8-28-2012

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THE EFFECTS OF ACETATE CONCENTRATIONS ON COMPETITION BETWEEN MICROBIAL POPULATIONS IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL FROM WASTEWATER

by

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Engineering

The University of New Mexico
Albuquerque, New Mexico

July, 2012

The Effects of Acetate Concentrations on Competition between

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ABSTRACT

Enhanced biological phosphorus removal (EBPR) is a popular modification of the activated sludge process, and has been studied and applied for decades. It was hypothesized that the bacteria responsible for EBPR, polyphosphate accumulating organisms (PAOs), have an advantage over their competitors, the glycogen accumulating organisms (GAOs). Because research EBPR systems have been primarily sequencing batch reactors (SBRs), which include rapid feed addition and relatively high substrate concentrations, while full scale systems often include complete mix reactors, which yield generally lower substrate concentrations due to instantaneous dilution of influent, evaluating this hypothesis has both fundamental and applied implications. The primary objectives of this research were to (1) evaluate the effects of carbon substrate (acetate) concentrations on PAO/GAO competition in conjunction with variable pH and temperature, and (2) evaluate whether GAOs tend to be causes of PAO failure or opportunists arising after PAO failure laboratory-scale sequencing batch reactor (SBR) EBPR systems were operated with synthetic feed, and acetate as the main carbon source

for more than 650 days. Experimental variables with respect to reactor operation include pH, temperature, and rate of acetate addition during each cycle feeding phase. Consistent with previous research, PAOs were dominant at higher pH values (7.4-8.4) when acetate was added rapidly (Stage 1), and when pH was decreased, EBPR failed (Stage 2). When the rate of acetate addition was then slowed to maintain low concentrations in the reactors (Stage 3), PAOs were able to once again dominate the system, supporting the hypothesis that PAOs may be favored by low acetate concentrations. Returning the system to an increased rate of acetate addition again led to PAO failure (Stage 4).

PAO failure in Stages 2 and 4 did not appear to be caused by GAOs, based on measurements of acetate concentrations, and it appeared that high acetate concentrations at low pH were themselves inhibitory to PAO metabolism. Batch tests with chemical inhibitors indicated that PAOs and GAOs likely use different transport systems to take up acetate, which provide PAOs the ability to scavenge low acetate concentrations through their particular transport mechanism, and inhibition was increased at lower acetate concentrations. Lower acetate concentrations did not provide an advantage of PAOs over GAOs when the temperature was increased from 22^{o} C to 29^{o} C. Lower acetate concentrations may be advantageous to EBPR process performance, as they increased the biomass polyphosphate content by 50% relative to higher acetate concentration conditions (22 °C, pH = 7.4-8.4). All the performance data were consistent with the Neisser staining, FISH and pyrosequencing analyses results.

TABLE OF CONTENTS

LIST OF ACRONYMS

ABM	Agent-Based Modeling
ATP	Adenosine triphosphate
CMFRs	Completely Mixed Flow Reactors
DO	Dissolved Oxygen
EBPR	Enhanced biological phosphorus removal
FISH	Fluorescence in situ hybridization
GAOs	Glycogen Accumulating Organisms
HRTs	Hydraulic resident times
NMR	Magnetic resonance
P	Phosphorus
PAOs	Polyphosphate Accumulating Organisms
PHAs	Polyhydroxyalkanoates

PHB	Poly-3-hydroxybutyrate
PHV	Poly-3-hydroxyvalerate
Pit	Phosphate inorganic transport
PMF	Proton motive force
PP	Polyphosphate
PPK	Polyphosphate kinase
PPX	Exopolyphosphatase
Pst	Phosphate specific transport
RAS	Return activated sludge
SBRs	Sequencing Batch Reactors
TCA	Tricarboxylic acid
VFAs	Volatile fatty acids
DCCD	N, N'-Dicyclohexylcarbodiimide
CCCP	carbonyl cyanide m-chlorophenylhydrazone

CHAPTER 1: INTRODUCTION

Increasing population growth and consumption in industrialized and developing countries is increasing demands on scarce water resources and the impacts of wastewater on the natural environment and human uses. Improvements to wastewater treatment technologies are needed to address these continuing needs. Particular pollutants of concern include nutrients such as nitrogen (N) and phosphorus (P), which can stimulate the growth of algae, which lead to eutrophication and damage aquatic ecosystems.

Technologies for phosphorus removal have received increased attention, which include biological removal and chemical precipitation. Chemical precipitation is usually achieved by addition of Al and Fe, however it can increase the treatment costs, including the precipitants used and sludge disposal.

Biological treatment using the enhanced biological phosphorus removal (EBPR) process is popular because of its ecological and economic advantages (Martin et al. 2006). EBPR is a modification of the activated sludge process in which high levels of phosphorus removal can be accomplished through the enrichment of Polyphosphate-Accumulating Organisms (PAOs), a group of bacteria that accumulates high levels of polyphosphate when subjected to anaerobic/aerobically cycling with short chain fatty acid feed, such as acetic acid (or acetate). The wasting of high polyphosphate biomass from a treatment system provides a high quantity of P removal from the liquid stream.

Glycogen accumulating organisms (GAOs) are a group of bacteria that is thought to compete with PAOs for short chain fatty acids, and so they have been identified as a potential cause of PAO failure in EBPR systems, and much research has been dedicated to better understanding environmental factors that affect whether PAOs or GAOs will dominate a given system (Fukase et al. 1985; Cech and Hartman 1990; Cech and Hartman 1993; Liu et al. 1994; Satoh et al. 1994; Nielsen et al. 1999; Crocetti et al. 2002; Wong et al. 2004).

Some bacteria are known to have a competitive advantage at high substrate (food source) concentrations (so-called “r-strategists”), while others have a competitive advantage at low substrate concentrations (“K-strategists”). There is has been little or no research to date on how substrate concentrations may affect PAO/GAO competition,

although this could be important, because such concentrations are affected by bioreactor design and other factors. Notably, most laboratory-scale research on EBPR system has been performed in sequencing batch reactors (SBRs), which typically yield relatively high substrate concentrations through much of their operation cycles, while full-scale systems more commonly utilize bioreactors with complete-mix reactor characteristics, producing lower substrate concentrations. Previous research on EBPR systems has suggested that PAOs may have a competitive advantage over GAOs due to a potential ability to take up substrate more rapidly at low concentrations, but this hypothesis has never been tested. If this were true, it would suggest that the importance of GAOs may have been exaggerated in much previous research, since SBR systems (with high substrate concentrations) have typically been used.

This project (1) evaluated the effects of carbon substrate (acetate) concentrations on PAO/GAO competition in conjunction with variable pH and temperature, (2) evaluate whether GAOs tend to be causes of PAO failure or opportunists arising after PAO failure, (3) study on the membrane transport mechanisms for the microorganisms in the EBPR process, which may be a key point for understanding PAOs and GAOs biokinetics and competition for substrate, (4) investigate the different acetate uptake rates under different acetate concentrations and pH condition by PAOs and GAOs, and (5) demonstrate the Pns/VSS and Prel/Ac uptake relationship with variable pH and acetate concentrations.

The research approach was to (1) operate several laboratory-scale sequencing batch reactors (SBRs) EBPR systems, all of them were operated with synthetic feed, and acetate as the main carbon source, but with pH, temperature and acetate addition as experimental variables, (2) conduct several batch experiments on biomass taken from the above SBRs by sampling prior to the addition of acetate feed at the beginning of the anaerobic phase to test the acetate uptake rate between PAOs and GAOs under variable pH and acetate concentrations, and the chemical inhibitors effects on the acetate uptake rate of PAOs and GAOs under variable acetate concentrations.

CHAPTER 2: LITERATURE REVIEW

2.1. Phosphorus discharge and eutrophication

Phosphorus (P) is an irreplaceable nutrient for all life forms. It is used in DNA, proteins, and cellular membranes. Discharge of P to sensitive receiving waters can result in eutrophication, which is one of the main causes of aquatic impairment in the United States (Sedlak 1991). Eutrophication the enrichment of an ecosystem with nutrients, typically nitrogen and phosphorus, leading to the excessive growth of phytoplankton species (algae), accumulation of decaying biomass, and depletion of dissolved oxygen. When this occurs, many species cannot survive, leading to the loss of macro-invertebrates (Oberholster et al. 2008; Beyene et al. 2009) and fish species (Campbell et al. 2005) in lakes, streams, and even in coastal areas. In some cases, this causes the development of mono-specific blooms of cyanobacteria (Oberholster et al. 2009). The harmful secondary metabolites of cyanobacterial blooms can have serious adverse effects on the health of humans and animals (Wiegand and Pflugmacher 2005). While eutrophication may occur naturally, human activities may induce or accelerate this process through increasing discharge of nutrients, which results in algae growth.

The most effective strategies of controlling algae growth are through the reduction of nutrient discharges (OECD 2008). Other options, such as the use of herbicides and algicides, are not as effective and present toxic hazards in the aquatic systems. Typical microalgae contains carbon (C), nitrogen (N), and P in the weight ratio of about 50:8:1 (Ian Woertz1 2009). Because algae are able to assimilate carbon from carbonate species, which are plentiful, and obtain energy from photosynthesis, so the strategies of controlling of algal growth can be accomplished by reducing the availability of N and/or P supply.

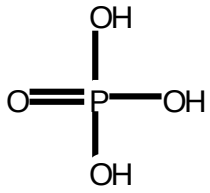
Either N or phosphorus P is usually the limiting nutrient to eutrophication, depending on local conditions. Even when nitrogen is the limiting factor, phosphorus may still be made to play the role of limiting factor through appropriate control (OECD 2008). A variety of natural and anthropogenic sources contribute P to lakes, rivers and

streams via many different pathways, including point sources such as municipalities and industries wastewater effluents, and diffuse sources, such as agricultural drainage (OECD 2008). Control of diffuse sources is always difficult, however, improved agricultural practices might decline the nutrients to water bodies. Control of point sources of pollution is usually much easier than non-point sources (OECD 2008), and so municipal wastewater treatment plants are facing increasingly stringent limits.

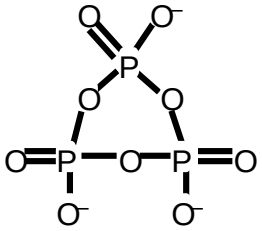
2.2. Characterization of P

P is a component of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), phospholipids, polyphosphate, and other biologically important molecules. P occurs in natural water and wastewaters most commonly as orthophosphate.

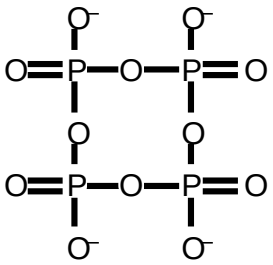
Soluble and particulate phosphorus forms of P are differentiated by whether or not they pass through a 0.45-μm membrane filter (Jenkins and Hermanowícz 1991). Soluble P can be classified as orthophosphates, condensed phosphates and organic phosphates (Table 2.1). Orthophosphate is the simplest in a series of phosphates, and exits as an independent unit without phosphate-phosphate bounds. Condensed phosphates, such as trimetaphosphate, tetrametaphosphate, and longer polymers of phosphate termed polyphosphates (PP), can be formed through dehydrating the orthophosphate radical (Snoeyink and Jenkins 1980) (Figure 2.1), . Pyrophosphate and tripolyphosphate are the simplest of PP, with just 2 and 3 monomeric units, respectively. Organic phosphates are phosphates which are bound to plant or animal tissue, and are formed primarily by biological processes, including sugar phosphates, phospholipids, and nucleotides (Snoeyink and Jenkins 1980).



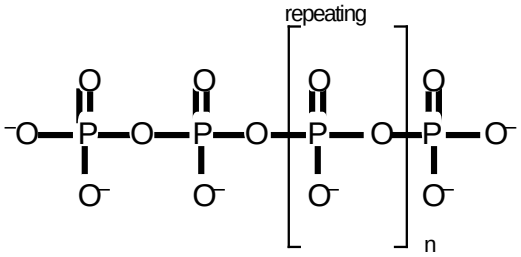
Orthophosphate



Trimetaphosphate



Tetrametaphosphate



Polyphosphate

n = 2 –1,000,000

Figure 2.1 Forms of phosphate

Table 2.1: Chemical forms of phosphate in the U.S.A. sewage (Jenkins and Hermanowícz 1991)

Phosphate Form	Typical Concentrations, mg P/L
Orthophosphate	3-4
Condensed Phosphates (pyrophosphate, tripolyphosphate, trimetaphosphate)	2-3
Organic Phosphates (sugar phosphates, phospholipids, nucleotides)	1
Total	6-8

2.3. Phosphorus removal from wastewater

2.3.1. Chemical precipitation

The main processes for removing phosphorus from wastewater are either through chemical precipitation or by biological processes. Chemical precipitation has the advantage of being reliable and simple to apply. Typical chemical precipitants include iron, alum, and lime (Donnert and Salecker 1999; Penetra et al. 1999). The major disadvantage of chemical precipitation is that it increases the volume of sludge produced (Tchobanoglous et al. 2003) and the precipitated sludge produced tends to be poorly settleable and dewaterable. Less sludge can be produced if alum is applied after secondary treatment, but this can result in increased costs (Strom 2006). For these reasons, adding chemicals in waste treatment should be minimized(van Loosdrecht et al. 1997).

2.3.2. Biological phosphorus removal

2.3.2.1. Bacterial cell composition

Bacteria are of the greatest numerical importance in the activated sludge system,

which are responsible for the biological removal of P from wastewater. The predominance of bacteria living in activated sludge is facultative, which means they are able to live in either the presence or absence of oxygen (Nelson and Cox 2008). P is present in microbial cells as a component of nucleic acids (RNA and DNA), nucleotides, phospholipids, sugar phosphates, Psol, and PP (Madigan et al. 1997).

Bacteria cells are normally composed of about 80 percent water and 20 percent dry material, of which 90 percent is organic and 10 percent is inorganic (Madigan et al. 1997). Typical values for the composition of bacteria cells are shown in Table 2.2.

Table 2.2: Typical composition of typical (non-PAO) bacteria cells (Madigan et al. 1997)

Constituent	**Percent of dry weight**
Protein	55.0
Polysaccharide	5.0
Lipid	9.1
DNA	3.1
RNA	20.5
Other (sugars, amino acids)	6.3
Inorganic ions	1.0
Element	
Carbon	50.0
Oxygen	22.0
Nitrogen	12.0
Hydrogen	9.0
Phosphorus	2.0
Sulfur	1.0
Potassium	1.0

Table 2.2: Typical composition of typical (non-PAO) bacteria cells (Madigan et al. 1997) (cont.)

Sodium	1.0
Calcium	0.5
Magnesium	0.5
Chlorine	0.5
Iron	0.2
Other trace element	0.3

2.3.2.2. Enhanced biological P removal

Enhanced biological P removal (EBPR) is a treatment process in which bacteria remove phosphorus from wastewater in amounts greater than typically required for cellular growth by accumulating large amounts of polyphosphate, which subsequently is removed from the process as a result of sludge wasting (Martin et al. 2006).

EBPR is most commonly obtained by cycling activated sludge biomass through anaerobic and aerobic bioreactors (Figure 2.2), which selects for PAOs thought to metabolize fatty acids as described below.

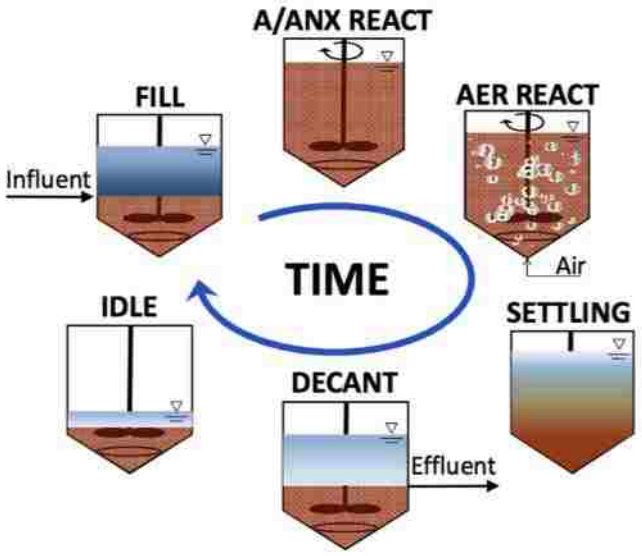


Figure 2.2 Activated sludge with anaerobic/aerobic reactor

As early as 1959, survey on EBPR was published by Srinath et al. (1959). The

observed EBPR was initially referred to as "Luxury uptake" (Levin and Shapiro 1965). Over 80 percent phosphorus removal was reported after aeration of the sludge. In the initial development of biological phosphorus removal, some researchers characterized this phosphorus removal in activated sludge systems either as chemical precipitation or adsorption on the biological sludge (Menar and Jenkins 1970). By the 1970's several wastewater treatment plants (Vacker et al. 1967; Milbury et al. 1971) reported phosphorus removal were around 90%, which provided empirical operating conditions favored biological phosphorus removal: plug flow tanks designs with wastewater addition only at inlet end and elevated dissolved oxygen (DO) concentrations at the end of aeration tank. These guidelines showed the importance of cycling sludge under alternating anaerobic/aerobic conditions and the need for substrate addition to the anaerobic zone (Stensel 1991).

Currently the existence of pure biological phosphorus removal is proven, but partial chemical precipitation and/or adsorption processes can occur simultaneously to be supplements of EBPR process (Bowker and Stensel 1990).

2.4. Metabolic Models of EBPR

2.4.1. Metabolic Models for Origin of Reducing Equivalents in EBPR

Several different metabolic models of EBPR have been proposed, which involve 3 microbial storage products: polyphosphate, glycogen and polyhydroxyalkanoates (PHAs). PHAs include poly-3-hydroxybutyrate (PHB) or poly-3-hydroxyvalerate (PHV). The roles of the compounds are described below.

In general, EBPR metabolism is thought to include the following steps:
during the anaerobic phase: EBPR organisms are capable of hydrolyzing intracellularly-stored polyphosphate to Psol, which is released from the cell. This metabolism produces energy through the cleavage of phosphoanhydride bonds. Then the energy gained is used by EBPR organisms to take up fatty acids and store them as PHAs (commonly PHB). While during the aerobic phase: Stored PHA is oxidized by EBPR organisms, producing energy and carbon substrates for metabolic reactions. The dissolved Psol is taken up by EBPR organisms and stored intracellularly as PP. EBPR organisms growth occur.

One difference in some of the proposed EBPR organisms' metabolic models is the source of reducing equivalents (e.g., reduced nicotinamide adenine dinucleotide [NADH]), which is necessary for PHA synthesis under anaerobic conditions (Schuler and Jenkins 2003a). Comeau et al (1986) and Wentzel et al (1986) suggested that the tricarboxylic acid (TCA) cycle as reducing equivalent source under anaerobic phase (Figure 2.3). In this model, the energy required for converting acetate to acetyl-CoA ATP is mainly from the degradation of polyphosphate. Some acetyl-CoA is cycled through the TCA cycle to provide reducing equivalents (NADH), which are then used to produce PHA from acetyl-CoA. Under aerobic conditions, stored PHA is converted to acetyl-CoA, which cycled to the TCA cycle to provide both reducing equivalents and ATP. Mino et al. (1987) proposed a modified model in which the internally stored glycogen generated reducing power (Figure 2.4). Figure 2.4 also incorporates the Mino Model (Mino 1987) with the energy requirement for transport of acetate. In either Comeau model or Mino model, stored PHA is degraded in the subsequent aerobic phase, which provides the energy and carbon for microorganisms growth and polyphosphorus recover (Comeau et al. 1986; Mino 1987). Smolders et al (1994) later developed a metabolic model based on the Mino model, compared to Mino model, which provided much more details about the energy and redox transformations occurred in the primary biochemical processes of EBPR organisms, and it also assumed that glycogen is the primary source for generating reducing powers. However, Pereira et al (1996) suggested both TCA cycle and glycogen generated reducing equivalents based on measurements using in vivo 13C and 31P nuclear magnetic resonance (NMR) (Figure 2.5). Hesselmann et al (2000) proposed that a split TCA cycle may be used by EBPR organisms to produce the reducing equivalents (Figure 2.6). The microorganisms that degrade PP for anaerobic uptake of VFAs have been named as polyphosphate-accumulating organisms (PAOs) (Mino et al. 1995).

