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The Influence of Carbon Source Types and Nitrate on the Performance of the Enhanced Biological Phosphorus Removal Systems

Shamim Ara Begum
University of Nevada, Las Vegas

THE INFLUENCE OF CARBON SOURCE TYPES AND NITRATE ON THE PERFORMANCE OF THE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL SYSTEMS

by

Shamim Ara Begum

Bachelor of Science
Bangladesh University of Engineering and Technology (BUET), Dhaka – Bangladesh
1997

Master of Science
International Institute for Infrastructural, Hydraulic and Environmental Engineering
(IHE), Delft - Netherlands
2003

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of the requirements for the

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Department of Civil and Environmental Engineering
Howard R. Hughes College of Engineering

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University of Nevada, Las Vegas
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Shamim Ara Begum

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Ph.D. in Engineering

[redacted]
Examination Committee Chair

[redacted]
Dean of the Graduate College

[redacted]
Examination Committee Member

[redacted]
Examination Committee Member

[redacted]
Graduate College Faculty Representative

[redacted]
Examination Committee Member

ABSTRACT

The Influence of Carbon Source Types and Nitrate on the Performance of the Enhanced Biological Phosphorus Removal Systems

by

Shamim Ara Begum

Dr. Jacimaria Ramos Batista, Examination Committee Chair
Associate Professor, Department of Civil and Environmental Engineering
University of Nevada, Las Vegas

This research focused on two issues in enhanced biological phosphorus removal (EBPR). The first issue encompassed the effect of glucose and butyrate on the microbiology of EBPR. The second issue addressed the influence of denitrification on EBPR. The first issue was investigated in SBRs system fed exclusively with glucose and butyrate. The second issue was investigated in batch tests using wastewater and different levels of acetate and propionate. In the case of first issue, the phosphorus removal was negatively affected by glucose and effective by using butyrate as a sole carbon source. Fluorescence In Situ Hybridization analysis revealed that *Candidatus* Accumulibacter phosphatis (CAP), a PAO was not selected in the glucose fed reactor. However, glucose selected for *Microlunatus phosphovorus*, a PAO, *Candidatus* Compatibacter phosphatis (CCP), a GAO and *Micropruina glycogenica*, a GAO. In the case of butyrate, CAP and *Defluvicoccus*-related tetrad-forming organisms, a GAO were selected in the reactor. However, butyrate did not select for CCP. The % RA of GAOs and % P removal did not show good correlation in the butyrate fed reactors, which might indicate the presence of

other potential GAOs not targeted in the present study. In the case of issue two, the addition of supplemental carbon source at stoichiometric level improved the phosphorus uptake under aerobic conditions when nitrate was introduced during the anoxic period. However, the addition of carbon source at a higher than stoichiometric level did not improve phosphorus uptake under the aerobic condition when denitrification was incorporated during the anoxic period of EBPR process. The results of this research have direct implications on the design of treatment plants to remove phosphorus from wastewaters.

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ABBREVIATIONS

ANOVA – One Way Analysis of Variance

AnRT – Anaerobic Retention Time

A/O – Anaerobic/Oxic

A^2/O – Anaerobic/Anoxic/Oxic

APAO – Actinobacterial Polyphosphate Accumulating Organism

APT – Activated Primary Tank

ATP – Adenosine Triphosphate

AUR – Acetate Uptake Rate

BLAST – Basic Local Alignment Search Tool

BOD - Biochemical Oxygen Demand

BOD_5 - Biochemical Oxygen Demand at day 5

BPR – Biological Phosphorus Removal

CAP – *Candidatus* Accumulibacter Phosphatis

CCP - *Candidatus* Competibacter Phosphatis

CLSM – Confocal Laser Scanning Microscope

CCWRD – Clark County Water Reclamation District

C/P – Carbon/Phosphorus

CMR – Completely Mixed Reactor

COD – Chemical Oxygen Demand

DBP – Disinfection by-Products

DIP – Dissolved Inorganic Phosphorus

DNA – Deoxyribonucleic Acid

DO – Dissolve Oxygen

DOC – Dissolved Organic Carbon

DPAO – Denitrifying Polyphosphate Accumulating Organism

DR – Dynamic Range

DSVI – Diluted Sludge Volume Index

EBPR – Enhanced Biological Phosphorus Removal

EDTA – Ethylenediaminetetraacetic Acid

FISH – Fluorescence In Situ Hybridization

FITC – Fluorescein-Isothiocyanate

HPLC – High Performance Liquid Chromatography

HRT – Hydraulic Retention Time

GAO – Glycogen Accumulating Organism

LPO – Lactate - Producing Organism

MAR-FISH – Microautoradiography with Fluorescence In Situ Hybridization

MCRT – Mean Cell Residue Time

MGD – Million Gallons per Day

MIG – *Micropruina glycogenica*

MLR – Mixed Liquor Return

MLSS – Mixed Liquid Suspended Solid

MLVSS – Mixed Liquid Volatile Suspended Solid

MP – *Microlunatus phosphovorus*

N – Nitrogen

NA – Numerical Aperture

NIH – National Institute of Health

NPDES – National Pollution Discharge Elimination System

OHO – Ordinary Heterotrophic Organism

OP – Orthophosphate

P – Phosphorus

PAO – Polyphosphate Accumulating Organism

PBS – Phosphate Buffer Saline

PHA – Polyhydroxyalkanoate

PHB – Polyhydroxybutyrate

PHV – Polyhydroxyvalerate

PolyP - Polyphosphate

PMT – Photomultiplier Tube

PP – Particulate Phosphorus

RA – Relative Abundance

RNA - Ribonucleic Acid

RPAO – Rhodocyclus- related Polyphosphate Accumulating Organism

S_{bsa} - Readily Biodegradable COD in the Anaerobic Reactor

S_{bsi} - Influent Readily Biodegradable COD Strength

SBH – Sludge Blanket Height

SBR – Sequencing Batch Reactor

SCFH – Standard Cubic Feet per Hour

$SCUR_{max}$ - Maximum Specific Carbon Uptake Rate

SCVFA - Short Chain Volatile Fatty Acid

SD – Standard Deviation

SDBS – Sodium Dodecylbenzene Sulfonate

$SDNR_{max}$ – Maximum Specific Denitrification Rate

SE – Standard Error

SLR – Solids Loading Rate

SOR – Surface Overflow Rate

SOP – Soluble Orthophosphate

S/N – Signal-to-Noise

$SPRR_{max}$ - Maximum Specific Phosphorus Release Rate

$SPUR_{max}$ - Maximum Specific Phosphorus Uptake Rate

SRT – Solid Retention Time

SS - Suspended Solid

sTOC – Soluble Total Organic Carbon

SVI – Sludge Volume Index

SWD – Side Water Depth

TFO – Tetrad-Forming Organism

TOC – Total Organic Carbon

TP – Total Phosphorus

TRITC – Tetramethyl-Rhodamine- Isothiocyanate

TSS – Total Suspended Solid

UASB – Upflow Anaerobic Sludge Blanket

UCT – University of Cape Town

USEPA – United State Environmental Protection Agency

VFA – Volatile Fatty Acid

VIP – Virginia Initiative Plant

VSS – Volatile Suspended Solid

WAS – Waste Activated Sludge

WW – Wastewater

WWTP – Wastewater Treatment Plant

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I would like to dedicate this dissertation to my parents: Mr. Golap Ali Pramanic and Mrs. Hazera Khatun, in return of their life-long hard work and sacrifices throughout my life.

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CHAPTER 1

INTRODUCTION

This research focuses on two issues in enhanced biological phosphorus removal (EBPR). The first issue encompasses the effect of the type of carbon sources (i.e. volatile fatty acids and non volatile fatty acids) on the microbiology of EBPR. The second issue deals with the influence of denitrification on EBPR. Few studies have been performed to investigate these two issues. The recent findings on these areas are summarized below to establish the knowledge gaps this dissertation attempted to address. Details on the specific findings can be found in the literature review portion of this proposal.

1.1. Issue One: Effect of Carbon Source Type on the Microbiological Performance of EBPR

In the last decade, enhanced biological phosphorus removal (EBPR) has become a very popular method to remove phosphorus from wastewater. Its popularity relates to low cost, low sludge generation and simple operation compared to traditional chemical phosphorus removal processes. At present, newly designed wastewater treatment plants all over the world are implementing EBPR systems and older plants are being refurbished to add biological phosphorus removal. It is well established that short chain volatile fatty acids (VFAs) (e.g. acetate, propionate, etc) play an important role in EBPR systems (Tasli, et al., 1997; Wang, et al., 2002; Hollender et al 2002; Cokgor et al., 2004; Pijuan, et al., 2004b; Oehmen et al, 2004, 2005a and 2006; Chen and Gu 2006) and several

models for VFA uptake and accumulation into bacteria have been proposed (Comeau et al., 1986; Grady et al., 1999). In addition, several researchers have been performed experiment to identify the influence of another carbon source type, glucose on phosphorus removal from wastewaters (Carucci et al., 1999; Sudiana et al., 1999; Hollender et al, 2002; Wang et al., 2002; Pijuan et al., 2004b; Machado, 2004; Oehmen et al., 2006).

It is known that the amount of VFAs present has significant influence on biological phosphorus removed from wastewaters (Ekama et al, 1983; Pitman, 1991; Elefsiniotis and Oldham 1993, Metcalf and Eddy, 2003). The quantities of VFAs that can be produced in the sewer lines depend on many factors including the composition of the wastewater and temperature. In colder regions, VFA generation is limited (Daigger et al., 1991; Skalsky and Daigger, 1995; Ferreiro, N. and Soto, M., 2003) and many plants may not have sufficient VFA present to obtain the desired phosphorus removal. Plants that have to remove phosphorus to very low levels must increase the amount of low molecular weight carbon source present in the influent wastewater. This can be accomplished by (a) increasing solids retention time in primary clarifiers to foster partial fermentation, (b) fermenting primary sludge, and (c) adding commercially available volatile fatty acids to the plant's influent. Because the type of carbon source present affects the percent phosphorus removal in EBPR systems, it is important to evaluate how the addition of a particular carbon source will affect the system.

While the biochemistry of EBPR systems has been intensively studied in the last decade and models have been proposed to explain phosphorus uptake and release, much less is known about the microbiology of EBPR systems. To date, only a few bacteria

have been found to perform EBPR (Crocetti et al., 2000; Pijuan et al., 2004a; Oehmen et al., 2005a; Nakamura et. al., 1995a; Wong et al., 2005; Kong et al., 2005) and they are referred to as Polyphosphate Accumulating Organisms (PAOs). There are also bacteria that have been found to deteriorate the performance of EBPR systems and they are known as Glycogen Accumulating Organisms (GAOs). All the PAO bacteria identified to date were present in full-scale or in laboratory enriched EBPR sludge and they have been detected using DNA probes. To date, scientists have been incapable of isolating a pure culture of bacteria that can perform EBPR. Fuhs and Chen (1975) first identified an organism named *Acinetobacter* by culture-dependent method, and considered it as a PAO due to the uptake of phosphorus by using energy from the stored polyhydroxybutyrate. However, recent research using molecular tools demonstrated that *Acinetobacter* are unable to accumulate PHA with associated phosphorus release under anaerobic conditions. Therefore, *Acinetobacter* do not act as PAOs in the EBPR process (Loosdrecht et al., 1997; Bond et al., 1999b; Kortstee et al., 2000; Blackall et al., 2002).

Because of the need to add additional carbon sources to EBPR systems that do not contain sufficient VFAs and given the performance of EBPR vary with VFA type, it is important to evaluate the effects of individual carbon type on the microbiology of EBPR systems. To the best of my knowledge, Kong et al. (2002a), Machado (2004) and Pijuan et al., (2004a) were the first researchers to report on the influence of carbon type on the microbiology of EBPR systems. In the last few years, other researchers have also contributed to this field (Wong et al., 2004, Beer et al., 2004, Oehmen et al., 2005a & 2005c, Lu et al., 2006, Oehmen et al., 2006, Meyer et al., 2006, Burow et al., 2007).

Acetate has been used as a sole carbon source to observe the microbial communities in EBPR system in several studies (Cech and Hartman, 1993; Mino et al., 1998; Machado, 2004; Pijuan, 2004a; Wong et al., 2004, Beer et al., 2004 and Oehmen et al., 2006). Cech and Hartman (1993) were probably the first researcher who observed the presence of G-bacteria in a reactor fed with acetate. Later, these G-bacteria became known as GAOs (Mino et al., 1998). Recently, Machado (2004) observed that *Candidatus Accumulibacter Phosphatis* (i.e. CAP), a PAO, was predominant over *Candidatus Competibacter Phosphatis* (i.e. CCP), a GAO, in an acetate fed reactor that showed good phosphorus removal. In contrast, Oehmen et al., (2006) indicated that the abundance of Competibacter was responsible for poor removal of phosphorus in a reactor fed with acetate. Similarly, Wong et al., (2004) observed the failure of a laboratory-scale sequencing membrane bioreactor fed with acetate. They identified the presence (i.e. 85% of total cells) of a tetrad-forming alphaproteobacteria (TFOs) which were closely related to *Defluvicoccus* and acted as GAOs. In addition, Beer et al., (2004) observed poor OP removal capacity of an acetate fed reactor dominated with *Sphingomonas*, a tetrad forming alphaproteobacteria, which behaved like a GAO.

Propionate might be a better carbon source compared to acetate in an EBPR system (Pijuan et al., 2004a; Oehmen et al., 2006). Many studies found that lower phosphorus effluent concentrations are obtained when propionate is used compared to acetate (Pijuan et al., 2004a; Oehmen et al., 2006). Pijuan et al., (2004a) and Oehmen et al., (2005a, and 2006) found that the Accumulibacter, a PAO, dominated (8-69% of total bacteria) over Competibacter, a GAO, (<1% of total bacteria) when propionate was used as a carbon source. Oehmen et al., (2005c) also observed that Competibacter takes up propionate at a

very slow rate compared to that of Accumulibacter. Oehmen et al., (2006) found a novel group of alphaproteobacterial GAOs in the presence of propionate, and verified that Accumulibacter can out-compete the alphaproteobacterial GAOs. However, Meyer et al., (2006) found the abundance of *Defluvicoccus vanus* related alphaproteobacteria (upto 55% of all bacteria) when propionate was used to enrich PAOs and GAOs. They suggested that these bacteria might play a significant role in the deterioration of an EBPR system fed with propionate. On the other hand, when acetate was used to enrich PAOs, Pijuan (2004) found a low abundance of Accumulibacter and Competibacter (i.e. only a few cells). In contrast, Oehmen et al., (2005c) found the abundance of Accumulibacter (13% to 65%) and Competibacter (24%). Moreover, Oehmen et al., (2006) found that mostly Competibacter dominated over Accumulibacter in presence of acetate. Therefore, it can be concluded that propionate can remove phosphorus better than acetate as a carbon source.

Butyrate might not select for Accumulibacter and Competibacter in an EBPR system (Machado, 2004; Pijuan, 2004; Oehmen et al., 2004). In a reactor fed with butyrate for 32 days, Machado (2004) observed large variation in the number of Accumulibacter but the phosphorus removal was above 80%. After 32 days, both the number of PAOs and the phosphorus removal decreased dramatically. In addition, Competibacter also decreased throughout the study period of 58 days. Pijuan (2004) switched carbon sources from acetate and propionate to butyrate and found that butyrate and phosphorus uptakes were low as well as the phosphorus release. Similarly, Oehmen et al., (2004) and Kong et al., (2004) detected that Accumulibacter did not assimilate butyrate.

Glucose, a non-VFA carbon source, plays a role in the EBPR system (Tasli et al., 1997; Jeon and Park, 2000; Wang et al., 2002; Machado, 2004). The performance of EBPR process has been shown to deteriorate with glucose-rich influents. It is thought that the deterioration of EBPR when glucose is the carbon source is related to the presence of microorganisms that use glycogen instead of polyphosphate as an energy source (Tasli et al., 1997; Wang et al., 2002). In contrast, several researchers found good phosphorus removal in glucose fed reactors (Jeon and Park, 2000; Wang et al., 2002). Wang et al., (2002) modified the operating conditions to obtain EBPR immediately after adding glucose. They increased the anaerobic retention time from 2 to 2.5 hours, glucose concentration in the influent from 150 to 200 mg C/L, and decreased aerobic retention time from 4 to 3.5 hours with low DO values (i.e. 2-3 mg O_2/L). As a result, the effluent phosphorus concentration was less than 1 mg/L for 3 months. Jeon and Park (2000) operated a sequencing batch reactor (SBR) fed with glucose for 250 days and observed that about 70-80 days were required to obtain phosphorus removal efficiency of 100%. Initially, phosphorus release and uptake were low but with time it reached 100% (Jeon and Park, 2000). Similarly, Machado (2004) observed that phosphorus removal improved at the end of 58 days run in a SBR fed with glucose. He revealed the absence of Accumulibacter and low levels of Competibacter at the end of the run. Kong et al. (2004) performed batch tests using sludge collected from three full-scale plants and suggested that Accumulibacter could not uptake glucose directly under anaerobic conditions. Therefore, glucose might not select Accumulibacter (Machado, 2004 and Kong et al., 2004) and Competibacter (Machado, 2004) in an EBPR system. However, Nakamura et al., (1995a) observed that *Microlunatus Phosphovorus* can uptake glucose

and release phosphorus under anaerobic conditions with subsequent uptake of phosphorus under aerobic conditions. Thus, *Microlunatus Phosphovorus* might be a possible PAO in presence of glucose. Kong et al., (2001 and 2002a) identified the abundance of *Micropruina glycogenica* in a SBR fed with a mixture of acetate and glucose and showed no phosphorus removal. They confirmed by batch tests that *Micropruina glycogenica* could consume glucose and might be a possible GAO when glucose is used as a carbon source.

1.1.1. Knowledge Gap and Hypothesis

The results of previous research described above demonstrate that there are several gaps that have to be addressed in order to understand how carbon source types affect the microbiology of EBPR system. Presently, there are not sufficient data on the type of PAOs and GAOs that can be involved in EBPR systems in the presence of butyrate and glucose as sole carbon source. Thomas et. al., (2003) and Batista and Eleuterio, (2004) found that butyrate was the third most abundant VFAs generated in a fermenter. In addition, butyrate is commercially available for purchase.

In a reactor fed with glucose, Machado (2004) found that phosphorus removal started to improve at the end of the run, and this was not associated with an increase of the number of *Candidatus Accumulibacter Phosphatis.* He suggested that the targeted PAO in the glucose fed reactor might have been replaced by other types of PAOs (e.g. *Microlunatus Phosphovorus* etc.) not targeted by the probes used.

Therefore, in this research, the influence of butyrate and glucose on the microbiology of an EBPR system was investigated using PAOs and GAOs probes, some of which have not been used in reactors fed exclusively butyrate and glucose. My hypothesis is that

butyrate and glucose will select for specific PAOs and GAOs not yet reported in systems fed exclusively with these carbon sources.

1.2. Issue Two: Influence of Denitrification and Different Carbon Sources on EBPR

The other issue to be addressed in this research is the influence of denitrification and different carbon sources on EBPR. In wastewater treatment systems that include enhanced biological phosphorus removal (EBPR), denitrification and phosphorus (P) release can occur simultaneously when sufficient carbon source is available (Ghekiere et al., 1991; Chuang et al., 1996; Patel et al., 2006; Zou et al., 2006). However, even when sufficient carbon source is available, phosphorus release rates have been reported to be negatively affected by denitrification (Zou et al., 2006; Lee et al., 2006). In EBPR, nitrate can interfere with phosphorus removal through sludge return and via internal mixed liquor return (Figure 1). In the case of sludge return, nitrate-rich sludge is returned from the clarifier to the anoxic tank as part of standard operation (Figure 1). In the case of internal mixed liquor return, nitrate-rich mixed liquor is purposively applied to the anoxic zone from the aerobic zone to promote denitrification (Figure 1). In both cases, returned nitrate can be converted to nitrogen gas if carbon sources are available. Available carbon sources include mainly volatile fatty acids (VFAs) and both Ordinary Heterotrophic Organisms (OHOs) and the phosphorus accumulating organisms (PAOs) will compete for VFAs in the presence of nitrate (Yagci et al., 2003; Zou et al., 2006; Yuan and Oleszkiewicz, 2008). Yagci et al. (2003) found that the amount of phosphorus release decreased by 25 mg/L in presence of nitrate at a low mass COD/N ratio of 3.8. Similarly, Chuang et al., (1996) observed that the specific phosphorus release rate in 30 min ($SPRR_{30}$) was 42% lower under anoxic condition compared to that under anaerobic

condition at a COD/N mass ratio of 1.7. However, Zou et al., (2006) noticed that the $SPRR_{30}$ was 42% in presence of nitrate even with a high COD/N ratio of 20. Moreover, Patel and Nakhla (2006) reported that phosphorus release occurred only when nitrate concentration was less than 1 mg/L at a high COD/N ratio of 85 and 42 using propionic acid and butyric acid as a carbon source, respectively.

It has been reported in the literature that when sufficient carbon sources are not present, some fractions of PAOs will use nitrate, as an electron acceptor for phosphorus uptake, instead of oxygen, under anoxic condition (Kerrn-Jespersen and Henze, 1993; Sorm et al., 1996, Merzouki et al., 2001, Yagci et al., 2003 and Peng et al., 2006). Malnou et al. was the first researcher to report that nitrate could serve as an electron acceptor for phosphorus uptake (Barker and Dold, 1996). Later, Vlekke et al (1988) and Zou et al., (2006) noticed that more stored carbon (i.e. PHB) was utilized for phosphate uptake in presence of nitrate compared to that of oxygen. Several investigators (Kerrn-Jespersen and Henze, 1993; Sorm et al., 1996 and Yagci et al., 2003) reported that the rate of phosphate uptake was lower (i.e. 1.7 to 5.5 mg PO_4-P/gVSS.hr) under anoxic condition than under aerobic conditions (i.e. 3.7 to 6.7 mg PO_4-P/g VSS. hr). Therefore, phosphorus uptake that happens in the anoxic zone, at the expense of nitrate, is not as effective (Kerrn-Jespersen and Henze, 1993; Kuba et. al., 1993, Sorm et al., 1996, Yagci et al., 2003 and Zou et al., 2006; Yuan and Oleszkiewicz, 2008). As a result of returning nitrate-containing stream, the performance of EBPR deteriorates: a) due to the lack of sufficient VFAs for denitrification and phosphorus release and b) inefficient phosphorus uptake in the anoxic zone by PAOs that utilize nitrate instead of oxygen as an electron acceptor.

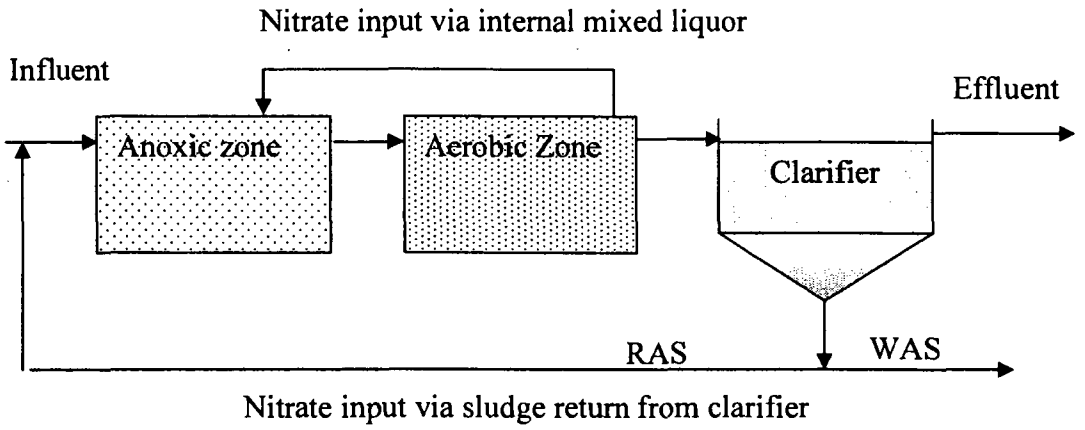


Figure 1.1: EBPR process with nitrate return in the anoxic zone

1.2.1. Knowledge Gap and Hypothesis

As previously outlined, numerous studies have been performed to evaluate the influence of denitrification on phosphorus release in the anoxic zone in the presence and in the absence of supplemental carbon source. Moreover, phosphorus uptake was investigated in the anoxic zone of EBPR using nitrate as an electron acceptor, and the results were also compared with the phosphorus uptake under the aerobic condition of EBPR in presence of oxygen as an electron acceptor. However, there is not enough evidence of the influence of denitrification, in the presence and in the absence of supplemental carbon source, on phosphorus uptake in the aerobic zone of EBPR systems. Therefore, in this research, investigations were performed using varying amounts of acetate and propionate to evaluate rates of phosphorus uptake under aerobic condition when denitrification is performed in the anoxic zone of EBPR systems. It is hypothesized that the addition of a carbon source will improve phosphorus uptake in the aerobic period when nitrate is introduced to the anoxic zone of the system.

1. 3. Objectives

The specific objectives of this research are:

1) To evaluate the microbial selection of GAOs and PAOs, using Fluorescence In-Situ Hybridization (FISH) analysis, in reactors fed exclusively with butyrate and glucose,

2) To investigate the influence of denitrification and various levels of acetate and propionate (i.e carbon sources) on the performance of EBPR systems.

CHAPTER 2

STATE OF KNOWLEDGE

2.1. Presence of Phosphorus and its Effects on Water Bodies

2.1.1. Presence of Phosphorus in Lakes, Rivers and Oceans

Phosphorus (P) concentration has increased in bodies of water due to the discharge from point and non-point sources. Point sources mainly include defective septic tanks, industry and wastewater treatment plant effluent. P loading of point sources depends on the population densities of humans, livestock and land use. Non-point phosphorus sources are surface runoff (Smith et al., 1999, McPherson et al., 2003), shallow sediments (Shaw and Prepas, 1990; Wetzel, 2001; Yiyong et al., 2004) and atmospheric deposition (Wetzel, 2001; McPherson et al., 2003). The different point and non-point sources of P are given in Table 2.1. The point and non-point discharge of phosphorus in lakes, rivers and oceans will be discussed in this section.

Table 2.1: Point and non-point sources recognized by statutes of the United States (Carpenters et al., 1998).

Sources	Components
Point	• Wastewater effluent (municipal and industrial) • Runoff and leachate from waste disposal sites • Runoff and infiltration from animal feedlots • Runoff from mines, oil fields, unsewered industrial sites • Storm sewer outfalls from cities with a population > 100000 • Overflows of combined storm and sanitary sewers • Runoff from construction sites > 2 ha
Non point	• Runoff from agriculture including return flow from irrigated agriculture • Runoff from pasture and range • Urban runoff from unsewered and sewered areas with a population <100,000 • Septic tank leachate and runoff from failed septic systems • Runoff from construction sites < 2 ha • Runoff from abandoned mines • Atmospheric deposition over a water surface • Activities on land that generate contaminants, such as logging, wetland conversion, construction, and development of land or waterways • Shallow sediments

Phosphorus in Lakes

Total phosphorus (TP) and dissolved inorganic phosphorus (DIP) (i.e. orthophosphate or soluble reactive phosphorus) are two important forms of phosphorus in fresh water lakes. Several lake analyses indicate that TP consists of mainly organic phosphorus, which contains almost 70%, or higher amounts of particulate organic fractions, and the rest is dissolved or colloidal organic phosphorus. The amount of DIP is only about 5% of total phosphorus (Wetzel, 2001). Total phosphorus concentration varies between 10 and 50 μg/L in most of the unpolluted lakes. However, the TP concentration can reach more

than 200 mg/L in some closed saline lakes. Normally, lakes, which are rich in organic matter or generated by the drainage of coastal areas such as in the southeastern United States, can contain high phosphorus (Wetzel, 2001).

Phosphorus can be supplied into lakes from point and non-point sources. Previously, point sources discharged a large amount of phosphorus into lakes. However, the phosphorus load from the point sources has been reduced due to the prohibition of phosphate use in detergents and the limit set on phosphorus concentration in treated effluent. As a result, non-point sources have become dominant sources of phosphorus loading in some lakes. Table 2.2 presents the phosphorus load from point and non-point sources in different lakes.

Table 2.2: Phosphorus load into lakes worldwide from point and non-point sources

Name of Lakes	Total phosphorus input from point sources (Kg/yr)	Total phosphorus input from non- point sources (Kg/yr)	References
Onondaga Lake, New York (1985 to 1990)	117,165 (70%)	35,405 (30%)	Heidtke et al., 1992
Hoedong lake, Korea peninsula	- (33%)	- (67%)	Choi and Koo, 1993
Western lake Michigan, USA	403,000 (1.2%)	30,597,000 (98.7%)	Robertson, 1996
Hillsdale lake, Kansas	5000 (7%)	65,000 (93%)	Juracek, 1998
Lake Mead, Nevada (1995 to 2000)	58,765 (70%)	24,820 (30%)	Du, 2002
Froyland lake, Norway	-	3200*	Bechmann et al., 2005

*Non-point sources (i.e. agricultural lands) were the main source of phosphorus and the value exceeded the acceptable limit of 2484 kg TP per year

Surface runoff is a main non – point source of phosphorus into lakes. Surface runoff includes a large amount of phosphorus from agricultural lands, domestic and industrial wastewater. The P concentration of surface runoff depends on P content in soil, topography, vegetation cover, land use, pollution, extent and amount of runoff (Wetzel, 2001). Atmospheric precipitation is another non point source of phosphorus loading in lakes. It was found that phosphorus concentration in rain and dry deposition varies from about 0.01 to 0.65 g per m^2 per year, whereas the acceptable value of lakes is 0.07 g per m^2 per year. If phosphorus concentration is 0.13 g per m^2 per year or higher, the lakes with average depth of less than 5 m are subjected to an eutrophication problem (Wetzel, 2001). Shallow sediments might be a potential source of phosphorus in lakes in case of limited external sources (Shaw and Prepas, 1990; Wetzel, 2001; Yiyong et al., 2004). Phosphorus can be consumed more easily from suspended sediments compared to deposited sediments for the growth of plant. Sharpley et al., (1995) reported that phosphorus concentration of two shallow hypereutrophic lakes in Indiana was raised by discharge of phosphorus from deposited sediment under anoxic conditions. Later, Sharpley et al., (1995) analyzed several lakes (Table 2.3) to identify particulate phosphorus (PP) in suspended and deposited sediments.

Table 2.3: Amount of PP transported in several lake tributaries and deposited in lake sediments (Sharpley et al., 1995)

Type of sediments	Location	Total P (g per Kg)	References
Suspended Sediment in tributaries	Lake Erie	0.6-1.5	Logan et. al., 1979
	Great Lakes	0.5-1.4	De Pinto et .al., 1981
	Indiana	0.2-0.7	Dorich et. al., 1985
	Amazon R.	0.4-1.1	Engle & Sarnelle, 1990
Deposited sediments	Wisconsin	0.6-3.9	Sagher et al., 1975
	Quebec	0.8-1.2	Carignan and Kalff, 1980
	Great Lakes	0.4-1.4	Williams et al., 1980
	Netherlands	0.4-4.8	Klapwijk et al., 1982

Phosphorus in Rivers

Point and non-point sources increase the phosphorus concentration in rivers. Caraco, (1995) analyzed 32 large rivers worldwide and found that point sources were the most significant contributor of soluble reactive phosphorus to rivers. Non-point fertilizer sources were also dominant in many systems. Normally, the rivers in the US receive phosphorus mostly from non –point sources due to ban of phosphate use in detergents and set a limit of phosphorus in the effluent from the wastewater treatment plants. McPherson et al., (2003) observed that total phosphorus concentrations were in the range of 0.01 to 2.22 mg P/L at all tested sites in the Mobile River Basin which include parts of Alabama, Georgia, Mississippi, and Tennessee. It was found that 42% of total phosphorus concentrations at all sites was higher than the USEPA limit of 0.1 mg/L set to avoid the growth of algae in the streams. This basin includes two streams draining the agricultural areas, two urban streams, and five large rivers with mixed land use. Parry,

(1998) reported that agricultural land has a great influence on the water quality problems in the rivers in USA and affected 60 % of the polluted rivers in miles.

Phosphorus in Oceans

Rivers are the major P contributors to the Oceans (Howarth et al., 1995). A large amount of P coming from rivers is in inorganic particulate form (Nixon, et al., 1996) and probably plays a significant role in regulating ocean's algal growth (Howarth et al., 1995). Howarth et al., (1995) reported the estimated phosphorus flux to the world's oceans (Table 2.4). In the table, natural values were calculated from relatively unperturbed rivers (i.e. before advent of agriculture) based on sediments records and modern values were determined from the rivers affected by human activities. According to Howarth et al., (1995), the P flux has increased from 8 x 106 Mg/yr to 22 x 106 Mg/yr by human activities (i.e. landscape and human wastewater). They also considered that atmosphere is another major source contributing P of 0.95 Tg per year. Therefore, TP input is 23 Tg per year to the oceans from rivers and the atmosphere.

Table 2.4: Amount of riverine input of P to the world's oceans at present (Modern) and before the beginning of agriculture (natural) (Howarth et al., 1995)

Types	Particulate P (Tg per year)	Dissolved P (Tg per year)	Total (Tg per year)	References
Modern	-	2	2	Meybeck, 1982
	-	-	24-38.5	Froelich et al., 1982
	30	2	32	GESAMP, 1987
	20	2	22	Howarth et al., 1995
Natural	20	1	21	Meybeck, 1982
	-	-	10	Froelich et al., 1982
	7	1	8	Howarth et al., 1995

2.1.2. Importance of Phosphorus Removal

Phosphorus is one of the limiting nutrients to control eutrophication in natural ecosystems (Du, 2002). Eutrophication is a process of excessive algal growth formation in water bodies because of excessive nutrients. It has been reported that algae has a fixed atomic composition (i.e. Carbon (C): Nitrogen (N): Phosphorus (P) = 106:16:1) known as the Redfield ratio under the proper growth conditions (Du, 2002). In principle, the limiting nutrient of water bodies can be estimated based on the Redfield ratio (Grobbelaar and House, 1995). If the N: P ratio is higher than the Redfield ratio in natural ecosystems, P is assumed to be the limiting nutrient for eutrophication (Du, 2002). However, eutrophication in most freshwaters such as lakes, reservoirs and streams has

been found to be P limited (Grobbelaar and House, 1995; Sharpley, et al., 1999; Du, 2002) due to least abundance of P among major nutrients needed for algal growth (Wetzel, 2001). The other nutrients such as N and C can easily transfer between the atmosphere and water. Besides, fixation of atmospheric N can be performed by some blue-green algae and is difficult to control in freshwaters (Sharpley, et al., 1999). Sometimes, in the coastal estuaries, the leaching of nitrate-N increases the N concentration resulting in P as a limiting nutrient (Howarth et al., 1995; Carpenter et al., 1998; Sharpley et al., 1999). Therefore, P input in the surface waters should be controlled for the reduction of eutrophication, the most widespread water pollution problem in the US and internationally (Carpenter et al., 1998). Eutrophication causes excessive algal growth, known as algal bloom, which has various detrimental effects:

Firstly, algal blooms deplete oxygen in water bodies causing the death of fish and loss of other aquatic habitats (Hallegraeff, 1993; Carpenter et al., 1998; Correll, 1998; Karim et al., 2002; Du, 2002). Oxygen depletion occurs due to high respiration of algae at night or in dim light during the day and most commonly bacterial respiration during decay of the bloom (Hallegraeff, 1993). Fish experience discomfort when DO is below 4 mg/l and transfer to a different area. The fish begin to die and shellfish will start to shut down when DO is below 3 mg/l. When DO is less than 30% saturation (i.e. about 2.0 mg/l in seawater during summer time) for one to four days, most biota will die, especially during summer months due to high metabolic rates (Karim et al., 2002).

Secondly, Algal blooms impair the drinking water quality by depleting oxygen, producing bad smells and tastes in the water due to the secretions of blue green algae (Du, 2002; Schreiter et al., 2001). In addition, it produces dissolved organic compounds

(DOCs), which have the tendency to form harmful disinfection by–products (DBPs). Moreover, the presence of DOCs after treatment can cause the growth of bacteria in the distribution system. Normally, DOCs are troublesome to eliminate from the water and require high coagulant doses depending on the solution pH and algal source (Widrig et al., 1996). As a result, the treatment cost of the drinking water supplies increases due to the presence of algal bloom (Du, 2002).

Thirdly, algal blooms have detrimental effects on tourism and the recreational use of water resulting in an economic loss (Lagos, 1998). For tourism and recreational purposes, good quality water (i.e. transparent, smell free and seawater color) is desirable. Algal blooms can severely discolor the seawater, known as 'red-tides,' and sometimes cause allergic reactions limiting tourism and recreational use of coastal areas (Zingone and Enevoldsen, 2000).

Fourthly, the algal (i.e. cyanobacterial) toxins are harmful for humans and animals. The toxins can be exposed to human and animals through drinking water supplies, recreational activities, and the food chain (Falconer, 1999; Fleming and Stephen, 2001). Humans can also be exposed to toxins through dialysis (Fleming and Stephen, 2001). The toxins including hepatotoxins, cytotoxins, and neurotoxins are difficult to remove from the drinking water and can easily enter the human body causing vomiting, blistering of the lips, sore throats, painful liver enlargement, constipation, bloody diarrhea, kidney damage, presence of blood in the urine, and dehydration depending on the type of toxins and concentration (Falconer, 1999). The toxins have also been recognized as a tumor promoting compound for humans and animals. It has been reported that animals are subjected to toxins in all continents except Antartica (Fleming and Stephen, 2001). If the

contaminated water comes in contact with human through bathing and other recreational purposes, it can cause eye and skin irritation, and also blistering all over the human body. A terrible incident occurred in Caruaru, Brazil because of the use of water containing cyanobacterial toxins (Falconer, 1999). It was reported that about 100 patients suffered from kidney dialysis showing visual problems, vomiting and nausea, and 50 of them died due to liver failure (Fleming and Stephen, 2001).

Finally, algal blooms cause fish and shellfish poisoning (Hallegraeff, 1993; Carpenter, et al., 1998; Lagos, 1998). These cause illness in humans and are sometimes responsible for the death of marine mammals (Carpenter, et al., 1998). Recently, six different syndromes related to shellfish poisoning were identified in the human body. The syndromes are called ciguatera, paralytic shellfish, neurotoxic shellfish, diarrhetic shellfish, Pfiesteria and amnesic shellfish poisoning. The human consumed the shellfishes, which were poisoned by micro algal toxins (Morris, 1999; Zingone and Enevoldsen, 2000) and suffered from neurological damage (Carpenter, et al., 1998).

Due to the above-mentioned problems, phosphorus concentration should be low in the water bodies. According to the U.S. Environmental Protection Agency, phosphorus concentrations should be 0.10 μg /l in the marine and estuarine water. In addition, the National Pollutant Discharge Elimination System (NPDES) limited the total phosphorus to under 0.1 mg/L in the municipal effluent or more than 99% phosphorus removal (Jiang et al, 2004).

2.2. Methods and Mechanisms of Phosphorus Removal

2.2.1. History of Enhanced Biological Phosphorus Removal (EBPR) System

In India, Srinath et al., (1959) first observed significant phosphorus removal in an aerated activated sludge tank. They found by batch tests that the rate of phosphorus removal was dependent on the rate of oxidation and concentration of activated sludge. Later, Alarcon (1961) also observed excess phosphorus removal in some plants in America. He observed by batch tests that almost complete removal of phosphorus was possible with sufficient aeration. However, several researchers (i.e. Randall et al., 1970, Marais et al., 1983 and Meganck and Faup, 1988) reported that phosphorus could be released due to aeration for a longer period.

In the mid 1960s, there was controversy about whether phosphorus removal was a chemical or biological process (Meganck and Faup, 1988). It was hypothesized that phosphorus might be precipitated as calcium hydroxyapatite due to high pH, which was a result of aeration and carbon dioxide stripping (Filho, 2001). However, Levin and Shapiro (1965) extensively explored the mechanism of phosphorus uptake and first proved that phosphorus removal was a biological process. The phosphate uptake occurred under aerobic conditions via ATP generation during oxidative phosphorylation and hampered due to the obstruction of oxidative phosphorylation by 2, 4-dichlorophenoxyacetic acid. Levin and Shapiro (1965), Shapiro (1967) and Randall et al., (1970) also proved that sludge microorganisms could uptake phosphorus in excess of their need for growth under aerobic conditions termed as "Luxury uptake." Besides, Fuhs and Chen (1975) first identified an organism named Acinetobacter that could store

polyhydroxybutyrate providing energy for phosphorus uptake in the presence of acetate. Later, Marais et al., (1983) also reported the biological nature of phosphorus uptake.

Phosphorus release was observed in some high-load conventional activated sludge plants in the U.S. It was thought that this release was due to about zero dissolved oxygen concentration in the inlet zone of aeration tanks involving plug-flow patterns (Marais, et al., 1983; Meganck and Faup, 1988). However, Shapiro (1967) first observed that phosphorus release was mostly affected by redox potential. Phosphorus was released rapidly when redox potential reached a value of 150 mv. In contrast, Randall et al., (1970) concluded by batch tests that phosphorus release was independent of redox potential. The release occurred instantly with a zero level of dissolved oxygen whereas redox potential declined after 40 to 60 minutes. Barnard (1974) first recommended a separate anaerobic zone for biological phosphorus removal. In contrast, Fuhs and Chen (1975) established that the anaerobic phase itself was not important for phosphate release instead low pH and addition of carbon source were more helpful. Finally, Barnard (1976) concluded that all plants removing excess phosphorus should have an anaerobic zone to release phosphorus in the form of orthophosphates with a minimum level of oxidation – reduction potential.

Until 1980s, different configurations of EBPR systems were developed. The most commonly used systems are A/O (Anaerobic/ Aerobic), A^2/O (Anaerobic/Anoxic/Aerobic), UCT (University of Cape Town), modified UCT, VIP (Virginia Initiative Plant), Phostrip and 5-stage Bardenpho processes. The similarities and differences of these systems are discussed below:

- A/O and Phostrip are the two processes used only for the removal of phosphorus. Phoredox is a biological removal process whereas Phostrip combines chemical and biological processes to remove phosphorus. A/O (Figure 2.1) is a mainstream process because anaerobic zone is placed in the main process stream. Phostrip (Figure 2.7) is a sidestream process because the anaerobic zone is located in a sidestream bioreactor. About 30 to 40% of the return activated sludge (RAS) is taken into the sidestream bioreactor for anaerobic treatment before returning to the aeration zone (Grady et al., 1999; Filho, 2001; Metcalf & Eddy, 2003).
- A^2/O, UCT, modified UCT, VIP and 5-stage Bardenpho processes remove both phosphorus and nitrogen. Since nitrification occurs in these processes, the RAS can supply nitrate-N to the anaerobic zone, which negatively affects the phosphorus removal process. To minimize this problem, the RAS is applied to the anoxic zone instead of anaerobic zone in the UCT, modified UCT, VIP processes. However, the RAS is directly applied to the anaerobic zone of A^2/O and 5-stage Bardenpho processes which were developed earlier.
- The 5-stage Bardenpho process (Figure 2.3) has a secondary anoxic zone, which is ineffective for denitrification purposes. However, A^2/O, UCT, modified UCT, VIP processes do not include the secondary anoxic zone.
- The basic design of A^2/O, UCT, modified UCT, VIP processes are similar with exception in their internal recycling of mixed liquor, and the divisions of different zones. In A^2/O (Figure 2.2) and UCT processes (Figure 2.4), there are one anaerobic, one anoxic and one aerobic zone. The mixed liquor is recycled from aerobic to anoxic internally in both processes for efficient nitrate removal.

Additionally, another recycle of mixed liquor is directed from the anoxic to the anaerobic zone in the UCT process to minimize the presence of nitrate described earlier. In the modified UCT process (Figure 2.5), the anoxic zone has two compartments in order to avoid poor settleability of the sludge when the TKN/COD ratio was greater than 0.11(Ekama et al., 1984). In the VIP process (Figure 2.6), all the zones consist of at least two completely mixed cells in series with different recycle systems (Grady et al., 1999; Metcalf & Eddy, 2003).

- The anaerobic retention time is from 0.5 to 1.5 hrs for A/O, A^2/O, and 5-stage Bardenpho processes, whereas UCT and VIP processes need 1 to 2 hrs. Besides, Phostrip process requires highest value of 8 to 12 hrs (Metcalf & Eddy, 2003).
- The aerobic retention time is lowest (i.e. 1 to 3 hrs) for A/O process whereas all other processes need minimum 4 hrs (Metcalf & Eddy, 2003).
- The required mixed liquid suspended solids are from 3000 to 4000 mg/L for A/O, A^2/O, UCT and 5-stage Bardenpho processes whereas VIP and Phostrip processes need 2000 to 4000 mg/L and 1000 to 3000 mg/L, respectively (Metcalf & Eddy, 2003).

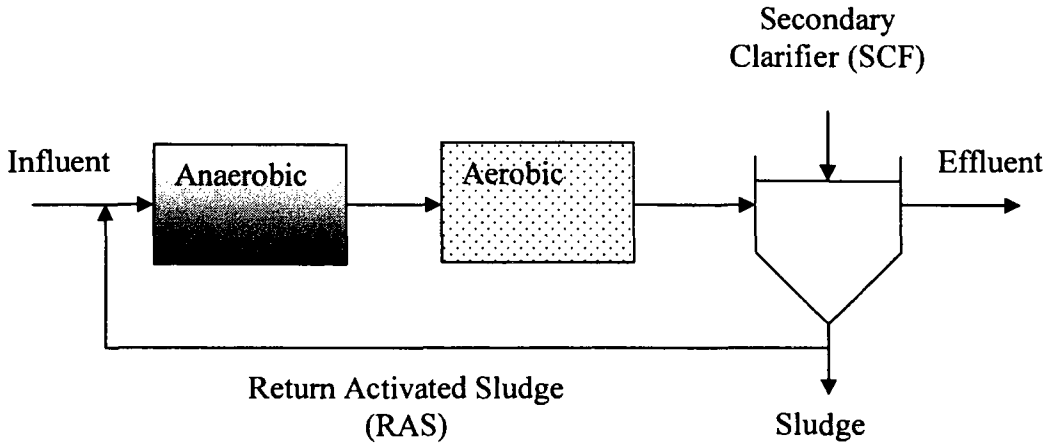


Figure 2.1 - A/O process (Modified from Metcalf & Eddy, 2003)

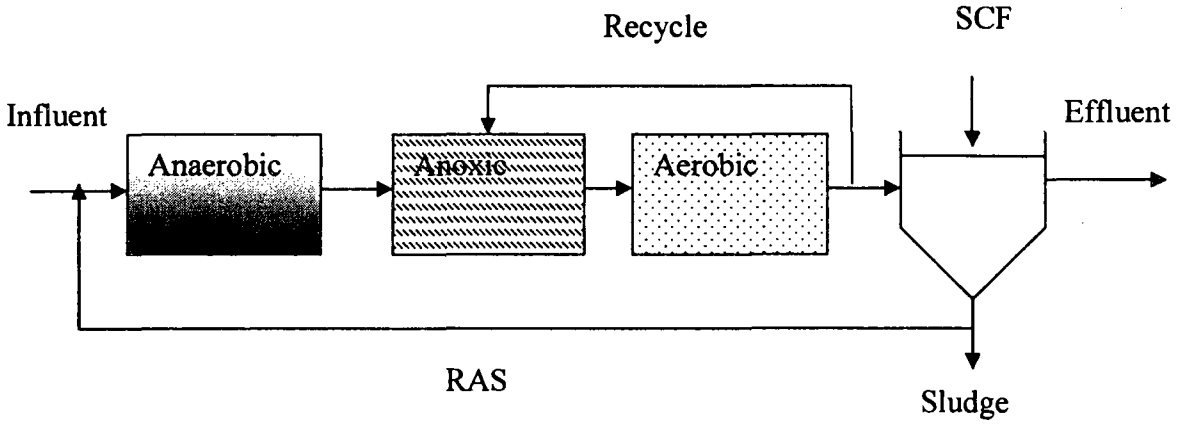


Figure 2.2: A^2/O process (Modified from Metcalf & Eddy, 2003)

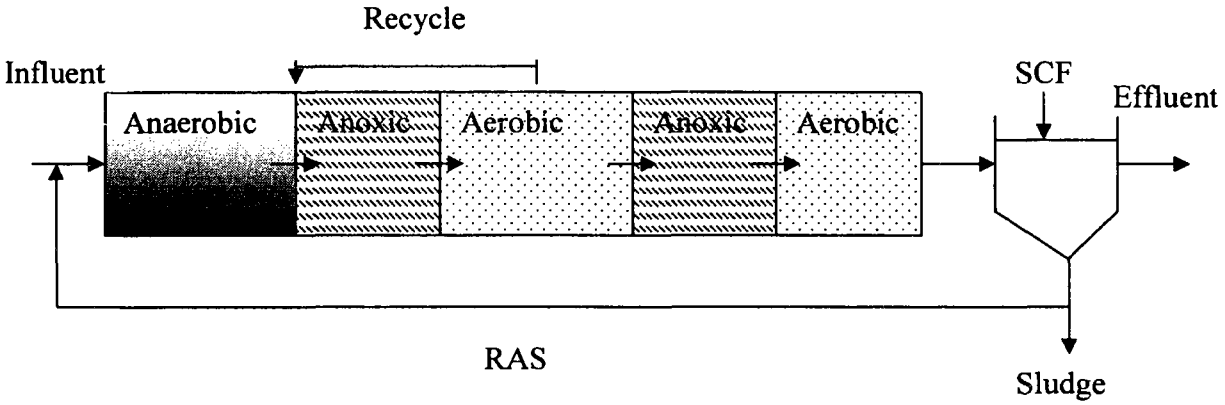


Figure 2.3: 5-Stage Bardenpho process (Modified from Metcalf & Eddy, 2003)

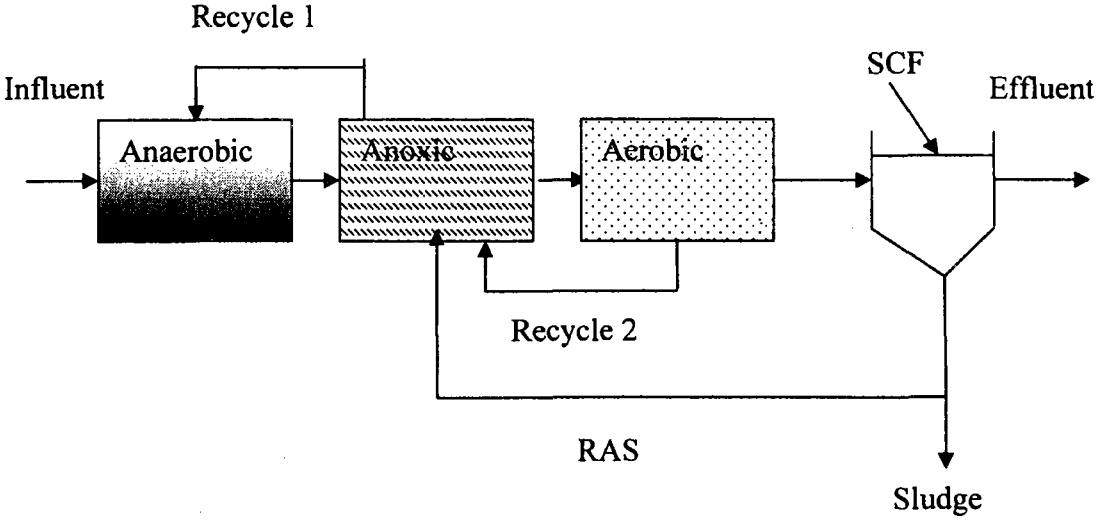


Figure 2.4: UCT process (Modified from Metcalf & Eddy, 2003)

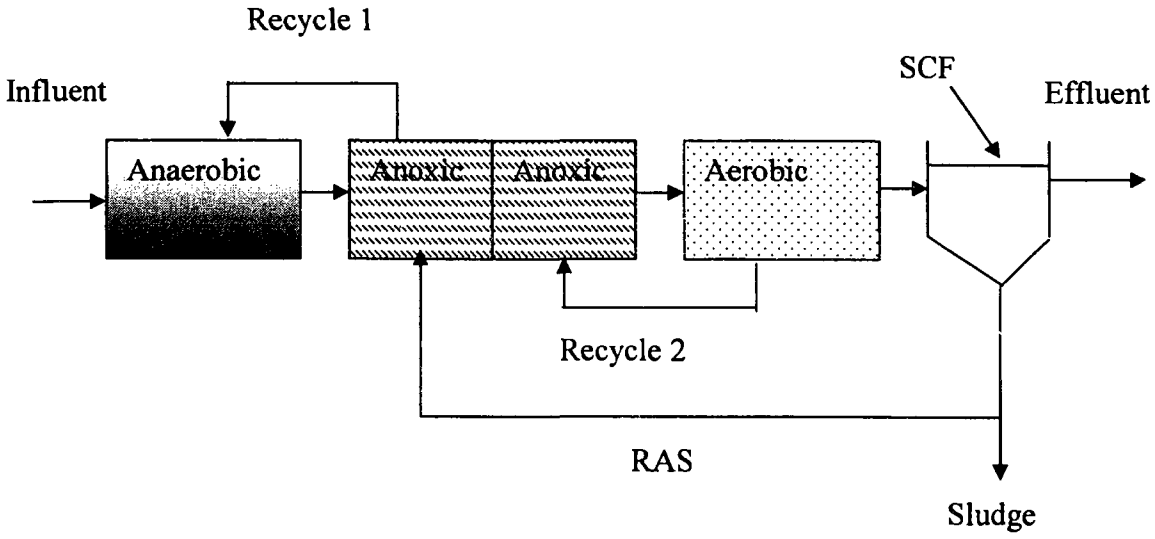


Figure 2.5: Modified UCT process (Modified from Metcalf & Eddy, 2003)

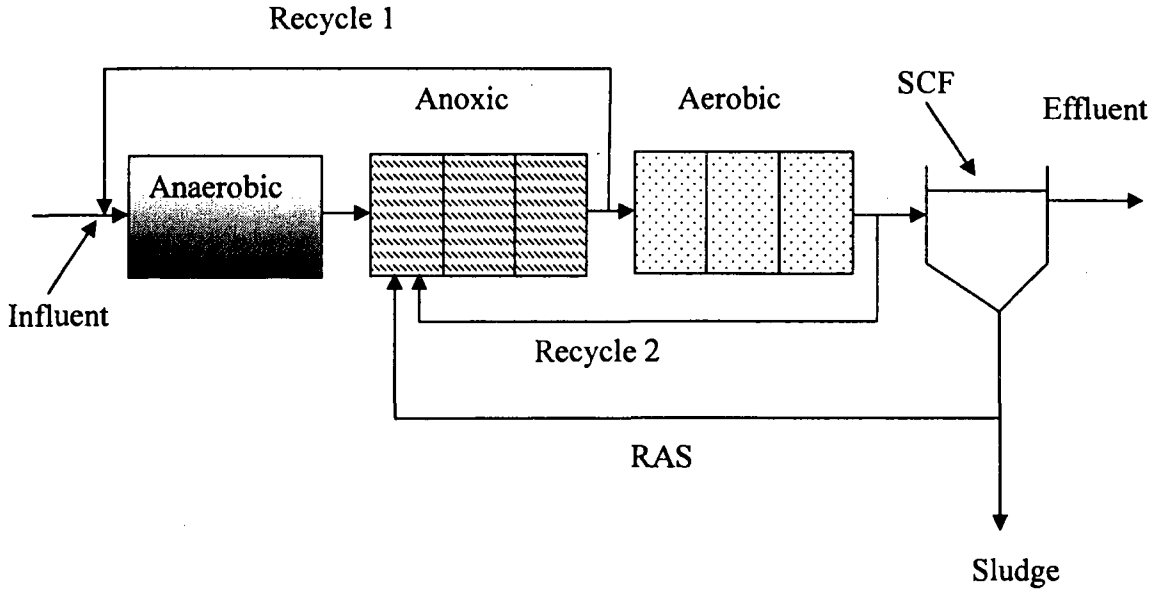


Figure 2.6: VIP process (Modified from Metcalf & Eddy, 2003)

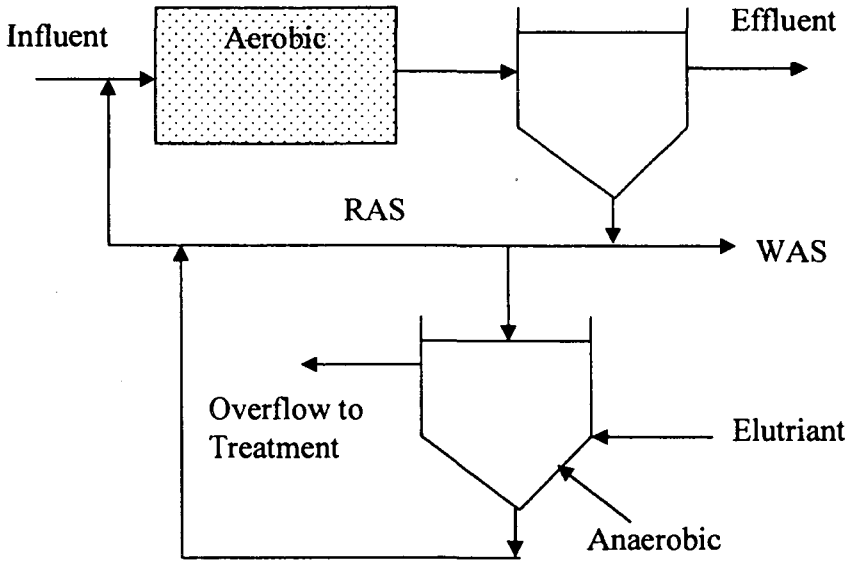


Figure 2.7: Phostrip process (Modified from Grady et al., 1999)

2.2.2. Requirement of Volatile Fatty Acids (VFAs) in the EBPR System

The presence of VFAs is important in the anaerobic zone of an EBPR system. Polyphosphate Accumulating Organisms (PAOs) store the VFAs as polyhydroxyalkanoates (PHAs) using energy from the hydrolysis of polyphosphate available as ATP in their cells. These PHAs supplied energy to uptake phosphate in the subsequent aerobic zone (Filho, 2001). Figure 2.8 shows the function of VFAs for the removal of phosphorus in the EBPR system. The VFAs are generated from the readily biodegradable organic carbon. The requirement of readily biodegradable organic carbon in the EBPR system can be expressed as chemical oxygen demand (COD) and biochemical oxygen demand (BOD). Marais and Ekama, (1976) reported that the COD value might be 2 times higher than the BOD_5 value for the municipal settled sewage. The

biodegradable COD might be 1.8 to 1.9 times higher than the BOD_5 value due to the presence of about 5 to 10 % unbiodegradable COD in the influent.

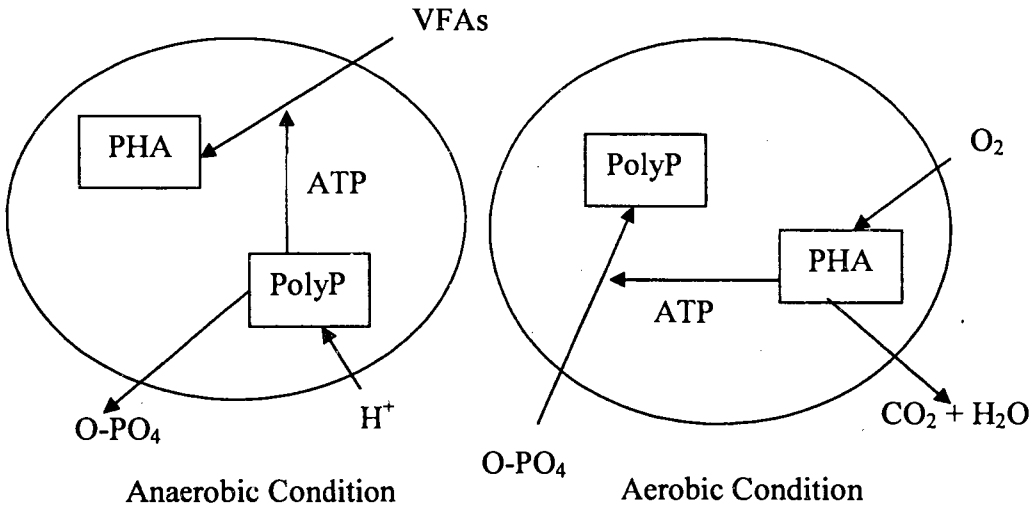


Figure 2.8: Role of VFAs in the EBPR process

(Modified from Seviour et al., 2003)

In the 1960s, it was thought that the presence of a carbon source might enhance phosphorus uptake (Levin and Shapiro, 1965). Randall et al., (1970) found through batch tests that the average mass ratio of initial COD to phosphate uptake was 48. The COD value was considered as a carbon source for normal metabolic uptake of phosphorus (Osborn and Nicholls, 1978). Rabinowitz, (1985) reported that the carbon storage as PHB in the anaerobic zone was essential for the metabolic activities of aerobic organisms. Short chain VFAs (SCVFAs) were the preferred substrate to store as PHB. Later, investigations were performed to identify the requirements of SCVFAs for better performance of the EBPR systems.

Several researchers (i.e. Marais et al., 1983; Ekama et al, 1983; Ekama et al., 1984; Meganck and Faup, 1988) reported that the phosphorus release was initiated when readily biodegradable COD in the anaerobic reactor (S_{bsa}) was above 25 mg/l for the Phoredox and UCT processes. Therefore, the minimum values of the influent readily biodegradable COD strength (S_{bsi}) should be from 50 to 62.5 mg COD/L to achieve S_{bsa} higher than 25 mg COD /l (Ekama et al, 1983). Pitman (1991) recommended that S_{bsi} should be above 100 mg/l for the smooth removal of phosphorus based on more than ten years experience at the Johannesburg plants involving the Phoredox/Bardenpho processes. He also suggested that special care must be taken if the values are below 50 mg COD /l. In case of the absence of nitrates, Elefsiniotis and Oldham (1993) reported that the VFA in the anaerobic zone requires 25 to 30 mg/l of acetic acid to stimulate the release of phosphorus.

Different sources (Metcalf and Eddy, 2003; Henze 1996; Abughararah and Randall, 1991) suggested the amount of carbon sources to remove 1 mg of phosphorus. Abu-ghararah and Randall (1991) suggested by pilot plant studies involving the UCT process that a minimum of 20 mg COD, equivalent to acetic acid was required to remove 1 mg of phosphorus. Lie et al., (1997) confirmed similar results by observing full-scale plants using the same process. However, about 7 to 10 mg of acetate can remove about 1 mg of phosphorus in biological phosphorus removal systems (Metcalf and Eddy, 2003). Similarly, Henze (1996) recommended by analyses full scale processes that about 10 mg of soluble easily degradable COD might be necessary to remove 1 mg of soluble phosphorus. Not only the carbon sources but also the phosphorus (P) concentration of

the influent has an effect on the performance of the EBPR process. Therefore, the influent COD to P ratio is considered a key parameter for the EBPR process.

The influent COD to P ratio controls P removal in the EBPR process (Henze, 1996). The increased ratio of COD to P can reduce P concentrations in the effluent (Randall et al., 1992) and also can cause the growth of GAOs (Randall et al., 1992; Liu et al., 1997; Yagci et al., 2003). On the other hand, a low ratio of COD to P can allow to dominate PAOs in the system, but sometimes, can increase phosphorus concentration in the effluent (Randall et al., 1992). For example, Randall et al., (1992) found that the average effluent concentration of TP was below 1 mg/L at a mass ratio of total COD to total P greater than 40 in full-scale plants. If the ratio was decreased to 21, the effluent concentration of TP increased to 1.98 mg/L. More phosphorus was removed with the ratio of 21. Liu et al., (1997) observed incomplete P removal in a sequencing batch reactor (SBR) at a C/P mass ratio of 50 because PAOs do not have sufficient poly-P as an energy source to uptake acetate, while GAOs have glycogen as energy source to uptake acetate. Therefore, GAOs became dominant over PAOs in the system at a C/P mass ratio of 50. Complete P removal was achieved at a C/P mass ratio of 5. They indicated that PAOs can store large amount of polyP at a high mass C/P ratio which helps to uptake acetate faster and out competes the GAOs. However, P removal was incomplete after 2 months. A stable and compete P removal was observed at a C/P mass ratio of 10 for 5 months. However, Mulkerrins et al., (2004) proposed that the total COD to total P ratio should be greater than 40 to get the effluent phosphorus levels of less than 1 mg/L based on the findings of Randall et al. (1992).

