance was comparable to or sometimes higher than in those sludge samples from EBPR plants. However, those PAOmix hybridized cells often appeared as singly loosely dispersed cells rather than clustered into densely packed microcolonies (Figure 7-3b).

The sludge samples were further analyzed using FISH with eight different published probes targeting important bacterial groups involving in EBPR process. Probe GB

targeted a yet-to-be-cultured GAO in the *Gammaproteobacteria*. This group was found as the second predominant group next to the *Rhodocyclus*-related PAO in all the sludge samples examined (Figure 7-3c), and abundances ranged from 10 to 31% of EUBmix-stained cells (Figure 7-1a). Samples Mikawasima_AO and Mikawasima_Pseudo-AO contained 31% of EUBmix-stained cells as GB-probe-binding cells, 2-3 times higher than those observed in other EBPR samples. FISH with GB subgroup-level probes further revealed that subgroups GB_2 (40±9% of total group GB cells), GB_4 (43±11%), and GB_6 (37±12%) were equally abundant in sample Mikawasima_AO. The abundance of the group GB cells in the three CAS samples varied from 3 to 11%.

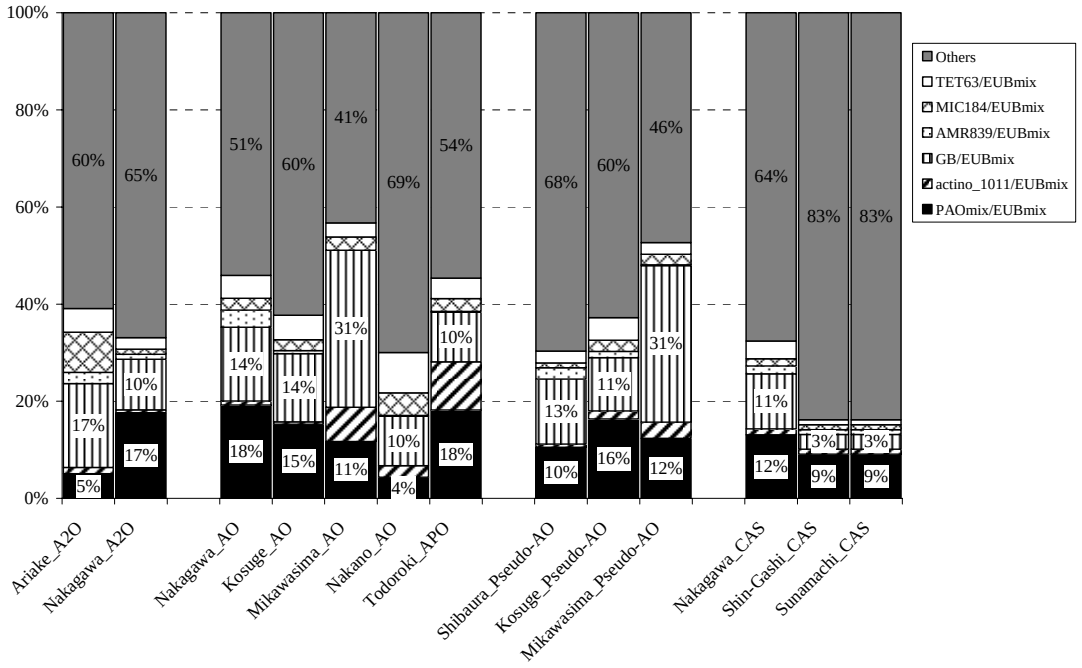
Probe actino_1011 (Liu *et al.*, 2001) targeting a rod-shaped bacterial morphotype related to *Tetrasphaera* in the *Actinobacteria* division was also used. Cells responding positively to this probe were only observed in samples Mikawasima_AO and Todoroki_APO with abundances of 7 and 10%, respectively. However, some of the bacterial cells targeted by probe actino_1011 were filamentous instead of rod shaped (Figure 7-3d).

Probes AMR839, MIC184, and TET63 have been designed to target putative GAO in different phylogenetic groups. Results indicated that AMR839-targeted cells accounted for less than 3% of EUBmix-stained cells in all the samples examined. MIC184-targeted cells were only observed in samples Ariake_A2O and Nakano_AO, and accounted for 8 and 5% of EUBmix-stained cells there, respectively. Comparing the AMR839- and MIC184-targeted cells, TET63-targeted cells were more abundant in particular with sample Nakano_AO (~ 8% of EUBmix-stained cells).

The occurrence of the *Defluvicoccus*-related TFO in these samples was examined using probes TFO_DF218, TFO_DF618, and TFO_862 designed recently (Wong *et al.*, 2004). None of the cell in these 13 samples hybridized with any of these probes. This observation agrees with an earlier report that the *Defluvicoccus*-related TFO are probably not the predominant populations in the full-scale WWTP plants (Wong *et al.*, 2004).

Overall, these oligonucleotide probes applied could only account for 30 to 60% of the microbial communities in the EBPR samples. The phylogenetic affiliation of those remaining microbial populations remains unknown.

(a)



(b)

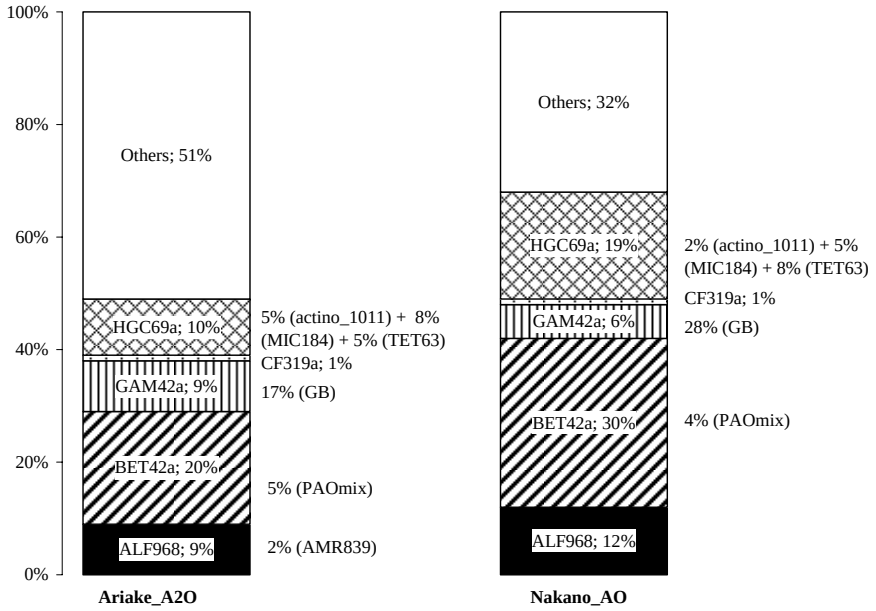


Figure 7-2 (a) Proportions of putative PAOs and GAOs in the 13 activated sludge samples determined by FISH; (b) microbial community composition of samples Ariake_A2O and Nakano_AO as revealed by FISH with group-specific oligonucleotide probes. The proportions of identified putative PAOs and GAOs were also indicated. All the values are presented as percentage of the relative cell area of respective probe against probe EUBmix.

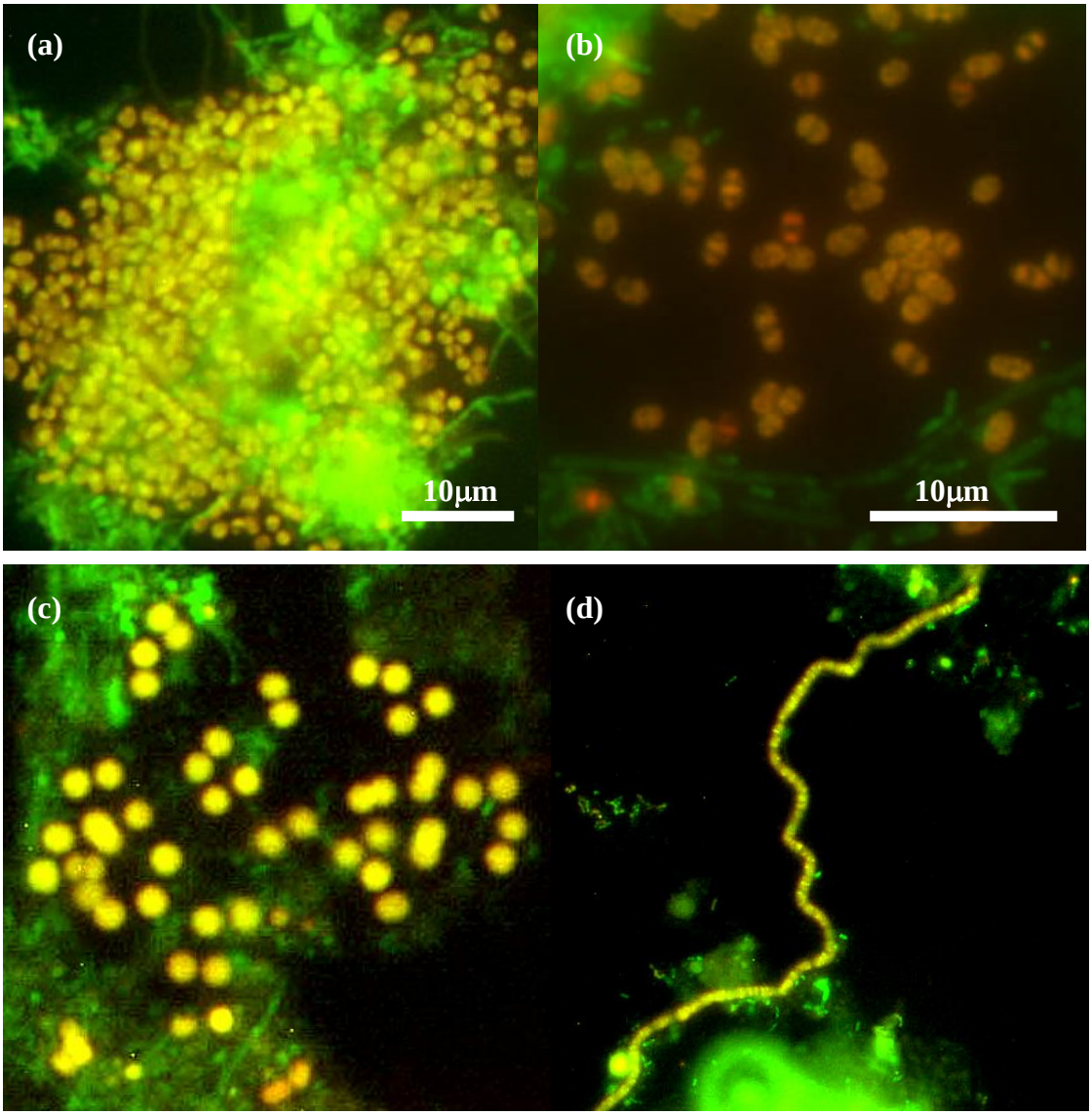


Figure 7-3 *In situ* hybridization of activated sludge samples with probes EUBmix (Cy5) and PAOmix (Cy3) on (a) Todoroki_APO and (b) Sunamachi_CAS respectively; (c) probes EUBmix(Cy5) and GB(Cy3) on Mikawasima_AO; (d) probes EUBmix (Cy5) and actino_1011 (Cy3) on Todroki_APO. Images were collected at two different filters depending on the fluorescent label and artificially colored and superimposed using Adobe Photoshop. The scale bar on panel a (10 μm) also applies to panels c and d.