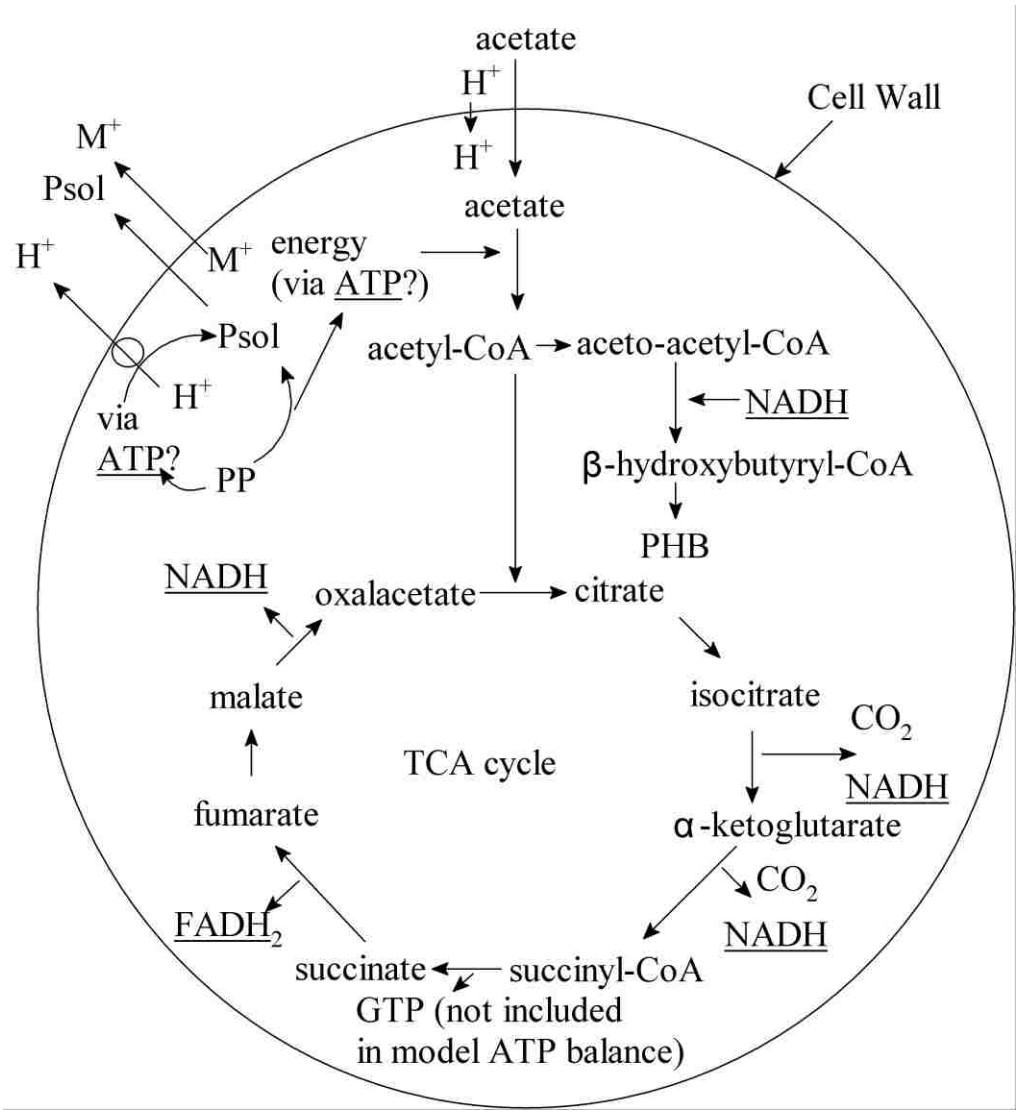


Figure 2.3: Comeau–Wentzel model of the EBPR anaerobic phase (Comeau et al. 1986)

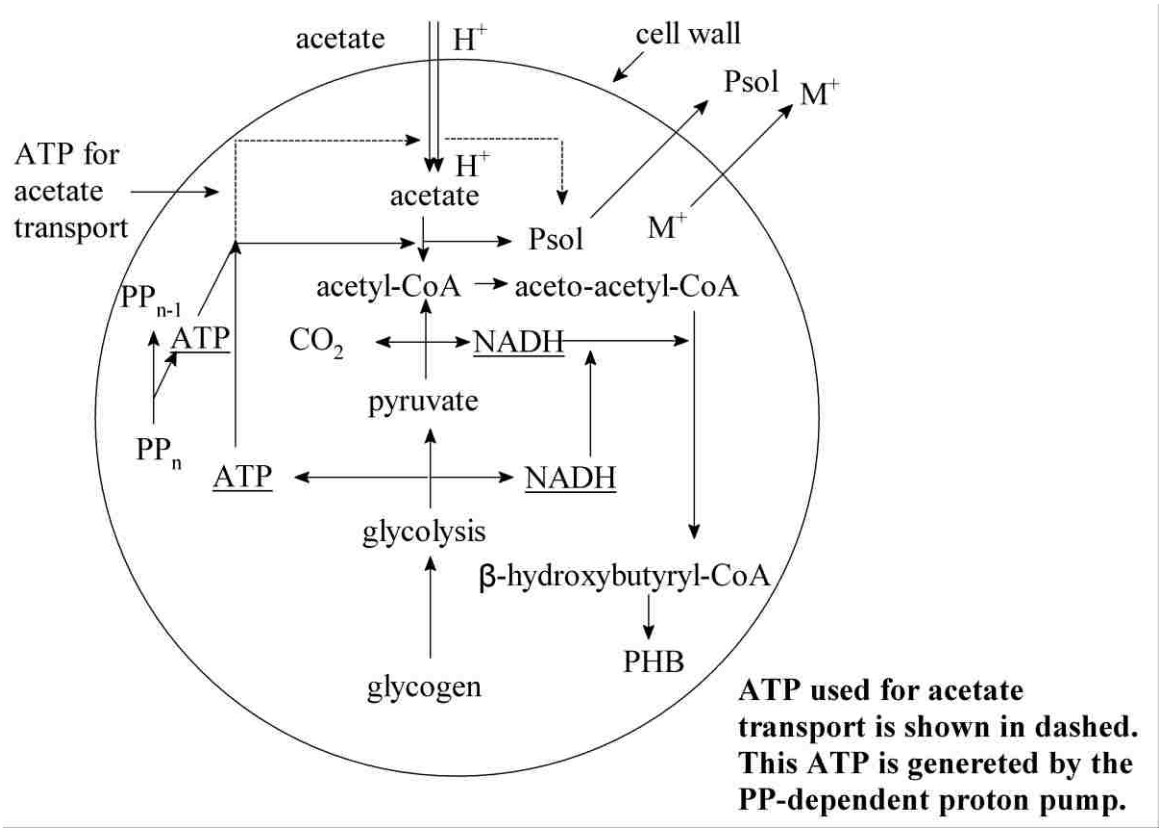


Figure 2.4: Mino model of the EBPR anaerobic phase (Mino 1987), including acetate transport ATP requirement)

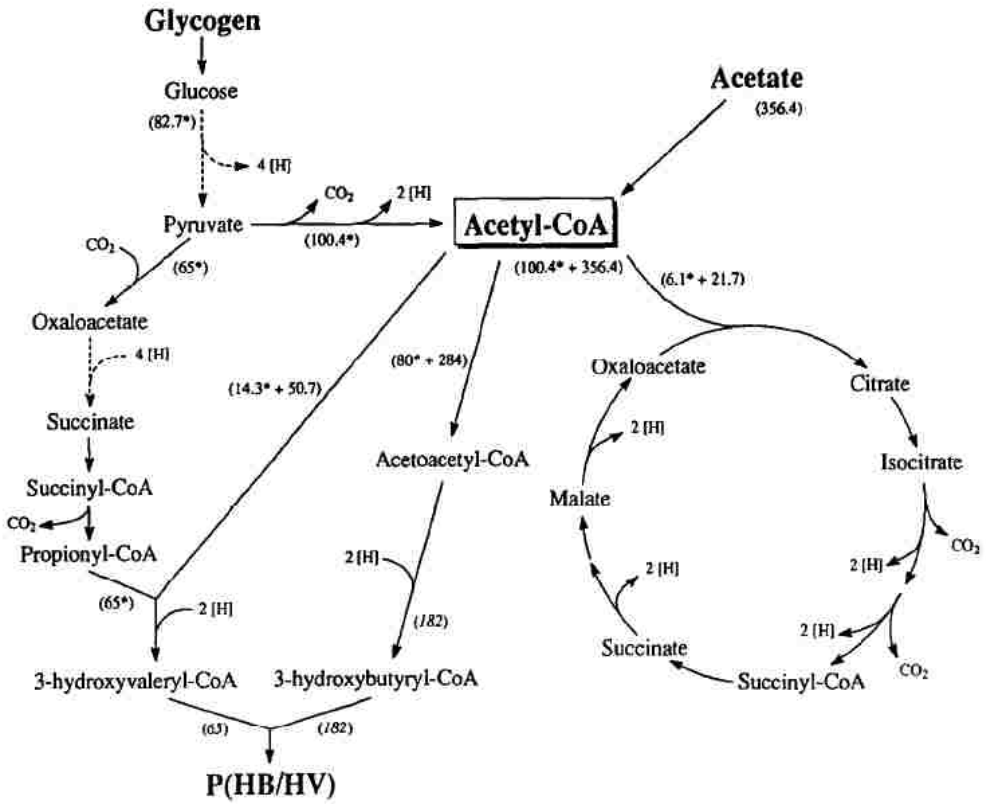


Figure 2.5: Pereira model of the EBPR anaerobic phase (Pereira et al. 1996)

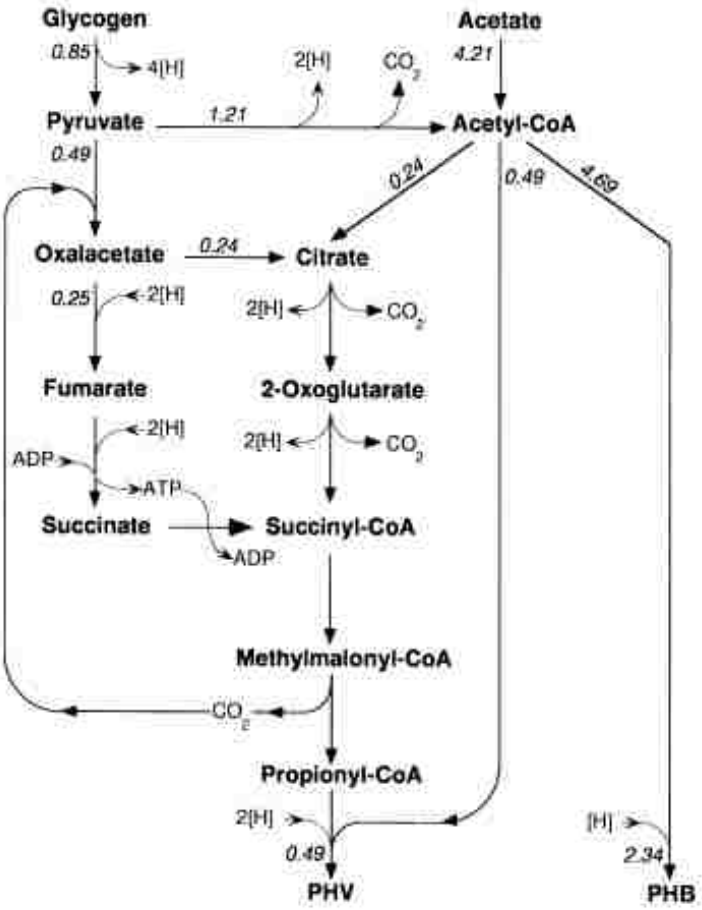


Figure 2.6: Hesselmann model of the EBPR anaerobic phase (Hesselmann et al. 2000)

It is possible that more than one of the above models is correct, with different PAOs using different pathways. Martin *et al.* (2006) proposed that the genes necessary for glycolysis, and the pathways of either the full TCA cycle (Figure 2.3) or the split TCA cycle (Figure 2.6) were present in *Accumulibacter* (a major EBPR organism).

2.4.2. Two proposed glycogen utilization mechanisms in EBPR

Contrasting experimental evidence supports either the Entner–Doudoroff (ED) or Embden-Meyerhof-Parnas (EMP) pathways for glycogen degradation have been detected. Mino *et al.* (1987) proposed that glycogen degradation was via the EMP pathway, producing the reducing equivalents. Wentzel (Wentzel et al. 1991) suggested that the glycogen hydrolysis occurred via Enter-Doudoroff (ED) pathway, which yielded less APT (2 APT) more than EMP (3 APT), and required higher polyphosphate degradation to

supply energy. Based on NMR results, Maurer (1997) and Hesselmann (2000) concluded that the ED pathway was active in PAOs. However, Erdal (2002) reported that glycogen degradation proceeded through the EMP pathway based on the test of the activity of phosphofructokinase, a key enzyme of the EMP pathway, in two EBPR systems. One possible reason for these contrasting results about the pathways for glycogen degradation is there are different bacteria in the microbial cultures of these different studies. However, it is surprising that different results were published in the investigations of Hesselmann *et al.* (2000) and Martin *et al.* (2006), where in both cases the cultures were mainly dominated by *Accumulibacter*. Another reasonable explanation is that there are several clusters of *Accumulibacter* that show different metabolic mechanisms. Further research is still needed in order to understand this issue (Oehmen et al. 2007).

2.4.3. Microbiology of Polyphosphate Accumulating Organisms (PAOs)

2.4.3.1. PAO metabolism: based on the Mino Model

EBPR is attributed to PAOs, which is an obligatory requirement to achieve high and stable EBPR (Mino et al. 1998). As noted, during the initial anaerobic phase (in the absence of oxygen, nitrite or nitrate), PAOs take up volatile fatty acids (VFAs), mainly acetate, and store them as polyhydroxyalkanoates (PHAs). The energy required for this transport is thought to be provided by two pathways: (i) the transport of acetate into the cell, which is supplied by their intracellular PP-dependent proton pump (discussed later) and (ii) the synthesis of acetyl-CoA, which is primarily supplied from the hydrolysis of the intracellular PP, which is released to the liquid as inorganic phosphate. The reducing power for these processes is obtained by the glycolysis of internally stored glycogen in accordance with the Mino model (Mino 1987). Glycogen also yields an additional amount of energy, performing as supplementary source of energy consumed by PAOs for VFA uptake and conversion to PHAs (Figure 2.4). In the subsequent aerobic phase, PAOs use their previously stored PHA as the energy source for biomass growth and glycogen and polyphosphate replenishment. As a result, net P removal is achieved through the removal of waste activated sludge containing a high polyphosphate content (Figure 2.7) (Schuler 1998).

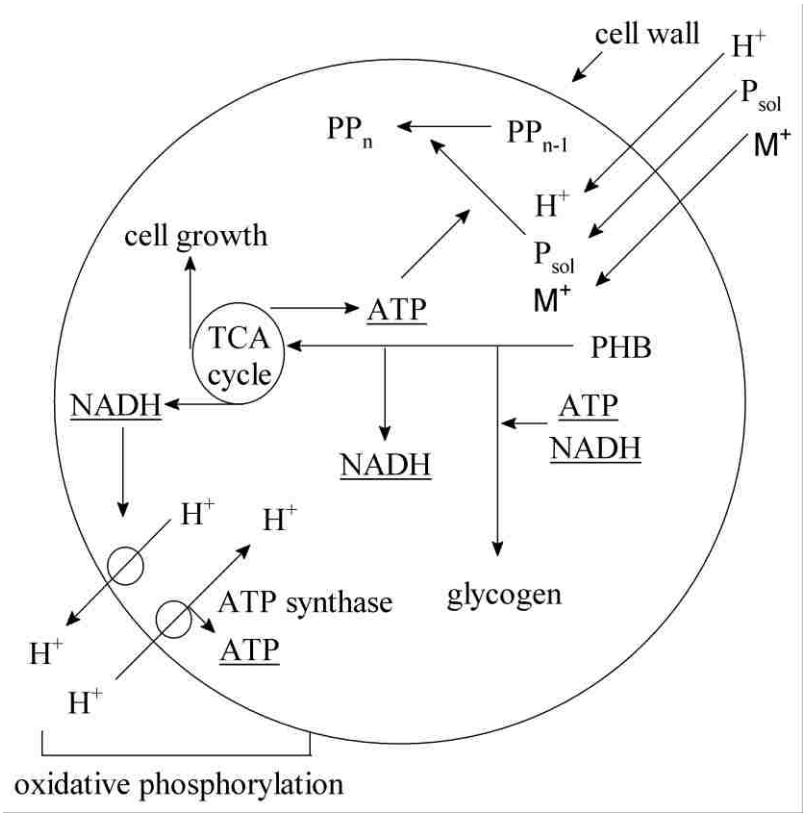


Figure 2.7: Aerobic Phase of the Phosphate Accumulating Organisms (PAOs) Metabolisms (based on Mino model (Mino 1987))

2.4.3.2. Identification of PAOs

Several studies have demonstrated that there are multiple bacteria that accumulate polyphosphate in EBPR system (Table 2.3). The first major investigation about PAOs was made by Fuhs and Chen (1975) (Table 2.3), based on microscopic observations of high level of phosphorus removal sludge. Fush and Chen (1975) also identified the predominant microorganisms associated with phosphorus removal in those sludges as *Acinetobacter spp.,* based on culture-dependent (agar plating) methods. Several other studies also reported the domination of *Acinetobacter spp.* in EBPR processes (Buchan 1983; Lotter 1985; Wentzel et al. 1986), also based on culture-dependent methods, and so *Acinetobacter spp.* was initially believed to be the an important PAO present in EBPR plants. However, Wagner *et al.* (1993) and Kampfer *et al.* (1996) observed that such methods have a bias towards bacteria that are culturable on the artificial media used, which represented only a small portion of bacteria in activated sludges can grow under

such conditions and thus can be detected.

Wagner *et al.* (1994) applied molecular microbiological techniques, including the design of an rRNA-targeted oligonucleotide probe specific for the *Acinetobacter spp.* and its application for fluorescence in situ hybridization (FISH), which showed that classical culture-dependent methods for bacterial counting are strongly selective for *Acinetobacter spp.*. Jenkins and Tandoi (1991) and van Loosdrecht *et al.* (1997) demonstrated that no pure cultures of *Acinetobacter spp.* have shown typical characteristics of EBPR sludge with high phosphorous removal ability. Based on these evidences (Table 2.3), Mino *et al.* (1998) proposed that *Acinetobacter spp.* need no longer be considered as the primary organisms responsible for the EBPR, although *Acinetobacter spp.* may participate in EBPR. There other organisms have been suggested to be PAOs, as shown in Table 2.3.

Many efforts have been made to isolate the PAOs primarily responsible for EBPR, however, all of them have failed (Mino et al. 1998). In spite of the difficulties to isolate microorganisms responsible for EBPR, the use of molecular techniques have helped identifying some organisms which are predominant in systems showing good EBPR performance (Bond et al. 1999). Molecular methods for identification of PAOs has attracted attention in the last fifteen years, including fluorescence in situ hybridization (FISH) analysis, which uses oligonucleotide probes to target bacterial ribosomes (typically 16s and 23s components of the rRNA gene) to determine PAOs species (de-Bashan and Bashan 2004) (Table 2.4). Bond et al (1999) applied FISH to show some sludges with good EBPR were contained many *Rhodocyclus*-related bacteria. Hesselmann et al (1999) named the subclass 2 *Betaproteobacteria* closely related to *Rhodocyclus* as ‘‘*Candidatus* Accumulibacter phosphatis’’. Hesselmann et al (1999) and Crocetti et al (2000) reported several FISH probes (PAO462, PAO651 and PAO846) for *Accumulibacter spp.* that target the organism at different areas of the 16S rRNA. These works verified that *Accumulibacter spp.* corresponded to the characteristic PAO phenotypes (Oehmen et al. 2007). Many detailed studies about the specific probes targeting *Acumulibacter spp. ssp.* have been reported by Crocetti *et al.* (2000). The PAOMIX probe mixture (a combination of the PAO462, PAO651 and PAO846 probes) is useful for quantifying *Acumulibacter spp. spp.* (Saunders 2005). *Accumulibacter* spp. was also found to be present at 4–22% of all Bacteria by FISH analyses in full-scale

EBPR plants (Oehmen et al. 2007). Taken together, these results suggest that *Accumulibacter* spp. is an important organism contributing to P removal in EBPR plants.

Several studies have sought to identify PAOs through detection of polyphosphate in-situ though combining imaging of FISH and PP staining (DAPI stain) methods. This approach has confirmed that not only *Accumulibacter* spp. and *Actinobacter* spp. contain polyphosphate granules (Wong et al., 2005). *Actinobacter* spp. have also been reported in full-scale plants (Kong et al. 2005; Beer et al. 2006), however their precise identity is still unclear, so further research is requested to clarify this point.

Table 2.3: Bacterial species that might be involved in EBPR (de-Bashan and Bashan 2004)

Bacterial genera or species	Evidence for involvement in EBPR	Evidence against involvement in EBPR	Reference
Acinetobacter spp.	1. Predominance in EBPR, based on isolation on agar plates. 2. Strains accumulate polyphosphate and PHAs under aerobic conditions.	1. Culture media detection method is selective for *Acinetobacter spp.* 2. Fluorescent antibody staining, quinone profile or fluorescent in situ hybridization with oligonucleotide probe specific for *Acinetobacter* showed that the species is not primarily responsible for EBPR and is present as a small percentage in the sludge (<10% of total bacteria). 3. No strain possesses the typical metabolic pathways of EBPR-like acetate uptake and its conversion to PHA and hydrolysis of polyphosphate and release of orthophosphate under anaerobic conditions.	(Fuhs and Chen 1975; Christensson et al. 1998; Mino et al. 1998)

Table 2.3: Bacterial species that might be involved in EBPR (de-Bashan and Bashan 2004) (cont.)