Some researchers (i.e. Tetreault et al., 1986; Randall et al., 1992) measured the required carbon sources as total BOD_5 or soluble BOD_5 (i.e. 37 to 55% of total BOD_5). Soluble BOD_5 is a better indicator compared to total BOD_5 because of presenting the readily degradable organic substrates available in the influent (Tetreault et al., 1986). Tetreault et al., (1986) reported by analyses the full-scale A/O processes that the threshold value of the soluble BOD_5 to soluble P ratio should be 12 to 15 to get soluble P less than 1 mg/L in the effluent. In addition, the BOD_5 to total P ratio should be greater than 20 to obtain similar results (Tetreault et al., 1986; Mulkerrins et al., 2004). If the ratios are below the above values, the effluent soluble P might increase up to 4.5 mg/l (Tetreault et al., 1986). The COD and BOD_5 values to remove P suggested by different researchers have been summarized in Table 2.5. Moreover, the ratio of COD to P used in different lab- scale SBR to investigate the performance of the EBPR process has been illustrated in Table 2.6.

Table 2.5: Required values of COD, BOD_5, COD/P and BOD_5/P to remove phosphorus in the EBPR systems

Parameters	Amount	Type of Study	EBPR process	P-Removal	References
COD (mg/L)	20	Pilot	UCT	To remove 1 mg of phosphorus	Abu-ghararah and Randall, 1991
Soluble easily degradable COD (mg/L)	10	Full-scale	Bardenpho, Phoredox, UCT	To remove 1 mg of soluble phosphorus	Henze, 1996
COD (mg/L)	20		UCT	To remove 1 mg of phosphorus	Lie et al., 1997
	26 to 34		A/O without nitrification, VIP, UCT		Grady et al., 1999
	34 to 43		A/O and A^2/O with nitrification		
	>43		Bardenpho		
BOD_5 (mg/L)	15 to 20	Full-scale	A/O without nitrification, VIP, UCT		Grady et al., 1999
	20 to 25		A/O and A^2/O with nitrification		
	>25		Bardenpho		
Acetate (mg/L)	7 to 10	-	BPR systems		Metcalf and Eddy, 2003
Total BOD_5 to total phosphorus ratio	>20	Full-scale	A/O	Effluent phosphorus concentration less than 1 mg/L	Tetreault et al., 1986; Mulkerrin et al., 2004
Soluble BOD_5 to soluble phosphorus ratio	12 to 15	Full-scale	A/O	Effluent phosphorus concentration less than 1 mg/L	Tetreault et al., 1986
Total COD to total phosphorus ratio	>40	Full-scale	A/O, A^2/O, VIP	Effluent phosphorus concentration less than 1 mg/L	Mulkerrin et al., 2004

Table 2.6: The COD to P ratio used in different lab-scale SBRs

COD:P Ratio	Carbon Source	% of P Removal	PAOs and GAOs	References
16(mass)	acetate	99	80% *Rhodocyclus* like *β-proteobacteria*	McMahon et al., 2002
14(mass)		98		
10 (mass)	acetate	-	65% *Accumulibacter* 24% *Competibacter*	Oehmen et al., 2004
14	Propionate	Substantial P removal	50 to 55% *Accumulibacter*	Pijuan et al., 2004a
15(mass)	Propionate	100	63% *Accumulibacter*	Oehmen et al., 2005a
10(mass)	acetate	-	65% *Accumulibacter* 24% *Competibacter*	Oehmen et al., 2005c
15(mass)	Propionate	>98	51 to 69% *Accumulibacter* <1% *Competibacter*	Oehmen et al., 2006
15(mass)	acetate	Unstable P removal	3 to 64% *Accumulibacter* 33 to 70% *Competibacter*	
20(mass)	acetate	100	-	Yagci et al., 2006
11.4(mass)		91		
9(mass)		91		
7(mass)		86		
20(mass)	acetate and propionate	99.5	>75 % *Accumulibacter*	Lu et al., 2006
15(mass)			91.5 ± 1.3 % *Accumulibacter*	
14(mass)	Acetate and casamino acid	99	>80 % *Accumulibacter*	He et al., 2006
10	Acetate and Propionate	88	-	Liu et al., 2007

2.2.3. Source of Volatile Fatty Acids (VFAs)

The volatile fatty acids (VFAs) are important for biological phosphorus removal (Lotter and Pitman, 1992). The presence of short chain volatile fatty acids (SCVFAs) in the anaerobic zone stimulates phosphorus release, which is the prerequisite of phosphorus uptake in the subsequent aerobic zone. The amount of SCVFAs is quite limited in domestic wastewater. When the wastewater does not contain enough SCVFAs, they can be added from the external sources (i.e. butyric acid, methanol, acetic acid, sodium acetate) (Jones et al., 1987; Canziani et al., 1995; Thomas et al, 2003; Chanona et al., 2006) or generated in the system itself via the fermentation process (Lotter and Pitman, 1992; Canziani et al., 1995; Chanona et al., 2006; Zeng et al., 2006). Generally, fermentation is the preferred option to produce SCVFAs from biodegradable organic matters, because the addition of external VFAs increases operational cost of the wastewater treatment plant (Elefsiniotis and Oldham 1993; Llabres, et al., 1999; Thomas et al., 2003; Zeng et al., 2006). The sources of VFAs including raw sewage, fermentation and external carbon have been illustrated in this section.

VFA Present in the Raw Sewage

The amount of VFA in the raw sewage is highly dependent on climate. Normally, the VFA content is higher at warm places compared to cold places. For example, the VFA is usually below 5 mg/L of acetic acid in Canada (Elefsiniotis and Oldham, 1993) whereas the VFA is 30 mg COD/L in Noosa region of Brisbane, Australia. The COD values for low, medium and high strength domestic wastewater are 250, 430 and 800 mg/L, respectively in the USA (Metcalf and Eddy, 2003). The corresponding readily biodegradable COD values are 50, 86 and 160 mg/L based on the assumption that readily

biodegradable COD is about 20% of total COD (Ekama et al, 1983). The major VFA components in the raw sewage are acetic and propionic acid (Chen et al., 2002; Thomas et al., 2003). Table 2.7 illustrates the total COD and VFAs values normally available in the raw domestic wastewater throughout the world.

Table 2.7: Total COD and VFAs found in raw domestic wastewater

Name of Places	Total COD (mg/L)	VFAs as acetic or propionic acid (mg/L)	VFAs as COD (mg/L)	References
Neveh Shaanan, Israel	394	27.9 (acetic acid) and 5.7 (propionic acid)	35.45	Narkis et al., 1980
Vancouver, Canada	181	-	-	Rabinowitz et al., 1987
Sjolundaverket, Sweden	440	-	60	Lie and Welander, 1997
Oresendsverket, Sweden	330*	-	37.5*	Lie and Welander, 1997
Istanbul, Turkey	410	-	-	Orhon et al., 1997
Loganholme, Australia	-	40 ± 10	57 ± 14**	Munch and Greenfield, 1998
Influent of Clark County Sanitation District WWTP in Las Vegas, NV (6/4/99 to 6/18/99)	-	15.3 (Acetic acid) and 2 (propionic acid)	-	Becker, 2000
East Orange County, Florida	416	41.6 (Acetic acid) and 15.2 (propionic acid)	-	Chen et al., 2002
Barcelona, Spain (01/09/99 to 30/12/99)	-	-	5.6	Barajas et al., 2002
Atakoy in Istambul, Turkey	440	-	-	Dulekgurgen et. al., 2006
Influent in Carraixet WWTP in Valencia, Spain	396***	-	10.9***	Chanona, et al., 2006

Note: * Mean values of weeks 1 and 2; ** Calculated based on that VFA as HAc is about 70% of VFA as COD; *** Mean values of experiments 1, 2, and 3

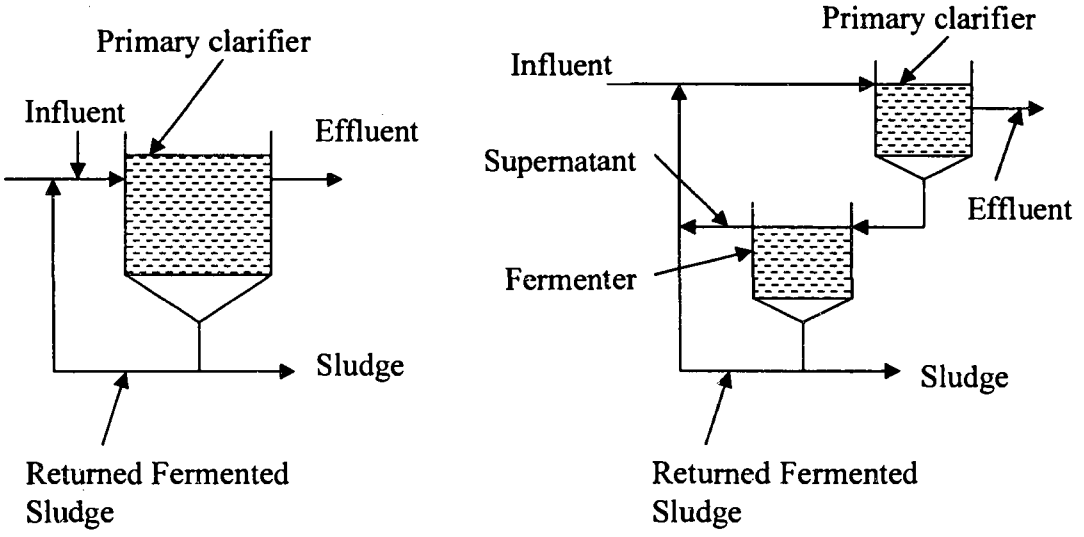
VFA Production by Fermentation

Fermentation (i.e. acidogenesis) is a process of forming SCVFAs from particulate material present in the wastewater. At first, particulate matter is transformed into soluble compounds, which produce simple monomers including fatty acids, amino acids and sugars through hydrolysis. These monomers are fermented and produce acetate, propionate, butyrate, hydrogen and CO_2. If the hydrogen concentration is low in the system (i.e. $H_2<10^{-4}$ atm), propionate and butyrate can be further fermented to acetate, hydrogen and CO_2 as the final products of fermentation (Metcalf and Eddy, 2003).

Fermentation is normally performed by installing prefermenters at the front end of a wastewater treatment plant. The prefermenters can produce SCVFAs independent of the influent carbon fluctuations (Mavinic et al., 2001). About 10 to 20 mg/l of excess VFA can be added to the influent wastewater by using pre-fermenters resulting in constant effluent phosphorus concentrations (Metcalf and Eddy, 2003). In addition, the prefermenter reduces the size of the anaerobic zone, and restricts secondary phosphorus release. In the mid 1970s in South Africa, Dr. James Barnard developed the prefermenters in association with the researchers of the University of Cape Town. Until now, prefermenters are not widely used in the USA (McCue, et al., 2003).

The prefermenters can be either on line (i.e. whole wastewater needs to be treated) or as a sidestream (i.e. the underflow from the primary clarifier is treated) option. The on-line prefermenter (Figure 2.9a) is a primary clarifier with a very high sludge blanket known as a Static Prefermenter (Barajas et al., 2002). On the other hand, the side stream prefermenter (Figure 2.9b) is a separate reactor, collecting only the underflow from the primary clarifier, followed by a thickener (McCue, et al., 2003). The solid retention time

(SRT) of the prefermenter is higher than that of primary clarifier (Barajas et al., 2002). The prefermenter is referred to as an Activated Primary Tank (APT) when the VFAs from the sludge blanket are recycled to the feed sludge in order to increase the production efficiency of the VFAs (Banister and Pretorius, 1998; McCue, et al., 2003). Chanona, et al., (2006) demonstrated that the VFA production improved due to the enlargement of the sludge blanket height and also by increasing the recirculation flow rates.



a. Online Fermenter b. Side Stream Fermenter

Figure 2.9: Fermenters used for VFAs production (Metcalf & Eddy, 2003)

The performance of the prefermenters has been reported to improve in recent years (Mavinic et al., 2001; Jiang et al., 2007). Mavinic et al., (2001) used ringlace fixed film media in the on-line prefermenters and found that the SCVFAs were 11 and 5.5 mg/L, as acetic acid from the screened raw wastewater and primary effluent, respectively with a hydraulic retention time (HRT) of 1 hour. The on-line fixed film prefermenter alone can

have the ability to remove 1-1.5 mg/L of phosphorus considering that the 6.5 mg SCVFA/L is required to remove 1 mg/L of phosphorus (Mavinic et al., 2001). The fixed film prefermenter is economic due to the requirement of low HRT (i.e. 1 hour) (Mavinic et al., 2001). Recently, Jiang et al., (2007) first an observed significant improvement of SCVFAs production using the waste activated sludge (WAS) with Sodium dodecylbenzene sulfonate (SDBS) used as a surfactant. The maximum amount of SCVFAs was 2599.1 mg COD/L with an optimum dosage of 0.02 g of SDBS per g of dry sludge at room temperature, whereas it was only 339.1 mg COD/L without the SDBS.

Various factors contribute to the performance of fermenters including SRT, temperature, solid content of primary sludge and pH. The SRT value should be selected properly to control methanogenic activity. Normally, the SRT values of 3 to 5 days are suitable depending on the temperature. If the SRT values are above 4 to 5 days, SCVFAs might be utilized in the methanogenic phase (Metcalf and Eddy, 2003). Elefsiniotis and Oldham (1993) demonstrated that the concentration of VFAs improved with the increase of SRT up to 10 days by using completely mixed reactor (CMR) including a clarifier and sludge recycle and an upflow anaerobic sludge blanket (UASB). They also indicated that the suitable pH for the production of propionic and butyric acid is from 4.3 to 4.6 and from 5.9 to 6.1, respectively. Engeler et al., (1998) established that the SRT values above 5 days had no beneficial effect on the fermentation of primary sludge at 20° C. Banister and Pretorius, (1998) and Jiang et al., (2007) also verified that the amount of VFAs did not increase significantly when the SRT values were higher than 6 days at room temperature. Zeng et al., (2006) illustrated that the production rate of VFAs improved if

the solids' concentration increased in the primary sludge. They also recommended that the optimum pH range should be from 6 to 7.

The VFAs, produced in the fermentation process, are acetate, propionate, butyrate and valeriate. Elefsiniotis and Oldham, (1993) observed the presence of iso-butyric, 3-methylbutyric, 2-methylbutyric and valeric in the fermenters. Engeler et al., (1998) found that acetate, propionate and n-butyrate are the main VFAs with percentages of 33%, 28% and 14% respectively, while iso-butyrate, methyl butyrate and n-valeriate constituted only 2 to 4% of dissolved COD. Thomas et al, (2003) demonstrated that the percentages of acetate, propionate, butyrate and valerate were 40%, 45%, 10% and 5%, respectively. Jiang et al., (2007) illustrated that the total SCVFAs consisted of 27.1 % acetic acid, 22.8% propionic acid, 20.1 % iso-valeric acid, 11.9 % iso-butyric acid, 10.4% n-butyric acid, and 7.7% n-valeric acid.

VFA Addition from External Sources

Limited information is available about the use of external carbon sources for biological phosphorus removal. Several researchers (i.e. Jones et al., 1987; Isaacs and Henze, 1995; Louzeiro et al., 2002; Thomas et al, 2003) investigated the effect of external carbon sources on the biological phosphorus removal system.

Jones et al., (1987) found by the lab-scale study that the descending order of magnitude of phosphorus release and uptake was butyric acid, ethanol, acetic acid, methanol and sodium acetate. The phosphorus release was higher with the increasing dosage of substrate addition. However, there was no significant difference in the ultimate removal of phosphorus. In addition, Jones et al., (1987) observed that the ultimate phosphorus removal only increased from 69 to 73% when the dosages of sodium acetate

were increased from 30 to 50 mg/L. Therefore, the optimum dosage of sodium acetate was considered as 30 mg/L.

Isaacs and Henze (1995) observed by the pilot test that acetate addition induced some phosphorus release. For example, the PO_4-P concentration increased by about 3 mg/L due to the addition of about 12 g COD/L as acetate. The overall phosphorus removal was improved during the experimental periods. However, they did not clearly indicate the effects of an external acetate addition for biological phosphorus removal.

Louzeiro et al., (2002) demonstrated, by analysis of a full scale SBR located at the Kent WWTP in Agassiz, BC, Canada, that the supplemental addition of methanol might not be utilized as a carbon source for the phosphorus release and uptake. Instead, phosphorus release started after denitrification of the nitrate by methanol. Therefore, methanol could facilitate the phosphorus removal process by removing nitrate from the system. Tam et al., (1992) also concluded that methanol had no significant impact on the biological phosphorus removal.

Thomas et al, (2003) studied the phosphorus removal performance in the Noosa WWTP by the supplemental addition of acetate and molasses due to low amount of VFAs present in the sewage (VFA-COD/P = 3.75). Acetate was supplied with or without fermentation and molasses were used with the fermenters. Poor phosphorus removal was observed when only acetate was added as an external carbon source. The acetate addition without the fermenter was costly and might favor the growth of GAOs in the system. In addition, Thomas et al, (2003) found that the use of molasses with the fermenters gave the best phosphorus removal and was the least costly.

2.2.4. Polyphosphate Accumulating Organisms (PAOs) in Lab-Scale EBPR Systems

In the 1970s, research on the microbiology of Enhanced Biological Phosphorus Removal (EBPR) system was started to identify the PAOs responsible for phosphorus removal (Loosdrecht et al., 1997). However, the PAOs involved have not been clearly identified to date. Much research is ongoing to identify the possible PAOs in the lab-scale and full-scale EBPR system. So far, the identified possible PAOs are *Acinetobacter* spp. (Fuhs and Chen, 1975), *Candidatus Accumulibacter phosphatis* (Kortstee et al., 2000; Blackall et al., 2002; Pijuan et al., 2004a; Oehmen et al., 2005a), *Microlunatus phosphovorus* (Nakamura et al., 1995a; Nakamura et al., 1995b; Kawahasaki, et al., 1999; Mino, 2000; Kortstee et al., 2000), *Lampropedia* (Stante et al., 1997; Mino, 2000), *Staphylococcus auricularis* (Choi and Yoo, 2000) and *Propionibacter pelophilus* (Crocetti et al, 2000).

Fuhs and Chen (1975) first suggested, by culture dependent methods, that bacteria of the genus *Acinetobacter*, which could store polyphosphate and polyhydroxybutyrate, were responsible for the removal of biological phosphate. *Acinetobacter* could only utilize the substrate and uptake excess phosphate under aerobic conditions. They were not able to prove the link between the anaerobic phosphate release and the growth of PAOs. Recent research using molecular tools demonstrated that *Acinetobacter* were unable to accumulate PHA with associated phosphorus release under anaerobic conditions. Therefore, *Acinetobacter* were not acting as PAOs in the EBPR process (Loosdrecht et al., 1997; Bond et al., 1999b; Kortstee et al., 2000; Blackall et al., 2002).

Candidatus Accumulibacter phosphatis played a major role as PAOs in the EBPR system (Kortstee et al., 2000; Blackall et al., 2002; Pijuan et al., 2004a; Oehmen et al., 2005a). The bacterium follows the typical characteristics of PAOs in the EBPR system. It can accumulate polyhydroxyalkanoates (PHA) and polyP under anaerobic and aerobic conditions, respectively. This bacterium was the first confirmed PAO among all other PAOs (Kortstee et al., 2000, Blackall et al., 2002). This bacterium could be found in abundance at a high ratio of phosphorus to carbon in the influent wastewater (Crocetti, et al., 2000). Later studies (Pijuan, et al., 2004a; Oehmen, et al., 2005a) showed that the *Candidatus Accumulibacter phosphatis* was the predominant PAO in the EBPR system fed with propionate. Machado (2004) also identified using FISH that this bacterium was dominant in the SBRs fed with acetate and propionate as a sole carbon source. He also observed this bacterium in the butyrate and valerate fed reactors while this was absent in glucose fed reactor.

Microlunatus phosphovorus has been identified recently as a PAO in the EBPR process (Nakamura et. al., 1995a; Nakamura, et al., 1995b; Kawaharasaki et al., 1999; Mino, 2000; Kortstee et al., 2000). *Microlunatus phosphovorus* strain NM-1 is a gram-positive, coccus-shaped, aerobic chemoorganotroph. The carbon sources of this organism are glucose, mannose, galactose, xylose, arabinose, saccharose, maltose, cellobiose, trehalose, melibiose, starch, sugar alcohol, pyruvate, casamino acids (Nakamura et al., 1995b, Mino, 2000). Nakamura et al. (1995a) found that the strain could release phosphate at a rate of 10 to 30 mg-P/g-cell. hr depending on the initial concentration of TOC under the anaerobic condition. Subsequently, it took up phosphate at a rate of about 10 to 20 mg-P/g-cell. h under an aerobic condition. In addition, Mino

(2000) observed that *Microlunatus phosphovorus* could uptake the carbon sources in anaerobic conditions by using polyP, which was stored in large amounts in aerobic conditions. However, it cannot uptake acetate nor produces PHA (Kortstee et al., 2000).

Lampropedia spp. has the key metabolic properties of PAOs (Mino, 2000). Pure culture of *Lampropedia* spp. was used to investigate whether this organism can remove phosphate from EBPR system. Experimental results indicated that gram-negative coccus shaped *Lampropedia* could uptake acetate with concomitant storage of PHB with an average yield of about 0.33 mg PHB per mg of HAc. The rate of PO_4-P release ranged from 1.7 to 3.6 mg PO_4-P per g of Volatile Suspended Solids (VSS) per hour with a pH of about 7.5. It was also found that the rate of phosphorus uptake under aerobic conditions was in the range of 0.25 to 0.54 mg PO_4-P per g of VSS per hour. Therefore, *Lampropedia* spp. is a PAO responsible for phosphorus removal in an EBPR system (Stante et al., 1997).

Choi and Yoo (2000) demonstrated that *Staphylococcus auricularis* acted as a PAO in the EBPR system. It could remove above 90% of phosphate from the influent phosphate concentration of 5 and 50 mg per liter. The phosphate removal was performed in a SBR, which was optimized for a cycle of 2h anaerobic and 4h aerobic conditions. Normally, a long time is needed to adapt the microbes for phosphorus removal, but *Staphylococcus auricularis* needed a short acclimatization period as it contained four times more phosphate (75 mg P per g of dry cell) compared to conventional activated sludge. Experimental results showed that this bacterium was releasing the phosphate under anaerobic conditions and removing the phosphate under aerobic conditions.

Propionibacter pelophilus, which is closely related to the group of β-proteobacteria, might be considered a possible PAO in the EBPR system (Crocetti et al, 2000). Crocetti et al, (2000) enriched PAOs in a SBR by increasing the phosphate concentration in the influent. They tried to find out the percentage of P content in the sludge and the corresponding cells of the PAOs. They noticed that there was a positive relationship between the percentage of P and the numbers of *Propionibacter pelophilus* cells in the sludge.

2.2.5. PAOs in Full-Scale EBPR Systems

Acinetobacter spp. was first identified as a PAO in EBPR process by Fuhs and Chen (1975). It was thought that *Acinetobacter* spp. was responsible for EBPR based on the culture-dependent identification methods. However, later it was decided, using the culture-independent methods (i.e. FISH, ubiquinone profiles), that they are probably not matched with model of PAOs for the removal of phosphorus in the EBPR systems. These bacteria cannot accumulate PHA by assimilating acetate and subsequent release of phosphorus by hydrolysis of polyP under anaerobic conditions (Mino, 2000; Mudaly et al., 2000; Tandoi, et. al., 1998; Loosdrecht, et al., 1997). Therefore, Acinetobacter spp. does not follow the pattern proposed for the typical PAOs. Nevertheless, some of these organisms (i.e. A. johnsonii 210A) can store polyP aerobically in the absence of extracellular energy sources (Tandoi et al., 1998, kortstee et al., 2000). Therefore, the presence of Acinetobacter in activated sludge can facilitate the removal of phosphate in the EBPR system (Tandoi et al., 1998).

Actinetobacterial PAO (APAO) is actively involved in the EBPR process for phosphorus removal from the wastewater. Recent investigations were performed to

identify the possible species of gram-positive APAOs in 10 full-scale EBPR plants using municipal and industrial wastewater. Clone library analysis and Microautoradiography together with Fluorescence in Situ Hybridization (MAR-FISH) were used to recognize the morphology and abundance of Actinobacteria in the EBPR system. The identified PAOs were closely related to the genus *Tetrasphaera* in the family of *Intrasporangiaceae* of the class *Actinobacteria.* Two different morphotypes of the APAO were identified and they were cocci growing in clusters of tetrad (targeted by probe Actino-221) and short rod (targeted by probe Actino-658). The coccus-APAO was closely related to *T. australiensis* and *T. japonica* and rod-PAO was very similar to *T. elongata.* The coccus-APAO was most abundant in industrial as well as domestic wastewater treatment plants (Kong et al., 2005).

The behavior of APAO was different from the typical PAOs. The APAOs consume certain amino acids such as Casamino acids anaerobically with subsequent release of phosphorus. They can uptake phosphorus and store it as polyP aerobically. However, APAO does not form PHA during uptake of organic substrate under anaerobic conditions. Still now, it is unclear how they store energy from anaerobic uptake of organic substrates to take up phosphorus aerobically. The results showed that about 3 to 35 % of all bacteria were APAO in all the investigated treatment plants (Kong et al., 2005 and 2006).

Another type of PAOs called *Rhodocyclus*-related PAOs (RPAO) is also found in full- scale EBPR wastewater treatment plants. They can efficiently consume organic substrates like acetate, propionate, pyruvate, some amino acids (i.e. glutamic acid) and other simple compounds under anaerobic conditions by using intracellular polyP. The

RPAO were mostly prominent about 9 to 17% in domestic plants and were rarely found (<3%) in the industrial plants (Kong et al., 2005 and 2006).

2.2.6. Effect of Glycogen Accumulating Organisms (GAOs) on the Performance of EBPR System

The presence of GAOs might cause the failure of EBPR systems due to the competition with PAOs for carbon sources (Cech and Hartman, 1993; Satoh et al., 1994; Bond et al., 1999b; Fang et al., 2002; Henze et al., 2002; Saunders et al., 2003; Kong et al, 2006; Burow et al., 2007). Normally, GAOs consume substrates by using intracellular glycogen as an energy source without releasing phosphorus and subsequently, store PHA under anaerobic conditions. The PHA is utilized to refill the glycogen without up taking phosphorus from the liquid under aerobic conditions (Crocetti et al., 2002; Saunders et al., 2003; Kong et al, 2006). Therefore, GAOs have no contribution to the removal of OP despite using the carbon sources. This section will present the results of laboratory and full-scale analysis regarding the OP removal performance of the EBPR process in the presence of GAOs.

2.2.6.1. Investigations of GAO's Abundance in Laboratory-Scale EBPR Systems

Several researchers (Bond et al., 1999a; Nielsen et al., 1999; Crocetti et al., 2002; Kong et al., 2002a; Beer et al., 2004; Wong et al., 2004; Oehmen et al., 2004 and 2006) revealed the dominance of GAOs in failed laboratory scale EBPR processes. They found diverse communities of GAOs, which might compete with PAOs and has been discussed in Table 2.8.

Table 2.8: Different types of GAOs found in the EBPR process

Carbon Sources	Types of GAOs	Abundance (%)	Shape and Size	References
Acetate	Novel group of γ-proteobacteria	35% of total population	Coccoid, 3 to 4 μm dia	Nielsen et. al., 1999
Acetate	β subclass of proteobacteria excluding β-1 and β-2	58% of total bacteria	Coccobacillus arranged in dense clusters, 2 μm dia	Bond et al., 1999a
Mixture of acetate and glucose	α proteobacteria γ- proteobacteria High G+C gram positive bacteria of the member of γ-proteobacteria	-21% of G-bacteria -10% of G-bacteria -30% of γ-proteobacteria	Coccoid in tetrad shape & cluster	Kong et al., 2002a
-	*Candidatus Competibacter Phosphatis*	92% in Q sludge* 28% in T sludge*	-	Crocetti et. al., 2002
Acetate	Most closely related to the *Sphingomonas* spp. of α-proteobacteria	71± 15 % of total cells	Cocci & tetrad forming bacteria, 1.2 to 1.5 μm dia	Beer et. al., 2004
Acetate	Closely related to *Defluvicoccus vanus* of α-1 proteobacteria	85± 7 % of total cells	Tetrad forming bacteria	Wong et al., 2004

*Q and T sludge had been collected from two deteriorated SBRs

It is interesting to note that Kong et al., (2002a) observed some of the α-proteobacteria assimilating glucose anaerobically. Beer et al., (2004) and Wong et al., (2004) proposed that the *Sphingomonas* and *Defluvicoccus* spp. could also be found in the full-scale EBPR process showing variable OP removal performance.

2.2.6.2. Investigations of GAO's Abundance in Full-Scale EBPR Systems

There is not sufficient information about the causes of failure in full-scale EBPR systems. Recently, Saunders et al., (2003) found that the presence of a large number of

competibacter and other possible GAOs augmented the requirement of carbon sources in full-scale EBPR plants. In addition, Kong et al., (2002b) reported that the members of γ-proteobacteria sometimes predominated (50% of total bacteria) in full-scale EBPR plants. However, Kong et al., (2002b) did not establish any correlation between the abundance of γ-proteobacteria and the performance of the EBPR system. Later studies of Kong et al, (2006) revealed that the γ-proteobacteria was GAOs which have very similar physiology like Rhodocyclus-related PAOs (RPAOs). The GAOs and RPAOs compete strongly with each other in full-scale EBPR plants as both are able to use organic substrates (i.e. acetate, propionate, and pyruvate). Furthermore, Burow et al., (2007) reported the presence of *Defluvicoccus* spp. as GAOs in full-scale EBPR plants. *Defluvicoccus* spp. could uptake acetate, propionate, pyruvate and glucose, and could not consume formate, butyrate, or ethanol under anaerobic and aerobic conditions. Sometimes, *Defluvicoccus* spp. was more abundant than the PAOs (Candidatus *Accumulibacter Phosphatis*) and other GAOs (*Candidatus Competibacter Phosphatis*) indicating competition.

2.2.7. Effect of Different Parameters on the Performance of EBPR System

In EBPR system, phosphorus release and uptake are important for better removal of phosphorus. The release of phosphorus is associated with the uptake of VFAs by PAOs from the wastewater and subsequent formation of PHA inside its cell under anaerobic conditions. The formation of PHA requires energy and reducing power, which are obtained from the hydrolysis of polyphosphate (poly-P) and glycogen, respectively. During the hydrolysis of poly-P, PAOs release phosphorus into the liquid. Under aerobic conditions, PAOs again uptake phosphorus to create poly-P and synthesize the glycogen

by utilizing stored PHA due to limitation of substrate (Smolders et al., 1995; Petersen et al., 1998; Chen et al., 2004).

Sometimes, the activity of PAOs is inhibited by the presence of GAOs, which compete with PAOs for the substrate. GAOs produce PHA by using substrate and required energy is collected from the degradation of glycogen. Therefore, GAOs do not take part in releasing of phosphorus and also subsequent uptake of it, causing deterioration of phosphorus removal in an EBPR system (Satoh et al., 1994; Filipe et al., 2001a; Zeng et al., 2002). The substrate uptake, PHA formation, degradation of glycogen, phosphorus release and uptake and also the presence of GAOs are all affected by different operational parameters (i.e. carbon sources, pH, temperature, nitrate/nitrite, solids retention time, dissolved oxygen, ions etc).

2.2.7.1. Carbon Source

Different carbon sources such as VFAs (i.e. acetate, propionate, butyrate and valerate etc.) and non-VFAs (i.e. glucose) are used in the EBPR system. These carbon sources have different types of responses on the EBPR system. More attention needs to be paid to understand the consequences of different carbon sources for the successful operation of the EBPR process (Pijuan et al., 2004b). Many researchers have analyzed the effects of different carbon sources on the performance of the EBPR process (Tasli et al., 1997; Jeon and Park, 2000; Wang et al., 2002; Hollender et al., 2002; Cokgor et al., 2004; Pijuan et al., 2004b; Oehmen et al, 2004, 2005a and 2006; Chen and Gu 2006). This section discusses phosphorus removal by using different carbon sources, comparison of consumption rates, glycogen degradation and formation for various carbon sources, PHA formation for different carbon sources, minimization of the growth of GAOs, effect of

carbon sources on mixed culture, glucose metabolism and conditions for improving EBPR process fed with glucose only.

Phosphorus Removal by Using Different Carbon Sources

Acetate gives best phosphorus removal efficiency when acetate, acetate/glucose and glucose are used as carbon sources (Hollender et al, 2002). Wang et al., (2002) also found similar results by using acetate and glucose separately. In contrast, propionate showed better results compared to acetate as a sole carbon source (Pijuan et al, 2004a; Oehmen et al., 2006). The microbiological analysis of the SBR fed with acetate indicates that the abundance of *Competibacter* causes high concentration of phosphorus in the effluent by using enriched culture of PAOs. In contrast, *Competibacter* are not detected in the propionate fed reactor while little amount of other GAOs may present. However, PAOs (*Accumulibacter*) out compete other GAOs in the propionate fed reactor. Similarly, the activity of PAOs dominant over GAOs in case of acetate added domestic wastewater (100 mg acetate COD/L and about 300 mg slowly biodegradable COD/L) compared to acetate (Cokgor et al., 2004).

Higher contents of propionic acid in the wastewater lead to long-term biological removal of soluble orthophosphate (SOP). Chen et al., (2004) indicated that the average efficiencies of SOP removal are 76.87% and 87.33% when the ratios of propionic to acetic acid are 0.16 and 2.06, respectively. This is due to higher SOP uptake capacity for the similar release of SOP, in case of higher ratio of propionic to acetic acid. In addition, the uptake of SOP per unit of PHAs is more effective for higher content of propionic acid. Chen et al., (2004) propose future research to identify the unquantified forms (i.e. 3H2MV) of PHAs when higher content of propionic acid is present.

The performance of the EBPR process deteriorates with glucose- rich influent. This is due to the dominance of microorganisms, which use glycogen instead of polyphosphate as the energy source (Tasli et al., 1997; Wang et al., 2002). In contrast, the better performance of phosphorus removal can be obtained in lab-scale SBR supplied with glucose as a sole carbon source (Carucci et al., 1999; Sudiana et al., 1999; Jeon and Park, 2000; Wang, et al., 2002). The consumption rate of glucose increases with the continuous operation of the SBR, although initial rate is slow. Due to the slow uptake of glucose, release and subsequent uptake of phosphorus also take time to improve. About 70-80 days is required to reach 100% efficiency of phosphorus removal (Jeon and Park, 2000).

Comparison of Consumption Rates

The comparison of consumption rates of different substrates has been investigated by different researchers (Lemos et al., 1998; Serafim et al 2002; Pijuan et al., 2004b). Lemos et al., (1998) show that the consumption rate was highest for acetate followed by propionate and butyrate when the carbon sources are used separately. Different results were obtained by Pijuan et al., (2004b), who found that the uptake rates for acetate and propionate are similar, and higher than those obtained by butyrate. However, propionate and butyrate maintain the similar uptake rates of single substrates while acetate uptake rate decreases significantly in a mixture of acetate, propionate, butyrate and glucose. Lemos et al., (1998) and Serafim et al., (2002) also report almost similar results that propionate is exhausted first followed by butyrate and lastly acetate when a mixture of acetate, propionate and butyrate is used. Chen and Gu (2006) indicate that the overall uptake rate of propionic acid is much faster than that of acetic acid in activated sludge,

which acclimated with a high propionic and acetic acid ratio. This may be due to the fact that propionic acid is metabolized more easily by PAOs than by other microorganisms (GAOs) (Pijuan et al., 2004b).

Glycogen Degradation and Formation for Various Carbon Sources

The glycogen degradation for substrates uptake depends on the types of carbon sources available in the anaerobic zone. The uptake of propionate needs less reducing power, resulting in less degradation of glycogen whereas the highest value is observed for the uptake of acetate (Pijuan et al., 2004b). However, production, instead of degradation, of glycogen is detected in case of glucose (Wang, et al., 2002; Pijuan et al., 2004b). Pijuan et al., (2004b) show that the ratio of glycogen degradation to substrate uptake is higher for individual substrates compared to a mixture of acetate, propionate, butyrate and glucose. This is because glucose in the mixed substrates acts as a source of reducing power resulting in less degradation of glycogen to store different VFAs as PHA.

PHA Formation for Different Carbon Sources

The formation of PHA is strongly influenced by different carbon sources. Acetate uptake leads to the highest formation of PHB (Lemos et al., 1998; Hollender et al., 2002; Pijuan et al., 2004b). Acetate also forms some amount of HV (Lemos et al., 1998). Propionate forms mainly PHV (Lemos et al., 1998; Pijuan et al., 2004b) and PH2HV (Pijuan et al., 2004b) while small amounts of PHB are also produced (Lemos et al., 1998). Butyrate produces a small amount of PHB and PHV with a higher amount of HB compared to HV (Lemos et al., 1998). Relatively lower amounts of PHA (i.e. PHB and PHV) are produced from glucose due to storage of glycogen (Pijuan et al., 2004b).

Acetate gives a higher yield of polymer per COD unit consumed leading to the best performance of an EBPR system (Lemos et al., 1998).

Minimization of the Growth of GAOs

The growth of GAOs in full-scale EBPR plants can be controlled by the change of carbon source from acetate to propionate. Oehmen et al, (2004 and 2005a) suggest that acetate enriched PAOs (i.e. *Accumulibacter*) are able to uptake propionate at a similar rate of acetate uptake. On the contrary, GAOs (i.e. *Competibacter*) enriched with the same conditions cannot uptake propionate effectively. Therefore, changing carbon source from acetate to propionate can provide a competitive advantage of PAOs over GAOs by minimizing the proliferation of GAOs in the EBPR system. When the carbon source is changed from acetate to propionate, an acclimation time is necessary for complete uptake of phosphorus due to initial slow oxidation rate of PHA. Therefore, further investigations are required to identify the appropriate acclimation period.

Effect of Carbon Sources on Mixed Culture

The effect of different carbon sources on the mixed culture of PAOs and GAOs has been identified by Zeng et al. (2003a) and Oehmen et al., (2006). Zeng et al., (2003a) found by using a mixed culture of PAOs (*Candidatus accumulibacter phosphatis*) and GAOs (*Candidatus Competibacter phosphatis*) that the amounts of acetate uptake are 0.68 and 0.32 mmol/L by PAOs and GAOs, respectively. In addition, the ratio of phosphorus release to acetate uptake for the mixed culture is 0.29, which is significantly lower than that of only PAO population (0.43). On the other hand, Oehmen et al., (2006) observed that *Accumulibacter* seem to dominate over alphaproteobacterial GAOs using propionate in a mixed culture of equal numbers of PAO and GAO. Further research is

needed to identify the diversity of novel GAOs in the mixed culture and their ability to compete with PAOs.

Glucose Metabolism

The metabolism of glucose is a two-stage process (Jeon and Park, 2000; Wang et al., 2002). At first, lactic acid (i.e. $C_3H_6O_3$) producing organisms (LPO) store glucose as a glycogen during anaerobic period. The required ATP necessary for this purpose is obtained from the formation of lactate by glycolysis of glucose. Then, polyphosphate accumulating organisms (PAOs) convert lactate to PHAs by the utilization of polyphosphate (Jeon and Park, 2000). In addition, Jeon et al., (2001) tried to investigate the metabolism of glucose together with acetate. They found that glucose and acetate were metabolized separately by using three organisms: acetate-using PAOs, lactate-producing organisms (LPOs), and lactate-using PAOs. Glucose is quickly transformed to lactic acid by LPOs so that acetate-using PAOs cannot use glucose as an energy source for synthesis of PHA from acetate. Finally, lactate- and acetate-using PAOs remove phosphorus independently by using lactate and acetate respectively.

Conditions for Improving EBPR Process Fed with Glucose Only

Three new operating procedures are required for successful operation of an EBPR system when glucose is present (Wang, et al., 2002): 1) larger reaction time in anaerobic period (from 2 to 2.5 h), 2) shorter reaction time in aerobic period (from 4 to 3.5h) with reduced in situ DO level (from 5-6 to 2-3 mg O_2/l) and 3) higher content of glucose in the influent (from 150 to 200 C-mg/l). The first procedure may induce an extended stressful situation for the discharge of PO_4-P from the bacteria. The second procedure may reduce the use of PHA during the aerobic period. Finally, the third procedure may stimulate

more PO_4-P release by metabolizing glucose and making available more organic carbon for PHA synthesis. Experimental results show that the effluent PO_4-P concentration becomes less than 1 mg/l by maintaining the three procedures.

2.2.7.2. pH

Intensive research has been performed to observe the impact of pH on the mechanisms under anaerobic and aerobic conditions and to find out optimum pH for better performance of an EBPR system. Some researchers focus mainly on the mechanism under anaerobic conditions, some give attention on both anaerobic and aerobic conditions and others emphasize the pH effect on the competition between PAOs and GAOs.

pH has significant effect on the metabolism of substrate and subsequent release of phosphorus under anaerobic conditions in an EBPR system (Smolders et al.,.1994; Cokgor et al., 2004; Liu et al., 1996a; Schuler and Jenkins, 2002; Serafim et al, 2002). Smolders, et al., (1994) demonstrate that the uptake of acetate needs less energy at low pH compared to high pH due to low electrical potential difference across the membrane of the cell at low pH. As a result, phosphate release becomes less at low pH than at high pH. According to Liu et al. (1996a), acetate uptake rate (AUR) is maximized at a pH of 7.3 ± 0.5 and release of phosphorus is highest in the pH range of 5.7 to 6.8. They recommend a pH value of 6.8 ± 0.7 for the acetate metabolism of PAOs. However, Schuler and Jenkins (2002) note similar AUR at pH 8 with non-soluble P/total suspended solids of 0.13-0.14 mg/mg. Moreover, phosphorus release rates increase up to the pH values of 7.5-8.0 and 7-7.5 for non-soluble P/total suspended solids of 0.13-0.14 mg/mg and 0.065-0.075 mg/mg, respectively. They suggest the optimum pH range of 7.4 to 8.5

for better performance of an EBPR system. Serafim et al, (2002) show that the phosphorus release and uptake considerably improve when pH is not controlled (pH raise from around 7.8 to 8.5) in a SBR fed with a mixture of acetate, propionate and butyrate. The efficiency of phosphorus removal is better without pH control compared to that with pH control (pH is around 7).

Under anaerobic conditions, the PHA and glycogen transformation are also affected by pH in the EBPR process (Filipe et al., 2001; Filipe et al. 2001a; Chen and Gu, 2006). Filipe et al. (2001) imply that the utilization of glycogen and storage of PHA are unaffected in the pH range of 6.5 to 8.0 in acetate fed EBPR process by using an enriched culture of PAOs. This result does not coincide with the findings of Filipe et al. (2001a) who study anaerobic metabolism of GAOs. In case of GAOs, the utilization of glycogen and storage of PHV increase with increasing pH in acetate fed reactor containing enriched culture of GAOs. Chen and Gu, (2006) show that the PHA and glycogen reduce linearly with higher pH values from 6.6 to 8.6 under anaerobic conditions with a mixture of propionate and acetic acids.

Recently, the influence of pH on both anaerobic and aerobic transformations of phosphorus has also been investigated (Pijuan et al., 2004a; Oehmen et al., 2005b and Chen and Gu, 2006). Pijuan et al., (2004a) performed experiment in the pH range of 6.5 to 8 with propionate in a SBR. They depict that the uptake rate of propionate increases by elevating the pH from 6.5 to 8 under anaerobic conditions while uptake rates of phosphorus are highest at pH 7.5 and 8 under aerobic conditions. Pijuan et al., (2004a) propose that the overall optimum pH is about 7.5. Chen and Gu (2006) find similar results for the uptake rate of propionic acid by using pH values from 6.6 to 8.6 with a

mixture of propionate and acetic acids. However, they establish that the uptake of phosphorus is low at either pH 6.6 or pH 8.6 under aerobic conditions. Chen and Gu (2006) recommend optimum pH of 7.1 to7.6 for better performance of EBPR system.

The activity of PAOs out-competes GAOs at high pH (Jeon et al., 2001; Serafim et al, 2002; Schuler and Jenkins, 2002; Oehmen et al., 2005b). Jeon et al., (2001) and Schuler and Jenkins (2002) observe that the metabolism of PAOs has a competitive advantage over the metabolism of GAOs at pH greater than 7 in a SBR fed with acetate. Serafim et al, (2002) find similar results by using a mixture of acetate, propionate and butyrate at a pH of 7.8 to 8.5. It is thought that PAOs have an excess energy, which is polyphosphate for uptaking the carbon at high pH while GAOs have only the glycogen as energy source causing the dominance of PAOs against GAOs. In addition, these findings coincide with the recent research of Oehmen et al., (2005b) who use pH values of 7 and 8 in either case of acetate and propionate.

2.2.7.3. Temperature

The effect of temperature on the EBPR process is discussed in this section. Temperature affects different aspects of an EBPR system: phosphorus release and uptake, stoichiometry and kinetics, microbial selection, PAOs response to different types of temperature shocks etc.

Mamais and Jenkins (1992) demonstrated that the rate of phosphate release and uptake, have similar dependency on temperature. These rates decreased from 1.5 to 1.7 times by each 10°C drop of temperature over the range of 10°C to 30°C. They also showed by batch test that the optimum temperatures for the rate of phosphorus release and uptake were within 10°C to 28°C and 28°C to 33°C, respectively. McClintock et al.,

(1993) reported that the phosphorus removal is best at the temperature of 20°C with MCRT of 5 days. Helmer and Kunst (1998) observe by mixed population of activated sludge that the phosphorus release and uptake decline with decrease in temperature from 20°C to 10°C.

The removal of phosphorus is achievable even at low temperature. Ydstebo et al., (2000) found that effluent P concentration of 0.6 mg/L is possible at 5°C with SRT of about 20 days in an EBPR system. Erdal et al., (2003a) also observed that phosphorus removal is good at 5°C and SRT of 18 days. However, poor performance of EBPR system can be observed due to rigid-like behavior of the cell membranes at cold temperature. Most cells can change their fatty acid compositions of the membranes as the temperature changes to keep it in normal fluidity state called homeoviscous adaptation. Erdal et al., (2003a) inferred that PAOs perform homeoviscous adaptation by raising the unsaturated to saturated fatty acid ratio in the side chains of fatty acids in the membrane. Therefore, they conclude that at low temperature, the failure of EBPR occur due to incompatible operational conditions such as low SRT and anaerobic detention time, excess electron acceptors etc. rather than the physical condition of cellular membrane.

Some researchers propose that temperature has no influence on the mechanism of phosphorus removal. Choi et al., (1998) indicate by batch test that the rate of phosphorus releases and uptake has no relation within the temperature range of 5°C to 20°C. Kumar et al., (1998) reported that more phosphorus is released at temperature 25°C than 10°C and aerobic uptake does not depend on anaerobic release of phosphorus. They identify that the VFA production becomes limited at 10°C compared to 25°C and consequently,

less release of phosphorus in the anaerobic zone. The effluent phosphorus concentration removal is less than 1 mg/L at both the temperatures of 10°C and 25°C.

The effect of temperature on the stoichiometry and kinetics of the EBPR process have been analyzed by several researchers (Brdjanovic, et al., 1997 and 1998; Erdal et al., 2003). Temperature has no impact on the stoichiometry of anaerobic process while little effect was observed in aerobic process. However, the rate of kinetic reactions under anaerobic and aerobic conditions increased with elevating temperature over the range of 5°C to 30°C by using enriched culture (Brdjanovic et al., 1997 and 1998). This result is similar to the finding of Erdal et al., (2003) who use temperature range of 5°C to 20°C with SRT of 18 days.

There are few studies showing the temperature effect on the microbial selection of the EBPR process (Helmer and Kunst, 1998; Erdal et al., 2003; Whang and Park, 2002 and 2006; Panswad et al., 2003). Helmer and Kunst (1998) indicate that at low temperature (5°C), facultative anaerobic bacteria (e.g. Aeromonas sp., Staphylococcus) increase compared to aerobic bacteria (e.g. Acinetobacter sp.). Facultative anaerobic bacteria gain energy through fermentation without hydrolysis of polyphosphate and thereby, no release of phosphorus in anaerobic phase. However, they store polyphosphate under aerobic phase causing higher uptake of phosphorus at low temperatures. Erdal et al., (2003) indicate that at low temperature (i.e. $\leq$ 10° C), the selection of PAOs occur over GAOs compared to high temperature (i.e. 20° C). This is due to preferable growth of PAOs over non-PAO at temperature of 10°C or less for psycrophilic nature of PAOs. As a result, PAOs can compete more efficiently for substrate than non-PAOs at low temperature, resulting in a large number of PAOs and better efficiency of phosphorus removal while

non-PAOs out-competed the PAOs at 20°C causing less removal of phosphorus. In contrast, Whang and Park (2002, 2006) demonstrate that PAOs dominate against GAOs in anaerobic/aerobic SBR at 20°C with SRT of 10 days whereas GAOs are thought to dominate after competing with PAOs for 20 days at 30°C with similar SRT. The reason is that the lower rate of anaerobic specific acetate uptake of PAO-dominated sludge (0.89 x 10^{-3} mg C/mg VSS. minute) compared to that of GAO- dominated sludge (1.34 x 10^{-3} mg C/mg VSS. minute) at 30°C (Whang and Park, 2002). Panswad et al., (2003) also got almost similar results. Their findings indicated that the optimum temperature for PAOs was 20°C or less. They also found that GAOs dominate between 25°C and 32.5°C whereas ordinary heterotrophic organisms predominate between 32.5°C and 35°C causing the failure of the EBPR system.

Panswad et al., (2003a) investigated the PAOs activity using two different temperature shocks (pulse and step) in the EBPR system. Temperature shock from 20°C to 35°C negatively affected the activities of PAOs either in pulse (5°C changes in every 5 days) or stepwise (1°C change per day) manner. However, stepwise increase from 30°C to 35°C had less impact compared to pulse wise increase of temperature. Conversely, decreasing pattern of temperature shock up to 20°C in both cases had positive effects on the activities of PAOs (Panswad et al., 2003).

2.2.7.4. Solids Retention Time (SRT)

The solids retention time is an important parameter responsible for the removal of phosphorus in the EBPR system. SRT is the average time for the activated sludge to be present in the system (Metcalf & Eddy, 2003). A long SRT value stimulates slow growing organisms and thus deteriorates the settling characteristics of solids in the

system. Again, short SRT values lead no growth of active biomass, which results in no removal of phosphorus from the system. This situation is called washout (Rittmann and McCarty, 2001). Therefore, this section describes the required SRT value based on solid settling characteristics, temperature, washout conditions, and interaction between PAOs and GAOs.

The influence of SRT on the settling characteristics of solids has been investigated by different researchers (Henze et al., 2002; Chang, et al, 2005). Henze et al., (2002), reveal that the settling of biomass is unstable when SRT is 4 days in a pilot scale A/O process. As a result, suspended solids concentration increase in the effluent causes elevated concentration of COD, nitrogen and phosphorus. Chang et al, (2005) also found in a pilot scale A/O system that the sludge settling characteristics were improved at SRT of 15 days than that of 5 days at 20° C. However, phosphorus removal was best at SRT of 10 days among the SRT values of 5 days, 10 days and 15 days. On the other hand, Henze et al., (2002) observe that SRT has no effect on the phosphorus removal of the EBPR plants when the ratio of VFA and TP is high in the raw wastewater.

SRT value of the EBPR system depends on the temperature because it controls the rate of biochemical reactions. Mamais and Jenkins (1992) indicate that above MCRT of 2.9 days, the EBPR system worked effectively and independently on MCRT over the temperature range of 13.5° to 20° C. The failure of EBPR system may occur at MCRT values of less than 2.9 days depending on temperature. In addition, Brdjanovic et al., (1998) also state that a longer resident time is required for the growth of desired organisms at low temperature due to slow down of the biochemical reactions rates. They demonstrate that the complete removal of phosphorus was possible for the arbitrary

chosen SRT values of 8 days, 16 days and 32 days at temperatures of 20°C, 10°C and 5°C, respectively. According to Henze, (1996) and Grady et al, (1999), the preferred SRT values are in the range of about 2 days to 3 days at 20° C and about 4 days to 5 days at 10° C for biological removal of phosphorus without nitrification in the A/O process.

Different researchers (Latawiec, 2000; Merzouki et al., 2001) have investigated the washout SRT values in the EBPR system. Latawiec D., (2000) observed that the washout of PAOs occurs at SRT less than 1.5 days with temperature range from 20°C to 23°C. The effluent phosphorus concentration is lowest (7.3 g P_{PO4}/ m^3) at SRT of 2 days over the range of 1.7-7 days because PAO are an important portion of activated sludge at SRT of 2 days. The effluent phosphorus concentration increases slowly with the increase of SRT (10.6 g P_{PO4}/ m^3 for SRT of 6 days) and increases rapidly (14.1 g P_{PO4}/ m^3) with the decrease of SRT to 1 day. However, Merzouki et al., (2001) observed in an anaerobic-anoxic SBR that the efficiency of phosphorus removal is better at SRT 15 days compared to 7.5 days due to washout of polyphosphate accumulating bacteria at 7.5 days.

SRT value affects the presence of PAOs and non-PAOs in the EBPR system (Matsuo, 1994; Whang and Park 2006). Matsuo, (1994) indicates by analysis of lab-scale A/O process that poor phosphorus removal is observed when anaerobic SRT (an-SRT) is low (i.e. 0.9 day) at 20 to 23°C due to proliferation of organisms, which are able to use anaerobic DOC whereas longer SRT value (i.e. 6.3 days) might help the PAOs against other heterotrophs for the uptake of anaerobic substrates. Matsuo (1994) observed that Filaments of N. limicola acts as a secondary contributor to EBPR at lower SRT. On the contrary, Whang and Park (2006) report that at SRT of 10 days and at 30°C, GAOs are able to out compete PAOs in the SBR due to higher anaerobic acetate uptake rate of

GAOs than PAOs. PAOs and GAOs exist together at SRT of 5 days with same temperature, resulting unstable phosphorus removal due to competition of GAOs with PAOs for acetate. However, PAOs dominate over GAOs for anaerobic acetate uptake when SRT is declined from 5 to 3 days results in improved and stable operation of EBPR system.

Since the room temperature of this research is 22± 2° C, the SRT values close to this temperature suggested by different researchers have been summarized in Table 2.9. The suggested SRT values vary from 2 days to 10 days based on the EBPR process. Table 2.9 shows the SRT values used in laboratory – scale SBR for phosphorus removal. The SRT values were from 8 days to 10 days at a temperature range of 20 to 25 ° C. The maximum SRT value of 10 days will be used in this research.

Table 2.9: SRT values used in laboratory-scale SBR

Temperature (° C)	SRT (days)	References
25	10	Jeon & Park, 2000
25 ± 1	8	Wang et al., 2002
23 to 24	9	Pijuan et al.,2004a
20	9	Chen et al., 2004
20	10	Cokgor et al., 2004
-	8	Oehmen et al., 2006
20 to 24	8	Lu et al., 2006

2.2.7.5. Nitrate/Nitrite

Nitrate

In EBPR, phosphorus removal capability can be reduced significantly by the addition of nitrate to the anaerobic zone (Metcalf and Eddy, 2003; Toerien et al, 1990; Mulder and

Rensink, 1987; Tetreault et al., 1986). This is because denitrifying bacteria/ heterotrophic bacteria consume VFAs in the denitrification process. Therefore, the availability of VFAs for PAOs to release phosphorus is reduced (Metcalf and Eddy, 2003; Toerien et al, 1990). At least 4 mg readily biodegradable COD is utilized for the reduction of 1 mg NO_3-N during denitrification process (Rittmann and McCarty, 2001). Nitrate concentration should not be higher than 2 mg N/l in the anaerobic reactor to avoid denitrification (Osborn and Nicholls, 1978).

Denitrification is a process where nitrate is reduced to nitrogen gas using four different enzymes. These enzymes are nitrate (NO_3^-) reductase, nitrite (NO_2^-) reductase, nitric oxide (NO) reductase and nitrous oxide (N_2O) reductase. Nitrate reductase converts NO_3^- to NO_2^-, nitrite reductase transfers NO_2^- to NO, nitric oxide reductase transforms NO to N_2O and finally, nitrous oxide reductase reduced N_2O to N_2 gas. The activities of these enzymes are controlled by the dissolved oxygen (DO) concentration. If the DO concentration is higher than 2.5 to 5 mg O_2/L, the genes of these enzymes are suppressed and are incapable of denitrification. On the other hand, their activities slow down when the DO concentration is above a few tenths of a mg O_2/L (Rittmann and McCarty, 2001).

Denitrification and phosphorus release can occur simultaneously when sufficient carbon source is available (Ghekiere et al., 1991; Chuang et al., 1996; Patel et al., 2006; Zou et al., 2006). However, even when sufficient carbon source is available, phosphorus release rates have been reported to be negatively affected by denitrification (Zou et al., 2006; Lee et. al., 2006). The specific phosphorus release rates in presence of nitrate have been reported to be 42% (COD =200 mg/L and NO_3-N = 10 mg/L) (Zou et al., 2006).

Moreover, Patel and Nakhla (2006) found that phosphorus release occurred only when nitrate concentration was less than 1 mg/L.

Recently, a few studies suggest that nitrate can be used as an electron acceptor in the anoxic zone of EBPR systems for phosphorus uptake by PAOs (Vlekke et al., 1988; Kuba et al., 1993; Sorm et al., 1996; Merzouki et al., 2001; Peng et al., 2006; Zou et al., 2006). Malnou et al. were the first researchers to report that nitrate could serve as an electron acceptor for phosphorus uptake (Barker and Dold, 1996). Later, Vlekke et al (1988) and Zou et al., (2006) noticed that more stored carbon (i.e. PHB) was utilized for phosphate uptake in presence of nitrate compared to that of oxygen. Several investigators (Kerrn-Jespersen and Henze, 1993; Sorm et al., 1996 and Yagci et al., 2003) reported that the rate of phosphate uptake was lower (i.e. 1.7 to 5.5 mg PO_4-P/gVSS.hr) under anoxic condition than under aerobic conditions (i.e. 3.7 to 6.7 mg PO_4-P/g VSS. hr). Therefore, the phosphorus removal efficiency was lower with nitrate compared to that of oxygen (Kuba et al., 1993 and Zou et al., 2006).

Normally, denitrification is performed by Ordinary Heterotrophic Organisms (OHOs) in a BNR system. Osaka et. al., (2006) found that the alpha and beta proteobacteria were present in a denitrifying batch reactor using acetate as a carbon source. The alphaproteobacteria include *Rhodobacteraceae* (e.g. *Paracoccus and Rhodobacter*) and the betaproteobacteria consist of *Comamonadaceae* (e.g. *Comamonas and Acidovorax*) and *Rhodocyclaceae* (e.g. *Thauera and Dechloromonas*). Parsons et al., (2007) observed that methanol-driven denitrification process could support methylotrophic bacteria. However, PAOs might also perform denitrification (Vlekke et al., 1988; Kuba et. al., 1993; Sorm et al., 1996; Merzouki et al., 2001; Peng et al., 2006; Zou et al., 2006). The

specific denitrification rate of PAOs (i.e. from 0.029 to 0.059 mg NO_3-N/ mg PAOA VSS) is about one-third of the specific denitrification rate of OHOs (i.e. from 0.114 to 0.185 mg NO_3-N/ mg OHOA VSS) (Hu et al., 2002). Therefore, if the nitrate load is in excess of the denitrification potential of OHOs, the Denitrifying Polyphosphate Accumulating Organisms (DPAOs) might have an opportunity to use nitrate and grow (Hu et al., 2002). Several researchers (Barker and Dold, 1996; Watchtmeister et al., 1997; Peng et al., 2006) indicated that DPAOs, which can perform denitrification and store phosphorus, are only a fraction of PAOs based on chemical analysis.

Recently, microbiological analysis in several studies points to the presence of some species of PAOs that act as DPAOs. Zeng et al., (2003) found by FISH analysis that *Accumulibater* was abundant in both anaerobic/aerobic and anaerobic/anoxic SBR fed with acetate. They noticed that *Accumulibacter* enriched under anoxic condition can uptake phosphorus immediately under aerobic condition. *Accumulibacter* enriched under aerobic conditions required lag time to use nitrate as an electron acceptor, and might therefore be considered a DPAO. Carvalho et al., (2007) also obtained similar results using acetate and propionate as a sole carbon source. However, they detected two different cell morphotypes (i.e. rods and cocci) and assumed that rod shape *Accumulibacter* is able to utilize nitrate, nitrite and oxygen as electron acceptors. Moreover, several investigators (i.e. Dabert et al., 2001; Ahn et al., 2002; Lee et al., 2003; Kong et al., 2004; Ahmed et al., 2007) identified that *Rhodocyclus* related PAOs could also perform denitrification in an anaerobic anoxic SBR fed with acetic acid.

Nitrite

The presence of nitrite influences the uptake of phosphorus under aerobic conditions (Meinhold et al., 1999; Saito, et al, 2004). The phosphate uptake was severely inhibited by the higher concentration of nitrite. The concentration range was from 5 to 8 mg NO_2-N/l above which phosphate uptake was hindered. The inhibiting action lasted for at least several hours after exposure to nitrite (Meinhold et al., 1999). Saito, et al, (2004) also found that nitrite, which was produced by denitrification, strongly repressed the uptake activity and growth of PAOs in aerobic period by using enriched culture of PAO bacteria. As a result, GAOs achieved the competitive advantage over PAOs. Niel et al., (1998) showed that the growth of PAOs might be hindered by nitrite, through its conversion to nitric oxide. The nitric oxide inhibits the activities of enzyme (i.e. adenylate kinase) in ATP formation from polyP resulting in a low acetate uptake and subsequently low phosphorus release and uptake.

2.2.7.6. Dissolved Oxygen

Dissolved Oxygen (DO) should be absent (i.e. between 0.0 and 0.2 mg/L) in the anaerobic zone because it could be utilized as an oxidizing agent for organic substrates in the denitrification process. Thus, organic substrates will not sufficient for phosphorus release under anaerobic condition, which is needed for phosphorus uptake under aerobic condition.

The uptake of phosphate has been reported to be influenced by the presence of DO in the aerobic zone (Levin and Shapiro, 1965). The uptake rate would be slow due to lack of adequate aeration (Pitmann et al., 1983). Several researchers (Daigger and Polson, 1991; Toerien et al., 1990; Griffiths et al., 2002) found that the optimum DO

concentration is from about 1.5 to 3 mg/l. According to Toerien et al., (1990) and Pitman, (1991), when DO value is lower than about 1 mg/L, nitrification might be repressed and sludge bulking might occur, resulting in the reduction of phosphorus removal. In contrast, higher DO value might decrease denitrification in the mixed liquor recycle streams which resulting in an increase of nitrate in the anaerobic zone and subsequent poor removal of phosphorus (Daigger and Polson, 1991). Griffiths et al., (2002) observed that the PAOs require the optimum DO value for their initial growth and phosphorus uptake while G bacteria can grow at DO concentrations above 4.5 to 5 mg/l. However, G bacteria, if established, are able to dominate over PAOs in the DO concentrations range of 2.5 mg/l to above 5 mg/l. Therefore, high DO concentration restricts the growth of PAOs.

2.2.8. Engineering Design of EBPR Systems

EBPR systems are now widely used in full-scale wastewater treatment plants. To achieve excellent performance, it is important to design the systems properly. Therefore, this section will focus on the design requirements of the major parts of the systems (i.e. size and shape of anaerobic and aerobic reactors, hydraulic retention times, types of aerators, mixed liquid suspended solids and final clarifiers). In addition, the design values of these parameters used in various wastewater treatment plants will be shown in Tables 2.11, 2.13 and 2.14.

Anaerobic Condition

Anaerobic conditions are necessary to release phosphorus in the anaerobic reactor. About 10-20% of the stored phosphorus is released in the anaerobic reactor (Grady et al., 1999). If oxygen or nitrate-N is present, the availability of COD reduces to release

phosphorus (Henze, 1996). Henze, (1996) observed that 16 g of substrate COD was utilized by 8 g oxygen or 2.8 g nitrate-N. Therefore, it is necessary to create in the anaerobic reactor an environment free from oxygen and nitrate (electron- acceptors). Tetreault et al., (1986) reported that the anaerobic condition was maintained in full-scale plants by reducing both DO and NO_3-N concentrations below 0.2 mg/L. Similar DO concentrations were also observed in the anaerobic zone of the Clark County Sanitation District Wastewater Treatment Plant in Las Vegas, Nevada (Filho, 2001).