7.3.4 Characteristics of Probe PAOmix Targeted Cells

The possible involvement of the *Rhodocyclus*-related PAO cells in P removal in these activated sludge plants was further examined by investigating their polyP accumulating traits and spatial organization. Using dual staining with probe PAOmix and DAPI, a considerable proportion of probe PAOmix positive cells were found to lack polyP granules. In terms of their spatial organization, the *Rhodocyclus*-related PAO cells inside a microcolony differed in the areal cell densities (Figures 6-3a and b). Comparatively high areal cell densities of *Rhodocyclus*-related PAO cells (0.13-0.19 cell μm^{-2}) were always found in the samples containing a high abundance of PAOmix-probe-binding cells. However, neither the number of *Rhodocyclus*-related PAO cells present in the microcolonies nor the diameters of these microcolonies could be established as a definite relationship with the probe PAOmix counts (Table 7-2).

The relationship between the *Rhodocyclus*-related PAO population or polyP-stained cell abundance and the sludge TP contents was determined using linear regression analyses. A statistically significant correlation between the polyP-stained cell abundance and the sludge TP contents was observed among 12 of the 13 samples (except from Ariake_A2O) (Figure 7-4). Results indicated that there was an increasing trend in the polyP-stained cell abundance as the sludge TP contents increased. However, the *Rhodocyclus*-related PAO population could be not correlated with the sludge TP contents in all the samples examined here.

Comparing the polyP-stained cell abundance with the *Rhodocyclus*-related PAO population, samples Ariake_A2O and Nakano_AO consisted of less than 15% of

polyP-stained cells hybridized by probe PAOmix (Figure 7-4). This suggested that considerable numbers of non-*Rhodocyclus*-related PAO were present in these samples.

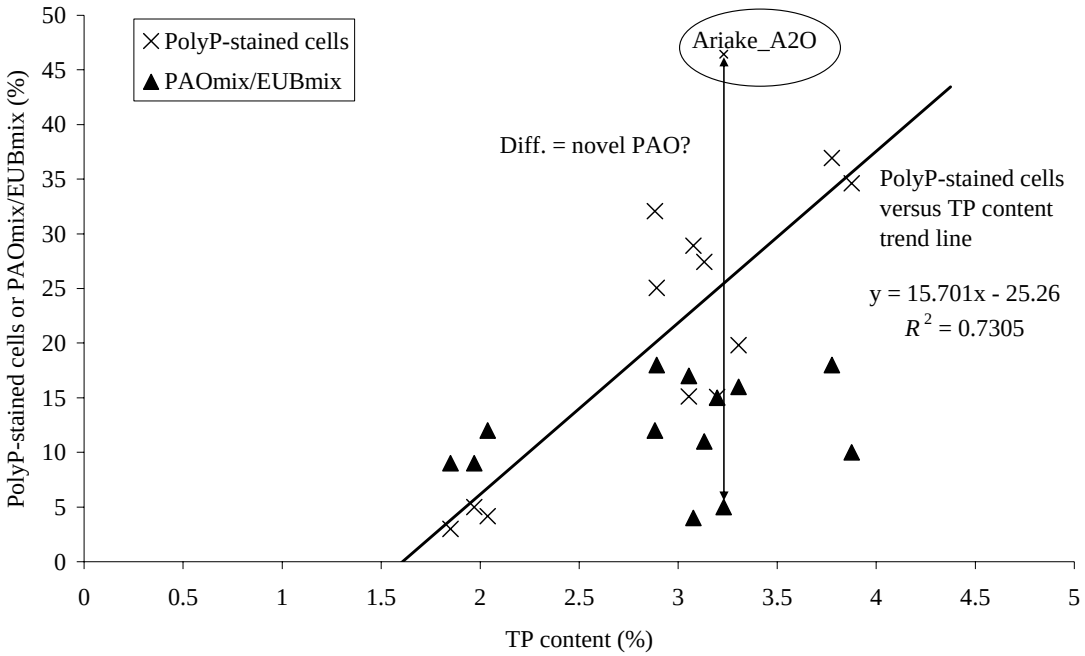


Figure 7-4 Correlations between the *Rhodocyclus*-related PAO population or polyP-stained cell abundance and the sludge TP contents.

Table 7-2 The number of cells, diameters, and areal cell densities of the *Rhodocyclus*-related PAO microcolonies. Standard deviations of the mean were also included.

Sample	Number of cell in microcolonies	Microcolonies diameter (μm)	Areal cell density of microcolonies (cell μm^{-2})
Ariake_A2O	17.5±7.3	10.3±4.2	0.27±0.17
Nakagawa_A2O	18.8±15.0	15.0±11.2	0.16±0.13
Nakagawa_AO	45.4±41.2	26.4±15.8	0.15±0.07
Kosuge_AO	31.5±24.4	28.0±20.4	0.13±0.05
Mikawasima_AO	21.6±17.1	23.9±10.5	0.06±0.05
Nakano_AO	15.6±9.6	14.6±5.5	0.11±0.07
Todoroki_APO	42.2±30.8	22.3±14.1	0.19±0.13
Shibaura_Pseudo-AO	53.5±27.3	26.2±20.7	0.13±0.10
Kosuge_Pseudo-AO	47.9±38.9	31.2±18.9	0.18±0.04
Mikawasima_Pseudo-AO	34.3±13.0	31.0±9.0	0.05±0.03
Nakagawa_CAS	32.5±16.9	36.4±22.4	0.05±0.04
Shin-Gashi_CAS	19.0±12.7	11.23±6.19	0.06±0.04
Sunamachi_CAS	5.8±5.5	6.0±4.0	0.05±0.02

7.3.5 Possible Bacterial Divisions of the PAO in Samples Ariake_A2O and Nakano_AO

The possible phylogenetic affiliations of the PAOs in samples Ariake_A2O and Nakano_AO were further determined by dual staining with group-specific oligonucleotide probes and polyP staining. Oligonucleotide probes targeting the bacterial divisions found in wastewater treatment process were used. In both samples, the *Betaproteobacteria* was the most abundant group accounting for 20±4% (Ariake_A2O) and 30±10% (Nakano_AO) of EUBmix-stained cells, followed by the *Actinobacteria*, 10±4% (Ariake_A2O) and 19±5% (Nakano_AO) of EUBmix-stained cells (Figure 7-2b). The *Alphaproteobacteria* and *Gammaproteobacteria* were present in an average abundance of 9±2% and 9±4%, respectively, in sample Ariake_A2O, and 12±6% and 6±3, respectively, in sample Nakano_AO. The CFB group and *Firmicutes* contributed to less than 1% of total populations in both samples. Combined with polyP

staining, many betaproteobacterial cells were found to accumulate a large amount of polyP in sample Nakano_AO. However, most of the polyP-stained cells in sample Ariake_A2O were not identified with any of these six group-specific probes although they can be detected with the domain *Bacteria* probes (EUBmix).

7.4 Discussion

7.4.1 Correlation between the PolyP-stained Cell Abundance and the Sludge TP Content

This study has shown that the sludge TP content proportionally corresponded to the EBPR activity or number of polyP-accumulating cells in all samples from these WWTPs. The typical sludge TP content ranged from 15-20 mg-P g^{-1}-SS in terms of the sludge dry weight for the CAS plants removing only small amounts of P to 40-50 mg-P g^{-1}-SS of the sludge dry weight for full-scale EBPR processes, but was much lower values reported for laboratory-scale EBPR processes (>15% P of cell dry weight) (Crocetti *et al.*, 2000). However, the chemical measurement for sludge TP contents can only reflect the amount of TP in a given activated sludge sample, but not the actual TP inside individual cells. Thus, DAPI stain was used qualitatively to show the accumulation of high polyP content inside a microbial cell. Although DAPI was reported to stain polyP and lipid granules, the fluorescence intensity observed from cells accumulating a large amount of polyP could be easily differentiated from those accumulating lipid granules (Kawaharasaki *et al.*, 1999). The comparatively high correlation (R^2 = 0.73) between the abundance of polyP-stained cells and the sludge TP contents suggested that the extent of polyP accumulation in a given sludge were affected by the abundance of PAO cells and the amount of polyP accumulated inside individual PAO cells.

7.4.2 Contribution of the *Rhodocyclus*-related PAO to EBPR

Despite the differences in process configuration and wastewater composition among these different plants, the *Rhodocyclus*-related PAO constituted more than 10% of EUBmix-stained cells in eight of the 10 EBPR samples, and thus should be considered important to the EBPR activity in them. Using the dual staining of FISH with probe PAOmix and DAPI, a considerable proportion of PAOmix-probe-binding cells appeared to contain little or no polyP granules. This finding agrees with other studies (Zilles *et al.*, 2002; Lee *et al.*, 2003; Kong *et al.*, 2004) and suggests that it is impractical to assess the EBPR activity in a WWTP by attempting to relate the numerical dominance of *Rhodocyclus*-related PAO to its polyP-accumulating capacity since a high proportion of *Rhodocyclus*-related PAO cells were not accumulating polyP or polyP contents of their cells were not so high that they are detectable as polyP granules. Unexpectedly, a high fraction of *Rhodocyclus*-related PAO cells were also detected in the CAS samples by FISH. However, these *Rhodocyclus*-related PAO cells appeared as singly loosely dispersed cells or scattered throughout the flocs, and none of these cells were DAPI stain positive for polyP accumulation. Their contribution, if any, to P removal remained unclear.

No statistically significant correlation was observed in this study and Lee *et al.* (2003) between the *Rhodocyclus*-related PAO population and the sludge TP contents in full-scale EBPR plants. A strong correlation ($R^2 > 0.9$), however, has been reported in communities analyzed from acetate-fed laboratory-scale sequencing batch reactors (SBRs) (Crocetti *et al.*, 2000; Kong *et al.*, 2002a). It seems from the present data that the *Rhodocyclus*-related PAO populations do not account totally for all the PAO populations present in full-scale EBPR systems.

7.4.3 Morphologic and Phylogenetic Groups of EBPR Communities

Diverse and distinct morphotypes of polyP containing cells were observed in the activated sludge samples examined in this study. This observation also agrees with other similar reports that a phylogenetically diverse spectrum of organisms may be involved in polyP accumulation (Mino *et al.*, 1998; Seviour *et al.*, 2003). With molecular techniques, members of several different bacterial divisions of microorganisms have been proposed as PAOs. These included the *Actinobacteria* (Wagner *et al.*, 1994; Bond *et al.*, 1999; Christensson *et al.*, 1998; Liu *et al.*, 2001), the *Alphaproteobacteria* (Kawaharasaki *et al.*, 2002); the *Betaproteobacteria* (Wagner *et al.*, 1994; Bond *et al.*, 1999; Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999; Lee *et al.*, 1999; Spring *et al.*, 2005), the *Gammaproteobacteria* (Nielsen *et al.*, 1999; Liu *et al.*, 2001); the CFB group (Dabert *et al.*, 2001) and even eukaryotes (Melasniemi and Hernesmaa, 2000). Data presented here add further supports to the view that EBPR may be mediated by different microbial populations under different conditions of process configuration and wastewater composition. Among the other putative PAOs, *N. limicola*-II like cells, a novel rod-shaped group targeted by probe acitno_1011, and GALO have been found to contain polyP but not PHA granules (Blackall *et al.*, 2000; Liu *et al.*, 2001; this study). It is possible that these bacterial groups could participate in phosphate metabolism in the EBPR processes but use mechanisms different from those proposed for PAOs (Seviour *et al.*, 2003).