Microlunatus phosphovorus	1. Bacteria accumulate large amounts of polyphosphate under aerobic conditions. 2. Capable of anaerobic uptake of glucose.	1. Bacteria do not take up acetate and do not accumulate PHA under anaerobic conditions. 2. A 16s rRNA-targeted oligonucleotide probe, specific to the species, showed that its population is <3% of total bacteria in the sludge, when PAO are about 9% of the total population.	(Nakamura et al. 1995; Kawaharasaki et al. 1998; Santos et al. 1999; Eschenhagen et al. 2003)
Lampropedia spp.	1. Bacteria possess key metabolic characteristics of PAO. Bacteria accumulate polyphosphate and poly β-hydroxybutyrate. 2. Bacteria take up acetate and store it as PHA with polyphosphate degradation and release of orthophosphate.	1. Has a unique sheet-like organization, which is uncommon in EBPR processes.	(Stante et al. 1997)
Accumulibacter spp.	1. Bacteria possess key metabolisms of PAOs. 2. Fluorescent in situ hybridization shows that the group dominates the EBPR process (>81% of the population).	No available data.	(Bond et al. 1999; Ahn et al. 2002; Eschenhagen et al. 2003; Lee et al. 2003)
Tetrasphaera japonica	Phosphate-accumulating cocci.	Microscopically similar to glycogen-accumulating bacteria from activated sludge.	(Maszenan et al. 2000; Eschenhagen et al. 2003)

Table 2.3: Bacterial species that might be involved in EBPR (de-Bashan and Bashan 2004) (cont.)

Tetrasphaera australiensis *Tessaracoccus bendigoensis*	1. Contains intracellular polyphosphate granules. 2. Morphologically similar to dominant microorganisms in activated sludge	Resembles glycogen-accumulating bacteria.	(Maszenan et al. 1999)
Paracoccus denitrificans	Polyphosphate-accumulating bacterium.	Does not need the alternating anaerobic/aerobic cycle for phosphate accumulation. Can accumulate PHB, but does not accumulate polyphosphate when cells are rich in PHBs.	(Barak and van Rijn 2000)
Burkholderia cepacia	Contains intracellular polyphosphate granules.	No available data.	(Mullan et al. 2002)
Agrobacterium spp. *Aquaspirillum spp. Micrococcus spp. Staphylococcus spp. Acidovorax spp. Microsphaera multipartite, Dechlorimonas spp.* *Unidentified yeast, Cytophaga-Flavobacteria group*	Found dominant numbers involved in EBPR processes.	No available data.	(Melasniemi et al. 1998; Kloeke and Geesey 1999; Merzouki et al. 1999; Melasniemi and Hernesmaa 2000; Ahn et al. 2002)

Table 2.4: 16S rRNA-targeted probes used for FISH identification of potential organisms in EBPR systems

Probe	Sequence 5'-3'	Target	Reference
Probes designed for potential PAOs			
PAO462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter*	(Crocetti et al. 2000)
RHC439	CNATTTCTTCCCCGCCGA	*Rhodocyclus*/*Accumulibacter*	(Hesselmann et al. 1999)
RHC175	TGCTCACAGAATATGCGG	Most *Rhodocyclus*	(Hesselmann et al. 1999)
PAO462b	CCGTCATCTRCWCAGGGTATTAAC	Most *Accumulibacter*	(Zilles et al. 2002)
PAO846b	GTTAGCTACGGYACTAAAAGG	Most *Accumulibacter*	(Zilles et al. 2002)
Actino-221a	CGCAGGTCCATCCCAGAC	*Actinobacteria*—potential PAOs	(Kong et al. 2005)
Actino-658a	TCCGGTCTCCCCTACCAT	*Actinobacteria*—potential PAOs	(Kong et al. 2005)
Probes designed for potential GAOs			
Gam1019	GGTTCCTTGCGGCACCTC	Some *Gammaproteobacteria*	(Nielsen et al. 1999)
Gam1278	ACGAGCGGCTTTTTGGGA	Some *Gammaproteobacteria*	(Nielsen et al. 1999)
GAOQ431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	(Crocetti et al. 2002)
GAOQ989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	(Crocetti et al. 2002)
GB	CGATCCTCTAGCCCACT	*Competibacter* (GB group)	(Kong et al. 2002)
GB_G1[a] (GAOQ989)	TTCCCCGGATGTCAAGGC	Some *Competibacter*	(Kong et al. 2002)
GB_G2[a]	TTCCCCAGATGTCAAGGC	Some *Competibacter*	(Kong et al. 2002)
TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus*-related organisms (cluster 1)	(Wong et al. 2004)
TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus*-related organisms (cluster 1)	(Wong et al. 2004)
DF988[a]	GATACGACGCCCATGTCAAGGG	*Defluviicoccus*-related organisms (cluster 2)	(Meyer et al. 2006)
DF1020[a]	CCGGCCGAACCGACTCCC	*Defluviicoccus*-related organisms (cluster 2)	(Meyer et al. 2006)

[a] Requires competitor or helper probes

2.4.4. Microbiology of Glycogen Accumulating Organisms (GAOs)

2.4.4.1. Microbiology characteristics of GAOs

As noted, glycogen accumulating organisms (GAOs) are widely thought to cause the failure of EBPR (Cech and Hartman 1990; Cech and Hartman 1993; Liu et al. 1994; Satoh et al. 1994; Nielsen et al. 1999; Crocetti et al. 2002; Wong et al. 2004). Early evidence of such competition was provided by Fukase *et al.* (1985), who reported that laboratory-scale organisms can compete with PAOs by taking up VFA under anaerobic conditions, without accumulating polyphosphate under aerobic conditions. Cech and Hartman (1990; 1993) reported that approximately 2-um size, cocci-shaped cells, often occurring with a tetrad morphology, were abundant in reactors when glucose and acetate were fed to a laboratory- scale EBPR reactor that had been previously fed with acetate as the primary carbon source. The culture continued to take up acetate anaerobically, while consuming CH, even after polyphosphate storage had ceased.

The organisms were initially called ‘‘G bacteria’’ because it was assumed that they were responsible for glucose uptake. Because of their common morphology, some of them are referred to as tetrad-forming organisms (TFOs) (Tsai and Liu 2002) out-compete PAOs by assimilating VFAs in the anaerobic zone, storing them as PHAs, but without P release under phosphorus limitation conditions (Tsai and Liu 2002). Because of the accumulation of glycogen in excess of that accumulated by PAOs, and their lack of polyphosphate accumulation, these organisms are now commonly referred to as GAOs defined as the phenotype of organisms that recover glycogen aerobically and degrade it anaerobically as their primary energy source for taking up carbon sources then storing them as PHAs Mino *et al.* (1995).

Reported GAOs include the *Gammaproteobacteria Candidatus Competibacter phosphatis* (Crocetti et al. 2002) and the *Alphaproteobacterial Defluviicoccus*- related organisms (Wong et al. 2004; Meyer et al. 2006). Recently, several FISH probes have been developed targeting GAOs (Table 2.4). Nielsen *et al.* (1999) first identified *Gammaproteobacteria* in a deteriorated EBPR system using DGGE analysis, and designed two FISH probes, Gam1278 and Gam1019 to cover the dominant sequences found. Crocetti *et al.* (2002) identified ‘*Competibacter’* in a deteriorated EBPR system, proposed two new FISH probes (GAOQ431, GAOQ989) to target the *Competibacter*;

Kong *et al.* (2002) designed ten FISH probes, which were divided into GB_G1 (identical to GAOQ989), GB_G2, and finally GB_1 – GB_7. All of these probes could targeted *Competibacter*, which have been frequently observed in the lab-scale cultures fed with acetate (Crocetti et al. 2002; Kong et al. 2002; Zeng et al. 2003) (Crocetti et al., 2002; Kong et al., 2002b; Zeng et al., 2003d; Oehmen et al., 2004).

Other groups of TFO GAOs belonging to the *Alphaproteobacteria* have been observed in lab-scale reactors. Wong *et al.* (2004) first demonstrated that *Alphaproteobacterial* GAOs might be related to *Defluviicoccus vanus* under a lab-scale sequencing membrane bioreactor, and also designed the two FISH probes (TFO_DF218 and TFO_ DF618) to target them. Meyer *et al.* (2006) found a new GAO belonging to the Alphaproteobacteria, which was also closely related to *Defluviicoccus. vanus*. Phylogenetic analysis showed that the sequences related to *Defluvvicoccus vanus* formed a monophyletic group with two distinct clusters. Cluster 1 included the sequences from Wong *et al.* (2004), cluster 2 were targeted by two new FISH probes (DF988, DF1020), which were designed by Meyer *et al* (2006). *Defluviicoccus*-related organisms were found to take up carbon sources such as acetate and propionate anaerobically, indicating that this group of organisms has the facility to compete with PAOs for anaerobic carbon uptake.

2.4.4.2. Metabolism of GAOs

Like PAOs, Glycogen-Accumulating Organisms (GAOs) take up VFAs anaerobically and convert them into storage compounds (PHA), but without polyphosphate degradation and P release, a distinctive feature of PAO. Instead, energy for PHA synthesis appeared to come from glycogen degradation, which is equally distinctive of the GAO (Figure 2.8). The reducing equivalents in GAOs are provided by glycogen hydrolysis, and GAOs have been reported to produce approximately 76% Poly-3-hydroxybutyrate (PHB) and 24% Poly-3-hydroxyvalerate (PHV) (C-mol basis) when grown on acetate, while PAOs produce mainly PHB (92%), with less PHV production (8%) (C-mol basis) (Burow et al. 2008). GAOs restore glycogen aerobically without phosphorus uptake and polyphosphate accumulation) (Figure 2.9). The possible presence of GAOs in cultures that were described as PAO-only cultures has been suggested as one

reason why the P release / Ac uptake ratios proposed in many studies have varied (Schuler and Jenkins 2003a).

To our knowledge, the presence of GAOs in EBPR systems has been observed most frequently in laboratory scale systems (Mino et al., 1998), but their presence has also been noted in full-scale systems (Cech and Hartman, 1993; Maszenan et al., 1998) (supported by FISH) (Table 2.5). In the lab-scale systems, substrate concentrations are typically much higher throughout most of the reaction phase than in completely mixed flow reactors (CMFRs), which are commonly used in full-scale systems (wastewater plant).

Table 2.5 Previous studies for GAOs

Lab studies	Full-scale studies
Liu et al (1994)	Saunders et al (2003)
Satoh et al (1994)	Thomas et al (2003)
Filipe et al (2001a)	Wong et al (2005)
Filipe et al (2001b)	
Crocetti et al (2002)	
Schuler and Jenkins (2002)	
Serafim et al (2002)	
Whang and Park (2002)	
Erdal et al (2003)	
Panswad et al (2003)	
Whang and Park (2006)	
Lopez-Vaquez et al (2009b)	

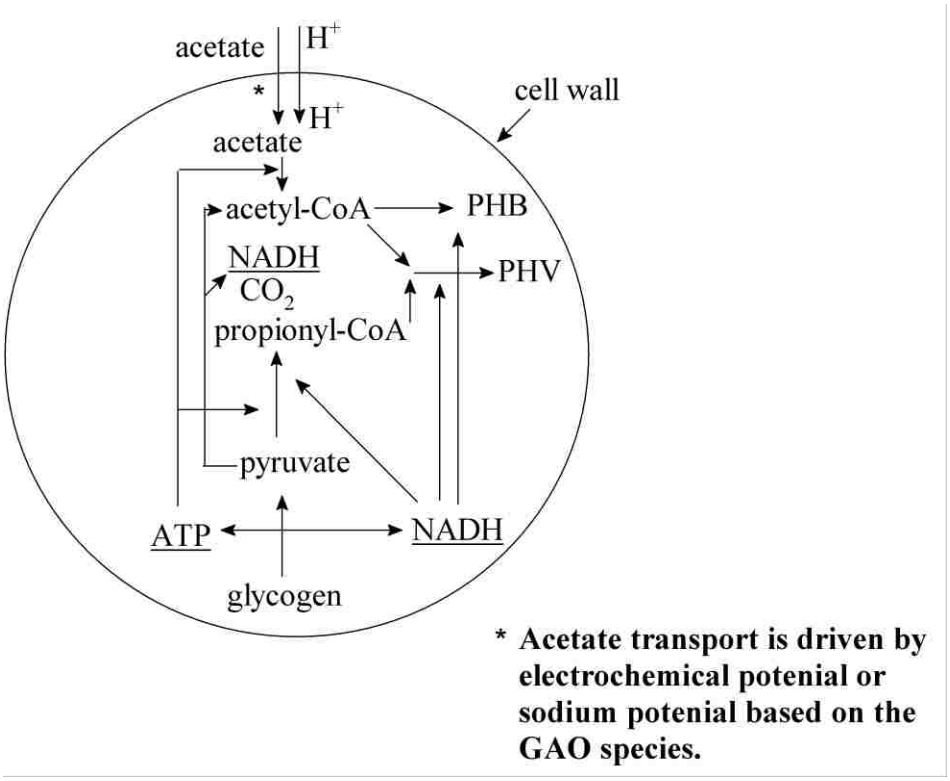


Figure 2.8: Anaerobic Phase of the Glycogen Accumulating Organisms (GAOs) Metabolisms (based on Satoh et al. (1992))

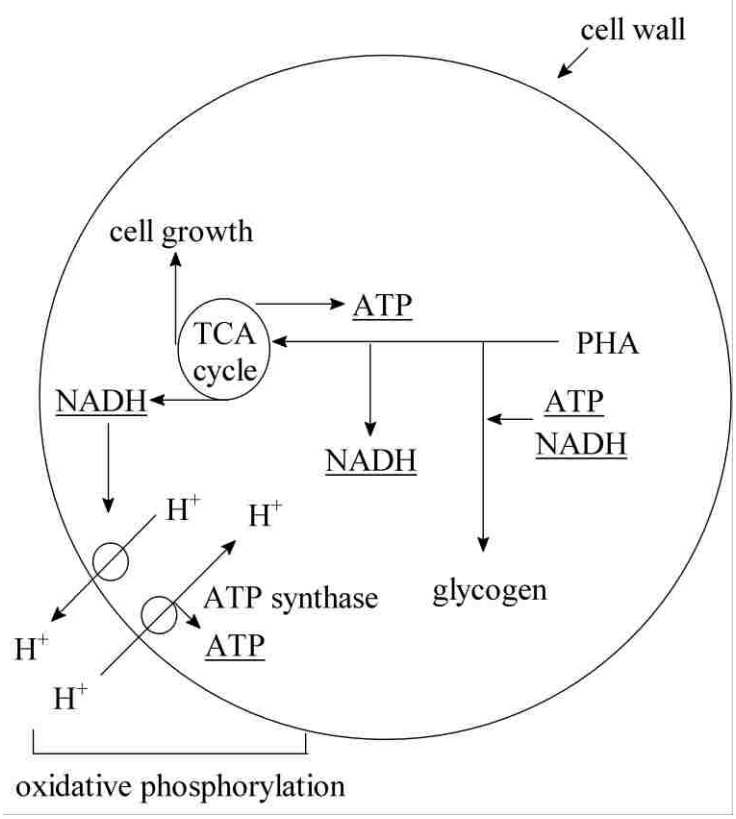


Figure 2.9: Aerobic Phase of the Glycogen Accumulating Organisms (GAOs) Metabolisms (based on Schuler (1998))

2.4.5 Mechanisms of Membrane Transport for PAOs and GAOs

As mentioned above, both PAOs and GAOs consume VFAs under anaerobic conditions, but only PAOs use this carbon to remove phosphorus from the wastewater; so competition for carbon between PAOs and GAOs is believed to be an important factor affecting the efficacy of the EBPR process (Mino et al. 1998; Saunders et al. 2003). The key point for understanding the competition for carbon may be the membrane transport mechanisms for PAOs and GAOs, but these have been studied only recently.

Schuler et al (2003b) calculated that PAO enriched cultures utilize much ATP for acetate uptake (approximately 0.51 mol ATP/ C-mol acetate, depending on which metabolic pathways and stoichiometric ratios are assumed) than a GAO enriched culture (approximately 0 mol ATP/ C-mol acetate). Based on these observations, it was hypothesized that GAOs take up acetate mostly by a diffusion based mechanism (passive transport), while PAOs take up acetate by active transport, in which case GAOs may rely

on higher acetate concentration to drive transport, while PAOs may therefore have a competitive advantage at lower acetate concentrations. In passive transport, the solute is transferred by diffusion, moving from high concentration to low concentration. In active transport, energy is expended by the cell for transport across the membrane. Active transport systems can be divided by two types: primary active transport and the secondary active transport. In primary active transport, a membrane transport protein binds to an external solute and releases it inside the cell by direct ATP hydrolysis or electron-transfer reactions (Nelson et al. 2008). In secondary active transport, energy is also used to transport molecules across a membrane; however, in contrast to primary active transport, the energy is created by coupled flow of two solutes, one of which (often H^+ or Na^+) flows down its electrochemical gradient as the other is pulled up its gradient (Nelson et al. 2008).

Sauders et al (2007) used selective inhibitors (N, N'-Dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide m-chlorophenylhydrazone (CCCP)) and highly-enriched cultures of a PAO, *Accumulibacter* spp., and a GAO, *Competibacter* spp. to study the mechanisms for the membrane transport processes. This research suggested that *Accumulibacter* spp. took up acetate through the acetate permease, ActP, which is primarily driven by a proton motive force (PMF) generated by the efflux of P through the phosphate inorganic transport (Pit) system (Figure 2.10). In *Competibacter* spp, the GAO, the acetate uptake appeared to be driven by a PMF generated by the ATPase and fumarate reductase. The ATP consumed by ATPase is generated by glycolysis and fumarate reductase catalyses (Figure 2.11). Burow et al (2008) assayed acetate and phosphorus transport mechanisms by using the same polyphosphate- accumulating organism as Saunders et al. (*Accumulibacter* spp.), but a different glycogen-accumulating organism, *Defluviicoccus* spp. Like Saunders et al., Burow et al. also concluded anaerobic acetate transport in *Accumulibacter* spp. was by the ActP.

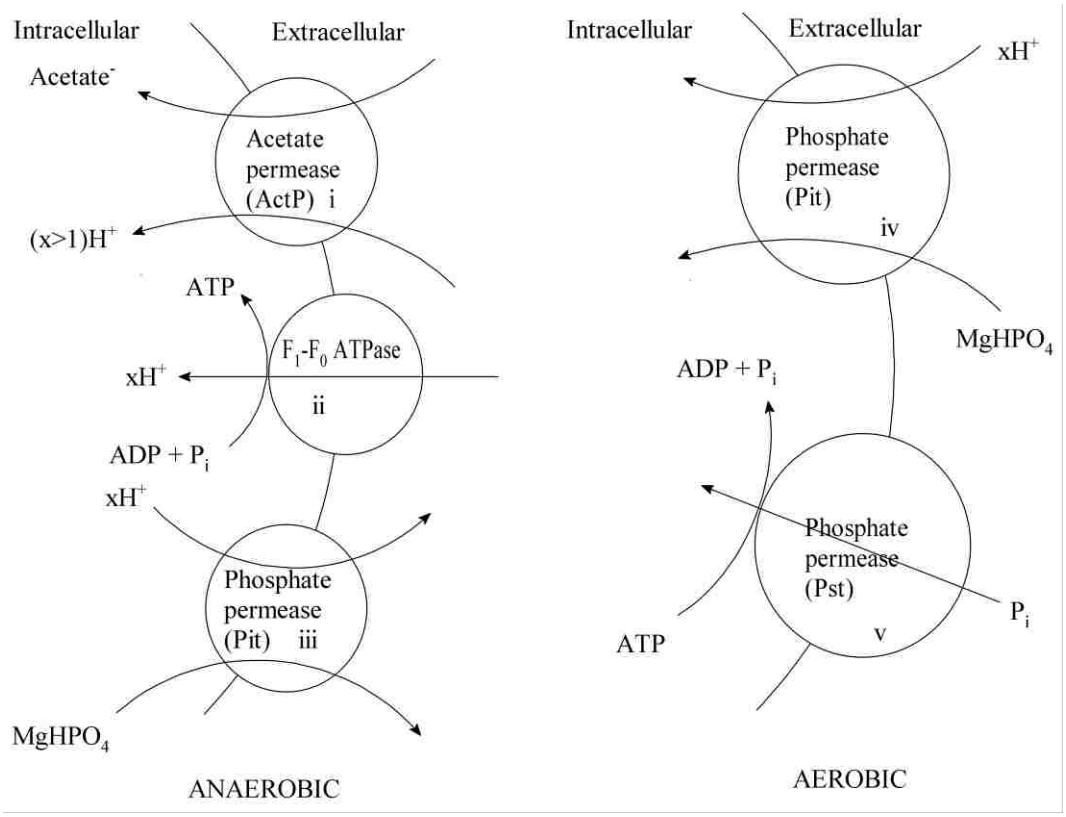


Figure 2.10 Diagram of the proposed mechanism of acetate uptake and phosphorus transport by *Accumulibacter* spp. (based on Saunders *et al.* (2007) and Burow *et al.* (2008)): (i) ActP-mediated symport of acetate is energized primarily by the electrical membrane potential. (ii) Energy is conserved by the Δp driven proton influx through an F_1F_0-ATPase. (iii) Pit efflux of H^+ in symport with inorganic P_i generates an electrochemical gradient and is used for acetate uptake. P_i uptake is driven by a high-affinity phosphate specific transporter (Pst) (iv), and a low-affinity phosphate transport system (Pit) at the beginning of the aerobic phase when P_i concentrations are high (v).