Anaerobic Retention Times

The anaerobic contact time permits the production and uptake of the short- chain volatile fatty acids (SCVFAs) by PAOs to release phosphorus in the EBPR process (Gerber et al, 1987; Stensel, 1991). Gerber et al., (1987) reported by batch tests that SCVFAs (i.e. acetate, propionate) released phosphorus in the aerobic conditions. The phosphorus uptake started after using all SCVFAs. Therefore, anaerobic contact time should be selected properly for the complete utilization of VFAs to release phosphorus in the anaerobic zone. The anaerobic retention time, which has been suggested by different researchers, are illustrated in Table 2.10. Osborn and Nicholls, (1978) reported that long retention time resulted in a large phosphorus release in the anaerobic zone and consequently, high phosphorus concentration was observed in the effluent. Besides, a long retention time (i.e. 3 hrs) would lead to the secondary release of phosphorus, which also hampers phosphorus removal (Barnard and Fothergill, 1998; Metcalf and Eddy, 2003). Randall et al., (1992) indicated by analyzing A/O, A^2/O, VIP, UCT processes that anaerobic retention time of 90 minutes is enough for extensive fermentation even at

temperatures of about 13.6° C. The anaerobic retention times used in different wastewater treatment plants will be given in Table 2.11.

In conclusion, the normal range of anaerobic retention time (AnRT) is from 0.5 hour to less than 2 hours in the absence of nitrate whereas the AnRT is from 2 to 3 hours in the presence of nitrate. A higher AnRT (i.e. 3 hrs) is avoided to prevent secondary phosphorus release.

Table 2.10: Suggested anaerobic retention times for mainstream processes

Process Types	Anaerobic Retention Time (hrs)	Considerations	References
A/O process	0.5-1.5	Based on average dry weather flow and sufficient for the selection of PAOs	Krichten et al., 1987; Metcalf & Eddy, 2003
	0.5-1	As a high loaded system	Meganck and Faup, 1988
A^2/O process	1.0	Based on controlled substrate addition and complete denitrification in the anoxic zone	Osborn and Nicholls, 1978
	2.0	Based on the presence of nitrate	
	0.5-1.5	-	Metcalf & Eddy, 2003
	2-4	Nitrate concentration in the recycle sludge	Meganck and Faup, 1988
A^2/O, UCT, modified UCT and Bardenpho processes	0.5-1	Based on good feed sewage quality	Pitman, 1991
A/O, A^2/O, UCT, modified UCT and Bardenpho processes	0.9-2	Based on soluble BOD and selection of PAOs in the mainstream	Tetreault, 1986; Daigger and Polson, 1991;
	1-3		Henze et al., 2002
UCT and 5-stage Bardenpho processes	0.5-2.5	Based on interference of recycled nitrate to create anaerobic condition	Meganck and Faup, 1988

Aerobic Retention Times

A sufficient detention time of the aerobic reactor is required for uptaking phosphorus from the solution (Stensel, 1991 and Pitman, 1991) and for efficient nitrification (Pitman, 1991). Meganck and Faup, (1988) proposed that the detention time of the aerobic tank should be from 1 to 3 hours for the A/O process. Stensel (1991) reported that the uptake rate of phosphorus was in the range of 10-30 mg P/hr-L. Therefore, a detention time of 1-2 hours might be reasonable since the anaerobic phosphorus release was about 20 -40 mg P /L. The aerobic retention times of different full-scale wastewater treatment plants are shown in Table 2.11.

Table 2.11: Hydraulic retention time of various full-scale wastewater treatment plants

Name of the Plants	Anaerobic (hr)	Anoxic (hr)	Aerobic (hr)	References
Largo, Florida	1	0.67	3.7	Liu and Liptak, 2000
Port Orange, Florida	1.3	1.3	7.2	Liu and Liptak, 2000
Warminster, Pennsylvania	1.5	1.1	6.8	Liu and Liptak, 2000
Clark County Sanitation District, WWTP, Nevada	1.06	-	4.2	Becker, 2000
York River WWTP, Virginia	2	-	4	Randall et al., 1992
East Boulevard WWTP, Michigan	1.7	-	6.4	Daigger and Polson, 1991
Largo WWTP, Florida	1.2	-	2.1	Meganck and Faup, 1988
Rilling Plant, Texas	3	-	3	Meganck and Faup, 1988
Reedy Creek Improvement District Main WWTP, Florida	1.9	-	3.8	Tetreault, 1986

Shape and Mixing of Anaerobic Reactors

The shape of the anaerobic reactor depends on the mixing conditions. T pically, the newly designed plants in the US have square tanks with a single mixer. In the case of retrofit applications, rectangular tanks with a single mixer have been used up to a length-to-width ratio of 1.5. However, multiple mixers have been used if the ratio exceeds 1.5 (Krichten et al., 1987).

The purpose of mixing is to disperse quickly the wastewater influent and return activated sludge throughout the reactor in order to obtain a homogenous mixture. Besides, mixing can keep mixed liquor solids in suspended conditions. During mixing, surface turbulence should be low to minimize oxygen transfer from the atmosphere to the liquid (Krichten et al., 1987; Randall, et al., 1992). Daigger and Polson (1991) suggested that the mixing energy of the anaerobic reactor should be about 10 W/m^3 with propeller type or submerged turbine mixers. Randall, et al., (1992) demonstrated that the mixing requirements of the activated sludge could be derived as a function of the mixed liquid suspended solids (MLSS) as follows:

$$P/V = 0.00094\ (\mu)^{0.3}\ (MLSS)^{0.298}$$

Where, $\mu = 1.0087$ at 20°C

P/V = Kilowatts per 1000 liters

MLSS = mg/L

Mixing can be performed using an axial flow turbine, which generates high pumping capacity. The turbine can supply power in the range of about 7.5 W/m^3 to 15 W/m^3. Recently, submersible electric motor-driven mixers are also available and can produce

power from 1 to 15 kw (Krichten et al., 1987). Therefore, the axial flow turbine and submersible electric motor-driven mixers are suitable to attain the mixing requirements.

Types of Aerators

Aeration can be performed using both mechanical surface aerators and diffused air systems (Krichten et al., 1987; Pitman, 1991). The surface aerators have a low capital cost, while the diffused air system is more cost-effective for power requirement. In addition, a diffused air system generates activated sludge with very good settling characteristics. Pitman (1991) reported that the Bushkoppies WWTP in Johannesburg incorporated a diffused air system, which always gives a diluted sludge volume index (DSVI) below 60 ml/g whereas Northern works of Goudkoppies used surface aerators showing a DSVI value up to 300 ml/g. However, diffused air systems are probably sensitive to inadequacies and defects of the fabrication materials, quality of installation, maintenance and process control.

Mechanical surface aerators and diffused air systems can be used in a tapered form to meet the variable oxygen demand in the aeration tank. Normally, a tapered aeration system, which has greater capacity at the inlet zone, should be used in the rectangular aeration tank to satisfy high oxygen demand (Pitman, 1991). A tapered power input profile is used in the surface aerator, whereas the number of diffusers is reduced along the length in the diffused air system (Krichten et al., 1987).

Aeration Tank Size

The size of the aeration tank depends on the type of the aerators. In case of a diffused air system, mixing pattern and aeration efficiency are important to select the size of the aeration tank. The width-to-depth ratio may be varied from 1:1 to 2.2:1 for the plug-flow

pattern with spiral-flow mixing (Rittmann and McCarty, 2001; Metcalf and Eddy, 2003). The minimum length-to-width ratio should be 5:1 for each channel in large plants. However, the ratio can be reduced in case of completely mixed systems (Metcalf and Eddy, 2003). To maximize the aeration efficiency, the wastewater depth should be from 4.5 to 7.5 m and the free board should be between 0.3 to 0.6 m in the aeration tank (Metcalf and Eddy, 2003), whereas Rittmann and McCarty, (2001) suggested a tank depth from 4.5 to 7.5 m. Becker, (2000) observed that the depth of the tank is 6.4 m in the Clark County Sanitation WWTP in Las Vegas, Nevada. In case of mechanical surface aerators, the depth and width of the aeration tank depend on the aerator size as shown in Table 2.12.

Table 2.12: Typical values of the depth and width of aeration tanks for mechanical surface aerators (Metcalf and Eddy, 2003)

Size of Aerator (KW)	Tank Depth (m)	Tank Width (m)
7.5	3-3.5	9-12
15	3.5-4	10-15
22.5	4-4.5	12-18
30	3.5-5	14-20
37.5	4.5-5.5	14-23
55	4.5-6	15-26
75	4.5-6	18-27

Mixed Liquid Suspended Solids (MLSS)

The MLSS should be selected properly to avoid poor settling of the activated sludge, which results in a poor effluent quality (Grady et al., 1999). Shapiro (1967) used sludge concentration in the range of 1700 to 15,800 mg/L to check the behavior of phosphate

release. The amount of phosphorus release was much higher in more concentrated sludge. However, the quantity of phosphorus release was basically the same for each unit of sludge. Pitman, (1991) established that the design values of MLSS should be from 3500 to 5000 mg/L without exception, whereas Grady et al., (1991) limited the MLSS concentration from 2000 to 5000 mg/L for activated sludge processes. Metcalf & Eddy, (2003) reported that the required MLSS should be from 3000 to 4000 mg/L for A/O, A^2/O, UCT and 5-stage Bardenpho processes whereas MLSS should be from 2000 to 4000 mg/L for VIP and 1000 to 3000 mg/L for Phostrip processes. Table 2.13 presents the MLSS values utilized in various wastewater treatment plants.

In summary, the MLSS value of the Palmetto Bardenpho Plant in Florida satisfied the requirement suggested by all researchers described here. The MLSS values of the DePere WWTP in Wisconsin and the Tri-City Water Pollution Control Plant in Oregon fulfilled the demand proposed by Grady et al., (1991) and Metcalf & Eddy, (2003), whereas the Reedy Creek Improvement District Main WWTP and the Blue Heron WWTF in Florida satisfied only the Grady et al., (1991) limit. Therefore, the MLSS values can be varied from 2000 to 5000 mg/L.

Table 2.13: MLSS values used in different wastewater treatment plants

Name of Plants	EBPR process	SRT, days	MLSS, mg/L	References
Palmetto Bardenpho Plant, Florida	5-stage Bardenpho	14-20	3500	Burdick et al., 1982
Reedy Creek Improvement District Main WWTP, Florida	A/O	7.2	2100	Tetreault, 1986
DePere WWTP, Wisconsin	A/O	10.6	3000	Tetreault, 1986
Tri-City Water Pollution Control Plant, Oregon	A/O	4-5	3300	Melcer et al., 1998
Blue Heron WWTF,	A^2/O process	10-11	2500	D'Amato et al., 1998

Final Clarifiers

The functions of final clarifiers are to generate well-clarified effluents with low suspended solids (SS) and BOD values and concentrated solids that are recycled to the anaerobic reactor (Narayanan et. al., 2000; Metcalf and Eddy, 2003). The total suspended solids (TSS) concentration in the effluent should be 10 mg/L or less to achieve phosphorus concentration below 1 mg/L, considering about 3 to 6 percent phosphorus in the solids (Albertson, 1992; Metcalf and Eddy, 2003). As a result, the BOD_5 of the effluents will be less or equal to 5 to 8 mg/L (Albertson, 1992).

The final clarifiers are normally either circular or rectangular in shape. Circular tanks have been built with diameters in the range of 3 to 60 m. However, the commonly used diameters are from 10 to 40 m. The radius of the tank should not be greater than five times the side water depth (Metcalf and Eddy, 2003). The minimum side water depth of

3 m and a bottom slope of 10% should be maintained in the circular conical bottom-scraped units (Pitman, 1991). The side water depth (SWD) should be from about 3.5 to 6 m for a modern design of large circular clarifiers (Hsu and Wilson, 1998; Metcalf and Eddy, 2003). In case of a rectangular tank, the length should not be more than 10 times its depth. In large plants, lengths of up to 90m have been used, and widths of up to 24 m have been used. The depth of the clarifier should be sufficient (i.e. 3.7 to 5.5 m) to maintain the sludge blanket lower than the effluent weirs (Metcalf and Eddy, 2003).

Sludge blanket height (SBH) is important in the final clarifier to avoid an anaerobic condition (Barnard, 1983; Tetreault et al., 1986; Metcalf and Eddy, 2003). Tetreault et al., (1986) suggested that the SBH should be 0.3 m whereas Metcalf and Eddy (2003) reported the desirable value from 0.3 to 0.9 m. Chavan (2003) recommended by analysis the Clark County Sanitation District Wastewater Treatment Plant in Las Vegas, Nevada that the SBH should be less than 0.76m to control the denitrification in the clarifiers. Narayanan et al., (2000) observed the close SBH (i.e. 0.8 m) in the Orange County's Wastewater Treatment Plant No.2 in Huntington Beach, California.

Surface overflow rate (SOR) and solids loading rates (SLR) are two important parameters used to control system performance. The recommended SOR and SLR values are from 0.68 to 1.36 m/h and 4.07 to 6.10 kg per m^2 per hr, respectively by the United States EPA in 1975 (Narayanan et al., 2000). Moreover, the typical value for an average SOR is from 0.67 to 1.17 m/h and SLR is from 5 to 8 kg per m^2 per hr (Metcalf and Eddy, 2003). It has been reported that SOR values are 1.21 m/h and 1.94 m/h for average flows of 10.5 million gallons per day (MGD) and 17.5 MGD respectively, in the Clark County Sanitation WWTP in Nevada (Becker, 2000). The values of some important

parameters of final clarifier used in several wastewater treatment plants are illustrated in Table 2.14.

Table 2.14: Values of parameters used for final clarifiers in various wastewater treatment plants

Name of Plants	Size (m)	SWD (m)	SOR (m/h)	References
Reedy Creek Improvement District Main WWTP, Florida	D = 3 to 4	3.9	0.63	Tetreault et al., 1986
DePere WWTP, Wisconsin	D = 3 to 4	4.3	0.83	Tetreault et al., 1986
Orange County's WWTP No.2 in Huntington Beach, California	L = 53 W = 18	4	0.76 to 1.10	Narayanan et al., 2000
Clark County Sanitation WWTP, Nevada	D = 42.7	4.3	1.21 & 1.94	Becker, 2000

In conclusion, the diameter for a circular tank can vary from 3 to 60 m whereas the length and width of a rectangular tank can be up to 90 m and 24 m, respectively. The minimum SWD is in the range from 3.5 to 6 m and the SBH varies from 0.3 to 0.9 m. The normal range of the SOR value is from 0.67 to 1.36 m/h.

2.3. Florescence In Situ Hybridization (FISH)-A Microbial Tool to Analyze Microbes in EBPR System

FISH is one of the most commonly used molecular methods, which identifies microorganisms in wastewater treatment plants. In FISH analysis, a specific fluorescently labeled oligonucleotide probe is used that can penetrate into a bacterial cell and form a hybrid with the complementary base sequence of the target nucleic acid within the intact cell. The oligonucleotide probe is a short sequence of nucleic acid,

which is complementary to a particular sequence of RNA. The probe is easy to detect under the microscope because it is labeled with a fluorescent dye called fluorochrome. Previously, fluorescein and rhodamine-derivatives including fluorescein-isothiocyanate (FITC) and tetramethyl-rhodamine-isothiocyanate (TRITC) were usually used as fluorochrome. However, the fluorescence intensity per mole of these dyes is low. In addition, they are sensitive to pH and bleaching. Recently, the dyes from cyanine series (i.e. Cy3, Cy5 etc.) are widely applied due to provide brighter straining and stable nature to photobleaching (Moter and Gobel, 2000; Bouvier and Giorgio, 2003). The advantages, disadvantages, considerations and applications of FISH analysis are discussed in this section.

2.3.1. Advantages of FISH Analysis

Over the last decade, FISH technique has become an important molecular tool to analyze microbial communities for environmental microbiologists and engineers. This methodology is used for various purposes such as identification and quantification of microbes, characterization of complex diversity of organisms, and identification of gene expression patterns of bacteria. This technique is now widely used for having a lot of advantages:

1. FISH is extensively used to understand the complex microbial community (Bouvier and Giorgio, 2003) because FISH can identify slow-growing, culturable as well as unculturable bacteria. FISH provides information related to the presence, number and spatial distribution of microorganisms (Moter & Gobel, 2000). FISH is a more rapid, economical and simple method than the culture

method (Reza et al., 2006) and is used for in situ studies (Bouvier and Giorgio, 2003).

2. FISH can identify the morphology of bacteria (Moter & Gobel, 2000; Bouvier and Giorgio, 2003; Reza et al., 2006). For example, FISH is used to observe the state of bacterial cell walls (Moter & Gobel, 2000).
3. FISH becomes a new research tool for discovering bacteria in activated sludge. FISH can easily recognize cells inside flocs (Hug et al, 2005). FISH can verify the cellular activity in inactive or dead cells of microbes in activated sludge or biofilms based on the degradation of rRNA. FISH can inspect the intact cells in samples microscopically without amplification of DNA or RNA (Wilderer et al., 2002). FISH is particularly suitable for correct enumeration of microorganisms in biofilms or aggregates (Daims et al., 2001). Coskuner (2002) also report that FISH together with confocal laser scanning microscopy can be used to understand the microbial ecology of activated sludge flocs and/or biofilm structure.
4. FISH appears to be a powerful tool for the quantification of target organisms (Coskuner, 2002). It can represent several different targets in a single hybridization step by using fluorescent probes labeled with dyes of varied emission wavelength (Moter & Gobel, 2000; Levsky and Singer, 2003).
5. FISH is a fast, straightforward and accurate technique to characterize the undiscovered diversity by identifying the related groups of bacteria. FISH can provide phylogenetic information by 16S rRNA sequences and thus differentiate the activities of various populations separately (Coskuner, 2002).

6. FISH provides information regarding the expression patterns and locations of genes in a single cell (Levsky and Singer, 2003). FISH is the favored option to predict the complicated aspects of gene expressions that cause diseases (Levsky and Singer, 2003).

2.3.2. Disadvantages of FISH Analysis

Although FISH technique has gained widespread popularity in the scientific community, it has a number of disadvantages as well. This section discusses various types of disadvantages such as FISH analysis sometimes gives false positive and false negative results (Gobel and Moter, 2000); it has systematic errors (Hug et al, 2005); it needs trained personnel and expensive equipment (Reza et al, 2006).

a) False Positive Results

FISH provides false positive results due to the presence of autofluorescent material. Autofluorescence is observed in various types of organisms such as, Pseudomonas, Legionella, Rhodospirillum, moulds and yeast (Moter & Gobel, 2000). The autofluorescence is often much stronger than the fluorescence of specific binding of probe (Coskuner, 2002). It reduces the signal-to-noise ratio and interferes with the specific fluorescent signal from the probe (Moter & Gobel, 2000; Hug et al, 2005). The problems associated with autofluorescence are removed 1) by using narrow-band filter and signal amplification system (Moter & Gobel, 2000), 2) by bleaching of fixed cells before hybridization and 3) by use of fluorescent dyes, which have emission wavelengths different from the autofluorescence (Coskuner, 2002).

False positive results are also caused by the lack of specificity of probe. Therefore, the checking of probe sequence is required on a regular basis by using new sequencing database for accurate and reliable FISH analysis (Moter & Gobel, 2000).

b) False Negative Results

Insufficient penetration of probe can cause false negative results in FISH analysis. If probe does not penetrate considerably into the bacterial cell, signal will be generated with low intensity (Moter & Gobel, 2000). It is uncertain that the oligonucleotide probe can penetrate all cell types and hybridize with the target rRNA sequence all the time although the cell becomes permeable successfully. Even cell penetration can be obtained by maintaining high stringency conditions (Coskuner, 2002).

Higher order structure of target or probe creates results with false negative. In case of higher order structure (three-dimensional rRNA), probe cannot reach equally to all sequences in the structure. The hybridization can be obstructed due to the presence of loop and hairpin structure and the interactions between rRNA and protein. As a result, probe can behave differently in FISH analysis (Moter & Gobel, 2000).

Low rRNA content is responsible for false negative results. Low rRNA content reduces the growth rate of bacterial cells, which causes low signal intensity during hybridization or false negative results (Moter & Gobel, 2000; Amann, 1995).

Photobleaching is another example of developing false negative results. Photobleaching means the rapid fadedness of fluorochromes due to excitation under the fluorescence microscope and the brightness of fluorochromes is permanently lost with time. The problem due to photobleaching can be avoided by using photo stable cyanine dyes, narrow band filter sets and antifading mounting media (Moter & Gobel, 2000).

FISH also gives false negative results by using of bacterial probes. Sometimes eubacterial probes binds non-specifically with bacterial 16S rRNA or cell components other than nucleic acids and gives false signal in FISH (Moter & Gobel, 2000).

Systematic error

FISH has systematic errors due to selective loss of bacteria by several centrifugation and washing steps. Thus, non-optimal hybridization can cause fewer amounts of bacteria and provide wrong results (Hug et al, 2005).

Trained personnel and Expensive Equipment

FISH technique necessitates skilled persons to run the FISH analysis in the laboratory. Then, a comparatively sophisticated instrument such as confocal microscope is required to take the images of the slides, which are obtained by FISH analysis (Reza et al, 2006).

2.3.3. Considerations for FISH Analysis

Some considerations are needed to improve the results of FISH analysis based on the discussion of the disadvantages of this technique.

1. The membrane of the cell should be sufficiently permeable for the proper penetration of the probes into the cell. The permeability of the cell depends on the proper fixation by using paraformaldehyde.
2. The optimum length of the probe should be 15-20 nucleotides in length. If the probe length is too short, it can hybridize to non-target sites and also can carry fewer labels. Again, if the probe is too long, it will increase the hybridization time.

3. The concentration of the formamide and sodium chloride should be appropriate to get the correct stringency conditions of the FISH analysis. Stringency means the reaction conditions (i.e. temperature, salt, and pH) that dictate the annealing of single-stranded DNA/DNA, DNA/RNA, and RNA/RNA hybrids. At high stringency, duplexes form by using two complementary strands and at low stringency, annealing occurs between strands with some degree of mismatch between bases.
4. Appropriate amount of EDTA should be used in the solution, which contains a large amount of divalent cations (i.e. Ca^{+2}, Mg^{+2}). Otherwise, the divalent cations (i.e. Ca^{+2}, Mg^{+2}) will bind with the probe and interfere in the analysis.
5. Proper filter set and signal amplification systems should be used to reduce the problem created by autofluorescence materials.
6. Specificity of the oligonucleotide probe should be maintained for the dependable and correct FISH analysis.
7. Photobleaching problem should be avoided to maintain brightness and longer life of fluorochromes.

2.3.4. Applications of FISH analysis

FISH for nucleic acids visualization has been developed from an old method called *in situ* hybridizations, which use probe labeled with radioisotopes instead of fluorescent dyes. In 1980, FISH was first applied to identify specific DNA sequence by using a probe prepared from RNA labeled on the 3' end with fluorophore (Levsky and Singer, 2003). In 1989, DeLong first used fluorescently labeled oligonucleotide for the recognition of single microbial cells (Moter & Gobel, 2000). The use of FISH has

increased dramatically in the 1990s (Levsky and Singer, 2003). The FISH technique is applied in different aspects of environmental engineering, subsurface microbiology and medicine.

Environmental Engineering

FISH is a fast, economical, precise and sensitive technique to recognize *Helicobacter* pylori, which is present in water (river and wastewater) and responsible for gastritis, peptic, and duodenal ulcers. When this organism causes infection, it creates gastric mucosa-linked lymphoid tissue lymphoma and adenocarcinoma (Moreno et al., 2003). FISH can identify this bacterium even if some amount of rRNA of this germ is present in the sample. The Hpy-probe, used in FISH technique is very precise and trustworthy to distinguish *H. pylori* from other related bacteria (Moreno et al., 2003; Reza et al., 2006). The results obtained by FISH are not influenced by the delay of the sample to transfer to the laboratory. The sensitivity and specificity of FISH to identify this bacterium collected from the antrum and corpus of the stomachs of dyspeptic patients are 98% and 100% respectively (Reza et al., 2006). Therefore, the diseases caused by *Helicobacter* pylori can be controlled as its presence can be recognized by using FISH (Moreno et al., 2003).

FISH is one of the most commonly used molecular methods to determine microorganisms in the wastewater treatment plants (Coskuner, 2002). FISH identifies the number and spatial distribution of ammonia- and nitrite oxidizing bacteria in nitrifying fluidized bed reactor and in activated sludge (Moter & Gobel, 2000). Among ammonia-oxidizing bacteria, *Nitrosomonas, Nitrosospira, Nitrosovibrio and Nitrosolobus* in activated sludge have been identified in an influent containing inorganic wastewater

(Satoh et al., 2006). FISH is applied to find out bacteria responsible for the removal of phosphorus in the EBPR system. FISH is also used to identify Actinomycetes, which are involved in filamentous foaming and methanogens in anaerobic digesters (Moter & Gobel, 2000; Coskuner, 2002). Recent combined use of FISH and microsensors permit analyses of bacterial communities and metabolic activities concurrently (Moter & Gobel, 2000).

FISH technique combined with microelectrodes provides the information regarding the community structures, spatial distributions and activities of nitrifying and denitrifying bacteria in the biofilms treating the industrial wastewater (Satoh et al., 2006). FISH can identity heterotrophic and ammonia oxidizing bacteria (i.e. *Nitrosomonas*) in the biofilm of about 300 μm when organic wastewater is fed into the system. Different operational conditions are suitable for growing different types of organisms, which can be detected by FISH and thus helps to select appropriate operational condition for better performance of the system (Aoi et al., 2000).

Subsurface Microbiology

FISH is a straightforward and cultivation independent method to determine the normal microbial communities in the subsurface. The microorganisms in the subsurface are normally attached to surfaces called biofilms, and the subsurface water often becomes oligotrophic. For example, this method identifies two gram-negative sulfate reducing bacteria (i.e. a *Desulfuromonas* and a *Desulfovibrio*- related population) in the biofilm of anaerobic fixed-bed bioreactor. Recently, specific probes have been used to determine the population of genus *Paracoccus* in the biofilms of a denitrifying sand filter. FISH can effectively investigate the bacterial community even in very oligotrophic

environments by using carbocyanine dye Cy3 and epifluorescence microscopes (Amann et al., 1997).

Medicine

In recent years, FISH has become an exciting and multipurpose research method (Heng et al., 1997). Moter & Gobel (2000) are hopeful that FISH will be a powerful tool for the diagnosis of human and animal infections microbiologically.

FISH is used for the examination of complex communities of microbe in oral cavity. This technique identifies more than 300 different bacterial species in the oral cavity. For example, oral infections (i.e. periodontitis and gingivitis) are linked with definite microbial consortia such as *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Prevotella intermedia* (Moter & Gobel, 2000).

FISH technique can be applied for the prenatal diagnosis of fetal chromosome disorders, primarily trisomies 21, 13, 18 and sex chromosome aneuploidies (Hulten et al., 2003; Pellestor et al., 2004). These risks are caused by maternal age, maternal serum screening programs and fetal ultrasonography (Hulten et al., 2003). This method is extensively used in the laboratories for the prenatal, postnatal and preimplantation diagnosis due to the simplicity of this method and the availability of numerous probes (Pellestor et al., 2004). Heng et al., (1997) tested 4500 patients for prenatal chromosome aneuploidies by FISH and found an overall detection rate of 73.3% with a 93.9% accuracy of results. Jalal et al., (2001) and Pellestor et al., (2004) established that multicolor fluorescent in situ hybridization (M-FISH) provides each chromosome with a unique color to detect congenital chromosomal abnormality. However, locus-specific

probes are required for subtle chromosomal anomalies (i.e. abnormal segments are 3 Mb or less).

2.4. Image Formation and Processing

In this research, digital images of PAOs and GAOs together with other bacteria were developed by using confocal laser scanning microscope (CLSM). The areas of images presenting PAOs and GAOs and whole bacterial community were determined using image processing software named ImageJ of National Institues of Health, USA. The relative abundance of PAOs and GAOs was calculated from ratios of the areas of PAOs and GAOs to whole bacterial community, respectively. Therefore, the basics of CLSM related to an image formation and factors affecting the quality of an image and also ImageJ software have been discussed in this section.

2.4.1. Image Formation

Murphy, (2001) reported that Marvin Minsky first discovered the principle of image formation of confocal microscope in 1957. To form an image, a laser beam providing the excitation light is reflected by a dichroic mirror and is focused into a small (ideally diffraction-limited) volume of a fluorescent specimen by the objective lens. The laser excites the fluorescence spot of the specimen and starts to emit a mixture of fluorescence light and captured by the objective lens. The fluorescence light is then transmitted through the dichroic mirror and is focused to the pinhole in front of a photomultiplier tube (PMT) detector. The light, which passes through the pinhole, is converted to an analog signal (voltage) in the detector and then transformed into pixels to form digital image by an analog-to-digital converter.

The pinhole aperture restricts the fluorescence light that does not come from the focal point as shown in the Fig 2.10. Thus, the pinhole blocks all of the out-of-focus points generating a true optical section with a thickness of about 0.2 μm (Edwards, 1999) and resulting in a formation of a sharper image compared to conventional fluorescence microscopy. The presence of pinhole also allows creating images of various z axis planes of the specimen. Since the detected light comes from a spot of the specimen, it forms one pixel of the created image. A complete image can be obtained pixel by pixel and line by line due to continuous scanning of the laser in a raster pattern over the specimen (Edwards, 1999). The brightness of the image pixel depends on the relative intensity of the fluorescence light identified by the detector. Thus, a confocal microscope can generate a very sharp image even of thick objects (Edwards, 1999).

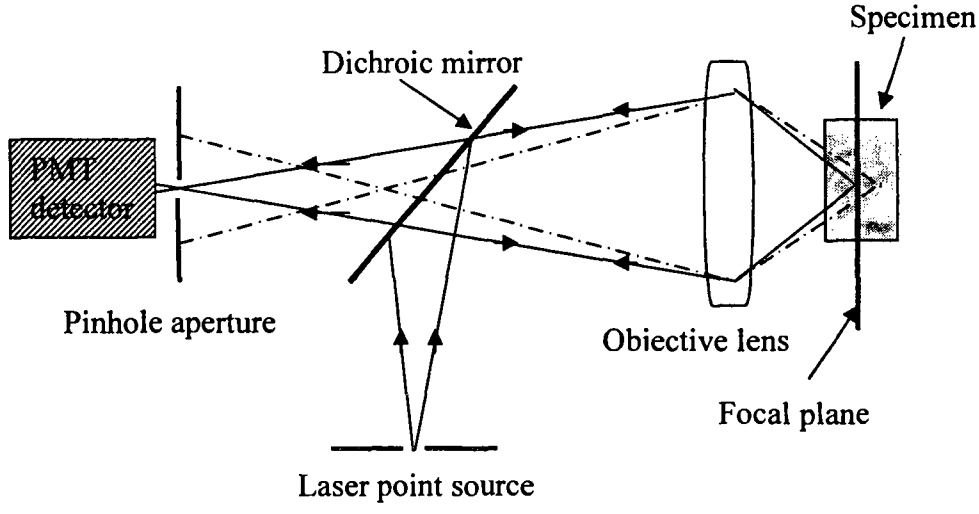


Figure 2.10: Principle of a confocal microscope (modified from Murphy, 2001)

2.4.2. Factors Affecting to the Image Quality

The quality of an image depends on four main factors. These factors include spatial resolution, temporal resolution, resolution of light intensity (dynamic range) and signal-to-noise ratio. In this research, all these factors are expected to have influence on the generation of images. Therefore, the influence of these factors on image formation is discussed in this section.

2.4.2.1. Spatial Resolution

Spatial resolution is the shortest resolvable distance between two points of an image. The resolution depends on numerical aperture of the objective lens, pinhole aperture of the detector, gain and offset of the detector, and wavelengths of excitation and fluorescence emission.

Numerical Aperture of the Objective Lens

The numerical aperture of the objective lens identifies the diffraction-limited spot size of the specimen and focused fluorescent spot size of the pinhole. The objective lens mainly controls the image quality of a confocal microscope. It properly focuses different color wavelengths onto the pinhole. Actually, the numerical aperture of the objective lens identifies the clarity and spatial resolution of the image, and focus length in the specimen. The focal plane thickness along the z-axis in the specimen can be calculated by $\lambda / (NA)^2$ where λ and NA are wavelength and numerical aperture, respectively. The objective lens with high NA reduces the thickness of the focal plane for the pinhole. Again, small pinhole aperture reduces the depth of the confocal optical section. Therefore, lens with high NA and pinhole aperture of small size together can develop the thinnest optical sections.

Pinhole Aperture of the Detector

The pinhole aperture should be adjusted properly because it has influence on the thickness of the focal plane, spatial resolution, brightness of an image, propensity of photodamage. If the size of pinhole aperture is small, optical section becomes thinnest improving horizontal resolution and contrast of the image by reducing out-of-focal-plane light. However, the detector receives less photon due to small pinhole aperture. It causes a longer exposure time of the specimen to the laser in order to get the desired signal-to-noise ratio resulting in considerable amount of photobleaching in the specimen. Photobleaching weakens the fluorescence that generates weak images. On the contrary, a big pinhole increases the photon flux, which decreases photobleaching problem and produces bright and less noisy images. The optimum average pinhole size can be obtained when the aperture gives 50% of maximum intensity. During this time, the pinhole transmits about 75% of the light of the Airy disk, and produces an image with 20% more resolution compared to a wide-field fluorescence system.

Gain and Offset of the Detector

The gain and offset of the detector should be adjusted properly so that light intensities of the image match the dynamic range of the detector. It confirms the presence of maximum gray levels in the photomultiplier tube (PMT). As a result, a photon signal can be represented as shades of gray in the range of black (no signal) to white (saturating signal) in the computer. The dynamic range of 10 or 12 bits means that 2^{10} (1024) or 2^{12} (4096) gray levels, respectively are available in the PMT. Therefore, the corresponding image files in the computer also include similar gray levels. By adjusting offset, a positive or negative voltage can be added to a signal of PMT in order to develop resulting

output signal of about zero volts (black). In contrast, by adjusting gain, input signal can be augmented by voltage multiplication method that provides the output signal with an increased gray level value. Normally, offset should be adjusted first followed by setting of gain. In practice, red and blue colors of the image represent the saturated pixels and black-level pixels, respectively. If a few red and blue pixels are observed in the image after proper adjustment, the whole dynamic range of the PMT is being utilized (Murphy, 2001).

Wavelengths of Excitation and Fluorescence Emission

The excitation wavelengths (λ_1) and emission wavelengths (λ_2) both have influence on the spatial resolution. The cut-off of spatial frequency is proportional to ($1/\lambda_1 + 1/\lambda_2$). Thus, resolution reduces with the increase of wavelength (Sheppard and Shotton, 1997).

2.4.2.2. Temporal Resolution

Temporal resolution depends on the rate of scanning and processing rate of the detector, analog-to-digital converter and computer (Murphy, 2001). Again, scan rate has direct influence on the photobleaching rate of the specimen and the signal-to-noise (S/N) ratio of the image. Slow scanning increases photobleaching rate of the fluorophore (Edwards, 1999) whereas faster scan rate decreases the S/N ratio. Slow scan rate permits the laser to remain for a long time on live fluorescence specimen causing damage to the cell. Normally, frames are received at a scanning rate of 2 frames per sec for an image of 512x512 pixels. However, rates of 100 frames per sec or above can be obtained for special images (Murphy, 2001).

2.4.2.3. Resolution of Light Intensity (Dynamic Range)

Dynamic range (DR) describes the number of gray levels of an image determined by the analog-to-digital converter. The detector DR determines the maximum amount of DR required to obtain an image that is from black (no signal) to white (saturating signal).

2.4.2.4. Signal-to-Noise Ratio

The degree of visibility or transparency of an image can be determined by the signal-to-noise (S/N) ratio. The S/N ratio is identified by using the intensities of the object and background for bright images whereas electronic noise is a dominant factor for dim images. A moderate to bright specimen can produce 50 -100 photons per pixel per sec which gives a S/N ratio of 25 for a sensitive confocal system. On the other hand, video and digital camera can have a maximum S/N ratio of 100 to several hundred. Therefore, the image quality of a bright specimen is not good or excellent in confocal system. The S/N ratio can be improved if the amount of light can be increased by decreasing the scan speed or opening the pinhole (Murphy, 2001).

2.4.3. Image Processing

Image processing is important to present and illustrate the details of an image to others (Murphy, 2001). To understand and measure specific features in an image, it is necessary to select the features by a particular range of brightness level using a histogram. The brightness histogram is a diagram of number of pixels with associated brightness values. For example, the brightness levels are up to 2^8 or 256 grey scale values for a typical 8-bit monochrome (black/grey/white image). The pixels within the selected range are considered to be a foreground and all other pixels are included in the backgrounds. This type of image can be represented as a binary image or two-level

image. Normally, black and white colors are used to express the pixels in the foreground and backgrounds, respectively. This process is known as thresholding and resulting images are binary images. Therefore, the purpose of binarization is to isolate the features from the backgrounds in order to measure them (Russ, 2002).

Sometimes, noise is present in the image and can make error in the measurement of specific features. The noise can be removed from the binary images by a combination of erosion followed by a dilation called opening. Erosion removes noisy pixels from features in an image, which have been selected by thresholding due to brightness value in the range of interest. They can have that brightness value accidentally due to a finite noise in the image. Erosion can remove all impertinent pixels resulting from point noise or line defects due to a single pixel. However, the classical erosion removes any pixel touching another pixel that is part of the background. As a result, a layer of pixels from the boundary of all features can be removed resulting in a reduction of size and sometimes, a break down of the features into parts. Therefore, dilation is performed to add pixels at the periphery of the features that have been deleted by erosion (Russ, 2002). After removing noisy pixels from the binary images, the area of the thresholded pixels is determined.

CHAPTER 3

WORK PLAN

3.1. Experimental Approach

The first hypothesis for this research is that butyrate and glucose will select for specific PAOs and GAOs not yet reported in systems fed exclusively with glucose and butyrate. To test hypothesis one, the laboratory-scale sequencing batch reactor (SBR) shown in Figures 3.1 and 3.2 was used. The SBR system contains two 8-liter sequencing batch reactors (SBRs). The SBRs were inoculated with activated sludge collected from the full-scale EBPR system of the Clark County Water Reclamation District (CCWRD) plant. They were fed with synthetic wastewater for a period of about 3 months per each carbon source. The performance of the system was evaluated by chemical analyses including orthophosphate (OP), carbon sources as soluble total organic carbon (sTOC), suspended solids (SS), volatile suspended solids (VSS), dissolved oxygen (DO), pH and microbiological analysis with Fluorescence in Situ Hybridization (FISH).

The second hypothesis for this research is that the use of supplemental carbon source would improve phosphorus uptake under aerobic conditions, in EBPR systems, when denitrification is introduced to the anoxic zone. To test hypothesis two, several batch tests (Figures 3.4 and 3.5), using wastewater from an actual wastewater treatment plant, were performed using acetate and propionate to identify the carbon source that would result in the best phosphorus removal. The phosphorus uptake under aerobic condition, in

presence of nitrate in the anoxic zone, was evaluated by measuring OP, NO_3-N, and sTOC with time during the batch tests.

3.2. Experimental Methods to Test Hypothesis One

3.2.1. Laboratory Set Up of SBRs

A laboratory setup has been built consisting of two 8-liter lab-scale SBRs. The operation of the reactors was automated using two timers, solenoid valves and peristaltic pumps. The reactors were connected to a feeding tank, air and nitrogen gas supply sources. Besides, a mixer was attached to each reactor, and an air/nitrogen diffuser was placed at the bottom of the reactor. Figures 3.1 and 3.2 show the schematic and experimental set up of one reactor established in the laboratory.

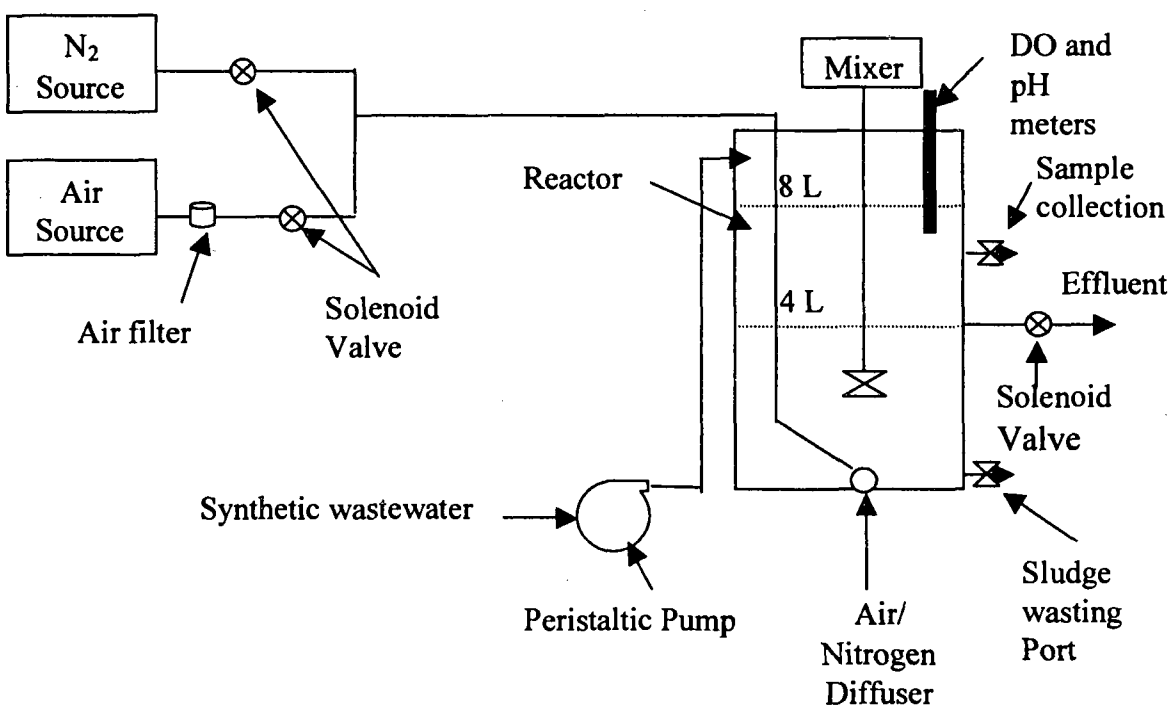


Figure 3.1 Schematic of one SBR in the laboratory

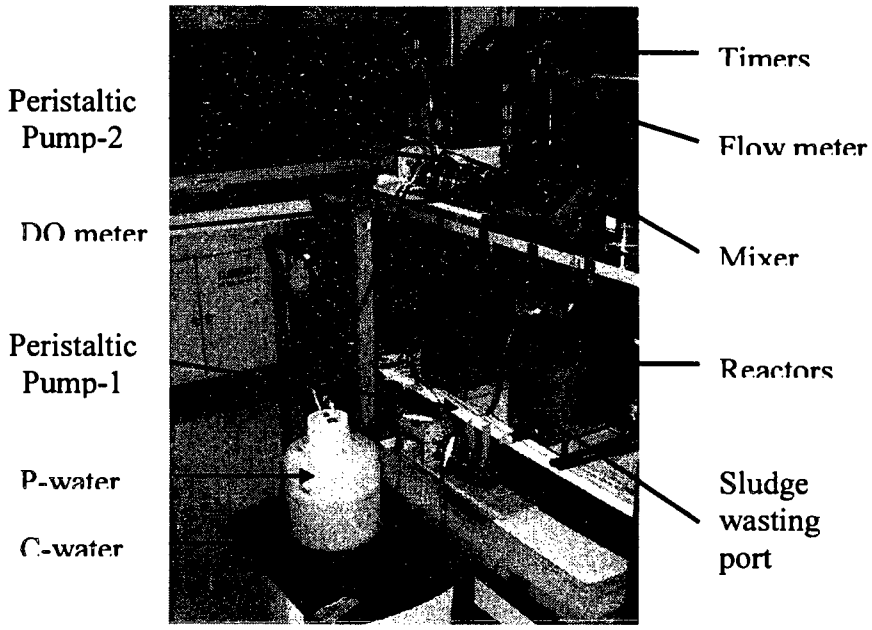


Figure 3.2: Experimental setup of the SBRs fed exclusively with glucose

3.2.2. Preliminary Design of SBRs

Reactors were run according to the preliminary design, summarized in Table 3.1. Adjustments of design parameters were performed during operation of SBRs as needed and are noted in the test results.

Table 3.1: Parameters used in the preliminary design of SBRs

Parameters	**Design value**
Influent flow	12 L/d
Influent COD	400 mg/L
Influent phosphorus	10 mg/L
Effluent COD	0.02 mg/L
Effluent phosphorus	3 mg/L
SRT	18 days
HRT	0.667 days (16 hours)
MLVSS	2500 mg/L
Volume of reactor	8 L
SVI	71 ml/g

3.2.3. Operation of SBRs

Four SBRs (B1 and B2 for butyrate and G1 and G2 for glucose) were operated under similar conditions using butyrate and glucose, separately, as sole carbon sources for a period of 92 days (butyrate) and 78 days (glucose). The run times were selected based on the results obtained by (Machado 2004). He observed that Competibacter decreased throughout the study period of 58 days. In addition, he found a large variation in the number of Accumulibacter whereas phosphorus removal was above 80% up to 32 days. Moreover, both the number of PAOs and the phosphorus removal decreased dramatically after 32 days. Due to time limitation, he was not able to identify the reasons of degradation of phosphorus removal after 32 days. Therefore, in this research, the SBRs were operated for 92 and 78 days to investigate the behavior and microbiology of the EBPR system using butyrate and glucose as a sole carbon source.

The SBRs were inoculated with biomass from an aeration basin of a local wastewater treatment plant that performs EBPR and were filled with synthetic wastewater (i.e. C-water) (Table 3.2) and phosphate solution (i.e. P-water) at flow rates of 254 ml/min and 40 ml/min, respectively. The SBRs were operated in 3 cycles of 8 hours. Each cycle had five steps, which were carried out in the following order: fill (15 minutes), anaerobic period (2.5 hours), aerobic period (4.5 hours), settle (30 minutes) and withdraw (15 minutes). At the beginning of the anaerobic period, N_2 gas was supplied and dispensed as bubbles into the reactors for 2 minutes at a flow rate of 13 standard cubic feet per hour (SCFH) in order to create anaerobic condition (i.e DO concentration less than 0.2 mg/L). During aerobic period, air was sparged into the reactors using ceramic stone diffuser at a flow rate of 8 SCFH to maintain a DO concentration greater than 2 mg/L. After settling

of sludge, 4 liters of wastewater was withdrawn from the reactors by gravity through effluent outlets and about 209 ml of settled sludge was collected through sludge wasting ports to maintain a SRT of 8 days at room temperature (22 ± 2° C). The average concentrations of mixed liquid suspended solids (MLSS) were 2431 and 2368 mg/L in B1 and B2 reactors, and 2393 mg/L and 2353 mg/L in G1 and G2 reactors, respectively. The percentage of MLVSS was about 70% in the butyrate reactors and 89% in the glucose reactors.

3.2.4. Preparation of Synthetic Wastewater for the SBRs

The C-water and P-water were prepared according to the synthetic wastewater's composition of Oehmen et. al.'s (2005a). The C-water contained carbon, nitrogen and nutrient sources whereas the P-water contained only phosphorus (Table 3.2). Both C and P-water were supplied into the reactors separately in order to avoid biodegradation in the tubing and precipitation of phosphorus.

Table 3.2: Composition of synthetic wastewater adapted from Oehmen et. al., (2005a). and Machado (2004)

Elements	**Amounts**
Carbon (i.e. propionate, butyrate, glucose)	400 mg COD/L
Nitrogen (i.e. NH_4Cl)	28 mg /L as N
Phosphorus (i.e. $NaHPO_4.H_2O$)	10 mg P /L for glucose 13.3 mg P/L for butyrate
Magnesium (i.e $MgSO_4.7H_2O$)	10 mg /L as Mg
Calcium (i.e. $CaSO_4.2H_2O$)	4 mg /L as Ca
Yeast Extract	1 mg/L
Trace elements*	0.3 ml/L

*Trace elements (g/L): 1.5 g $FeCl_3.2H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 $MnCl_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$ and 10 g EDTA)

3.2.5. Sample Collection, Preservation and Analysis

Samples were collected and preserved according to Table 3.3 and Table 3.4.

3.2.5.1. Collection and Preservation of Samples

Six hundred eighty-ml of sample were collected during a cycle (Table 3.3). To avoid phosphorus release after a sample was taken, about 15 ml samples were immediately filtered through 0.45 μm syringe filter (GHP Acrodisc, PALL, East Hills, NY) and refrigerated. The samples were analyzed on the same day of collection or preserved when necessary. The preservation techniques used for different analysis are presented in Table 3.4. In addition, Table 3.5 describes a weekly schedule for sample analysis.

Table 3.3: Schedule for sample collection during a cycle of the SBR run

Time (min)	Sample volume (ml) for different tests				Actual volume (ml)	Replicate volume (ml)	Total volume (ml)	Phase total (ml)
	OP*	sTOC**	SS/VSS***	FISH****				
Anaerobic phase								380
0	5	2	-	-	15	15	30	
30	5	2	-	-	15	15	30	
60	5	2	-	-	15	15	30	
90	5	2	-	-	15	15	30	
120	5	2	-	-	15	15	30	
150	5	2	50	-	115	115	230	
Aerobic phase								300
30	5	2	-	-	15	15	30	
60	5	2	-	-	15	15	30	
120	5	2	-	-	15	15	30	
180	5	2	-	-	15	15	30	
270	5	2	-	3	20	20	40	
Withdraw phase	5	2	50	-	70	70	140	
Grand total:								680

Table 3.4: Techniques for sample preservation

Type of Test	**Type of Sample**	**Preservation of Sample**
OP	Centrifuged/ Filtered mixed liquor (4000 rpm for 15 minutes/ 0.45 μm membrane filter)	Store at 4° C (maximum storage 48 hours)
sTOC	Centrifuged/ Filtered mixed liquor (4000 rpm for 15 minutes/ 0.45 μm membrane filter)	Adjust sample pH between 2 - 3 using 2 N HCl and store at 4° C (maximum storage 28 days)
SS/VSS	Mixed liquor	Analyze samples in the same day (no preservation)
NH_3-N	Mixed liquor	Store at 4° C (maximum storage 48 hours)
NO_3-N	Mixed liquor	Store at 4° C (maximum storage 48 hours)
NO_2-N	Mixed liquor	Store at 4° C (maximum storage 48 hours)
FISH	Mixed liquor	Fix, centrifuge and store at -20° C in an ethanol solution

Table 3.5: Weekly schedule for sample analysis collected from laboratory-scale SBRs

Day	Chemical analysis					Microbiological analysis
	pH	DO	OP	SS/VSS	sTOC	FISH
1	√	√	√	√	√	√
2				√		
3	√	√	√	√	√	√
4				√		
5	√	√	√	√	√	√
6				√		
7						

3.2.5.2. Analysis of Samples

3.2.5.2.1. Chemical Analysis

Samples were analyzed for orthophosphate (OP), soluble total organic carbon (sTOC), and suspended and volatile suspended solids (SS/VSS) at the Environmental Engineering Laboratory of UNLV. Orthophosphate, nitrate-N, nitrite-N, and ammonia-N were measured using HACH (Loveland, Colorado) kits with a DR/3000 spectrophotometer. Soluble TOC was analyzed according to the Standard Method 5310-B (Eaton et al., 2005) using a TOC Analyzer (Model TOC-$V_{CPH/CPN}$, SHIMADZU). Standard Methods 2540D and 2540E were used to measure total suspended solids (SS) and volatile suspended solids (VSS), respectively using a 47mm Whatman GF/C microfiber glass filter. Filtered samples were utilized for OP and sTOC analysis whereas the unfiltered samples were used for TP and SS/VSS analysis. SS/VSS was measured on the same day of sample collection. Dissolved Oxygen (DO) was measuring using an YSI Model 54A DO meter. The pH was measured using an Accumet, AR25 pH meter.

3.2.5.2.2. Microbiological Analysis

a) FISH procedure

Unfiltered samples were used for Fluorescence in Situ Hybridization (FISH) analysis. The procedure for FISH analysis was adopted from Amann (1995) and modified by De Los Reyes (2003). For gram-negative bacteria, 3ml of the sample were mixed with 9 ml of 4% ice-cold paraformaldehyde (96%, Fisher Scientific) in a 15 ml plastic centrifuge tube (VWR) for fixation. Fixation is required to maintain the morphological integrity of the cells and to minimize the auto-fluorescence (Amann, 1995). The sample was then kept in the refrigerator at 4°C for 2.5 hours. After fixation, the sample was washed two

times with 1 x phosphate buffer saline (PBS) solution (1 volume of 3 x PBS; 390 mM NaCl in 30 mM $NaPO_4$ buffer and 2 volume DI water) by spinning the sample in a centrifuge (SORVALL, Legend RT) at 2000 rpm for 5 minutes to remove the fixative solution. The sample was stored in the refrigerator at -20°C by adding 1x PBS/ethanol (1:1) solution. For gram-positive bacteria, 1 volume of sample was mixed with 1 volume of 50% ethanol (v/v) (95%, IBI-Scientific, IA) and the sample was stored at –20°C (Kong et. al., 2005 and personal communication with Simon McIlroy, referred by Dr. R. Seviour, Biotechnology research center, La Trobe University, Bendigo, Victoria, Australia). Next, 3 µl of sample was applied into three wells of a 6-well Teflon-coated microscope slide (Cel Line, Portsmouth, NH) to immobilize the cell. The sample of the slide was air dried for about 45-50 minutes. Afterwards, it was dehydrated by successive dipping into 50%, 80% and 95% ethanol in staining jars (3 minutes per step) and air dried for 8-10 minutes. Next, 8 µl hybridization buffer, 1 µl of EUB bacteria probe and 1 µl the desired PAO or GAO probes were applied to three wells. The details of the hybridization buffer are given in Table 3 in Chapters 6 and 7. To hybridize the samples, the slide was inserted into a properly sealed moist chamber, which was kept in an oven (Millipore, Billerica, MA) at 46°C for 60-120 minutes without shaking. The moist chamber was built in-house using a 50 ml centrifuge tube and a piece of Whatman filter paper wetted with 0.5 ml of hybridization buffer. A properly sealed moist chamber is necessary for hybridization to avoid evaporation of hybridization solution, which leads to nonspecific binding of the fluorescent probe to the cells (Amann, 1995). After hybridization, the sample was washed with 50 ml wash solution in a water bath (Model AP-152 from SOILTEST, Lake Bluff, IL) at 48°C for 20 minutes. The details of the wash solution are

given in Table 3 in Chapters 6 and 7. The slide was dipped again in 50 ml centrifuge tube containing ice-cold deionized water for 3 seconds and air dried until all water droplets are removed. Finally, the slide was mounted with a microcover glass (24 X 60mm, VWR Scientific) by using Citifluor mountant media (Ted Pella Inc., Redding, CA). The basic steps for FISH analysis are illustrated in Figure 3.3.

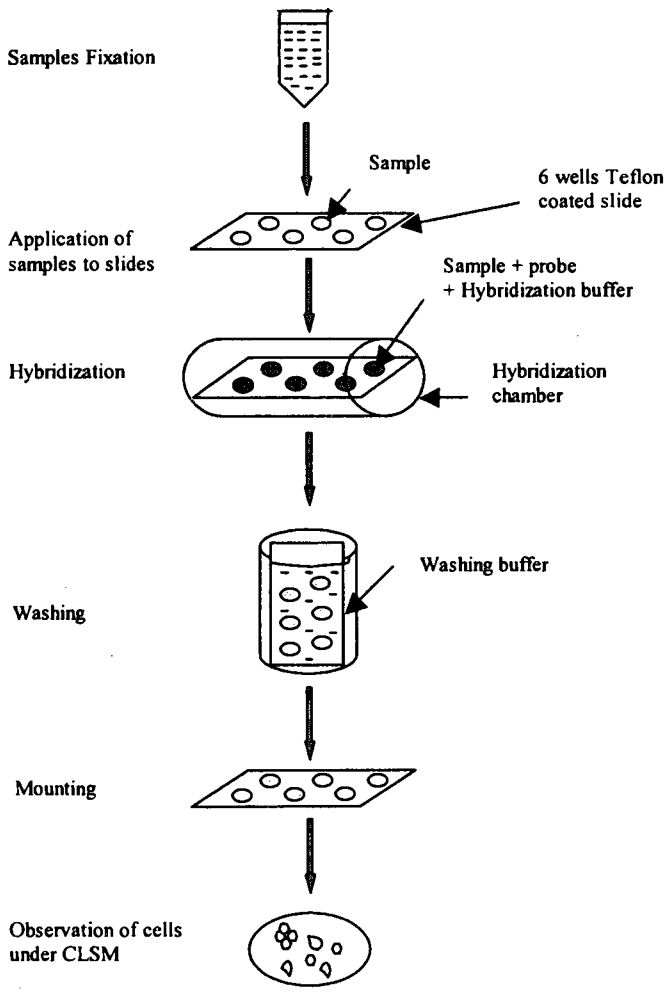


Figure 3.3: Steps of sample preparation and hybridization for FISH analysis (Source: modified from Moter and Gobel, 2000)

In the FISH analysis, the PAOMIX probe (i.e. PAO462, PAO651, PAO846) (Crocetti et. al., 2000) and MP2 (Kawaharasaki et al., 1999) were used to detect *Candidatus Accumulibacter Phosphatis* and *Microlunatus Phosphovorus* (i.e. PAOs) respectively. Moreover, GAOMIX probes (i.e. GAOQ431, GAOQ989) (Crocetti et. al., 2002) and MIC184 (Kong et. al., 2001) were utilized for targeting *Candidatus Competibacter Phosphatis* and *Micropruina glycogenica* (i.e. GAOs) respectively. In addition, in the butyric acid reactor, two other probes, TFOmix (tetrad forming organisms mix – TFO_DF218 and TFO_DF618) and Actino (Actino_658) were used (Table 3.8). The probes for targeting PAOs and GAOs were labeled by Cy3. The EUBMIX probe (i.e. EUB338, EUB338-II and EUB338-III) (Crocetti et. al., 2002) labeled with Cy5 was used to target the entire bacterial community, because only domain-specific probe EUB 338 is not sufficient to detect all bacteria (Daims et. al., 1999). Cy3 and Cy5 are fluorescent dyes of cyanine group with fluorescent color orange-red and near infrared, respectively use to visualize the microbes under the microscope. All probes used in this research were purchased from Integrated DNA Technology, Inc. (Coralville, IA) with HPLC purification. The details of these oligonucleotide probes are given in Table 3.8.

b) Specificity test of GAO and PAO Probes

To assure that the purchased probes were specific to the targeted GAOs and PAOs, two measures were taken. First, the purchased sequence was checked with the microbial sequence database BLAST (Basic Local Alignment Search Tool) available from NIH online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligonucleotide sequences were compared with a database of sequences (i.e. Nucleotide collection, nr/nt) to detect sequences with high identity. This operation was performed for all probes and a match

varying from 87-95%, 93-100% and 94-100% was found for PAO462, Actino_658 and EUB338 probes, respectively. All other probes matched 100%. The second check of specificity was performed using an enrichment culture available in our laboratory that is known to degrade perchlorate. A sample of the culture was taken and prepared for FISH as described above. Every probe to be used was tested with the sample and no hybridization occurred for all GAO and PAO probes while the EUB bacteria hybridized.

c) Microscopy and microbial quantification

For a sample, twenty digital images (ten images/well) of PAOs and GAOs were captured from two wells using confocal laser scanning microscope (Zeiss LSM510, Axioplan 2) using Argon (488, 514 nm) and HeNe (633 nm) lasers for the excitation of dyes Cy3 and Cy5, respectively. The emission filters for Cy3 and Cy5 were 530-600 nm and 650 nm, respectively. 400X magnification was used to observe the microbes under the microscope. To quantify the organisms, the images were analyzed using the software ImageJ available free of charge from NIH (National Institute of Health). For an image, the % relative abundance (RA) of PAOs/GAOs was calculated based on the ratio of the area of PAOs/GAOs to the entire bacterial population. The average % RA value of twenty images was considered as the final % RA of PAOs/GAOs in the entire bacterial population. Statistical analysis was performed between the mean %RA of ten images of two wells in a slide based on a two-tailed independent-samples t test at a 95% confidence interval. The null hypothesis was that there was no difference between means % RA of PAOs/GAOs in two wells of a slide. The alternate hypothesis was that there was difference in the mean %RA of PAOs/GAOs in two wells of a slide. In addition, the final % RA (i.e. mean of twenty images) of PAOs/GAOs between G1 and G2 reactors was

compared statistically for each sampling day. Moreover, to compare the % RA of PAOs/GAOs in replicate reactors fed with glucose, mean value of %RA of PAOs/GAOs of each reactor was determined and compared statistically as described above. Finally, the standard error of mean %RA of PAOs/GAOs was determined between replicate reactors fed with glucose.

3.3. Experimental Methods to Test Hypothesis Two

3.3.1. Experimental set up of batch tests

Batch tests were performed to investigate hypothesis two of this research using actual wastewater from a full-scale EBPR system. The batch tests were set-up in a laboratory located about 300 feet from the wastewater treatment plant, to minimize any variation due to wastewater storage and transportation. The experimental set-up used for the batch tests is illustrated in Figures 3.4 and 3.5. A three-liter glass reactor was utilized for the batch tests. The reactor was covered with a glass plate containing three openings that housed a DO meter, air inlet, and a sampling port. The reactor was placed on a magnetic plate for mixing with a magnetic stirrer. During the aerobic period, air was supplied to the reactor through a narrow tube connected to a pressure pump fitted with a flow meter, which regulated the flow rate of air into the reactor. A ceramic air dispenser was used at the end of the tube to disperse the air uniformly into the reactor. A DO meter (YSI Model 54A) was connected to the reactor for continuous monitoring of the DO concentration.

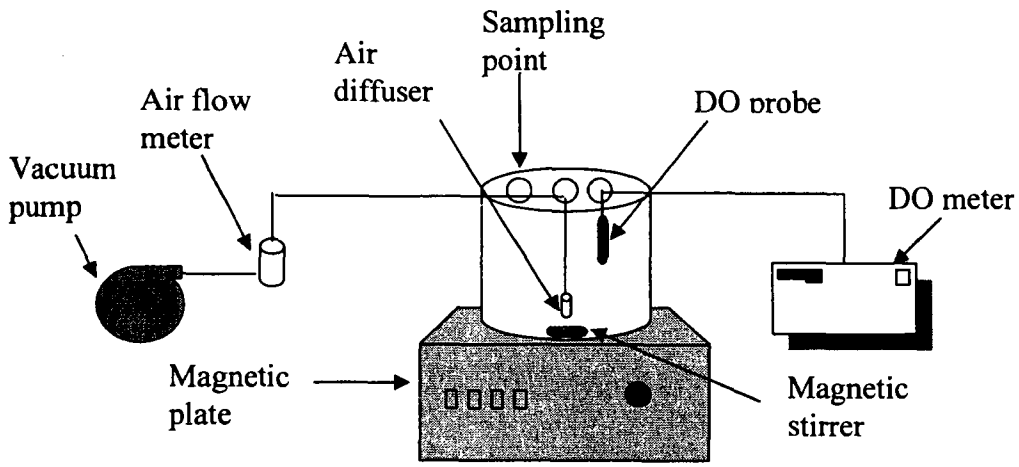


Figure 3.4: Schematic of the set up used in the batch tests

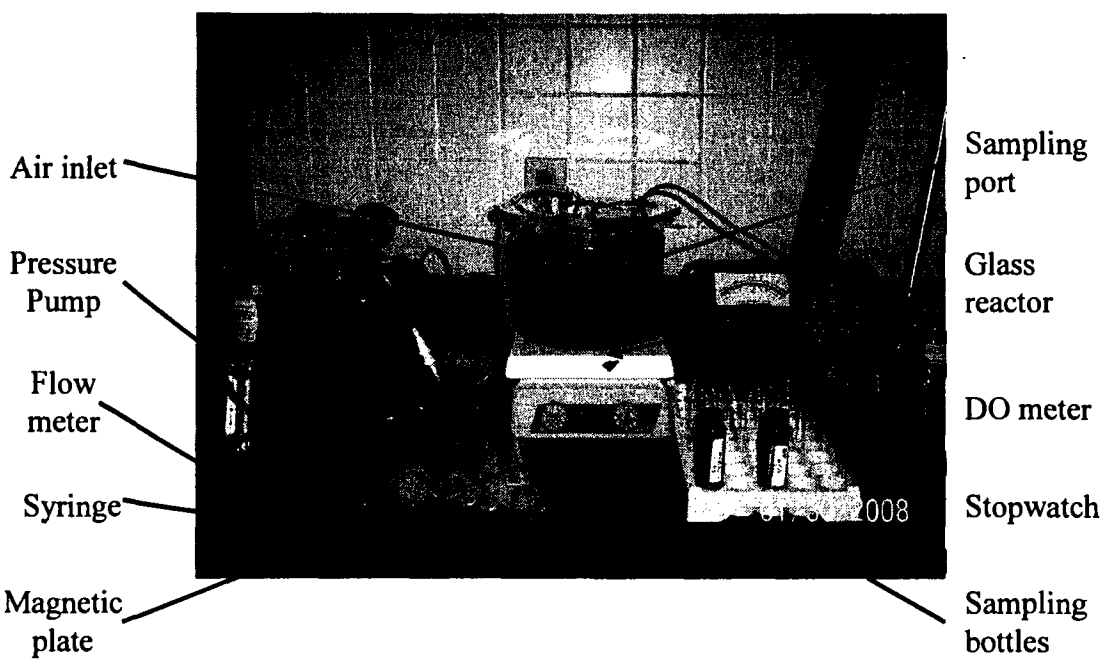


Figure 3.5: Experimental setup assembled for the batch tests

3.3.2. Experimental procedure of batch tests

The experimental set-up simulates a typical EBPR system containing several aerobic, anaerobic and anoxic zones and liquor return to accomplish denitrification (Figure 3.6). A 1,500 mL sample (equivalent to 1.3 Q return flow) of mixed liquor return (MLR) and 1,146 ml (Q) of mixed liquor from the end of ANA2 zone (Figure 3.6) was collected during the experiments. This mixture represents the MLR/Q ratio used in the full-scale plant. The samples were mixed for 40 minutes (i.e. the total HRT of anoxic condition) in the absence of oxygen and aerated for about 140 minutes (i.e total HRT of aerobic condition). The duration of anoxic and aerobic periods was similar to those of a full-scale wastewater treatment plant. The DO concentration under aerobic condition varied from 5 to 7 mg/L, which corresponds to those of zones AE 5 to AE 6 of basin AB #9.