Two of the samples examined here probably containing other PAOs besides the *Rhodocyclus*-related organisms, were examined further. In sample Ariake_A2O, most of the polyP-stained cells could not be identified by any of the probes used here which targeted bacteria frequently reported in activated sludge systems. Similar findings were

also reported by Lee *et al.*, (2003). This suggests that these polyP-stained cells were from other bacterial divisions or had 16S rRNA sequences not targeted by the probes used. In sample Nakano_AO, the possible PAO could be members of the *Betaproteobacteria* other than in the *Rhodocyclus*-related group since most of the polyP-stained cells were found to be *Betaproteobacteria*.

7.4.4 Involvement of Putative PAOs and GAOs in Full-scale EBPR Processes

Of the eight oligonucleotide probes targeting the putative PAOs and GAOs, probe GB was the most dominating group in all the samples. Although the presence of GB group in anaerobic-aerobic system has been well documented (Nielsen *et al.*, 1999; Dabert *et al.*, 2001; Liu *et al.*, 2001; Kong *et al.*, 2002b), their ecological function in EBPR is still unclear. Both Nielsen *et al.* (1999) and Liu *et al.* (2001) have observed that this group can accumulate PHA and occasionally polyP in several EBPR systems. In contrast, Dabert *et al.* (2001) observed a marked increase in the GB group in the microbial community in an SBR during the transition from good to poor EBPR capacity. Blackall *et al.* (2002) proposed that members of the subgroup GB_6 had a phenotype consistent with them being GAOs. When Kong *et al.* (2002b) extensively examined the distributions of group GB and its subgroups in 13 different laboratory-scale and full-scale systems with or without EBPR activity, they found that it was 1-4 times more abundant in most EBPR systems investigated (contributing 10-50% of total cells) than in the CAS systems (< 10%). However, no such trend was observed in this study. Phylogenetic analysis based on 16S rRNA sequences further divided this GB group into seven subgroups (Kong *et al.*, 2002b). In addition to subgroups GB_4 and GB_6 which have been frequently found in the anaerobic-aerobic systems (Crocetti *et al.*, 2002; Kong *et al.*, 2002b), subgroup GB_2 was also present in sample

Mikawasima_AO in high numbers. Further studies using microautoradiography and PHA staining together with the GB subgroups probes are necessary to understand the physiological roles of these individual subgroups in EBPR and CAS systems (Lee *et al.*, 1999; Liu *et al.*, 2001). It was also noted that the inconsistencies of probes GAM42a and GB counts in samples Ariake_A2O and Nakano_AO were attributed to the specificity of probes BET42a and GAM42a which differed from each other by a single central nucleotide and were originally designed based on the small number of 23S rRNA sequences available (Yeates *et al.*, 2003). FISH analyses with combined probes of BET42a and GB, or GAM42a and GB were performed to validate such an incongruity. Results revealed that cells targeted by probe GB also hybridized with probe BET42a but not with probe GAM42a. This study further suggested that unlike the *Rhodocyclus*-related PAO and GB group, *Micropruina glycogenica* and members of the genera *Defluvicoccus*, *Amaricoccus*, and *Tetrasphaera* are not the dominant populations in either full-scale EBPR or CAS systems in Japan. Their absence could be because the substrate complements and process configuration used in the laboratory-scale experiments, where these populations were detected (Maszenan *et al.*, 2000; Kong *et al.*, 2001; Wong *et al.*, 2004) were much simpler than those in full-scale WWTPs.

7.5 Conclusions

This study has examined the composition of the bacterial communities in EBPR process in nine different full-scale WWTPs in Japan and attempted to identify those populations involved in P removal. The findings indicated that only 30 to 60% of the populations there could be phylogenetically identified by FISH analyses using EBPR-related oligonucleotide probes described in the literature. The role of "*Candidatus*

Accumulibacter phosphates" (*Rhodocyclus*-related organisms) in EBPR activity observed in full-scale processes appeared to be less prominent than in laboratory-scale EBPR systems and their abundance was not proportional to the sludge TP contents. This study also revealed that the EBPR microbiology is more complex in full-scale EBPR systems than in laboratory-scale systems fed with synthetic sewage, and other non-*Rhodocyclus*-related organisms may also be possible to involve in EBPR activity in these processes.

Chapter 8

Conclusions

8.1 Conclusions

8.1.1 Operation of Activated Sludge Processes

Two laboratory-scale sequencing membrane bioreactors (MBRs) fed with acetate as the sole carbon source were operated under identical cyclic anaerobic and aerobic conditions to mimic enhanced biological phosphorus removal (EBPR) processes for 260 and 474 days, respectively. Using the applied conditions that should select for polyphosphate accumulating organisms (PAOs) [high phosphorus (P):total organic carbon (TOC) ratio], biomass composition and chemical analysis data suggested that microbial consortia exhibiting the typical characteristics of glycogen accumulating organism (GAO) with no EBPR activity were enriched instead. Small cocci/coccobacilli occurring mostly in pairs, tetrads, or aggregates [the so-called tetrad-forming organisms (TFOs)] were the most dominant populations observed in samples taken at approximately day 65 and onwards. Based on size and cluster formation patterns, various morphotypes of TFO were observed and recognized in these two MBRs. Most TFOs showed positive response to Sudan black B staining, but to different extents.

8.1.2 *Defluviicoccus*-related Tetrad-Forming Organisms in EBPR Processes

Microbial communities with no EBPR activity were phylogenetically analyzed by culture-independent molecular methods. 16S rRNA gene clone libraries specific for the *Alphaproteobacteria* indicated that most 16S rRNA gene clones (> 30% of total clones) were closely affiliated with *Defluviicoccus vanus* forming a cluster within subgroup 1 of the *Alphaproteobacteria*. Combined polyhydroxyalkanoate (PHA) staining and fluorescence *in situ* hybridization (FISH) with specific probes designed for the members of the *Defluviicoccus* cluster suggested diversity within this TFO cluster, and

that these TFOs were newly identified GAO in EBPR systems. The occurrence of the *Defluviicoccus*-related TFOs in 21 full-scale EBPR and non-EBPR systems (8 samples in Chapter 4 and 13 samples in Chapter 7) was further evaluated using FISH and the designed probes. The low abundance of the *Defluviicoccus*-related TFOs in the examined samples (results in Chapters 4 and 7) suggested that they were not the key populations responsible for the deterioration of full-scale EBPR processes. This difference could be due to the unfavorable growth conditions for the *Defluviicoccus*-related populations in the full-scale EBPR plants. In particular, the MBR system used here, where the biomass concentration and organic loading were much higher and lower, respectively, than other conventional gravity liquid-solid separation systems.

8.1.3 Physiological Traits of the *Defluviicoccus* Cluster

Using microautoradiography (MAR) in combination with FISH and PHA staining, the *Defluviicoccus*-related TFOs present in an acetate-fed anaerobic-aerobic sequencing MBR without EBPR activity appeared to behave as GAOs. These *Defluviicoccus*-related TFOs could take up and transform acetate, lactate, propionate, and pyruvate, but not aspartic acid and glucose, as PHA under anaerobic conditions. In contrast, their *in situ* substrate utilization patterns differed from those reported for pure cultures of *D. vanus* and GAOs for glucose uptake. These findings reveal unexpected levels of physiological, phylogenetic, and morphological diversity within members of the *Defluviicoccus* cluster. In addition, the definition of GAOs may be broadened to include microorganisms that can take up one or more types of substrates and transform them into storage compounds other than PHA, and can proliferate in the EBPR processes.

8.1.4 Microbial Interaction of *Defluviicoccus* Organisms and Other GAOs

Microbial succession involving a gammaproteobacterial lineage GB (GB group) and the *Defluviicoccus*-related TFOs occurred in the community of a laboratory-scale deteriorated EBPR system. Competition for carbon substrate (i.e., acetate) was anticipated to take place among them. The reasons for this competition was not clear but the long solids residence times (> 7-8 d) and long hydraulic retention times (> 12 h) were considered to be the important factors to cause the proliferation of the *Defluviicoccus*-related organisms over the GB group.

8.1.5 Microbial Community Structure in Japanese EBPR Processes

The microbial communities of full-scale EBPR plants differ markedly from those developed in laboratory-scale anaerobic-aerobic reactors fed with a synthetic sewage. Only 30-60% of the populations there could be phylogenetically identified by FISH analyses using EBPR-related oligonucleotide probes described in the literature. The role of '*Candidatus* Accumulibacter phosphates' (*Rhodocyclus*-related organisms) in EBPR activity observed in full-scale processes appeared to be less prominent than in laboratory-scale EBPR systems and their abundance was not proportional to the sludge total P contents. This study also revealed that the EBPR microbiology is more complex in full-scale EBPR systems than in laboratory-scale systems fed with synthetic sewage, and other non-*Rhodocyclus*-related organisms may also possibly be involved in EBPR activity in these processes.

8.2 Recommendations

During the course of this study, it became apparent that there are several areas requiring further research. Limits of the present study have also identified future

research needs. The following information is required not only for a better understanding of the microbial ecology of EBPR process, but also for an effective removal of P in full-scale EBPR plants.

8.2.1 Ecophysiological and Phylogenetical Diversity of PAOs and GAOs

As the PAO and GAO populations in EBPR systems now appear to be phylogenetically diverse, more studies of these physiological groups are needed to clarify their diversity and help elucidate their ecophysiology. In this regard, i*n situ* characterization approaches like MAR-FISH and MAR-PHA should be continuously employed. Development of novel molecular techniques is further required to directly link the substrate uptake and carbon polymer storage (PHA or others) with glycogen consumption under anaerobic conditions. Considering that some PAO/GAO may store substances other than PHA (Kong *et al.*, 2005), a detailed chemical characterization of any storage material present in the community could be resolved with ^{13}C nuclear magnetic resonance analysis of biomasses in these systems. In addition, more efforts using *in situ* physiological techniques are needed to further elucidate the microbial interactions between these GAOs and PAOs, and within different members of GAOs or PAOs in laboratory- and full-scale EBPR systems.

8.2.2 Applicability of Laboratory Findings to Full-scale EBPR

This study has used semi-quantitative FISH analysis to determine the community composition of samples of biomass from full-scale EBPR plants, and together with DAPI staining to see which populations were storing polyP in aerobic reactor samples. Only a single sample of biomass was analyzed with FISH from each plant. It might be argued that this approach would not accommodate the probability that all cells would

be behaving at the single sample time in a similar metabolic fashion. However, these data sufficient in the view to suggested that the bacterial communities of full-scale EBPR plants may differ markedly to those developing in laboratory-scale anaerobic-aerobic reactors fed with a synthetic sewage containing acetate, and add support to data from other similar recent work (Lee *et al.*, 2003; Kong *et al.*, 2005). In particular, the low abundance of the *Rhodocyclus*-related PAOs and *Defluviicoccus*-related TFOs in some of the examined samples suggested that they were not necessarily to be the key populations responsible for the promotion and deterioration of full-scale EBPR processes, respectively. Cautions should be exercised to directly apply laboratory findings on full-scale EBPR plants.

8.2.3 EBPR Metabolism with Substrates Other Than Acetate

Full-scale activated sludge systems often deal with a wide range of organic matter including carboxylic acids, sugars, and amino acids. The importance of carbon sources other than VFAs for the proliferation of PAOs or GAOs is not, however, clear at present. A wide range of organic matter other than VFAs to be utilized by PAOs and GAOs and simultaneous formation of storage polymers other than PHA has been recognized (Satoh *et al.*, 1998). Investigation of organic substrate metabolisms should not thus be limited to acetate only. In particular, deterioration of the EBPR in laboratory-scale systems has been observed and attributed to the carbon source supplied during the anaerobic phase of the EBPR process (Cech and Hartman, 1993).