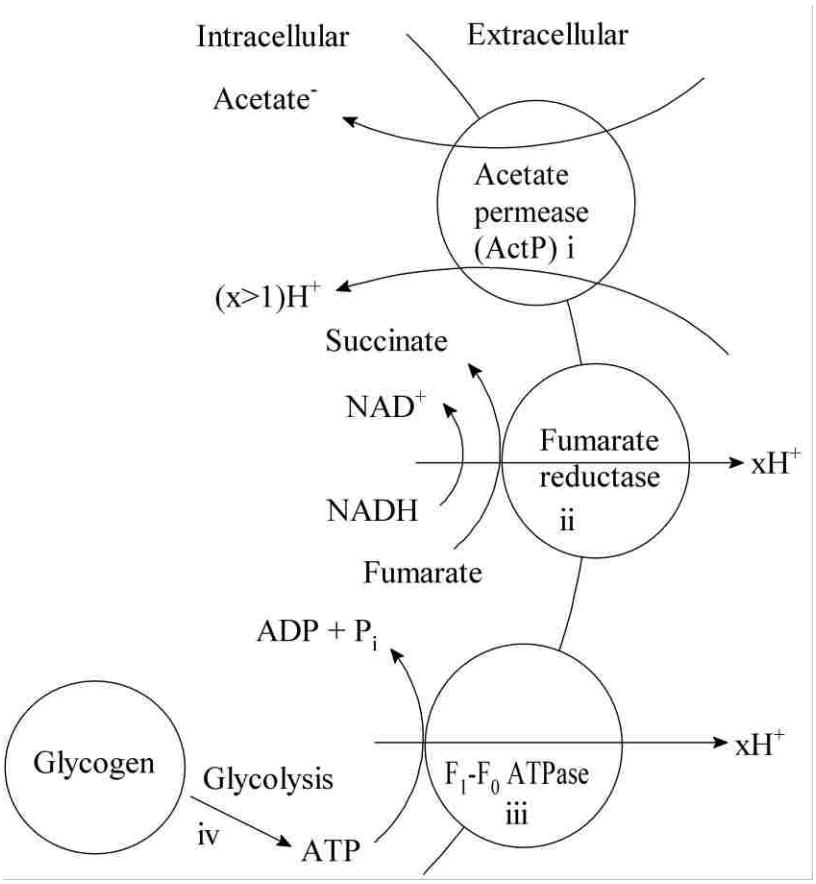


Figure 2.11 The proposed mechanism of acetate uptake by *Competibacter* (based on Saunders et al. (2007)) Acetate uptake (i) is primarily driven by a PMF generated by the ATPase (iii) and fumarate reductase (ii). The ATPase consumes ATP generated by glycolysis (iv).

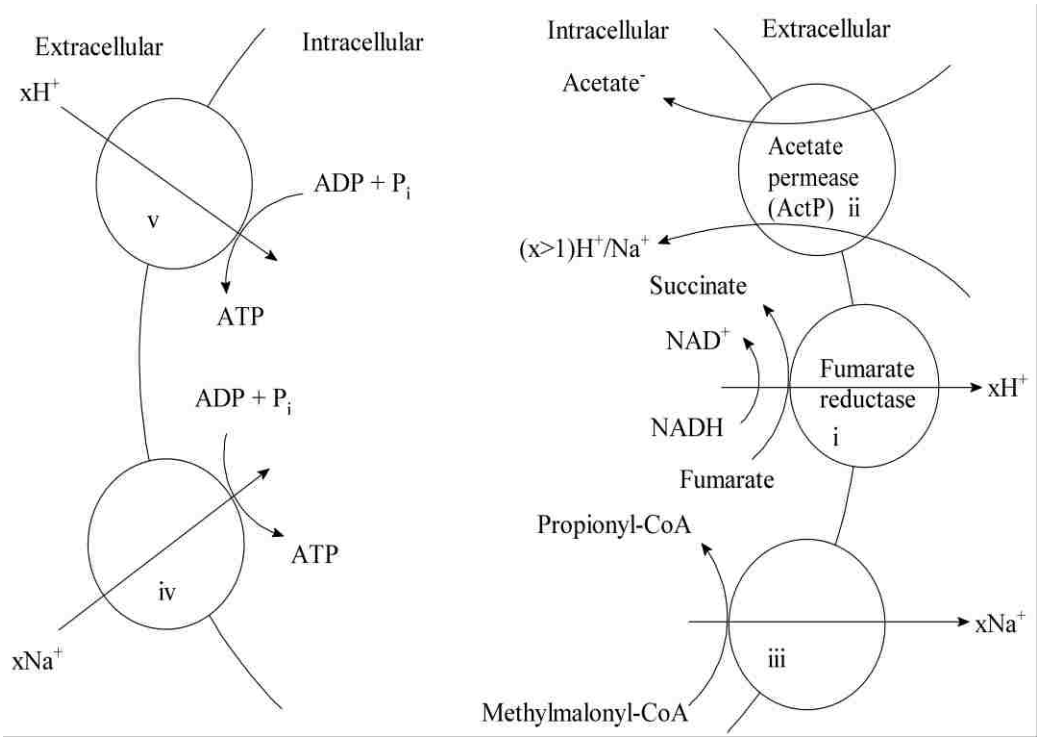


Figure 2.12 The proposed mechanism of acetate uptake by *Dfluviicoccus* (based on Burow et al. (2008)) (i) The fumarate reductase system generates an electrochemical potential. (ii) Permease-mediated symport of acetate is energized by the membrane potential and likely the sodium potential. (iii) Methylmalonyl-CoA decarboylation may generate a sodium potential. Energy is likely conserved by a Na+-translocating ATPase (iv) and by a proton translocating ATPase (v).

The model for acetate uptake in *Defluviicoccus* spp. proposed by Burow et al (2008) (Figure 2.12) is that the fumarate reductase system generates an electrochemical potential, acetate is transported by the chemiosmotic membrane potential and likely the sodium potential, while energy is conserved by ATPase. After using the selective inhibitor, carbonyl cyanide m-chlorophenylhydrazone (CCCP), a PMF disruptor, Burow et al (Burow et al. 2008) observed that active acetate transport contributes comparatively more to overall acetate uptake in *Accumulibacter* spp. than in *Defluviicoccus* spp., which suggests that *Defluviicoccus* spp utilize less energy for acetate than do *Accumulibacter* spp..

It was hypothesized hat *Defluviicoccus* spp. benefit from higher acetate concentrations, which would facilitate low energy transport mechanisms, such as diffuse transport, while *Accumulibacter* spp, the PAO, may be better provide a competitive advantage over *Defluviicoccus* spp., the GAO, under the low acetate conditions common in full-scale EBPR plants.

Burow et al (2008) also studied inorganic phosphorus transport mechanism under aerobic conditions (Figure 2.10). There are two major P transport systems, the phosphate inorganic transport (Pit) system and phosphate-specific transport systems (Pst). Pst has a higher affinity for soluble phosphorus and is active at low soluble phosphorus concentrations. Pit has lower substrate specificity than Pst and acts when the soluble phosphorus is high. Burow et al (2008) found the inorganic phosphorus uptake is driven by Pst system, and Pit system at the beginning of the aerobic phase when inorganic phosphorus concentrations are high. Both Pst and Pit systems are active for inorganic phosphorus uptake, Pst is the primary system, however only Pit system performs effect on inorganic phosphorus release. Kornberg et al (1999) proposed that polyphosphate kinase (PPK) can convert polyphosphate and ADP to ATP and a yeast exopolyphosphatase (PPX) can hydrolyze polyphosphate to inorganic P under anaerobic condition.

2.5 The competition between PAOs and GAOs

Successful operation of the EBPR process depends on numerous environmental factors including pH (Smolders et al. 1994; Liu et al. 1996; Filipe et al. 2001b; Schuler and Jenkins 2002), aeration (Brdjanovic et al. 1998a), solids residence time (SRT; (Brdjanovic et al. 1998b)), presence of nitrate (Puig et al. 2007), presence of nitrite (Saito et al. 2004; Oehmen et al. 2007), and temperature (Brdjanovic et al. 1998c; Panswad et al. 2003), many of which may affect competition between PAOs and GAOs. There are also many reported occasions where the phosphorus removal deteriorated due to what is believed to be microbial competition of GAOs with PAOs (Oehmen et al. 2007).

2.5.1 Influent carbon effects on competition

One factor affecting the PAO–GAO competition is the influent P/COD ratio, as influent P content will limit polyphosphate storage, and PAO activity as well (CITE MY 2003 PAPER). A high P/C ratio (e.g. 0.14 mg P/mg COD) in the wastewater feed tends to favor the growth of PAOs and a low P/COD ratio (e.g. 0.005 mgP/mCOD) should be more favorable to the growth of GAOs (Dai et al. 2007).

The VFAs acetic and propionic acid are known to be excellent substrates for PAOs (Hesselmann et al. 1999; Pijuan et al. 2004). Recent studies have suggested that

propionate may be a more favorable substrate than acetate for successful EBPR performance in long-term operation, as it may produce better net P removal and enriched biomass by PAO (*Accumulibacter* spp.) over GAO (*Competibacter* spp.) (Pijuan et al. 2004; Oehmen et al. 2005b). Oehmen *et al.* (2005b) used acetate and propionate as carbon sources, and compared the uptake results by PAOs and GAOs. The results suggested that GAOs (*Competibacter* spp.) have a slower response to a change in carbon source as compared to PAOs (*Accumulibacter* spp.), which might be used as a selective advantage for PAOs in full-scale EBPR plants.

In contrast, the *Defluviicoccus*-related GAOs have been observed to to compete with PAOs for propionate, leading to the deterioration of P removal performance (Oehmen et al., 2005a; Meyer et al., 2006). Dai (2006) also found that *Defluviicoccus spp.* took up propionate at a faster rate than acetate. These results suggest that a propionate carbon source may not provide PAOs a selective advantage over GAOs.

Wastewater is a heterogeneous and complex mixture, which often reaches the treatment plant without complete acid fermentation (Pijuan et al. 2004) and including large amounts of non-VFA compounds. The most widely studied carbon substrate other than VFAs, has been the glucose. Glucose is believed to deteriorate EBPR systems when it is fed as the carbon source (Cech and Hartman 1990; Cech and Hartman 1993), however, it can be fermented to produce VFAs. Other studies have evaluated alternative substrates, such as malate, lactate (Satoh et al. 1996), starch (Randall et al. 1994), mixtures of acetate and peptone (Liu et al. 1997) and ethanol (Puig et al. 2008). The experimental results demonstrated that PAOs appear to have mechanisms that allow the use of non-VFA compounds without prior fermentation by heterotrophic bacteria (Pijuan et al. 2004).

2.5.2 The effect of pH

Numerous studies have focused on pH effect on EBPR, and they have consistently found that higher pH within the range 7 to 8.5 generally improves EBPR and leads to higher anaerobic P release/acetate uptake ratios (Smolders et al. 1994; Liu et al. 1996; Jeon et al. 2001; Schuler and Jenkins 2002; Pijuan et al. 2004). Jeon et al (2001) found that the P release at the initial anaerobic stage increased with pH from 6.0 to 8.5 in

batch tests. Filipe et al (2001a) (2001b) (2001c)reported the effect of pH on the anaerobic metabolism of PAOs and GAOs in acetate wastewater, observed that P removal increased as pH increased from 7.1 to 7.6, and the P release by PAOs increased linearly with pH in the range pH 6.5 to 8.0, but neither PHA synthesis nor glycogen degradation by PAOs were affected by external pH, and suggested that PAOs have a competitive advantage over GAOs above pH 7.25. Schuler et al (2002) proposed that the optimal EBPR occurred pH between 7.4 and 8.5. Serafim et al (2002) reported that the P release and uptake rates were higher at pH 8.0 than at pH 7.0. Oehmen et al (2005a) also showed that when pH was increased from 7 to 8, PAOs appeared to out-compete GAOs, and a considerable improvement of the P removal was also observed. Pijuan et al. (2004) found that the P release and uptake rates increased with pH ranging from 6.5 to 8.0 in an SBR. Zhang et al (2005) proposed that a change of pH of the mixed liquor from 7 to 6.5 led to a complete loss of phosphate-removing capability and a drastic change of microbial populations from PAOs to GAOs, verified by FISH.

The reason the anaerobic P release/acetate uptake ratio increases with increasing pH has been explained as follows (Smolders et al. 1994): assuming the that the internal pH of the cell is constant, when the ambient pH is increased there is a corresponding increase in the electrochemical potential difference across the cell membrane, which leads to decline the PMF required used for acetate transport (Figure 2.10). More energy is therefore needed for acetate transport when the external pH is high, which is generated through an increase in polyphosphate degradation (Smolders et al. 1994; Schuler and Jenkins 2002).

Filipe et al. (2001a) and (2001c) studied the stoichiometry and kinetics of acetate uptake by enriched cultures of PAOs and GAOs as a function of pH. They observed that the rate of acetate uptake by GAOs was significantly decreased when the pH of the medium was increased, but that the uptake rate for PAOs was essentially independent of the pH for the range studied (6.5 to 8.0). Consistent with other pH studies, this suggested that the control of maintaining high pH in the anaerobic zone of an EBPR system provides a strategy for minimizing the growth of GAOs.

These studies consistently demonstrated that increased pH can improve P removal performance by selecting for PAOs over GAOs. However, there is still a limitation,

Schuler and Jenkins (2002) proposed that the acetate uptake rate decreased when pH was controlled above 8.5; Oehmen *et al.* (2005a) observed that all VFAs uptake rate, P release rate and P uptake rate decreased after the pH was increased to 8.5; Liu *et al.* (1996) also suggested that above pH 8.0, both acetate uptake rate and P release rate started to decrease.

2.5.3 Temperature

Temperature has also been demonstrated to be important factor in the competition between PAOs and GAOs and the related stability of EBPR systems. Whang et al (2002) reported that PAOs were enriched in an SBR at 20 °C, however, when temperature was increased to 30 °C, GAOs were able to out-compete PAOs and enriched in the EBPR system, based on the advantage of acetate uptake. Temperature variation between 20 °C and 35 °C affected the microbial community of the EBPR system (Panswad et al. 2003). This study concluded that the PAOs were possibly psychrophilic microorganisms and would dominate in the system only at 20 °C or even lower. The GAOs were rather mesophilic organisms with optimum temperature between 25 °C and 32.5 °C. Lopez-Vazquez et al. (2007) demonstrated that GAO had obvious advantages over PAO to take up substrate at temperature higher than 20 °C. Below 20 °C, PAOs and GAOs had similar maximum acetate uptake rates. However, the lower maintenance requirements of PAOs at temperature lower than 20 °C could provide they advantage over GAOs. Accordingly, PAO could be considered to be psychrophilic microorganisms while the behavior of GAOs was more close to be mesophilic. These studies were consistent with the observations related to the good EBPR process at WWTP operated under cold weather conditions. They also clarify the proliferation of GAO, which indicates the poor EBPR process, in treatment plants from hot districts or treating high-temperature wastewater.

Jones et al (1996) and Li et al (2010) reported that at 5 °C, EBPR activity was reduced as compared to higher temperatures (15 °C). Li et al. also suggested that EBPR will decreased as the temperature increased, however, the optimal temperature for PAOs is 15 °C, which suggested that there was a limitation, too low temperature could have a negative effect on EBPR. However, Erdal et al. (2003) found a higher P removal performance and a corresponding decrease in glycogen from biomass taken at 5 °C as

compared at 20 °C, and suggested that the low temperature favored the growth of PAOs over GAOs. They also suggested that PAOs were able to dominate the system at 5 °C by changing their metabolic pathway.

Lopez-Vazquez et al. (2009b) also noted that from 20 to 30 °C, *Competibacter* could successfully compete with PAO based on their higher anaerobic acetate uptake rate. However, the *Competibacter* had a lower biomass growth rate than PAOs, which required a minimum aerobic SRT longer than required by PAOs (the difference is larger at temperatures lower than 20 °C), so potentially, short aerobic SRT could be applied in lab-scale systems to enrich PAO cultures.

Ren et al. (2011) showed that the temperature shock had more serious effect on PAOs culture on GAOs in the enriching process. Under sudden and substantially temperature variation, from 22 ± 1 °C to 29 ± 1 °C and then to 14 ± 1 °C, the domination of PAOs was declined. After temperature shock, PAOs' competitive advantages at low temperature that suggested in other studies did not occur in their reseach. As mesophilic microorganism, GAOs adapted the temperature variation soon and dominated the system at 14 ± 1 °C in the end.

The results described above concluded that GAOs more likely to dominate EBPR systems at higher temperatures, and PAOs tend to dominate at lower temperatures. This indicates that EBPR plants may be more challenging in warm climates.

2.5.4 Other factors affecting competition between PAOs and GAOs

Schuler (2005) performed agent based (distributed state) modeling of EBPR systems, and reported that increasing hydraulic residence times (HRTs) tend to increase diversity of state distributions, because larger HRTs provide more time for distributed state development and could therefore increase negative distributed state effects, and this led to decrease predicted EBPR performance. The term state was used to describe the microbial storage product (PP, glycogen, and PHA) of an individual bacterium content. It was proposed that hydraulic characteristics that affect HRT distributions, such as mixing conditions and numbers of reactors in series are critical to EBPR system performance (Schuler 2006). It was concluded that EBPR may be improved by minimizing state distributions, which can be done by utilizing plug flow, using SBRs, or increasing

numbers of completely mixed reactors in series.

It has also been observed that P uptake is inhibited by the presence of nitrite (Kuba et al. 1996; Saito et al. 2004). Saito et al. (2004) observed that an increase in the *Competibacter* population coincided with the accumulation of nitrite in the anoxic phases, and proposed that it may be a factor that provides GAOs an advantage over PAOs. The growth rate of PAOs was also inhibited when the nitrite was present.

The effect of acetate concentrations on PAOs and GAOs competition has not been studied previously. This factor may be important because reactor type and configuration can greatly affect acetate concentrations. The presence of GAOs in EBPR systems has been observed most frequently in laboratory scale systems (Mino et al., 1998), but their presence has also been noted in full-scale systems (Cech and Hartman, 1993; Maszenan et al., 1998) (Table 2.5). In the lab-scale systems, substrate concentrations are typically much higher throughout most of the reaction phase than in completely mixed flow reactors (CMFRs), which are commonly used in full-scale systems (wastewater plant).

Although GAOs have been found to be common in EBPR systems, their presence does not necessarily indicate EBPR failure, as in one study of six full-scale EBPR systems (Saunders et al. 2003). Although most of the plants studied contained GAOs, they were less numerous than the PAOs, even more some data showed GAOs were there but they were failed, which would suggest that it's possible for a full-scale system to operate with an insignificant GAO population (Saunders et al. 2003). This was consistent with the previous observation that the presence of GAOs may indicate well-functioning EBPR systems, since the low effluent P concentrations in these system indicates P limitation, and, as noted above, this would limit PAO activity and is one factor linked to increased GAO populations (Schuler and Jenkins, 2003a).

It has been hypothesized that PAOs may take up acetate more rapidly than GAOs at low acetate concentrations, which could give them a competitive advantage over GAOs under such conditions. Schuler (2003b) suggested this based on measurements of enriched PAO and GAO cultures, with stoichiometric calculations showing that PAOs appear to produce surplus ATP during the EBPR anaerobic phase that could be used for acetate transport, while GAO ATP production appeared to be sufficient for internal transformations of acetate to polyhydroxalkanoates, with little or no excess ATP

available for acetate transport. Also, as described above, Burow et al. (2008) suggested that active transport contributes comparatively more to overall acetate uptake in *Accumulibacter* spp. (PAOs) than in *Defluviicoccus spp.* (GAOs). To date, the hypothesis that PAOs have a competitive advantage over GAOs at low acetate concentrations has not been experimentally evaluated.

2.6 Typical EBPR process configurations

A variety of process configurations have been widely implemented for cdomestic and industrial wastewater to perform EBPR. These include Phoredox (A/O) (Figure 2.13), A2/O (Figure 2.14), Five-stage Bardenpho (Figure 2.15), UCT (University of Cape Town) (Figure 2.16) (Metcalf and Eddy 2002).

The A/O (Figure 2.13) process is a basic configuration for biological phosphorus removal consists of an anaerobic zone followed by an aerobic zone. Barnard (1974) was the first to clarify the need for anaerobic contacting between activated sludge and influent wastewater before aerobic degradation to accomplish biological phosphorus removal. The A/O process is a patented version of Phoredox by Air Products and Chemicals, Inc., and the main difference is the use of multiple-staged anaerobic and aerobic reactors. In this process there is no nitrification, and the anaerobic retention time is 30 min to 1 h to provide the selective conditions for the biological phosphorus removal. The SRT of the aerobic zone mixed liquor is 2 to 4 d, depending on the temperature.