Two carbon sources, acetate and propionate were used to investigate the phosphate uptake under aerobic condition in the presence of denitrification. In the case of acetate and propionate, the minimum concentrations to be used were selected from stoichiometric carbon source requirements based on thermodynamic computations for nitrate, 3.66 mg acetate/mg NO_3-N and 2.82 mg propionate/mg NO_3-N, respectively (Rittmann and McCarty, 2001). The acetate and propionate concentrations were also increased to five and ten times the stoichiometric ratios need for denitrification. Duplicate tests were performed for varying amount of acetate and propionate. Tables 3.6 and 3.7 summarize the operational conditions and wastewater composition of the various batch tests. The batch tests were performed at different days and wastewater samples were used immediately after collection of mixed liquor from the aeration basins. In some occasions, the solids concentration varied in the basins during the experimental period.

The variation of MLSS concentration was considered in the analysis of the results obtained. All tests were performed in duplicate and average values and their standard deviations are reported. The average values of duplicate tests were used in reporting the results.

Table 3.6: Operational conditions of batch tests

Batch Test No.	Replicate	Carbon source	Sampling Location and Volume	Reaction Condition and Time
B1	1 2	Wastewater without external carbon	MLR-1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B2	1 2	Acetate (X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B3	1 2	Acetate (5X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B4	1 2	Acetate (10X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B5	1 2	Propionate (X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B6	1 2	Propionate (5X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B7	1 2	Propionate (10X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min

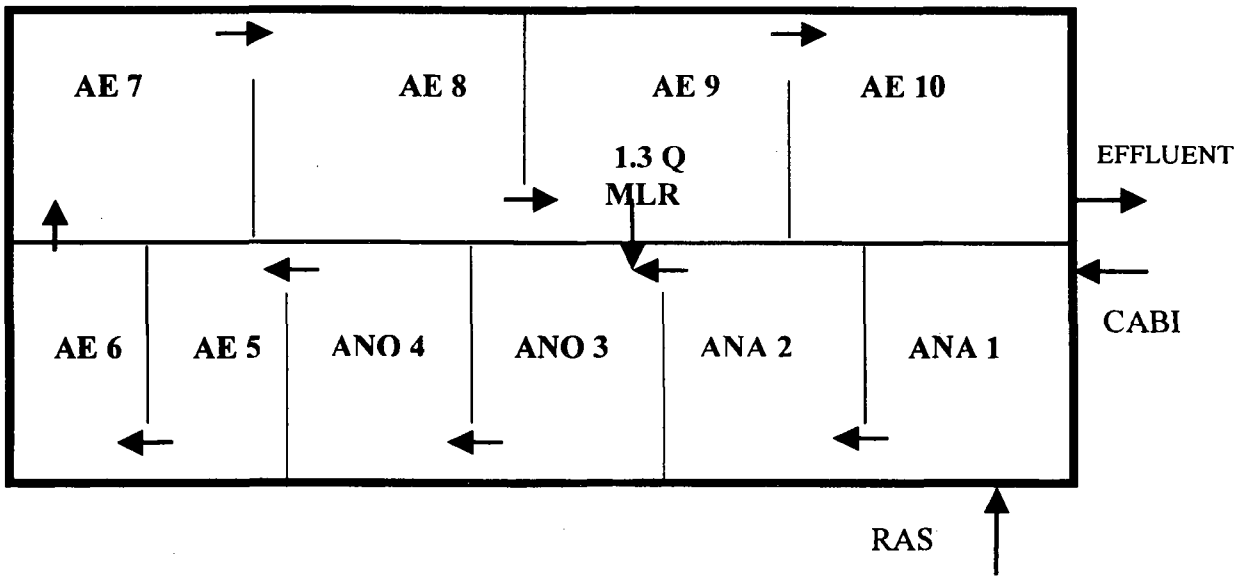


Figure 3.6: Schematic of Aeration Basin # 9 of CCWRD plant used for the EBPR with denitrification

Table 3.7: Composition of the mixture of wastewater and supplemental carbon sources

Parameters	Units	Acetate			Propionate			WW only
		X	5X	10X	X	5X	10X	-
NO_3-N	mg/L	8.5	10	6.8	7.7	11	8.7	10.3
OP	mg P/L	8.5	4.6	4.4	7.4	6.9	3.9	8.2
COD	mg/L	64	220	398	59	244	392	27
C/N ratio	-	7.6	22	59	7.7	22	45	3
MLSS	mg/L	3.414	2.66	3.546	3.22	3.196	2.45	3.499
VSS	mg/L	2.73	2.13	2.84	2.58	2.56	1.96	3

Table 3.8: Oligonucleotide probes used in this research

Probe	Specificity	rRNA Target site	Sequence (5'-3')	FA* (%)	Reference
EUB338	Bacteria		GCTGCCTCCCGTAGGGT	30	Crocetti et al., 2002
EUB338-II	*Planctomycetales*	16S, 338-355	GCAGCCACCCGTAGGTGT		Crocetti et al., 2002
EUB338-III	*Verrucomicrobiales*	16S, 338-355	GCTGCCACCCGTAGGTGT		Crocetti et al., 2002
			PAOs		
PAO462	*Candidatus* Accumulibacter phosphatis	16S, 462-485	CCGTCATCTACWCAGGGTATTA AC	30	Crocetti et al., 2000
PAO651	*Candidatus* Accumulibacter phosphatis	16S, 651-668	CCCTCTGCCAAACTCCAG	30	Crocetti et al., 2000
PAO846	*Candidatus* Accumulibacter phosphatis	16S, 846-866	GTTAGCTACGGCACTAAAAGG	30	Crocetti et al., 2000
MP2	*Microlunatus phosphovorus*	16S , 68-87	GAGCAAGCTCTTCTGAACCG	10	Kawaharasaki et al., 1999
Actino_658	Actinobacterial PAO		TCCGGTCTCCCCTACCAT	40	Kong et al., 2005
			GAOs		
TFO_DF218	Defluvicoccus-related TFO in α-proteobacteria	16S, 218-235	GAAGCCTTTGCCCCTCAG	25-35	Wong et al., 2004
TFO_DF618	Defluvicoccus-related TFO in α-proteobacteria	16S, 618-635	GCCTCACTTGTCTAACCG	25-35	
MIC184	*M. glycogenica*		CATTCCTCAAGTCTGCC	20	Kong et al., 2001
GAOQ431	*Candidatus Competibacter Phosphatis*	16S, 431-448	TCCCCGCCTAAAGGGCTT	35	Crocetti et al., 2002
GAOQ989	*Candidatus Competibacter Phosphatis*	16S, 989-1006	CACCTCCCGACCACATTT	35	Crocetti et al., 2002

* FA-Formamide

3.4. Analytical Methods

The analytical methods used to analyze the samples were: orthophosphate, soluble TOC, SS/VSS analysis, NH_3-N, NO_3-N, NO_2-N and FISH analysis.

a) Orthophosphate (OP)

Orthophosphate was measured using the PhosVerR 3 Method 8048, Test'N TubeTM procedure of HACH (Loveland, CO). This procedure is similar to Method 365.2 of USEPA and Standards Method 4500-P E (ascorbic acid method). In this method, 4 ml deionized (DI) water were mixed with 5 ml of centrifuged sample (4000 rpm for 15 minutes) into a test tube. To prepare a blank, 5 ml DI were used instead of the sample. The reagent from a P-RGT labeled pillow was added to the mixture. The tube was capped properly and shaken for 20 seconds to mix the solution. If the sample contained phosphate, a blue color would develop. After five minutes, OP was measured by using a Hach DR/3000 spectrophotometer at a wavelength of 890 nm. Manual mode was used for the analysis. A concentration factor of 1.8 or 5.52 was used for mg/L as P or PO_4, respectively.

b) Soluble Total Organic Carbon (sTOC)

Soluble TOC was analyzed according to Standard Method 5310-B (Eaton et al., 2005). Samples were measured for NPOC using a Total Organic Carbon (TOC) Analyzer (Model TOC-$V_{CPH/CPN}$, SHIMADZU). The TOC analyzer was controlled by computer software named TOC-Control V. Samples were acidified to obtain pH between 2 to 3. Samples were filtered through 0.45 μm membrane filter before analysis. Five points (i.e. 0 mg/L, 1 mg/L, 2 mg/L, 4 mg/L and 5 mg/L) calibration curve were prepared

using standard solutions of potassium hydrogen phthalate. An auto sampler (ASI-V, SHIMADZU) was used for the automatic injection of samples.

c) Solids

Standard methods 2540D and 2540E were used to measure total suspended solids (SS) and volatile suspended solids (VSS) respectively. Appropriate volume of sample was filtered through a 47 mm Whatman glass microfiber filter GF/C. The filter with settled solids was dried at 103-105°C for 1 hour to measure total suspended solids. Later, the sample was ignited in the muffle furnace at 550°C for half an hour to measure volatile suspended solids.

d) Nitrate Nitrogen (NO_3-N)

Nitrate nitrogen was measured using the Chromotropic Acid Method 10020, Test'N Tube TM procedure of HACH. In this method, 1 ml sample (DI for blank) was added to each vial. The vial was capped and inverted ten times to mix. The content of one NitraVer X reagent B powder pillow was added to the vial and again inverted ten times to mix. The reaction time was 5 minutes. A yellow color generated if nitrate nitrogen was present. The concentration of NO_3-N was measured by a HACH DR/4000 spectrophotometer and 410 nm wavelength.

e) Ammonia Nitrogen (NH_3-N)

Two methods were used for the measurement of ammonia. When the expected concentration was high, ammonia nitrogen was measured using the Salicylate Method 10031, Test'N Tube TM procedure of HACH. On the other hand, Salicylate Method 10023, Test'N Tube TM procedure of HACH was used for low concentration of NH_3-N. In case of Salicylate Method 10031, 0.1 ml sample (DI for blank) was added to each vial.

The contents of an ammonia salicylate and an ammonia cyanurate reagent powder pillows were added to each vial. The vial was capped tightly and mixed thoroughly to dissolve the powder, and placed in a rack for the reaction time of 20 minutes. If ammonia was present, a green color developed. In case of Salicylate Method 10023, sample preparation was similar to the Salicylate Method 10031 except sample (DI for blank) volume was 2 ml. The concentration of NH_3-N was measured by a HACH DR/4000 spectrophotometer at 655 nm wavelength.

f) Nitrite Nitrogen (NO_2-N)

Nitrate nitrogen was measured using the Diazotization Method 10019, Test'N Tube TM procedure of HACH. In this method, 5 ml sample (DI for blank) was added to each vial. The vial was capped and shaken properly to mix the powder, and placed in a rack for 20 minutes. A pink color developed if nitrite was present. The concentration of NO_3-N was measured by a HACH DR/4000 spectrophotometer at a wavelength of 507 nm.

CHAPTER 4

DATA ANALYSIS AND QA/QC

4.1. Introduction

In this research, chemical and microbiological analysis was performed to study the performance of the laboratory-scale EBPR systems. In addition, chemical analysis was performed to investigate the impact of denitrification and different substrate levels on phosphorus uptake in EBPR process. Chemical analyses included orthophosphate (OP), soluble total organic carbon (sTOC), mixed liquid suspended solids (MLSS), mixed liquid volatile suspended solids (MLVSS), nitrate –nitrogen (NO_3-N), ammonia – nitrogen (NH_3-N), and nitrite-nitrogen (NO_2-N). Microbiological analysis included FISH analysis to identify specific PAOs and GAOs involved in laboratory-scale SBRs fed with butyrate and glucose.

4.2. Analysis of Chemical and Microbiological Data to Meet Hypothesis One

4.2.1. Chemical Performance Data

Chemical parameters analyzed were OP, soluble TOC, MLSS and MLVSS. In an EBPR system, phosphorus release and uptake occurred under anaerobic and aerobic conditions respectively, and thus, the overall phosphorus removal efficiency was determined by using OP concentration of the liquid phase. Soluble TOC indicated the amount of carbon source available in the EBPR system. Soluble TOC consumed and

stored as polyhydroxyalkanoates (PHA) under anaerobic condition, and consequently, phosphorus was released in a typical EBPR system.

For each cycle of EBPR described in Table 3.3, OP and sTOC values were plotted against time (i.e. hour) to observe the performance of an EBPR system. The percent phosphorus removal in a cycle was determined based on the influent and effluent OP concentration and plotted against time (i.e. day). The mean percent removal of OP between the replicate reactors fed with butyrate (B1 and B2) and glucose (G1 and G2) was compared using a two-tailed Independent-samples t Test assuming a 95% confidence level ($p <= 0.05$). The null hypothesis was that the difference in mean OP removal percentage between the replicate reactors (i.e. B1 and B2) was zero. The alternate hypothesis was that the mean OP removal percentages between the replicate reactors were different. The standard error of the mean OP removal percentage was calculated for the reactors fed with butyrate and glucose.

4.2.2. Microbiological Performance Data

The microbiological performance of the lab-scale SBRs fed with butyrate and glucose was evaluated using FISH analysis. Firstly, PAOs and GAOs, not yet reported in systems fed exclusively with butyrate and glucose, were targeted using specific PAOs and GAOs probes in FISH analysis. In addition, total bacterial population was targeted by using EUB338, EUB338-II and EUB338-III probes. Secondly, a confocal laser scanning microscope was used to capture five and ten digital pictures from two wells of a slide containing 6 wells for butyrate and glucose fed reactors, respectively. Thus, a total of ten and twenty pictures were captured for each PAOs and GAOs in butyrate and glucose fed reactors, respectively. Thirdly, PAOs, GAOs and the whole bacterial population were quantified in terms of their area by using the image processing software ImageJ (National

Institute of Health). Fourthly, these data were transferred to Microsoft Excel where the percentage of relative abundance (% RA) of PAOs and GAOs was calculated. Percent relative abundance indicates the ratio of the total areas of PAOs/GAOs to the whole bacterial population. Finally, the % RA of PAOs and GAOs was plotted against time for the reactors fed with butyrate and glucose.

To compare the % RA of PAOs/GAOs in the replicate reactors fed with butyrate and glucose, the mean value of the %RA of PAOs/GAOs of each reactor was determined and compared using a two-tailed Independent-samples t Test assuming a 95% confidence level. The null hypothesis is that there was no difference between the mean percent RA of PAOs/GAOs in the replicate reactors. The alternate hypothesis is that there was difference in mean %RA of PAOs/GAOs in the replicate reactors. PAOs and GAOs were analyzed separately. Moreover, the mean value of the %RA of PAOs/GAOs at different date of duplicate reactors was determined and compared using a two-tailed Independent-samples t Test assuming a 95% confidence level described above. Furthermore, the mean value of the %RA of PAOs/GAOs was determined for duplicate samples for the same date and compared using a two-tailed Independent-samples t Test assuming a 95% confidence level described above.

4.2.3. Integrating Chemical and Microbiological Data

The combination of chemical and microbiological data represented the actual scenario of the performance of an EBPR system. It was expected that good phosphorus removal was related to the abundance of the targeted PAOs whereas poor phosphorus removal was associated with the abundance of targeted GAOs. If not, the carbon types would not select the targeted PAOs and GAOs. Therefore, the correlation was made between the percent OP removal and the relative abundance of PAOs by using a linear regression

analysis. Similarly, the correlation was made between the percent OP removal and the relative abundance of GAOs.

4.3. Analysis of Chemical Performance Data to Meet Hypothesis Two

In the CCWRD process laboratory, duplicate tests were performed for each batch test. The parameters to be monitored with time were OP, soluble TOC, NO_3-N, NH_3-N and NO_2-N (described in Section 3.3) and they were plotted for each batch test. Standard deviation between the results of duplicate tests was calculated. To identify the carbon source that would give the best phosphorus uptake, One-way Analysis of Variance (i.e. ANOVA) test was performed among the batch tests performed using wastewater with acetate and propionate as a carbon source and without supplemental carbon source assuming 95% confidence level. The null hypothesis of the test was that the mean values of percent OP removal were similar. The alternate hypothesis was that at least two mean values were different. In addition, denitrification rate, phosphorus release and uptake rates were tabulated for each batch tests.

4.4. Quality Assurance/Quality Control (QA/QC)

The QA/QC evaluation is important to ensure the quality of the results obtained from different experiments in this research. The quality of the analysis can be assured by minimizing the systematic error (i.e. instrumental, procedure and human errors) whereas the quality can be controlled by checking the accuracy, precision and detection limit of the methods used in the analysis.

4.4.1. Chemical Performance Data

4.4.1.1. Quality Assurance

The critical parameters that were analyzed were orthophosphate (OP), soluble total organic carbon (sTOC), mixed liquid suspended solids (MLSS), mixed liquid volatile suspended solids (MLVSS), nitrate –nitrogen (NO_3-N), ammonia –nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), temperature, dissolved oxygen (DO) and pH. The OP concentration of the effluent was directly involved in the determination of the performance of an EBPR system. In addition to phosphorus, NO_3-N was also a key parameter to observe the performance of a BNR system. DO concentration was measured to maintain the anaerobic and aerobic conditions of an EBPR system. Solids concentration was required to maintain the required solids retention time (SRT) of the system. Soluble TOC was monitored to identify whether there was sufficient carbon source for phosphorus release under anaerobic condition, which was important for subsequent phosphorus uptake. The goal was to measure all parameters accurately. However, sometimes error could occur due to instrumental and procedural problems. Therefore, following precautions were undertaken and recorded to maintain the quality of the analyses:

1. The analytical balance used to prepare solutions was calibrated weekly. In the UNLV Environmental Engineering Laboratory the balances are calibrated every six month by an outside contractor. In addition, the analytical balance was checked weekly by using 5 g and 50 g standard weights.
2. The calibration of micropipettes was monitored every week. The accuracy of the pipettes used was verified by weighing, on an analytical balance, various volumes of water withdrawn with the pipette. If the weight of the water in gram is equal to

the volume of the water in ml, the calibration of micropipette is correct. Otherwise, the micropipette was sent for calibration with an independent contractor.

3. All glass micro fiber filters for solids analysis were preserved in the desiccator prior to use to avoid moisture interferences.
4. Aluminum dishes used for solids analysis were preignited at 550°C for one hour to avoid weight loss due to ignition.
5. DO and pH meters were calibrated before every use. DO meter was calibrated using 100 percent air saturation method. In this method, temperature and altitude factor of the place were used to calibrate the DO meter. The pH meter was calibrated using two pH buffer solutions (i.e. 7 and 10). If the slope was above 90%, the pH meter was working accurately.
6. Acidification was used in all TOC analysis to assure inorganic carbon was not present. The pH of samples to be analyzed for TOC was lowered to 2-3 using hydrochloric acid (HCl). To assure all TOC measurements were performed at low pH values, HCl acidified water was used.
7. During laboratory-scale SBR and batch experiments, anaerobic (i.e. DO < 0.2 mg/L) and aerobic condition (i.e. DO > 2 mg/L) were maintained properly. For laboratory-scale SBR, nitrogen gas was supplied into the reactors for two minutes to obtain anaerobic condition, and air was diffused during the whole aerobic period (i.e. 4.5 hours) to achieve the aerobic condition. For laboratory-scale batch experiments in the CCWRD Process laboratory, the mixed liquor was kept in a three-liter glass reactor, which was covered tightly to avoid oxygen entrance. The

goal was to obtain dissolved oxygen concentrations below 0.2 mg/L within two minutes. To establish aerobic conditions, air was provided to the reactor using a pressure pump and a ceramic air diffuser. Dissolved oxygen concentration was measured using a DO meter during batch tests. A sampling schedule for the SBR experiment is described in Table 3.3.

8. During batch tests in the CCWRD Process Laboratory, mixed liquor was mixed very slowly using a magnetic stirrer operating at a rotating speed of # 2 (i.e. 2000 rpm) to avoid the air entrapment. The collected samples were stored immediately on ice chests until the analysis was performed to avoid the degradation of ammonia and phosphorus.
9. The influent tubes of laboratory-scale SBRs that were carrying synthetic wastewater from the buckets were placed inside metal rods to avoid flotation in the synthetic wastewater feed buckets.

4.4.1.2. Quality Control

The quality of the chemical analysis was evaluated based on the accuracy, precision and detection limit described in Table 4.1. The accuracy of the measurements was determined by using standard solutions, and precision was evaluated by using replicates. The estimated detection limit, as given by the equipment manufacturer, was followed during the analysis (Table 4.1).

Table 4.1: Accuracy, precision and detection limit of the methods used in the analysis

Parameters	Method	Standard used to check accuracy	Precision*	Detection Limit
OP	8048 (HACH/DR 4000)	1 mg P/L and 0.02 mg/L PO_4^{3-}	95% confidence limit	0.02 mg/L PO_4^{3-}
NO_3-N	10020 (HACH/DR 4000)	10 mg/L NO_3-N and 0.2 mg/L NO_3-N	95% confidence limit	0.2 mg/L NO_3-N
NH_3-N	10031 (HACH/DR 4000)	10 mg/L NH_3-N and 0.6 mg/L NH_3-N	95% confidence limit	0.6 mg/L NH_3-N
NO_2-N	10019 (HACH/DR 4000)	0.25 mg/L NO_2-N and 0.0013 mg/L NO_2-N	95% confidence limit	0.0013 mg/L NO_2-N
sTOC**	5310-B	1, 2, 4 and 5 mg/L	Standard deviation is 0.1	200 µg/L

*Duplicate samples were monitored for each parameter in every five samples.
** Standard sample was analyzed in every five samples for accuracy check.

4.4.2. Microbiological Performance Data

4.4.2.1. Quality Assurance

The percentage of relative abundance (% RA) was the critical parameter to quantify the PAOs and GAOs using FISH analysis. The %RA was determined by the ratio of the areas of the images representing PAOs/GAOs to the total bacterial population. Therefore, the effectiveness of hybridization technique, digital image capture using confocal laser scanning microscope and image analysis using ImageJ software significantly affected the number of the % RA. The following safety measures were undertaken to avoid error during quantification of PAOs and GAOs:

1. Fixation of samples was performed immediately after collection from the lab scale SBRs and within two hours after collection from the full-scale EBPR plant.

2. Probes and fixed samples were preserved at -20°C. One thermometer was placed in the refrigerator, and temperature was monitored weekly.
3. Temperature of the hybridization oven was fixed at 46°C, and monitored weekly by placing a thermometer inside the oven to obtain the desired temperature before analysis.
4. Temperature of the washing water bath (i.e. 48°C) was recorded with a thermometer before analysis.
5. Duration of the Hybridization process and rinsing of the samples were monitored carefully using a stop- watch during FISH analysis.
6. Digital images, which were captured using the confocal laser scanning microscope, were analyzed by an undergraduate student. A random check was performed weekly to check the quality of the analysis performed by the undergraduate student and to correct any errors that might have occurred. The inspection was performed by checking one image in every ten images.

4.4.2.2. Quality Control

To control the quality of the microbiological analysis, duplicate slide was prepared and analyzed to quantify the PAOs and GAOs using FISH analysis.

For the quality assurance of the chemical and microbiological analyses, a form has been created and given in Table 5.2. The form was completed every week and discrepancies were discussed with Dr. Batista.

Table 4.2: Quality Assurance Form

Parameters	Date	Values	Comments
Analytical Balance Calibration		Weighs =	
Micropipettes Calibration		Weighs =	
DO Meter Calibration		DO conc.=	
pH Meter Calibration		Slope=	
Refrigerator Temperature		(°C) =	
Hybridization Oven Temperature		(°C) =	
FISH Washing Water Bath Temperature		(°C) =	
Digital Image Analysis		(%RA) =	
Dilution Water		pH =	
Glass Micro fiber Filter		-	
Aluminum Dish		-	

CHAPTER 5

THE IMPACTS OF NITRATE AND DIFFERENT SUBSTRATE LEVELS ON PHOSPHORUS UPTAKE IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL

5.1. Abstract

In this study, simultaneous denitrification and phosphorus (P) removal were investigated in batch tests using actual wastewater from a full-scale treatment plant and different levels of acetate and propionate as supplemental carbon sources. Without supplemental carbon source, denitrification occurred at low rate and P release and P uptake were negatively affected (i.e. P removal of only 59.7 %). When acetate and propionate were used, denitrification and P release occurred simultaneously under anoxic conditions. For acetate and propionate at a C/N ratio of 7.6 (stoichiometric ratio), P release was negatively affected by denitrification. The maximum specific denitrification rate ($SDNR_{max}$), maximum specific phosphorus uptake rate ($SPUR_{max}$) and maximum specific carbon uptake rate ($SCUR_{max}$) were 4.4 mg N (g VSS)$^{-1}h^{-1}$, 7.4 mg P (g VSS)$^{-1}h^{-1}$ and 20.5 mg sTOC (g VSS)$^{-1}h^{-1}$, respectively. The rates for propionate were also similar. For acetate, percent P removal and denitrification were very similar for C/N ratios of 22 (5X stoichiometric) and 59 (10X stoichiometric). For propionate, both % P removal and denitrification deteriorated for C/N ratios of 22 (5X stoichiometric) and 45 (10X stoichiometric). In addition, P uptake in the aerobic zone was not complete at higher C/N

ratios, which might be due to insufficient aerobic retention time to account for the higher P release. In addition it was observed that excess carbon source was consumed in the aerobic zone, but P was not taken up. This implies that PAO bacteria will utilize the excess carbon source in the aerobic zone rather than their polyhydroxyalkanoate (PHA) reserves. In summary, in EBPR systems where denitrification is introduced, P removal will be negatively impacted. The degree of the impact depends upon the amount of carbon source added. Greater P release in the anoxic zone occurs with the addition of carbon source to foster denitrification. Longer aerobic retention times need to be provided for P uptake to accommodate for the larger P release. In addition, care must be exercised during operation to assure free carbon source does not reach the aerobic zone of the EBPR system. It is concluded from the results of this research that P uptake will deteriorate if free carbon source is carried from the anoxic zone to the aerobic zone; the phosphate accumulating bacteria (PAOs) will utilize the free carbon source first rather than PHA (polyhydroxyalkanoates) accumulated during the anoxic period and they will not take up P under aerobic conditions.

5.2. Introduction

In wastewater treatment systems that include enhanced biological phosphorus removal (EBPR), denitrification and P release can occur simultaneously when sufficient carbon source is available (Ghekiere et al., 1991; Chuang et al., 1996; Patel et al., 2006; Zou et al., 2006). However, even when sufficient carbon source is available, P release rates have been reported to be negatively affected by denitrification (Zou et al., 2006; Lee et al., 2006). In EBPR, nitrate can interfere with P removal through sludge return via internal mixed liquor return (Figure 5.1). In the case of sludge return, nitrate-rich sludge

is returned from the clarifier to the anoxic tank as part of standard operation (Figure 5.1). In the case of internal mixed liquor return, nitrate-rich mixed liquor is purposively applied to the anoxic zone from the aerobic zone to promote denitrification (Figure 1). In both cases, returned nitrate can be converted to nitrogen gas if carbon sources are available. Available carbon sources include mainly volatile fatty acids (VFAs) and both Ordinary Heterotrophic Organisms (OHOs) and the phosphorus accumulating organisms (PAOs) will compete for VFAs in the presence of nitrate (Yagci et al., 2003; Zou et al., 2006; Yuan and Oleszkiewicz, 2008). Yagci et al. (2003) found that the amount of P release decreased by 25 mg/L in presence of nitrate at a low mass COD/N ratio of 3.8. Similarly, Chuang et al., (2003) observed that the specific phosphorus release rate in 30 min ($SPRR_{30}$) was 42% lower under anoxic condition compared to that under anaerobic condition at a COD/N mass ratio of 1.7. However, Zou et al., (2006) noticed that the $SPRR_{30}$ was 42% in presence of nitrate even with a high COD/N ratio of 20. Moreover, Patel and Nakhla (2006) reported that P release occurred only when nitrate concentration was less than 1 mg/L at a high COD/N ratio of 85 and 42 using propionic acid and butyric acid as a carbon source, respectively.

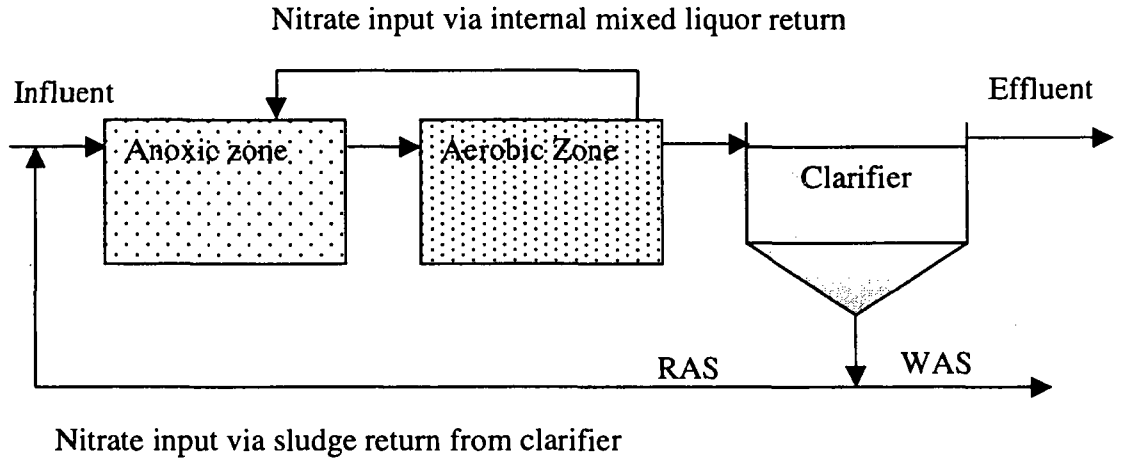


Figure 5.1: Schematic of EBPR process with internal nitrate return in the anoxic zone

It has been reported in the literature that when sufficient carbon sources are not present, some fractions of PAOs will use nitrate, as an electron acceptor for P uptake, instead of oxygen, under anoxic condition (Kerrn-Jespersen and Henze, 1993; Sorm et al., 1996, Merzouki et al., 2001, Yagci et al., 2003 and Peng et al., 2006). Malnou et al. was the first researcher to report that nitrate could serve as an electron acceptor for P uptake (Barker and Dold, 1996). Later, Vlekke (1988) and Zou et al., (2006) noticed that more stored carbon (i.e. PHB) was utilized for P uptake in presence of nitrate compared to that of oxygen. Several investigators (Kerrn-Jespersen and Henze, 1993; Sorm et al., 1996 and Yagci et al., 2003) reported that the rate of P uptake was lower (i.e. 1.7 to 5.5 mg PO_4-P/gVSS.hr) under anoxic condition than under aerobic conditions (i.e. 3.7 to 6.7 mg PO_4-P/g VSS. hr). Therefore, P uptake that happens in the anoxic zone, at the expense of nitrate, is not as effective (Kerrn-Jespersen and Henze, 1993; Kuba et. al., 1993, Sorm et al., 1996, Yagci et al., 2003 and Zou et al., 2006; Yuan and Oleszkiewicz, 2008). In summary, return of nitrate-rich stream in EBPR will deteriorate P release due

to the lack of sufficient VFAs and may result in an inefficient P uptake by PAOs that utilize nitrate as an electron acceptor in the anoxic zone.

As previously outlined, numerous studies have been performed to evaluate the influence of denitrification on P release in the anoxic zone in the presence and in the absence of supplemental carbon source. Moreover, P uptake was investigated in the anoxic zone of EBPR using nitrate as an electron acceptor, and the results were also compared with the P uptake under the aerobic condition of EBPR in presence of oxygen as an electron acceptor. However, there is not enough evidence of the influence of denitrification, with and without supplemental carbon source, on P uptake in the aerobic zone of EBPR systems. Therefore, in this research, investigation was performed using varying amount of acetate and propionate to evaluate the phosphorus uptake under aerobic condition when denitrification is performed in the anoxic zone of EBPR systems. It is hypothesized that the addition of a carbon source will improve phosphorus uptake in the aerobic period when nitrate is introduced to the anoxic zone of the system.

5.3. Materials and Methods

5.3.1. Experimental set up of batch tests

Batch tests were performed to investigate the hypothesis of this research using actual wastewater from a full-scale EBPR system. The batch tests were set-up in a laboratory located about 300 feet from the wastewater treatment plant, to minimize any variation due to wastewater storage and transportation. The experimental set-up used for the batch tests is illustrated in Figures 5.2 and 5.3. A three-liter glass reactor was utilized for the batch tests. The reactor was covered with a glass plate containing three openings that

housed a DO meter, air inlet, and a sampling port. The reactor was placed on a magnetic plate for mixing with a magnetic stirrer. During the aerobic period, air was supplied to the reactor through a narrow tube connected to a pressure pump fitted with a flow meter, which regulated the flow rate of air into the reactor. A ceramic air dispenser was used at the end of the tube to disperse the air uniformly into the reactor. A DO meter (YSI Model 54A) was connected to the reactor for continuous monitoring of the DO concentration.

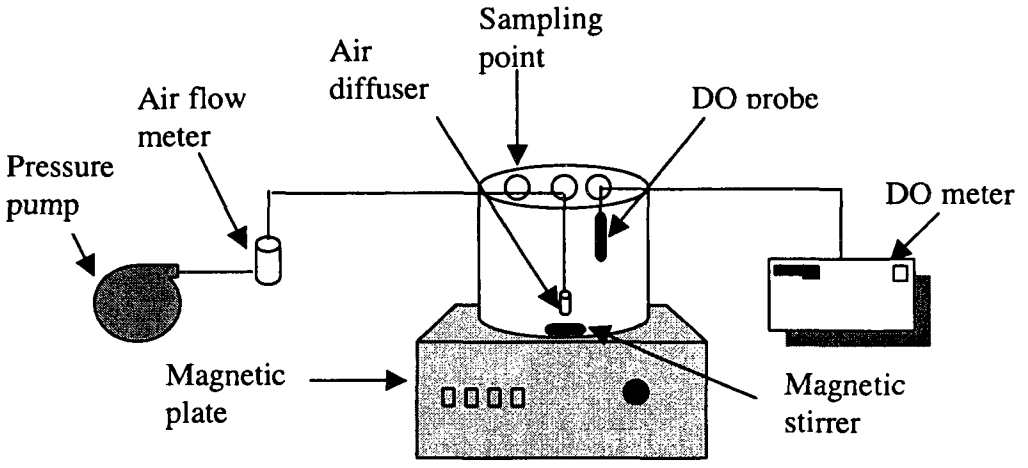


Figure 5.2: Schematic of the setup used in the batch tests

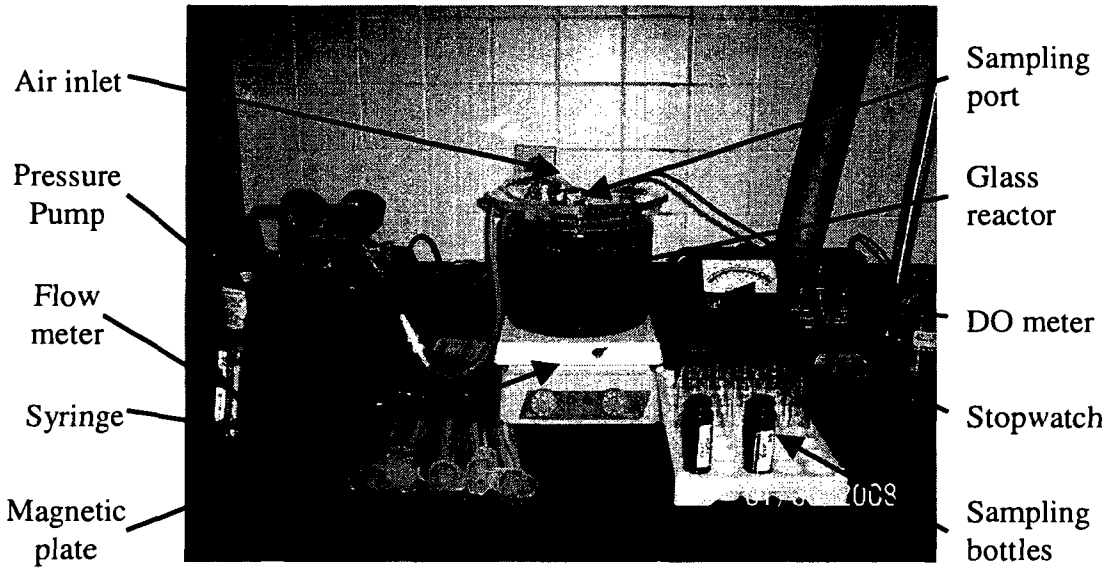


Figure 5.3: Experimental setup assembled for the research in the operation and control laboratory of a full-scale wastewater treatment plant.

5.3.2. Experimental procedure of batch tests

The experimental set-up simulates a typical EBPR system containing several aerobic, anaerobic and anoxic zones and liquor return to accomplish denitrification (Figure 5.4). A 1,500 mL sample (equivalent to 1.3 Q return flow) of mixed liquor return (MLR) and 1,146 ml (Q) of mixed liquor from the end of ANA2 zone (Figure 5.4) was collected during the experiments. This mixture represents the MLR/Q ratio used in the full-scale plant. The samples were mixed for 40 minutes (i.e. the total HRT of anoxic condition) in the absence of oxygen and aerated for about 140 minutes (i.e total HRT of aerobic condition). The durations of anoxic and aerobic periods were similar to those at the local full-scale wastewater treatment plant. The DO concentration under aerobic condition varied from 5 to 7 mg/L, which corresponds to those of zones AE 5 to AE 6 of basin AB #9. Different carbon sources (i.e. acetate and propionate) were used to identify the phosphate uptake under aerobic condition when dentrification occurred under anoxic

condition. In the case of acetate and propionate, the minimum concentrations to be used were selected from stoichiometric carbon source requirements based on thermodynamic computations for nitrate, 3.66 mg acetate/mg NO_3-N and 2.82 mg propionate/mg NO_3-N, respectively (Rittmann and McCarty, 2001). The detail calculations are given in Appendix I. The acetate and propionate concentrations were also increased to five and ten times stoichiometric ratios needed for denitrification. Table 5.1 summarizes the operational conditions of the various batch tests. The batch tests were performed at different days and wastewater samples were used immediately after collection of mixed liquor from the aeration basins. In some occasions, the solids concentration varied in the basins during the experimental period. The variation of MLSS concentration was considered in the analysis of the results obtained. All batch tests were performed in duplicate and average values and their standard deviations are reported. The average values of duplicate tests were used in the plots presented.

The composition of the mixture of wastewater and supplemental carbon sources used in the batch tests is shown in Table 5.2. The average C/N ratios were 7.6 and 22 when acetate and propionate were used at stoichiometric and five times stoichiometric ratios, respectively. Moreover, the average C/N ratios were 59 for acetate and 45 for propionate at ten times stoichiometric ratios.

Table 5.1: Operational conditions of batch tests

Batch Test No.	Replicate	Carbon source	Sampling Location and Volume	Reaction Condition and Time
B1	1 2	Wastewater without external carbon	MLR-1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B2	1 2	Acetate (X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B3	1 2	Acetate (5X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B4	1 2	Acetate (10X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B5	1 2	Propionate (X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B6	1 2	Propionate (5X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min
B7	1 2	Propionate (10X mg/L)	MLR - 1500 ml; ANA2 -1146 ml	Anoxic: 40 min Aerobic: 140 min

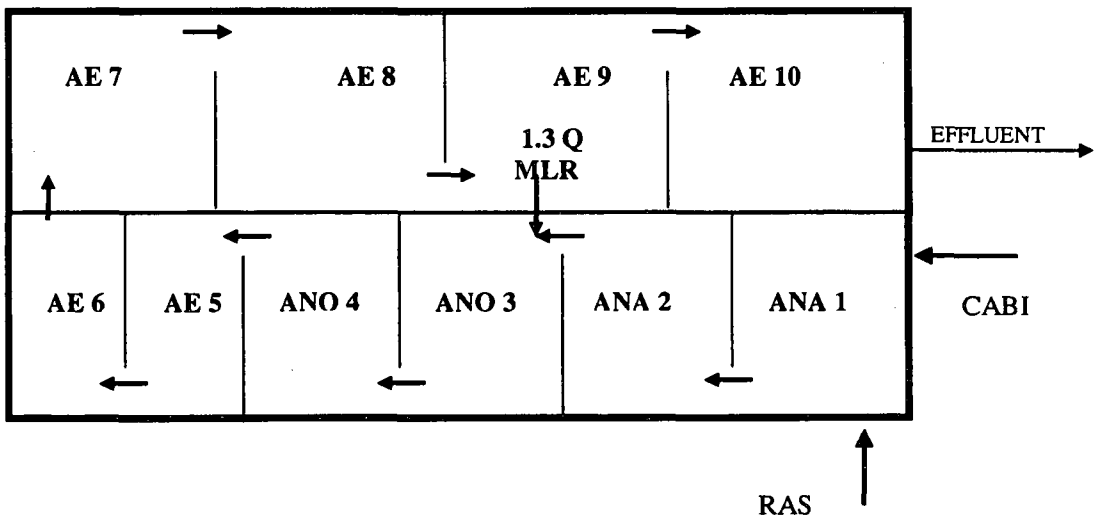


Figure 5.4: Schematic of EBPR/Denitrification System in full-scale wastewater treatment plant

Table 5.2: Composition of the mixture of wastewater and supplemental carbon sources used in the batch tests

Parameters	Units	Acetate			Propionate			WW only
		X	5X	10X	X	5X	10X	-
NO_3-N	mg/L	8.5	10	6.8	7.7	11	8.7	10.3
OP	mg P/L	8.5	4.6	4.4	7.4	6.9	3.9	8.2
COD	mg/L	64	220	398	59	244	392	27
C/N ratio	-	7.6	22	59	7.7	22	45	3
MLSS	mg/L	3.414	2.66	3.546	3.22	3.196	2.45	3.499
VSS	mg/L	2.73	2.13	2.84	2.58	2.56	1.96	3

5.3.3. Sample collection, preservation and analysis

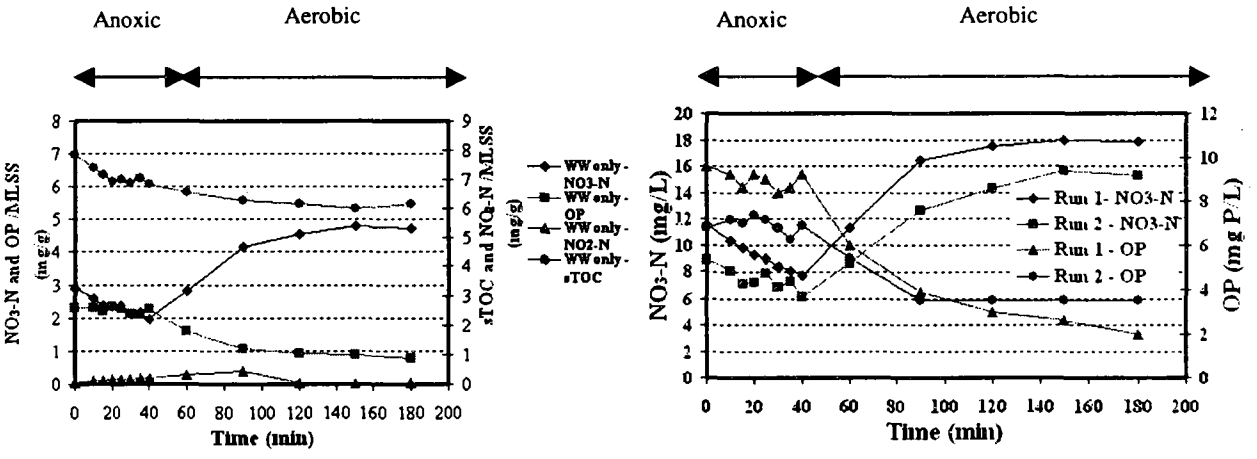
Twenty-ml samples were collected using a syringe every 5 minutes under anoxic condition and every 20 minutes under aerobic condition. The samples were filtered immediately through 0.45 μm syringe filter (GHP Acrodisc, PALL) to prevent secondary P-release. The samples were analyzed on the same day of collection or preserved if necessary. The samples were monitored for OP, NO_3-N, NH_3-N, NO_2-N and sTOC. OP, NO_3-N, NH_3-N and NO_2-N were measured using HACH kit (Loveland, Colorado) and a DR-4000 spectrophotometer. Soluble TOC were measured using a total carbon analyzer (Model TOC-$V_{CPH/CPN}$, SHIMADZU).

5.4. Results

5.4.1. Kinetics of denitrification and P removal without supplemental carbon source

In the absence of supplemental carbon source, denitrification occurred at a slow rate of 2.1 mg N/(gVSS) $^{-1}$ h $^{-1}$ using the carbon source present in the wastewater (i.e. C/N ratio of 3) (Figure 5). Patel and Nakhla (2006) also observed a slow denitrification rate of 1.3 mg N/(gVSS) $^{-1}$ h $^{-1}$ at a C/N ratio of 8.3 using municipal wastewater. In the present research, nitrate concentration decreased only from 2.9 ± SD 0.3 to 2 ± SD 0.2 mg N/g MLSS during the anoxic period. Moreover, there was no noticeable P release under anoxic condition due to the lack of carbon source. The amount of carbon present in the wastewater used in the batch tests was low because part of it had been utilized in zones 1 and 2 for P release and to reduce nitrate contained in the activated sludge return from the clarifier (Figure 5.4). As the samples used in batch test were a mixture of mixed liquor collected from the end of anaerobic period (Zone 2) and the end of aerobic period (Zone 9), the initial P and nitrate-N concentration were 2.3 ± SD 0.4 mg P/g MLSS and 2.9 ± SD 0.3 mg N/g MLSS, respectively. The nitrate concentration at the end of the anoxic period was 6.9 mg NO_3-N/L, corresponding to 32% removal (Figure 5.5b). The initial P concentration did not change during the anoxic period. However, P uptake was observed at a specific P uptake rate (SPUR $_{max}$) of 2.9 mg P/(gVSS) $^{-1}$ h $^{-1}$ under aerobic conditions resulting in an effluent P concentration of about 3.25 mg/L (59.7% removal) (Figure 5.5b). These results demonstrate that if denitrification is introduced, without supplemental carbon source, P removal will be affected because insufficient P is released in the anoxic zones negatively affecting the uptake of P in the aerobic zone. That is, if sufficient P is not released in the anoxic zone, the bacteria entering the aerobic zone will

not contain sufficient polyhydroxyalkanoates stored within the cells to be utilized to take-up P in the aerobic zone. The detail results of batch tests are given in Appendix II.



(a) (b)

Figure 5.5: Variation of NO_3-N, NO_2-N, OP and sTOC using wastewater without supplemental carbon source. Average values of duplicate tests are shown in Figure (a). Duplicate tests are shown in Figure (b)

5.4.2. Effect of denitrification on P removal with supplemental acetate at varying C/N ratios

When acetate was added to supplement the carbon source present in the wastewater, denitrification and P release occurred simultaneously with the use of acetate (Figure 5.6). This observation is supported by the findings of several researchers (i.e. Yagci et al., 2003; Patel et. al., 2006; Zou et. al., 2006; Ahmed et. al., 2007), who also obtained simultaneous denitrification and P release using acetate. Concentration variations of P-release, NO_3-N, NH_3-N, NO_2-N and sTOC obtained in the batch studies are also shown in Figure 5.6. At the stoichiometric C/N ratio of 7.6, denitrification and P release occurred simultaneously in the first 20 minutes with the consumption of almost the entire sTOC added. The sTOC concentration decreased from 8.1 ± SD 1.0 mg sTOC /g MLSS to 3.5 ± SD 0.6 mg sTOC /g MLSS.

After 20 minutes, P release stopped and the P concentration was about 5.7 ± 0.4 mg P/g MLSS at the end of anoxic period. However, denitrification continued up to the end of anoxic condition. The effluent P concentration and the concentration of nitrate at the end of the anoxic zone were 1.15 mg P/L and 2.02 mg NO_3-N/L, respectively, corresponding to 94% P removal and 76% nitrate removal (Table 5.3).

At a C/N ratio of 22 (5X stoichiometric ratio), denitrification was complete within 30 minutes whereas P release continued until the end of anoxic condition resulting in a P value of 11.1 ± SD 0.6 mg P/g MLSS. Moreover, the sTOC value reached below 5 mg sTOC/g MLSS after 90 min. The nitrate concentration after 30 minutes anoxic period was 0.4 mg NO_3-N/L, representing 96% nitrate removal (Table 5.3). The P concentration in the effluent was 9.7 mg P/L and a P removal of 67.2% (Table 5.3). However, the P uptake was not complete under aerobic condition.

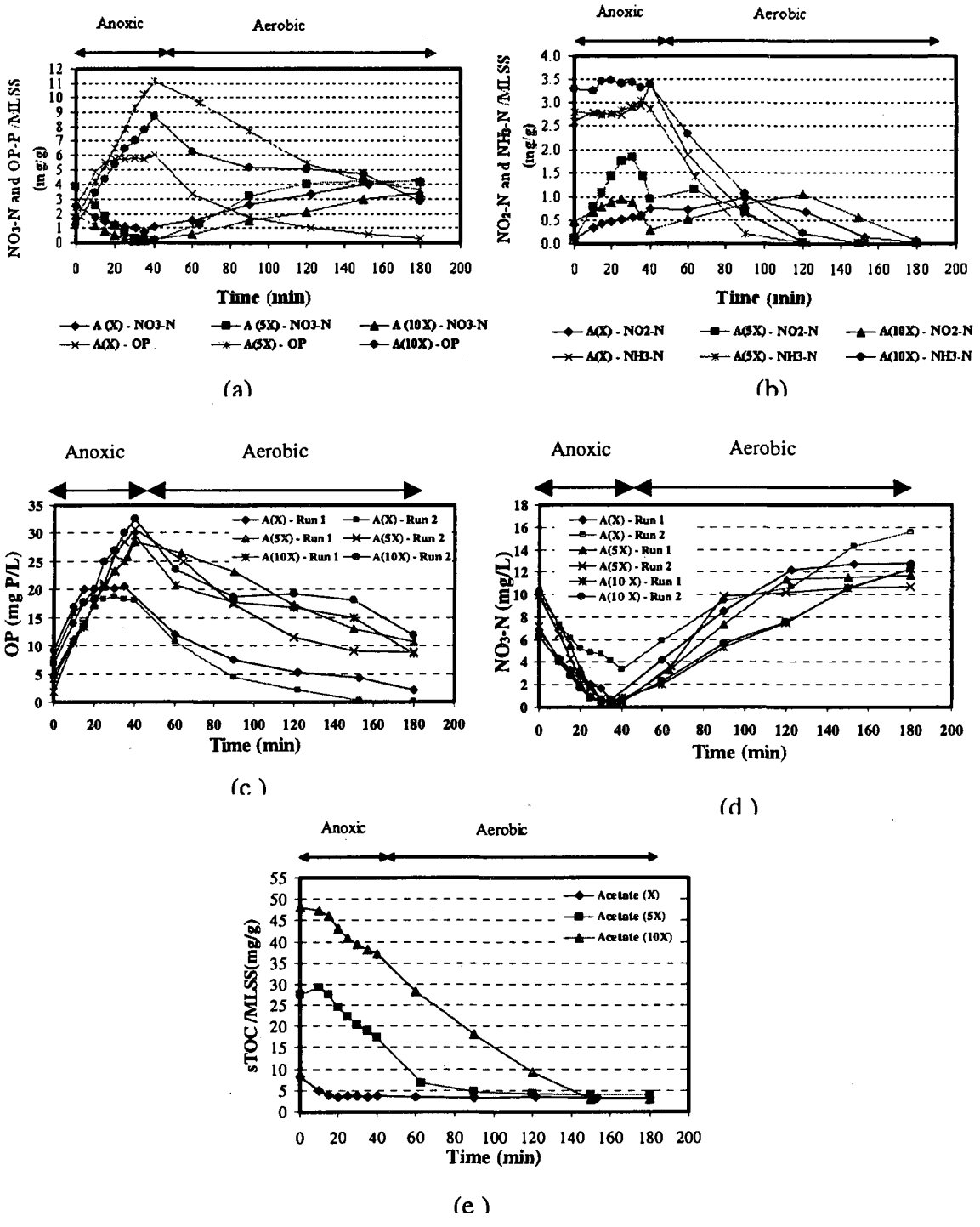


Figure 5.6: Variation of NO_3N, OP, NO_2-N, NH_3-N and sTOC using acetate. Average values of duplicate tests are shown in Figures (a), (b), and (e). Duplicate tests are shown in Figures (c) and (d)

At a C/N ratio of 59 (10X stoichiometric), the nitrate concentration reached a low level within 25 minutes. Release of P was observed until the end of anoxic condition in presence of high amount of sTOC (i.e. 37.2 ± SD 1.1 mg sTOC/g MLSS). The nitrate concentration in the end of the anoxic period was 0.4 mg NO_3-N/L, corresponding to 94% nitrate removal. The P concentration in the effluent was 10.13 mg P/L, representing 67.3% (Table 5.3). The % denitrification and P removal for C/N ratio of 22 and C/N ratio of 59 were very similar, demonstrating that there is no benefit in increasing the C/N ratio to 10 X stoichiometric. Similar to the C/N ratio of 22, P uptake was incomplete at a C/N ratio of 59. This may be due to insufficient aerobic retention time to allow the bacteria to take-up phosphorus. It may also be due to the presence of excess sTOC entering the aerobic zone. The results show sTOC being used in the aerobic zone. It is possible that when sTOC is freely available in the aerobic zone, the PAO bacteria will use it as the carbon source, instead of breakdown the PHA's stored within the cell during the anoxic period. This suggestion is supported by the fact P is poorly taken-up in the aerobic zone. The detail results of batch tests are given in Appendix II.

5.4.3. Effect of denitrification on P removal using supplemental propionate at varying C/N ratios

Denitrification and P release occurred simultaneously when propionate was used as a carbon source in this study. These results are similar to the observations made by Patel et. al., (2006). However, Patel and Nakhla (2006) found that, in the presence of propionate, the P release started only after the nitrate concentration was below 0.8 mg/L. The variation of P release, NO_3-N, NH_3-N, NO_2-N and sTOC are shown in Figure 5.7. At a C/N ratio of 7.7 (Stoichiometric ratio), P release occurred within 10 min whereas the

denitrification process continued under anoxic conditions. The P value increased to 4.0 ± SD 1.0 mg P/g MLSS at the end of anoxic period. The P uptake was almost completed and the final P concentration in the effluent and the final nitrate concentration at the end of the anoxic zones were 0.63 mg P/L and 1.95 mg NO_3-N/L, respectively (Figures 5.7c and 5.7d). These correspond to 95% removal of P and 74.7% removal of nitrate (Table 5.3).

At a C/N ratio of 22 (5X stoichiometric), denitrification was not completed even though sufficient sTOC was present in the end of anoxic condition (i.e. 15.4 ± SD 2.1 mg sTOC/g MLSS). However, P release continued until the end of anoxic period resulting in a P value of 8.0 ± SD 0.1 mg P/g MLSS. The percent removal of P and nitrate were 58.1% and 63.8 %, respectively (Table 5.3). The effluent P concentration was 10.75 mg P/L and the nitrate concentration in the end of the anoxic zone was 4.0 mg NO_3-N/L (Table 5.3).

At a C/N ratio of 45 (10X stoichiometric ratio), P release and denitrification patterns were similar to that of the C/N ratios of 22 under the anoxic period. However, the P value was less (i.e. 6.3 ± SD 0.6 mg P/g MLSS) at the end of anoxic period at a C/N ratio of 45 compared to that of the C/N ratio of 22. The removal of nitrate was only 46% with a nitrate concentration of 4.65 mg at the end of the anoxic zone (Table 5.3). Significant P release was observed, however, P uptake did not take place in the aerobic zone, resulting in a very high effluent P concentration (18 mg P/L) (Table 5.3). These results demonstrate that both P removal and denitrification are negatively affected by propionate concentrations above stoichiometric ratios. The detail results of batch tests are given in Appendix II.

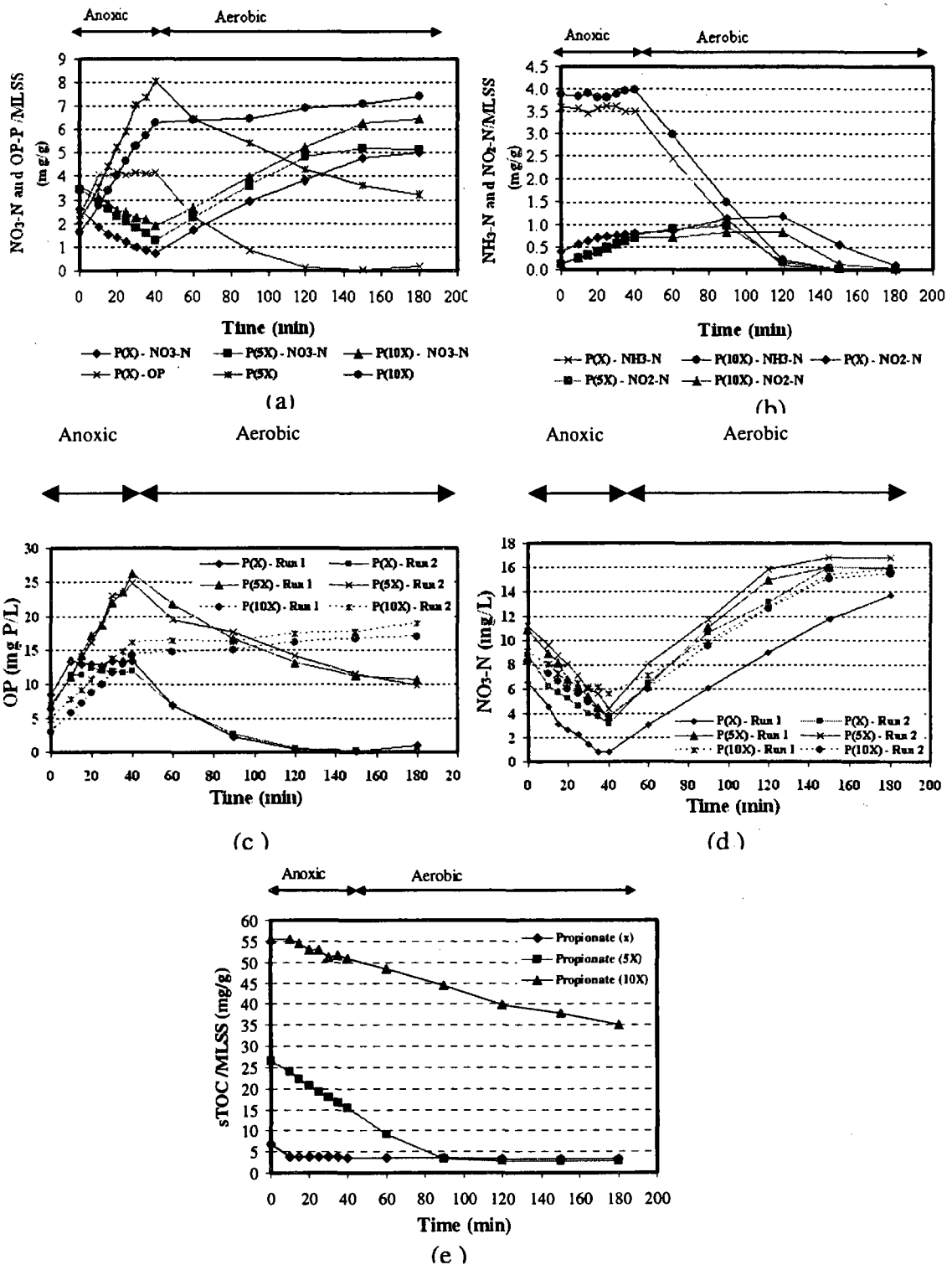


Figure 5.7: Variation of NO_3-N, OP, NO_2-N, NH_3-N and sTOC in batch tests of EBPR and denitrification using propionate. Average values of duplicate tests are shown in Figures (a), (b) and (e). Duplicate tests are shown in Figures (c) and (d).

In a study using propionate, Patel and Nakhla (2006) found P uptake under aerobic condition at a high C/N ratio of 83, and the P concentration was 2 mg/L in the effluent. In the present study, at a C/N ratio of 45, sufficient carbon was present and consumed during the aerobic period but P uptake was not observed. As mentioned earlier, a possible explanation for this observation is the greater advantage to PAOs when they use freely available carbon source rather than using stored polyhydroxyalkanoates (PHAs). In the anoxic zone, the PAOs released phosphorus and accumulated PHA. The accumulated PHA is utilized in the aerobic zone as a carbon source to uptake phosphate. However, if another carbon source (i.e excess acetate or propionate) is available, there is no motivation for the PAO bacteria to use the internal carbon source reserves. Zou et. al., (2006) also observed that the presence of high residual TOC at the end of anoxic period might reduce the uptake of P in EBPR systems.

5.4.4. Comparison of the effect of acetate and propionate at similar C/N ratios

The results of this research indicate that at a C/N ratio of 7.6 (stoichiometric), denitrification negatively affected P release because there was insufficient acetate and propionate to support both denitrification and P release. It was found that P release stopped within 20 min and 10 minutes, for acetate and propionate, respectively, whereas denitrification continued until the end of anoxic condition. About the same amount of nitrate (i.e. 1.8 mg N/g MLSS) was denitrified in both cases. However, P release was higher in presence of acetate (i.e. 3 mg P/g MLSS) compared to that of propionate (i.e. 1.8 mg P/g MLSS). The effluent P concentrations were 1.15 mg P/L and 0.63 mg P/L for acetate and propionate, respectively. However, the % P removal was similar, 94% for acetate and 95% for propionate.

At a C/N ratio of 22 (5X stoichiometric), 3.7 mg NO_3-N/g MLSS was denitrified using acetate whereas 2.2 mg NO_3-N/g MLSS was denitrified using propionate and there was still carbon source remaining at the end of anoxic period. The P release was higher for acetate (i.e. 9.4 mg P/g MLSS) compared to that of propionate (i.e. 5.8 mg P/g MLSS). The effluent P concentrations were 9.7 mg P/L and 10.75 mg P/L for acetate and propionate, respectively. However, % nitrate removal was much greater for acetate (96%) than for propionate (63.8%).

When acetate was used at higher C/N ratios (10X stoichiometric), both % P removal and denitrification, were not improved as compared to those for 5X stoichiometric ratios. Indeed, at higher C/N ratios of propionate, both denitrification and % P removal deteriorated.

In the present study, the effluent P concentration increased with higher releases of P for both acetate and propionate. Oppositely, Patel and Nakhla (2006) observed the lowest effluent P concentration with the highest amount of P release for acetic acid. However, the nitrate concentrations of their wastewater and the P content of their sludge were different from the ones used in this study and the results cannot be directly compared.

5.4.5. Kinetics of denitrification and P removal at different C/N ratio using acetate

The kinetics of denitrification, P release and uptake using acetate at a different C/N ratio are illustrated in Figure 5.8. In the present study, the maximum specific denitrification rate ($SDNR_{max}$) was highest (i.e. 9.3 mg N/(g MLSS) $^{-1}$ h $^{-1}$) at a C/N ratio of 22 (5X stoichiometric). Zou et al., (2006) found that $SDNR_{max}$ was 3 mg N/(g MLSS) $^{-1}$ h $^{-}$1 at a C/N ratio of 20. Chuang et al., (1996) found that the $SDNR_{max}$ was 4 mg N/(g

MLSS) $^{-1}$ h $^{-}$1 at a C/N ratio of 6 whereas Ahmed et. al., (2007) observed that the $SDNR_{max}$ was 36.2 mg N/(g VSS) $^{-1}$ h $^{-}$1 at a C/N ratio of 6.5. As can be seen from the reported data, there is significant variation in the reported $SDNR_{max}$. A potential reason for this variation is the different nitrate and P concentrations used in the various studies. In the present study, similar denitrification rates (i.e. 5 mg N/(gVSS) $^{-1}h^{-1}$) were obtained at C/N ratios of 7.6 and 59, which is comparable with the findings of Patel and Nakhla (2006) (4.5 mg N/(gVSS) $^{-1}h^{-1}$) at a C/N ratio of 50.