8.2.4 Isolation of Representative Culture

Knowing the phylogeny of organisms does not often help in predicting its physiology, isolation of microorganisms responsible for effective and deteriorated EBPR should be

continued. Though isolation of representative microorganisms from EBPR has been recognized to be very difficult due to no suitable isolation methods, new techniques should be developed. Once a representative culture is isolated, the physiological and metabolic characteristics of this culture should be extensively studied in order for the verification of various biochemical models proposed for EBPR.

References

1. Abu-Ghararah, Z.H. and Randall, C.W. (1991). The effect of organic compounds on biological phosphorus removal. Water Sci. Technol. 23(Kyoto), 585-594.
2. Adam, C., Gnirss, R., Lesjean, B., Buisson, H., and Kraume, M. (2002). Enhanced biological phosphorus removal in membrane bioreactors. Water Sci. Tech. 46(4-5), 281-286.
3. Ahn, J., Daidou, T., Tsuneda, S., and Hirata, A. (2002). Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. Water Res. 36, 403-412.
4. Ahn, K.-H., Song, K.-G., Cho, E., Cho, J., Yun, H., Lee, S., and Kim, J. (2003). Enhanced biological phosphorus and nitrogen removal using a sequencing anoxic/anaerobic membrane bioreactor (SAM) process. Desalination 157, 345-352.
5. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25, 3389-3402.
6. Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919-1925.
7. Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143-169.

8. Anderson, A.J. and Dawes, E.A. (1990). Occurrence, metabolism, metabolic role, industrial use of bacterial polyalkanoates. Microbiol. Rev. 54, 450-472.

9. Andreasen, K. and Nielsen, P.H. (1997). Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. Appl. Environ. Microbiol. 63, 3662-3668.

10. Andreeva, N.A., Lichko L.P., Kulakovskaya T.V., and Okorokov, L.A. (1993). Biochemistry (Moscow), 58, 737-744.

11. APHA (1998). Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association.

12. Arun, V., Mino, T., and Matsuo, T. (1989). Metabolism of carboxylic acid located in and around the glycolytic pathway cycle in the biological phosphate removal process. Water Sci. Technol. 21(4-5), 363-374.

13. Arvin, E. and Kristensen, G.H. (1985). Exchange of organics, phosphate and cations between sludge and water in biological phosphorus and nitrogen removal processes. Water Sci. Technol. 17(11-12), 147-162.

14. Auling, G., Pilz, F., Busse, H-J., Karrasch, M., Streichan, M., and Schon, G. (1991). Analysis of the polyphosphate-accumulating microflora in phosphorus-eliminating, anaerobic-aerobic activated sludge systems by using diaminopropane as a biomarker for rapid estimation of *Acinetobacter* spp. Appl. Environ. Microbiol. 57, 3685-3692.

15. Ault-Riche, D., Fraley, C.D., Tzeng, C.M., and Kornberg, A. (1998). Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. J. Bacteriol. 180, 1841-1847.

16. Barker, P.S. and Dold, P.L. (1996). Denitrification behavior in biological excess phosphorus removal activated sludge systems. Water Res. 30, 769-780.

17. Barak, Y. and van Rijn, J. (2000). Atypical polyphosphate accumulation by the denitrifying bacterium *Paracoccus denitrificans*. Appl. Environ. Microbiol. 66, 1209-1212.

18. Barnard, J.L. (1974). Cut P and N without chemicals. Water Wasters Eng. 11, 33-44.

19. Barnard, J.L. (1976). A review of biological phosphorus removal in activated sludge. Water SA 2, 136-144.

20. Barnard, J.L., Stevens, G.M., and Leslie, P.J. (1985). Design strategies for nutrient removal plant. Water Sci. Technol. 17(11-12), 233-242.

21. Baetens, D., Vanrolleghem, P.A., van Loosdtrecht, M.C.M., and Hosten, L.H. (1999). Temperature effects in bio-P removal. Water Sci. Technol. 39(1), 215-225.

22. Beer, M., Kong, Y., and Seviour, R.J. (2004). Are some putative glycogen accumulating organisms (GAO) in anaerobic:aerobic activated sludge systems members of the *α-Proteobacteria*? Microbiology 150, 2267-2275.

23. Bernard, J.L., Stevens, G.M., Leslie, P.J. (1985). Design strategies for nutrient removal plants. Water Sci. Technol. 17, 233-242.

24. Blackall, L.L., Seviour, E.M., Bradford, D., Rossetti, S., Tandoi, V., and Seviour, R.J. (2000). '*Candidatus* Nostocoida limicola', a filamentous bacterium from activated sludge. Int. J. Syst. Evol. Microbiol. 50, 703-709.

25. Blackall, L.L., Crocetti, G.R, Saunders, A.M., and Bond, P.L. (2002). A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. Antonie van Leeuwenhoek 81, 681-691.

26. Bond, P.L., Hugenholtz, P., Keller, J., and Blackall, L.L. (1995). Bacterial community structures of phosphate-removing and non-phosphate-removing

activated sludges from sequencing batch reactors. Appl. Environ. Microbiol. 61, 1910-1916.

27. Bond, P.L., Erhart, R., Wagner, M., Keller, J., and Blackall, L.L. (1999a). Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. Appl. Environ. Microbiol. 65, 4077-4084.

28. Bond, P.L., Keller, J., and Blackall, L.L. (1999b). Bio-P and non-bio-P bacteria identification by a novel microbial approach. Water Sci. Technol. 39(6), 13-20.

29. Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Dore, J., Delgenes, P., Moletta, R., and Wagner, M. (2000). Ecological study of a bioaugmentation failure. Environ. Microbiol. 2, 179-190.

30. Brdjanociv, D., Hooijmans, C.M., van Loosdrecht, M.C.M., Alaerts, G.J., and Heijnen, J.J. (1996). The dynamic effect of potassium limitation on biological phosphorus removal. Water Res. 30, 2323-2328.

31. Brdjanociv, D., van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., and Heijnen, J.J. (1997). Temperature effects on physiology of biological phosphorus removal. J. Environ. Eng. ASCE 123, 144-153.

32. Brdjanovic, D., Slamet, A., van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G. J., and Heijnen, J.J. (1998a). Impact of excessive aeration on biological phosphorus removal from wastewater. Water Res. 32, 200-208.

33. Brdjanovic, D., Slamet, A., van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G. J., and Heijnen, J.J. (1998b). Influence of temperature on BPR: process and molecular ecological studies. Water Res. 32, 1035-1058.

34. Buchan, L. (1981). The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal. Water SA 7, 1-7.

35. Carucci, A., Majone, M., Ramadori, R., and Rosetti, S. (1994). Dynamics of phosphorus and organic substrates in anaerobic and aerobic phases of a sequencing batch reactor. Water Sci. Technol. 30(6), 237-246.

36. Carucci, A., Lindrea, K., Majone, M., and Ramadori, R. (1995). Dynamics of the anaerobic utilization of organic substrates in an anaerobic/aerobic sequencing batch reactor. Water Sci. Technol. 31(2) 35-43.

37. Carucci, A., Kühni, M., Brun, R., Carucci, G., Koch, G., Majone, M., and Siegrist, H. (1999). Microbial competition for the organic substrates and its impact on EBPR systems under conditions of changing carbon feed. Water Sci. Technol. 39(1) 75-85.

38. Cech, J.S. and Hartman, P. (1990). Glucose induced breakdown of enhanced biological phosphate removal. Environ. Technol. 11, 651-656.

39. Cech, J.S. and Hartman, P. (1993). Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. Water Res. 27, 1219-1225.

40. Chang, W.C., Chiou, R.J., and Ouyang, C.F. (1996). The effect of residual substrate utilization on sludge settling in an enhanced biological phosphorus removal process. Water Sci. Technol. 34(1-2), 425-430.

41. Chen, C.-L., Macarie, H., Ramirez, I., Olmos, A., Ong, S.L., Monroy, O., and Liu, W.-T. (2004a). Microbial community structure in a thermophilic anaerobic hybrid reactor degrading terephthalate. Microbiology 63, 3429-3440.

42. Chen, Y., Randall, A.A., and McCue, T. (2004b). The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. Water Res. 38, 27-36.

43. Christensson, M., Blackall, L.L., and Welander, T. (1998). Metabolic transformation and characterization of the sludge community in an enhanced biological phosphorus removal system. Appl. Microbiol. Biotechnol. 99, 226-234.

44. Chua, A.S.M., Eales, K., Mino, T., and Seviour, R.J. (2004). The large PAO cells in full-scale EBPR biomass samples are not yeast spores but possibly novel members of the *β-Proteobacteria*. Water Sci. Technol. 50(6), 123-130.

45. Chuang, S.-H., Ouyang, C.-F., and Wang, Y.-B. (1996). Kinetic competition between phosphorus release and denitrification on sludge under anoxic conditions. Water Res. 30, 2961-2968.

46. Chuang, S.-H., Ouyang, C.-F., Yuang, H.-C., and You, S.-J. (1998). Phosphorus and polyhydroxyalkanoates variation in a combined process with activated sludge and biofilm. Water Sci. Technol. 37(4-5), 593-597.

47. Cloete, T.E. and Steyn, P.L. (1988). Combined membrane filter-immunofluorescent technique for the *in situ* identification and enumeration of *Acinetobacter* in activated sludge Water Res. 22, 961-909.

48. Comeau, Y., Hall, K.J., Hancock, R.E.W., and Oldham, W.K. (1985). Biochemical model for enhanced biological phosphorus removal in Proceedings of University of British Columbia Conference on New Directions and Research in Waste Treatment and Residuals Management, pp. 324-346, University of British Columbia, Vancouver, Canada.

49. Comeau, Y., Hall, K.J., Hancok, R.E.W., and Oldham, W.K. (1987). Dynamics of carbon reserves in biological dephosphatation of wastewater. In: Advances in Water Pollution Control: Biological Phosphate Removal from Wastewaters (Ramadori, R., eds), pp. 39-55. Pergamon Press, Oxford.

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

51. Crocetti, G.R., Banfield, J.F., Keller, L., Bond, P.L., and Blackall, L.L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology 148, 3353-3364.

52. Dabert, P., Sialve, B., Delgenes, J.-P., Moletta, R., and Godon, J.-J. (2001). Characterization of the microbial 16S rDNA diversity of an aerobic phosphorus removal ecosystem and monitoring of its transition to nitrate respiration. Appl. Microbiol. Biotechnol. 55, 500-509.

53. Daims, H., Brühl, A., Amann, R.I., Schlerfer, K.H., and Wagner, M. (1999). The domain-specific probe Eub338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434-444.

54. Dawes, E.A. and Senior, P.J. (1973). Energy reserve polymers in microorganisms. Adv. Microbiol. Physiol. 10, 135-266.

55. Dawes, E.A. (1986). Microbial Energetics. Blackie, New York.

56. Dawes, E.A. (1992) Storage polymers in prokaryotes. In: Prokaryotic structure and function: a new perspective. (Mohan, S., Dowand, C., and Cole, J.A. eds), pp. 81-122, Cambridge University Press, Cambridge.

57. de-Bashan, L.E. and Bashan Y. (2004). Recent advances in removing phosphorous from wastewater and its future use as fertilizer (1997-2003). Water Res. 38, 4222-4246.

58. Deinema, M.H., Habets, L.H.A., Scholten, J., Turkstra, E., and Webers, H.A.A.M. (1980). The accumulation of polyphosphate in *Acinetobacter* spp. FEMS Microbiol. Lett. 9, 275-279.

59. Ekama, G., Marais, G., and Siebritz, I. (1984). Biological Excess Phosphorous Removal, in Theory, Design and Operation of Nutrient Removal Activated Sludge Process, Water Research Commission, Pretoria, South Africa.