The proprietary A^2/O process (Figure 2.14) is a modification of the A/O process and provides an anoxic zone for denitrification. The detention period in the anoxic zone is approximately 1 h. The anoxic zone is deficient in dissolved oxygen, but chemically bound oxygen in the form of nitrate or nitrite is introduced by recycling nitrified mixed liquor from the aerobic section. Use of the anaerobic zone in the return activated sludge.

First achieved phosphorus removal in a mainstream process later called the Bardenpho process (Figure 2.15). A four phase anoxic-aerobic-anoxic-aerobic configuration, originally designed for nitrogen removal, was used. Sludge from the secondary clarifier and the mixed liquor from the first aerobic basin are recirculated to the first anoxic reactor. Including an anaerobic reactor ahead of the first anoxic reactor in the Bardenpho configuration, fed with sludge from this first anoxic reactor, created

favorable conditions for phosphorus removal. This configuration described in is known as the 5-stage Bardenpho configuration, or the modified Bardenpho process. The 5-stage process uses a longer SRT (10 to 20 days) than an A^2/O process, and thus increases the carbon oxidation capability.

The UCT process (Figure 2.16) stands for University of Cape Town in South Africa. The UCT process was developed to minimize the effect of nitrate in weaker wastewaters in entering the anaerobic contact zone, which is similar to the A^2/O process with two exceptions. The Return Activated Sludge (RAS) is recycled to the anoxic stage instead of the aeration stage, and the internal recycle is from the anoxic stage to the anaerobic stage to avoid any negative effects on the initial phosphorus removal efficiency by nitrate present in the return sludge.

In our study, the Sequencing Batch Reactors (SBRs) are a main configuration to culture the different bacteria. SBR is considered an alternative technology to conventional processes for removing nutrients from wastewater and are widely and commonly used recently (Mace and Mata-Alvarez 2002). The SBR is a fill-and-draw activated sludge system for wastewater treatment. While in continuous systems the reaction and settling occur in different reactors, in SBR all the processes are conducted in a single reactor following a sequence of fill, reaction, settling and draw phases, with repeating cycle. The SBRs have a higher flexibility and controllability, allowing for more rapid adjustment to changing wastewater characteristics, which converts the conventional wastewater treatment process from space-course to time-course, which substantially reduces space occupation (Metcalf and Eddy 2002). An SBR in the case of EBPR systems, SBRs consist of a single reactor that cycles through feed (wastewater addition), anaerobic (mixing with aeration), aerobic (mixing with aeration), settling (no mixing), and draw (effluent withdrawal) phases, after which the cycle starts over (Figure 2.17).

One important difference between laboratory and full-scale systems is that laboratory studies of EBPR have typically utilized SBRs (Figure 2.17) due to their ease of construction and operation, while full-scale systems typically include CMFRs (Figures 2.13-2.19). This is important for this proposal because SBRs, due to their batch nature, have inherently high substrate (acetate or other VFA) concentrations at the beginning of each feed cycle. CMFRs, however, have inherently lower substrate concentration due to

their completely mixed and continuous nature, in which case influent is immediately diluted, and is equal to the effluent substrate concentration.

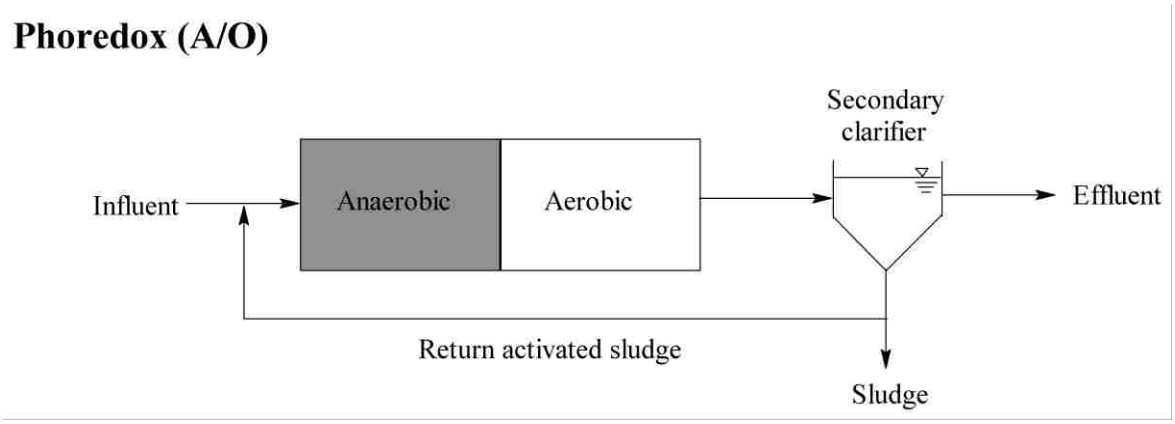


Figure 2.13 A/O process

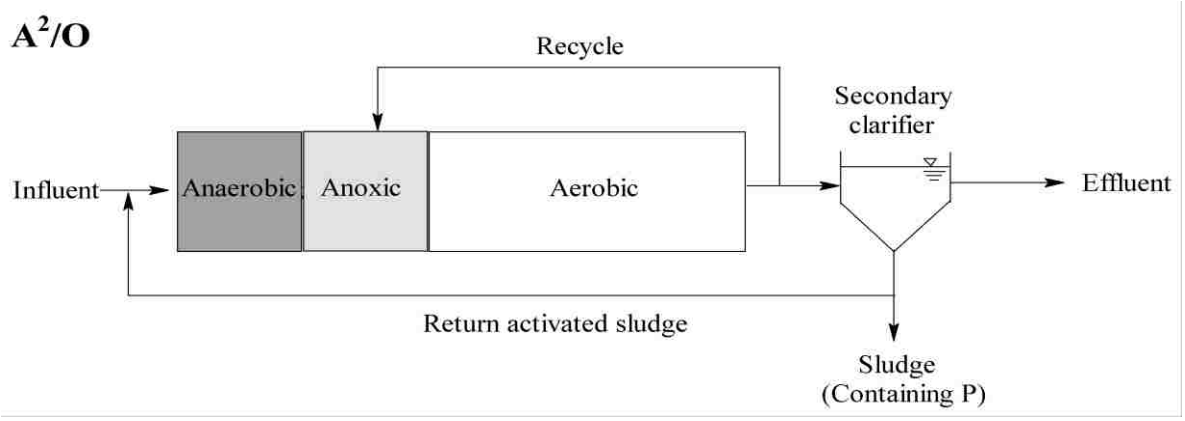


Figure 2.14 A^2/O process

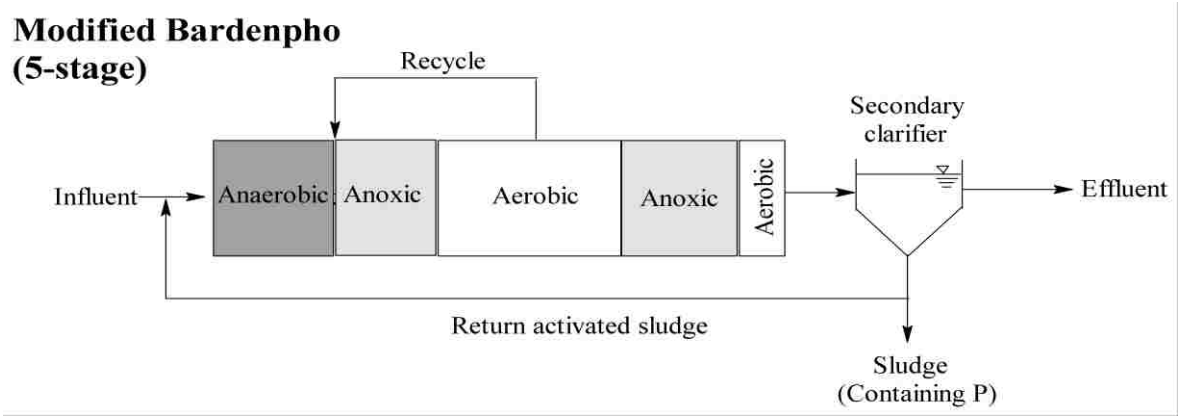


Figure 2.15 Modified Bardenpho (5-stage) process

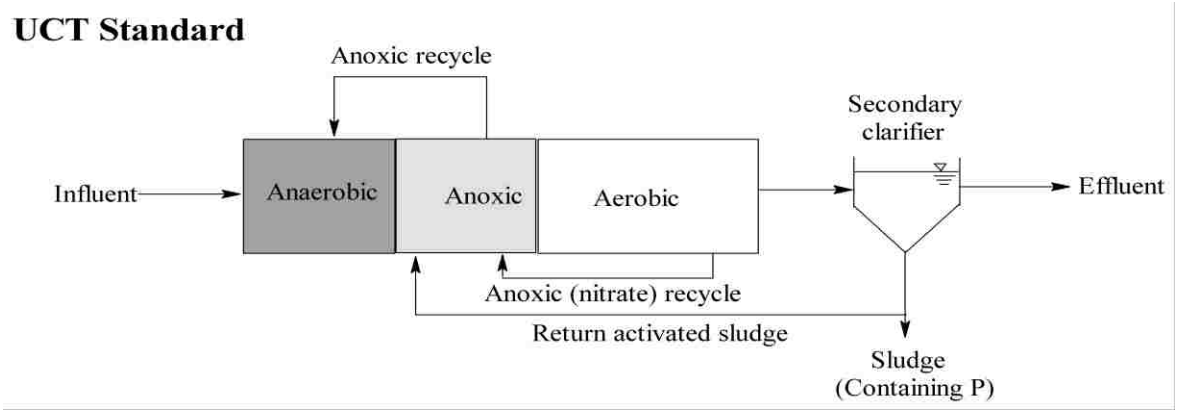


Figure 2.16 UCT standard process

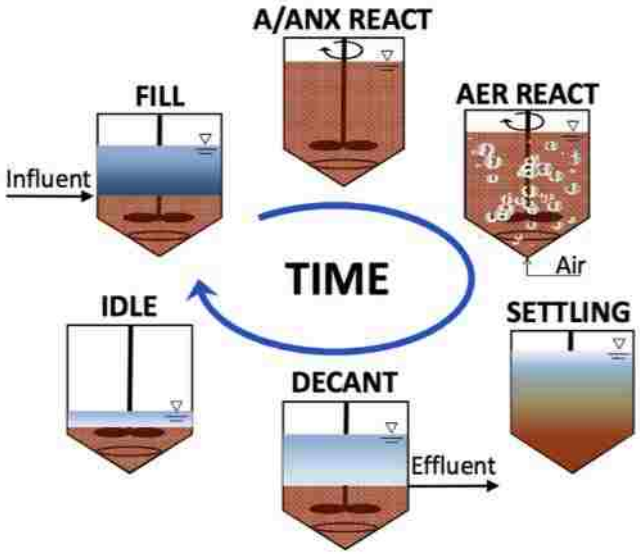


Figure 2.17 SBR process

CHAPTER 3 RESEARCH OBJECTIVES AND HYPOTHESIS

- Construct and operate lab-scale SBR systems for enrichment of cultures dominated by PAOs and GAOs through changing the pH.
- Determine and evaluate the anaerobic acetate transport mechanisms of PAOs and GAOs using batch tests with different acetate concentrations and with added chemical inhibitors targeting different acetate transport mechanisms.
- Evaluate whether PAOs are favored by low acetate concentrations by maintaining low acetate concentrations (using a syringe pump) in a lab-scale reactor.
- Determine PAOs and GAOs kinetics, acetate uptake rate under different substrate concentrations and pH.
- Determine the temperature effect on PAOs and GAOs.
- Compare Pns/VSS under different operation conditions (high pH high substrate concentrations, low pH low substrate concentrations and high pH low substrate concentrations).
- Identify PAOs and GAOs by Neisser staining, FISH and pyrosequencing.

The hypothesis was that GAOs benefit from higher acetate concentrations, which would facilitate low energy transport mechanisms, such as diffuse transport, while the PAO, may be better provide a competitive advantage over the GAO, under the low acetate conditions common in full-scale EBPR plants.

This research is expected to provide both fundamental contributions to the understanding of EBPR metabolisms, and it should also provide information important to full scale systems. Since acetate concentrations are generally low in full-scale wastewater treatment plants due to common use of CMFRs, this research may suggest that GAOs competition is less important in full-scale systems than previously reported. The transport mechanisms tests and kinetics tests will provide a clearer understanding of PAO and GAO biokinetics and competition, the various Pns/VSS under different conditions will give us a new method to improve the EBPR, while the temperature effect on PAOs and GAOs provides evidences that PAO could be considered to be psychrophilic microorganisms while GAO appear to be mesophilic, which contributes to understand and support the practical observations related to the stability of the EBPR process at

WWTP operated under cold weather conditions.

CHAPTER 4 MATERIALS AND METHODS

4.1 Reactor configuration and operation

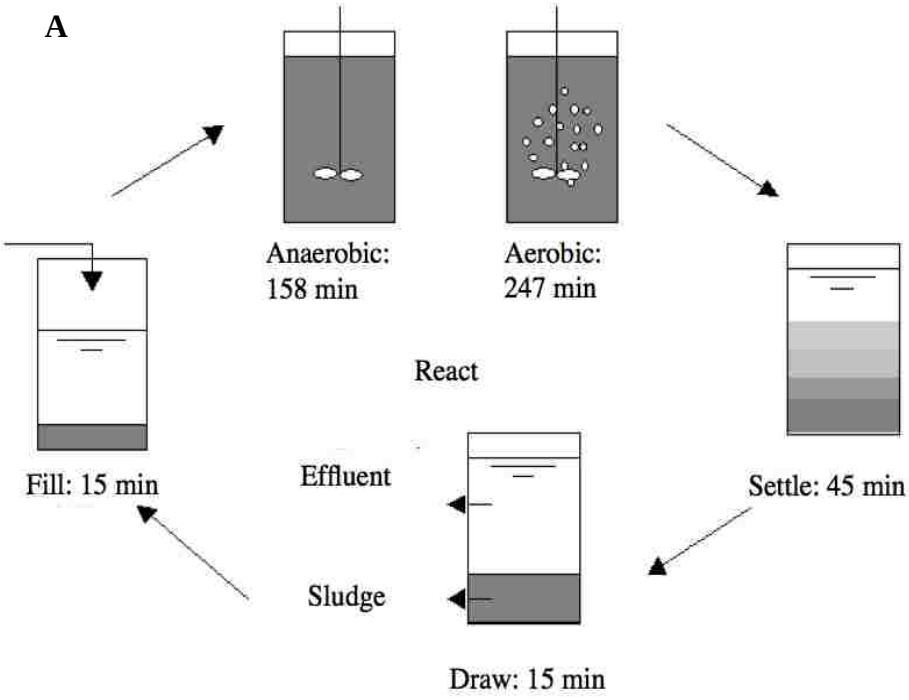
Three anaerobic-aerobic 2 L SBRs were operated (Reactor 1 and Reactor 2 were operated with an 8 h cycle, Reactor 3 was operated as 6 h cycle) (Figure 4.1), as following: Reactor 1 and Reactor 2: Settle phase (45 minutes): no mixing to allow biomass to settle; Draw phase (15 minutes): withdraw 1 L of settled supernatant (50% of volume); Fill phase (15 minutes): add 200 mL of inorganic nutrient feed, 200ml of organic feed and 600 mL DI H_2O, mixing on. The reactor contents were also deoxygenated by bubbling with N_2 gas; Anaerobic phase (158 minutes): 200 mL organic feed was added in the first 10 min; Aerobic phase (247 minutes): bubble ambient air through a porous diffuser.

Reactor HRT's of 16 h, were maintained by withdrawing 1 L of the reactor contents each cycle (3 L/d) and replacing it with 1 L fresh nutrient feed. Operation of feed and effluent pumps, air and nitrogen flow, and mixing was controlled with a programmable controller (Model CD-4, Chrontrol Corp., San Diego, CA). A solids residence time (SRT) of 7 days was maintained by manually withdrawing a portion of the mixed reactor contents once per day during the same cycle immediately prior to the settle phase.

For Reactor 3: Settle phase (40 minutes): no mixing to allow biomass to settle; Draw phase (15 minutes): withdraw 1 L of settled supernatant (50% of volume); Fill phase (15 minutes): add 200 mL of inorganic nutrient feed, 200ml of organic feed and 600 mL DI H2O, mixing on. The reactor contents were also deoxygenated by bubbling with N2 gas; Anaerobic phase (130 minutes): 200 mL organic feed was added in the first 10 min; Aerobic phase (160 minutes): bubble ambient air through a porous diffuser.

Reactor HRT's of 12 h, were maintained by withdrawing 1 L of the reactor contents each cycle (4 L/d) and replacing it with 1 L fresh nutrient feed. Operation of feed and effluent pumps, air and nitrogen flow, and mixing was controlled with a programmable controller (Model CD-4, Chrontrol Corp., San Diego, CA). SRT of 5 days was maintained by manually withdrawing a portion of the mixed reactor contents once per day during the same cycle immediately prior to the settle phase. Attached growth on

the sides of all the 3 reactors was minimized by regular cleaning with a brush. All the 3 reactors were seeded with mixed liquor taken from the City of Albuquerque Wastewater Plant (the Southside Water Reclamation Facility).



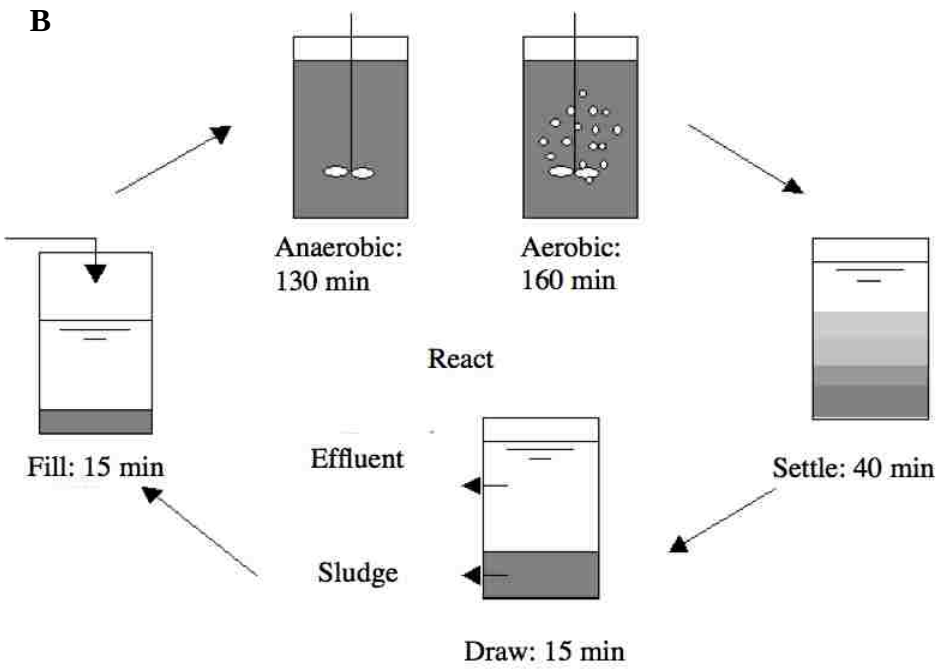


Figure 4.1 (A) Phases in 8 hour sequencing batch reactor cycle, (B) Phases in 6 hour sequencing batch cycle

4.2. Synthetic feed and pH control

Synthetic feed was added as two separate 5X concentrated solutions, the carbon feed and the nutrient feed to reduce the volume of media requiring autoclaving. 200 mL of each of these, with 600 mL deionized water, were added each cycle. The carbon feed, described below, included acetate as the primary carbon source (Table 4.1). The nutrient feed included a nitrification inhibitor (N-Allylthiourea, 98%), and phosphorus (P) in excess so as not to limit PAO activity (Table 4.1). The nutrient feed and carbon feed were added separately. During the nutrient feed addition, the reactor contents were deoxygenated by bubbling with N_2 gas for 15 min, after that the carbon feed was added. Both the carbon and nutrient feeds were autoclaved for 15 min. at 121 °C prior to use. The pH was continuously controlled in the ranges listed below with an auto pH-controller (Chemcadet pH meter/controller, Cole-Parmer Instrument Company, Vernon Hills, IL) by the addition of either a 0.1 M HCl solution or a 0.4 M Na_2CO_3 solution.

The stock carbon feed solution consisted of 937 mg acetate as acetic acid/L (1,000

mg acetate chemical oxygen demand [COD]/L) and 300 mg casamino acids/L (300 mg casamino acids COD/L) (Table 4.1). Per L of reactor volume, 93.7 mg acetate (as acetic acid) (100 mg acetate COD), 30 mg casamino acids (30 mg casamino acids COD) were added each cycle. The influent P/COD ratio was 0.14 mg/mg. The rate of acetate addition was an experimental variable, as described below.