The specific phosphorus release rate ($SPRR_{max}$) increased by 2.6 mg P/(gVSS) $^{-1}$ h $^{-1}$ (from 15 mg P/(gVSS) $^{-1}$ h $^{-1}$ to 17.6 mg P/(gVSS) $^{-1}$ h $^{-1}$) with the increase of C/N ratio from 7.6 to 22, respectively. However, $SPRR_{max}$ decreased at a high C/N ratio of 59 compared to that of the C/N ratio of 22. Similar result (i.e. 17 mg P/(gVSS) $^{-1}$ h $^{-1}$) was obtained by Ahmed et. al., (2007) at a C/N ratio of 6.5. Moreover, Patel et al., (2006) found 20 mg P/(gVSS) $^{-1}$ h $^{-1}$) and Chuang et. al., (1996) obtained 10.9 mg P/(g MLSS) $^{-1}$ at a similar C/N ratio of 6. Zou et al., (2006) obtained $SPRR_{max}$ of 4.2 mg P/(g MLSS) $^{-1}$ h $^{-1}$ at a C/N ratio of 20. The $SPRR_{max}$ rates reported in the literature indicate that at C/N ratios between 6.0-8.0, the $SPRR_{max}$ varies between 11 and 20 mg P/g VSS. hr. Higher C/N ratios such as the one utilized in this research and by Zou et. al., (2006) seem to have no positive effect on P release. A potential reason for this observation is the amount of phosphate that is accumulated within the PAO's. Although there are reports of EBPR sludge with 15% P (Machado, 2004), most EBPR sludge will contain 4-6% P (Hesselman et. al., 2000; Patel and Nakhla, 2006).

In this study, the lowest maximum specific phosphorus uptake rate ($SPUR_{max}$) was found when $SDNR_{max}$ and $SPRR_{max}$ reached the highest level at a C/N ratio of 22. The

$SPUR_{max}$ was 1.7 times higher than the $SDNR_{max}$ at C/N ratios of 7.6 and 59 whereas $SPUR_{max}$ was 58 % of $SDNR_{max}$ at a C/N ratio of 22. The $SPUR_{max}$ was 50%, 31% and 66% of the $SPRR_{max}$ at a C/N ratio of 7.6, 22 and 59, respectively. Overall, the SPURmax decreased with the increase of SPRRmax. Therefore, the worst P effluent quality was obtained with higher releases of P in the anoxic zone.

At a C/N ratio of 22 (5X stoichiometric), the maximum specific carbon uptake rate ($SCUR_{max}$) value reached a maximum level with the highest $SDNR_{max}$ of 9.3 mg $N/(gVSS)^{-1}\ h^{-1}$) and $SPRR_{max}$ of 17.6 mg $P/(gVSS)^{-1}\ h^{-1}$), as expected. The lowest $SCUR_{max}$ value was associated with the lowest $SDNR_{max}$ and $SPRR_{max}$. A good linear correlation was found between $SCUR_{max}$ and $SDNR_{max}$ ($R^2 = 0.86$) and $SPRR_{max}$ ($R^2 = 0.90$). Therefore, the acetate consumed was used for P release and denitrification.

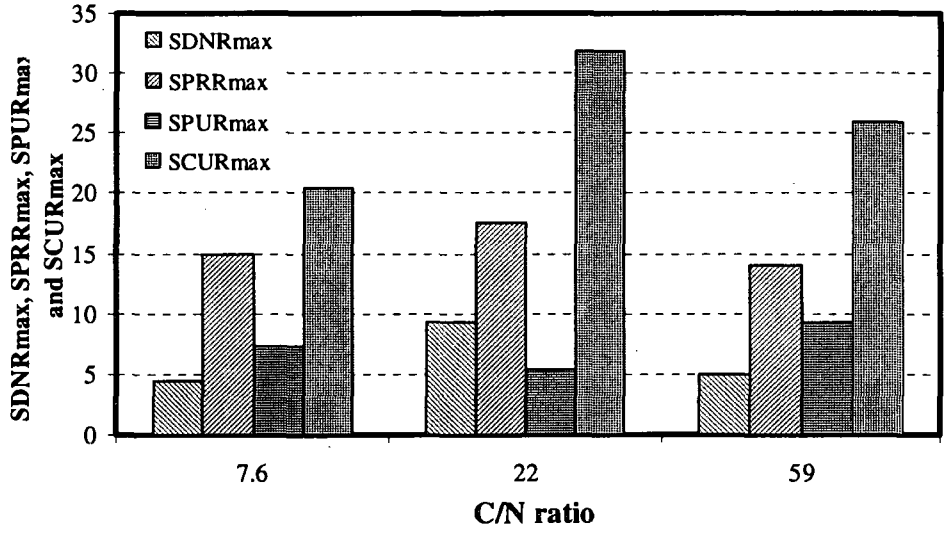


Figure 5.8: Comparison of the kinetics of denitrification and P removal at different C/N ratio using acetate

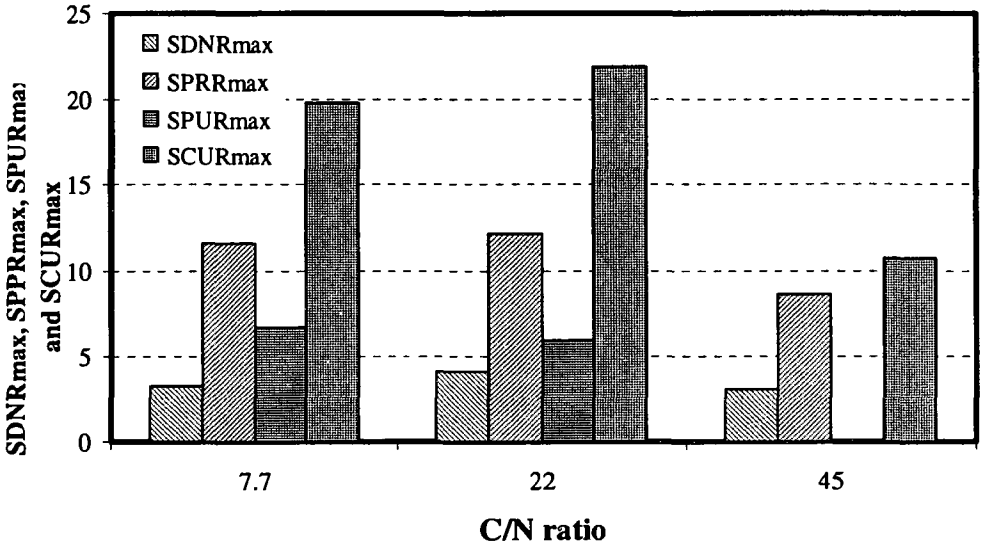


Figure 5.9: Comparison of the kinetics of denitrification and P removal at different C/N ratio using propionate

5.4.6. Kinetics of denitrification and P removal at different C/N ratio using Propionate

Figure 5.9 reveals the kinetics of denitrification and P removal using propionate with varying C/N ratio. A $SDNR_{max}$ of 3.3 mg N/(gVSS $^{-1}$ h $^{-1}$ at C/N ratios of 7.7 and the highest $SDNR_{max}$ (i.e. 4.1 mg N/(gVSS) $^{-1}$ h $^{-1}$) was obtained at a C/N ratio of 22 in this study. Patel and Nakhla (2006) observed a $SDNR_{max}$ of 1.6 mg N/(gVSS) $^{-1}$ h $^{-1}$ using propionate at a high C/N ratio of 83. Patel et. al (2006) found an $SDNR_{max}$ of 3.23 mg N/(gVSS) $^{-1}$ h $^{-1}$ at a C/N ratio of 3.6. Similar to the findings for acetate the $SDNR_{max}$ is not increased when very high concentrations of substrate are added. Stoichiometric ratios seem sufficient.

The $SPRR_{max}$ did not improve significantly when the C/N ratio was increased from 7.7 to 22. In addition, the lowest P release rate (i.e. 8.6 mg P/(gVSS) $^{-1}$ h $^{-1}$) was observed at a high C/N ratio of 45. In contrast, Patel et al., (2006) found the similar $SPRR_{max}$ at a low C/N ratio of 2. Patel and Nakhla (2006) obtained the $SPRR_{max}$ of only 0.71 mg

P/(gVSS) $^{-1}$ h $^{-1}$ using a high C/N ratio of 83. In this investigation, the P uptake rate reduced with increased denitrification and increased P release rate, except for a C/N ratio of 45. The $SPUR_{max}$ was lower than the $SPRR_{max}$ at different C/N ratios in the present study.

5.4.7. Comparison of the kinetics of denitrification and P removal using acetate and propionate

At a C/N ratio of 7.6, the $SDNR_{max}$ was similar for acetate and propionate. In addition, the $SPUR_{max}$ was also similar for acetate and propionate. However, the $SPRR_{max}$ increased by 3.4 mg P/(gVSS) $^{-1}$ h $^{-1}$ for acetate compared to that of propionate. The $SCUR_{max}$ value implies that the consumption of acetate and propionate were also similar.

At a C/N ratio of 22, acetate provided 2.3 times higher $SDNR_{max}$ compare to that of propionate as a substrate. Moreover, the $SPRR_{max}$ was also increased by 5.4 mg P/(gVSS) $^{-1}$ h $^{-1}$ with acetate. However, the $SPUR_{max}$ was similar (i.e. 5.4 mg P/(gVSS) $^{-1}$ h $^{-1}$) for both carbon sources. The $SCUR_{max}$ value was 1.5 times higher for acetate compared to that of propionate, which explains the reasons for higher $SDNR_{max}$ and $SPRR_{max}$ when acetate was utilized as a substrate.

It is interesting to note that at a high C/N ratio of 59 with acetate, the $SPUR_{max}$ reached a highest value of 9.3 mg P/(gVSS) $^{-1}$ h $^{-1}$ whereas at a high C/N ratio of 45 using propionate, P uptake did not take place, instead P release was observed with a $SPRR_{max}$ of 0.6 mg P/(gVSS) $^{-1}$ h $^{-1}$. The $SDNR_{max}$ and $SPRR_{max}$ were higher with an elevated consumption of carbon source for acetate compared to that of propionate.

5.4.8. Percentage of P removal with denitrification using acetate and propionate

The percentage of P removal using wastewater with and without supplemental carbon source at different C/N ratio is summarized in Table 5.3. The percentage of P removal was about 95% for both acetate and propionate at a C/N ratio of 7.6. At this C/N ratio for acetate and propionate, the nitrate concentration was about 2 mg N/L at the end of anoxic period. In addition, the $SDNR_{max}$ was about 4 mg N/(gVSS) $^{-1}$ h $^{-1}$ for both acetate and propionate. The amount of P released was lower compared to that at other C/N ratios resulting in low effluent P concentration, even though the $SPUR_{max}$ was not highest for acetate.

The percentage of P removal was below 70% for the wastewater without a supplemental carbon source and also with the addition of acetate and propionate at a higher rate. These results indicate that the addition of a higher amount of carbon source to foster denitrification does not result in improved P removal.

Table 5.3: The performance of P and nitrogen removal at different C/N ratio using acetate and propionate

Carbon Source	Amount	P_{max} (mg P/L)	P_f (mg P/L)	% P removal	N_i (mg N/L)	N_f (mg N/L)	% N removal
Acetate	X	19.38	1.15	94	8.46	2.02	76
	5X	29.6	9.7	67.2	10	0.4	96
	10X	31	10.13	67.3	6.73	0.4	94
Propionate	X	12.75	0.625	95.1	7.7	1.95	74.7
	5X	25.63	10.75	58.1	11.2	4.05	63.8
	10X	15.19	18	-	8.62	4.65	46

Note: P_{max} = Maximum P concentration under anoxic period
P_f = Effluent P concentration
N_i = Initial NO_3-N concentration under anoxic period
N_f = NO_3-N concentration at the end of anoxic period

The major goal of this study was to investigate how denitrification and associated carbon source addition affected the uptake of P in the aerobic zones of EBPR systems. One-way ANOVA test was performed to investigate how P removal was impacted by the addition of acetate and propionate at different ratios. The % P removal without supplemental carbon source was used as the control for the analysis. This control was compared with % P removal using different levels of acetate (X, 5X and 10X) and propionate (X and 5X) to identify levels of acetate and propionate, which can provide better P removal than the control. The carbon level of 10X for propionate was not considered in this analysis because there was no P removal at this propionate level. Moreover, the overall % P removal using acetate and propionate was compared with the control to evaluate which carbon source can yield better % P removal. The results of the ANOVA analysis are depicted in Table 5.4. For the comparison of the wastewater with acetate, the *P* values were 0.177, 1.0, 0.999, 0.195, 0.203 and 1.00 when comparing means for the control, wastewater (i.e. WW) and acetate (i.e. A) (X), WW and A (5X), WW and A (10X), A (X) and A (5X), A (X) and A (10X), and A (5X) and A (10X), respectively. All *P* values were higher than 0.05. These results indicate that there was no significant difference in % P removal using the wastewater and different acetate levels at 95% confidence level. For propionate, *P* values were 0.148, 0.852 and 0.097 for WW and propionate (X), WW and propionate (5X), and propionate (X) and propionate (5X), respectively. All *P* values were higher than 0.05, implying that there was no significant difference in % P removal using propionate at different levels at 95% confidence level.

For the overall comparison between acetate, propionate and wastewater, the *P* values were 0.753, 0.730 and 0.993 for WW and acetate, WW and propionate, and acetate and

propionate, respectively. These results show that the % P removal, obtained using the wastewater only as carbon source, was not significantly different (at 95% confidence level) from that when acetate and propionate were used. Therefore, statistical analysis reveals that P removal from the wastewater does not improve when acetate and propionate at different carbon levels are used. Additionally, the results show that acetate and propionate result in similar P removal from the wastewater.

Table 5.4: Results of multiple comparisons by Tukey Test in one-way ANOVA analysis

Parameters	Mean difference in % P removal	P value
Wastewater and Acetate (X)	28.50	0.177
Wastewater and Acetate (5X)	1.10	1.000
Wastewater and Acetate (10X)	1.55	0.999
Acetate (X) and Acetate (5X)	27.40	0.195
Acetate (X) and Acetate (10X)	26.95	0.203
Acetate (5X) and Acetate (10X)	0.45	1.000
Wastewater and propionate (X)	29.40	0.148
Wastewater and propionate (5X)	6.15	0.852
propionate (X) and propionate (5X)	35.55	0.097
Wastewater and acetate (mean of X, 5X and 10X)	10.38	0.753
Wastewater and propionate (mean of X, 5X and 10X)	11.63	0.730
Acetate and propionate (mean of X, 5X and 10X)	1.24	0.993

5.5. Discussion

In this research, investigation was performed using varying amount of acetate and propionate to evaluate the P uptake under aerobic condition when denitrification is introduced into the anoxic zone of EBPR systems.

The results of this research showed that, at statistically significant levels, there is no improvement in overall P removal in the EBPR system when additional carbon source is added to foster denitrification. Furthermore the results show that both acetate and propionate have the same effect in the system. Therefore, the introduction of denitrification has always the potential to negatively affect P removal.

For acetate, the data also revealed that, addition of supplemental carbon source at and above C/N stoichiometric ratios, results in greater degree of denitrification and lower % P removal. For propionate, both, % P removal and denitrification decrease with increasing C/N ratios.

The biochemistry of P removal may offer a potential explanation for the results obtained in this research. When supplemental carbon source is added to EBPR/denitrification systems, larger amounts of P will be released and higher levels of denitrification will be observed. However, PAO bacteria needs sufficient time in the aerobic zone to take up the P released in the anoxic zone. If sufficient retention time is not available, the effluent phosphate concentrations will be high and may not meet desired levels. In addition, if too much supplemental carbon is added and free carbon is carried to the aerobic zone, PAO may use this carbon, instead of using the polyhydroxyalkanoates stored during the anoxic period. This will result in no P uptake during the aerobic period and P removal will deteriorate.

The results of this research have implications in the design and operation of EBPR systems with denitrification. As mentioned earlier, denitrification can be incorporated to EBPR systems by internal return of nitrate-rich sludge from the end of the aerobic zone to anoxic zones of the plant. The degree of denitrification to be obtained is dependent upon the amount of carbon source, mostly volatile fatty acids, present in the system. The bacteria that perform P removal and denitrification compete for the VFAs present in the wastewater and hence P removal and denitrification will be affected by the amount of carbon source present. If high levels of both P removal and denitrification are desired, supplemental carbon source must be added. The results of this research point to the following implications:

1) EBPR systems will perform best when denitrification is not incorporated to them.

2) When denitrification is incorporated to EBPR systems, care should be taken to add stoichiometric amounts of supplemental carbon sources to achieve the desired denitrification level. Addition of higher carbon source than needed negatively affects the effluent P concentrations because more P is released and because PAO bacteria may use the free carbon available, instead of using PHAs.

3) If the desired denitrification level promotes high P release, the designer should increase the aerobic retention time of the EBPR system to accommodate for the larger amount of P that must be taken up in the aerobic zone. Obviously that will not only result in greater capital cost from building a larger tank, but also in higher operating cost because of the longer aeration period needed.

5.6. Conclusions

The following conclusions can be drawn from the present study:

When denitrification is incorporated into EBPR process, without supplemental carbon source, P uptake will be adversely affected due to insufficient P release under anoxic period. As a result, low amounts of P will be removed from the wastewater. In the present study, only 59.7% P removal was observed without supplemental carbon source.

With acetate and propionate at different C/N ratios, denitrification and P release occurred simultaneously.

At a C/N ratio of 7.6 for acetate and propionate, denitrification negatively affected P release because of insufficient acetate and propionate. The effluent P concentration was lowest with % P removal of 94% and 95% for acetate and propionate. The % N removal was 76% and 74.7% for acetate and propionate, respectively. In addition, the $SDNR_{max}$, $SPUR_{max}$ and $SCUR_{max}$ were similar for both acetate and propionate.

At a C/N ratio of 22 and 59 for acetate, % P removal and % N removal were very similar. This indicates that increase of C/N ratio from 22 to 59 is not useful. For propionate, % P removal and denitrification were deteriorated by the increase of C/N ratio from 7.7 to 22 and 45.

Acetate provided higher $SDNR_{max}$ and $SPRR_{max}$ with an elevated consumption of carbon source compared to that of propionate.

At a C/N ratio of 22, $SDNR_{max}$, $SPRR_{max}$ and $SCUR_{max}$ reached the highest level for both acetate and propionate.

The higher P release under anoxic period resulted in higher effluent P concentration for both acetate and propionate.

Statistical analysis shows that P removal does not improve from the wastewater using acetate and propionate at different carbon levels. Moreover, similar P removal can be obtained using both acetate and propionate.

The results of this research have implications to engineering design of EBPR/denitrification systems to treat wastewater. Longer aerobic retention time should be provided to accommodate for greater P release. Care must be taken, during operation, to avoid excess carbon source in the aerobic zone of the system. It is suggested that P uptake is hindered by the presence of free carbon source.

CHAPTER 6

THE EFFECTS OF GLUCOSE ON THE MICROBIAL SELECTION OF ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL SYSTEMS

6.1. Abstract

In this research, the microbial selection of enhanced biological phosphorus removal (EBPR) system was investigated in a laboratory-scale sequencing batch reactor fed exclusively with glucose as a sole carbon source. Fluorescence In Situ Hybridization (FISH) analysis was performed to target two polyphosphate accumulating organisms (PAOs) (i.e. *Candidatus* Accumulibacter phosphatis *and Microlunatus phosphovorus*) and two glycogen accumulating organisms (GAOs) (i.e. *Candidatus* Competibacter phosphatis and *Micropruina glycogenica*). The results show that glucose might not select for *Candidatus* Accumulibacter phosphatis. However, *Microlunatus phosphovorus*, *Candidatus* Competibacter phosphatis and *Micropruina glycogenica* might be selected by glucose. The highest % relative abundance (RA) of *Candidatus* Accumulibacter phosphatis was about 42% and occurred at the beginning of the experimental period when phosphorus removal was efficient. However, the % RA of these bacteria started to decrease, and reached below 4% at the end of the reactor run. The maximum % RA of *Microlunatus phosphovorus*, *Candidatus* Competibacter phosphatis and *Micropruina glycogenica* was about 21%, 37%, 17%, respectively. From two weeks running at higher glucose concentration, it seems that higher glucose concentration might be detrimental

for *Microlunatus phosphovorus* and *Micropruina glycogenica*. Results of this research also indicate a dominance of GAOs over PAOs when EBPR systems are fed with glucose. It seems, as suggested by previous researchers, that the low phosphorus release is caused by lower pH, resulting from glucose metabolism during the anaerobic period. As a consequence of low phosphorus release, PAOs contain insufficient polyhydroxyalkanoates to uptake phosphorus during the aerobic period. As a result; phosphorus removal deteriorates. Therefore, glucose is not a strong candidate carbon source to be supplemented to EBPR systems that do not contain sufficient volatile fatty acids.

6.2. Introduction

In the last decade, enhanced biological phosphorus removal (EBPR) has become a very popular method to remove phosphorus (P) from wastewater. Its popularity relates to low cost, low sludge generation and simple operation compared to traditional chemical phosphorus removal processes. At present, newly designed wastewater treatment plants all over the world are implementing EBPR systems and older plants are being refurbished to add biological phosphorus removal. It is well established that short chain volatile fatty acids (VFAs) (e.g. acetate, propionate, etc) are used as carbon sources and play an important role in EBPR systems (Hollender et al 2002; Cokgor et al., 2004; Pijuan, et al., 2004b; Machado 2004; Oehmen et al, 2004, 2005a and 2006; Chen and Gu 2006) and several models for VFA uptake and accumulation into bacteria have been proposed (Comeau et al., 1986; Grady et al., 1999). In addition, another carbon source, glucose has been experimented with in EBPR system to identify P removal performance (Tasli, et al.,

1997; Carucci et. al., 1999; Sudiana et al., 1999; Jeon and Park, 2000; Jeon et. al., 2001; Wang, et al., 2002).

VFAs are formed as wastewater travels sewer lines and the amount of VFA present in individual plants depends on the wastewater characteristics, travel time, and temperature. The wastewater of some plants does not contain sufficient VFA to support P removal, mainly during winter when VFA generation rates are much slower than during summer. Supplemental carbon source is therefore needed. Because of the need to add additional carbon source to EBPR systems with insufficient carbon and given the performance of EBPR vary with carbon source type, it is important to evaluate the effects of individual carbon type on the microbiology of EBPR systems. Kong et al. (2001, 2002a), Machado (2004) and Pijuan (2004) were the first researchers to report on the influence of carbon type on the microbiology of EBPR systems. In the last few years, several other researchers have also contributed knowledge to this area (Wong et al., 2004, Beer et al., 2004, Oehmen et al., 2005a & 2005c, Lu et al., 2006, Oehmen et al., 2006, Meyer et al., 2006, Burrow et al., 2007). To date, only a few bacteria have been found that can perform EBPR (Crocetti et al., 2000; Pijuan et al., 2004a; Oehmen et al., 2005a; Nakamura et. al., 1995a; Wong et al., 2005; Kong et al., 2005) and they are referred to as Polyphosphate Accumulating Organisms (PAOs). There are also bacteria that have been found to promote the deterioration of EBPR performance and they are known as Glycogen Accumulating Organisms (GAOs).

Glucose, a non-VFA carbon source, plays a role in the EBPR system (Tasli et al., 1997; Jeon and Park, 2000; Wang et al., 2002; Machado, 2004). The performance of EBPR process has been shown to deteriorate with glucose-rich influents. It is thought

that the deterioration of EBPR when glucose is the carbon source is related to the presence of microorganisms that use glycogen instead of polyphosphate as an energy source (Tasli et al., 1997; Wang et al., 2002) in the anaerobic zone of EBPR. In contrast, several researchers found good P removal in glucose fed reactors (Jeon and Park, 2000; Wang et al., 2002). Wang et al., (2002) modified the reactor's operating conditions to obtain EBPR immediately after adding glucose. They increased anaerobic retention time from 2 to 2.5 hours, glucose concentration in the influent from 150 to 200 mg C/L, and decreased aerobic retention time from 4 to 3.5 hours with low DO values (i.e. from 5-6 to 2-3 mg O_2/L). As a result, the effluent P concentration was less than 1 mg/L for 3 months. Jeon and Park (2000) operated a sequencing batch reactor (SBR) fed with glucose for 250 days and observed that about 70-80 days were required to obtain P removal efficiency of 100%. Initially, P release and uptake were low but with time it reached 100% (Jeon and Park, 2000). Similarly, Machado (2004) observed that P removal improved at the end of 58 days run in a SBR fed with glucose. He revealed the absence of Accumulibacter and low levels of Competibacter at the end of the run. Kong et al. (2004) performed batch tests using sludge collected from three full-scale plants and suggested that Accumulibacter could not uptake glucose directly under anaerobic conditions. Therefore, glucose might not select Accumulibacter (Machado, 2004 and Kong et al., 2004) and Competibacter (Machado, 2004) in an EBPR system. Nakamura et al., (1995a) observed that *Microlunatus phosphovorus* can uptake glucose and release P under anaerobic conditions with subsequent uptake of P under aerobic conditions. Thus, *Microlunatus phosphovorus* might be a possible PAO in presence of glucose. Kong et al., (2001 and 2002a) identified the abundance of *Micropruina glycogenica* in a

SBR fed with a mixture of acetate and glucose and showed no P removal. They confirmed by batch tests that *Micropruina glycogenica* could consume glucose and might be a possible GAO when glucose is used as a carbon source.

The results of previous research described above demonstrate that there are several gaps that have to be addressed in order to understand how carbon source types affect the microbiology of EBPR system. Presently, there are not sufficient data on the type of PAOs and GAOs that can be involved in EBPR systems in presence of glucose as a sole carbon source. Therefore, in this research, the influence of glucose on the microbiology of an EBPR system will be investigated using glucose as a sole carbon source. It is hypothesized that the use of PAO and GAO probes will detect for PAOs and GAOs involved in EBPR systems fed with glucose as a sole carbon source.

In this research, two PAO bacteria, which are *Candidatus* Accumulibacter phosphatis (CAP), and *Microlunatus phosphovorus* (MP) and two GAO bacteria, which are *Candidatus* Competibacter phosphatis (CCP) and *Micropruina glycogenica* (MIG) were targeted using oligonucleotide probes in a sequencing batch reactor fed exclusively with glucose. CAP, MP, CCP and MIG were targeted using probes PAOMIX (i.e. PAO462, PAO651 and PAO846), MP-2, GAOMIX (i.e. GAOQ431 and GAOQ989) and MIG-184, respectively.

6.3. Materials and Methods

6.3.1. Laboratory set up of SBRs

A laboratory setup was built consisting of two 8-liter lab-scale SBRs. The operation of the reactors was automated using two timers, solenoid valves and two peristaltic

pumps. The reactors were connected to a feeding tank, air and nitrogen gas supply sources. Besides, a mixer was attached to each reactor, and an air/nitrogen diffuser was placed at the bottom of the reactor. Figures 6.1 and 6.2 show the schematic and experimental set up of one reactor established in the laboratory.

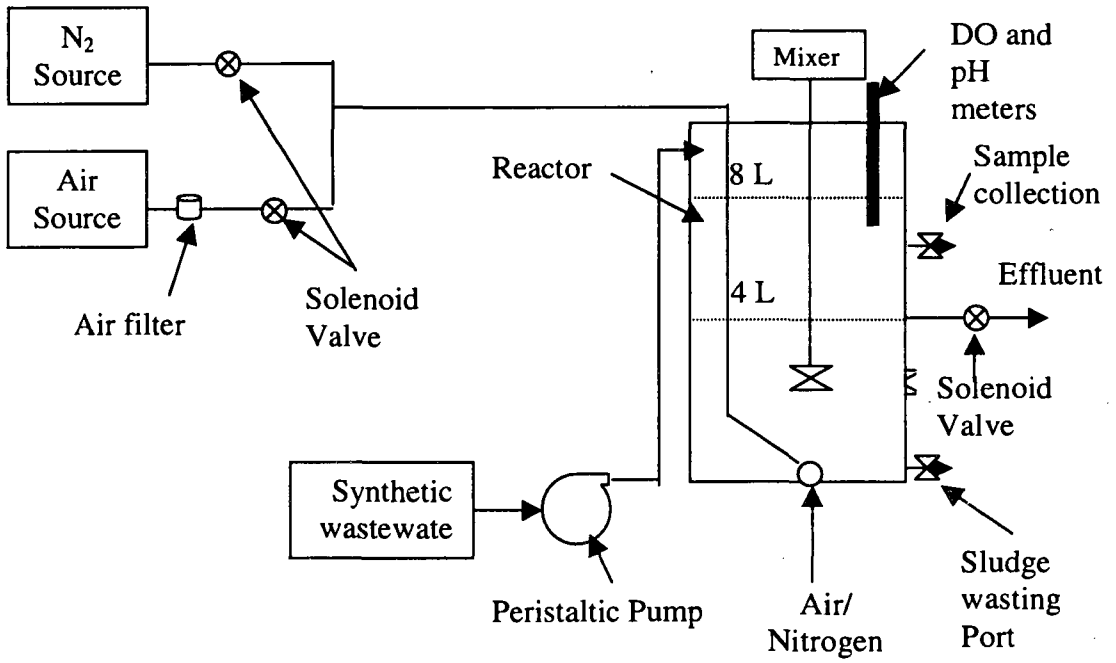


Figure 6.1: Schematic of the setup of one SBR in the laboratory

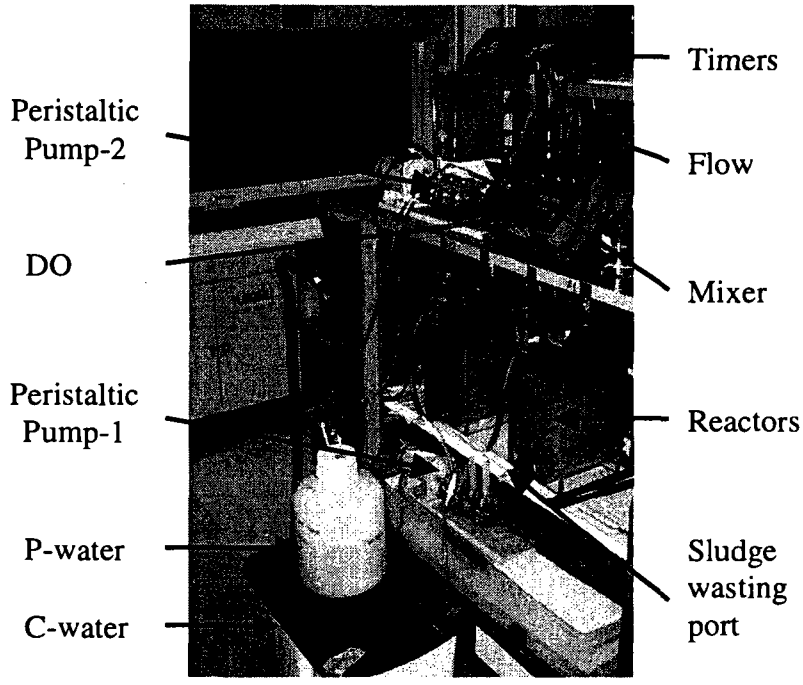


Figure 6.2: Experimental setup of the SBRs fed exclusively with glucose

6.3.2. Preliminary design of SBRs

Reactors were run according to the preliminary design, summarized in Table 6.1. Adjustments of design parameters were performed during operation of SBRs as needed and are noted in the test results.

Table 6.1: Parameters used in the preliminary design of SBRs

Parameters	Design value
Influent flow	12 L/d
Influent COD	200 mg/L
Influent phosphorus	10 mg P/L
Effluent COD	0.02 mg/L
Effluent phosphorus	5.26 mg/L
SRT	8 days
HRT	0.667 days (16 hours)
MLVSS	2500 mg/L
Volume of reactor	8 L
SVI	71 ml/g

6.3.3. Operation of SBRs

Two SBRs (G1 and G2) were operated under similar operating conditions using exclusively with glucose as a sole carbon source for a period of 78 days. This run time was selected based on the results obtained by (Machado 2004). He observed that the P removal improved in the reactor fed with glucose at the end of 58 days of run. Due to time limitation, he was unable to identify whether the improvement in P removal in the reactor fed with glucose was stable. Therefore, in this research, the SBRs were operated for 78 days to investigate the behavior and microbiology of the EBPR system using glucose as a sole carbon source.

The SBRs were inoculated with biomass from an aeration basin of a local wastewater treatment plant that performs EBPR and were filled with synthetic wastewater (i.e. C-water) (Table 6.2) and phosphate solution (i.e. P-water) at flow rates of 254 ml/min and 40 ml/min, respectively. The SBRs were operated in 3 cycles of 8 hours. Each cycle had five steps, which were carried out in the following order: fill (15 minutes), anaerobic period (2.5 hours), aerobic period (4.5 hours), settle (30 minutes) and withdraw (15 minutes). At the beginning of the anaerobic period, N_2 gas was supplied and dispensed as bubbles into the reactors for 2 minutes at a flow rate of 13 standard cubic feet per hour (SCFH) in order to create anaerobic condition (i.e DO concentration less than 0.2 mg/L). During aerobic period, air was sparged into the reactors using a ceramic stone diffuser at a flow rate of 8 SCFH to maintain a DO concentration greater than 2 mg/L. After settling of sludge, 4 liters of wastewater was withdrawn from the reactors by gravity through effluent outlets and about 209 ml of settled sludge was collected through sludge wasting port to maintain a SRT of 8 days at room temperature (22 ± 2° C). The average

concentrations of mixed liquid suspended solids (MLSS) were 2393 and 2353 mg/L, whereas the average concentration of mixed liquid volatile suspended solids (MLVSS) were 2111 and 2086 in G1 and G2 reactors, respectively. The percentage of MLVSS was about 89% in both reactors.

6.3.4. Preparation of synthetic wastewater for the SBRs

The C-water and P-water were prepared according to the synthetic wastewater's composition of Oehmen et. al., (2005a). The C-water contained carbon, nitrogen and nutrient sources whereas the P-water contained only phosphorus (Table 6.2). The reactor was run for 63 days using the concentrations shown in Table 6.2. After that only glucose and P concentrations were increased to 200 mg C/L (i.e. 535 mg COD/L) and 15 mg P/L, respectively for the last two weeks of the run to observe P removal variation in the reactors. Both C and P-water were supplied into the reactors separately in order to avoid biodegradation in the tubing and precipitation of phosphorus.

Table 6.2: Composition of synthetic wastewater adapted from Oehmen et. al., (2005a)

Parameters	Amounts
Carbon (glucose)	200 mg COD/L
Nitrogen (i.e. NH_4Cl)	27 mg /L
Phosphorus (i.e. $NaHPO_4.H_2O$)	10 mg /L as P
Magnesium (i.e $MgSO_4.7H_2O$)	43 mg /L
Calcium (i.e. $CaCl_2.2H_2O$)	20 mg /L
Peptone	12 mg /L
ATU	0.525 mg /L
Trace elements*	0.14 ml/L

* Trace elements (g/L): 1.5 g $FeCl_3.2H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 $MnCl_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$ and 10 g EDTA)

6.3.5. Collection and preservation of samples

Six hundred eighty-ml of sample were collected during a cycle. To avoid P release after a sample was taken, about 15 ml samples were immediately filtered through 0.45 μm syringe filter (GHP Acrodisc, PALL) and refrigerated. The samples were analyzed on the same day of collection or preserved when necessary.

6.3.5.1. Chemical analysis

Samples were analyzed for orthophosphate (OP), soluble total organic carbon (sTOC), and suspended and volatile suspended solids (SS/VSS) as per Standard Methods. OP was measured using HACH kit (PhosVerR 3 Method 8048) with a DR/3000 spectrophotometer. Soluble TOC was analyzed according to the Standard Method 5310-B (Eaton et al., 2005) using a TOC Analyzer (Model TOC-$V_{CPH/CPN}$, SHIMADZU). Standard Methods 2540D and 2540E were used to measure total suspended solids (SS) and volatile suspended solids (VSS), respectively using a 47mm Whatman GF/C microfiber glass filter. Filtered samples were utilized for OP and sTOC analysis whereas the unfiltered samples were used for SS/VSS analysis. SS/VSS was measured on the same day of sample collection. DO (YSI Model 54A) and pH (Accumet, AR25) were measured at the time of sample collection.

The mean % removal of P between the replicate reactors fed with glucose (G1 and G2) was compared using a two-tailed Independent-samples t Test assuming a 95% confidence level ($p <= 0.05$). The null hypothesis was that the difference in mean P removal percentage between the replicate reactors (i.e. G1 and G2) was zero. The alternate hypothesis was that the mean P removal percentages between the replicate

reactors were different. Standard error of mean P removal percentage was calculated for reactors fed with glucose.

6.3.5.2. Microbiological analysis

a) FISH procedure

Unfiltered samples were used for Fluorescence in Situ Hybridization (FISH) analysis. The procedure for FISH analysis was adopted from Amann (1995) and modified by De Los Reyes (2003). For gram-negative bacteria, 3ml of the sample were mixed with 9 ml of 4% ice-cold paraformaldehyde (96%, Fisher Scientific, Suwanee, GA) in a 15 ml plastic centrifuge tube (VWR) for fixation. Fixation is required to maintain the morphological integrity of the cells and to minimize the auto-fluorescence (Amann, 1995). The sample was then kept in the refrigerator at 4^0C for 2.5 hours. After fixation, the sample was washed two times with 1 x phosphate buffer saline (PBS) solution (1 volume of 3 x PBS; 390 mM NaCl in 30 mM $NaPO_4$ buffer and 2 volume DI water) by spinning the sample in a centrifuge (SORVALL, Legend RT) at 2000 rpm for 5 minutes to remove the fixative solution. The sample was stored in the refrigerator at -20^0C by adding 1x PBS/ethanol (1:1) solution. For gram-positive bacteria, 1 volume of sample was mixed with 1 volume of 50% ethanol (v/v) (95%, IBI-Scientific, IA) and the sample was stored at –20°C (Kong et. al., 2005 and personal communication with Simon Mcllroy, referred by Dr. R. Seviour, Biotechnology research center, La Trobe University, Bendigo, Victoria, Australia). Next, 3 µl of sample was applied into three wells of a 6-well Teflon-coated microscope slide (Cel Line, Portsmouth, NH) to immobilize the cell. The sample of the slide was air dried for about 45-50 minutes. Afterwards, it was dehydrated by successive dipping into 50%, 80% and 95% ethanol in staining jars (3

minutes per step) and air dried for 8-10 minutes. Next, 8 µl hybridization buffer, 1 µl of EUB bacteria probe and 1 µl the desired PAO or GAO probes were applied to three wells. The details of hybridization buffer are given in Table 6.3. To hybridize the samples, the slide was inserted into a properly sealed moist chamber, which was kept in an oven (Millipore, Billerica, MA) at 46^0C for 60-120 minutes without shaking. The moist chamber was built in-house using a 50 ml centrifuge tube and a piece of Whatman filter paper wetted with 0.5 ml of hybridization buffer. A properly sealed moist chamber is necessary for hybridization to avoid evaporation of hybridization solution, which leads to nonspecific binding of the fluorescent probe to the cells (Amann, 1995). After hybridization, the sample was washed with 50 ml wash solution in a water bath (Model AP-152 from SOILTEST, Lake bluff, IL) at 48^0C for 20 minutes. The details of wash solution are given in Table 6.3. The slide was dipped again in 50 ml centrifuge tube containing ice-cold deionized water for 3 seconds and air dried until all water droplets are removed. Finally, the slide was mounted with a microcover glass (24 X 60mm, VWR Scientific) by using Citifluor mountant media (Ted Pella Inc., Redding, CA). The basic steps for FISH analysis are illustrated in Figure 6.3.

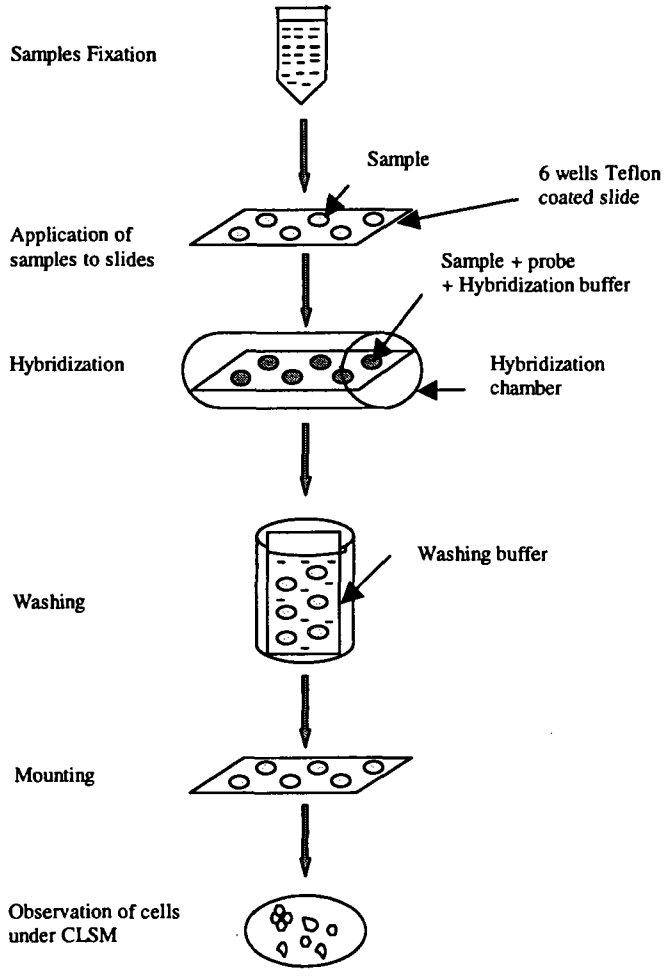


Figure 6.3: Steps of sample preparation and hybridization for FISH analysis

(Source: modified from Moter and Gobel, 2000)

Table 6.3: The ingredients of hybridization buffer and wash solution

Name of bacteria	Hybridization buffer					
	FA (µl)	MQ (µl)	5M NaCl (µl)	1 M Tris/HCl (pH = 8.0) (µl)	10% SDS (µl)	0.5 M EDTA (µl)
MP2 (% FA = 10)	200	1400	360	40	2	-
MIC184 (% FA = 20)	400	1200	360	40	2	-
CAP & CCP (% FA = 35)	700	900	360	40	2	-
	Wash solution *					
MP2 (% FA = 10)	-	-	4500	1000	50	-
MIC184 (% FA = 20)			2150	1000	50	500
CAP & CCP (% FA = 35)	-	-	700	1000	50	500

* The washing buffer was made in 50 ml tubes and was filled up to 50 ml using dH_2O

In the FISH analysis, the PAOMIX probe (i.e. PAO462, PAO651, PAO846) (Crocetti et. al., 2000) and MP2 (Kawaharasaki et al., 1999) were used to detect *Candidatus* Accumulibacter phosphatis and *Microlunatus phosphovorus* (i.e. PAOs) respectively. Moreover, GAOMIX probes (i.e. GAOQ431, GAOQ989) (Crocetti et. al., 2002) and MIC184 (Kong et. al., 2001) were utilized for targeting *Candidatus* Competibacter phosphatis and *Micropruina glycogenica* (i.e. GAOs) respectively. The probes for targeting PAOs and GAOs were labeled by Cy3. The EUBMIX probe (i.e. EUB338, EUB338-II and EUB338-III) (Crocetti et. al., 2002) labeled with Cy5 was used to target the entire bacterial community, because only domain-specific probe EUB 338 is not enough to detect all bacteria (Daims et. al., 1999). Cy3 and Cy5 are fluorescent dyes of

cyanine group with fluorescence color orange-red and near infrared, respectively use to visualize the microbes under microscope. All probes used in this research were purchased from a company named Integrated DNA Technology, Inc. (Coralville, IA) with HPLC purification. The details of these oligonucleotide probes are given in Table 6.4.

b) Specificity test

To assure that the purchased probes were specific to the targeted GAOs and PAOs, two measures were taken. First, the purchased sequence was checked with the microbial sequence database BLAST (Basic Local Alignment Search Tool) available from NIH online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligonucleotide sequences were compared with a database of sequences (i.e. Nucleotide collection, nr/nt) to detect sequences with high identity. This operation was performed for all probes and a match varying from 87-95% and 94-100% was found for PAO462 and EUB338 probes, respectively. All other probes matched 100%. The second check of specificity was performed using an enrichment culture available in our laboratory that is known to degrade perchlorate. A sample of the culture was taken and prepared for FISH as described above. Every probe to be used was tested with the sample and no hybridization occurred for all GAO and PAO probes while the EUB bacteria hybridized.

c) Microscopy and microbial quantification

For a sample, twenty digital images (ten images/well) of PAOs and GAOs were captured from two wells using confocal laser scanning microscope (Zeiss LSM510, Axioplan 2) using Argon (488, 514 nm) and HeNe (633 nm) lasers for the excitation of dyes Cy3 and Cy5, respectively. The emission filters for Cy3 and Cy5 were 530-600 nm

and 650 nm, respectively. 400X magnification was used to observe the microbes under the microscope. To quantify the organisms, the images were analyzed using the software ImageJ available free of charge from NIH (National Institute of Health). For an image, the % relative abundance (RA) of PAOs/GAOs was calculated based on the ratio of the area of PAOs/GAOs to entire bacterial population. The average % RA value of twenty images was considered as the final % RA of PAOs/GAOs in the entire bacterial population. Statistical analysis was performed between the mean % RA of ten images taken from two duplicate wells in a slide based on a two-tailed Independent-samples t Test at a 95% confidence level. The null hypothesis was that there was no difference between means % RA of PAOs/GAOs in two wells of a slide. The alternate hypothesis was that there was difference in the mean %RA of PAOs/GAOs in two wells of a slide. In addition, the final % RA value (i.e. mean of twenty images) of PAOs/GAOs between G1 and G2 reactors were compared statistically for each sampling day. Moreover, to compare the % RA of PAOs/GAOs in replicate reactors fed with glucose, mean value of %RA of PAOs/GAOs of each reactor was determined and compared statistically described above. Finally, the standard error of mean %RA of PAOs/GAOs was determined between replicate reactors fed with glucose.

Table 6.4: Oligonucleotide probes used in this research

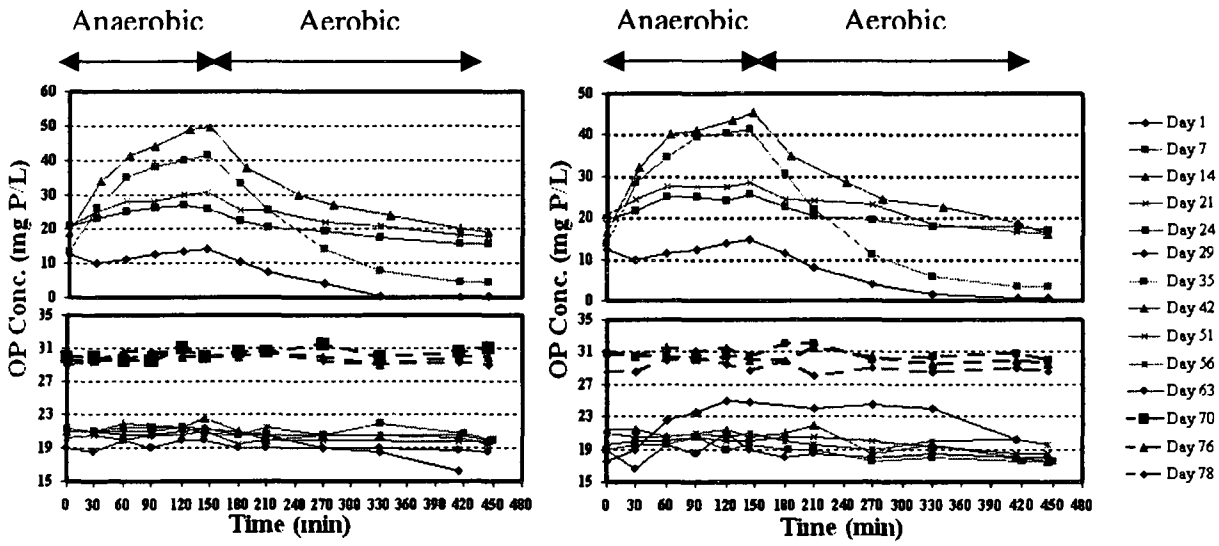
Probe	Specificity	rRNA Target site	Sequence (5'-3')	(%) Formamide	Reference
EUB338	Many but not all *Bacteria*	16S, 338-355	GCTGCCTCCCGTAGGGT	0-70	Crocetti et. al., 2002
EUB338-II	*Planctomycetales*	16S, 338-355	GCAGCCACCCGTAGGTGT	0-50	Crocetti et. al., 2002
EUB338-III	*Verrucomicrobiales*	16S, 338-355	GCTGCCACCCGTAGGTGT	0-50	Crocetti et. al., 2002
			PAOs		
PAO462	*Candidatus* Accumulibacter phosphatis	16S, 462-485	CCGTCATCTACWCAGGGTATTAAC	35	Crocetti et. al., 2000
PAO651	*Candidatus* Accumulibacter phosphatis	16S, 651-668	CCCTCTGCCAAACTCCAG	35	Crocetti et. al., 2000
PAO846	*Candidatus* Accumulibacter phosphatis	16S, 846-866	GTTAGCTACGGCACTAAAAGG	35	Crocetti et. al., 2000
MP2	*Microlunatus phosphovorus*	16S , 68-87	GAGCAAGCTCTTCTGAACCG	10	Kawaharasaki et al., 1999
			GAOs		
GAOQ431	*Candidatus* Competibacter phosphatis	16S, 431-448	TCCCCGCCTAAAGGGCTT	35	Crocetti et. al., 2002
GAOQ989	*Candidatus* Competibacter phosphatis	16S, 989-1006	CACCTCCCGACCACATTT	35	Crocetti et. al., 2002
MIC184	*M. glycogenica*	16S, 184-200	CATTCCTCAAGTCTGCC	20	Kong et al., 2001

6.4. Results

6.4.1. Overall performance of glucose fed reactor

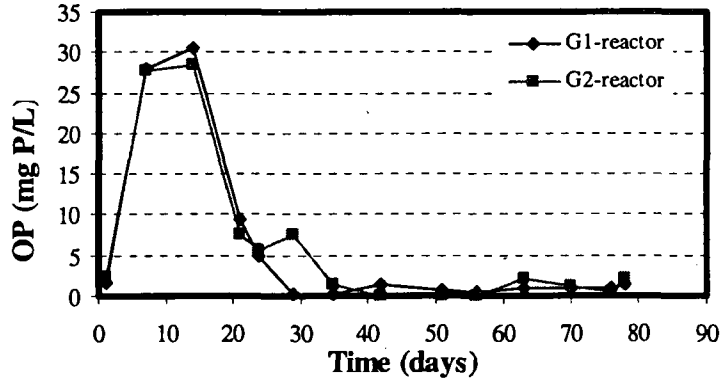
Two SBRs (G1 and G2) were operated using glucose as a sole carbon source under similar operating conditions for 78 days. The OP profiles of both reactors are shown in Figures 6.4a and 6.4b. The top lines show profiles for days 1 to 24. The bottom lines depict profiles for days 29 to 63. The middle dashed lines show profiles for days 70 to 78, after the concentrations of P and glucose were increased from their initial values. The results show that initially the system operated well, but it increasingly got worst with complete deterioration after the 29th day. Initially, both P release and uptake were observed during the anaerobic and aerobic periods, respectively. However, the effluent P concentration increasingly got worst. The maximum OP concentration was above 45 mg P/L at the end of anaerobic period at day 14. The corresponding effluent P concentration was above 16.6 mg P/L.

Based on research results reported by Wang et al. (2002), an attempt was made to improve P removal performance of the glucose fed reactors. The concentrations of glucose and P were increased in the reactors from days 64 to 78. The results show no noticeable P release and uptake in the system during this period (dashed lines in Figures 6.4a and 6.4b). Figure 6.4c shows the daily net phosphorus release (mg P/L) during the entire experimental period. Similar to the trend seen in Figures 6.4a and 6.4b, the data show good P release up to day 14 and then a significant decrease in P release up to the end of the experimental period. The maximum net P release of 30 mg P/L was found at day 14



(a) OP profiles in G1

(b) OP profiles in G2

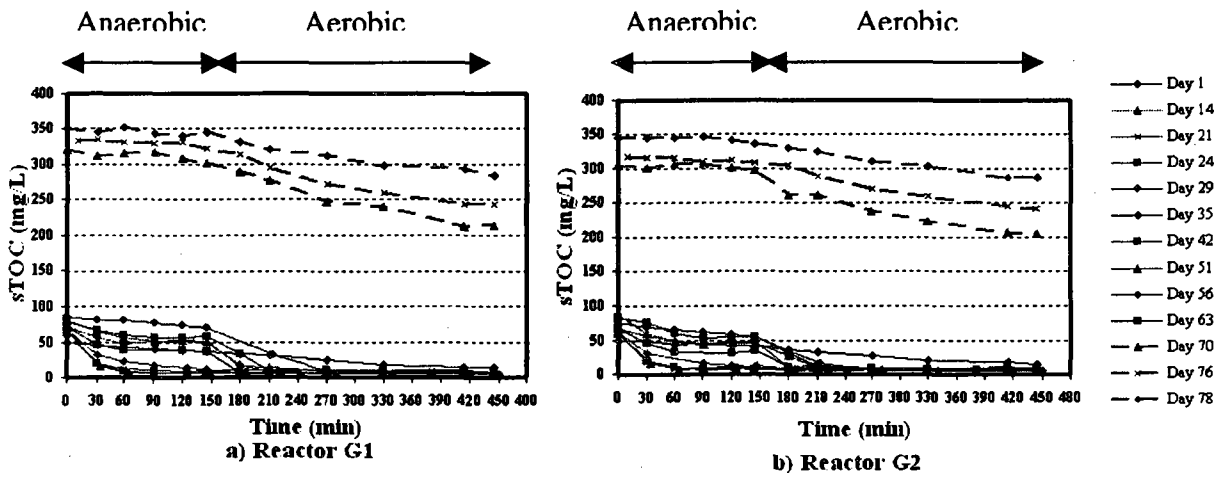


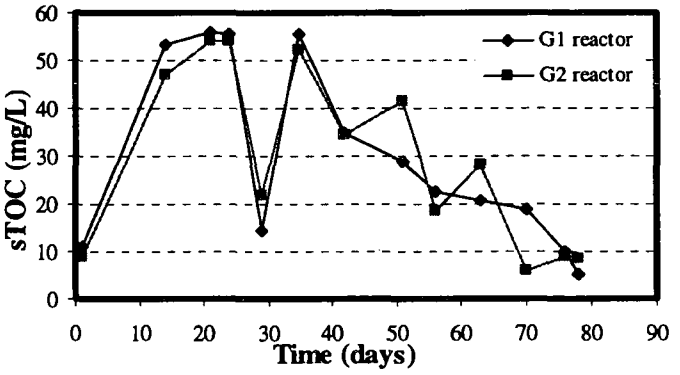
(c) Daily net OP release in G1 and G2 reactors

Figure 6.4: Orthophosphate profiles in reactors G1 (a) and G2 (b) showing P release and uptake during anaerobic and aerobic periods, respectively. Daily net P release in G1 and G2 reactors during the entire experimental period (c).

Figures 6.5a and 6.5b show the profiles of sTOC in the reactors during the run. From days 1 to 24, complete uptake of sTOC was observed within 60 minutes. Then, the sTOC uptake during the anaerobic period started to decrease significantly coinciding with the decrease in P release (Figures 6.4a to 6.4c). After P release started decreasing in the anaerobic period, higher sTOC concentrations were observed and as a consequence higher sTOC concentrations were seen in the aerobic period. However, during the anaerobic period, the sTOC was consumed without any P release. The net sTOC consumption in the anaerobic period for the entire run is shown in Figure 6.5c. A maximum consumption of about 55 mg sTOC/L was observed at day 24. After day 35, significant decrease occurred.

Figures 6.6a and 6.6b show the variation of P-release/C-uptake ratio and % P removal during the reactor run, respectively. The detail calculations for P-release/C-uptake ratio and % P removal are presented in Appendix III. Following the same pattern seen with the net P release, the P-release/C-uptake ratio increase up to day 14 to a maximum of 0.23 moles P/moles C, and then it continuously decreased until the end of the experimental period. The % P removal in the reactors decreased from 90% in the first days to 55% in day 7. After day 7, P removal started deteriorating and the % P removal reached about 10% because P accumulated from one cycle to another. At day 14, the P release was the greatest and uptake was the poorest, resulting in negative P removal. Statistical analysis shows that the % P removal in G1 and G2 reactors was not significantly different ($p = 0.772$) based on a two-tailed Independent-samples t Test at a 95% confidence level. The standard errors of mean of G1 and G2 reactors were 8.1 ± SE 10.4 and 12.2 ± SE 9.5, respectively.





(c) Daily net sTOC consumption in G1 and G2 reactors

Figure 6.5: Soluble TOC profiles in reactors G1 (a) and G2 (b). Net sTOC consumption in G1 and G2 reactors during the anaerobic period (c)

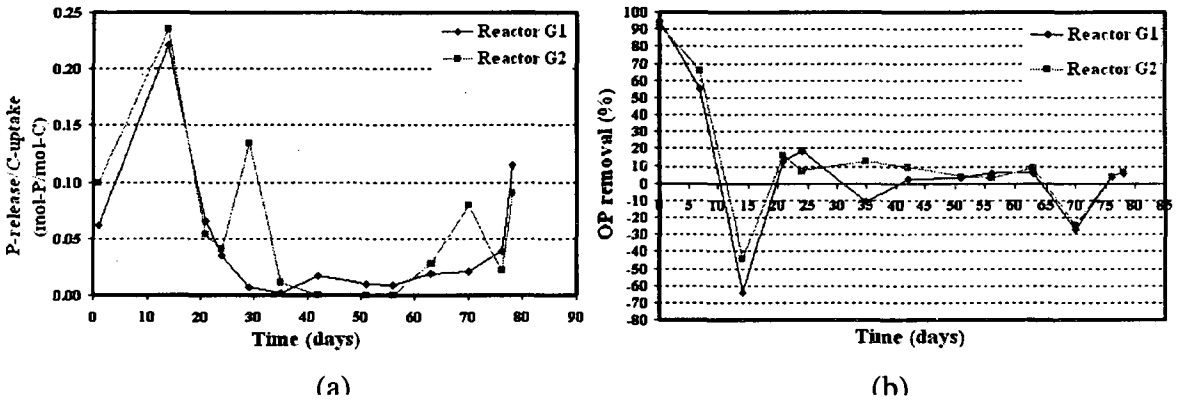


Figure 6.6: Variation of P-release /C-uptake ratio (a) and % P removal (b) during the experimental period

Similar to the present study, other researchers (i.e. Tasli et al., 1997; Wang et al., 2002; Machado, 2004) found that the performance of the EBPR process deteriorated with glucose-rich influent. However, other researchers have changed operating parameters that resulted in improved conditions in reactors fed with glucose. Machado (2004) also observed a very low P release and uptake in the reactor fed with glucose as a sole carbon source during his experimental period of 58 days. Machado (2004) found that the maximum P concentration was 25 mg P/L at the end of anaerobic period, and the effluent P concentration varied from 2.8 to 10.5 mg/L during the reactor run. Other researchers (i.e. Tasli et al., 1997; Jeon and Park, 2000; Wang et. al., 2002) also noticed that P release decreased in the presence of glucose under anaerobic conditions. Jeon and Park (2000) noticed that the consumption rate of glucose increased with continuous operation of an SBR reactor, although the initial rate was slow. In their study, due to the slow uptake of glucose, it took time to improve the P release and subsequently, P uptake. They reported that about 70-80 days were required to reach 100% efficiency of P removal. Wang, et al.,

(2002) report an effluent P concentration of less than 1 mg/l for three months, after increasing the anaerobic retention time, glucose concentration, and decreasing aerobic retention time. In addition, they maintained a pH of 7 ± 0.1.

6.4.2. Microbial species present in the reactors fed glucose

FISH analysis revealed the presence of two PAOs (*Candidatus* Accumulibacter phosphatis (CAP), *Microlunatus phosphovorus* (MP)), and two GAOs (*Candidatus* Competibacter phosphatis (CCP) and *Micropruina glycogenica* (MIG)) in the reactors. Statistical analysis for all species investigated show that there exists no significant different, at 95% confidence level, between the mean % RA of CAP ($p = 0.971$), mean % RA of MP ($p = 0.633$), mean % RA of CCP ($p = 0.886$) and mean % RA of MIG ($p = 0.861$) in G1 and G2 reactors based on a two- tailed Independent- samples t Test. That is the species were present in the duplicate reactors G1 and G2. In addition, statistical analysis was performed between the mean % RA of specific bacteria in two wells of a slide considering duplicate sample, and between the mean % RA of specific bacteria at different days in G1 and G2 reactors. The details of the statistical analysis are given in Appendix IV.

The variation of % RA of CAP, a PAO, during the reactor run is shown in Figure 6.7a. The images of CAP captured using confocal laser scanning microscope (CLSM) at different days are depicted in Figure 6.8a. It was observed that % RA of CAP was 13% at the start up of the reactor, and increased to about 42% in 14 days. Then, the % RA of CAP started to reduce, and reached below 4% at the end of the run. Machado (2004) was the first researcher who used PAOMIX probe to target CAP in a SBR fed exclusively with glucose. He found, using FISH analysis, that the CAP was about 8% in the first day

of run. Then, % RA of CAP started to reduce, and was absent after 20 days in the reactor fed with glucose as a sole carbon source at a COD/P ratio of 20. Machado (2004) concluded that glucose is detrimental to CAP. Moreover, Kong et al. (2004), using FISH-MAR analysis with sludge collected from three full-scale wastewater plants, suggest that CAP cannot uptake glucose directly under anaerobic conditions. Therefore, glucose might not select for the CAP as also shown in this research. Since the sludge seed sample used in this research was collected from a full-scale treatment plant that performs EBPR with VFAs, CAP was originally present, but feeding exclusively glucose did not select for CAP and that explains the reduction in their relative abundance with time.

The variation of % RA of MP, a PAO, is illustrated in Figure 6.7b and the images of MP captured using CLSM during the reactor run are given in Figure 6.9a. The changing pattern in the % RA of MP for G1 reactor was comparable to that for G2 reactor. Initially, % RA of MP was below 4%. However, % RA of MP was about 7% until day 63. The highest % RA of MP was about 21% and 13% in G1 and G2 reactors, respectively at day 51. The % RA of MP was below 2% with the increase of glucose concentration for the last 14 days. Not much research has been performed on the variation of % RA of MP in a SBR fed exclusively with glucose as a carbon source. Researchers (Nakamura et. al., 1995a; Nakamura, et al., 1995b; Kawaharasaki et al., 1999) identified that MP might act as a PAO in the EBPR process. Nakamura et al., (1995b) and Mino, (2000) reported that MP strain NM-1 could utilize glucose as a carbon source. In addition, Nakamura et al. (1995a) found that, when fed glucose, the strain

could uptake (20 mg-P/g-cell. hr) and release (10 to 30 mg-P/g-cell. hr) P, depending on the influent concentration of TOC.