60. Elhottová, D., Tříska, J., Šantrůčková, H., Květoň, J., Šantrůček, J. M., and Šimková, M. (1997). Rhizosphere microflora of winter wheat plants cultivated under elevated CO_2. Plant Soil 197, 251-259.

61. Erdal, U.G., Erdal, Z.K., and Randall, C.W. (2003). The competition between PAOs (phosphorous accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on system performance. Water Sci. Technol. 47(11), 1-8.

62. Erhart, R., Bradford, D., Seviour, R.J., Amann, R.I., and Blackall, L.L. (1997). Development and use of fluorescent *in situ* hybridization probes for the detection and identification of '*Microthrix parvicella*' in activated sludge. Syst. Appl. Microbiol. 20, 310-318.

63. Eschenhagen, M., Schuppler, M., and Roske, I. (2003). Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. Water Res. 37, 3224-3232.

64. Falvo, A., Levantesi, C., Rossetti, S., Seviour, R.J., and Tandoi, V. (2001). Synthesis of intracellular storage polymers by *Amaricoccus kaplicensis*, a tetrad forming bacterium present in activated sludge. J. Appl. Microbiol. 91, 299-305.

65. Fang, H.H.P., Zhang, T., and Liu, Y. (2002). Characterization of an acetate-degrading sludge without intracellular accumulation of polyphosphate and glycogen. Water Res. 36, 3211-3218.

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

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

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

69. Filipe, C.D.M., Daigger, G.T. and Grady, C.P.L. (2001d). pH as a key factor in the competition between glycogen-accumulating and phosphate-accumulating organisms. Water Environ. Res. 73, 223-232.

70. Fleit, E. (1995). Intracellular pH regulation in biological excess phosphorus removal systems. Water Res. 29, 1787-1792.

71. Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., and Amann, R.I. (1998). Flow cytometric analysis of the in situ accessibility of *Escherichia*

coli 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. 64, 4973-4982.

72. Fuhs, G.W. and Chen, M. (1975). Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microbial. Ecol. 2(2), 119-138.

73. Fukase, T. Shibata, M., and Miyaji, Y. (1982). Studies on the mechanism of biological phosphorus removal. Translate by Mardon, C.J. Chem. Technol. Jap. J. Water Pollut. Res. 5, 309-317.

74. Fukase, T., Shibata, M., and Miyaji, Y. (1985). Factors affecting biological removal of phosphorous. Water Sci. Technol. 17(11-12), 187-198.

75. Furumai, H., Kazmi, A.A., Furuya, Y., and Sasaki, K. (1999). Effect of sludge retention time (SRT) on nutrient removal in sequencing batch reactors. J. Environ. Sci. Health 34, 317-328.

76. Gieseke, A., Purkhold, U., Wagner, M., Amann, R.I., and Schramm, A. (2001). Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl. Environ. Microbiol. 67, 1351-1362.

77. Gottschalk, G. (1986). Bacterial Metabolism, 2nd ed., Springer-Verlag, NY.

78. Gray, N.D., Miskin, I.P., Kornilova, O., Curtis, T.P., and Head, I.M., (2002). Occurrence and activity of *Archaea* in aerated activated sludge wastewater treatment plants. Environ. Microbiol. 4, 158-168.

79. Groben, R., Doucette, G.J., Kopp, M., Kodama, M., Amann, R.I., and Medlin, L.K. (2000). 16S rRNA targeted probes for the identification of bacterial strains isolated from cultures of the toxic dinoflagellate *Alexandrium tamarense*. Microb. Ecol. 39, 186-196.

80. Guschin, D.Y., Mobarry, B.K., Proudnikov, D., Stahl, D.A., Rittmnn, B.E., and Mirzabekov, A.D. (1997). Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. 63, 2397-2402.

81. Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Sr. 41, 95-98.

82. Hanada, S., Liu, W.-T., Shintani, T., Kamagata, Y., and Nakamura, K. (2002). *Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium from activated sludge. Int. J. Syst. Evol. Microbiol. 52, 883-887.

83. Head, I.M., Saunders, J.R., and Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb. Ecol. 35, 1-21.

84. Hesselmann, R.P.X., Werlen, C., Hahn, D., van der Meer, J.R., and Zehnder, A.J.B. (1999). Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. Syst. Appl. Microbiol. 22, 454-465.

85. Hesselmann, R.P.X., von Rummel, R., Resnick, S.M., Hany, R., and Zehnder, A.J.B. (2000). Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res. 34, 3487-3494.

86. Hiraishi, A., Masamune, K., and Kitamura, H. (1989). Characterization of the bacterial population structure in an anaerobic:aerobic activated sludge system on the basis of respiratory quinone profiles. Appl. Environ. Microbiol. 54, 197-210.

87. Hiraishi, A. and Morishita, Y. (1990). Capacity of polyphosphate accumulation of predominant bacteria in activated sludge showing enhanced phosphate removal. J. Ferment. Bioeng. 69, 368-371.

88. Jenkins, D. and Tandoi, V. (1991). The applied microbiology of enhanced biological phosphate removal – accomplishments and needs. Water Res. 25, 1471-1478.

89. Jenkins, D., Richard, M.G., and Daigger, G.T. (2004). *Manual on the causes and control of activated sludge bulking and foaming*, 3rd ed., Lewis Publishers, USA.

90. Jeon, C.O., Lee, D.S., and Park, J.M. (2000). Morphological characteristics of microbial sludge performing enhanced biological phosphorous removal in a sequencing batch reactor fed with glucose as a sole carbon source. Water Sci. Technol. 41(12), 79-84.

91. Jeon, C.O. and Park, J.M. (2000). Enhanced biological phosphorous removal in a sequencing batch reactor fed with glucose as a sole carbon source. Water Res. 34, 2160-2170.

92. Jeon, C.O., Lee, D.S., and Park, J.M., (2001a). Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: characteristics of carbon metabolism. Water Environ. Res. 73, 295-300.

93. Jeon, C.O., Lee, D.S., Lee, M.W., and Park, J.M. (2001b). Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor. Water Environ. Res. 73, 301-306.

94. Jeon, C.O., Lee, D.S., and Park, J.M. (2003). Microbial communities in activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor. Water Res. 37, 2195-2205.

95. Jones, M. and Stephenson, T. (1996). The effect of temperature on enhanced biological phosphorous removal. Environ. Technol. 17, 965-976.

96. Juni, E. (1978). Genetic and physiology of *Acinetobacter*. Ann. Rev. Microbiol. 32, 349-371.

97. Kämpfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M., and Amann, R.I. (1996). Characterization of bacterial communities from activated sludge: culture-dependent numerical identification versus *in situ* identification using group- and genus- specific rRNA-targeted oligonucleotide probes. Microbial Ecol. 32, 101-121.

98. Kane**,** M.D., Poulsen, L.K., and Stahl, D.A. (1993). Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences**.** Appl. Environ. Microbiol. 59, 682-686.

99. Kawaharasaki, M, Kanagawa, T., Tanaka, H., and Nakamura, K. (1998). Development and application of 16S rRNA-targeted probe for detection of the phosphate accumulating bacterium *Microlunatus phosphovorus* in an enhanced biological phosphorus removal process. Water Sci. Technol. 37(4-5), 481-484.

100. Kawaharasaki, M., Tanaka, H., Kanagawa, T., and Nakamura, K. (1999). *In situ* identification of polyphosphate accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4',6-diamidino-2-sphenylindol (DAPI) at a polyphosphate-probing concentration. Water Res. 33, 257-265.

101. Kawaharasaki, M., Manome, A., Kanagawa, T., and Nakamura, K. (2002). Flow cytometric sorting and RFLP analysis of phosphate accumulating bacteria in an

enhanced biological phosphorous removal system. Water Sci. Tech. 46(1-2), 139-144.

102. Keasling, J.D. and Hupf, G.A. (1996). Genetic manipulation of polyphosphate metabolism affects cadmium tolerance in *Escherichia coli*. Appl. Environ. Microbiol. 62, 743-746.

103. Keasling, J.D., van Dien, S.J., Trelstad, P., Renninger, N., and McMahon, K. (2000). Application of polyphosphate metabolism to environmental and biotechnological problems. Biochemistry 65, 324-331.

104. Keyhani, S., Lopez, J.L., Clark, D.S., and Keasling, J.D. (1996). Effect of polyphosphate on cadmium tolerance in the cyanobacterium *Anacystis nidulans* R2. Microbios. 88, 105-114.

105. Kim, H.Y., Schlictman, D., Shanker, S., Xie, Z., Chakrabarty, A.M., and Kornberg, A. (1998). Alginate, inorganic polyphosphate, GTP and ppGpp synthesis co-regulated in *Pseudomonas aeruginosa*: Implications for stationary-phase survival and synthesis of RNA/DNA precursors. Mol. Microbiol. 27, 717-725.

106. Kong, Y., Beer, M., Seviour, R.J., Lindrea, K.C., and Rees, G.G. (2001). Structure and functional analysis of the microbial community in an aerobic:anaerobic sequencing batch reactor (SBR) with no phosphorous removal. Syst. Appl. Microbiol. 24, 597-609.

107. Kong, Y., Beer, M., Seviour, R.J., Lindrea, K.C., and Rees, G.N. (2002a). Functional analysis of microbial communities in sequencing batch reactor fed with different phosphorous/carbon (P/C) ratios. Microbiology 148, 2299-2307.

108. Kong, Y., Ong, S.L., Ng, W.J., and Liu, W.-T. (2002b). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge process. Environ. Microbiol. 4, 753-757.

109. Kong Y., Nielsen, J.L., and Nielsen, P.H. (2004). Microautoradiographic study of *Rhodocyclus*-related polyphosphate accumulating bacteria in full-scale enhanced biological phosphorous removal plants. Appl. Environ. Microbiol. 70, 5383-5390.

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

111. Kong Y., Xia, Y., Nielsen, J.L., and Nielsen, P.H. (2006). Ecophysiology of a group of uncultured gammaproteobacterial glycogen-accumulaitng organisms in full-scale enhanced biological phosphorous removal wastewater treatment plants. Environ. Microbiol. 8, 479-489.

112. Kortstee, G.J.J., Appeldoorn, K.J., Bonting, C.F.C., van Niel, E.W.J., and van Veen, H.J. (2000). Ecological aspects of biological phosphorous removal in activated sludge systems. In: Advances in Microbial Ecology (Schink, B., eds), Vol. 16, pp. 169-200. Kluwer Academic/Plenum Publishers, New York.

113. Kuba, T., Smolders, G., van Loosdrecht, M.C.M., and Heijnen, J.J. (1993). Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. Water Sci. Technol. 27(5-6), 241-252.

114. Kuba, T., Wachtmeister, A., van Loosdrecht, M.C.M., and Heijnen, J.J. (1994). Effect of nitrate on phosphorus release in biological phosphorus removal systems. Water Sci. Technol. 30(6), 263-269.

115. Kuba, T., van Loosdrecht, M.C.M., and Heijnen, J.J. (1996). Phosphorus and nitrogen removal with minimum COD requirement by integration of denitrification in a two-sludge system. Water Res. 30, 1702-1710.

116. Kulaev, I.S. (1979). The Biochemistry of Polyphosphates. Wiley, Chichester, UK.

117. Kulaev, I.S. (1985). Some aspects of environmental regulation of microbial phosphorous metabolism. FEMS Symp. 23, 1-25.

118. Kumar, S., Tamura, K., and Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5, 150-163.

119. LaPara, T.M., Zakharova, T., Nakatsu, C.H., and Konopka, A. (2001). Functional and structural adaptations of bacterial communities growing on particulate substrates under stringent nutrient limitation. Microb. Ecol. 44, 317-326.

120. Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H., and Wagner, M. (1999). Combination of fluorescent *in situ* hybridization and microautoradiography – a new tool for structure-function analyzes in microbial ecology. Appl. Environ. Microbiol. 65, 1289-1297.

121. Lee, N., la Cour Janssen, J., Aspegren, H., Henze, M., Nielsen, P.H., and Wagner, M. (2002). Population dynamics in wastewater treatment plants with enhanced biological phosphorous removal operated with and without nitrogen removal. Water Sci. Technol. 46(1-2), 163-170.

122. Lee, N., Nielsen, P.H., Aspegren, H., Henze, M., Schleifer, K.-H., and la Cour Janssen, J. (2003). Long-term population dynamics and *in situ* physiology in

activated sludge systems with enhanced biological phosphorous removal operated with and without nitrogen removal. Syst. Appl. Microbiol. 26, 211-227.

123. Lee, S.Y. (1996). Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49, 1-14.

124. Lemos, P.C., Viana, C., Salguero, E.N., Ramos, A.M., Crespo, J.P.S.G., and Reis, M.A.M. (1998). Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. Enz. Microbial Technol. 22, 662-671.

125. Lesjean, B., Gnirss, R., Adam, C., Kraume, M., and Luck, F. (2003). Enhanced biological phosphorous removal process implemented in membrane bioreactors to improve phosphorous recovery and recycling. Water Sci. Technol. 48(1), 87-94.

126. Levantesi, C., Serafim, L.S., Crocettii, G.R., Lemos, P.C., Rossetti, S., Blackall, L.L., Reis, M.A.M., and Tandoi, V. (2002). Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. Environ. Microbiol. 4, 559-569.

127. Levin, G.V. and Shapiro, J. (1965). Metabolic uptake of phosphorous by wastewater organisms. J. Water Pollut. Contr. Fed. 37, 800-821.

128. Liu, Y.H. (1998). Relation between sludge carbohydrate content and biological phosphate removal. Water Environ. Res. 32, 1635-1641.

129. Liu, W. -T., Mino, T., Nakamura, K., and Matsuo, T. (1994). Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic-aerobic activated sludge with a minimized polyphosphate content. J. Ferment. Bioeng. 77, 535-540.

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

131. Liu, W.-T., Mino, T., Nakamura, K., and Matsuo, T. (1996b). Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. Water Res. 30, 75-82.

132. Liu, W.-T., Marsh, T.L., Cheng, H., and Forney, L.J. (1997a). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of 16S ribosomal DNA. Appl. Environ. Microbiol. 63**,** 4516-4522.

133. Liu, W.-T., Nakamura, K., Matsuo, T., and Mino, T. (1997b). Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors - effect of P/C feeding ratio. Water Res. 31, 1430-1438.

134. Liu, W.-T., Marsh, T.L., and Forney, L.J. (1998). Determination of the microbial diversity of anaerobic-aerobic activated sludge by a novel molecular biological technique. Water Sci. Technol. 37(4-5), 417-422.

135. Liu, W.-T., Linning, K.D., Nakamura, K., Mino, T., Matsuo, T., and Forney, L.J. (2000). Microbial community changes in biological phosphorous-removal systems on altering sludge phosphorous content. Microbiology 156, 1099-1107.

136. Liu, W.-T., Nielsen, A.T., Wu, J.-H., Tsai, C.-S., Matsuo, Y., and Molin, S. (2001). *In situ* identification of polyphosphate and polyhydroxyalkanoate accumulating traits for microbial populations in a biological phosphorus removal process. Environ. Microbiol. 3, 110-122.

137. Liu, W.-T., Hanada, S., Marsh, Y., Kamagata, Y., and Nakamura, K. (2002a). *Kineosphaera limosa* gen. nov. sp. nov., a new gram-positive polyhydroxyalkanoate-accumulating coccus, isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 52, 1845-1849.

138. Liu, W.-T., Huang, C.H., Hu, J.Y., Song, L., Ong, S.L., and Ng, W.J. (2002b). Denaturing gradient gel electrophoresis polymorphism for rapid 16S rDNA screening and microbial diversity study. J. Biosci. Bioeng. 93**,** 101-103.

139. Lötter, L.H. (1985). The role of bacterial phosphate metabolism in enhanced phosphorous removal from the activated sludge process. Water Sci. Technol. 17(11-12), 127-138.

140. Lötter L.H. and Dubery I.A. (1989). Metabolic regulation of β-hydroxybutyrate dehydrogenase in *Acinetobacter calcoaceticus* var *lwoffi*. Water SA 15, 65-70.

141. Louie, T.M., Mah, T.J., Oldham, W., and Ramey, W.D. (2000). Use of metabolic inhibitors and gas chromatography/mass spectroscopy to study poly-β-hydroxyalkanoates metabolism involving cryptic nutrients in enhanced biological phosphorus removal systems. Water Res. 34, 1507-1514.

142. Loy, A., Daims, H., and Wagner, M. (2002). Activated sludge – molecular techniques for determining community composition. In: Encyclopedia of Environmental Microbiology (Bitton, G., Ed.), pp. 26-43. Wiley Scientific, New York.

143. Ludwig, W., Strunk, O., Westram, R., and 29 other authors (2004). ARB: a software environment for sequence data. Nucleic Acids Res. 32(4), 1363-1371.

144. Maidak, B.L., Cole, J.R., Lilburn, T.G., and 7 other authors (2001). The RDP-II (Ribosomal Database Project). Nucleic Acids Res. 29**,** 173-174.

145. Manga, J., Ferrer, J., Garcia-Usach, F., and Seco, A. (2000). A modification to the activated sludge model No. 2 based on the competition between phosphorus-accumulating-organisms and glycogen-accumulating organisms. Proc. 1st World Conf. of IWA, 3(Wastewater Treatment Plants), 300-307.

146. Manz, W., Amann, R.I., Ludwig, W., Wagner, M., and Schleifer, K.H (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. 15**,** 593-600.

147. Manz, W., Amann, R.I., Ludwig, W., Vancanneyt, M., and Schleifer, K.H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum C*ytophage-Flavobacter-Bacteroides* in the natural environment. Microbiology 142, 1097-1106.

148. Marais, G.v.R., Lowenthal, R.E., and Siebritz, I.P. (1983). Observations supporting phosphate removal by biological excess uptake – a review. Water Sci. Technol. 15(3-4), 15-42.

149. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Rees, G.N., and McDougall, B.M. (1997). *Amaricoccus* gen. nov., a gram-negative coccus occurring in regular packages and tetrads, isolated from activated sludge biomass, and descriptions of *Amaricoccus veronensis* sp. nov., *Amaricoccus tamworthensis* sp. nov., *Amaricoccus macauensis* sp. nov. and Amaricoccus kaplicensis sp. nov. Int. J. Syst. Bacteriol. 47, 727-734.

150. Maszenan, A.M., Seviour, R. J., Patel, B. K. C., Schumann, P., and Rees, G. N. (1999). *Tessaracoccus bendigoenisis* gen. nov., sp. nov., a gram-positive coccus occurring in regular packages or tetrads, isolated from activated sludge biomass. Int. J. Syst. Bacteriol. 48, 459-468.

151. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., Burghardt, Y., Tokiwa, Y., and Stratton, H.M. (2000a). Three isolates of novel polyphosphate accumulating gram positive cocci, obtained from activated sludge belong to a new genus *Tetrasphaera* gen. nov. & description of two new species *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov. Int. J. Syst. Evol. Microbiol. 50, 593-603.

152. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., and Wanner, J. (2000b). A fluorescently labeled r-RNA targeted probe for the *in situ* detection of G-bacteria of the genus *Amaricoccus* in activated sludge. J. Appl. Microbiol. 88, 826-835.

153. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., and Schumann, P. (2002). *Quadricoccus australiensis* gen. nov., sp. nov., a *β-proteobacterium* from activated sludge biomass. Int. J. Syst. Evol. Microbiol. 52, 223-228.

154. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Janseen, P.H., and Wanner, J. (2005). *Defluvicoccus vanus* gen. nov., sp. nov., a novel Gram-negative coccus/coccobacillus in the '*Alphaproteobacteria*' from activated sludge. Int. J. Syst. Evol. Microbiol. 55, 2105-2111.

155. Matsuo, Y., Kitagawa, M., Tanaka, T., and Miya, A. (1982). Sewage and nightsoil treatments by anaerobic-aerobic activated sludge process for biological phosphate removal. Proc. Environ. Sani. Eng. Res. 19, 82-87 (in Japanese).

156. Matsuo, T., Mino, T., and Satoh, H. (1992). Metabolism of organic substances in anaerobic phase of biological phosphate uptake process. Water Sci. Technol. 25, 83-92.

157. Matsuo, Y. (1994). Effect of the anaerobic solids retention time on enhanced biological phosphorus removal. Water Sci. Technol. 30(6), 193-202.

158. Maurer, M. (1997). Intracellular carbon flow in phosphorus accumulating organisms from activated sludge systems. Water Res. 31, 907-917.

159. McClintock, S., Randall, C.W., and Pattarkine, V. (1992). The effects of temperature and mean cell residence time on enhanced biological phosphorus removal. Environmental Engineering, the Proceedings of the 1991 Specialty Conference on Environmental Engineering, ASCE, 319-324.

160. McGrath, J.W. and Quinn, J.P. (2000). Intracellular accumulation of polyphosphate by the yeast *Candida humicola* G-1 in response to acid pH. Appl. Environ. Microbiol. 66, 4068-4073.

161. McGrath, J.W., Cleary, S., Mullan, A., and Quinn, J.P. (2001). Acid-stimulated phosphate uptake by activated sludge microorganisms under aerobic laboratory conditions. Water Res. 35, 4317-4322.

162. McGrath, J.W. and Quinn, J.P. (2003). Microbial phosphate removal and polyphosphate production from wastewaters. In: Advances in Applied Microbiology, Vol. 52. (Laskin, A.I., Bennett, J.W., and Gadd, G.M., Eds), pp 75-100. Elsevier, California, USA.

163. McMaholm, K.D., Dojka, M.A., Pace, N.R., Jenkins, D., and Keasling, J.D. (2002). Polyphosphate kinase from activated sludge performing enhanced biological phosphorus removal. Appl. Environ. Microbiol. 68**,** 4971-4978.

164. Mecalf and Eddy, Inc. (2003). Wastewater Engineering: Treatment and Reuse. McGraw-Hill, 4th edition, New York, USA.

165. Meier, H., Amann, R.I., Ludwig, W., Schleifer, K.-H, (1999). Specific oligonucleotide probes for *in situ* detection of a major group of Gram-positive bacteria with low G+C content. Syst. Appl. Microbiol. 22, 186-196.

166. Meinhold, J., Arnold, E. and Isaacs, S. (1999a). Effect of nitrite on anoxic phosphate uptake in biological phosphorus removal activated sludge. Water Res. 33(8), 1871-1883.

167. Meinhold, J., Filipe, C.D.M., Daigger, G.T., and Isaacs, S. (1999b). Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. Water Sci. Technol. 30(1), 31-42.

168. Melasniemi, H., Hernesmaa, A., Pauli, A.S.-L., Rantanen, P., and Salkinoja-Salonen, M. (1998). Comparative analysis of biological phosphate removal (BPR) and non-BPR activated sludge bacterial communities with particular reference to *Acinetobacter*. J. Ind. Microbiol. Biotechnol. 21, 300-306.

169. Melasniemi, H. and Hernesmaa, A. (2000). Yeast spores seem to be involved in biological phosphate removal: a microscopic *in situ* case study. Microbiology 146, 701-707.