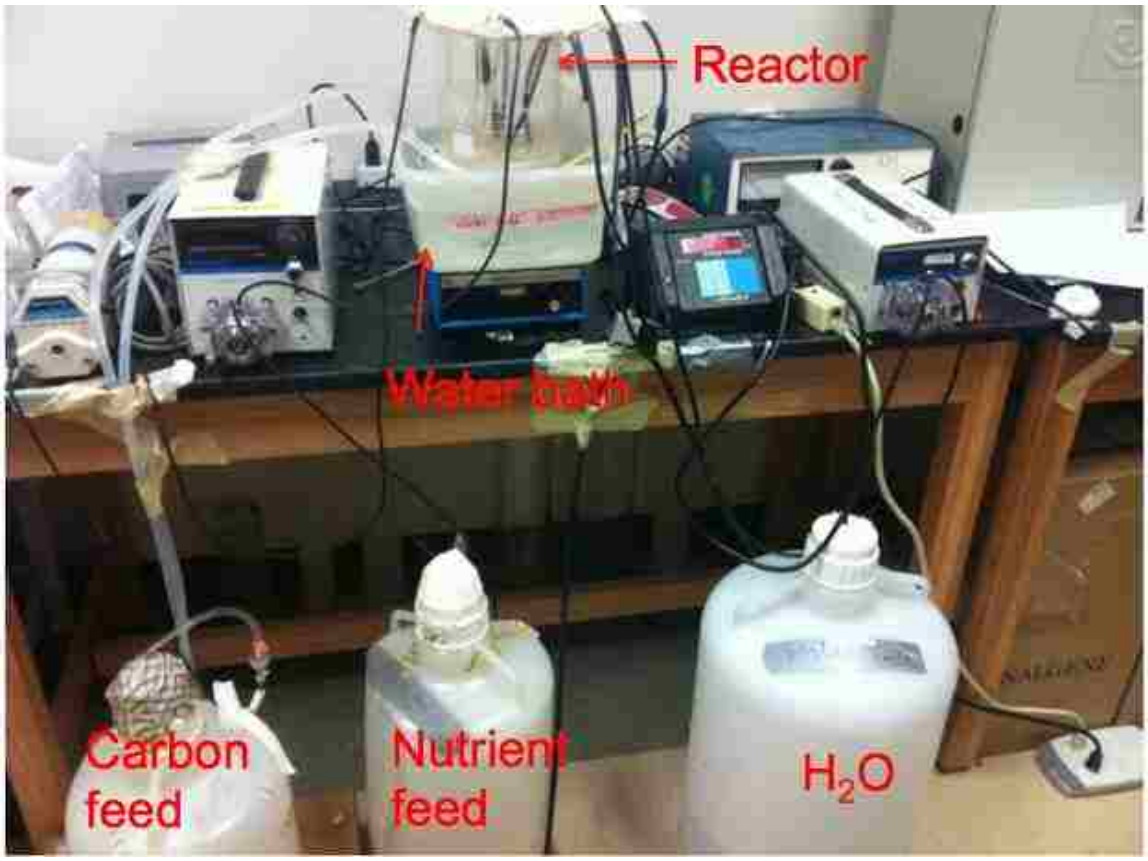


Figure 4.2 SBR and feed

Table 4.1 Reactor synthetic feed

Feed	Stock solution		Net influent concentration	
Carbon feed	dilution = 0.2 mL/mL influent*			
CH_3COONa-$3H_2O$	2124	mg/L	425	mg/L
as CH_3COOH	937.1	mg/L	187	mg/L
Casamino acids	150	mg/L	30	mg/L
Total COD	1300	mg/L	230	mg/L
Nutrient feed	dilution = 0.2 mL/mL influent			
KCl	583.5	mg/L	116.7	mg/L
NH_4Cl	594.5	mg/L	118.9	mg/L
$MgCl_2$-$6H_2O$	1093	mg/L	218.6	mg/L
$MgSO_4$-$7H_2O$	72	mg/L	14.4	mg/L
$CaCl_2$	229.5	mg/L	45.9	mg/L
Yeast extract	41.5	mg/L	8.3	mg/L
Trace element solution	1	mL/L	0.2	mL/L
$FeSO_4$ solution	0.75	mL/L	0.15	mL/L
Phosphate NaH_2PO_4	712.25	mg/L	142.45	mg/L
Trace element stock solution (included in nutrient feed)				
H_3BO_3	305	mg/L	0.061	mg/L
$ZnSO_4$-$7H_2O$	1525	mg/L	0.305	mg/L
KI	75	mg/L	0.015	mg/L
$CuSO_4$-$5H_2O$	305	mg/L	0.061	mg/L
$Co(NO_3)2$-$6H_2O$	375	mg/L	0.075	mg/L
Na_2MoO_4-$2H_2O$	155	mg/L	0.031	mg/L
$MnSO_4$-H_2O	1710	mg/L	0.342	mg/L
$FeSO_4$ stock solution (included in nutrient feed)				
$FeSO_4Z7H_2O$	2026.67	mg/L	0.304	mg/L

4.3. Aeration control

As noted, anaerobic conditions were maintained by bubbling with N_2 gas through a porous diffuser for 15 min before the addition of acetate. Aerobic conditions were maintained by continuous bubbling ambient air through 3 porous diffusers. Anaerobic and aerobic conditions were verified by continuous measurements using an in- reactor

oxygen electrode (M1016-0770, New Brunswick Scientific, Edison, NJ), a dissolved oxygen meter (Model DO-40, New Brunswick Scientific) and a strip chart recorder (Model 288, Rustrak Corporation, Manchester, NH).

4.4. Reactor operation

4.4.1. Reactor 1 operation

The Reactor 1 was operated sequentially in four stages, with pH and rates of acetate addition as experimental variables (Table 4.2). Stage 1 was similar to typical operation used in some previous laboratory-scale SBR studies (Schuler et al. 2001; Filipe et al. 2001b; Filipe et al. 2001c; Schuler and Jenkins 2002; Liu et al. 2007) that enriched for PAOs , with rapid acetate feed addition at the beginning of the anaerobic phase (concentrated acetate feed occurred over 10 minutes) and relatively high pH conditions (7.4-8.4). During Stage 2, rapid acetate addition was maintained, but pH was decreased to the range 6.4 to 7.0 by changing the settings on the pH controller. The objective of Stage 2 was to induce EBPR failure, in accordance with previous studies suggesting that GAOs can out-compete PAOs at lower pH values (Filipe et al. 2001b). During Stage 3 pH was unchanged, but the rate of acetate feed was decreased (acetate addition occurred over 120 minutes rather than 10 minutes), with the objective of decreasing acetate concentrations in the reactor to test whether such conditions may give PAOs a competitive advantage over GAOs under pH conditions that would otherwise favor GAOs. In Stage 4 the reactor conditions were returned to those of Stage 2 to determine whether any observed changes in going from Stage 2 to 3 were reversible. The temperature was controlled at 22 ± 1 ^{o} C through all the 4 stages.

Table 4.2 Reactor 1 operating conditions in difference stages

Stage	Rate and duration of concentrated acetate feed addition	Relative acetate concentration in anaerobic phase	pH	Day range, from first inoculation (duration in days)
1	20 mL/min; 10 min	high	7.4-8.4	301 to 418 (117)
2	20 mL/min; 10 min	high	6.4-7.0	419 to 463 (44)
3	1.7 mL/min; 120 min	low	6.4-7.0	464 to 583 (119)
4	20 mL/min; 10 min	high	6.4-7.0	584 to 624 (40)

4.4.2. Reactor 2 operation

The Reactor 2 was operated sequentially in 5 stages, with the various temperatures, acetate addition rate, and system cycles. Stage 1 was identical to stage 1 in the Reactor 1, with rapid acetate feed addition at the beginning of the anaerobic phase (concentrated acetate feed occurred over 10 minutes), relatively high pH conditions (7.4-8.4), and temperature was controlled at 22 ± 1 ^{o} C. During Stage 2, the only changed condition was temperature, which was increased to 29 ± 1 ^{o} C equipped using a water bath (NESLAB-RTE7, Thermo ELECTRON CORPORATION). In Stage 3, the acetate feed flow rate was decreased so that it was added over the course of 112 minutes, rather

than over 10 minutes as in Stages 1 and 2, with the other operations maintained in the same as Stage 2. In Stage 4, the reactor was operated with a 4 h cycle (shorter than 8 h cycle in the stage 1and 2) and seeded with 200 ml reactor 1 sludge (high PAO biomass), with the other operations were still maintained in the same as Stage 3.

4.4.3. Different operations for Reactor 3

Reactor 3 differed from Reactors 1 and 2 in that the cycle length was 6 h, rather than 8 h, as mentioned above. Corresponding, the HRT was changed to 12h, and the SRT was changed to 5 days. Reactor 3 was operated sequentially in 2 stages, temperature as an experimental variable. Stage 1 was almost identical to stage 1 in the Reactor 1, with rapid acetate feed addition at the beginning of the anaerobic phase (concentrated acetate feed occurred over 10 minutes) and relatively high pH conditions (7.4-8.4), except the cycle length was decreased to 6 hours, HRT was decreased to 12h, and SRT was decreased to 5 days. During Stage 2 the only changed condition was the rate of acetate feed, which was decreased (acetate addition occurred over 120 minutes rather than 10 minutes), with the objective of decreasing acetate concentrations in the reactor to test whether such conditions may give PAOs better condition to grow, the temperature was controlled at 22 ± 1 ^{o} C in both 2 stages.

4.4.4. Batch experiments to measure acetate uptake rate between PAOs and GAOs under variable pH and acetate concentrations

Several batch experiments were conducted on biomass taken from both the PAO- and GAO-dominated SBRs (Stage-3 and Stage-4) by sampling prior to the addition of acetate feed at the beginning of the anaerobic phase. Samples (200 mL) were placed in 250 mL graduated cylinders. Anaerobic condition was maintained by bubbling through an air stone with N_2. The batch tests were conducted at both pH 6.5 and 7.5, and acetate was added either as a pulse (as a single dose with a pipettor), or it was added slowly with a syringe pump at 0.167ml/min to obtain rates of acetate uptake across a range of acetate concentrations for 30 min. Mixing was by a magnetic stir bar and bubbling of N_2. The temperature was controlled at 22 ± 1 ^{o} C.

4.4.5. Inhibition batch tests

Inhibition batch tests were conducted with chemical inhibitors to assess mechanisms of acetate uptake under different acetate concentrations. The samples were all taken from the reactor 1 (for PAOs test, samples were taken from stage-1, for GAOs test, samples were taken from stage-2) at the end of the aerobic period. The tests used 200 mL samples transferred to 250 mL flasks, mixed with a magnetic stirrer. The concentration of N, N'-Dicyclohexylcarbodiimide (DCCD) in the initial test was 50 mM, while the carbonyl cyanide m-chlorophenylhydrazone (CCCP) was 100 mM, as noted. DCCD is an inhibitor of the ATPase, while CCCP is a PMF disruptor (Saunders et al. 2007). All samples were preincubated with DCCD and CCCP for 30 min to allow the reaction to take place and bubbled with N_2 gas for 30 minutes before acetate addition and throughout the test to maintain anaerobic conditions. 200 mg/L of batch test acetate was added either as a pulse using a pipette, which provided relatively high acetate concentrations, or 100mg/L of acetate was added as slowly (using a syringe pump, rate was 0.167ml/min), which provided relatively low acetate concentration. The batch tests were controlled at pH 7.5 $\pm$0.2 for PAOs tests, pH 6.5 $\pm$0.2 for GAOs tests and continuously stirred. Parallel tests were conducted with no inhibitor addition as controls. Rates of acetate and phosphorus release were determined sampling at 30 and 60 min after acetate addition. The temperature was controlled at 22 $\pm$1 ^{o} C.

4.5. Analytical methods

4.5.1. Total suspended solids (TSS) and volatile suspended solids (VSS)

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured on samples taken at the end of the aerobic phase by Standard Methods 2540B and 2540E, respectively (APHA 1992). Unless otherwise noted, suspended solids samples were measured on the samples taken at the end of the aerobic phase.

4.5.2. Phosphate

Soluble orthophosphate (Psol) was on GF/C-filtered samples by the vanado-molybdate colorimetric method (Standard Methods, Method 4500-P C, (APHA 1992)), measured on the samples taken at the end of anaerobic and aerobic phases. Total

orthophosphate (Pt) was by the persulfate digestion method (Standard Methods, Method 4500-P B.5, (APHA 1992)). Non-soluble phosphate (Pns) was calculated as (total P - Psol) for samples taken at the end of the aerobic phase.

4.5.3. Acetate

Acetate was analyzed on GF/C-filtered samples by flame ionization detector gas chromatography (FID GC), using a J&W Scientific DB-FFAP 0.53 mm capillary column with 2 µL injection volumes. Samples were acidified with concentrated phosphoric acid and stored at 4 ^{o} C prior to analysis. The carrier gas was nitrogen with a flow rate of 15 mL/min, hydrogen flow rate was 20 mL/min. and the air flow rate was 250 mL/min. to the FID. Oven temperature began at 90 ^{o} C ramped to 110 ^{o} C at 50 ^{o} C /m, remained at 110 ^{o} C for 30 sec., and then ramped to 130 ^{o} C at 50 ^{o} C / min. Injector temperature was 250 ^{o} C.

4.5.4. Neisser staining

Neisser Staining was as described in (1981) for microscope visualization of polyphosphates stored in microbial cells. Unless noted otherwise, stained samples were examined under oil immersion at 1000x magnification with direct illumination (bright field).

4.5.5. Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was carried out on fixed samples of biomass according to the method of from The University of Tokyo. Fluorescence *in situ* hybridization (FISH) probes used in this study were: PAO462, PAO641 and PAO846 to target *Accumulibacter* (Crocetti et al. 2000); GAOQ989 (Crocetti et al. 2002) and GB_G2 (Kong et al. 2002) to target *Competibacter*; DF1MIX (TFO_DF218 plus TFO_DF618) for the cluster 1 *Defluviicoccus spp.* (Wong et al., 2004), and DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) for cluster 2 *Defluviicoccus spp.* (Meyer et al. 2006). All microscopic examinations were carried out using Olympus BX51 microscope. Fluorescence *in situ* hybridization quantification was carried out by digital image analysis of FISH images using Image-J. Samples were hybridized with the Cy3-labelled PAOMIX probes and FAM-labelled EUBMIX probes for *Accumulibacter* spp. identification;

samples were hybridized with the FAM-labelled *Competibacter* probes and TAMRA-labelled EUBMIX probes for *Competibacter* spp. identification; samples were hybridized with the Cy5-labelled DFMIX probes and FAM-labelled or TAMRA-labelled EUBMIX probes for *Defluviicoccus* spp. identification.

4.5.6. Pyrosequencing analyses

Pyrosequencing analyses were performed by Research and Testing Laboratories (RTL) (Lubbock, TX). Biomass samples were washed in phosphate buffer solution and shipped frozen. Total genomic DNA was extracted from fecal samples using a QIAamp stool DNA mini kit and its manufacturers suggested methods (Qiagen, Valencia, CA). DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). All DNA samples were adjusted to 100ng/ µ l. A 100ng (1 µ a aliquot of each samples DNA was used for a 50 µ l PCR reaction.

The 16S universal Eubacterial primers the primers 28f (GAG TTT GAT CNT GGC TCA G) and 519r (GTN TTA CNG CGG CKG CTG) were used for amplifying the 600bp region of 16S rRNA genes. HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used for PCR under the following conditions: 94 ^{o} C for 3 minutes followed by 30 cycles of 94 ^{o} C for 30 seconds; 60 ^{o} C for 40 seconds and 72 ^{o} C for 1 minute; and a final elongation step at 72 ^{o} C for 5 minutes. A secondary PCR was performed for 454 amplicon sequencing under the same condition by using designed special fusion primers with different tag sequences. After secondary PCR all amplicon products from different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). In preparation for FLX sequencing (Roche, Nutley, New Jersey), the DNA fragments' size and concentration were accurately measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA). All FLX procedures were performed using Genome Sequencer FLX System manufacturer's instructions (Roche, Nutley, New Jersey).

Custom software written in C# within a Microsoft.NET (Microsoft Corp, Seattle,

WA) development environment was used for all post sequencing processing. Discussion of software code is outside the scope of this report; however, a brief description of the algorithm follows. Quality trimmed sequences obtained from the FLX sequencing run were processed through a custom scripted bioinformatics pipeline. In short, quality trimmed sequencing reads were originated directly from FLX sequencing run output files. Tags were obtained from the multi-FASTA file into individual sample specific files based upon the tag sequence. Tags which did not have 100% homology to the sample designation were not considered. Sequences which were less than 150 bp after quality trimming were not considered. The resultant individual sample after parsing the tags into individual FASTA files were compiled by CAP3 (Huang and Madan 1999). The ace files created by CAP3 were then processed to produce a secondary FASTA file containing the tentative consensus (TC) sequences of the association with the number of reads integrated into each consensus. TC were required to have at least 3-fold coverage. The resulting TC FASTA for each sample was then evaluated using BLASTn (Altschul et al. 1990) against a database of high quality 16s bacterial sequences (Research and Testing Laboratory, Lubbock, TX).

Based upon the above BLASTn derived sequence identity (percent of total length query sequence which aligns with a given database sequence) and validated using taxonomic distance methods the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level, 80 and 85% at the class and 77% to 80% at phyla. After resolving based upon these parameters, the percentage of each bacterial and Fungal ID will be individually analyzed for each sample providing relative abundance information within and among the individual samples based upon relative numbers of reads within each. Evaluations presented at each taxonomic level, including percentage compilations represent all sequences resolved to their primary identification or their closest relative (Callaway et al. 2010; Finegold et al. 2010; Olafson et al. 2010; Pitta et al. 2010; Smith et al. 2010; Stephenson et al. 2010; Williams et al. 2010; Bailey et

al. 2010a; Gontcharova et al. 2010a; Bailey et al. 2010b; Gontcharova et al. 2010b; Andreotti et al. 2011; Handl et al. 2011; Ishak et al. 2011).

CHAPTER 5. ACETATE CONCENTRATION EFFECTS ON EBPR

The primary objective of this part of the research was to determine the effect of low acetate concentrations on PAO/GAO competition under low pH conditions previously thought to favor GAOs. As noted, these effects may be important because research systems have historically been conducted in SBRs, which yield higher substrate concentrations than do the completely mixed systems typically used in full-scale systems. The research approach was to operate Reactor 1 with variable pH values and rates of acetate addition to manipulate the reactor acetate concentrations, and to monitor change in reactor performance and dominance by PAOs and GAOs, as described in the below section (Table 5.1).

5.1 Stage 1: High pH, rapid acetate addition

Stage 1 consisted of high pH (7.4-8.4) and relatively rapid acetate addition over 10 minutes (Table 5.1), which was similar to conventional SBR operation used in previous studies (Schuler et al. 2001). Concentrations of acetate and phosphorus during a typical anaerobic/aerobic cycle are shown in Figure 5.1. Acetate concentrations at the end of the 10 minute acetate addition period were approximately 79 mg/L (Figure 5.1), which was less than the total 100 mg of acetate added/L of reactor volume due to acetate uptake during the feed addition. Acetate uptake was complete after approximately 60 minutes. Figure 5.1 also shows increasing P concentrations, since PAOs could degrade their intracellular PP, release to the liquid as inorganic phosphate.