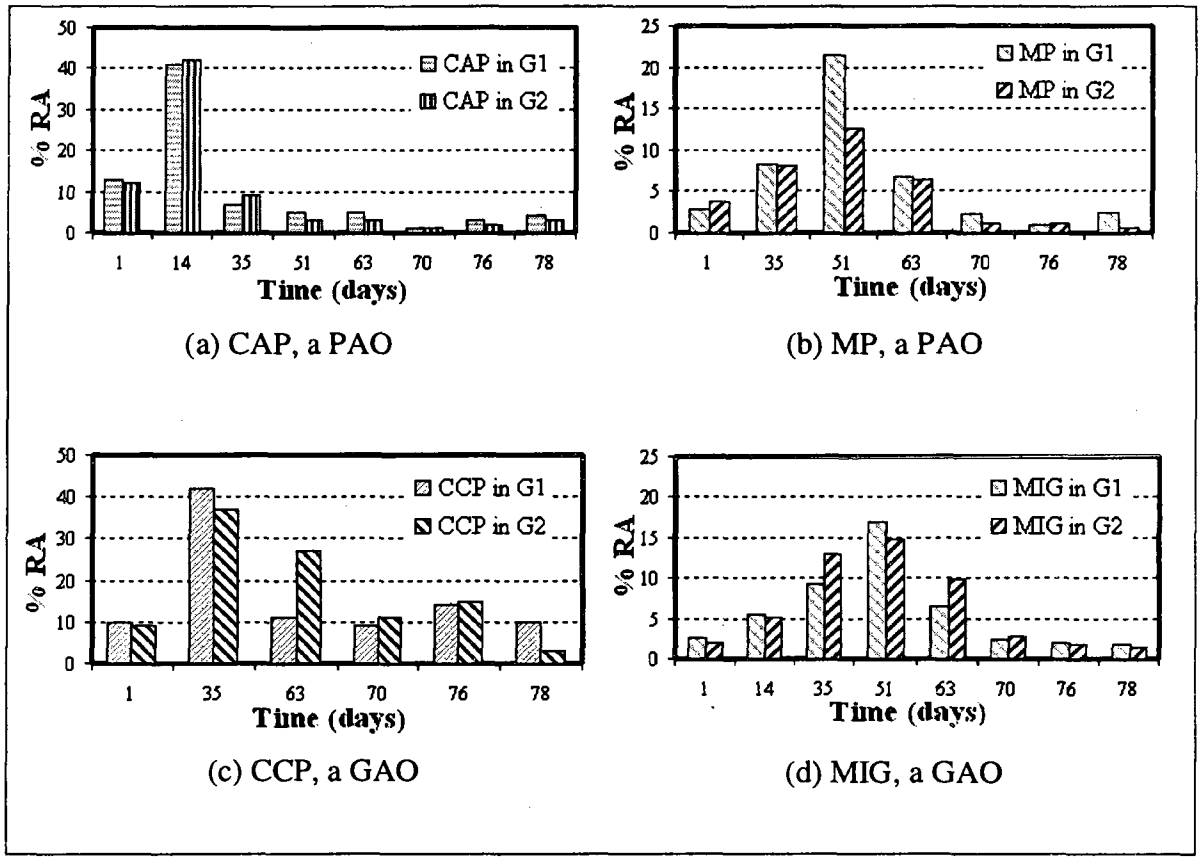


Figure 6.7: Variation of % RA of (a) CAP, (b) MP, (c) CCP and (d) MIG in reactors G1 and G2 fed with glucose.

The results of this research indicate that MP can be selected in glucose fed reactors. From two weeks running at higher glucose concentration, it seems that higher glucose concentration might be detrimental for MP. Initially, percentage of MP was less because the seed of the SBR was collected from a full-scale treatment plant, and there was insufficient glucose in the wastewater to enrich for MP. The % RA of MP increased in

presence of glucose in this study. However, increased glucose concentration reduced their abundance at the end of the experimental period.

The % RA of CCP, a GAO, of G1 and G2 reactors are given Figure 6.7c, and the images of CCP captured using CLSM throughout the reactor run are shown in Figure 6.8b. Initially, the % RA of CCP was about 10%, and increase to above 37% at day of 35. After that, the % RA of CCP was stable at about 10% until the end of the run. The % RA of CCP was higher compared to that of other microbes targeted in this study. There is not sufficient evidence about the presence of CCP in a reactor fed exclusively with glucose. However, Machado (2004) observed that the % RA of CCP was 25% at the first day of the run, and then it was increased to about 35% within 7 days. However, the % RA of CCP followed a decreasing pattern and finally, reached a value of 7% at the end of the run of 58 days. The results of this study indicate that CCP is selected in EBPR systems when glucose is fed as a carbon source.

Figure 6.7d shows that %RA of MIG, a GAO was low (i.e. about 2 %) initially. However, the % RA of MIG slowly reached to a maximum level of about 17% in 51 days. After 63 days, % RA of MIG decreased to about 1% until the end of the run. In the present study, the images of MIG captured using CLSM throughout the reactor run are shown in Figure 6.9b. Kong et al., (2001 and 2002a) identified the abundance of *Micropruina glycogenica* in a SBR fed with a mixture of acetate and glucose and showed no P removal. About 22% of total bacteria were *Micropruina glycogenica* (Kong et. al., 2001). The identified *Micropruina glycogenica* were small cocci in clusters or sheets. They confirmed by batch tests that *Micropruina glycogenica* could consume glucose and might be a possible GAO when glucose is used as a carbon source (Kong et al., 2002a).

The results of this study indicate that glucose selects for this GAO, MIG, up to a certain concentration. It seems that MIG is negatively affected by higher glucose concentration.

In summary, CAP, a PAO dominated over all other bacteria targeted in the present study until day 14. Subsequently, CCP, a GAO, out-competed all other bacteria during reactor run. MP and MIG slowly increased into the reactor, and were present until 63 days. However, their abundance was below 3% for the last 14 days, which might be an indication that higher glucose concentration is not suitable for MP and MIG.

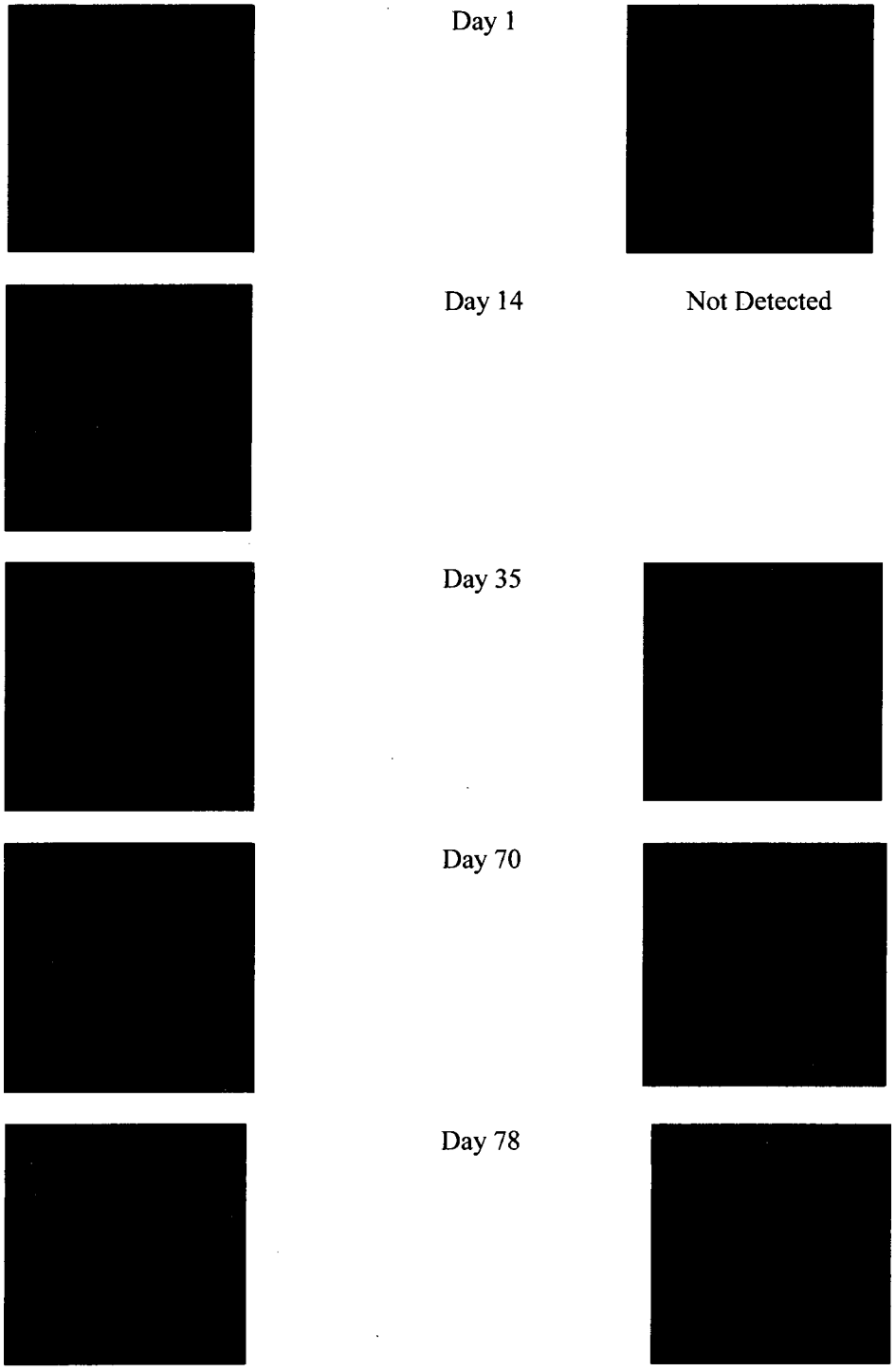


Figure 6.8: Images of CAP and CCP captured using CLSM at different day. CAP and CCP were labeled by Cy3 (channel 2) and eubacteria was labeled by Cy5 (channel 1). Pinholes for channels 1 and 2 were 337 μm and 338 μm, respectively (Scaling: X = 0.45 μm and Y = 0.45 μm).

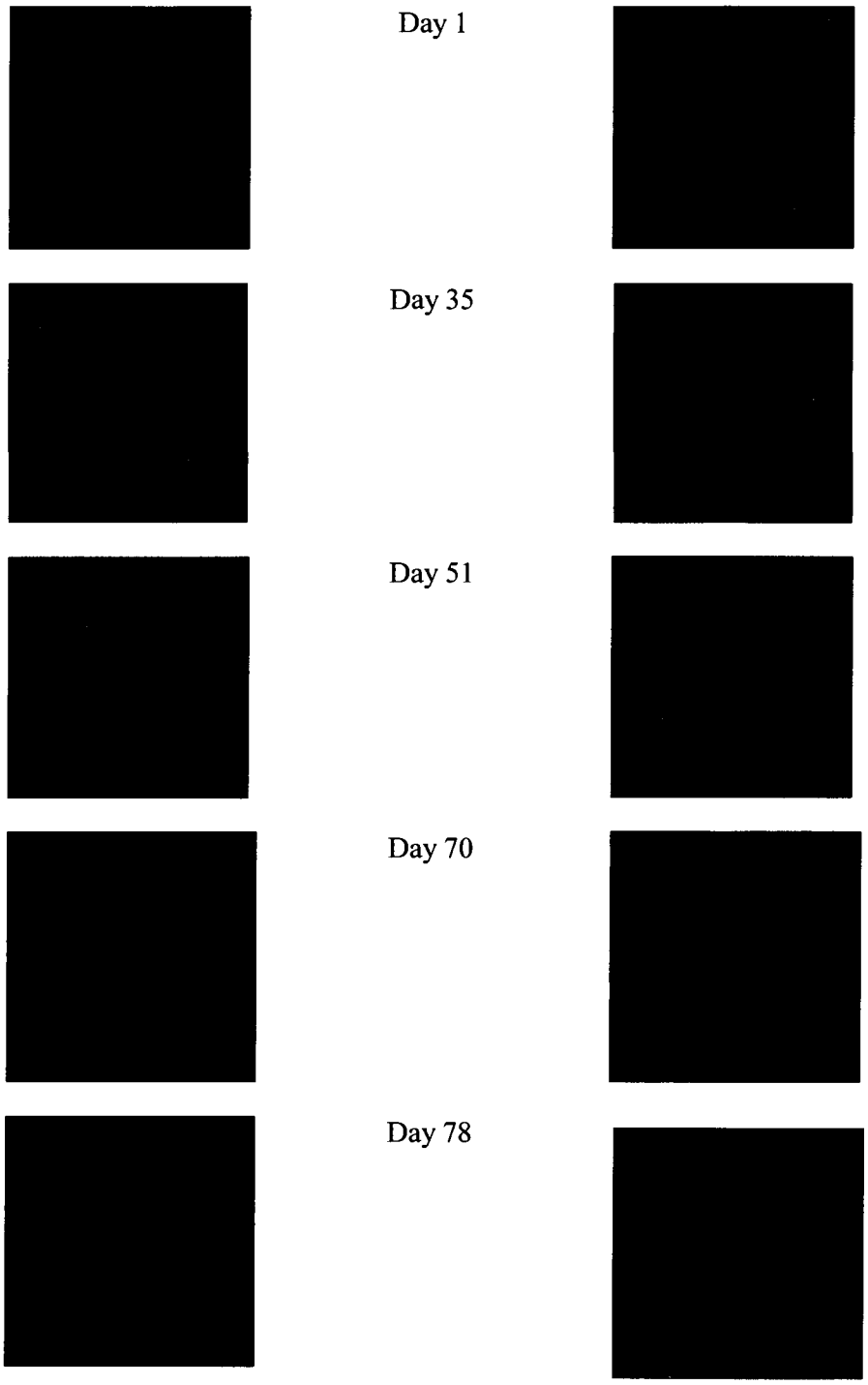


Figure 6.9: Images of MP and MIG captured using CLSM at different day. MP and MIG were labeled by Cy3 (channel 2) and eubacteria was labeled by Cy5 (channel 1). Pinholes for channels 1 and 2 were 337 μm and 338 μm, respectively (Scaling: X = 0.45 μm and Y = 0.45 μm).

6.4.3. Effect of pH in glucose fed reactor

The variation of pH in G1 and G2 reactors is given in Figure 6.10. In general, the pH decreased in the anaerobic zone and increased in the aerobic zone. In Figure 6.10 the continuous lines represent the variation of pH up to 63 days, whereas the dash lines showed the variation of pH for the last 14 days after increasing glucose concentration in the reactors. The pH values decreased under anaerobic conditions in the present study, which coincides with the findings of Jeon and Park (2000). They also observed the reduction of pH value under anaerobic conditions using glucose as a sole carbon source. Jeon and Park (2000) indicated that glycogen formation from glucose required ATP, which is provided by the generation of lactate through glycolysis of glucose, resulting pH decrease under anaerobic conditions. In the present research, the average pH values observed are summarized in Table 6.5 for anaerobic and aerobic conditions.

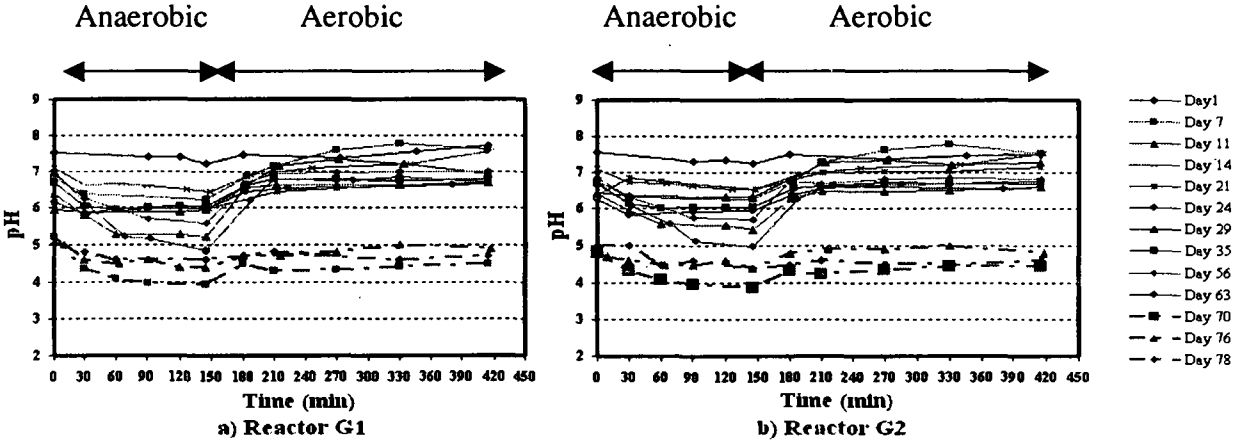


Figure 6.10: pH profiles in reactors G1 and G2

In G1 reactor, the average pH values were about 6.3 ± 0.6 and 7 ± 0.3 based on the results of 10 days under anaerobic and aerobic conditions, respectively, until day 63. After glucose concentration was increased, pH values decreased to 4.5 ± 0.2 and 4.6 ± 0.2 under anaerobic and aerobic conditions, respectively (Table 6.5). The average pH values of G2 reactors were comparable to that of the G1 reactors.

Table 6.5: Variation of pH under anaerobic and aerobic conditions in G1 and G2 reactors

Reactor	Influent parameters	Anaerobic	Aerobic
G1	COD = 200 mg/L; P = 10 mg P/L	6.3 ± 0.6	7 ± 0.3
	COD = 535 mg/L; P = 15 mg P/L	4.5 ± 0.2	4.6 ± 0.2
G2	COD = 200 mg/L; P = 10 mg P/L	6.3 ± 0.54	7 ± 0.38
	COD = 535 mg/L; P = 15 mg P/L	4.5 ± 0.25	4.6 ± 0.26

It has been reported that the activity of PAOs out-competes GAOs at high pH (Jeon et al., 2001; Serafim et al, 2002; Schuler and Jenkins, 2002; Oehmen et al., 2005b). Jeon et al., (2001) and Schuler and Jenkins (2002) observe that the metabolism of PAOs had a competitive advantage over the metabolism of GAOs at pH values greater than 7 in a SBR fed with acetate. Serafim et al, (2002) found similar results by using a mixture of acetate, propionate and butyrate at a pH of 7.8 to 8.5. It is thought that PAOs have excess energy, which is polyphosphate for up taking the carbon at high pH while GAOs have only glycogen as energy source causing the dominance of PAOs against GAOs. At high pH, electrical potential difference across the membrane of the cell is high, which requires more energy to uptake carbon sources (Smolders et. al., 1994). As PAOs have more

energy source (i.e. polyphosphate and glycogen) compared to GAOs (i.e. glycogen), PAOs might dominate over GAOs at high pH. Moreover, Pijuan et al., (2004a) proposed that the overall optimum pH for EBPR is about 7.5. Similarly, Chen and Gu (2006) recommended an optimum pH of 7.1 to7.6 for a better performance of EBPR system. However, in the present study, the pH value was about 6.3 and 7 under anaerobic and aerobic conditions, respectively. This might be a reason for the observed dominance of GAOs over PAOs after day of 14 of the experimental period, resulting in poor P removal in the glucose fed reactors.

6.5. Discussion

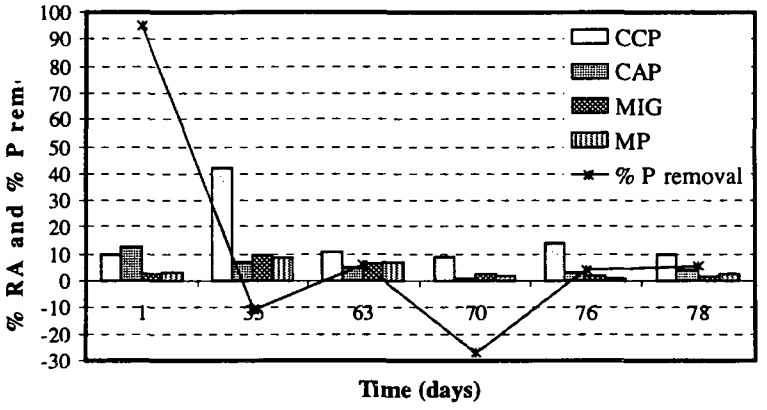
The variation of % P removal with the corresponding % RA of various microbes targeted in the present study is illustrated in Figures 6.11a and 6.11b. The patterns of P removal are similar in both, G1 and G2, reactors except for one day. The % RA of various PAOs and GAOs in both reactors is summarized in Table 6.6.

Table 6.6: % RA of PAOs and GAOs present in the reactors

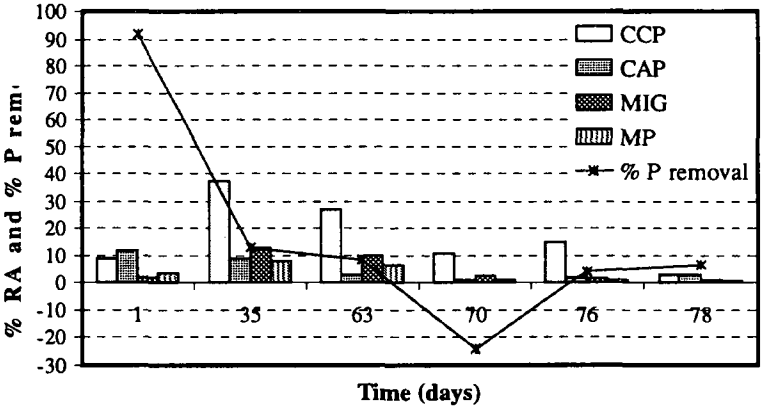
Day	G1 Reactor						G2 Reactor					
	% RA of PAO			% RA of GAO			% RA of PAO			% RA of GAO		
	CAP	MP	Total	CCP	MIG	Total	CAP	MP	Total	CCP	MIG	Total
1	13	3	16	10	3	13	12	4	16	9	2	11
35	7	8	15	42	9	51	9	8	17	37	13	50
63	1	7	8	11	7	18	1	6	7	27	10	37
70	1	2	3	9	2	11	1	1	2	11	3	14
76	3	1	4	14	2	16	2	1	3	15	2	17
78	4	2	6	10	2	12	3	1	4	3	1	4

It was found that the GAOs dominated over all other bacteria during the experimental period except at the first day. The % RA of PAOs was higher compared to GAOs at day 1 because the seed for the reactors was collected from a well-operated full-scale EBPR system where VFA is present at sufficient amounts. In the full-scale plant mentioned above, the effluent P concentration is below 0.1 mg P/L, indicating that the PAOs might dominate over GAOs in the plant. In the present study, it was also observed that the % of P removal was above 90% at day 1 with a higher % RA of PAOs compared to that of GAOs (Figure 6.11) in both reactors. Afterwards, the % P removal was reduced to a low level with the higher % RA of GAOs compared to that of PAOs. Moreover, there was no P removal at day 35 in G1 reactor due to the presence of a very low amount of PAOs compared to GAOs (Table 6.6). In addition, P removal was also not observed at day 70 in

both reactors, and it might be due to the change in operational concentration of the reactors (i.e. high glucose and P concentration).



(a)



(b)

Figure 6.11: Percentage of P removal with % RA of different microbe in a) reactor G1 and b) reactor G2

Presence of GAOs might cause the failure of EBPR systems due to the competition with PAOs for carbon sources (Cech and Hartman, 1993; Satoh et al., 1994; Bond et al., 1999b; Fang et al., 2002; Saunders et al., 2003; Kong et al, 2006; Burow et al., 2007). Normally, GAOs consume substrates by using intracellular glycogen as an energy source

without releasing P and storing PHA under anaerobic conditions. The PHA is utilized to refill the glycogen without up taking P from the liquid under aerobic conditions (Crocetti et al., 2002; Saunders et al., 2003; Kong et al, 2006). Therefore, GAOs have no contribution to the removal of P despite using the carbon source.

In summary, the results of this research indicate a dominance of GAOs over PAOs when EBPR systems are fed with glucose. It seems, as suggested by other researchers, that the low release of P is caused by lower pH, resulting from glucose metabolism, which is established under the anaerobic period. As a consequence of low P release under the anaerobic period, PAOs do not contain sufficient polyhydroxyalkanoates (PHAs) to support P uptake under the aerobic period. As a result, P removal deteriorates. Therefore, glucose is not a strong candidate carbon source to be supplemented to EBPR systems that do not contain sufficient VFAs.

6.6. Conclusions

The conclusions of this research are as follows:

1) The performance of biological P removal was found to be negatively affected by glucose, when glucose was fed as a sole carbon source.
2) Initially, when P release and uptake were performing well, PAOs dominated over GAOs in the EBPR system, resulting in higher P removal from the system. With time, GAOs out-competed PAOs and there was no P release and uptake in the reactor.

3) *Candidatus* Accumulibacter phosphatis is not selected in the glucose fed reactor. The highest % RA of these bacteria was 41% at day 14 and it was then reduced to below 4% at the end of reactor run.

4) Glucose selects for *Microlunatus phosphovorus* in glucose fed reactors. However, the data obtained from a two weeks reactor run with a high glucose concentration indicates that higher glucose concentration might be detrimental to these bacteria. The highest % RA of these bacteria measured was about 21%.

5) Glucose selects for targeted *Candidatus* Competibacter phosphatis given the high abundance of these bacteria detected during the experimental period. The % RA of *Candidatus* Competibacter phosphatis varied from about 10% to about 41% during the experimental period.

6) *Micropruina glycogenica* was selected in reactors fed glucose up to a certain glucose concentration. The maximum % RA of these bacteria found in this research was about 17%. It seems that *Micropruina glycogenica* is negatively affected by higher glucose concentration.

7) Glucose is not a suitable carbon source for the EBPR systems that contain insufficient VFAs. The low pH generated from glucose metabolism causes less phosphorus to be released and as a consequence PAOs do not have sufficient PHAs to take up OP during the aerobic period. As a result, P removal deteriorates.

CHAPTER 7

THE EFFECT OF BUTYRATE ON THE MICROBIAL SELECTION OF ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL SYSTEMS

7.1. Abstract

In this research, the microbial selection of enhanced biological phosphorus removal (EBPR) system was investigated in a laboratory –scale sequencing batch reactor fed exclusively with butyrate as a sole carbon source for 92 days. As reported in the few studies available, butyrate uptake in the anaerobic period was slow and phosphorus release occurred during the entire period. Polyphosphate accumulating organisms (i.e. *Candidatus* Accumulibacter phosphatis and actinobacterial PAOs) and glycogen accumulating organisms (i.e. *Candidatus* Competibacter phosphatis and *Defluvicoccus* related tetrad-forming alphaproteobacteria) were identified using Fluorescence In Situ Hybridization (FISH) analysis. The results show that *Candidatus* Accumulibacter phosphatis and *Defluvicoccus* related tetrad-forming alphaproteobacteria were selected in the butyrate fed reactor. However, butyrate did not select for *Candidatus* Competibacter phosphatis. The % RA of *Candidatus* Accumulibacter phosphatis increased from 13% to 50% during the experimental period except the last day. The % RA of *Defluvicoccus* related tetrad-forming alphaproteobacteria increased from 8% to 16% during the experimental period. The % RA of *Candidatus* Competibacter phosphatis decreased from 8% to below 2% during the experimental period. FISH analysis showed the

presence of actinobacteial PAOs in the butyrate fed reactor. The results also show that phosphorus removal was efficient at the beginning of the experimental period with associated higher % RA of PAOs in the system. However, after about 40 days, phosphorus removal deteriorated and there was no good correlation with the % P removal and % RA of GAOs targeted in the present research. This indicates the potential presence of other GAOs, not targeted in the present research, in the system. In addition, the results point to need to increase anaerobic periods in EBPR systems fed with butyrate. If the anaerobic period is increased, more PHA will be stored and therefore phosphorus uptake in the aerobic period will increase, if sufficient PAOs are present and GAOs are absent.

7.2. Introduction

Enhanced biological phosphorus removal (EBPR) has become a very popular method to remove phosphorus (P) from wastewater. Its popularity is due to low cost, low sludge generation and simple operation compared to traditional chemical P removal processes. New and existing wastewater treatment plants all over the world are implementing EBPR systems for P removal. It is well established that short chain volatile fatty acids (VFAs) (e.g. acetate, propionate, butyrate, etc) play an important role in EBPR systems (Tasli, et al., 1997; Wang, et al., 2002; Hollender et al 2002; Cokgor et al., 2004; Pijuan, et al., 2004b; Oehmen et al, 2004, 2005a and 2006; Chen and Gu 2006) and several models for VFA uptake and accumulation into bacteria have been proposed (Comeau et al., 1986; Grady et al., 1999).

It is known that the amount and type of VFAs present has significant influence on biological phosphorus removed from wastewaters (Ekama et al, 1983; Pitman, 1991; Elefsiniotis and Oldham 1993, Metcalf and Eddy, 2003). The quantities of VFAs that can be produced in sewer lines depend on many factors including the composition of the wastewater and temperature. In colder regions, VFA generation is limited (Daigger and Polson1991; Skalsky and Daigger, 1995; Ferreiro and Soto, 2003) and many plants may not have sufficient VFA to obtain the desired P removal. Plants that have to remove P to very low levels must increase the amount of VFAs present in the influent wastewater. This can be accomplished by (a) increasing solids retention time in primary clarifiers to foster partial fermentation, (b) fermenting primary sludge, and (c) adding commercially available volatile fatty acids to the plant's influent. Because of the need of supplemental VFAs to EBPR systems that do not contain sufficient amounts and given the performance of EBPR vary with VFA type, it is important to evaluate the effects of individual VFA type on the microbiology of EBPR systems.

Presently, not much is known about the microbiology of EBPR systems that use different VFAs. Only few bacteria have been found to perform EBPR (Crocetti et al., 2000; Pijuan et al., 2004a; Oehmen et al., 2005a; Wong et al., 2005; Kong et al., 2005) and they are referred to as Polyphosphate Accumulating Organisms (PAOs). There are also bacteria that have been found to deteriorate the performance of EBPR systems, known as Glycogen Accumulating Organisms (GAOs). Machado (2004) and Pijuan (2004a) were the first researchers to report on the influence of VFA types on the microbiology of EBPR systems. In the last few years, several other researchers have also contributed knowledge to this area (Wong et al., 2004, Beer et al., 2004, Oehmen et al.,

2005a & 2005c, Lu et al., 2006, Oehmen et al., 2006, Meyer et al., 2006, Burow et al., 2007).

Acetate has been used as a sole carbon source to observe the microbial communities in EBPR system in several studies (Cech and Hartman, 1993; Mino et al., 1998; Machado, 2004; Pijuan, 2004; Wong et al., 2004, Beer et al., 2004 and Oehmen et al., 2006). Cech and Hartman (1993) were probably the first researcher who observed the presence of G-bacteria in a reactor fed with acetate. Later, these G-bacteria became known as GAOs (Mino et al., 1998). Machado (2004) observed that *Candidatus Accumulibacter Phosphatis* (i.e. Accumulibacter), a PAO, was predominant over *Candidatus Competibacter Phosphatis* (i.e. Competibacter), a GAO, in acetate fed reactors, which showed good P removal. In contrast, Oehmen et al., (2006) indicated that P removal was poor in a reactor fed with acetate due to the abundance of Competibacter. Similarly, Wong et al., (2004) observed the failure of a laboratory-scale sequencing membrane bioreactor fed with acetate. They identified the presence (i.e. 85% of total cells) of a tetrad-forming alphaproteobacteria (TFOs) which were closely related to *Defluvicoccus* and acted as GAOs. In addition, Beer et al., (2004) observed poor P removal capacity of an acetate fed reactor dominated with *Sphingomonas* of tetrad forming alphaproteobacteria, which behaved like a GAO.

Propionate might be a better carbon source compared to acetate in an EBPR system (Pijuan, 2004; Oehmen et al., 2006). Many studies found that lower P effluent concentrations are obtained when propionate is used compared to acetate (Pijuan, 2004a; Oehmen et al., 2006). Pijuan et al., (2004a) and Oehmen et al., (2005a and 2006) found that the Accumulibacter, a PAO, dominated (8-69% of total bacteria) over

Competibacter, a GAO, (<1% of total bacteria) when propionate was used as a carbon source. Oehmen et al., (2005b) also observed that Competibacter can uptake propionate at a very slow rate compared to that of Accumulibacter. Oehmen et al., (2006) found a novel group of alphaproteobacterial GAOs in the presence of propionate, and verified that Accumulibacter can out-compete the alphaproteobacterial GAOs. However, Meyer et al., (2006) found the abundance of *Defluvicoccus vanus* related alphaproteobacteria (upto 55% of all bacteria) when propionate was used to enrich PAOs and GAOs. They suggested that these bacteria might play a significant role in the deterioration of an EBPR system fed with propionate. On the other hand, when acetate was used to enrich PAOs, Pijuan, (2004) found a low abundance of Accumulibacter and Competibacter (i.e. only a few cells). In contrast, Oehmen et al., (2005c) found the abundance of Accumulibacter (13% to 65%) and Competibacter (24%). Moreover, Oehmen et al., (2006) found that mostly Competibacter dominated over Accumulibacter in presence of acetate. Therefore, it can be concluded that propionate promotes better P removal because it selects for more PAO bacteria over GAO than acetate.

Butyrate might not select for Accumulibacter (Machado, 2004; Oehmen et al., 2004; Kong et al., 2004) and Competibacter (Machado, 2004; Oehmen et al., 2004) in EBPR systems. In a reactor fed with butyrate for 58 days, Machado (2004) observed large variation in the number of Accumulibacter, a PAO, but the P removal was above 80%. After 32 days, both the number of PAOs and the P removal decreased dramatically. In addition, the number of Competibacter also decreased throughout the study period of 58 days. Pijuan (2004) switched carbon sources from acetate and propionate to butyrate and found that butyrate and P uptake were low as well as the P release. Moreover, Oehmen et

al., (2004) detected that Accumulibacter assimilated butyrate at a very slow rate (i.e. 1 mmol C/L in 120 min) in a cycle study of a PAOs enriched SBR using acetate. Furthermore, kong et al., (2004) obtained that butyrate was not taken up by Accumulibacter in a microautoradiography combined fluorescence in situ hybridization (MAR-FISH) analysis.

The results of previous research described above demonstrate that there are several gaps that have to be addressed in order to understand how individual VFA types affect the microbiology of EBPR system. Presently, there are not sufficient data on the type of PAOs and GAOs that are involved in EBPR systems in presence of butyrate as a sole carbon source. Therefore, in this research, the influence of butyrate on the microbiology of an EBPR system will be investigated. It is hypothesized that the use of PAO and GAO DNA probes will detect for PAOs and GAOs involved in EBPR systems fed with butyrate as a sole carbon source. In this research, PAOs (i.e. *Candidatus Accumulibacter Phosphatis* and Actinobacteria) and GAOs (i.e. *Candidatus Competibacter Phosphatis* and Defluvicoccus *vanus* related alphaproteobacteria) will be targeted with probes specific for these bacteria.

7.3. Materials and Methods

7.3.1. Laboratory set up of SBRs

A laboratory setup was built consisting of two 8-liter lab-scale SBRs. The operation of the reactors was automated using two timers, solenoid valves and peristaltic pumps. The reactors were connected to a feeding tank, air and nitrogen gas supply sources. Besides, a mixer was attached to each reactor, and an air/nitrogen diffuser was placed at

the bottom of the reactor. Figures 7.1 and 7.2 show the schematic and experimental set up of one reactor in the Environmental Engineering Laboratory.

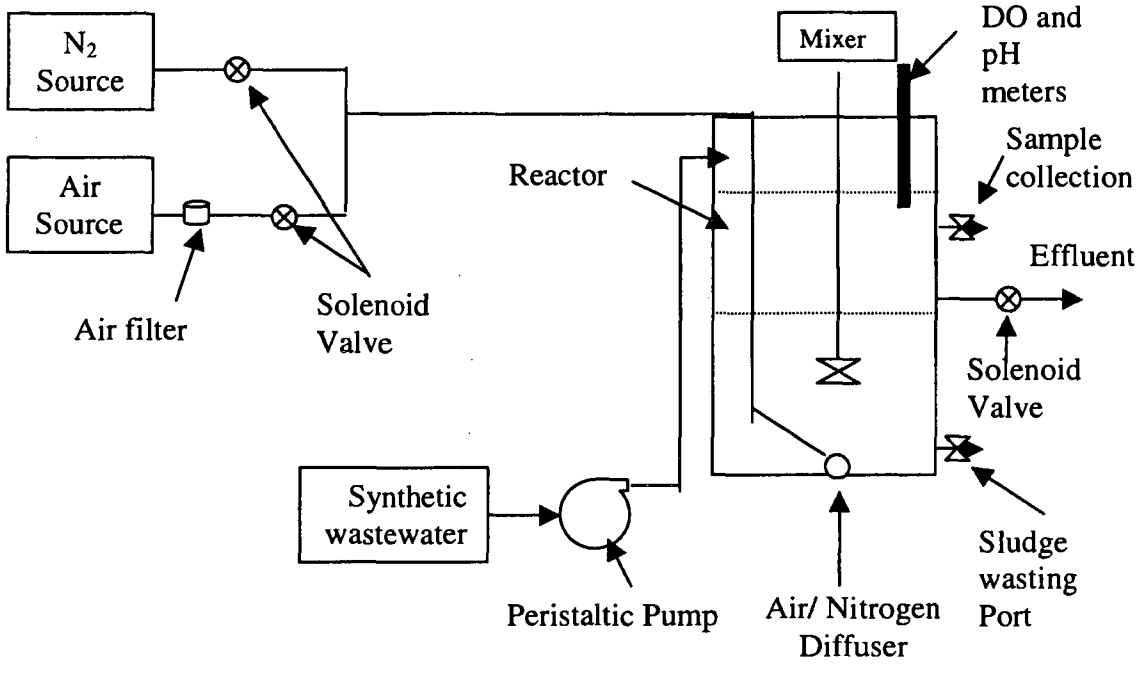


Figure 7.1: Schematic of the setup of one SBR in the laboratory

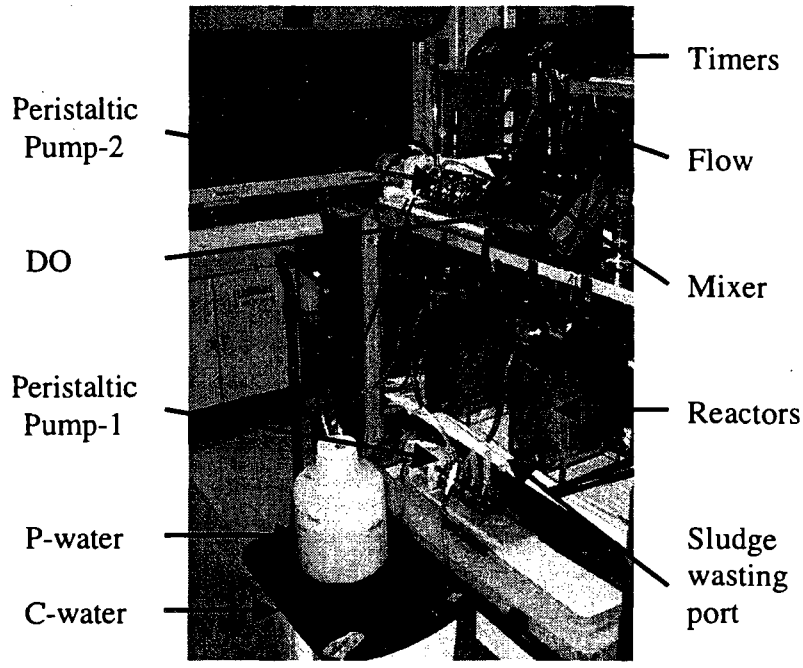


Figure 7.2: Experimental setup of the SBRs assembled in the Environmental Engineering Laboratory at UNLV

7.3.2. Preliminary design of SBRs

Reactors were run according to the preliminary design, summarized in Table 1. Details of the design are given in the Appendix V.

Table 7.1: Parameters used in the preliminary design of SBRs

Parameters	Design value
Influent flow	12 L/d
Influent COD	200 mg/L
Influent phosphorus	13.33 mg P/L
Effluent COD	0.02 mg/L
Effluent phosphorus	8.59 mg/L
SRT	8 days
HRT	0.667 days (16 hours)
MLVSS	2500 mg/L
Volume of reactor	8 L
SVI	71 ml/g

7.3.3. Operation of SBRs

Two SBRs (B1 and B2) were operated under similar conditions using butyrate as a sole carbon source for a period of 92 days. This run time was selected based on the results obtained by (Machado 2004). He observed that Competibacter decreased throughout the study period of 58 days. In addition, he found a large variation in the number of Accumulibacter whereas P removal was above 80% up to 32 days. Moreover, both the number of PAOs and the P removal decreased dramatically after 32 days. Due to time limitation, he was not able to identify the reasons of degradation of P removal after 32 days. Therefore, in this research, the SBRs were operated for 92 days to investigate the behavior and microbiology of the EBPR system using butyric acid as a sole carbon source.

The SBRs were inoculated with biomass from the aeration basin of a local wastewater treatment plant that performs EBPR and were filled with synthetic wastewater (i.e. C-water) (Table 7.2) and phosphate solution (i.e. P-water) at flow rates of 254 ml/min and 40 ml/min, respectively. The SBRs were operated in 3 cycles of 8 hours. Each cycle had five steps, which were carried out in the following order: fill (15 minutes), anaerobic period (2.5 hours), aerobic period (4.5 hours), settle (30 minutes) and withdraw (15 minutes). At the beginning of the anaerobic period, N_2 gas was supplied and dispensed as bubbles into the reactors for 2 minutes at a flow rate of 13 standard cubic feet per hour (SCFH) in order to create anaerobic condition (i.e DO concentration less than 0.2 mg/L). During the aerobic period, air was spurge into the reactors using a ceramic stone diffuser at a flow rate of 8 SCFH to maintain a DO concentration greater than 2 mg/L. After settling of sludge, 4 liters of wastewater were withdrawn from the reactors by gravity

through effluent outlets and about 209 ml of settled sludge was collected through sludge wasting ports to maintain a SRT of 8 days at room temperature (22 ± 2° C). The average concentrations of mixed liquid suspended solids (MLSS) were 2431 and 2368 mg/L in B1 and B2 reactors, respectively. The percentage of MLVSS was about 70% in both reactors. The pH values were 7 ± 0.09 and 7.6 ± 0.1 in B1, and 7 ± 0.12 and 7.5 ± 0.1 in B2 for anaerobic and aerobic period, respectively.

7.3.4. Preparation of synthetic wastewater for the SBRs

The C-water and P-water were prepared according to the synthetic wastewater's composition of Oehmen et. al., (2005a). The C-water contained carbon, nitrogen and nutrient sources whereas the P-water contained only phosphorus (Table 7.2). Both C and P-water were supplied into the reactors separately in order to avoid biodegradation in the tubing and precipitation of phosphorus.

Table 7.2: Composition of synthetic wastewater adapted from Oehmen et. al., (2005a)

Parameters	Amounts
Carbon (glucose)	200 mg COD/L
Nitrogen (i.e. NH_4Cl)	27 mg /L
Phosphorus (i.e. $NaHPO_4.H_2O$)	13.33 mg /L as P
Magnesium (i.e $MgSO_4.7H_2O$)	43 mg /L
Calcium (i.e. $CaCl_2.2H_2O$)	20 mg /L
Peptone	12 mg /L
ATU	0.525 mg /L
Trace elements*	0.14 ml/L

* Trace elements (g/L): 1.5 g $FeCl_3.2H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 $MnCl_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 $ZnSO_4.7H_2O$, 0.15 g $CoCl_2$. $6H_2O$ and 10 g EDTA)

7.3.5. Collection and preservation of samples

Five hundred forty-ml of sample were collected during a cycle. To avoid P release after a sample was taken, about 15 ml samples were immediately filtered through 0.45 μm syringe filter (GHP Acrodisc, PALL) and refrigerated. The samples were analyzed on the same day of collection or preserved when necessary.

7.3.5.1. Chemical analysis

Samples were analyzed for orthophosphate (OP), soluble total organic carbon (sTOC), and suspended and volatile suspended solids (SS/VSS) as per Standard Methods (Eaton et al., 2005). OP was measured using HACH kit (PhosVerR 3 Method 8048) and a DR/3000 spectrophotometer. Soluble TOC was analyzed according to Standard Method 5310-B (Eaton et al., 2005) using a TOC Analyzer (Model TOC-$V_{CPH/CPN}$, SHIMADZU). Standard Methods 2540D and 2540E were used to measure total suspended solids (SS) and volatile suspended solids (VSS), respectively using a 47mm Whatman GF/C microfiber glass filter. Filtered samples were utilized for OP and sTOC analysis whereas the unfiltered samples were used for SS/VSS analysis. SS/VSS was measured on the same day of sample collection. Dissolved oxygen was measured using a YSI Model 54A) DO meter. The pH was measured using an Accumet, AR25 pH meter.

The mean % removal of P between the replicate reactors fed with butyrate (B1 and B2) was compared using a two-tailed Independent-samples t Test assuming a 95% confidence level ($p <= 0.05$). The null hypothesis was that the difference in mean P removal percentage between the replicate reactors (i.e. B1 and B2) was zero. The alternate hypothesis was that the mean P removal percentages between the replicate

reactors were different. Standard error of mean P removal percentage was calculated for reactors fed with butyrate.

7.3.5.2. Microbiological analysis

a) FISH procedure

Unfiltered samples were used for Fluorescence in Situ Hybridization (FISH) analysis. The procedure for FISH analysis was adopted from Amann (1995) and modified by De Los Reyes (2003). For gram-negative bacteria, 3ml of the sample were mixed with 9 ml of 4% ice-cold paraformaldehyde (96%, Fisher Scientific, Suwanee, GA) in a 15 ml plastic centrifuge tube (VWR) for fixation. Fixation is required to maintain the morphological integrity of the cells and to minimize the auto-fluorescence (Amann, 1995). The sample was then kept in the refrigerator at 4^0C for 2.5 hours. After fixation, the sample was washed two times with 1 x phosphate buffer saline (PBS) solution (1 volume of 3 x PBS; 390 mM NaCl in 30 mM $NaPO_4$ buffer and 2 volume DI water) by spinning the sample in a centrifuge (SORVALL, Legend RT) at 2000 rpm for 5 minutes to remove the fixative solution. The sample was stored in the refrigerator at -20^0C by adding 1x PBS/ethanol (1:1) solution. For gram-positive bacteria, 1 volume of sample was mixed with 1 volume of 50% ethanol (v/v) (95%, IBI-Scientific, IA) and the sample was stored at –20°C (Kong et. al., 2005 and personal communication with Simon Mcllroy, referred by Dr. R. Seviour, Biotechnology research center, La Trobe University, Bendigo, Victoria, Australia). Next, 3 µl of sample was applied into three wells of a 6-well Teflon-coated microscope slide (Cel Line, Portsmouth, NH) to immobilize the cell. The sample of the slide was air dried for about 45-50 minutes. Afterwards, it was dehydrated by successive dipping into 50%, 80% and 95% ethanol in staining jars (3

minutes per step) and air dried for 8-10 minutes. Next, 8 µl hybridization buffer, 1 µl of EUB bacteria probe and 1 µl the desired PAO or GAO probes were applied to three wells. The details of hybridization buffer are given in Table 7.3. To hybridize the samples, the slide was inserted into a properly sealed moist chamber, which was kept in an oven (Millipore, Billerica, MA) at 46^0C for 60-120 minutes without shaking. The moist chamber was built in-house using a 50 ml centrifuge tube and a piece of Whatman filter paper wetted with 0.5 ml of hybridization buffer. A properly sealed moist chamber is necessary for hybridization to avoid evaporation of hybridization solution, which leads to nonspecific binding of the fluorescent probe to the cells (Amann, 1995). After hybridization, the sample was washed with 50 ml wash solution in a water bath (Model AP-152 from SOILTEST, Lake Bluff, IL) at 48^0C for 20 minutes. The details of wash solution are given in Table 7.3. The slide was dipped again in 50 ml centrifuge tube containing ice-cold deionized water for 3 seconds and air dried until all water droplets were removed. Finally, the slide was mounted with a microcover glass (24 X 60mm, VWR Scientific) by using Citifluor mountant media (Ted Pella Inc., Redding, CA). The basic steps for FISH analysis are illustrated in Figure 7.3.

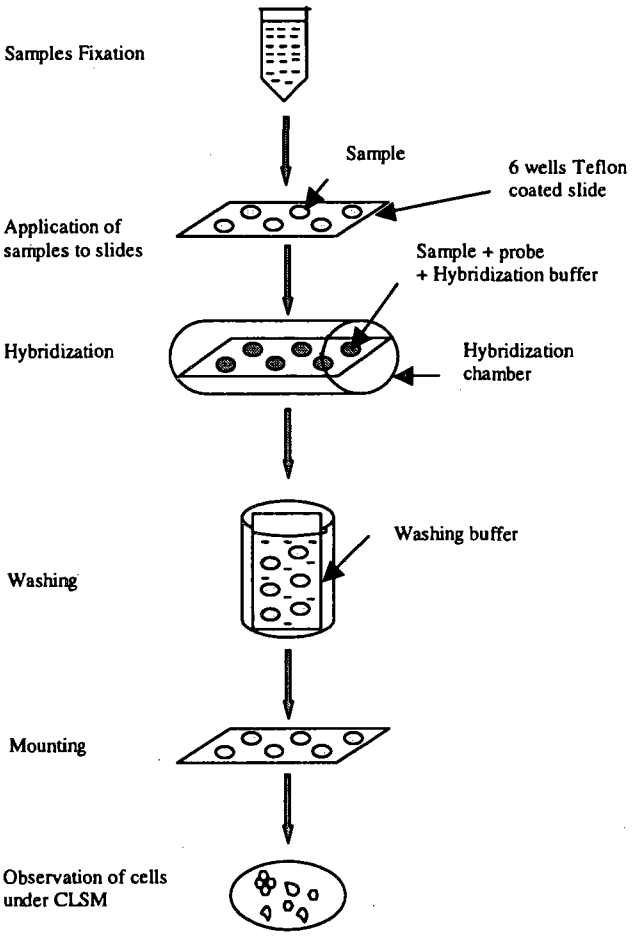


Figure 7.3: Steps of FISH analysis
(Source: modified from Moter and Gobel, 2000)

Table 7.3: The ingredients of hybridization buffer and wash solution

Name of bacteria	Hybridization buffer					
	FA (µl)	MQ (µl)	5M NaCl (µl)	1 M Tris/HCl (pH = 8.0) (µl)	10% SDS (µl)	0.5 M EDTA (µl)
Actino-PAO (% FA = 40)	800	800	360	40	2	-
TFO, CAP & CCP (% FA = 35)	700	900	360	40	2	-
	Wash solution *					
Actino-PAO (% FA = 40)	-	-	460	1000	50	500
TFO, CAP & CCP (% FA = 35)	-	-	700	1000	50	500

* The washing buffer was made in 50 ml tubes and was filled up to 50 ml using dH_2O

In the FISH analysis, the PAOMIX probe (i.e. PAO462, PAO651, PAO846) and Actino_658 were used to detect *Candidatus Accumulibacter Phosphatis* (Crocetti et. al., 2000) and Actinobacerial PAOs (i.e. PAOs) (Kong et. al., 2005), respectively. Moreover, GAOMIX probes (i.e. GAOQ431, GAOQ989) and TFOMIX (i.e. TFO_DF 218 and TFO_DF 618) were utilized for targeting *Candidatus Competibacter Phosphatis* (Crocetti et. al., 2002) and a group of *Defluvicoccus* related alphaproteobacteria (i.e. GAOs) (Wong et al., 2004) respectively. The probes for targeting PAOs and GAOs were labeled by Cy3. The EUBMIX probe (i.e. EUB338, EUB338-II and EUB338-III) (Crocetti et. al., 2002) labeled with Cy5 was used to target the entire bacterial community, because only domain-specific probe EUB 338 is not sufficient to detect all bacteria (Daims et. al., 1999). Cy3 and Cy5 are fluorescent dyes of cyanine group with fluorescence color orange-red and near infrared, respectively use to visualize the microbes under

microscope. All probes used in this research were purchased from a company named Integrated DNA Technology, Inc. (Coralville, IA) with HPLC purification. The details of these oligonucleotide probes are given in Table 7.4.

b) Specificity test

To assure that the purchased probes were specific to the targeted GAOs and PAOs, two measures were taken. First, the purchased sequence was checked with the microbial sequence database BLAST (Basic Local Alignment Search Tool) available from NIH online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligonucleotide sequences were compared with a database of sequences (i.e. Nucleotide collection, nr/nt) to detect sequences with high identity. This operation was performed for all probes and a match varying from 87-95%, 93-100% and 94-100% was found for PAO462, Actino_658 and EUB338 probes, respectively. All other probes matched 100%. The second check of specificity was performed using an enrichment culture available in our laboratory that is known to degrade perchlorate. A sample of the culture was taken and prepared for FISH as described above. Every probe to be used was tested with the sample and no hybridization occurred for all GAO and PAO probes while the EUB bacteria hybridized.

c) Microscopy and microbial quantification

For a sample, ten digital images (five images/well) of PAOs and GAOs were captured from two wells using confocal laser scanning microscope (Zeiss LSM510, Axioplan 2) using Argon (488, 514 nm) and HeNe (633 nm) lasers for the excitation of dyes Cy3 and Cy5, respectively. The emission filters for Cy3 and Cy5 were 530-600 nm and 650 nm, respectively. 400X magnification was used to observe the microbes under the microscope. To quantify the organisms, the images were analyzed using the software

ImageJ available free of charge from NIH (National Institute of Health). For an image, the % relative abundance (RA) of PAOs/GAOs was calculated based on the ratio of the area of PAOs/GAOs to entire bacterial population. The average % RA value of ten images was considered as the final % RA of PAOs/GAOs in the entire bacterial population. Statistical analysis was performed between the mean % RA of five images taken from two replicate wells in a slide based on a two-tailed Independent-samples t Test at a 95% confidence level. The null hypothesis was that there was no difference between means % RA of PAOs/GAOs in two wells of a slide. The alternate hypothesis was that there was difference in the mean %RA of PAOs/GAOs in two wells of a slide. In addition, the final % RA value (i.e. mean of ten images) of PAOs/GAOs between B1 and B2 reactors were compared statistically for each sampling day. Moreover, to compare the % RA of PAOs/GAOs in replicate reactors fed with butyrate, mean value of %RA of PAOs/GAOs of each reactor was determined and compared statistically described above. Finally, the standard error of mean %RA of PAOs/GAOs was determined between replicate reactors fed with butyrate.

Table 7.4: Oligonucleotide probes used in this research

Probe	Specificity	rRNA Target site	Sequence (5'-3')	(%) Formamide	Reference
EUB338	Many bacteria but not all	16S, 338-355	GCTGCCTCCCGTAGGGT	0-70	Crocetti et. al., 2002
EUB338-II	*Planctomycetales*	16S, 338-355	GCAGCCACCCGTAGGTGT	0-50	Crocetti et. al., 2002
EUB338-III	*Verrucomicrobiales*	16S, 338-355	GCTGCCACCCGTAGGTGT	0-50	Crocetti et. al., 2002
			PAO probes		
PAO462	*Candidatus Accumulibacter phosphatis*	16S, 462-485	CCGTCATCTACWCAGGGTATTAAC	35	Crocetti et. al., 2000
PAO651	*Candidatus Accumulibacter phosphatis*	16S, 651-668	CCCTCTGCCAAACTCCAG	35	Crocetti et. al., 2000
PAO846	*Candidatus* Accumulibacter phosphatis	16S, 846-866	GTTAGCTACGGCACTAAAAGG	35	Crocetti et. al., 2000
Actino_658	Actinobacterial PAO		TCCGGTCTCCCCTACCAT	40	Kong et al., 2005
			GAO probes		
GAOQ431	*Candidatus Competibacter Phosphatis*	16S, 431-448	TCCCCGCCTAAAGGGCTT	35	Crocetti et. al., 2002
GAOQ989	*Candidatus Competibacter Phosphatis*	16S, 989-1006	CACCTCCCGACCACATTT	35	Crocetti et. al., 2002
TFO_DF218	Defluvicoccus-related TFO in α-proteobacteria	16S, 218-235	GAAGCCTTTGCCCCTCAG	25-35	Wong et al., 2004
TFO_DF618	Defluvicoccus-related TFO in α-proteobacteria	16S, 618-635	GCCTCACTTGTCTAACCG	25-35	Wong et al., 2004

7.4. Results

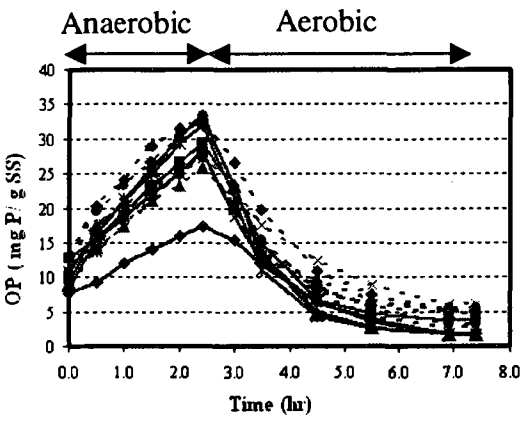
7.4.1. Overall Performance in reactors fed with butyric acid

Two SBRs (B1 and B2) were operated using butyrate as a sole carbon source under similar operating conditions for 92 days. The OP profiles of both reactors are shown in Figures 7.4a and 7.4b. P release and uptake were observed during the anaerobic and aerobic period, respectively. P released occurred during the entire anaerobic period. However, P uptake was not completed during the aerobic period. The system operated well for the first 6 weeks. At week 7, due to leakage in B1 reactor, some amount of solids was lost from the reactor. Moreover, at week 8, about half of the solids were lost from both reactors due to malfunction of the timers that control the operation of both reactors. Statistical analysis using a two-tailed Independent-samples t Test shows at the 95% confidence level that there was no significant difference in overall % P removal between two reactors (P= 0.088). The mean % P removal was 59.7% and 45.9% with a standard error of 5.89 and 5.09 in B1 and B2 reactors, respectively. Even though the overall % P removal was not significantly different, the P release, P uptake and % P removal varied with time due to the variation of solids concentration (Figure 7.5). Therefore, the analysis in this paper will follow reactor B2 because it operated with less disturbance.

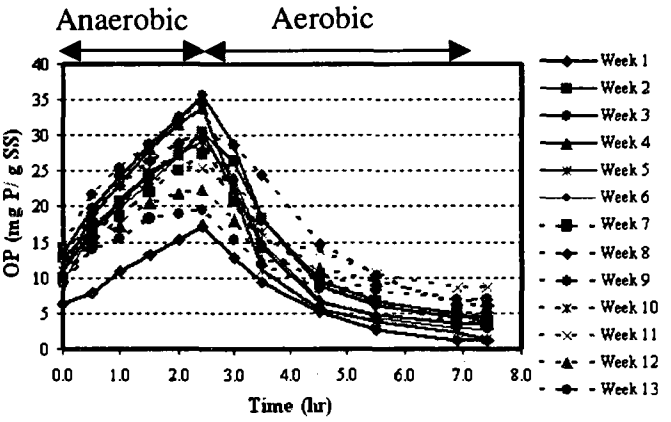
After 6 weeks, the effluent P concentration started to increase, and continued until 11^{th} week. The effluent P concentration increased from 3.6 to 8.6 mg P/g SS from week 7 to week 11, respectively. However, after 11 weeks, the effluent P concentration reduced and reached a value of 4.94 mg P/g SS at the end of the experimental period. This might be due to a low P release under anaerobic conditions. As a result, the effluent

P concentration was lower. The highest specific P concentration was 35.5 mg P/g SS at week 3 during the anaerobic period. In the present study, the highest P concentration was 28.4 mg P/g SS, which is similar to the results obtained by Machado (2004). Machado (2004) found that the maximum P concentration was 27.95 mg P/g SS. In the present study, the effluent P concentration fluctuated from 5 to 19 mg P/L. However, Machado (2004) obtained effluent P concentration of less than 1 mg P/L until 43 days, and afterwards it increased to a level of 14 mg P/L.

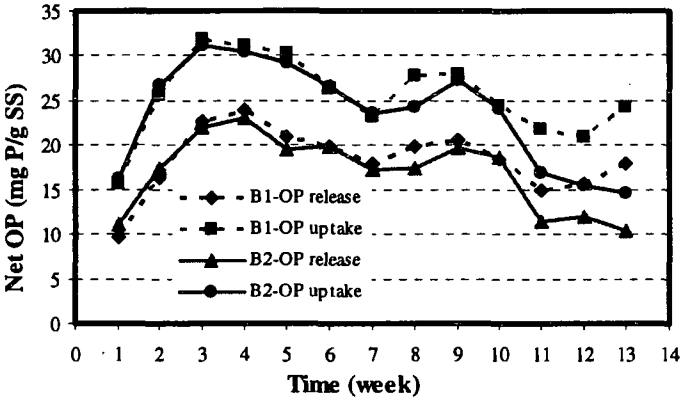
Figure 7.4c shows the weekly net specific P release and uptake during the anaerobic and aerobic periods, respectively. The amount of P uptake always exceeded the amount of P release, indicating good operation of the EBPR system. Moreover, net specific P release and uptake increased until week four of the experimental period. The highest net specific P release was about 23 mg P/g SS, which is close to the value obtained by Machado (2004). Machado (2004) found that the highest net specific P release was about 22.2 mg P/ g SS. After four weeks, net specific P release and uptake showed a decreasing pattern except during weeks 9 and 10.



(a) Weekly OP profiles in B1 reactor



(b) Weekly OP profiles in B2 reactor



(c) Weekly net specific P release and uptake in B1 and B2 reactors

Figure 7.4: Weekly OP profiles in reactors B1 (a) and B2 (b) showing P release and uptake during anaerobic and aerobic periods, respectively. Weekly net specific P release and uptake in B1 and B2 reactors during the experimental period (c)

Figure 7.5 shows the weekly variation of suspended solids concentration in both reactors during the experimental period. The suspended solids concentration was stable until 5 weeks. Afterwards, there were fluctuations in suspended solids concentration due to leakages and electrical issue related to the automated operation of the reactors.

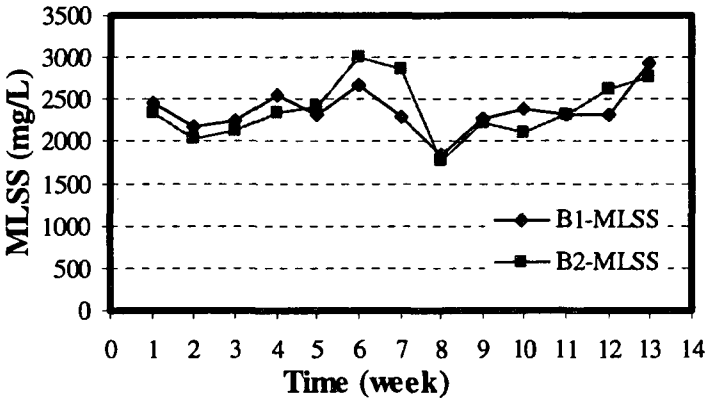
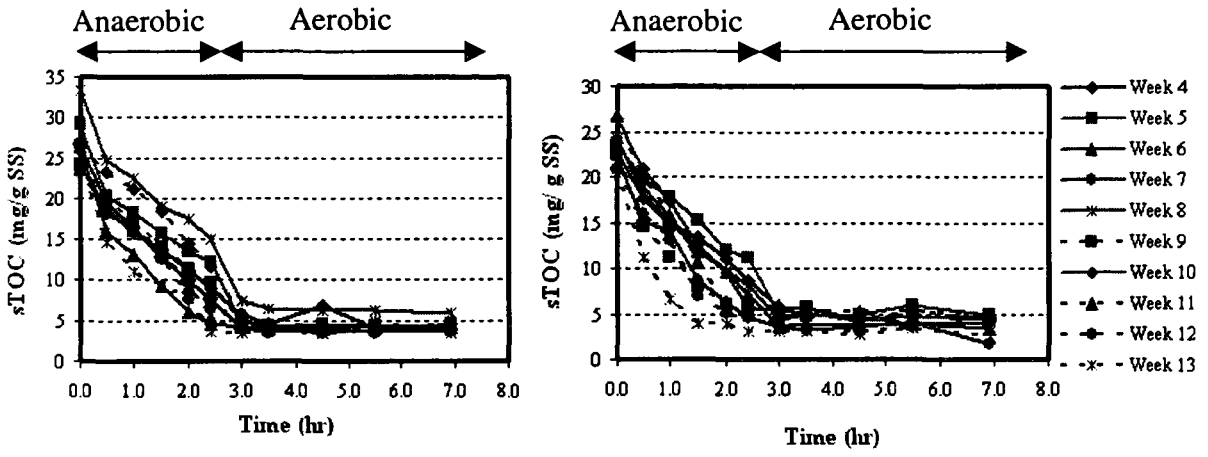
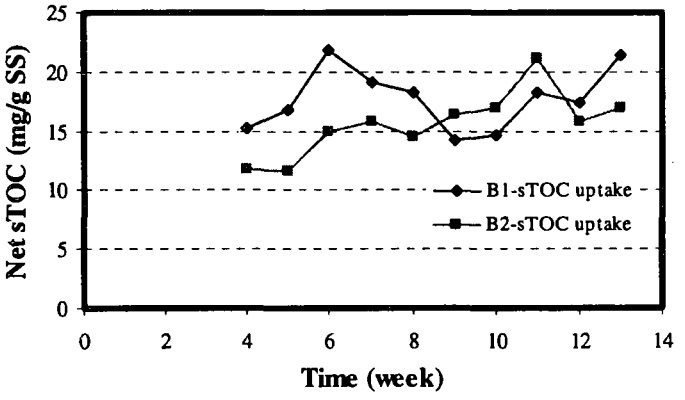


Figure 7.5: Suspended solids concentration in B1 and B2 reactors during the experimental period

Figures 7.6a and 7.6b depict the sTOC profiles of both reactors. The uptake of butyrate was not completed during the anaerobic period of 2.5 hours. Almost all butyrate was consumed within 3 hours. This explains the P release during the whole anaerobic period. Machado (2004) also observed that butyric acid was taken up during the whole anaerobic period, and carbon uptake was almost completed within 180 min. In the present research, the average utilization rate of butyrate was 0.014 mmol C/gVSS/min assuming a linear uptake of carbon source. However, Machado (2004) found that average carbon uptake rate was 0.029 mmol C/g VSS/min, which is two times higher compared to the rate obtained in the present study. This is likely due to a higher influent sTOC concentration (i.e. Machado's about 60 mg sTOC/L and this study 45.15 mg sTOC/L) that would give faster kinetics. During the aerobic period, no uptake of butyrate was observed. Figure 7.6c represents the weekly net specific sTOC uptake during the anaerobic period. After 5 weeks, net specific sTOC consumption varied. This may be due to the changes in microbiology of the system and it will be discussed later in this paper. Even though there was sTOC consumption, P release decreased under anaerobic condition.