170. Menar, A.B. and Jenkins, D. (1970). Fate of phosphorous in waste treatment processes: The enhanced removal of phosphate by activated sludge, in 24th Industrial Waste Treatment Conference, pp. 655-667. Purdue University, Lafayette, Ind.

171. Merzouki, M., Delgenes, J.-P., Bernet, N., Moletta, R., and Benlemlih, M. (1999). Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors. Curr. Microbiol. 38, 9-17.

172. Milbury, W.F., McCauley, D., and Hawthorne, C.H. (1971). Operation of conventional activated sludge for maximum phosphorus removal. J. Water Pollut. Contr. Fed. 43, 1890-1901.

173. Mino, T., Kawakami, T., and Matsuo, T. (1984). Location of phosphorus in activated sludge and function of intracellular polyphosphates in biological phosphorus removal process. Water Sci. Technol.17 (2-3), 93-106.

174. Mino, T., Kawakami, T., and Matsuo, T. (1985). Behaviour of intracellular polyphosphate in the biological phosphate removal process. Water Sci. Technol. 17(11-12), 11-21.

175. Mino T., Tsuzuki Y., and Matsuo T. (1987). Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process. In: Biological Phosphate Removal from Wastewaters (Ramadori, R., ed.), pp 27-38, Pergamon Press, Oxford.

176. Mino, T., van Loosdrecht, M.C.M., and Heijnen, J.J. (1998). Microbiology and biochemistry of enhanced biological phosphate removal process. Water Res. 32, 3193-3207.

177. Mino, T. (2000). Microbial selection of polyphosphate-accumulating bacteria in activated sludge wastewater treatment processes for enhanced biological phosphate process. Biochemistry (Moscow) 65, 405-413.

178. Miyamoto-mills, J., Larson, J., Jenkins, D., and Owen, W. (1983). Design and operation of a pilot scale biological phosphate removal plant at the Central Contra Costa Sanitary District. Water Sci. Tech. 15(3-4), 153-179.

179. Morgenroth, E. and Wilderer, P.A. (1998). Modeling of enhanced biological phosphorous removal in a sequencing batch biofilm reactor. Water Sci. Technol. 37(4-5), 583-587.

180. Mudaly, D.D., Atkinson, B.W., and Bux, F. (2000). Microbial community profile of a biological excess phosphorous removal (BEPR) activated sludge system using a cultivation-independent approach. Water SA 26, 343-352.

181. Mulkerrins, D., Dobson, A.D.W., and Colleran, E. (2004). Parameters affecting biological phosphate removal from wastewaters. Environ. International 30, 249-259.

182. Mullan, A., Quinn, J.P., and McGrath, J.W. (2002). Enhanced phosphate uptake and polyphosphate accumulation in *Burkholderia cepacia* grown under low-pH conditions. Microbiol. Ecol. 44, 69-77.

183. Murnleitner, E., Kuba, T., van Loosdrecht, M.C.M., and Heijnen, J.J. (1997). An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnol. Bioeng. 54, 434-450.

184. Nakamura, K., Masuda, K., and Mikami, E. (1991). Isolation of a new type of polyphosphate accumulating bacterium and its phosphate removal characteristics. J. Ferment. Bioeng. 71, 258-263.

185. Nakamura, K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Matsuda, K., and Kamagata, Y. (1995a). *Microlunatus phosphovorus* gen. nov. sp. nov., a new gram-positive polyphosphate accumulating bacterium isolated from activated sludge. Int J. Syst. Bacteriol. 45, 17-22.

186. Nakamura, K., Ishikawa, S., and Kawaharasaki, M. (1995b). Phosphate uptake and release activity in immobilized polyphosphate accumulating bacterium *Microlunatus phosphovorus* strain NM-1. J. Ferment. Bioeng. 80, 377-382.

187. Neef, A., Zaglauer, A., Meier, H., Amann, R.I., Lemmer, H., and Schleifer, K.-H. (1996). Population analysis in a denitrifying sand filter: conventional and *in situ* identification of *Paracoccus* spp. in methanol-fed biofilms. Appl. Environ. Microbiol. 62, 4329-4339.

188. Neef, A., Witzenberger, R., and Kämpfer, P. (1999). Detection of *Sphingomonads* and *in situ* identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. J. Indust. Microbiol. Biotechnol. 23, 261-267.

189. Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

190. Nicholls, H.A. and Osborn, D.W. (1979). Bacterial stress, a prerequisite for biological removal of phosphorous. J. Water Pollu. Cont. Fed. 51, 557-569.

191. Nielsen, A.T., Liu, W.-T., Filipe, C., Grady, L.J., Molin, S., and Stahl, D.A. (1999). Identification of novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. Appl. Environ. Microbiol. 65, 1251-1258.

192. Ohtake, H., Takahashi, K., Tsuzuky, Y., and Toda, K. (1985). Uptake and release of phosphate by a pure culture of *Acinetobacter calcoaceticus*. Water Res. 19, 1587-1594.

193. Okada, M., Lin, C.K., Katayama, Y., and Murakami, A. (1992). Stability of phosphorous removal and population of bio-P bacteria under short term disturbances in sequencing batch reactor activated sludge process. Water Sci. Technol. 26(3-4), 483-491.

194. Okunuki, S., Kawaharasaki, M., Tanaka, H., and Kanagawa, T. (2004). Changes in phosphorous removing performance and bacterial community structure in an enhanced biological phosphorous removal reactor. Water Res. 38, 2432-2438.

195. Onuki, M. Satoh, H., and Mino, T. (2002). Analysis of microbial community that performs enhanced biological phosphorous removal in activated sludge fed with acetate. Water Sci. Technol. 46(1-2), 145-154.

196. Ostle, A.G. and Holt, J.G. (1982). Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. 44, 238-241.

197. Ouverney, C.C. and Fuhrman, J.A. (1999). Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell type *in situ*. Appl. Environ. Microbiol. 65, 1746-1752.

198. Panswad T, Doungchai, A, and Anotai, J. (2003). Temperature effect on microbial community of enhanced biological phosphorus removal system. Water Res. 37, 409-415.

199. Patel, J., Nakhla, G., and Margaritis, A. (2005). Optimization of biological nutrient removal in a membrane bioreactor system. J. Environ. Eng. ASCE 131, 1021-1029.

200. Pereira, H., Lemos, P.C., Reis, M.A.M., Crespo, J.P.S.G., Carrondo, M.J.T., and Santos, H. (1996). Model for carbon metabolism in biological phosphorus removal processes based on in vivo ^{13}C-NMR labeling experiments. Water Res. 30, 2128-2138.

201. Pitman, A.R. (1991). Design considerations for nutrient removal activated sludge plants. Water Sci. Technol. 23(4-6), 781-790.

202. Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., and Blackall, L.L. (2004). Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnol. Bioeng 85, 56-87.

203. Randall, C.W., Barnard, J.L., and Stensel, H.D. (1992). Design and retrofit of wastewater treatment plants for biological nutrient removal. Lancaster: Technomic Publishing, 25-78.

204. Randall, A.A., Benefield, L.D., and Hill, W.E. (1994). The effect of fermentation products on enhanced biological phosphorus removal, polyphosphate storage and microbial population dynamics. Water Sci. Tech. 30(6), 213-219.

205. Randall, C.W. and Chapin, R.W. (1997). Acetic acid inhibition of biological phosphorous removal. Water Environ. Res. 69, 955-960.

206. Rao, N.N., Liu, S., and Kornberg, A. (1998). Inorganic polyphosphate in *Escherichia coli*: The phosphate regulon and the stringent response. J. Bacteriol. 180, 2186-2193.

207. Reddy, M. (1998). Biological and chemical systems for nutrient removal. WEF, special publication.

208. Rensink, J.J. (1981). Biologische Defosfatering en procesbepalende Factoren, presented at the NVA Symposium, Amersfoort, The Netherlands.

209. Reyes, G.D., So, R.S., and Ulep, M.M. (1997). Isolation, screening, and identification of bacteria for poly-β-hydroxybutyrate (PHB) production. In D. L. WISE (Ed.) Global Environmental Biotechnology, Elsevier Science, Boston, Massachusetts, USA, pp. 737-748.

210. Roller, C., Wagner, M., Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1994). *In situ* probing of Gram-positive bacteria with high G+C content using 23S rRNA-targeted oligonucleotides. Microbiology 140, 2849-2858.

211. Rosenberger, S., Witzig, R., Manz, W., Kraume, M., and Szewszyk, U. (2000). Operation of different membrane bioreactors: experimental results and physiological state of the microorganisms. Water Sci. Technol. 41(10/11), 269-277.

212. Saito, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Bio. Evol. 4**,** 406-425.

213. Santos, M.M., Lemos, P.C., Reis, M.A.M., and Santos, H. (1999). Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium *Microlunatus phosphovorus*. Appl. Environ. Microbiol. 65, 3920-3928.

214. Sathasivan, A., Mino, T., and Matsuo, T. (1993). A deeper look on acetic acid metabolism in bio-P organism. Proc. Annual Conf. Jap. Soc. Water Environ., 27.

215. Satoh H., Mino T., and Matsuo T. (1990). Biochemical mechanism of anaerobic glutamate uptake in the enhanced biological phosphorus removal process. In: Proc. IAWPRC Biennial Conf., pp. 531-534, Kyoto, Japan.

216. Satoh, H., Mino, T., and Matsuo, T. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal process. Water Sci. Technol. 26(5-6) 933-942.

217. Satoh, H., Mino, T., and Matsuo, T. (1994). Deterioration of enhanced biological phosphorous removal by the domination of microorganisms without polyphosphate accumulation. Water Sci. Technol. 30(11), 203-211.

218. Satoh, H., Mino, T., and Matsuo, T. (1998). Anaerobic uptake of glutamate and aspartate by enhanced biological phosphorus removal activated sludge. Water Sci. Technol. 37(4-5), 579-582.

219. Saunders, A.M., Oehmen, A., Blackall, L.L., Yuan, Z., and Keller, J. (2003). The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water Sci. Technol. 47(11), 37-43.

220. Schramm, A. and Amann, R.I. (1999). Nucleic-acid based techniques for analyzing the diversity, structure and dynamics of microbial communities in activated sludge. In: Environmental Processes 1 (Winter, J., Ed.), pp. 85-108. Wiley-VCH, Weinheim.

221. Schlegel, H.G. (1993). General Microbiology, 7th edition, Cambridge University Press, Cambridge, UK.

222. Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M. S.M., Metzger, J.W., Schleifer, K.-H., and Wagner, M. (2000). Molecular evidence for genus-level diversity of bacteria of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. 23, 93-106.

223. Schuler, A.J., Jenkins, D., and Ronen, P. (2001). Microbial storage products, biomass density, and settling properties of enhanced biological phosphorous removal activated sludge. Water Sci. Technol. 43(1), 172-180.

224. Schuler, A., Onuki, M., Satoh, H., and Mino, T. (2002). Density separation and molecular methods to characterize enhanced biological phosphorus removal system populations. Water Sci. Technol. 46(1-2), 195-198.

225. Schuler, A.J. and Jenkins, D. (2002). Effects of pH on enhanced biological phosphorus removal metabolisms. Water Sci Technol. 46(4-5), 171-178.

226. Schwieger, F. and Tebbe, C.C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl. Environ. Microbiol. 64, 4870-4876.

227. Sell, R. (1981). Low temperature biological phosphorous removal. Presented at the 54th Annual Conference of the Water Pollution Control Federation, Detroit, Michigan. Air Products and Chemicals, Inc. Allentown, PA, USA.