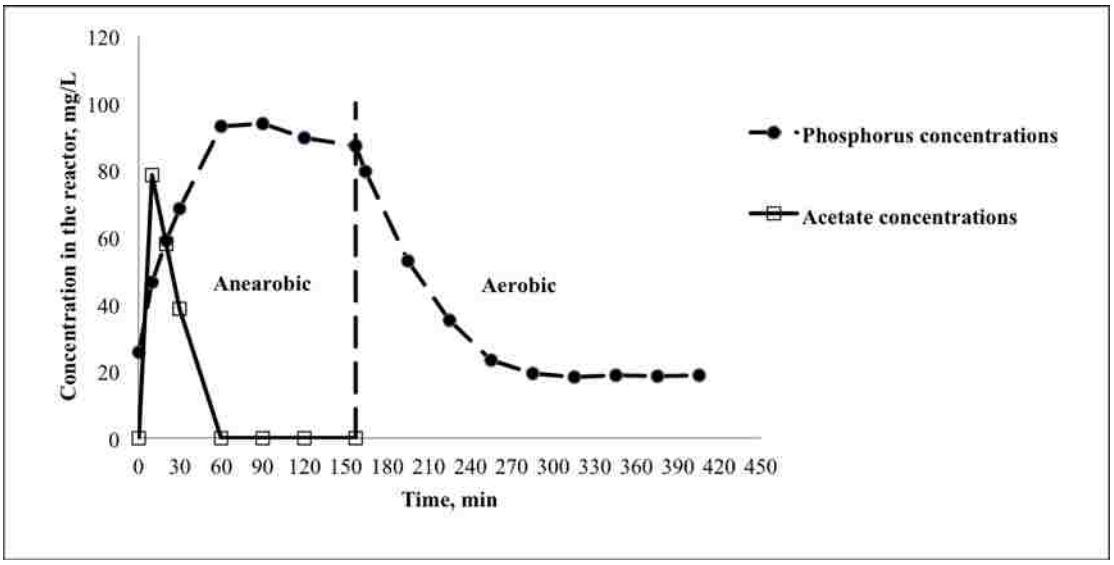


Figure 5.1 Acetate and phosphorus profiles throughout anaerobic and aerobic phases with rapid acetate addition (Sample taken on day 315)

The reactor conditions and performance during the four stages are summarized in Table 5.1 Several measurements indicated that the Stage 1 culture was highly enriched with PAOs, consistent with previous studies that used similar conditions to enrich for PAOs (Schuler et al. 2001). First, the biomass had a relatively high phosphorus content (non-soluble P/VSS [Pns/VSS] of 0.20 ±0.016 mg/mg, [Pns/TSS] of ±0.007 mg/mg Table 5.1), compared to typical bacterial biomass content at Pns/TSS of 0.02 mg/mg (Bond et al. 1998; Schuler and Jenkins 2003a), and this was evidence for high amounts of polyphosphate storage. Second, the relatively high average anaerobic P release/acetate uptake (Prel/Ac) ratio values during Stage 1 indicated PAO dominance. This ratio has been previously suggested as an indicator of relative PAO and GAO activity, with higher values indicating greater relative PAO activity, since PAOs release P during acetate uptake but GAOs do not (Schuler and Jenkins 2003a). The Stage 1 Prel/Ac ratio of 0.65 ± 0.09 mol/C-mol was greater than the 0.5 mol/C-mol value suggested by Schuler and Jenkins (2003a) as evidence for PAO dominance.

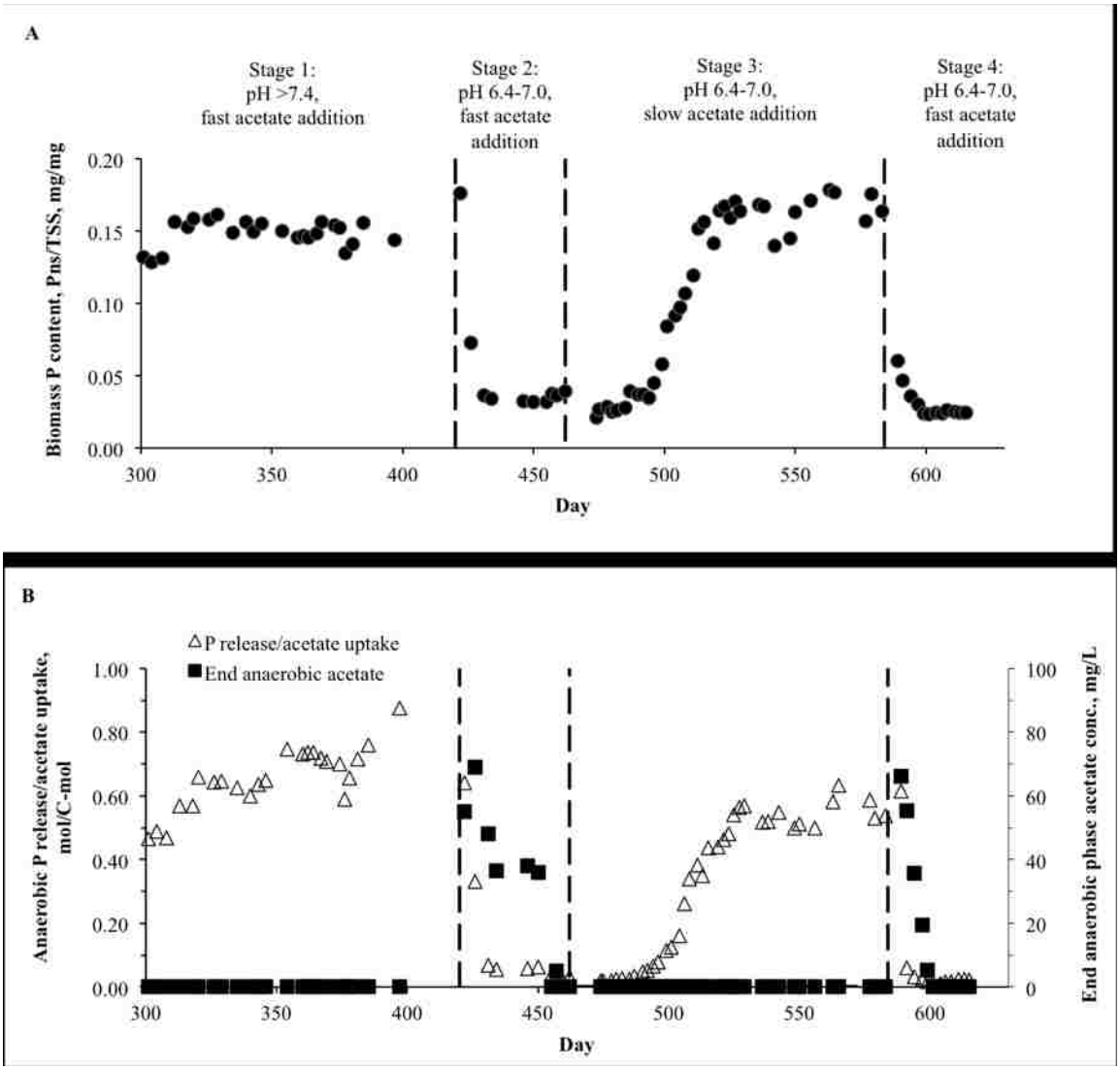


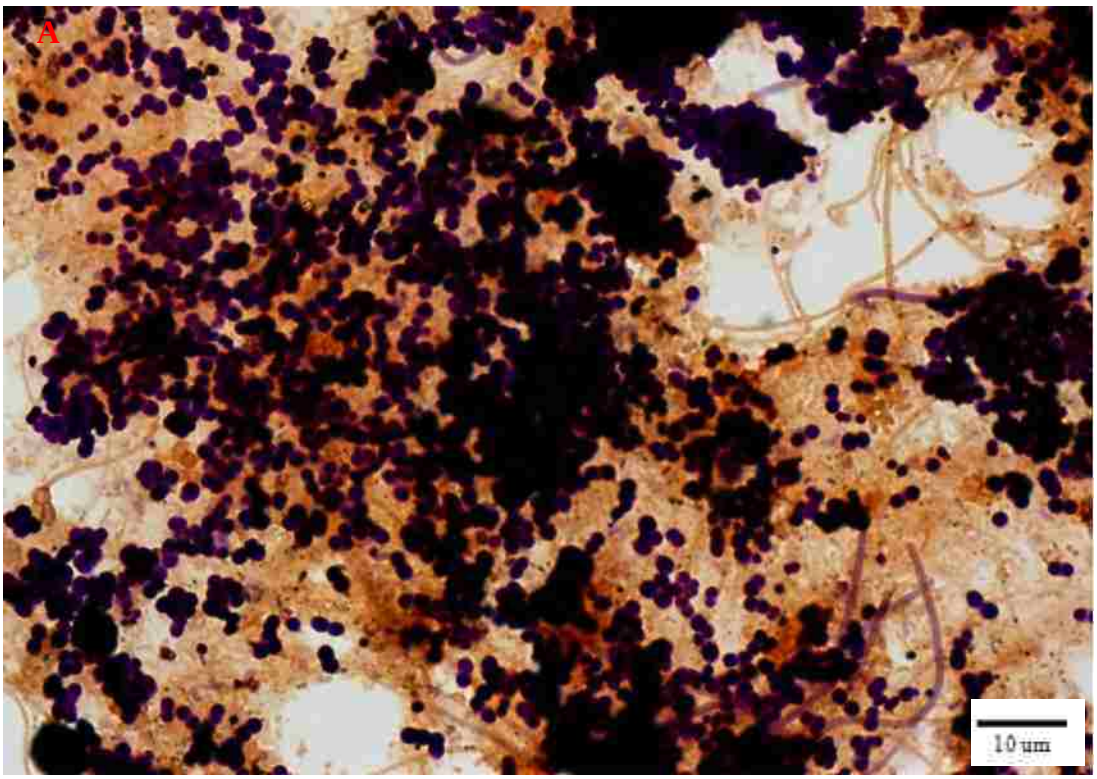
Figure 5.2 Reactor performance with respect to (A) phosphorus content of the biomass (Pns/VSS), and (B) anaerobic P release/acetate uptake and end anaerobic acetate concentrations during four Stages of operation with variable pH and rates of acetate addition

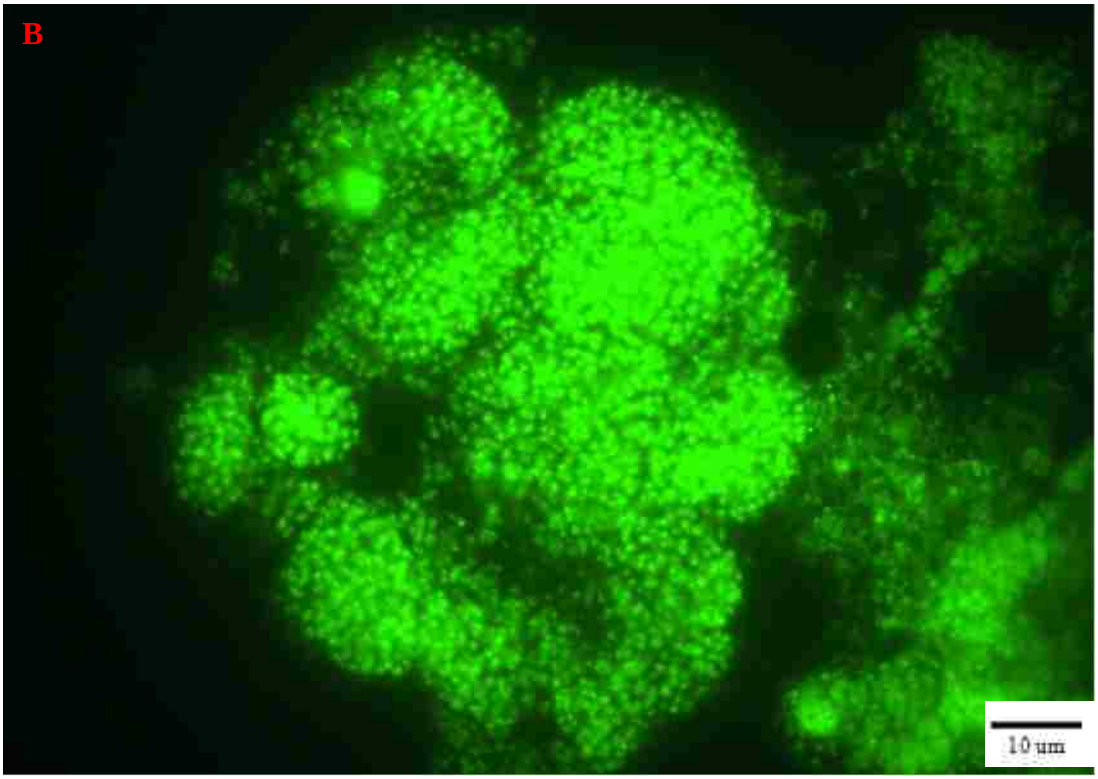
Table 5.1 Reactor characteristics over 4 stages

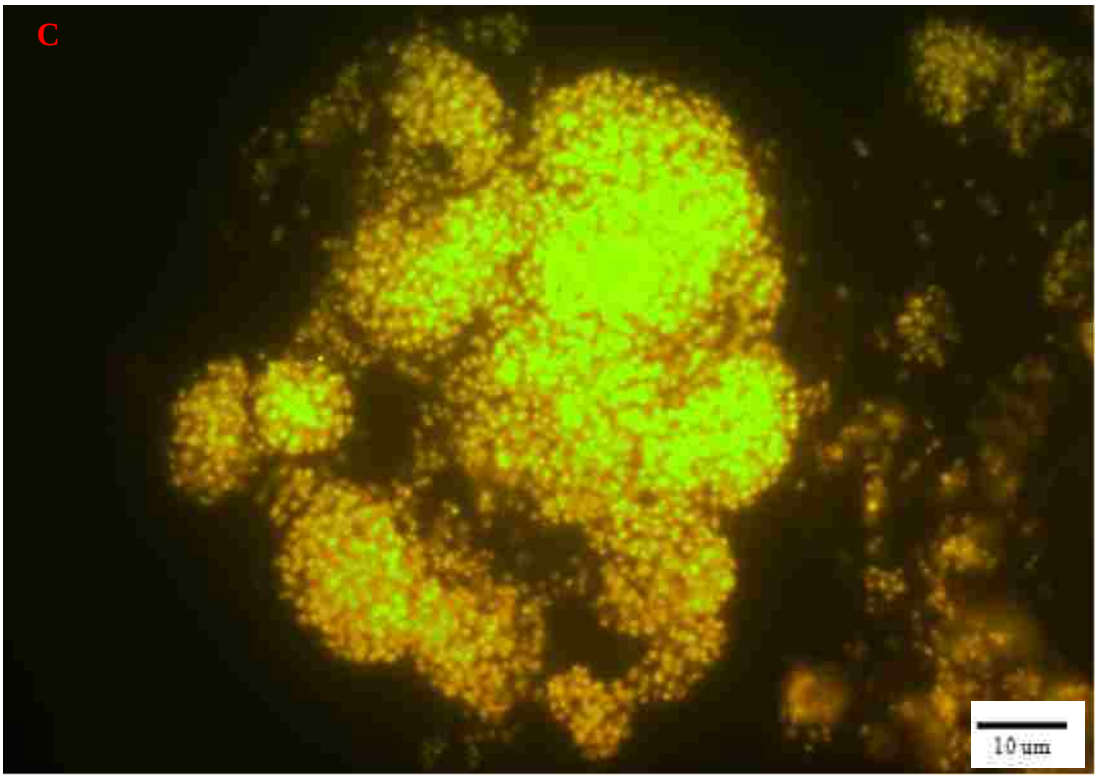
Stage	Rate and duration of concentrated acetate feed addition/pH	Relative acetate concentration in anaerobic phase	Pns/TSS (mg/mg)	Pns/VSS (mg/mg)	Anaerobic P release/acetate uptake (mol/C-mol)	Day range (duration)
1	20 ml/min; 10 min/7.4-8.4	high	0.15 ±0.009	0.20 ±0.016	0.65 ±0.053	301 to 418 (117)
2	20 ml/min; 10 min/6.4-7.0	high	0.04 ±0.003*	0.04 ±0.004*	0.04 ±0.012*	419 to 463 (44)
3	1.7 ml/min; 120 min/6.4-7.0	low	0.16 ±0.01*	0.28 ±0.02*	0.55 ±0.043*	464 to 583 (119)
4	20 ml/min; 10 min/6.4-7.0	high	0.02 ±0.001*	0.03 ±0.001*	0.02 ±0.005*	584 to 624 (40)

* Average value after stage reached to a new steady state

Third, microscopic examination confirmed that the Stage 1 conditions enriched for a highly PAO-dominated culture. Neisser staining indicated a large quantity of polyphosphate-containing cocci (Figure 5.3A), which is consistent with the PAO phenotype. Fourth, FISH analyses indicated 82 ± 11% of the bacteria were positive for the PAOmix probe targeting *Accumulibacter spp.* (Figures 5.3B and 5.3C) in samples taken during Stage 1. *Competibacter spp.* and cluster 1 *Defluviicoccus spp.* were absent. Cluster 2 *Defluviicoccus spp.* were present at 4 ± 2% of the bacteria.







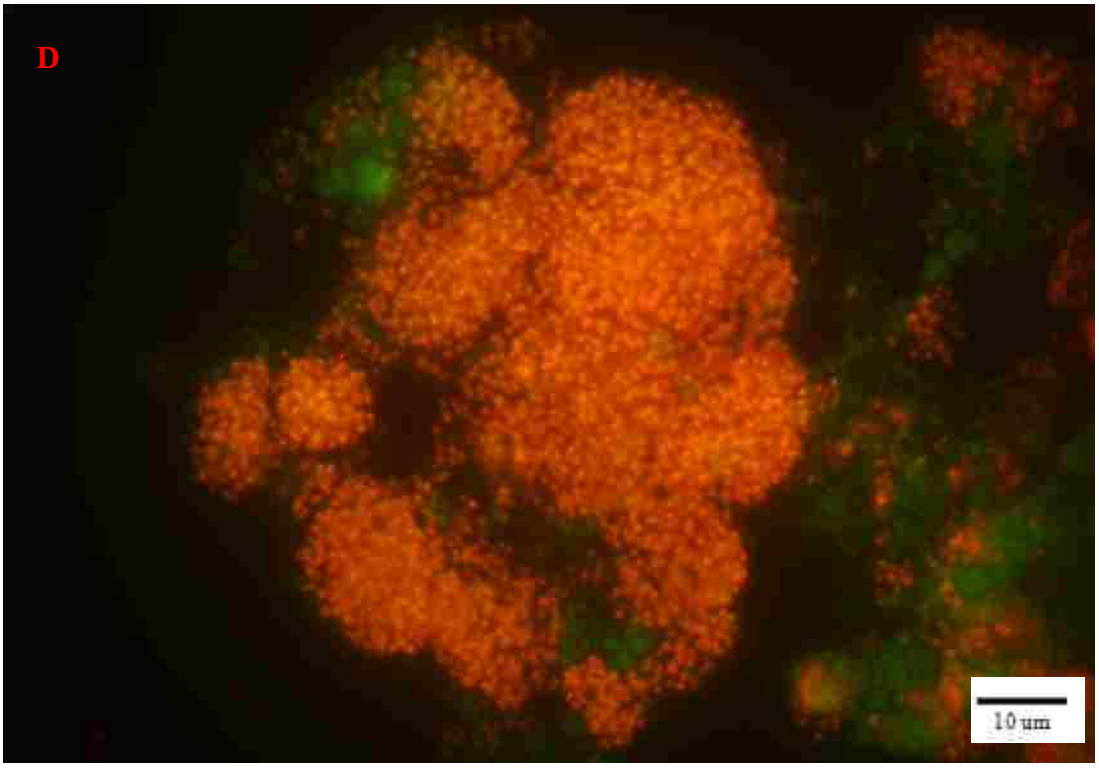


Figure 5.3 Typical Neisser Staining and FISH results on activated sludge samples taken Stage 1 (Day 385). Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser stain, dark cells were PAOs, suggesting the accumulation of polyphosphate storage, (B*)* FISH with EUBMIX probe (FAM) targeting most bacteria (green), (C) FISH with PAOMIX (Cy3) specific for *Accumulibacter spp*. (orange), and (D) Overlay (B) with (C), *Accumulibacter spp*. appear orange.

5.2 Stage 2: Low pH, rapid acetate addition

Stage 2 was initiated by decreasing the reactor pH to the range 6.4-7.0 on Day 419, while maintaining the same high rate of acetate feed addition (Table 5.1). This change led to a decline in PAO activity, as indicated by a drop in the Pns/VSS and Prel/Ac values after the change was made (Figure 5.2A and Table 5.1). Another indication that PAO activity decreased was that acetate began to "leak" into the aerobic phase after the pH was decreased, with end anaerobic acetate concentrations increasing from zero to a maximum of 69 mg/L 7 days after the pH change (Figure 5.2B), indicating that only 31 mg/L was anaerobic taken up of the total 100 mg/L acetate added at the beginning of the anaerobic phase.