(a) Weekly sTOC profiles in B1 reactor (b) Weekly sTOC profiles in B2 reactor

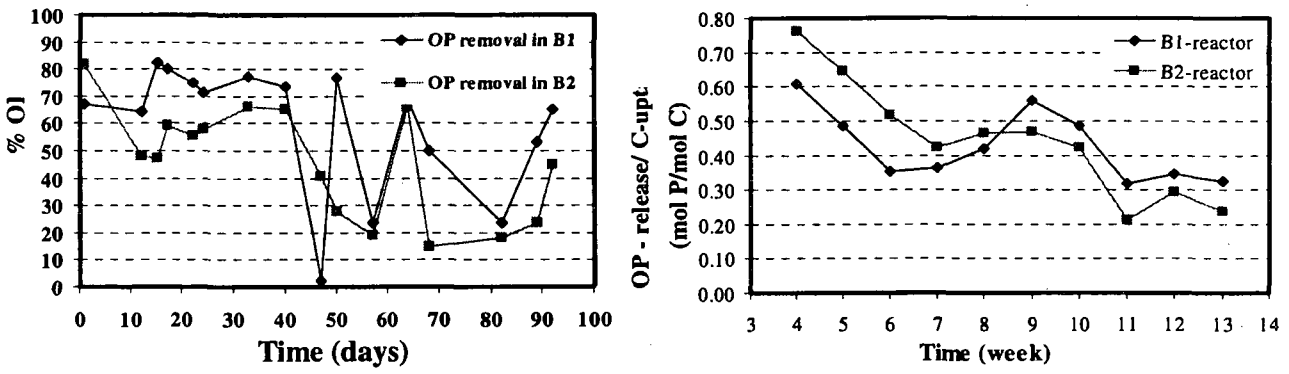


(c) Weekly net specific sTOC consumption in B1 and B2 reactors

Figure 7.6: Weekly sTOC profiles in reactors B1 (a) and B2 (b). Weekly net specific sTOC consumption in B1 and B2 reactors during the anaerobic period (c)

Figures 7.7a and 7.7b depict the % P removal and the P-release/C-uptake ratio, respectively during the experimental period. The detail calculations of the % P removal and the P-release/C-uptake ratio are presented in Appendix III. The % P removal reached a highest level of above 65% at day 40 (i.e. about 6 weeks). Afterwards, % P removal started to decrease until 82 days (i.e. 11 weeks). However, % P removal again improved to about 45% at the end of the experimental period. This is likely due to an improved

effluent P concentration from week 11 to 13 shown in Figure 7.4b. The P release/C-uptake ratio showed a decreasing trend from week 4, which coincided the P release pattern illustrated in Figure 7.4c. The P-release/C-uptake ratio was stable from week 8 to 10, which might be due to a slight increase of net specific P release during this period. The highest P release/C uptake ratio of above 0.60 was observed at week 4.

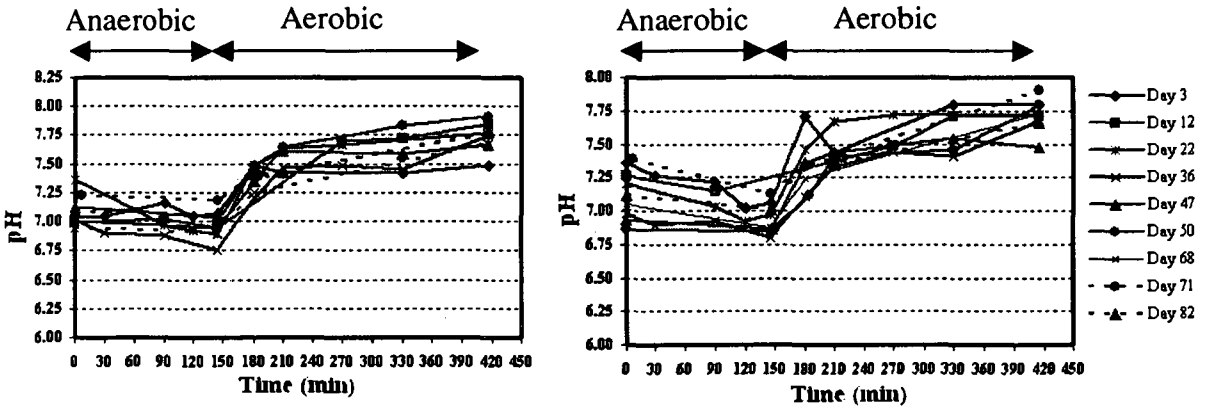


a) % OP removal

b) P-release/C-uptake ratio

Figure 7.7: Variation of % P removal (a) and P-release/C-uptake ratio (b)

The profiles of pH obtained during the reactor run are shown in Figures 7.8a and 7.8b. In both reactors, pH values were stable during the anaerobic period and increased in first 60 min of aerobic period, and then became stable up to the end of aerobic period. The slight increase of pH at the beginning of an aerobic cycle is due to the release of CO_2 from mixed liquor. The average pH values were similar in both reactors during the anaerobic (i.e. pH of 7 ± 0.1) and aerobic (i.e. B1 = 7.6 ± 0.1 and B2 = 7.5 ± 0.1) periods, which were expected.



(a) pH profiles in B1 reactor (b) pH profiles in B2 reactor

Figure 7.8: pH profiles in B1 (a) and B2 (b) reactors during the experimental period

7.4.2. Microbial communities and P removal in reactors fed with butyric acid

FISH analysis showed the presence of *Candidatus Accumulibacter Phosphatis* (CAP), *Defluvicoccus*-related tetrad-forming alphaproteobacteria (TFO) and *Candidatus Competibacter Phosphatis* (CCP) in both reactors (Figures 7.9a to 7.9c). Statistical analysis for all species investigated show that there was no significant difference, at 95% confidence level, between the mean % RA of CAP ($P = 0.449$), mean % RA of CCP ($P = 0.664$), and mean % RA of TFO ($P = 0.07$) in B1 and B2 reactors based on a two- tailed Independent-samples t Test. In addition, statistical analysis was performed between the mean % RA of specific bacteria in two replicate wells of a slide, and between the mean % RA of specific bacteria at different days in G1 and G2 reactors. The details of the statistical analysis are given in Appendix IV.

The abundance of CAP, a PAO, observed during the reactor run is shown in Figure 7.9a. The images of CAP captured using confocal laser scanning microscope (CLSM) are shown in Figure 7.10a. The % RA of CAP increased continuously from 13% to 50% until day 64 (i.e. 9 weeks). At the end of the run, day 92, the % RA of CAP reduced to 34%.

Even though % RA of CAP improved in the reactor until 64 days, % P removal was above 60 % only up to 40 days (Figure 7.7a). Then, % P removal decreased sharply until 57 days, and was almost stable up to 82 days. It is also observed from Figure 7.9a that increase of % P removal at the end of reactor run was not associated with the increase of CAP. The correlation between % RA of CAP and % P removal was poor (R^2 = 0.07) throughout the reactor run.

A similar trend on the % RA for CAP was observed in the study of Machado (2004). However, in Machado's study the % RA of CAP did not change until day 19, but good phosphorus release and uptake was observed. He observed significant CAP increase by day 32 still with good P removal. From day 32 to day 58 the % CAP decreased to below 5% and P removal deteriorated. Oehmen et al., (2004) suggested that butyrate might not select for CAP because uptake rate of butyrate was very slow (i.e. 0.0083 mmol C/L min) in two cycles of a SBR, which was enriched with CAP (i.e. 65% of total bacteria) using acetate. However, in this study, CAP was selected by butyrate. It might be due to a faster uptake rate of butyrate (i.e. 0.021 mmol C/L.min). In both, Machado's and this study, the SBRs were fed directly with butyric acid. Contrary to the findings of the present research, kong et al., (2004), based on MAR-FISH analysis, performed using batch tests on sludge from three full-scale wastewater plants, reported that butyrate was not taken up by CAP.

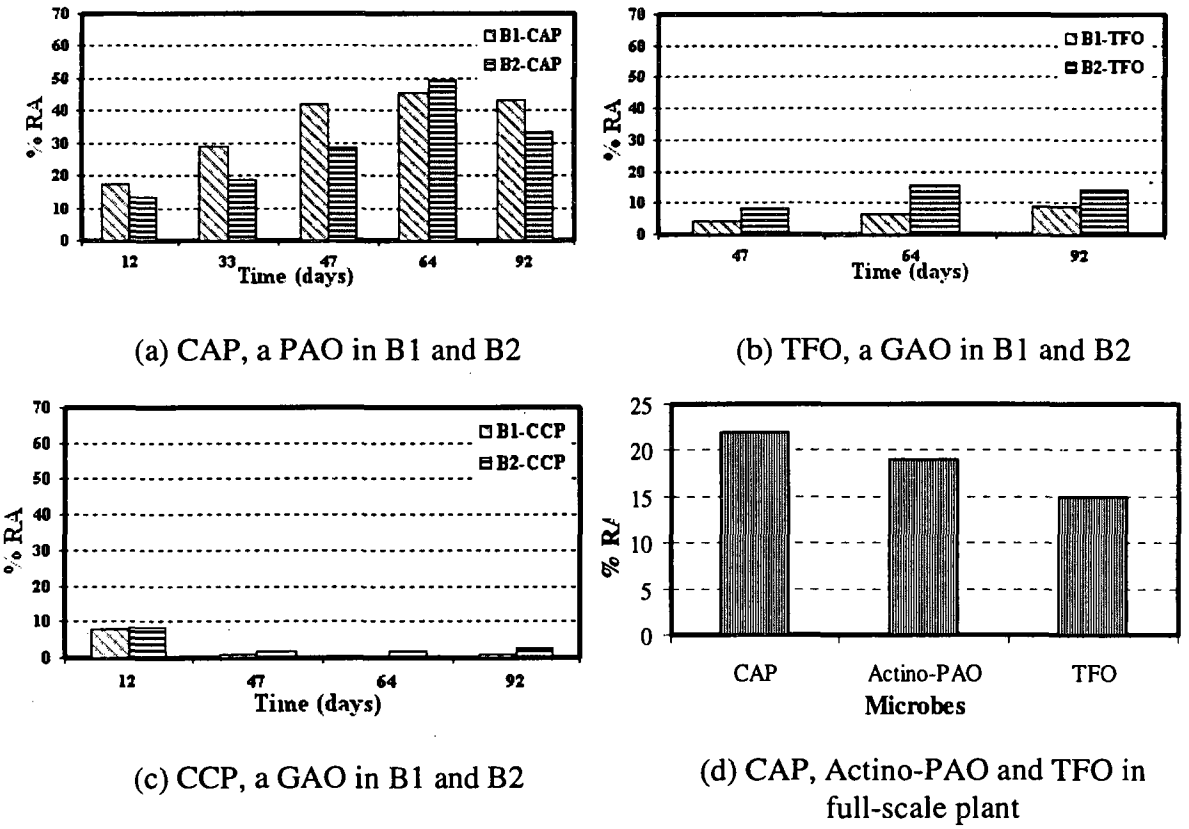
Figure 7.9b shows the % RA of *Defluvicoccus*-related alphaproteobacteria (i.e. TFO), a GAO, during the experimental period. The abundance of TFO increased from 8% to 16% from day 47 to day 64 and then it remained stable at 16% up to the end of run. The increase in TFO with time can be supported by the micrographs depicted in Figure 7.11a.

Increase of % RA of this GAO might have a contribution to reduce P release /C-uptake ratio described in Figure 7.6b. However, % P removal and % RA of TFO showed a poor correlation (R^2 = 0.58), which indicate the presence of other GAOs not targeted in the present study. This study is the first report on the presence of TFO in SBRs fed exclusively with butyrate. In contrast to the present study, Burow et al., (2007) reported, using FISH-MAR analysis, that butyrate was not consumed by *Defluvicoccus* spp. targeted by the similar probes used in the present research. Wong et. al., (2004) obtained about 8 to 20 % of TFOs in a failed membrane bioreactor fed with acetate as a sole carbon source.

Butyric acid might not select for *Candidatus Competibacter Phosphatis* (CCP), a GAO, which can be supported by the results illustrated in Figure 7.9c. The % RA of CCP was 8% at day of 12. However, the % RA of CCP dropped to below 2% in 47 days and remained same until the reactor run. The images of CCP shown in Figure 7.10b also confirm the low abundance of CCP in the reactor. Poor correlation (R^2 = 0.02) was observed between % P removal and % RA of CCP during the experimental period. Machado (2004) also obtained similar results. He found that % RA of CCP was about 25% at first day of run. However, % RA of CCP decreased at a linear rate of 0.6 %/day throughout the reactor run. Similarly, Oehmen et al., (2004) observed that almost no butyrate was taken up in two cycle studies in a SBR enriched with CCP (i.e. 53 % of total bacteria) using acetate. Therefore, butyrate might not select CCP.

The presence of actinobacterial PAOs (i.e. Actino-PAO) was also investigated in the present research. Figure 7.11b shows the images of Actino-PAO captured by using CLSM after FISH Analysis. Two types of bacteria were targeted using probe Actino-658.

One type was a short rod that occurs in clumps, and another type was coccus in clusters of tetrads. In the present study, Actino-658 targeted mostly coccus in clusters of tetrads. Unfortunately, the % RA for the Actino-PAO could not be calculated because the images of the hybridized bacterial population (i.e. EUBMIX probe) were not clearly visible under the microscope. It is believed this was the result of the % ethanol used for fixing the bacteria to the microscope slides.



(a) CAP, a PAO in B1 and B2

(b) TFO, a GAO in B1 and B2

(c) CCP, a GAO in B1 and B2

(d) CAP, Actino-PAO and TFO in full-scale plant

Figure 7.9: % RA of CAP (a), TFO (b) and CCP (c) in reactors fed with butyrate. % RA of CAP, Actino-PAO and TFO found in the local full-scale wastewater treatment plant from where seed was collected (d)

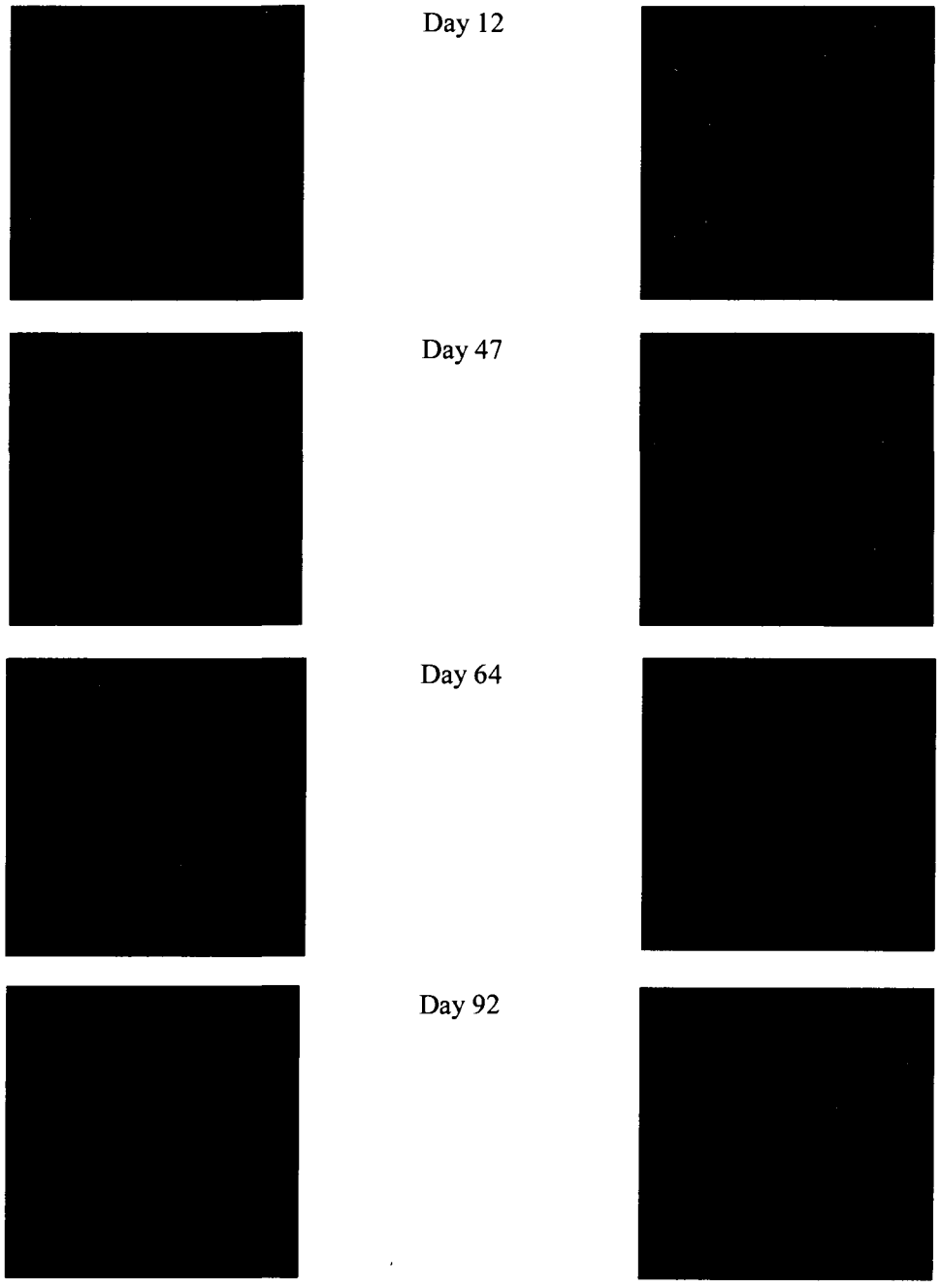


Figure 7.10: Images of CAP (red) and CCP (red) with time captured using CLSM in a SBR fed with butyrate. CAP and CCP were labeled by Cy3 (channel 2) and eubacteria was labeled by Cy5 (channel 1). Pinholes for channels 1 and 2 were 337 μm and 338 μm, respectively (Scaling: X = 0.45 μm and Y = 0.45 μm).

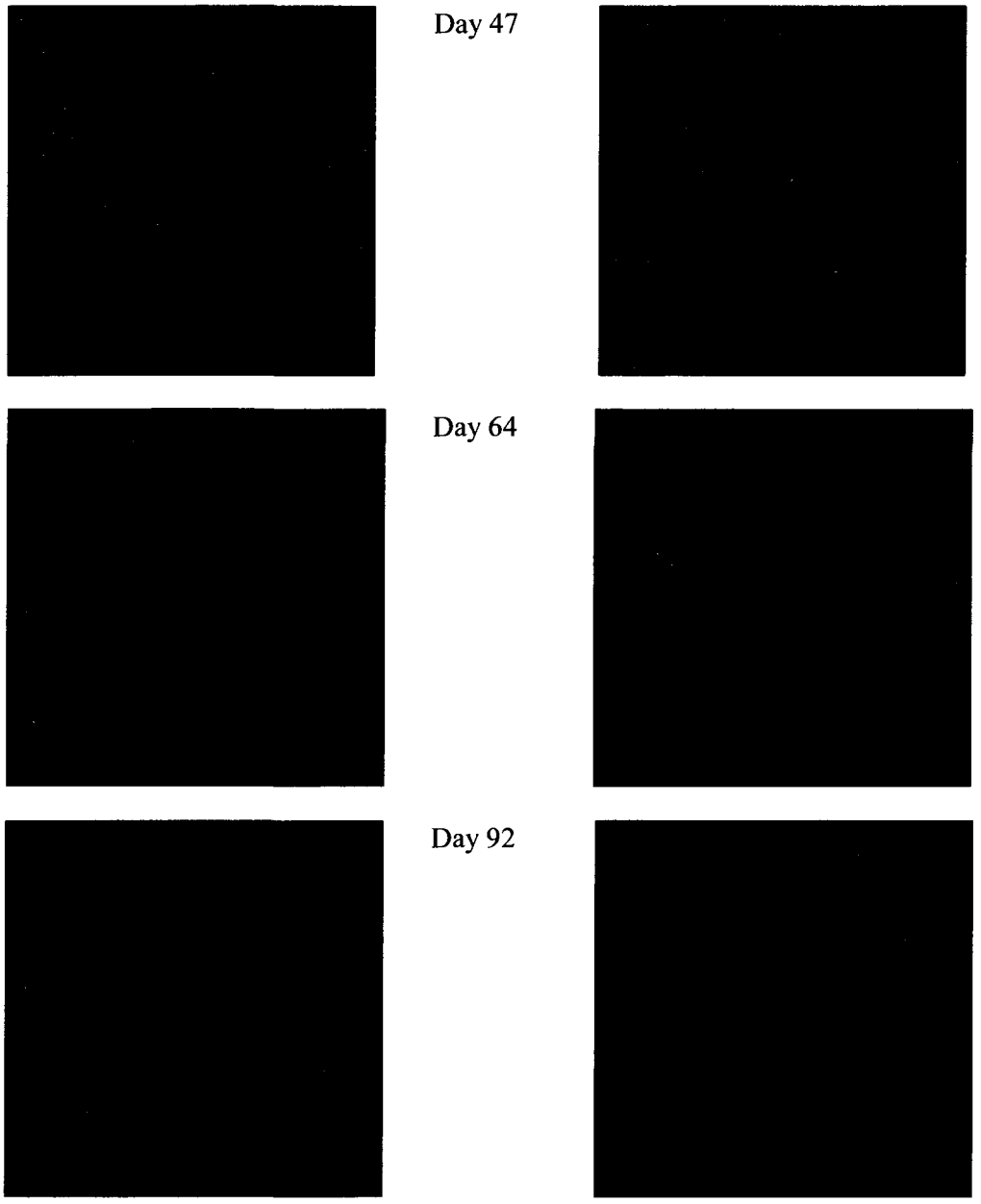


Figure 7.11: Images of TFO and actino-PAO captured using CLSM during the experimental period. TFO and Actino-PAO were labeled by Cy3 (channel 2) and eubacteria was labeled by Cy5 (channel 1). Pinholes for channels 1 and 2 were 337 μm and 338 μm, respectively (Scaling: X = 0.45 μm and Y = 0.45 μm).

7.5. Discussion

Initially, P removal performance was good using butyrate as a sole carbon source. P release was observed with associated uptake of butyrate under anaerobic conditions, and subsequently, P was taken up under the aerobic condition during reactor run. However, butyrate uptake rate was slow as demonstrated by increasing P release during the entire anaerobic period. Oehmen et al. (2004) also found that butyrate uptake rate was slow. Therefore, butyrate fed EBPR systems may need longer anaerobic period than the currently used for systems where acetate is the major volatile fatty acids.

If not sufficient butyrate is taken up, because of the slow rate, then the microbial cells entering the aerobic period will have free butyrate that they can utilize, instead of utilizing the stored PHAs. If that occurs, then the amount of P taken up during the aerobic period will be less and the overall P removal will be negatively affected.

The amount of P release and uptake increased at the beginning of run, which indicate higher PAOs activity in the reactor. FISH analysis also showed that % RA of PAOs was higher compared to GAOs, targeted in the present study. However, later in the experimental period, the amount of P release and P uptake reduced even though butyrate was consumed, resulting in low P removal. This might be an indication of the presence of GAOs in the reactor fed with butyric acid. The abundance of PAOs (i.e. CAP) and GAOs (i.e. TFO and CCP) with associated % P removal during reactor run has been summarized in Table 7.5.

Table 7.5: % P removal with the abundance of PAOs and GAOs in B2 reactor

Day	% RA of TFO	% RA of CCP	Total % RA of GAOs	% RA of PAO	% P removal
12	-	8.3	8.3	13.4	47.95
33	-	-	-	18.8	66.02
47	8.31	1.6	9.9	28.7	40.54
64	15.8	1.8	17.6	49.6	66.00
92	14.42	2.8	17.2	33.5	45.05

Because the seed sludge used in the SBR runs was collected from an operating EBPR system, several PAOs and GAOs were present, at different percentages. The probes for CAP and Actino-PAO, PAOs and TFO, a GAO were used in full-scale plant to determine their relative abundance. Samples were collected every 2-3 weeks for a period of six month. Figure 7.9d shows the RA of the bacteria in the full-scale plant. The most abundant was the PAO, CAP (22 ± SD 4.2 %), followed by PAO-Actino (19 ± SD 1.7 %), and GAO –TFO (15 ± SD 1.0 %). The concentration of suspended solids in the SBR was close to that found in the full-scale plant, therefore, the % of GAOs and PAOs found in day 1 in the SBRs (Figures 7.9a, 7.9b and 7.9c) is compatible with that shown in Figure 7.9d.

In the SBR, the % RA of CAP increased continuously during the reactor run except last day, and % RA of CCP was low from day 47. This indicates that butyrate might select CAP and might not select CCP. However, % P removal and P release to C-uptake ratio revealed that GAOs were growing in the system. This can also be seen from % RA of TFOs in reactor. Initially its abundance was low, and later, showed an increasing trend in the reactor. However, % RA of TFO did not improve above 16% and there was not good correlation between % RA and % P removal. Therefore, TFO is not likely to be a

key population deteriorating P removal performance of the reactor. There might be other GAOs, not targeted in present study, present in reactor as P-release and C-uptake ratio continuously reduced during reactor run. In addition, it is possible that P removal deteriorated because of slow uptake of butyrate during the anaerobic period and excess butyrate entering the aeration. As mentioned earlier, that will results in less PHA formation and therefore less P uptake in the aerobic zone.

There is not enough evidence on the microbial selection using butyrate as a sole carbon source. However, Meyer et. al., (2006) found that *Defluvicoccus vanus*, a putative GAO, was 51 and 55% of all bacteria in a lab-scale deteriorated bioreactor using propionate. In addition, Beer et. al., (2004) obtained that *Sphingomonas* in alpha-proteobacteria was 71 ± 15% of total cell area in a lab-scale SBR with poor P removal capacity using acetate as a sole carbon source. Therefore, *Defluvicoccus vanus* and *Sphingomonas* in alpha-proteobacteria could be present in reactor fed with butyric acid. This possibility requires further investigation.

7.6. Conclusions

The following conclusions can be drawn from the findings of this research:

1) Butyrate might select for *Candidatus* Accumulibacter phosphatis, a PAO because the % RA of these bacteria increased continuously from 13% to 50% until day 64. Even though the % RA decreased at the last day of the experimental period of 92 days, the % RA of these bacteria was about 34%.

2) *Candidatus* Competibacter phosphatis, a GAO is not selected in the butyrate fed reactor. The highest % RA of these bacteria was 8% at day 12, and reduced below 2% for the rest of the experimental period.

3) Butyrate selects for *Defluvicoccus* related tetrad-forming organisms, a GAO. The % RA of these bacteria increased from 8% to 16% from days 47 to 64, and then remained stable at 16% up to the end of the experimental period. However, these bacteria might not be a key population deteriorating P removal performance of the reactor. The % RA of these bacteria and % P removal did not show good correlation (i.e. $R^2 = 0.58$), which might indicate the presence of other GAOs not targeted in the present study.

4) FISH analysis showed the presence of actinobacterial PAOs in the butyrate fed reactor. However, the % RA could not be quantified with accuracy.

5) Initially, the P removal performance was effective using butyrate as a sole carbon source. The P release and uptake increased at the beginning of the run indicate higher PAOs activity in the reactor, which was also confirmed by using FISH analysis. However, after six weeks, P removal performance (i.e. P release, P uptake and % P removal) deteriorated.

6) Results of this research indicate that anaerobic periods need to be increased in EBPR systems fed with butyrate. If the anaerobic period is increased, more PHA will be stored and therefore P uptake in the aerobic period will increase, if sufficient PAOs are present and GAOs are absent.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions

The first issue addressed in this research focused on the effect of glucose and butyrate on the microbiological performance of enhanced biological phosphorus removal (EBPR) systems. To investigate the first issue, the hypothesis was that glucose and butyrate will select for specific PAOs and GAOs not yet reported in systems fed exclusively with glucose and butyrate. The following conclusions can be drawn from the results obtained in the investigation of the first issue:

A) In the Case of Glucose Fed Reactors

1) The performance of biological P removal was found to be negatively affected by glucose, when glucose was fed as a sole carbon source.
2) Initially, when P release and uptake were performing well, PAOs dominated over GAOs in the EBPR system, resulting in higher P removal from the system. With time, GAOs out-competed PAOs and there was no P release and uptake in the reactor.
3) *Candidatus* Accumulibacter phosphatis is not selected in the glucose fed reactor. The highest % RA of these bacteria was 41% at day 14 and it was then reduced to below 4% at the end of reactor run.

4) Glucose selects for *Microlunatus phosphovorus* in glucose fed reactors. However, the data obtained from a two weeks reactor run with a high glucose concentration indicates that higher glucose concentration might be detrimental to these bacteria. The highest % RA of these bacteria measured was about 21%.

5) Glucose selects for targeted *Candidatus* Competibacter phosphatis given the high abundance of these bacteria detected during the experimental period. The % RA of *Candidatus* Competibacter phosphatis varied from about 10% to about 41% during the experimental period.

6) *Micropruina glycogenica* was selected in reactors fed glucose up to a certain glucose concentration. The maximum % RA of these bacteria found in this research was about 17%. It seems that *Micropruina glycogenica* is negatively affected by higher glucose concentration.

7) Glucose is not a suitable carbon source for the EBPR systems that contain insufficient VFAs. The low pH generated from glucose metabolism causes less phosphorus to be released and as a consequence PAOs do not have sufficient PHAs to take up OP during the aerobic period. As a result, P removal deteriorates.

B) In the Case of Butyrate Fed Reactors

1) Butyrate might select for *Candidatus* Accumulibacter phosphatis, a PAO because the % RA of these bacteria increased continuously from 13% to 50% until day 64. Even though the % RA decreased at the last day of the experimental period of 92 days, the % RA of these bacteria was about 34%.

2) *Candidatus* Competibacter phosphatis, a GAO is not selected in the butyrate fed reactor. The highest % RA of these bacteria was 8% at day 12, and reduced below 2% for the rest of the experimental period.

3) Butyrate selects for *Defluvicoccus* related tetrad-forming organisms, a GAO. The % RA of these bacteria increased from 8% to 16% from days 47 to 64, and then remained stable at 16% up to the end of the experimental period. However, these bacteria might not be a key population deteriorating P removal performance of the reactor. The % RA of these bacteria and % P removal did not show good correlation (i.e. $R^2 = 0.58$), which might indicate the presence of other GAOs not targeted in the present study.

4) FISH analysis showed the presence of actinobacterial PAOs in the butyrate fed reactor. However, the % RA could not be quantified with accuracy.

5) Initially, the P removal performance was effective using butyrate as a sole carbon source. The P release and uptake increased at the beginning of the run indicate higher PAOs activity in the reactor, which was also confirmed by using FISH analysis. However, after six weeks, P removal performance (i.e. P release, P uptake and % P removal) deteriorated.

6) Results of this research indicate that anaerobic periods need to be increased in EBPR systems fed with butyrate. If the anaerobic period is increased, more PHA will be stored and therefore P uptake in the aerobic period will increase, if sufficient PAOs are present and GAOs are absent.

From the above conclusions, it can be pointed out that the hypothesis related to issue one was validated because glucose selects for *Microlunatus phosphovorus*, a PAO and

Micropruina glycogenica, a GAO, and butyrate selects for *Defluvicoccus* related tetrad-forming organisms, a GAO. In addition, actinobacterial PAOs were also detected in the samples collected from butyrate fed reactors. However, the % RA of actinobacterial PAOs was not quantified with accuracy.

The second issue focused on this research is the influence of denitrification and different carbon source types on EBPR. It was hypothesized that the addition of a supplemental carbon source will improve phosphorus uptake in the aerobic period when nitrate is introduced during the anoxic period of the system. The following conclusions can be drawn from the present study regarding to issue two:

1) When denitrification is incorporated into EBPR process, without supplemental carbon source, P uptake will be adversely affected due to insufficient P release under anoxic period. As a result, low amounts of P will be removed from the wastewater. In the present study, only 59.7% P removal was observed without supplemental carbon source.
2) With acetate and propionate at different C/N ratios, denitrification and P release occurred simultaneously.
3) At a C/N ratio of 7.6 for acetate and propionate, denitrification negatively affected P release because of insufficient acetate and propionate. The effluent P concentration was lowest with % P removal of 94% and 95% for acetate and propionate. The % N removal was 76% and 74.7% for acetate and propionate, respectively. In addition, the $SDNR_{max}$, $SPUR_{max}$ and $SCUR_{max}$ were similar for both acetate and propionate.

4) At a C/N ratio of 22 and 59 for acetate, % P removal and % N removal were very similar. This indicates that increase of C/N ratio from 22 to 59 is not useful. For propionate, % P removal and denitrification were deteriorated by the increase of C/N ratio from 7.7 to 22 and 45.

5) Acetate provided higher $SDNR_{max}$ and $SPRR_{max}$ with an elevated consumption of carbon source compared to that of propionate.

6) At a C/N ratio of 22, $SDNR_{max}$, $SPRR_{max}$ and $SCUR_{max}$ reached the highest level for both acetate and propionate.

7) The higher P release under anoxic period resulted in higher effluent P concentration for both acetate and propionate.

8) Statistical analysis shows that P removal does not improve from the wastewater using acetate and propionate at different carbon levels. Moreover, similar P removal can be obtained using both acetate and propionate.

9) The results of this research have implications to engineering design of EBPR/denitrification systems to treat wastewater. Longer aerobic retention time should be provided to accommodate for greater P release. Care must be taken, during operation, to avoid excess carbon source in the aerobic zone of the system. It is suggested that P uptake is hindered by the presence of free carbon source.

The hypothesis regarding the second issue addressed in this research was only partially demonstrated. The addition of supplemental carbon source at stoichiometric level improved the phosphorus uptake under aerobic conditions when nitrate was introduced during the anoxic period. However, the addition of carbon source at a higher

than stoichiometric level did not improve phosphorus uptake under the aerobic condition when denitrification was incorporated during the anoxic period of EBPR process.

8.1.1. Implications of the Findings of this Research

The results of this research have implications on the design and operation of EBPR systems, as outlined below:

a) Glucose is not a suitable carbon source for EBPR systems. Low pH generated from glucose metabolism causes less phosphorus to be released. As a result, PAOs do not have sufficient PHAs to take up phosphorus during the aerobic period, and phosphorus removal deteriorates.

b) The anaerobic period needs to be increased in EBPR systems fed with butyrate. If the anaerobic period is increased, more PHA will be stored and therefore, phosphorus uptake in the aerobic period will be improved, if sufficient PAOs are present and GAOs are absent.

c) EBPR systems will perform best when denitrification is not incorporated to them.

d) Supplemental carbon source must be added if high levels of both phosphorus removal and denitrification are desired.

e) Care should be taken to add stoichiometric amounts of supplemental carbon source to achieve the desired denitrification level.

f) If desired denitrification level results in more phosphorus release, then designers may increase the aerobic period to allow for uptake of extra phosphorus released under the anoxic period.

8.2. Recommendations for Future Works

The following recommendations are made for future work:

1) Performed research with higher glucose and phosphorus concentration for a longer period to identify the reasons for the reduction of % RA of *Microlunatus phosphovorus* and *Micropruina glycogenica.*
2) Increase the anaerobic retention time in the reactor fed with butyrate and investigate phosphorus uptake.
3) Investigation of other potential GAOs present in the reactor fed with butyrate (e.g. *Sphingomonas spp.*).
4) Identification of the effect of denitrification on EBPR using multi cycle denitrification batch tests with PHA measurement to confirm that less PHA formation results in EBPR system failure.

APPENDIX I

THERMODYNAMIC COMPUATIONS FOR NITRATE

Calculation for the requirement of Acetate:

0.1438 mole nitrate-nitrogen (NO_3-N) needs 0.125 mole of Acetate (CH_3COO^-) (Ritmann & McCarty, 2001)

So, 1 mole of NO_3-N need (0.125/0.1438) = 0.8693 mole of CH_3COO^-

Molecular weight of NO_3 = 14 + 48 = 62 g

1 mole of NO_3 has 14 g of Nitrogen (N)

Molecular weight of CH_3COO^- = 59 g

1 mole NO_3-N (14 g) need 0.8693 mole (0.8693 * 59) = 51.3 g of CH_3COO^-

So, 1 g of NO_3-N need (51.3/14) = 3.66 g of CH_3COO^-

Volume of wastewater from ANA 2 = 1146 ml

Volume of wastewater from AE 9 = 1500 ml

Total Volume of wastewater = 1146 + 1500 = 2646 ml

NO_3-N concentration in the 2646 ml of wastewater = 9.2 mg/L

Consider, NO_3-N concentration = 10 mg/L

Total amount of NO_3-N in 2646 ml of wastewater = (10*2646/1000) = 26.46 mg NO_3-N

Required CH_3COO^- = (3.66 * 26.46) = 96.84 mg

Consider, required CH_3COO^- = 100 mg

So, the required concentration of CH_3COO^- = (100 * 1000/2646) = 37.8 mg/L for NO_3-N concentration of 10 mg/L

Used chemical was Sodium Acetate and molecular formula is = $CH_3COONa.\ 3H_2O$

Molecular weight of $CH_3COONa.\ 3H_2O$ = 136 g

So, Required $CH_3COONa.\ 3H_2O$ = (136 *100/59) = 231 mg = 0.231 g

Calculation for the requirement of Propionate:

1 g of NO_3-N need 4 g of BOD_L (Ritmann &McCarty, 2001) Which is about 4 g of COD

So, 4 g of COD/ g of NO_3-N

We know,

1.42 mg of COD/mg of Propionate

So, 4 g of COD = (4/1.42) = 2.82 g of Propionate

So, 2.82 g of Propionate/ g of NO_3-N

Volume of wastewater from ANA 2 = 1146 ml

Volume of wastewater from AE 9 = 1500 ml

Total Volume of wastewater = 1146 + 1500 = 2646 ml

NO_3-N concentration in the 2646 ml of wastewater = 7.0 mg/L

Total amount of NO_3-N in 2646 ml of wastewater = (7*2646/1000) = 18.522 mg NO_3-N

Required Propionate = (2.82 * 18.522) = 52.23 mg

The concentration of required Propionate = (52.23 * 1000 / 2646) = 19. 74 mg /L

So, required Propionate is 19. 74 mg /L for 7.0 mg/L of NO_3-N

Used chemical was Sodium Propionate and molecular formula is = CH_3CH_2COONa

Molecular weight of CH_3CH_2COONa = 96 g

Molecular weight of $CH_3CH_2COO^-$ = 73 g

Required Sodium Propionate = (96 * 52.23/73) = 68.7 mg

The available Sodium Propionate is 99%

So, the required Sodium Propionate = (100 * 68.7/99) = 69.4 mg

Consider, the required Sodium Propionate = 70 mg = 0.07 g

APPENDIX II

DETAIL RESULTS OF BATCH TESTS

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

NO3-N Analysis:

Run 1:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3667 mg/L
Std. = 10.1 mg/L

Date	Time (min)	NO3-N (mg/L)	Duplicate NO3-N (mg/L)	Mean NO3-N Conc. (mg/L)
3/31/2008	0	11.6	11.5	11.55
	10	10.1	10.5	10.3
	15	9.9	9.7	9.8
	20	9.5	9	9.25
	25	9.1	8.8	8.95
	30	8.3	8.4	8.35
	35	8.3	7.9	8.1
	40	7.5	7.9	7.7
	60	11.6	10.9	11.25
	90	16.3	16.6	16.45
	120	17.6	17.4	17.5
	150	18	17.9	17.95
	180	17.9	17.8	17.85
	ANA 2=	0.8	1.3	1.05
	AE 9=	19.3	20.4	19.85

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

NO3-N Analysis:

Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3331 mg/L
Std. = 10.1 mg/L

Date	Time (min)	NO3-N (mg/L)	Duplicate NO3-N (mg/L)	Mean NO3-N Conc. (mg/L)
4/2/2008	0	8.9	8.8	8.85
	10	8.4	7.7	8.05
	15	7	7.2	7.1
	20	7	7.4	7.2
	25	8	7.8	7.9
	30	6.9	6.7	6.8
	35	7.5	7	7.25
	40	6.2	6.1	6.15
	60	8.2	9.1	8.65
	90	12.6	12.7	12.65
	120	14.5	14.2	14.35
	150	15.8	15.4	15.6
	180	15.2	15.4	15.3
	ANA 2=	1.1	0.9	1
	AE 9=	15.9	15.8	15.85

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

Nitrite Analysis:
Run 1:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3667 mg/L
mg/L as
Std. = 0.2373 NO_2-N Standard of 0.25 mg/L)

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Dilution factor	NO_2-N Conc. 1 (mg/L)	NO_2-N Conc. 2 (mg/L)	Mean NO_2-N Conc. (mg/L)
3/31/2008	0	0.0250	0.0246	10	0.25	0.2460	0.248
	10	0.0401	0.0488	10	0.401	0.4880	0.4445
	15	0.0508	0.0496	10	0.508	0.4960	0.502
	20	0.0679	0.0692	10	0.679	0.6920	0.6855
	25	0.0693	0.0752	10	0.693	0.7520	0.7225
	30	0.0782	0.0824	10	0.782	0.8240	0.803
	35	0.0853	0.0857	10	0.853	0.8570	0.855
	40	0.0889	0.0903	10	0.889	0.9030	0.896
	60	0.1360	0.1371	10	1.36	1.3710	1.3655
	90	0.1600	0.1535	10	1.6	1.5350	1.5675
	120	0.0076	0.0086	10	0.076	0.0860	0.081
	150	0.0036	0.0047	10	0.036	0.0470	0.0415
	180	0.0089	0.0072	10	0.089	0.0720	0.0805
	ANA 2	0.0077	0.0071	11	0.0847	0.0781	0.0814
	AE 9	0.0343	0.0385	12	0.4116	0.4620	0.4368

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

Nitrite Analysis:
Run 2:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3331 mg/L
Std. = 0.2373 mg/L as NO_2-N Standard of 0.25 mg/L)

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Dilution factor	NO_2-N Conc. 1 (mg/L)	NO_2-N Conc. 2 (mg/L)	Mean NO_2-N Conc. (mg/L)
4/2/2008	0	0.0085	0.0103	10	0.085	0.1030	0.094
	10	0.0336	0.0319	10	0.336	0.3190	0.3275
	15	0.0296	0.029	10	0.296	0.2900	0.293
	20	0.0388	0.0382	10	0.388	0.3820	0.385
	25	0.0397	0.0416	10	0.397	0.4160	0.4065
	30	0.0400	0.0377	10	0.4	0.3770	0.3885
	35	0.0454	0.0477	10	0.454	0.4770	0.4655
	40	0.0491	0.0487	10	0.491	0.4870	0.489
	60	0.0987	0.0907	10	0.987	0.9070	0.947
	90	0.1440	0.1453	10	1.44	1.4530	1.4465
	120	0.0060	0.0064	10	0.06	0.0640	0.062
	150	0.0055	0.0059	10	0.055	0.0590	0.057
	180	0.0055	0.0098	10	0.055	0.0980	0.0765
	ANA 2	0.0048	0.0068	11	0.0528	0.0748	0.0638
	AE 9	0.0114	0.0173	12	0.1368	0.2076	0.1722

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

sTOC analysis:
Run 1:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3667 mg/L

Date	Time (min)	sTOC (mg/L)
3/31/2008	0	12.12
	10	11.12
	15	10.66
	20	10.53
	25	11.07
	30	10.57
	35	10.80
	40	10.66
	60	9.87
	90	9.57
	120	9.29
	150	8.97
	180	8.93
	ANA 2	17.14
	AE 9	8.43

sTOC analysis:
Run 2:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3331 mg/L

Date	Time (min)	sTOC (mg/L)
4/2/2008	0	11.38
	10	10.99
	15	10.73
	20	10.20
	25	9.94
	30	10.02
	35	10.22
	40	9.86
	60	9.74
	90	9.16
	120	9.15
	150	8.93
	180	9.37
	ANA 2	15.87
	AE 9	8.29

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

OP Analysis:
Run 1:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. = 3667 mg/L
Std. = 1.01 mg/L (Standard of 1 mg P/L)

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Dilution factor	OP Conc. 1 (mg P/L)	OP Conc. 2 (mg P/L)	Mean OP Conc. (mg P/L)
3/31/2008	0	0.39	0.38	25	9.75	9.50	9.63
	10	0.37	0.37	25	9.25	9.25	9.25
	15	0.34	0.35	25	8.50	8.75	8.63
	20	0.37	0.37	25	9.25	9.25	9.25
	25	0.37	0.35	25	9.25	8.75	9.00
	30	0.33	0.34	25	8.25	8.50	8.38
	35	0.35	0.34	25	8.75	8.50	8.63
	40	0.37	0.37	25	9.25	9.25	9.25
	60	0.25	0.23	25	6.25	5.75	6.00
	90	0.16	0.15	25	4.00	3.75	3.88
	120	0.13	0.11	25	3.25	2.75	3.00
	150	0.11	0.10	25	2.75	2.50	2.63
	180	0.08	0.08	25	2.00	2.00	2.00

Denitrification using wastewater collected from ANA2 and AE9 under the anoxic and aerobic condition

OP Analysis:
Run 2:
ANA2 = 1146 ml
AE 9= 1500 ml
MLSS Conc. 3331 mg/L
Std. = 0.86 mg/L (Standard of 1 mg P/L)

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Dilution factor	Correction	Actual Read 1 (mg P/L)	Actual Read 2 (mg P/L)	OP Conc. 1 (mg P/L)	OP Conc. 2 (mg P/L)	Mean OP Conc. (mg P/L)
4/2/2008	0	0.12	0.14	25	0.14	0.26	0.28	6.5	7	6.75
	10	0.13	0.16	25	0.14	0.27	0.30	6.75	7.5	7.13
	15	0.15	0.13	25	0.14	0.29	0.27	7.25	6.75	7.00
	20	0.15	0.16	25	0.14	0.29	0.30	7.25	7.5	7.38
	25	0.14	0.15	25	0.14	0.28	0.29	7	7.25	7.13
	30	0.13	0.13	25	0.14	0.27	0.27	6.75	6.75	6.75
	35	0.13	0.09	25	0.14	0.27	0.23	6.75	5.75	6.25
	40	0.14	0.13	25	0.14	0.28	0.27	7	6.75	6.88
	60	0.08	0.07	25	0.14	0.22	0.21	5.5	5.25	5.38
	90	0.00	0.00	25	0.14	0.14	0.14	3.5	3.5	3.50
	120	0.00	0.00	25	0.14	0.14	0.14	3.5	3.5	3.50
	150	0.00	0.00	25	0.14	0.14	0.14	3.5	3.5	3.50
	180	0.00	0.00	25	0.14	0.14	0.14	3.5	3.5	3.50
	ANA 2	0.40	0.47	25	0.14	0.54	0.61	13.5	15.25	14.38
	AE 9	0.01	0.00	25	0.14	0.15	0.14	3.75	3.5	3.63

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 35 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.38	mg C/L
MLSS Conc. =	3790	mg/L
ANA 2=	0	mg/L
AE 9=	13.8	mg/L

Date	Time (min)	NO_3-N (mg/L)
1/14/2008	0	6.9
	10	4.3
	15	3.3
	20	2.8
	25	2
	30	1.7
	35	0.7
	60	4.2
	90	8.5
	122	12.2
	153	12.7
	180	12.8

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.38	mg/L
MLSS Conc. =	3038	mg/L
ANA 2=	0.4	mg/L
AE 9=	16.8	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Mean NO_3-N (mg/L)
1/30/2008	0	10.5	9.6	10.05
	10	7.3		7.3
	15	6.1		6.1
	20	5.2		5.2
	25	4.9		4.9
	30	4.7		4.7
	35	4.1		4.1
	40	3.3	3.4	3.35
	60	5.9		5.9
	90	9.4		9.4
	122	10.7		10.7
	153	14.3		14.3
	180	15.6	15.6	15.6

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 1:
Aeration starts at 35 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3790	mg/L
ANA 2=	19	mg/L
AE 9=	0.6	mg/L as N

Date	Time (min)	NH_3-N (mg/L)
1/14/2008	0	8.20
	10	8.30
	15	8.60
	20	8.50
	25	8.80
	30	8.40
	35	8.70
	60	5.30
	90	1.80
	122	0.04
	153	0.03
	180	0.03

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3038	mg/L
ANA 2=	25.5	mg/L
AE 9=	0	mg/L as N

Date	Time (min)	NH_3-N (mg/L)	Duplicate of NH_3-N (mg/L)	Mean NH_3-N (mg/L)
1/30/2008	0	9.10	9.4	9.25
	10	10.40		10.40
	15	10.00		10.00
	20	10.00		10.00
	25	9.50		9.50
	30	10.8		10.80
	35	10.9		10.90
	40	10.50	10.2	10.35
	60	7.20		7.20
	90	2.40		2.40
	122	0.00		0.00
	153	0.00		0.00
	180	0.00		0.00

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

OP analysis:
Run 1:
Aeration starts at 35 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3790	mg/L
ANA 2=	19.5	mg P/L
AE 9=	2.9	mg P/L

Date	Time (min)	Read 1 (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
1/14/2008	0	0.37	25	9.25
	10	0.68	25	17.00
	15	0.80	25	20.00
	20	0.80	25	20.00
	25	0.82	25	20.50
	30	0.81	25	20.25
	35	0.82	25	20.50
	60	0.48	25	12.00
	90	0.76	10	7.60
	122	0.52	10	5.20
	153	0.43	10	4.30
	180	0.22	10	2.20

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

OP analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3038	mg/L
ANA 2=	21.25	mg P/L
AE 9=	0	mg P/L

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
1/30/2008	0	0.31	0.31	0.31	25	7.75
	10	0.63		0.63	25	15.75
	15	0.70		0.70	25	17.50
	20	0.74		0.74	25	18.50
	25	0.73		0.73	25	18.25
	30	0.75		0.75	25	18.75
	35	0.73		0.73	25	18.25
	40	0.72	0.73	0.725	25	18.13
	60	0.43		0.43	25	10.75
	90	0.45		0.45	10	4.50
	122	0.21		0.21	10	2.10
	153	0.03		0.03	10	0.30
	180	0.01	0.01	0.01	10	0.10

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 1:
Aeration starts at 35 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3790	mg/L
ANA 2=	0.0620	mg/L as NO_2-N
AE 9=	0.4740	mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
1/14/2008	0	0.0346	10	0.35
	10	0.1033	10	1.03
	15	0.1408	10	1.41
	20	0.1528	10	1.53
	25	0.1695	10	1.70
	30	0.1784	10	1.78
	35	0.1995	10	2.00
	60	0.2331	10	2.33
	90	0.2873	10	2.87
	122	0.0466	10	0.47
	153	0.0066	10	0.07
	180	0.0064	10	0.06

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
MLSS Conc. =	3038	mg/L
ANA 2=	0.0350	mg/L as NO_2-N
AE 9=	0.7765	mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Dupplicate of NO_2-N	Mean NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
1/30/2008	0	0.0455	0.0466	0.04605	10	0.46
	10	0.1166		0.1166	10	1.17
	15	0.1444		0.1444	10	1.44
	20	0.1711		0.1711	10	1.71
	25	0.1807		0.1807	10	1.81
	30	0.1961		0.1961	10	1.96
	35	0.2061		0.2061	10	2.06
	40	0.2238	0.2226	0.2232	10	2.23
	60	0.2551		0.2551	10	2.55
	90	0.3547		0.3547	10	3.55
	122	0.3737		0.3737	10	3.74
	153	0.0711		0.0711	10	0.71
	180	0.0086	0.0087	0.00865	10	0.09

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

sTOC analysis:
Run 1:
Aeration starts at 35 min

MLSS Conc. =	3790	mg/L
ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
ANA2 =	15.52	mg/L
AE 9=	8.77	mg/L

Date	Time	Reading (mg/L)
1/14/2008	0	28.17
	10	15.48
	15	13.02
	20	11.59
	25	11.72
	30	12.44
	35	11.36
	60	11.18
	90	10.67
	122	11.66
	153	11.86
	180	11.54

Denitrification using wastewater and Acetate (stoichiometric need, X) under the anoxic and aerobic condition

sTOC analysis:
Run 2:
Aeration starts at 40 min

MLSS Conc. =	3038	mg/L
ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	37.8	mg/L
carbon =	15.8	mg/L
ANA2 =	20.24	mg/L
AE 9=	8.68	mg/L

Date	Time	sTOC (mg/L)
1/30/2008	0	26.68
	10	17.10
	15	13.71
	20	12.05
	25	12.65
	30	11.97
	35	12.56
	40	11.25
	60	11.40
	90	11.08
	120	11.22
	150	10.38
	180	10.13

Denitrification using wastewater and Acetate (5 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =	76.88	mg/L
MLSS Conc. =	2648	mg/L
ANA 2=	0	mg/L
AE 9=	18.45	mg/L
Std. =	10.4	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Mean NO_3-N (mg/L)
11/19/2008	0	10.8	10.4	10.6
	10	6.9	7.6	7.3
	15	5.4	5.5	5.5
	20	3.5	3.3	3.4
	25	1.9	1.8	1.9
	30	0.8	0.6	0.7
	36	0.5	0.6	0.6
	40	0.4	0.7	0.6
	63	3.4	3.2	3.3
	90	7.4	7.2	7.3
	120	11.3	11.4	11.4
	150	11.6	11.5	11.6
	180	11.7	11.6	11.7

Denitrification using wastewater and Acetate (5 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =	76.88	mg/L
MLSS Conc. =	2671	mg/L
ANA 2=	0.1	mg/L
AE 9=	18.9	mg/L
Std. =	10.4	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Mean NO_3-N (mg/L)
11/20/2008	0	10.2	9.7	10.0
	10	6.5		6.5
	15	4.3		4.3
	20	2.7		2.7
	25	1.6		1.6
	30	0.6	0.8	0.7
	35	0.6		0.6
	40	0.4		0.4
	64	3.6		3.6
	90	9.8		9.8
	120	10.3	10.1	10.2
	150	10.6		10.6
	180	10.7		10.7

Denitrification using wastewater and Acetate (5 X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
MLSS Conc. =	2648	mg/L
ANA 2=	21.15	mg/L
AE 9=	0.189	mg/L as N
Std. =	9.5	mg/L (for 10 mg/L)

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
11/19/2008	0	7.60	7.3	7.45
	10	7.70	7.6	7.65
	15	7.50	6.9	7.2
	20	7.40	7.2	7.3
	25	7.50	7.2	7.35
	30	8.10	8.20	8.15
	36	7.40	8.8	8.1
	40	7.10		7.10
	63	4.70	4.1	4.4
	90	1.10	1.094	1.097
	120	0.07	0.005	0.039
	150	0.01	0.082	0.048
	180	0.08	0.074	0.0755

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

NH_3-N Analysis:
Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Acetate = 189 mg/L
MLSS Conc. = 2671 mg/L
ANA 2= 18.7 mg/L
AE 9= 0.03 mg/L as N
Std. = 10.7 mg/L (for 10 mg/L)

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
11/20/2008	0	7.500	7.300	7.40
	10	7.100		7.10
	15	7.400		7.40
	20	7.500		7.50
	25	7.800		7.80
	30	7.100	7.500	7.30
	35	8.100	8.200	8.15
	40	8.200		8.20
	64	3.000		3.00
	90	0.028		0.03
	120	0.051	0.033	0.04
	150	0.016		0.02
	180	0.021		0.02

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

OP analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =		mg/L
MLSS Conc. =	2648	mg/L
ANA 2=	8.63	mg P/L
AE 9=	1.05	mg P/L
Std. =	1.02	mg P/L

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
11/19/2008	0	0.20	0.2	0.2	25	5.00
	10	0.45	0.45	0.45	25	11.25
	15	0.56	0.56	0.56	25	14.00
	20	0.69	0.69	0.69	25	17.25
	25	0.84	0.8	0.82	25	20.50
	30	0.93	0.94	0.935	25	23.38
	36	1.04	1.03	1.035	25	25.88
	40	1.13	1.14	1.135	25	28.38
	63	1.07	1.04	1.055	25	26.38
	90	0.94	0.92	0.93	25	23.25
	120	1.74	1.72	1.73	10	17.30
	150	1.31	1.28	1.295	10	12.95
	180	1.07	1.05	1.06	10	10.60

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

OP analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =		mg/L
MLSS Conc. =	2671	mg/L
ANA 2=	9.50	mg P/L
AE 9=	0.3	mg P/L
Std. =	1.02	mg P/L

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
11/20/2008	0	0.17	0.16	0.165	25	4.13
	10	0.43		0.43	25	10.75
	15	0.56		0.56	25	14.00
	20	0.70		0.70	25	17.50
	25	0.84		0.84	25	21.00
	30	1.01	1.08	1.045	25	26.13
	35	1.14		1.14	25	28.50
	40	1.23		1.23	25	30.75
	64	1.00		1.00	25	25.00
	90	0.70		0.70	25	17.50
	120	1.15	1.14	1.145	10	11.45
	150	0.91		0.91	10	9.10
	180	0.88		0.88	10	8.80

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

NO_2-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =		mg/L
MLSS Conc. =	2648	mg/L
ANA 2=	0.0310	mg/L as NO_2-N
AE 9=	0.5040	mg/L as NO_2-N
Std. =	0.2533	mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Dupplicate of NO_2-N (mg/L)	Mean NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
11/19/2008	0	0.0532	0.0521	0.05265	10	0.53
	10	0.2180		0.2180	10	2.18
	15	0.2935		0.2935	10	2.94
	20	0.3781		0.3781	10	3.78
	25	0.4656		0.4656	10	4.66
	30	0.5138	0.5059	0.50985	10	5.10
	36	0.4307		0.4307	10	4.31
	40	0.3287	0.3320	0.33035	10	3.30
	63	0.3493	0.3455	0.3474	10	3.47
	90	0.3180		0.3180	10	3.18
	120	0.0128	0.0132	0.013	10	0.13
	150	0.0027		0.0027	10	0.03
	180	0.0023		0.0023	10	0.02

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

NO_2-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
carbon =		mg/L
MLSS Conc. =	2671	mg/L
ANA 2=	0.0310	mg/L as NO_2-N
AE 9=	0.5040	mg/L as NO_2-N
Std. =	0.2533	mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Duplicate of NO_2-N (mg/L)	Mean NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
11/20/2008	0	0.0114	0.0108	0.0111	10	0.11
	10	0.2012		0.2012	10	2.01
	15	0.2883		0.2883	10	2.88
	20	0.3788		0.3788	10	3.79
	25	0.4597		0.4597	10	4.60
	30	0.4686	0.4635	0.46605	10	4.66
	36	0.3272		0.3272	10	3.27
	40	0.1777	0.1742	0.17595	10	1.76
	63	0.2699	0.2674	0.26865	10	2.69
	90	0.0435		0.0435	10	0.44
	120	0.0021	0.0017	0.0019	10	0.02
	150	0.0017	0.0026	0.00215	10	0.02
	180	0.0024		0.0024	10	0.02

Denitrification using wastewater and Acetate (5 X) under anoxic and aerobic condition

sTOC analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
MLSS Conc. =	2648	mg/L
ANA 2=	14.80	mg /L
AE 9=	7.64	mg /L

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual sTOC (mg/L)
11/19/2008	0	4.72		4.72	20	94.36
	10	3.89		3.89	20	77.70
	15	3.71		3.71	20	74.24
	20	3.32		3.32	20	66.38
	25	2.98		2.98	20	59.62
	30	2.81	2.75	2.78	20	55.61
	36	2.56		2.56	20	51.10
	40	2.44		2.44	20	48.70
	63	1.16		1.16	20	23.24
	90	0.77		0.77	20	15.32
	120	0.61	0.62	0.61	20	12.27
	150	0.56		0.56	20	11.14
	180	0.57		0.57	20	11.48

Denitrification using wastewater and Acetate (5X) under anoxic and aerobic condition

sTOC analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	189	mg/L
MLSS Conc. =	2671	mg/L
ANA 2=	12.63	mg /L
AE 9=	7.056	mg /L

Date	Time (min)	Read 1 (mg/L)	Read 2 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual sTOC (mg/L)
11/20/2008	0	2.606	2.631	2.619	20	52.37
	10	3.860		3.860	20	77.20
	15	3.578		3.578	20	71.56
	20	3.219		3.219	20	64.38
	25	2.931		2.931	20	58.62
	30	2.597	2.621	2.609	20	52.18
	35	2.457		2.457	20	49.14
	40	2.195		2.195	20	43.90
	63	0.614		0.614	20	12.28
	90	0.473		0.473	20	9.46
	120	0.487	0.477	0.482	20	9.64
	150	0.503		0.503	20	10.06
	180	0.503		0.503	20	10.06

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

OP analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml		
AE 9=	1500	ml		
Acetate =	378	mg/L		
carbon =	154	mg/L		
Acetate =	2.31	g		
MLSS Conc. =	3497	mg/L		
ANA 2=	11.14	mg P/L	AE 9=	0.14 mg P/L
Std. =	0.86	mg/L		

Date	Time (min)	Read 1 (mg/L)	Duplicate of Read 1 (mg/L)	Mean Read	Dilution factor	Actual PO_4-P (mg/L)
2/7/2008	0	0.08	0.07	0.075	25	1.88
	10	0.42		0.42	25	10.50
	15	0.53		0.53	25	13.25
	20	0.71		0.71	25	17.75
	25	0.82		0.82	25	20.50
	30	0.92	0.93	0.925	25	23.13
	35	1.00		1.00	25	25.00
	40	1.18		1.18	25	29.50
	60	0.83		0.83	25	20.75
	90	0.71		0.71	25	17.75
	120	0.69	0.65	0.67	25	16.75
	150	0.60		0.60	25	15.00
	180	0.34		0.34	25	8.50

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

OP analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml		
AE 9=	1500	ml		
Acetate =	378	mg/L		
carbon =	154	mg/L		
Acetate =	2.31	g		
MLSS Conc. =	3595	mg/L		
ANA 2=	16.5	mg P/L	AE 9=	0 mg P/L
Std. =	1.02	mg/L		

Date	Time (min)	Read 1 (mg/L)	Duplicate of Read 1 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
2/14/2008	0	0.27	0.28	0.275	25	6.88
	10	0.56		0.56	25	14.00
	15	0.71		0.71	25	17.75
	20	0.80		0.80	25	20.00
	25	1.00		1.00	25	25.00
	30	1.08	1.07	1.075	25	26.88
	35	1.20		1.20	25	30.00
	40	1.30		1.30	25	32.50
	60	0.94		0.94	25	23.50
	90	0.75		0.75	25	18.75
	120	0.77	0.77	0.77	25	19.25
	150	0.72		0.72	25	18.00
	180	0.47		0.47	25	11.75

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3497	mg/L
ANA 2=	0.3	mg/L
AE 9=	14	mg/L
Std. =	10	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO3-N (mg/L)	Mean NO_3-N (mg/L)
2/7/2008	0	7	7.3	7.15
	10	4.1		4.1
	15	3.1		3.1
	20	2		2
	25	0.9		0.9
	30	0.4		0.4
	35	0.5		0.5
	40	0.8		0.8
	60	2		2
	90	5.3		5.3
	120	7.3	7.6	7.45
	150	10.6		10.6
	180	12.3		12.3

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3595	mg/L
ANA 2=	0.4	mg/L
AE 9=	11.3	mg/L
Std. =	10.1	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO3-N (mg/L)	Mean NO_3-N (mg/L)
2/14/2008	0	6.2	6.4	6.3
	10	4		4
	15	2.8		2.8
	20	1.7		1.7
	25	0.8		0.8
	30	0.7	0.7	0.7
	35	0.4		0.4
	40	0.4	0.7	0.55
	60	2.3		2.3
	90	5.6		5.6
	120	7.6	7.5	7.55
	150	10.5		10.5
	180	12.3		12.3

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NH_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3497	mg/L
ANA 2=	24.6	mg/L
AE 9=	0	mg/L as N
Std. =	7.3	mg/L (for 10 mg/l)

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
2/7/2008	0	10.20	9.5	9.85
	10	9.80		9.80
	15	10.90		10.90
	20	10.90		10.90
	25	10.90		10.90
	30	10.30	10.70	10.5
	35	10.50		10.50
	40	10.70		10.70
	60	7.20		7.20
	90	2.20		2.20
	120	0.00	0	0
	150	0.00		0.00
	180	0.00		0.00

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NH_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3595	mg/L
ANA 2=	21.1	mg/L
AE 9=	5.9	mg/L as N
Std. =	9.5	mg/L (for 10 mg/l)