228. Serafim, L. S., Lemos, P. C., Levantesi, C., Tandoi, V., Santos, H., and Reis, M. A. M. (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. J. Microbiol. Meth. 51, 1-18.

229. Seviour, E.M., Williams, C.J., Seviour, R.J., Soddell, J.A., and Lindrea, K.C. (1990). A survey of filamentous bacterial populations from foaming activated sludge plants in eastern states of Australia. Water Res. 24, 493-498.

230. Seviour, R.J., Mino, T., and Onuki, M. (2003). The microbiology of biological phosphorous removal in activated sludge systems. FEMS Microbiol. Rev. 27, 99-127.

231. Shapiro, J. (1967). Induced rapid release and uptake of phosphate by microorganisms. Science 155, 1269-1271.

232. Shehab, O., Deininger, R., Porta, F., and Wojewski, T. (1996). Optimizing phosphorous removal at the Ann Arbor wastewater treatment plant. Water Sci. Technol. 34(1-2), 493-499.

233. Shintani, T., Liu, W.-T., Hanada, S., Kamagata, Y., Miyaoka, S., Suzuki, Y., and Nakamura, K. (2000) *Micropruina glycogenica* gen. nov., sp. nov., a new gram-positive glycogen-accumulating bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 50, 201-207.

234. Smolders, G.J.F., van der Meij, J., van Loostrecht, M.C.M., and Heijnen, J.J. (1994). Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol. Bioeng. 44, 837-848.

235. Smolders, G.J.F., van der Meij, J., van Loostrecht, M.C.M., and Heijnen, J.J. (1995). A structured metabolic model for the anaerobic and aerobic

stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol. Bioeng. 47, 277-287.

236. Soddell, J.A. (1999). Foaming *In Microbiology of Activated Sludge* (Eds R.J. Seviour & L.L. Blackall), Kluwer Academic Publishers, Dordrecht1, 161-202.

237. Spring, S., Wagner, M., Schumann, P., Kämpfer, P. (2005). *Malikia granosa* gen. nov., sp. nov., a novel polyhydroxyalkanoate- and polyphosphate-accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. Int. J. Syst. Evol. Microbiol. 55, 621-629.

238. Srinath, E.G., Sastry, C.A., and Pillai, S.C. (1959) Rapid removal of phosphorus from sewage by activated sludge. Experientia 15, 339-340.

239. Stante, L., Cellamare, C. M., Malaspina, F., Bortone, G., and Tiche, A. (1997). Biological phosphorus removal by pure culture of *Lampropedia* spp. Water Res. 31, 1317-1324.

240. Steinbüchel, A. and Valentin, H.E. (1995). Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiology Letters 128, 219-228.

241. Stephenson, T., Judd, S., Jefferson, B., and Brindle, K. (2000). Membrane Bioreactors for Wastewater Treatment. IWA Publishing, London, UK.

242. Streichan, M., Golecki, J.R., and Schön, G. (1990). Polyphosphate-accumulating bacteria from sewage plants with different processes for biological phosphorous removal. FEMS Microbiol. Ecol. 73, 113-124.

243. Sudiana, I.M., Mino, T., Satoh, H., and Matsuo, T. (1998). Morphology, *in situ* characterization with rRNA targeted probes and respiratory quinine profiles of enhanced biological phosphorous removal sludge. Water Sci. Technol. 38(8-9), 69-76.

244. Sudiana, I.M., Mino, T., Satoh, H., Nakamura, K., and Matsuo, T. (1999). Metabolism of enhanced biological phosphorus removal and non-enhanced biological phosphorus removal sludge with acetate and glucose as carbon source. Water Sci. Technol. 39(6), 29-35.

245. Tammink, H., Petersen, B., Issacs, S., and Henze, M. (1996). Recovery of biological phosphorous removal after periods of low organic loading. Water Sci. Technol. 34(1-2), 1-8.

246. Tandoi, V., Beccari, M., Ramadori, R., and Annicchiarica, S.L. (1987). *Acinetobacter* sp. growth in alternate anaerobic-aerobic conditions. Proc. IAWPRC Int. Conf. on Biological Phosphate Removal from Wastewaters, Rome, Italy, Adv. Water Pollut. Cont. (ed. Ramadori).

247. Tandoi, V., Majone, M., May, J., and Ramadori, R. (1998). The behaviour of polyphosphate accumulating *Acinetobacter* isolates in an anaerobic:aerobic chemostat. Water Res. 32, 2903-2912.

248. Thompson, J.D., Higgins, D.G., and Gibson, T.G. (1994). CLUSTER_W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22**,** 4673-4680.

249. Toerien, D.F., Gerber, A., Lötter, L.H., and Cloete, T.E. (1990). Enhanced biological phosphorous removal in activated sludge systems. Adv. Microbiol. Ecol. 11, 173-230.

250. Tsai, C.-S. and Liu, W.-T. (2002). Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorous removal systems. Water Sci. Technol. 46(1-2), 179-184.

251. Tunlid, A. and White, D.C. (1992). Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of the microbial community in soil, pp. 229-262. *In* J. M. Bollag, and G. Stotzky (ed.), Soil biochemistry, vol. 7. Marcel Dekker, Inc, New York, N.Y.

252. Ubukata, Y. and Takii, S. (1994). Induction ability of excess phosphate accumulation for phosphate removing bacteria. Water Res. 28, 247-249.

253. Ubukata, Y. and Takii, S. (1998). Some physiological characteristics of a phosphate removing bacterium, *Microlunatus phosphovorus*, and a simplified isolation and identification method for phosphate-removing bacteria. Water Sci. Technol. 38(1), 149-157.

254. Ujang, Z., Salim, M.R., and Khor, S.L. (2002). The effect of aeration and non-aeration time on simultaneous organic, nitrogen and phosphorous removal using an intermittent aeration membrane reactor. Water Sci. Technol. 46(9), 193-200.

255. Urbain, V., Mobarry, B.K., de Silva, D.G., Stahl, D.A., Rittmann, B.E., and Manem, J. (1998). Integration of performance, molecular biology and modeling to describe the activated sludge process. Water Sci. Technol. 37(3), 223–229.

256. Vacker, D., Connell, C. H., and Well, W. N. (1967). Phosphate removal through municipal wastewater treatment at San Antonio, Texas, J. Water Pollut. Contr. Fed. 39, 750-771.

257. van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D., and Heijnen, J.J. (1997). Biological phosphorus removal processes. Appl. Microbiol. Biotechnol. 48, 289-296.

258. van Ommen Kloeke, F. and Geesey, G.G. (1999). Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs. Microbial. Ecol. 38, 201-214.

259. van Wazer, J.R. and Holst, K.A. (1950). Structures and properties of condensed phosphates: I. Some general considerations about phosphoric acids. J. Am. Chem. Soc. 72, 639-644.

260. Vocks, M., Adam, C., Lesjean, B., Gnirss, R., and Kraume, M. (2005). Enhanced post-denitrification without addition of an external carbon source in membrane bioreactors. Water Res. 39, 3360-3368.

261. Wagner, M., Amann, R.I., Lemmer, H., and Schleifer, K.-H. (1993). Probing activated sludge with oligonucleotides specific for Proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59, 1520-1525.

262. Wagner, M., Erhart, R., Manz, W., Amann, R.I., Lemmer, H., Wedl, D., and Schleifer, K.-H. (1994). Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated. Appl. Environ. Microbiol. 60, 792-800.

263. Wallner, G., Amann, R.I., and Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for low cytometric identification of microorganisms. Cytometry 14, 136-143.

264. Wang, J.C. and Park, J.K. (2001). Effect of anaerobic-aerobic contact time on the change of internal storage energy in two different phosphorous-accumulating organisms. Water Environ. Res. 436-443.

265. Wang, J.C., Park, J.K., Whang, L.M. (2001). Comparison of fatty acid composition and kinetics of phosphorous-accumulating organisms and glycogen-accumulating organisms. Water Environ. Res. 73, 704-710.

266. Wang, N., Peng, J., and Hill, G. (2002). Biochemical model of glucose induced enhanced biological phosphorus removal under anaerobic condition. Water Res. 36, 49-58.

267. Weller, R., Glöckner, F.O., and Amann, R.I. (2000). 16S rRNA oligonucleotide probes for the *in situ* detection of members of the phylum *Cytophaga-Flavobacterium-Bacteroides*. Syst. Appl. Microbiol. 23, 107-114.

268. Wells, W.N. (1969). Differences in phosphate uptake rates exhibited by activated sludges. J. Water Pollut. Contr. Fed. 41, 765-771.

269. Wentzel, M.C. Loewenthal, R.E., Ekama, G.A., and Marais, G.v.R. (1988). Enhanced polyphosphate organism cultures in activated sludge - Part I: Enhanced culture development. Water SA 14, 81-92.

270. Wentzel, M.C, Ekama, G.A., Lowenthal, R.E., Dold, P.L., and Marais, G.v.R. (1989a). Enhanced polyphosphate organism cultures in activated sludge - Part II: Experimental behaviour. Water SA. 15, 71-88.

271. Wentzel, M.C, Ekama, G.A., Lowenthal, R.E., Dold, P.L., and Marais, G.v.R. (1989b). Enhanced polyphosphate organism cultures in activated sludge - Part III: Kinetic model. Water SA. 15, 89-102.

272. Wentzel, M.C., Lötter, L.H., Ekama, G.A., Loewenthal, T.E., and Marais, G.v.R (1991). Evaluation of biochemical models for biological excess phosphorus removal. Water Sci. Technol. 23(Kyoto), 567-576.

273. WHO (2002). Eutrophication and health. World Health Organization Regional Office for Europe.

274. Wintzingerode, F.V., Goebel, U.B., and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213-229.

275. Wong, M.-T. and Liu, W.-T. (2006). Microbial succession of glycogen accumulating organisms in an anaerobic-aerobic membrane bioreactor with no phosphorous removal. Water Sci. Technol. 54(1), 29-37.

276. Wong, M.-T. and Liu, W.-T. (2007). Ecophysiology of *Defluviicoccus*-related tetrad-forming organisms in an anaerobic-aerobic activated sludge process. Microbiol., accepted.

277. Wong, M.-T., Tan, F.M., Ng, W.J., and Liu, W.-T. (2004). Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. Microbiology 150, 3741-3748.

278. Wong, M.-T., Mino, T., Seviour, R.J., Onuki, M., and Liu, W.-T. (2005). *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Res. 39, 2901-2914.

279. Yagci, N., Artan, N., Ubay Cokgör E., Randall, C.W., and Orhon, D. (2003). Metabolic model for acetate uptake by a mixed cultura of phosphate and glycogen accumulating organisms under anaerobic conditions. Biotechnol. Bioeng. 84, 360-373.

280. Yall, I. Boughton, W. H. Knudsen, R. C., and Sinclavi, N. A. (1970). Biological uptake of phosphorus by activated sludge. Appl. Microbial. 20, 145-150.

281. Yeates, C., Saunders, A.M., Crocetti, G.R., and Blackall, L.L. (2003). Limitations of the widely used GAM42a and BET42a probes targeting bacteria in the *Gammaproteobacteria* radiation. Microbiology 149, 1239-1247.

282. Zeng, R., Yuan, Z., van Loosdrecht, M.C.M., and Keller, J. (2002). Proposed modifications to metabolic model for glycogen-accumulating organisms under anaerobic conditions. Biotechnol. Bioeng. 80, 277-279.

283. Zilles, J.L., Peccia, J., Kim, M.-W., Hung, C.-H., and Noguera, D.R. (2002). Involvement of *Rhodocyclus*-related organisms in phosphorous removal in full-scale wastewater treatment plants. Appl. Environ. Microbiol. 68, 2763-2769.