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
2/14/2008	0	13.70	13.5	13.6
	10	13.40		13.40
	15	13.60		13.60
	20	13.80		13.80
	25	13.40		13.40
	30	13.6	14.1	13.85
	35	13.10		13.10
	40	13.40		13.40
	60	9.30		9.30
	90	5.40		5.40
	120	1.60	1.7	1.65
	150	0.00		0.00
	180	0.30		0.30

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NO_2-N Analysis:

Run 1:

MLSS Conc. = 3497 mg/L

ANA2 = 1146 ml

AE 9= 1500 ml

Acetate = 378 mg/L

carbon = 154 mg/L

Acetate = 2.31 g

ANA 2= 0.0450 mg/L as NO_2-N AE 9= 3.1070 mg/L as NO_2-N

Std. = 0.2569 mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO2-N (mg/L)	Mean NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
2/7/2008	0	0.1380	0.1391	0.13855	10	1.39
	10	0.2208		0.2208	10	2.21
	15	0.2540		0.2540	10	2.54
	20	0.2849		0.2849	10	2.85
	25	0.3205		0.3205	10	3.21
	30	0.3334	0.3336	0.3335	10	3.34
	35	0.2612		0.2612	10	2.61
	40	0.1469		0.1469	10	1.47
	60	0.2167		0.2167	10	2.17
	90	0.3227		0.3227	10	3.23
	120	0.3860	0.3888	0.3874	10	3.87
	150	0.1381		0.1381	10	1.38
	180	0.0088		0.0088	10	0.09

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

NO_2-N Analysis:
Run 2:
MLSS Conc. = 3595 mg/L
ANA2 = 1146 ml
AE 9= 1500 ml
Acetate = 378 mg/L
carbon = 154 mg/L
Acetate = 2.31 g
ANA 2= 0.0690 mg/L as NO_2-N AE 9= 3.4250 mg/L as NO_2-N
Std. = 0.2527 mg/L as NO_2-N

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO2-N (mg/L)	Mean NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)
2/14/2008	0	0.1987	0.1957	0.1972	10	1.97
	10	0.2676		0.2676	10	2.68
	15	0.3111		0.3111	10	3.11
	20	0.3373		0.3373	10	3.37
	25	0.3498		0.3498	10	3.50
	30	0.2875	0.2833	0.2854	10	2.85
	35	0.1743		0.1743	10	1.74
	40	0.0655		0.0655	10	0.66
	60	0.1494		0.1494	10	1.49
	90	0.2658		0.2658	10	2.66
	120	0.3610	0.3588	0.3599	10	3.60
	150	0.2576		0.2576	10	2.58
	180	0.0376		0.0376	10	0.38

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

sTOC analysis:
Run 1:

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3497	mg/L
ANA 2=	25.73	mg/L
AE 9=	8.832	mg/L

Date	Time (min)	sTOC (mg/L)
2/7/2008	0	166.30
	10	167.50
	15	164.70
	20	156.60
	25	146.30
	30	139.30
	35	134.90
	40	132.70
	60	98.72
	90	63.51
	120	32.62
	150	8.86
	180	8.71

Denitrification using wastewater and Acetate (10 X) under anoxic and aerobic condition

sTOC analysis:
Run 2:

ANA2 =	1146	ml
AE 9=	1500	ml
Acetate =	378	mg/L
carbon =	154	mg/L
Acetate =	2.31	g
MLSS Conc. =	3595	mg/L
ANA 2=	24.99	mg/L
AE 9=	10.24	mg/L

Date	Time (min)	sTOC (mg/L)
2/14/2008	0	174.60
	10	168.00
	15	162.50
	20	149.10
	25	143.90
	30	139.20
	35	134.80
	40	130.90
	60	101.80
	90	65.38
	120	33.10
	150	12.24
	180	12.36

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146 ml
AE 9=	1500 ml
Propionate =	19.74 mg/L
Sodium Propionate (99%) =	0.07 g
MLSS Conc. =	4013 mg/L
ANA 2=	0.2 mg/L
AE 9=	12.2 mg/L
Std. =	10.5 mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Mean NO_3-N (mg/L)
3/5/2008	0	6.6	6.6	6.6
	10	4.5		4.5
	15	3.1		3.1
	20	2.6		2.6
	25	2.2		2.2
	30	1.5	1.3	1.4
	35	0.8		0.8
	40	0.8		0.8
	60	3		3
	90	6.1		6.1
	120	8.8	9.1	8.95
	150	11.7		11.7
	180	13.7	13.8	13.75

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	22.6	mg/L
Sodium Propionate (99%) =	0.08	g
MLSS Conc. =	2427	mg/L
ANA 2=	0.8	mg/L
AE 9=	16.4	mg/L
Std. =	11	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Mean NO_3-N (mg/L)
3/12/2008	0	9	8.6	8.8
	10	6.3		6.3
	15	5.7		5.7
	20	5.2		5.2
	25	4.6		4.6
	30	4.2	3.7	3.95
	35	3.7		3.7
	40	3.1		3.1
	60	6.5		6.5
	90	10.6		10.6
	120	13.3	13.1	13.2
	150	16		16
	180	16		16

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

OP analysis:
Run 1:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	19.74	mg/L
Sodium Propionate (99%) =	0.07	g
MLSS Conc. =	4013	mg/L
ANA 2=	21	mg P/L
AE 9=	0.5	mg P/L
Std. =	1.07	mg/L

Date	Time (min)	Read 1 (mg/L)	Duplicate of Read 1 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
3/5/2008	0	0.34	0.34	0.34	25	8.50
	10	0.52	0.55	0.535	25	13.38
	15	0.52	0.52	0.52	25	13.00
	20	0.52	0.52	0.52	25	13.00
	25	0.52	0.5	0.51	25	12.75
	30	0.54	0.54	0.54	25	13.50
	35	0.55	0.51	0.53	25	13.25
	40	0.54		0.54	25	13.50
	60	0.28		0.28	25	7.00
	90	0.09		0.09	25	2.25
	120	0.02	0.01	0.015	25	0.38
	150	0.01	0.00	0.005	25	0.13
	180	0.04		0.04	25	1.00

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

OP analysis:
Run 2:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	22.6	mg/L
Sodium Propionate (99%) =	0.08	g
MLSS Conc. =	2427	mg/L
ANA 2=	17.75	mg P/L
AE 9=	0	mg P/L
Std. =	1.01	mg/L

Date	Time (min)	Read 1 (mg/L)	Duplicate of Read 1 (mg/L)	Mean Read (mg/L)	Dilution factor	Actual PO_4-P (mg/L)
3/12/2008	0	0.26	0.25	0.255	25	6.38
	10	0.46		0.46	25	11.50
	15	0.46		0.46	25	11.50
	20	0.50		0.50	25	12.50
	25	0.48		0.48	25	12.00
	30	0.48	0.48	0.48	25	12.00
	35	0.47		0.47	25	11.75
	40	0.48		0.48	25	12.00
	60	0.28		0.28	25	7.00
	90	0.11		0.11	25	2.75
	120	0.02	0.02	0.02	25	0.50
	150	0.01		0.01	25	0.25
	180	0.01		0.01	25	0.25

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 1:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	19.74	mg/L
Sodium Propionate (99%) =	0.07	g
MLSS Conc. =	4013	mg/L
ANA 2=	24.2	mg/L
AE 9=	0.9	mg/L as N
Std. =	9.9	mg/L

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
3/5/2008	0	10.50	10	10.25
	10	10.60		10.60
	15	9.90	9.9	9.9
	20	10.60		10.60
	25	10.90	10.2	10.55
	30	10.8	10.7	10.75
	35	10.70		10.70
	40	10.80		10.80
	60	7.20		7.20
	90	3.00		3.00
	120	0.33	0.338	0.334
	150	0.02		0.02
	180	0.03		0.03

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 2:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	22.6	mg/L
Sodium Propionate (99%) =	0.08	g
MLSS Conc. =	2427	mg/L
ANA 2=	26.4	mg/L
AE 9=	0.3	mg/L as N
Std. =	10.2	mg/L

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
3/12/2008	0	11.30	11.2	11.25
	10	11.00		11.00
	15	10.80		10.80
	20	11.00		11.00
	25	11.20		11.20
	30	11	11.2	11.1
	35	10.50		10.50
	40	10.70	10.3	10.5
	60	7.50		7.50
	90	3.60		3.60
	120	0.28	0.272	0.2765
	150	0.00		0.00
	180	0.00		0.00

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

sTOC analysis:
Run 1:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	19.74	mg/L
Sodium Propionate (99%) =	0.07	g
MLSS Conc. =	4013	mg/L
ANA 2=	18.99	mg/L
AE 9=	9.829	mg/L

Date	Time (min)	sTOC (mg/L)
3/5/2008	0	20.80
	10	13.27
	15	13.10
	20	13.05
	25	13.14
	30	12.93
	35	12.24
	40	12.36
	60	12.47
	90	11.63
	120	11.50
	150	11.13
	180	11.43

Denitrification using wastewater and propionate (stoichiometric need, X) under the anoxic and aerobic condition

sTOC analysis:
Run 2:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	22.6	mg/L
Sodium Propionate (99%) =	0.08	g
MLSS Conc. =	2427	mg/L
ANA 2=	16.29	mg/L
AE 9=	9.046	mg/L

Date	Time (min)	sTOC (mg/L)
3/12/2008	0	20.21
	10	10.76
	15	10.90
	20	10.61
	25	10.27
	30	10.70
	35	11.15
	40	10.24
	60	9.97
	90	9.53
	120	9.41
	150	9.04
	180	9.39

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	76.11	mg/L
Sodium Propionate (99%) =	0.101	g
	0.506	g
MLSS Conc. =	3329	mg/L
Std. =	10.45	mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Average NO_3-N (mg/L)
5/5/2008	0	11	10.8	10.90
	10	9.1	8.6	8.85
	15	8.2	8.2	8.20
	20	6.6	7	6.80
	25	6.2	6.2	6.20
	30	5.2	5.6	5.40
	35	4.4	4.5	4.45
	40	3.7	3.7	3.70
	60	6.4	6.2	6.30
	90	11	11.2	11.10
	120	14.8	15.1	14.95
	150	16.2	15.9	16.05
	180	15.9	16	15.95
	ANA 2	0.4	0.4	0.40
	AE 9	19.1	18	18.55

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 79.84 mg/L
Sodium Propionate (99%) = 0.106 g
0.53 g
MLSS Conc. = 3062 mg/L
Std. = 10.5 mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Average NO_3-N (mg/L)
5/7/2008	0	11.3	11.1	11.20
	10	9.5	9.7	9.60
	15	8.7	8.7	8.70
	20	8.2	7.8	8.00
	25	7	7.3	7.15
	30	6.1	6	6.05
	35	5.6	5.6	5.60
	40	4.7	4.1	4.40
	60	8.1	7.9	8.00
	90	12	11.5	11.75
	120	16	15.7	15.85
	150	16.8	16.9	16.85
	180	16.9	16.8	16.85
	ANA 2	0.5	0.5	0.50
	AE 9	19.8	19.9	19.85

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

OP analysis:
Run 1:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 76.11 mg/L
Sodium Propionate (99%) = 0.101 g
0.506 g
MLSS Conc. = 3329 mg/L
Std. = 0.965 mg/L

Date	Time (min)	PO_4^{3-}-P (mg/L)	Duplicate PO_4^{3-}-P (mg/L)	Dilution factor	Actual PO_4^{3-}-P (mg/L)	Actual Duplicate PO_4^{3-}-P (mg/L)	Mean PO_4^{3-}-P (mg/L)
5/5/2008	0	0.30	0.25	25	7.50	6.25	6.88
	10	0.45	0.44	25	11.25	11	11.13
	15	0.54	0.6	25	13.50	15	14.25
	20	0.71	0.66	25	17.75	16.5	17.13
	25	0.77	0.73	25	19.25	18.25	18.75
	30	0.85	0.9	25	21.25	22.5	21.88
	35	0.96	0.93	25	24.00	23.25	23.63
	40	1.03	1.08	25	25.75	27	26.38
	60	0.87	0.87	25	21.75	21.75	21.75
	90	0.69	0.65	25	17.25	16.25	16.75
	120	0.53	0.52	25	13.25	13	13.13
	150	0.47	0.44	25	11.75	11	11.38
	180	0.44	0.42	25	11.00	10.5	10.75
	ANA 2	0.62	0.7	25	15.50	17.5	16.50
	AE 9	0.01	0.01	25	0.25	0.25	0.25

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

OP analysis:
Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 79.84 mg/L
Sodium Propionate (99%) = 0.106 g
0.53 g
MLSS Conc. = 3062 mg/L
Std. = 0.965 mg/L

Date	Time (min)	PO_4^{3-}-P (mg/L)	Duplicate PO_4^{3-}-P (mg/L)	Dilution factor	Actual PO_4^{3-}-P (mg/L)	Actual Duplicate PO_4^{3-}-P (mg/L)	Mean PO_4^{3-}-P (mg/L)
5/7/2008	0	0.28	0.28	25	7.00	7	7.00
	10	0.46	0.44	25	11.50	11	11.25
	15	0.58	0.54	25	14.50	13.5	14.00
	20	0.65	0.64	25	16.25	16	16.13
	25	0.75	0.76	25	18.75	19	18.88
	30	0.95	0.9	25	23.75	22.5	23.13
	35	0.94	0.93	25	23.50	23.25	23.38
	40	1.00	0.99	25	25.00	24.75	24.88
	60	0.79	0.77	25	19.75	19.25	19.50
	90	0.72	0.7	25	18.00	17.5	17.75
	120	0.57	0.56	25	14.25	14	14.13
	150	0.46	0.47	25	11.50	11.75	11.63
	180	0.39	0.4	25	9.75	10	9.88
	ANA 2	0.60	0.63	25	15.00	15.75	15.38
	AE 9	0.05	0.05	25	1.25	1.25	1.25

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 1:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 76.11 mg/L
Sodium Propionate (99%) = 0.101 g
0.506 g
MLSS Conc. = 3329 mg/L
Std. = 0.248 mg/L

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)	Actual Duplicate NO_2-N (mg/L)	Mean NO_2-N (mg/L)
5/5/2008	0	0.044	0.044	10	0.443	0.443	0.44
	10	0.089	0.088	10	0.887	0.877	0.88
	15	0.112	0.109	10	1.123	1.089	1.11
	20	0.146	0.146	10	1.460	1.464	1.46
	25	0.173	0.173	10	1.732	1.731	1.73
	30	0.201	0.203	10	2.006	2.026	2.02
	35	0.231	0.232	10	2.313	2.322	2.32
	40	0.260	0.260	10	2.604	2.597	2.60
	60	0.304	0.298	10	3.037	2.978	3.01
	90	0.326	0.323	10	3.256	3.229	3.24
	120	0.048	0.048	10	0.481	0.475	0.48
	150	0.003	0.003	10	0.032	0.034	0.03
	180	0.004	0.003	10	0.036	0.032	0.03
	ANA 2	0.003	0.003	10	0.032	0.034	0.03
	AE 9	0.072	0.072	10	0.722	0.723	0.72

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	79.84	mg/L
Sodium Propionate (99%) =	0.106	g
	0.53	g
MLSS Conc. =	3062	mg/L
Std. =	0.2489	mg/L

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)	Actual Duplicate NO_2-N (mg/L)	Mean NO_2-N (mg/L)
5/7/2008	0	0.0323	0.0319	10	0.3230	0.3190	0.32
	10	0.0704	0.0722	10	0.7040	0.7220	0.71
	15	0.0980	0.0973	10	0.9800	0.9730	0.98
	20	0.1197	0.1183	10	1.1970	1.1830	1.19
	25	0.1453	0.1429	10	1.4530	1.4290	1.44
	30	0.1656	0.1644	10	1.6560	1.6440	1.65
	35	0.1933	0.1935	10	1.9330	1.9350	1.93
	40	0.2200	0.2201	10	2.2000	2.2010	2.20
	60	0.2737	0.2737	10	2.7370	2.7370	2.74
	90	0.3036	0.3026	10	3.0360	3.0260	3.03
	120	0.0587	0.0600	10	0.5870	0.6000	0.59
	150	0.0036	0.0042	10	0.0360	0.0420	0.04
	180	0.0030	0.0038	10	0.0300	0.0380	0.03
	ANA 2	0.0039	0.0039	10	0.0390	0.0390	0.04
	AE 9	0.0531	0.0526	10	0.5310	0.5260	0.53

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

sTOC Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	76.11	mg/L	
Sodium Propionate (99%) =	0.101	g	
	0.506	g	(5 times)
MLSS Conc. =	3329	mg/L	
Std. =	2.26	mg/L	(for 2.5 mg/L)

Date	Time (min)	Dilution factor	Reading 1 (mg/L)	Reading 2 (mg/L)	Mean Reading (mg/L)	Actual sTOC (mg/L)
5/5/2008	0	8	10.21	10.08	10.15	81.16
	10	8	9.00	9.15	9.07	72.59
	15	8	8.56	8.56	8.56	68.50
	20	8	8.00	7.86	7.93	63.44
	25	8	7.28	7.34	7.31	58.49
	30	8	6.65	6.72	6.68	53.44
	35	5	9.90	9.82	9.86	49.29
	40	5	9.27	9.21	9.24	46.19
	60	5	5.11	5.04	5.07	25.36
	90	5	1.60	1.60	1.60	8.01
	120	5	1.49	1.50	1.50	7.48
	150	5	1.73	1.69	1.71	8.57
	180	5	1.59	1.52	1.55	7.77
	ANA2	5	3.06	3.07	3.06	15.31
	AE9	5	1.45	1.40	1.43	7.14

Denitrification using wastewater and propionate (5 X) under the anoxic and aerobic condition

sTOC Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	79.84	mg/L	
Sodium Propionate (99%) =	0.106	g	
	0.53	g	(5 times)
MLSS Conc. =	3062	mg/L	
Std. =	4.8	mg/L	(for 5 mg/L)

Date	Time (min)	Dilution factor	Reading 1 (mg/L)	Reading 2 (mg/L)	Mean Reading (mg/L)	Actual sTOC (mg/L)
5/7/2008	0	10	8.82	8.78	8.80	87.98
	10	10	8.06	7.93	7.99	79.91
	15	10	7.20	7.31	7.26	72.56
	20	10	6.82	6.85	6.84	68.37
	25	10	6.37	6.47	6.42	64.24
	30	10	6.16	5.95	6.05	60.52
	35	10	5.61	5.60	5.60	56.05
	40	10	5.14	5.19	5.17	51.67
	60	5	6.32	6.38	6.35	31.76
	90	5	2.46	2.47	2.46	12.31
	120	5	2.03	2.04	2.03	10.16
	150	5	1.89	2.02	1.95	9.76
	180	5	1.83	1.83	1.83	9.16
	ANA2	5	3.19	3.18	3.19	15.93
	AE9	5	1.69	1.64	1.67	8.33

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 1:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 236.9 mg/L
Sodium Propionate (99%) = 0.083 g
0.833 g (10X)
MLSS Conc. = 2450 mg/L
Std. = 10.1 mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Average NO_3-N (mg/L)
2/10/2009	0	9	9.1	9.05
	10	8		8.00
	15	7.3		7.30
	20	6.6		6.60
	25	6.5		6.50
	30	6.3	6.1	6.20
	35	6.2		6.20
	40	5.6		5.60
	60	7.1		7.10
	90	9.8		9.80
	120	12.7	12.7	12.70
	150	15.4		15.40
	180	15.8		15.80
	ANA 2	0.6		0.60
	AE 9	15.8		15.80

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NO_3-N Analysis:
Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 236.9 mg/L
Sodium Propionate (99%) = 0.0833 g
0.8326 g (10X)
MLSS Conc. = 2403 mg/L
Std. = 10.5 mg/L

Date	Time (min)	NO_3-N (mg/L)	Duplicate NO_3-N (mg/L)	Average NO_3-N (mg/L)
2/11/2009	0	8.4	8.1	8.25
	10	7.3		7.30
	15	6.7		6.70
	20	6		6.00
	25	5.6		5.60
	30	5	4.9	4.95
	35	4.3		4.30
	40	3.7		3.70
	60	6		6.00
	90	9.5		9.50
	120	12.7	12.5	12.60
	150	15.1		15.10
	180	15.5		15.50
	ANA 2	0.8		0.80
	AE 9	14.8		14.80

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

OP analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.083	g	
	0.833	g	(10X)
MLSS Conc. =	2450	mg/L	
Std. =	0.99	mg/L	(for 1 mg P/L)

Date	Time (min)	PO_4^{3-}-P (mg/L)	Duplicate PO_4^{3-}-P (mg/L)	Dilution factor	Actual PO_4^{3-}-P (mg/L)	Actual Duplicate PO_4^{3-}-P (mg/L)	Mean PO_4^{3-}-P (mg/L)
2/10/2009	0	0.11	0.13	25	2.75	3.25	3.00
	10	0.23		25	5.75		5.75
	15	0.29		25	7.25		7.25
	20	0.35		25	8.75		8.75
	25	0.40		25	10.00		10.00
	30	0.47	0.47	25	11.75	11.75	11.75
	35	0.52		25	13.00		13.00
	40	0.57		25	14.25		14.25
	60	0.59		25	14.75		14.75
	90	0.60		25	15.00		15.00
	120	0.65	0.64	25	16.25	16	16.13
	150	0.67		25	16.75		16.75
	180	0.68		25	17.00		17.00
	ANA 2	0.41		25	10.25		10.25
	AE 9	0.00		25	0.00		0.00

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

OP analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.0833	g	
	0.8326	g	(10X)
MLSS Conc. =	2403	mg/L	
Std. =	0.99	mg/L	(for 1 mg P/L)

Date	Time (min)	PO_4^{3-}-P (mg/L)	Duplicate PO_4^{3-}-P (mg/L)	Dilution factor	Actual PO_4^{3-}-P (mg/L)	Actual Duplicate PO_4^{3-}-P (mg/L)	Mean PO_4^{3-}-P (mg/L)
2/11/2009	0	0.20	0.19	25	5.00	4.75	4.88
	10	0.31		25	7.75		7.75
	15	0.37		25	9.25		9.25
	20	0.43		25	10.75		10.75
	25	0.50		25	12.50		12.50
	30	0.56	0.54	25	14.00	13.5	13.75
	35	0.59		25	14.75		14.75
	40	0.65		25	16.13		16.13
	60	0.66		25	16.38		16.38
	90	0.65		25	16.25		16.25
	120	0.70	0.69	25	17.50	17.25	17.38
	150	0.71		25	17.75		17.75
	180	0.76		25	19.00		19.00
	ANA 2	0.35	0.39	25	8.75		8.75
	AE 9	1.49	1.52	2	2.98		2.98

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.083	g	
	0.833	g	(10X)
MLSS Conc. =	2450	mg/L	
Std. =	0.248	mg/L	

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)	Actual Duplicate NO_2-N (mg/L)	Mean NO_2-N (mg/L)
2/10/2009	0	0.017	0.017	10	0.170	0.171	0.17
	10	0.045		10	0.445		0.45
	15	0.062		10	0.622		0.62
	20	0.080		10	0.796		0.80
	25	0.095		10	0.952		0.95
	30	0.115	0.114	10	1.151	1.141	1.15
	35	0.131		10	1.309		1.31
	40	0.150		10	1.503		1.50
	60	0.147		10	1.467		1.47
	90	0.182		10	1.824		1.82
	120	0.198	0.199	10	1.980	1.987	1.98
	150	0.039		10	0.388		0.39
	180	0.003		10	0.031		0.03
	ANA 2	0.004		10	0.042		0.04
	AE 9	0.007		10	0.073		0.07

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NO_2-N Analysis:
Run 2:
Aeration starts at 40 min
ANA2 = 1146 ml
AE 9= 1500 ml
Propionate = 236.9 mg/L
Sodium Propionate (99%) = 0.0833 g
0.8326 g (10X)
MLSS Conc. = 2403 mg/L
Std. = 0.2434 mg/L

Date	Time (min)	NO_2-N (mg/L)	Duplicate NO_2-N (mg/L)	Dilution factor	Actual NO_2-N (mg/L)	Actual Duplicate NO_2-N (mg/L)	Mean NO_2-N (mg/L)
2/11/2009	0	0.049	0.049	10	0.488	0.492	0.49
	10	0.080		10	0.804		0.80
	15	0.101		10	1.010		1.01
	20	0.119		10	1.186		1.19
	25	0.139		10	1.385		1.39
	30	0.160	0.159	10	1.596	1.591	1.59
	35	0.178		10	1.783		1.78
	40	0.190		10	1.896		1.90
	60	0.193		10	1.926		1.93
	90	0.223		10	2.233		2.23
	120	0.207	0.206	10	2.068	2.060	2.06
	150	0.017		10	0.174		0.17
	180	0.003		10	0.031		0.03
	ANA 2	0.003		10	0.033		0.03
	AE 9	0.080		10	0.804		0.80

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 1:

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.083	g	
	0.833	g	(10X)
MLSS Conc. =	2450	mg/L	
ANA 2=	23	mg/L	
AE 9=	0	mg/L as N	
Std. =	10.1	mg/L	

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
2/10/2009	0	10.00	9.3	9.65
	10	9.50		9.50
	15	9.90		9.90
	20	9.30		9.30
	25	9.30		9.30
	30	9.4	9.2	9.3
	35	9.70		9.70
	40	9.70		9.70
	60	7.20		7.20
	90	3.70		3.70
	120	0.73	0.731	0.7305
	150	0.00		0.00
	180	0.00		0.00

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

NH_3-N Analysis:
Run 2:

ANA2 =	1146	ml
AE 9=	1500	ml
Propionate =	236.9	mg/L
Sodium Propionate (99%) =	0.0833	g
	0.8326	g (10X)
MLSS Conc. =	2403	mg/L
ANA 2=	21.9	mg/L
AE 9=	0.445	mg/L as N
Std. =	9.4	mg/L

Date	Time (min)	NH_3-N (mg/L)	Duplicate NH_3-N (mg/L)	Mean NH_3-N (mg/L)
2/11/2009	0	9.20	9.3	9.25
	10	9.10		9.10
	15	9.10		9.10
	20	9.20		9.20
	25	9.20		9.20
	30	9.6	9.4	9.5
	35	9.50		9.50
	40	9.60		9.60
	60	7.30		7.30
	90	3.50		3.50
	120	0.29	0.281	0.2855
	150	0.00		0.00
	180	0.00		0.00

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

sTOC Analysis:
Run 1:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.083	g	
	0.833	g	(10X)
MLSS Conc. =	2450	mg/L	

Date	Time (min)	Dilution factor	Reading 1 (mg/L)	Reading 2 (mg/L)	Mean Reading (mg/L)	Actual sTOC (mg/L)
2/10/2009	0	20	7.00	6.83	6.91	138.29
	10	20	6.78		6.78	135.54
	15	20	6.70		6.70	134.04
	20	20	6.62		6.62	132.46
	25	20	6.57		6.57	131.36
	30	20	6.47	6.19	6.33	126.58
	35	20	6.35		6.35	126.92
	40	20	6.30		6.30	126.00
	60	20	5.98		5.98	119.52
	90	20	5.51		5.51	110.18
	120	20	5.03	4.90	4.96	99.23
	150	20	4.67		4.67	93.30
	180	20	4.39		4.39	87.78
	ANA2	4	3.58		3.58	14.33
	AE9	4	1.82		1.82	7.28

Denitrification using wastewater and propionate (10 X) under the anoxic and aerobic condition

sTOC Analysis:
Run 2:
Aeration starts at 40 min

ANA2 =	1146	ml	
AE 9=	1500	ml	
Propionate =	236.9	mg/L	
Sodium Propionate (99%) =	0.0833	g	
	0.8326	g	(10X)
MLSS Conc. =	2450	mg/L	

Date	Time (min)	Dilution factor	Reading 1 (mg/L)	Reading 2 (mg/L)	Mean Reading (mg/L)	Actual sTOC (mg/L)
2/11/2009	0	20	6.76	6.63	6.69	133.85
	10	20	6.83		6.83	136.54
	15	20	6.64		6.64	132.70
	20	20	6.34		6.34	126.84
	25	20	6.40		6.40	127.96
	30	20	6.34	6.13	6.23	124.68
	35	20	6.29		6.29	125.78
	40	20	6.16		6.16	123.16
	60	20	5.88		5.88	117.52
	90	20	5.39		5.39	107.74
	120	20	4.91	4.69	4.80	95.90
	150	20	4.62		4.62	92.38
	180	20	4.17		4.17	83.36
	ANA2	4	3.69		3.69	14.75
	AE9	4	1.94		1.94	7.74

APPENDIX III

CALCULATION OF % P REMOVAL AND P-RELEASE/C-UPTAKE IN GLUCOSE AND BUTYRATE FED REACTORS

Calculation of % P removal in G1 reactor

Date	Day	Influent OP Conc. (mg/L)	Influent OP flow (L)	OP -In (mg)	P_{bf} (mg/L)	mixed liquor before feed (L)	OP amount before feed (mg)	Total initial OP (mg)	P_{in} (mg/L)	P_{out} (mg/L)	% P removal (mg/L)
10/15/2008	0	395	0.2	79.00	-	-	-	79.00	9.88	0.5	94.94
10/21/2008	7	390	0.2	78.00	0.5	4	2	80.00	10.00	4.5	55.00
10/28/2008	14	372.5	0.2	74.50	4.5	4	18	92.50	11.56	19	-64.32
11/4/2008	21	440	0.2	88.00	19	4	76	164.00	20.50	18	12.20
11/7/2008	24	410	0.2	82.00	18	4	72	154.00	19.25	15.8	17.92
11/18/2008	35	395	0.2	79.00	16.3	4	65.2	144.20	18.03	20	-10.96
11/25/2008	42	420	0.2	84.00	20	4	80	164.00	20.50	20	2.44
12/4/2008	51	417.5	0.2	83.50	20	4	80	163.50	20.44	19.8	3.12
12/9/2008	56	432.5	0.2	86.50	19.8	4	79.2	165.70	20.71	19.5	5.85
12/16/2008	63	397.5	0.2	79.50	19.5	4	78	157.50	19.69	18.5	6.03
12/23/2008	70	605	0.2	121.00	18.5	4	74	195.00	24.38	31	-27.18
12/29/2008	76	630	0.2	126.00	31	4	124	250.00	31.25	30	4.00
12/31/2008	78	630	0.2	126.00	30	4	120	246.00	30.75	29	5.69

Note: P_{bf} = OP in the reactor before feed

Calculation of % P removal in G2 reactor

Date	Day	Influent OP Conc. (mg/L)	Influent OP flow (L)	OP -In (mg)	P_{bf} (mg/L)	mixed liquor before feed (L)	OP amount before feed (mg)	Total initial OP (mg)	P_{in} (mg/L)	P_{out} (mg/L)	% P removal (mg/L)
10/15/2008	0	395	0.2	79.00	-	-	-	79.00	9.88	0.8	91.90
10/21/2008	7	390	0.2	78.00	0.8	4	3.2	81.20	10.15	3.5	65.52
10/28/2008	14	372.5	0.2	74.50	3.5	4	14	88.50	11.06	16	-44.63
11/4/2008	21	440	0.2	88.00	16	4	64	152.00	19.00	16	15.79
11/7/2008	24	410	0.2	82.00	16	4	64	146.00	18.25	17	6.85
11/18/2008	35	395	0.2	79.00	20.3	4	81.2	160.20	20.03	17.5	12.61
11/25/2008	42	420	0.2	84.00	17.5	4	70	154.00	19.25	17.5	9.09
12/4/2008	51	417.5	0.2	83.50	17.5	4	70	153.50	19.19	18.5	3.58
12/9/2008	56	432.5	0.2	86.50	18.5	4	74	160.50	20.06	19.5	2.80
12/16/2008	63	397.5	0.2	79.50	19.5	4	78	157.50	19.69	18	8.57
12/23/2008	70	605	0.2	121.00	18	4	72	193.00	24.13	30	-24.35
12/29/2008	76	630	0.2	126.00	30	4	120	246.00	30.75	29.5	4.07
12/31/2008	78	630	0.2	126.00	29.5	4	118	244.00	30.50	28.5	6.56

Calculation of % P removal in B1 reactor

Date	Day	Influent OP Conc. (mg/L)	Influent OP flow (L)	OP -In (mg)	P_{bf} (mg/L)	SWW in the reactor (L)	OP amount before feed (mg)	Total initial OP (mg)	Influent OP Conc. (mg/L)	P_{out} (mg/L)	% P removal (mg/L)
7/11/2008	1	550	0.2	110.00				110.00	13.75	4.5	67.27
7/22/2008	12	570	0.2	114.00	5	4	20	134.00	16.75	6	64.18
7/25/2008	15	570	0.2	114.00	6	4	24	138.00	17.25	3	82.61
7/27/2008	17	537.5	0.2	107.50	3	4	12	119.50	14.94	3	79.92
8/1/2008	22	550	0.2	110.00	4.5	4	18	128.00	16.00	4	75.00
8/3/2008	24	550	0.2	110.00	4	4	16	126.00	15.75	4.5	71.43
8/12/2008	33	537.5	0.2	107.50	4	4	16	123.50	15.44	3.5	77.33
8/19/2008	40	597.5	0.2	119.50	4.5	4	18	137.50	17.19	4.5	73.82
8/26/2008	47	570	0.2	114.00	4.3	4	17.2	131.20	16.40	16	2.44
8/29/2008	50	545	0.2	109.00	16	4	64	173.00	21.63	5	76.88
9/5/2008	57	597.5	0.2	119.50	12	4	48	167.50	20.94	16	23.58
9/12/2008	64	560	0.2	112.00	7.5	4	30	142.00	17.75	5.5	69.01
9/16/2008	68	570	0.2	114.00	5.5	4	22	136.00	17.00	8.5	50.00
9/30/2008	82	560	0.2	112.00	10	4	40	152.00	19.00	14.5	23.68
10/7/2008	89	562.5	0.2	112.50	14.5	4	58	170.50	21.31	10	53.08
10/10/2008	92	550	0.2	110.00	10	4	40	150.00	18.75	6.5	65.33

Calculation of % P removal in B2 reactor

Date	Day	Influent OP Conc. (mg/L)	Influent OP flow (L)	OP -In (mg)	P_{bf} (mg/L)	SWW in the reactor (L)	OP amount before feed (mg)	Total initial OP (mg)	Influent OP Conc. (mg/L)	P_{out} (mg/L)	% P removal (mg/L)
7/11/2008	1	550	0.2	110.00				110.00	13.75	2.5	81.82
7/22/2008	12	570	0.2	114.00	8	4	32	146.00	18.25	9.5	47.95
7/25/2008	15	570	0.2	114.00	9.5	4	38	152.00	19.00	10	47.37
7/27/2008	17	537.5	0.2	107.50	10	4	40	147.50	18.44	7.5	59.32
8/1/2008	22	550	0.2	110.00	8.5	4	34	144.00	18.00	8	55.56
8/3/2008	24	550	0.2	110.00	8	4	32	142.00	17.75	7.5	57.75
8/12/2008	33	537.5	0.2	107.50	5.5	4	22	129.50	16.19	5.5	66.02
8/19/2008	40	597.5	0.2	119.50	7.5	4	30	149.50	18.69	6.5	65.22
8/26/2008	47	570	0.2	114.00	8.5	4	34	148.00	18.50	11	40.54
8/29/2008	50	545	0.2	109.00	11	4	44	153.00	19.13	13.8	27.84
9/5/2008	57	597.5	0.2	119.50	12.8	4	51.2	170.70	21.34	17.3	18.92
9/12/2008	64	560	0.2	112.00	12	4	48	160.00	20.00	6.8	66.00
9/16/2008	68	570	0.2	114.00	6.8	4	27.2	141.20	17.65	15	15.01
9/30/2008	82	560	0.2	112.00	18.3	4	73.2	185.20	23.15	19	17.93
10/7/2008	89	562.5	0.2	112.50	19	4	76	188.50	23.56	18	23.61
10/10/2008	92	550	0.2	110.00	18	4	72	182.00	22.75	12.5	45.05

Calculation of carbon uptake in G1 reactor

Sample ID	Time (min)	10/15	10/28	11/4	11/7	11/12	11/18	11/25	12/4	12/9	12/16	12/23	12/29	12/31
B1-9:30	0	48.48	62.82	66.64	62.82	85.92	67.22	72.14	80.68	71.46	78.78	320.28	332.6	351.24
B1-10:00	30	45.48	15.38	19.14	19.5	81.82	32.28	45.84	65.02	56.02	66.8	311.8	334.08	345.92
B1-10:30	60	41.62	7.5	11.88	8.46	81.26	22.28	38.16	55.84	47.78	59.52	315.76	331.28	352.08
B1-11:00	90	39.82	7.44	9.9	6.62	78.2	17.84		50.78	51.1	55.94	316.64	330	343.04
B1-11:30	120	38.38	6.96	9.28	6.82	74.52	13.3	38.22	52.75	51.34	55.6	308.12	331.92	340.76
B1-11:55	145	37.54	9.36	10.53	7.4	71.34	11.81	37.28	51.93	48.95	57.88	301.5	322.7	346.16
B1-12:30	180	33.92	6.5	10.6	7.86		5.91	7.64	16.3	16.78	33.48	289	313.24	330.76
B1-1:00	210	31.12	5.38	11.3	7.2	31.12	6.06	7.14	13.56	6.86	9.24	276.64	293.96	320.48
B1-2:00	270	25.24	5.62	10.2	7.08	9.676	6.38	6.86	9.14	6.98	10.02	246.68	271.88	312.24
B1-3:00	330	18.22	5.26	9.34	5.42	7.68	6.54	7.9		7.16	8.26	239.32	258.68	298.2
B1-4:25	415	14.83	7.15	8.04	5.73	6.805	5.33	7.75	10.24	8.34	6.95	210.9	242.92	292.32
B1-4:55	445	14.32	5.48	6.3	6.3		5.52	5.86	7.32	7.04	7.8	213.72	243.76	283.24
C-uptake (mg/L)		10.94	53.46	56.11	55.42	14.58	55.41	34.86	28.75	22.51	20.9	18.78	9.9	5.08
C-uptake (mol/L)		0.00091	0.00446	0.00468	0.0046	0.00122	0.00462	0.00291	0.0024	0.00188	0.0017	0.00157	0.00083	0.0004

Calculation of P-release/ C-uptake ratio in G1 reactor

Sample ID	Time (min)	10/15	10/28	11/4	11/7	11/12	11/18	11/25	12/4	12/9	12/16	12/23	12/29	12/31
		1	14	21	24	29	35	42	51	56	63	70	76	78
B1-9:30	0	12.5	19	21	20.75	21	21.25	21	20.25	21	19	30	29.5	29
B1-10:00	30	10	34	25	23	21	21	21	20.5	21	18.5	30	29.5	29.5
B1-10:30	60	11	41.5	28	25	20.5	21.5	22	20	21	20	29.5	30.5	29.5
B1-11:00	90	12.5	44	28	26	20.5	21.5		20.5	21	19	29.5	30.5	30.5
B1-11:30	120	13.5	49	30	27	21	21.5	21.5	20.5	21.5	20	31	30	30.5
B1-11:55	145	14.25	49.5	30.5	25.75	21.25	20.75	22.5	21	21.25	20	30	30	30.25
B1-12:30	180	10.5	37.5	25.5	22.5		20.5	21	19.5	20.5	19	30.5	30	30
B1-1:00	210	7.5	30	25.5	20.5	20.5	20.5	20	20	21.5	19	30.5	30.5	30.5
B1-2:00	270	4	27	22	19.5	19	20.5	20.5	20	20.5	19	31.5	30	29.5
B1-3:00	330	0.5	24	21	17.5	18.5	22	20.5		20.5	19	30	29	29
B1-4:25	415	0	20	18.25	15.75	16.25	20.75	20.5	19.75	20.25	18.75	30.5	30	29.25
B1-4:55	445	0.5	19	18	15.75		20	20	19.75	19.5	18.5	31	30	29
P-release (mg/L)		1.75	30.5	9.5	5	0.25	0.25	1.5	0.75	0.5	1	1	1	1.5
P-release (mol/L)		0.00006	0.00098	0.00031	0.00016	0.00001	0.00001	0.00005	0.00002	0.00002	0.00003	0.00003	0.00003	0.00005
C-uptake (mol/L)		0.000912	0.00446	0.00468	0.00462	0.00122	0.0046	0.00291	0.0024	0.00188	0.0017	0.00157	0.000825	0.00042
P-release/C-uptake (mol P/mol C)		0.06	0.22	0.07	0.03	0.01	0.00	0.02	0.01	0.01	0.02	0.02	0.04	0.11

Calculation of carbon uptake in G2 reactor

Sample ID	Time (min)	10/15	10/28	11/4	11/7	11/12	11/18	11/25	12/4	12/9	12/16	12/23	12/29	12/31
B1-9:30	0	50.2	60.16	62.38	60.86	76.54	62.78	68.4	86.96	66.88	83.8	304.36	316.92	344.88
B1-10:00	30	49.9	16.86	18.36	19.72	69.08	31.02	46.34	59.9	55.94	76.24	301.84	315.76	345.96
B1-10:30	60	43.82	7.22	9.36	10.64	65.1		33.1	48.08	48.90	60.94	307.72	315.52	345.2
B1-11:00	90	45.22	8.38	8.8	7.08	61.32	15.88		44.24	46.44	53.52	306.72	310.96	347.8
B1-11:30	120	41.6	6.66	11.28	8.7	57.96	13.42	30.6	45.82	46.74	54.14	301.04	313.16	340.68
B1-11:55	145	41.44	13.24	8.35	6.89	54.74	10.68	33.99	45.45	48.33	55.67	298.26	308	336.34
B1-12:30	180	37.4	7.3	8.68	6.66		5.54	10.08	27.56	10.08	30.72	260.52	305.44	329.84
B1-1:00	210	33.76	6.22	10.34	6.18	16.74	7.52	9.84	9.24	12.72	10.9	260.4	287.72	324.64
B1-2:00	270	27.32	6.5	10	7.14	5.58	7.02	9.44	8.84	8.50	8.9	237.92	270.4	311.08
B1-3:00	330	19.46	5.22	9.58	7.28	5.64	5.92	7.92		9.76	6.92	223.12	258.8	302.48
B1-4:25	415	18.94	5.71	9.83	7.63	5.37	5	7.71	8.8	8.59	10.44	205.54	245.34	286.24
B1-4:55	445	15.02	5.3	7.7	6.14		5.56		7.39	8.98	7.24	203.72	241.92	286.52
C-uptake (mg/L)		8.76	46.92	54.03	53.97	21.8	52.1	34.41	41.51	18.55	28.13	6.1	8.92	8.54
C-uptake (mol/L)		0.00073	0.00391	0.0045	0.0045	0.00182	0.00434	0.00287	0.00346	0.00155	0.0023	0.00051	0.00074	0.0007

Calculation of P-release/ C-uptake ratio in G2 reactor

Sample ID	Time (min)	10/15	10/28	11/4	11/7	11/12	11/18	11/25	12/4	12/9	12/16	12/23	12/29	12/31
		1	14	21	24	29	35	42	51	56	63	70	76	78
B1-9:30	0	12.5	16.75	20.75	19.75	17.5	19	21.5	19.5	21	19	30.75	31	28.5
B1-10:00	30	10	32	24.5	21.5	19	19.5	21.5	20	20.5	16.5	30.5	30.5	28.5
B1-10:30	60	11.5	40	27.5	25	22.5	19.5	20.5	20	20.5	19.5	30.5	31.5	30
B1-11:00	90	12.5	41	27.5	25	23.5	20.5		20.5	21	18.5	30	31	30.5
B1-11:30	120	14	43.5	27.5	24	25	19	21.5	20	20.5	21	30.5	31.5	29.5
B1-11:55	145	14.75	45.25	28.25	25.5	24.75	19.5	20.5	20	21	19	30.5	30	28.75
B1-12:30	180	11.5	35	24.5	22.5		19	21	20.5	20	18	32	30	30
B1-1:00	210	8	28.5	24	20.5	24	19	22	20.5	19.5	18.5	32	31.5	28
B1-2:00	270	4	24.5	23	19.5	24.5	17.5	18.5	20	19	18	30	30.5	29
B1-3:00	330	1.5	22.5	18.5	18	24	18	19.5		20	18.5	30.5	29.5	28.5
B1-4:25	415	0.75	18.75	16.75	17.75	20.25	17.5	18	18.5	20.25	18	30.75	29.9	29
B1-4:55	445	0.75	16	16	17		17.5	17.5	18.5	19.5	18	30	29.5	28.5
P-release (mg/L)		2.25	28.5	7.5	5.75	7.5	1.5	0	0	0	2	1.25	0.5	2
P-release (mol/L)		0.0001	0.0009	0.0002	0.0002	0.0002	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0001
C-uptake (mol/L)		0.00073	0.00391	0.0045	0.0045	0.00182	0.0043	0.00287	0.00346	0.0015	0.0023	0.00051	0.0007	0.00071
P-release/C-uptake (mol P/mol C)		0.10	0.24	0.05	0.04	0.13	0.01	0.00	0.00	0.00	0.03	0.08	0.02	0.09

Weekly OP profiles in B1 reactor

Time (hr)	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13
0.0	7.77	12.84	10.94	8.99	10.89	8.21	10.06	12.99	12.78	8.83	12.82	10.25	9.33
0.5	9.23	15.64	16.31	17.35	16.36	15.64	14.60	20.27	19.43	13.65	17.82	14.20	15.70
1.0	11.99	19.52	21.06	20.73	21.24	18.76	18.47	23.96	23.21	17.07	20.52	17.38	18.86
1.5	14.05	23.34	26.02	25.71	24.93	22.09	21.82	29.04	26.54	20.94	22.68	21.10	22.30
2.0	15.96	26.65	30.24	30.60	29.30	25.22	24.96	31.50	30.37	25.26	25.38	23.27	24.82
2.4	17.36	29.28	33.57	32.82	31.87	28.15	27.96	32.80	33.38	27.22	27.81	25.82	27.30
3.0	15.31	22.62	22.91	23.32	21.50	18.76	19.64	26.68	23.10	20.11	22.95	20.66	20.11
3.5	11.75	15.00	13.62	12.95	10.95	12.12	13.08	19.65	15.35	13.84	17.55	12.59	15.03
4.5	6.57	6.68	4.93	4.79	4.37	6.25	8.72	10.88	7.81	7.60	12.42	9.72	8.55
5.5	3.47	4.68	2.72	2.79	2.47	3.91	5.78	7.46	6.04	4.25	8.91	6.78	5.55
6.9	1.82	3.80	1.75	1.86	1.76	1.76	4.80	5.21	5.45	2.81	6.07	4.90	3.24
7.4	1.79	3.68	1.66	1.75	1.64	1.76	4.80	4.94	5.45	2.81	6.07	4.81	3.10

Weekly OP profiles in B2 reactor

Time (hr)	WeeK 1	WeeK 2	WeeK 3	WeeK 4	WeeK 5	WeeK 6	WeeK 7	WeeK 8	WeeK 9	WeeK 10	WeeK 11	WeeK 12	WeeK 13
0.0	6.26	12.93	13.52	10.85	11.04	9.37	9.94	13.11	14.49	10.07	13.86	10.24	9.14
0.5	7.81	16.87	19.53	18.68	16.71	16.39	14.90	19.11	21.54	15.47	17.15	15.27	13.96
1.0	10.92	20.54	24.19	23.14	20.71	19.81	18.43	22.82	25.36	19.69	19.97	17.24	15.60
1.5	13.36	24.34	28.66	28.14	24.72	23.03	22.01	26.29	28.74	24.42	23.26	20.29	18.22
2.0	15.32	27.09	32.26	31.59	27.14	27.03	25.03	28.84	32.53	27.65	25.38	21.73	18.88
2.4	17.26	30.31	35.55	33.93	30.56	29.28	27.22	30.46	34.23	28.64	25.38	22.27	19.52
3.0	12.96	26.40	28.60	22.46	22.18	24.01	20.60	28.61	23.70	21.83	22.56	18.14	15.41
3.5	9.43	18.36	18.21	14.46	11.24	14.93	14.21	24.34	18.11	16.33	15.51	12.57	11.87
4.5	5.12	9.53	8.79	6.58	5.59	7.02	9.49	14.86	10.49	10.01	13.86	11.31	8.47
5.5	2.68	6.65	6.11	4.90	3.86	4.39	6.68	10.46	8.21	6.90	10.57	9.70	6.75
6.9	1.24	4.94	4.37	3.57	2.30	2.83	3.61	6.12	6.89	4.65	8.58	7.00	4.99
7.4	1.10	3.60	4.37	3.45	1.40	2.73	3.61	6.12	6.89	4.60	8.58	6.82	4.94

Calculation of P-release/C-uptake ratio in B1 reactor

Parameters	Weeks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
OP release (mg P/g SS)	9.59	16.44	22.63	23.83	20.98	19.94	17.90	19.80	20.60	18.39	14.98	15.57	17.97
OP release (mol P/g SS)	0.0003	0.0005	0.0007	0.0008	0.0007	0.0006	0.0006	0.0006	0.0007	0.0006	0.0005	0.0005	0.0006
sTOC uptake (mg/ gSS)				15.21	16.78	21.85	19.04	18.27	14.25	14.67	18.30	17.34	21.43
sTOC uptake (mol/ gSS)				0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.002	0.001	0.002
P -release/C-uptake (mol P/mol C)				0.61	0.48	0.35	0.36	0.42	0.56	0.49	0.32	0.35	0.32

Calculation of P-release/C-uptake ratio in B2 reactor

Parameters	Weeks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
OP release (mg P/g SS)	11.00	17.38	22.02	23.08	19.52	19.91	17.29	17.36	19.73	18.57	11.51	12.03	10.37
sTOC uptake (mg/ gSS)				11.73	11.67	14.89	15.87	14.49	16.33	17.00	21.05	15.80	16.93
OP release (mol P/g SS)	0.0004	0.0006	0.00071	0.0007	0.0006	0.0006	0.0006	6E-04	0.0006	0.0006	0.0004	0.0004	0.00033
sTOC uptake (mol C/ gSS)				0.001	0.001	0.0012	0.0013	0.001	0.0014	0.0014	0.0018	0.0013	0.00141
OP -release/C-uptake (mol P/mol C)				0.76	0.65	0.52	0.42	0.46	0.47	0.42	0.21	0.29	0.24

APPENDIX IV

STATISTICAL ANALYSIS OF THE % RA OF PAO and GAO IN GLUCOSE AND BUTYRATE FED REACTORS

Statistical analysis of the % RA of PAOs and GAOs in glucose fed reactors:

Table 1 – Comparison of the overall % RA of PAOs and GAOs between G1 and G2 reactors

Name of microbe	Reactors	Mean (μ_1, μ_2)	P value (95% CI)
PAOMIX	G1 and G2	9.38, 9.13	0.971
GAOMIX	G1 and G2	15.9, 17	0.886
MIC 184	G1 and G2	5.81, 6.29	0.861
MP2	G1 and G2	6.39, 4.83	0.633

Table 2 - Comparison of two wells for GAOMIX in G1and G2 reactors at different dates

Date	G1 reactor		G2 reactor	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	10.2, 9.58	0.767	9.63, 9.07	0.704
11-18-08	40.73, 43.69	0.377	32.14, 41.45	0.068
12-16-08	9.36, 9.08	0.889	25.17, 28.60	0.574
12-16-08-D	10.74, 13.51	0.482	-	-
12-23-08	7.11, 10.09	0.155	11.92, 9.62	0.257
12-29-08	15.51, 11.75	0.202	13.69, 16.94	0.368
12-31-08	8.75, 11.55	0.197	3.78, 2.63	0.101

Table 3 - Comparison of two wells for PAOMIX in G1and G2 reactors at different dates

Date	G1 reactor		G2 reactor	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	13.60, 12.24	0.469	12.45, 10.81	0.489
10-28-08	44.12, 37.37	0.296	43.53, 39.68	0.614
11-18-08	7.89, 6.82	0.618	9.51, 7.97	0.543
12-4-08	5.14, 4.48	0.595	2.66, 2.94	0.771
12-16-08	0.913, 1.21	0.429	1.61, 1.21	0.379
12-23-08	1.05, 0.955	0.804	1.09, 1.76	0.277
12-29-08	3.07, 3.79	0.593	2.76, 1.92	0.214
12-31-08	3.97, 3.25	0.491	2.50, 2.69	0.805

Table 4 - Comparison of two wells for MIC-184 in G1and G2 reactors at different dates

Date	G1 reactor		G2 reactor	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	3.40, 1.77	0.03	2.45, 1.42	0.276
10-28-08	5.58, 5.51	0.977	5.48, 4.71	0.526
11-18-08	9.83, 8.73	0.683	13.39, 12.53	0.805
12-4-08	15.24, 18.57	0.322	13.03, 16.40	0.282
12-16-08	4.96, 4.00	0.481	8.16, 11.49	0.246
12-16-08-D	8.27, 8.66	0.863	-	-
12-23-08	3.04, 1.72	0.141	1.94, 3.45	0.07
12-29-08	2.15, 1.70	0.490	1.51, 2.09	0.423
12-31-08	1.05, 2.34	0.097	1.46, 1.23	0.683

Table 5 - Comparison of two wells for MP-2 in G1and G2 reactors at different dates

Date	G1 reactor		G2 reactor	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	2.55, 3.04	0.555	2.95, 4.68	0.257
11-18-08	10.30, 6.25	0.012	8.10, 8.01	0.976
12-4-08	18.85, 23.90	0.255	11.53, 13.66	0.392
12-16-08	7.86, 6.29	0.440	6.33, 6.56	0.899
12-16-08-D	6.47, 6.21	0.874	-	-
12-23-08	1.47, 2.90	0.076	1.03, 1.26	0.478
12-29-08	0.79, 1.09	0.311	1.21, 1.14	0.852
12-31-08	2.46, 2.16	0.507	0.496, 0.749	0.109

Table 6 - Comparison of PAOMIX and MP-2 in G1and G2 reactors at different dates

Date	PAOMIX of G1 and G2		MP2 of G1 and G2	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	12.92, 11.63	0.385	2.80, 3.81	0.244
10-28-08	40.75, 41.6	0.860	-	-
11-18-08	7.36, 8.74	0.393	8.27, 8.06	0.898
12-4-08	4.8, 2.80	0.011	21.37, 12.60	0.001
12-16-08	1.06, 1.41	0.227	6.71, 6.44	0.806
12-23-08	1.0, 1.43	0.233	2.18, 1.14	0.021
12-29-08	3.43, 2.34	0.142	0.94, 1.18	0.323
12-31-08	3.61, 2.59	0.117	2.4, 0.62	0.000

Table 7 - Comparison of GAOMIX and MIG-184 in G1and G2 reactors at different dates

Date	GAOMIX of G1 and G2		MIG-184 of G1 and G2	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
10-15-08	9.89, 9.35	0.665	2.59, 1.94	0.291
10-28-08	-	-	5.55, 5.10	0.722
11-18-08	42.21, 36.79	0.083	9.28, 12.96	0.092
12-4-08	-	-	16.91, 14.72	0.335
12-16-08	10.67, 26.89	0.000	6.48, 9.82	0.02
12-23-08	8.60, 10.77	0.139	2.38, 2.69	0.615
12-29-08	13.63, 15.31	0.463	1.93, 1.80	0.796
12-31-08	10.15, 3.20	0.000	1.69, 1.35	0.468

Statistical analysis of the % RA of PAOs and GAOs in butyrate fed reactors:

Table 1 – Comparison of overall % RA of PAOs and GAOs between B1 and B2 reactors

Name of microbe	Reactors	Mean (μ_1, μ_2)	P value (95% CI)
CAP	B1 and B2	35.38, 28.8	0.449
CCP	B1 and B2	2.53, 3.63	0.664
TFO	B1 and B2	6.47, 12.84	0.07

Table 2 - Comparison of two wells for CAP in B1and B2 reactors at different dates

Date	B1 reactor (W1 & W2)		B2 reactor (W1 & W2)	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
7-22-08	17.76, 16.90	0.759	10.66, 16.15	0.047
8-12-08	25.81, 32.07	0.536	19.82, 17.72	0.512
8-26-08	42.31, 41.76	0.950	28.87, 28.43	0.878
9-12-08	48.76, 41.77	0.225	52.09, 47.03	0.580
10-10-08	47.04, 39.71	0.459	37.88, 29.05	0.137

Table 3 - Comparison of two wells for CCP in B1and B2 reactors at different dates

Date	B1 reactor (W1 & W2)		B2 reactor (W1 & W2)	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
7-22-08	8.81, 7.09	0.390	6.51, 10.08	0.283
8-26-08	0.75, 0.63	0.625	1.79, 1.39	0.387
9-12-08	0.62, 0.48	0.502	2.64, 0.92	0.198
10-10-08	1.29, 0.55	0.189	2.41, 3.13	0.383

Table 4 - Comparison of two wells for TFO in B1and B2 reactors at different dates

Date	B1 reactor (W1 & W2)		B2 reactor (W1 & W2)	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
8-26-08	5.72, 2.72	0.066	8.02, 8.60	0.869
9-12-08	5.68, 7.1	0.540	18.12, 13.55	0.581
10-10-08	7.15, 10.42	0.305	12.69, 16.14	0.248

Table 5 - Comparison of CCP and TFO in B1and B2 reactors at different dates

Date	CCP in B1 and B2		TFO in B1 and B2	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
7-22-08	7.95, 8.29	0.856	-	-
8-26-08	0.69, 1.60	0.002	4.22, 8.31	0.037
9-12-08	0.55, 1.78	0.075	6.39, 15.84	0.029
10-10-08	0.92, 2.77	0.001	8.79, 14.42	0.014

Table 6- Comparison of CAP and Actino in B1and B2 reactors at different dates

Date	CAP in B1 and B2		Actino in B1 and B2	
	Mean (μ_1, μ_2)	P value (95% CI)	Mean (μ_1, μ_2)	P value (95% CI)
7-22-08	17.33, 13.40	0.056	-	-
8-12-08	28.94, 18.77	0.053	-	-
8-26-08	42.03, 28.65	0.006	-	-
9-12-08	45.27, 49.56	0.407	-	-
10-10-08	43.38, 33.47	0.086	-	-

APPENDIX V

PRELIMINARY DESIGN OF SBR

a) Some typical values:

True yield (mg VSS_a/mg BOD_L), Y = 0.45

Maximum specific rate of substrate utilization (mg BOD_L/ mg VSS_a.d), q^ = 20

Endogenous decay co-efficient, b = 0.15

Fraction of biodegradable active biomass, f_d = 0.8

Concentration of substrate gives one-half of maximum growth rate, k = 1

b) Effluent standard:

BOD_5 < 20 mg/L

Suspended solids (SS) < 20 mg/L

Phosphorus (P) < 1 mg P/L

c) Limiting SRT value:

$[\theta_x^{min}]_{lim}$ (day) = 1/(Yq^ -b) = 0.11

d) Minimum substrate concentration in the reactor:

S_{min} (mg COD/L) = $kb[\theta_x^{min}]_{lim}$ =0.02

e) Design SRT:

θ_x (day) = 8 (Oehmen et al., 2005a)

Safety factor, SF = θ_x / $[\theta_x^{min}]_{lim}$ = 70.8

f) Effluent substrate concentration:

S (mg COD/L) = $(k (1 + b \theta_x))/\theta_x * (Yq^{\wedge} -b) - 1) = 0.03$ (< 20 mg/L) OK

g) Effluent phosphorus concentration:

P_s = P content in sludge (mg P/ mg VSS) = 0.0935

COD/P = 15

COD_{in} (mg/L) = 199.95

P_{in} (mg/L) = 13.33

P_{out} (mg/L) = $P_{in} - ((P_s) Y [1 + (1 - f_d) b\theta_x] (\Delta BOD_L)/(1 + b\theta_x)) = 8.59$

h) Hydraulic retention time (HRT):

Fill volume per cycle, V_f (L) = 4

Number of cycle per day, n = 3

Influent flow, Q (L/d) = 12

Reactor volume, V (L) = 8

HRT, θ (d) = 0.667

HRT, θ (hr) = 16

i) MLVSS and MLSS:

S_{in} (mg COD/L) = 199.95

S (mg COD/L) = 0.03

Inert biomass concentration, X_i^0 (mg/L) = 25 (Metcalf and Eddy, 2003)

MLVSS, X_v (mg/L) = 2500 (assume)

MLVSS is 80 to 90% of MLSS

MLSS (mg/L) = 2941 (Consider 85%)

j) Active biomass:

X_a (mg VSS_a/L) = $(X_v - (\theta_x/\theta)* X_i^0)/(1 + (1 - f_d) * b * \theta x) = 1774$

k) Solids wasted:

Effluent flow, Q^e = Qi (L/d) = 12

Effluent SS concentration, X_{ss}^e (Mg SS/L) = 0 (assume)

Effluent SS mass flow rate, $Q^e X_{ss}^e$ (mg/day) = 0

Total solids mass, MLSS * V (mg) = 23529

$(MLSS*V)/\Theta_x$ (mg/d) = 2941

Solids wasted rate, $Q^w X_{ss}^w$(mg/day) = ($(MLSS*V)/\Theta x) - (Q^e * X_{ss}^e) = 2941$

SVI (ml/g) = 71 (assume)

SS concentration in the settling sludge, X_{ss}^w (mg/L) = 10000

Solids wasted rate, Q_w (ml/day) = $Q^w X_{ss}^w/X_{ss}^w = 294$

l) Nutrients Requirement:

Yield, Y mg VSS_a/mg COD) = 0.45

Influent COD (mg/L) = 199.95

Active biomass, M_a (mg VSS_a) = 89.9775

P content in biomass (mg P/mg VSS) = 0.0935

Formula of cell = $C_5H_7O_2NP_{0.34}$

Molecular wt, M_w(g) = 113

N content in cell, N_c (%) = 12.4

P content in cell, P_a (mole) = 0.34

P content in cell, P_c (%) = ((31*Pa/Mw)*100) =9.3

N required (mg/L) = $((M_a*N_c)/100) = 11.1$

P required (mg/L) = $((M_a*P_c)/100)$ = 8.4

Biomass P/C rato (molar) =$(P_a/5)$ = 0.068

m) Butyrate:

Butyrate (mg COD/L) = 199.95

Butyrate, B_m (mg /L) = (1.75 mg of COD/mg of butyrate) = 228.6

P content (mmole) = $(P_{in}/31)$ = 0.43

C content (mmole) = $(4*B_m/87)$ = 10.51

Feed P/C ratio (molar) = 0.041

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VITA

Graduate College
University of Nevada, Las Vegas

Shamim Ara Begum

Local Address:
4255 Tamarus Street # 421
Las Vegas, NV 89119

Permanent Address:

Village: Baradanga (Road No. 1)
Post Office: Rajbari
Police Station: Rajbari
District: Rajbari
Country: Bangladesh

Degree:

Bachelor of Science, Civil Engineering, 1997
Bangladesh University of Engineering and Technology (BUET), Bangladesh

Master of Science, Sanitary Engineering, 2003
International Institute for Infrastructural, Hydraulic and Environmental Engineering (IHE), Delft - Netherlands

Special Honors and Awards:

- Acquired Carollo Engineers Graduate Scholarship ($1300) for Fall 2008.
- Received Nevada Power Fellowship ($15,000) for the academic year 2006-2007.
- Acquired Graduate and Professional Student Association (GPSA) grant for Fall 2005 ($500), Summer 2007 ($750) and Fall 2008 ($600) from UNLV.
- Obtained Graduate Research Training (GREAT) Assistantship in Summer 2005 from UNLV. The -assistantship provided $1,333 per month for three months and paid tuition fee for 3 credits of course.
- Accepted Fellowship (€898 per month) of the Netherlands Fellowship Programme from Oct. 2001 to April 2003.

Publications:

B. Petrusevski, S. K. Sharma, E. Hossain, S. A. Begum, J. C. Schippers, "Field Testing of IHE Arsenic Removal Filter in Bangladesh." Proceedings of the International Water Association (IWA) Asia-Pacific Regional Conference, Bangkok, Thailand, October 20-22, 2003.

S. A. Begum and M. A. Ali, "Estimation of Arsenic Affected Population in Bangladesh," Proceedings of the First International Conference and Annual Paper Meet on Civil Engineering, S. M. Seraj, M. A. Ali and B. Ahmed eds., Organized by Institution of Engineers, Bangladesh (IEB) and FEISCA, IEB Chittagong Centre, 2-3 November, 2001.

Dissertation Title: The Influence Of Carbon Source Types And Nitrate On The Performance Of The Enhanced Biological Phosphorus Removal Systems

Dissertation Examination Committee:
Chair Person, Dr. Jacimaria Batista, Ph. D
Committee Member, Dr. Thomas Piechota, Ph. D
Committee Member, Dr. David E. James, Ph. D
Committee Member, Dr. Dick Tandy, Ph. D
Graduate Faculty Representative, Dr. Jeffery Q. Shen, Ph. D