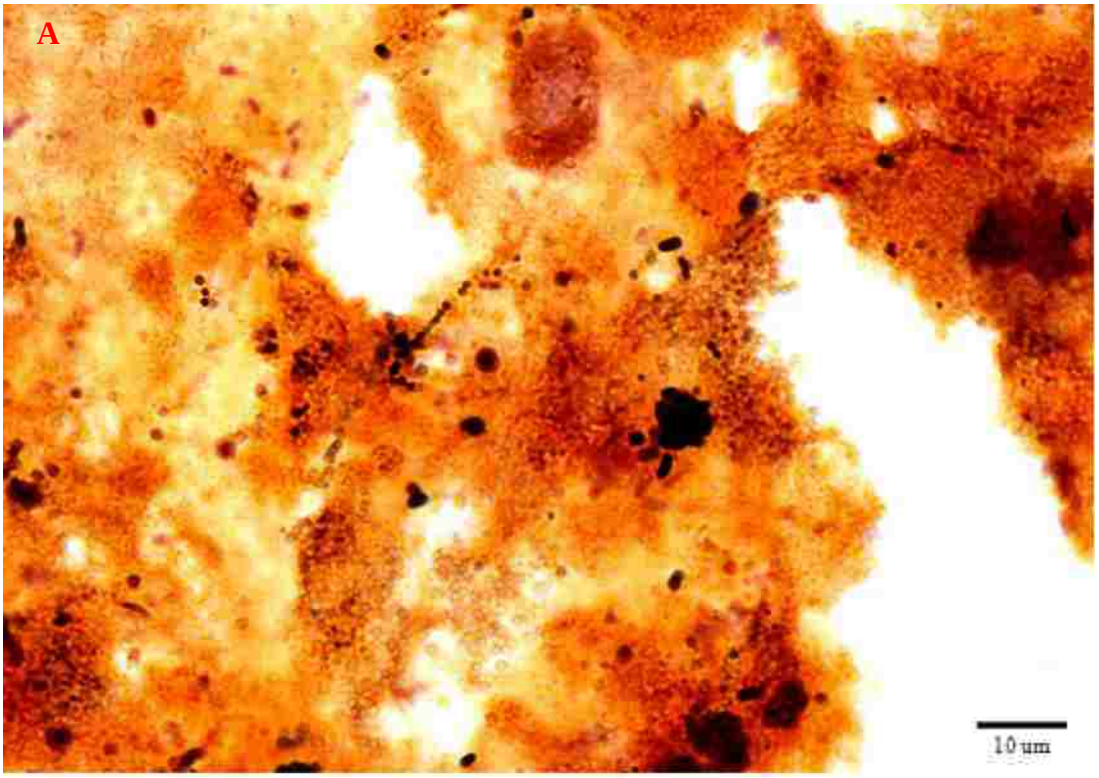
The presence of acetate in the reactor at the end of the anaerobic phase after the pH decrease (Figure 5.2B) suggested that increased competition from GAOs for this

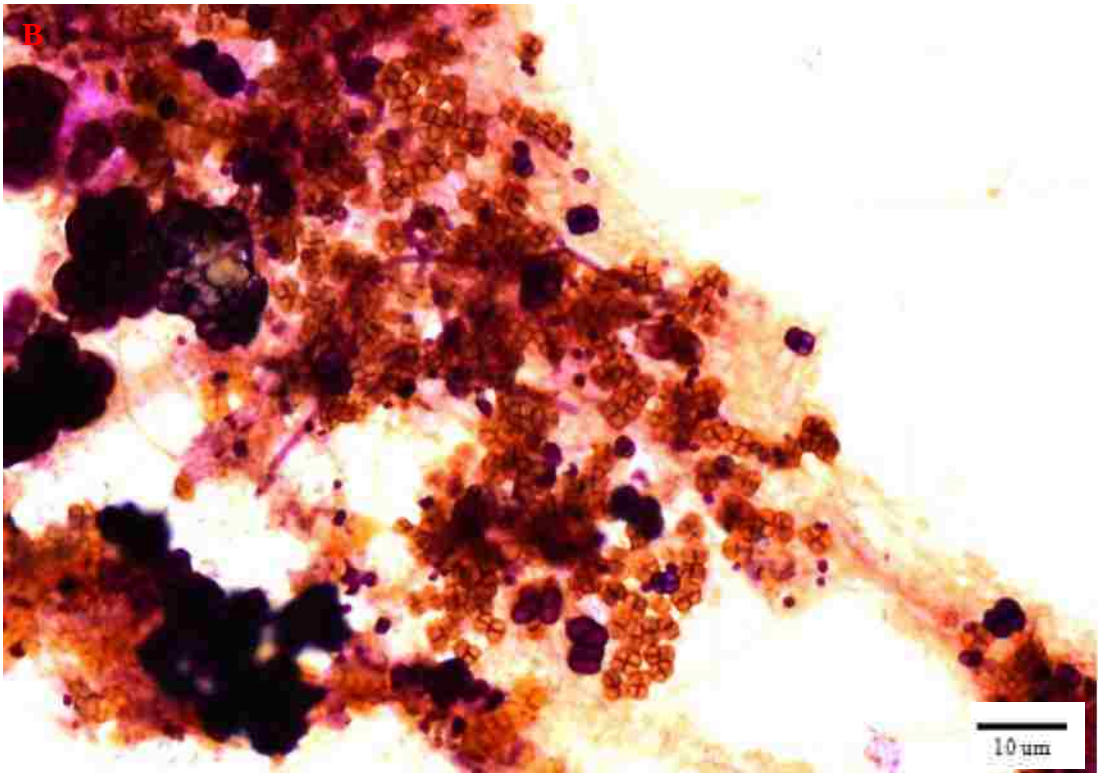
substrate was not the immediate cause of the PAO decline, since acetate was plentiful for both groups during the anaerobic phase after Stage 2 began. Anaerobic acetate uptake gradually recovered thereafter, until it was once again complete on Day 455, 36 days after the pH was reduced to begin Stage 2 (Figure 5.2B).

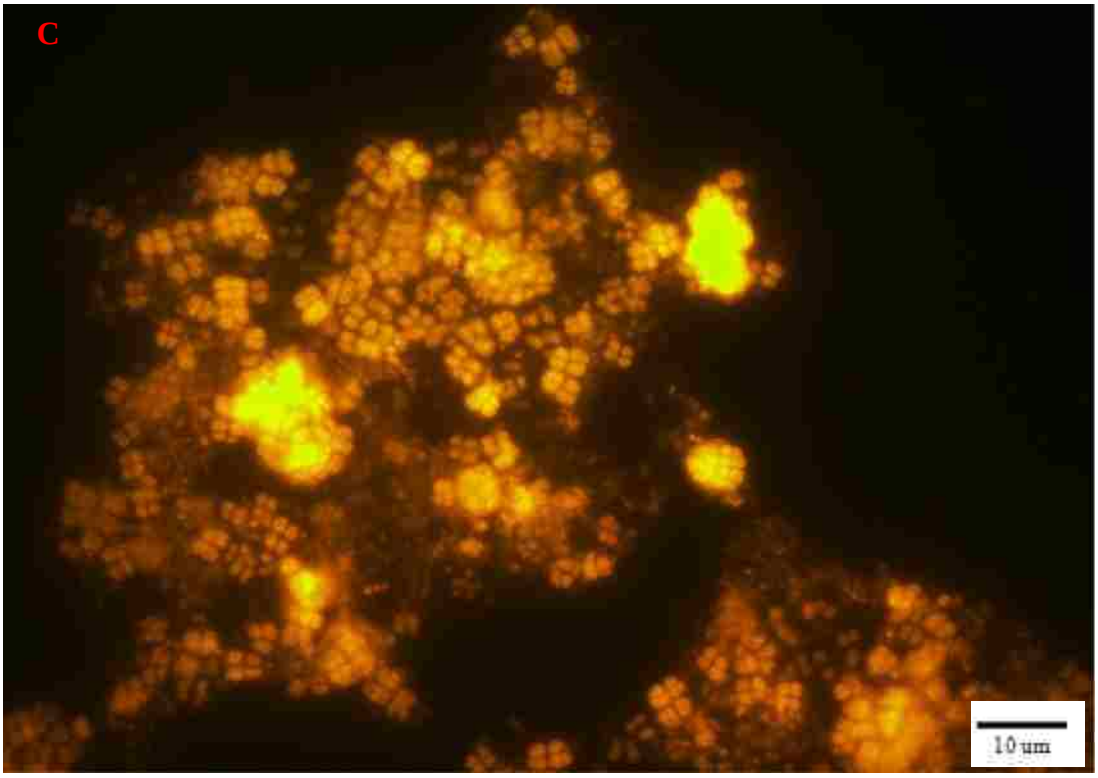
After day 455 and through the end of Stage 2 (day 463), the average Pns/VSS, Pns/TSS and Prel/Ac uptake values were 0.04 ±0.004 mg/mg, 0.04 ±0.003 mg/mg and 0.04 ±0.01 mol/C-mol, respectively (Table 5.1); these low values indicated that the GAOs had begun to dominate the culture after day 455. That GAO activity increased only after the PAOs had failed suggested that the GAOs were opportunists that were able to take up the newly-available acetate availability after the PAO failure, rather than being the initial cause of the PAO failure. Bacteria with tetrad morphology only appeared in late Stage 2 (after approximately day 455), which supported the idea that GAOs only began to dominate the culture at this time. Quantitative FISH showed that cluster 2 *Defluviicoccus spp.* represented 33 ±7% of the bacteria, Cluster 1 *Defluviicoccus spp*. comprised about of 2.1 ±0.7% the bacteria. *Competibacter spp*. and *Accumulibacter spp.* were absent (Figure 5.4C and 5.4D) in the later stage 2.

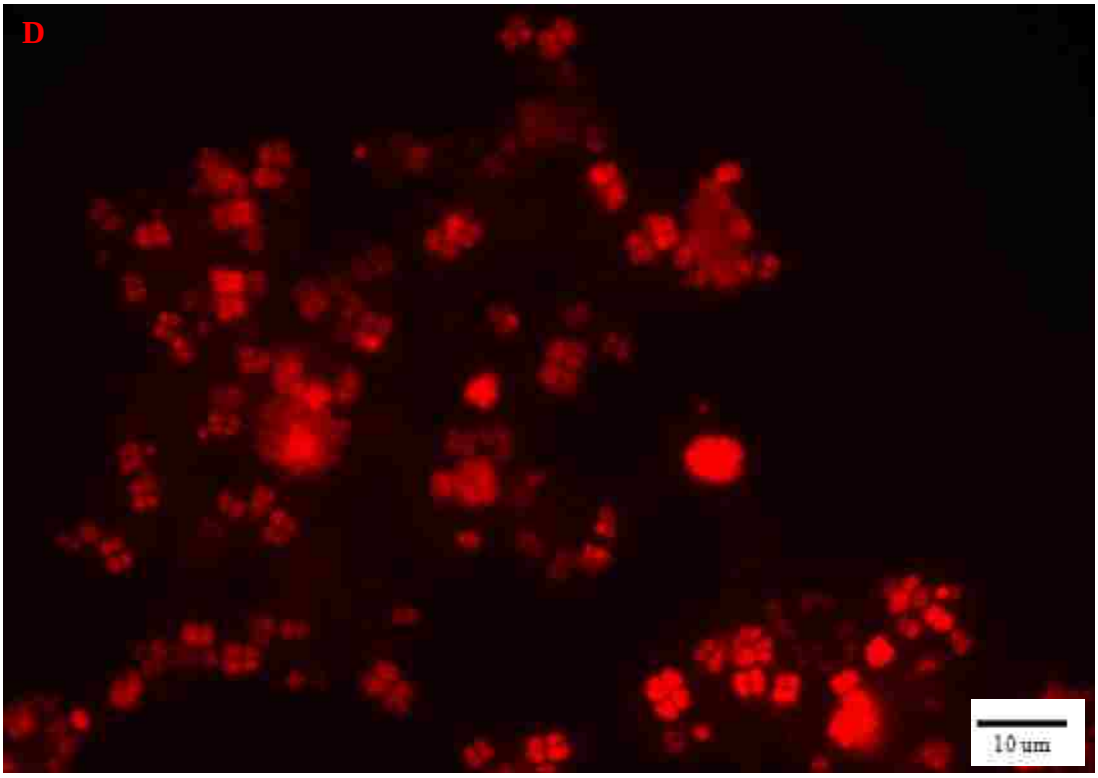
Stages 1 and 2 confirmed thatdecreasing the reactor pH from 7.4-8.4 to 6.4-7.0 led to a loss of phosphate-removing capability and a change of microbial populations from PAOs to GAOs. This result was consistent with previous research (Liu et al. 1996; Jeon et al. 2001; Filipe et al. 2001b; Schuler and Jenkins 2002; Serafim et al. 2002; Oehmen et al. 2005a) suggesting the higher pH values may favor PAOs over GAOs.

Different trends of pH variation during the anaerobic phases, were observed in the PAO-dominated Stage 1 and the GAO-dominated late Stage 2. In the case of PAO enriched culture, pH tended to decrease (from approximately 7.7 to 7.4) during the anaerobic phase, while in the GAO dominated culture,), pH tended to increase (from approximately 6.4 to 6.6) during the anaerobic phase. Both system pH values tended towards neutral pH at the end of anaerobic time regardless whether the initial pH was alkaline or acidic which have been attributed to intracellular pH regulation (Padan et al. 1981). The pH generally increased in both the Stage 1 and late Stage 2 systems during the aerobic phase, possibly due to the stripping of CO_2 during the aeration.









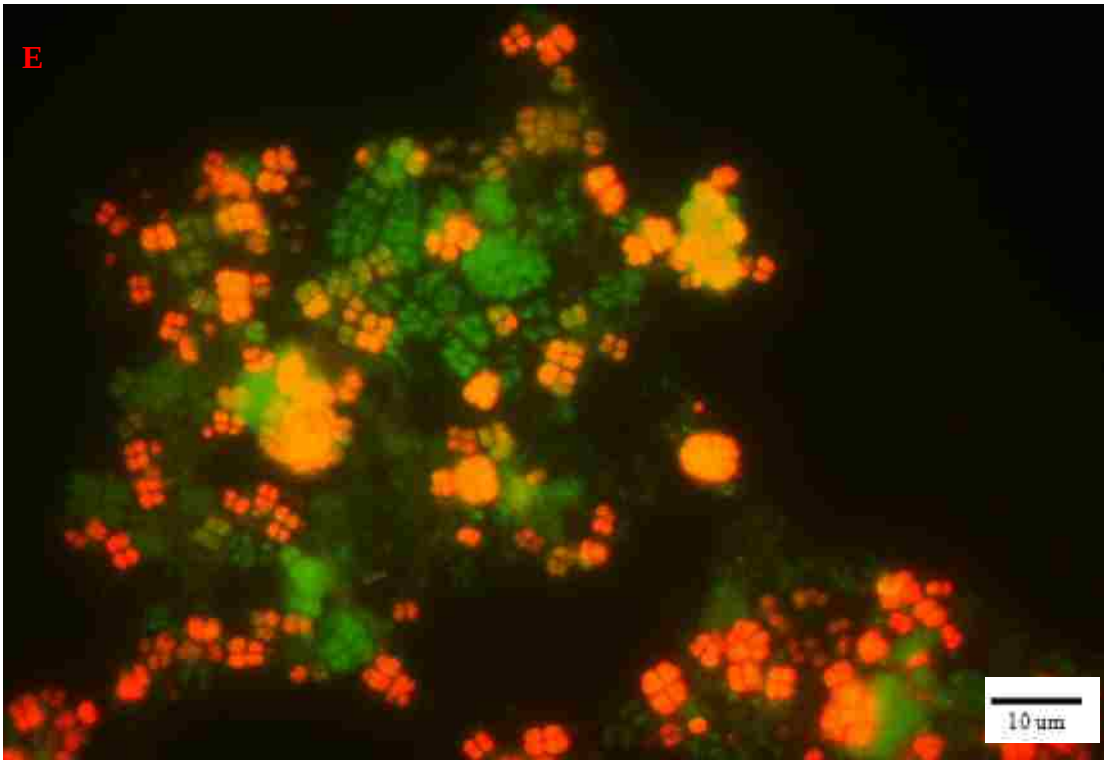


Figure 5.4 Neisser Staining and FISH results on reactor samples taken Stage 2. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser stain of early Stage 2 sample (day 447), when anaerobic acetate uptake was not complete, showing few to no PAOs or tetrads-forming GAOs (B) Neisser stain for late Stage 2 day 460), after anaerobic acetate uptake was complete, showing many tetrads typical of some GAOs, (C) FISH image of late Stage 2 (day 462) sample with probe EUBMIX (TAMRA) targeting most bacteria (orange), (D) FISH image of late Stage 2 (day 462) sample with probes DF2MIX (DF988, DF1020 (Cy5) specific for cluster 2 *Defluviicoccus spp*. (red), and (E) Overlay (C) with (D), *Defluviicoccus spp*. appear orange.

5.3 Stage 3: Low pH, slow acetate addition

In Stage 3, the acetate feed flow rate was decreased so that it was added over the course of 112 minutes, rather than over 10 minutes as in Stages 1 and 2, with the pH maintained in the same range as Stage 2 (6.4-7.0). The decreased rate of acetate feed addition accomplished the goal of maintaining low acetate concentrations during the anaerobic phase, with acetate concentrations less than the detection limit during the anaerobic and aerobic phases. Acetate was never detected at the end of the anaerobic phase, indicating that anaerobic acetate uptake was complete through Stage 3 (Figures 5.2 and 5.5).

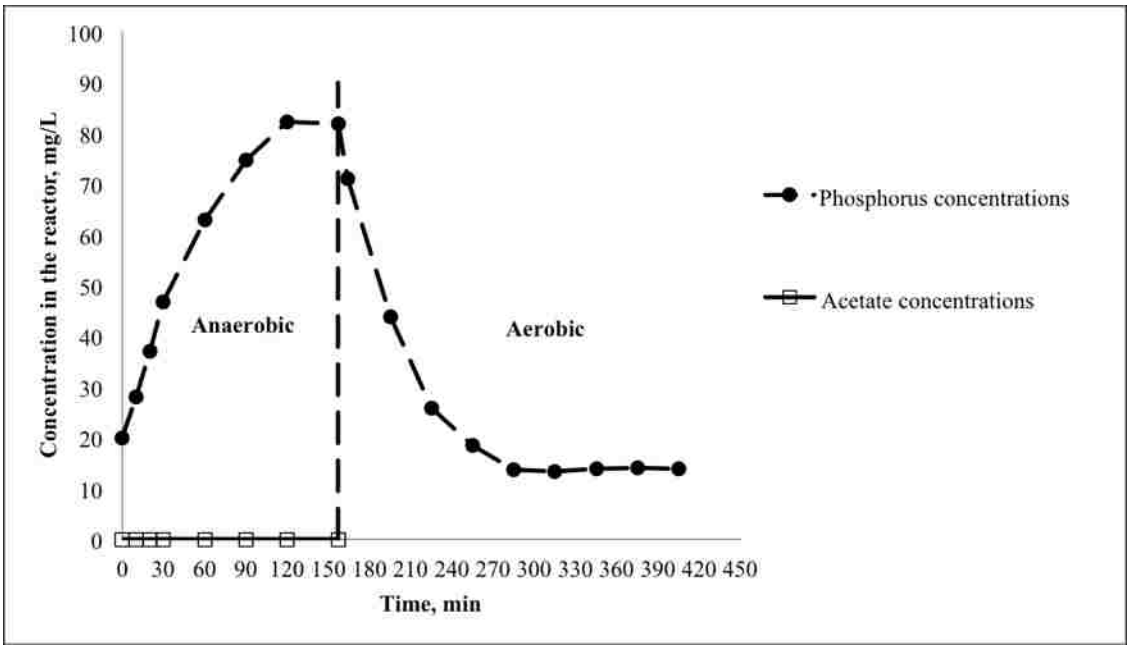
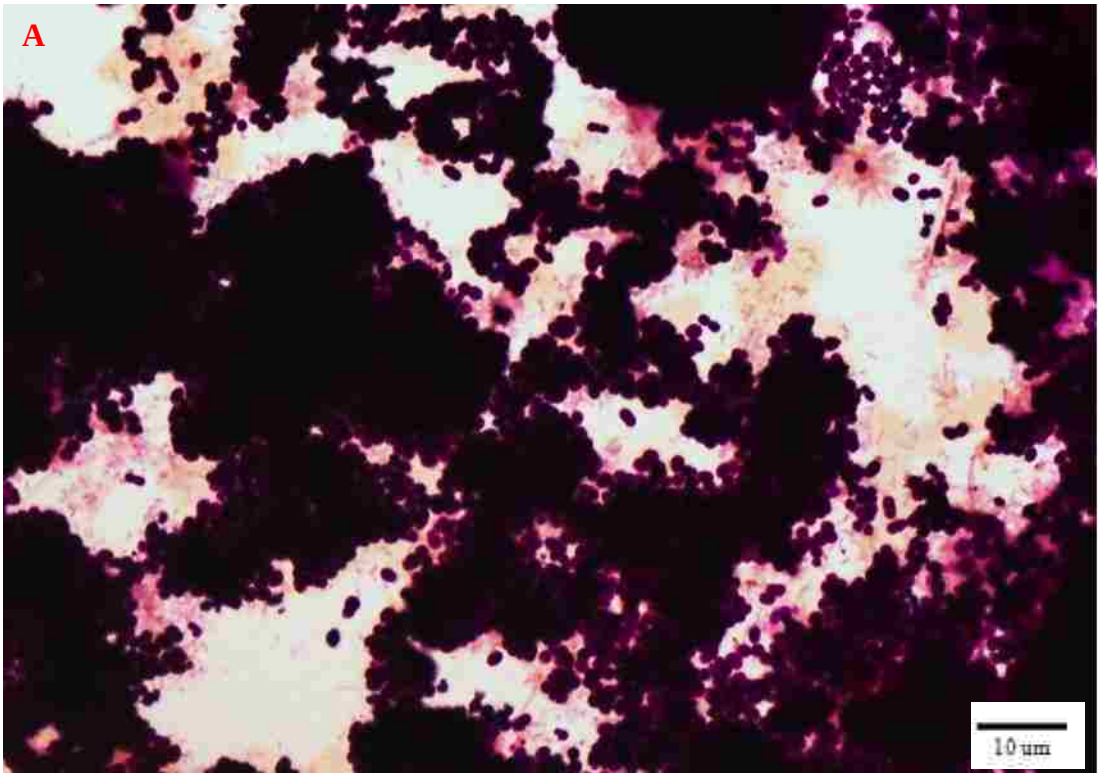
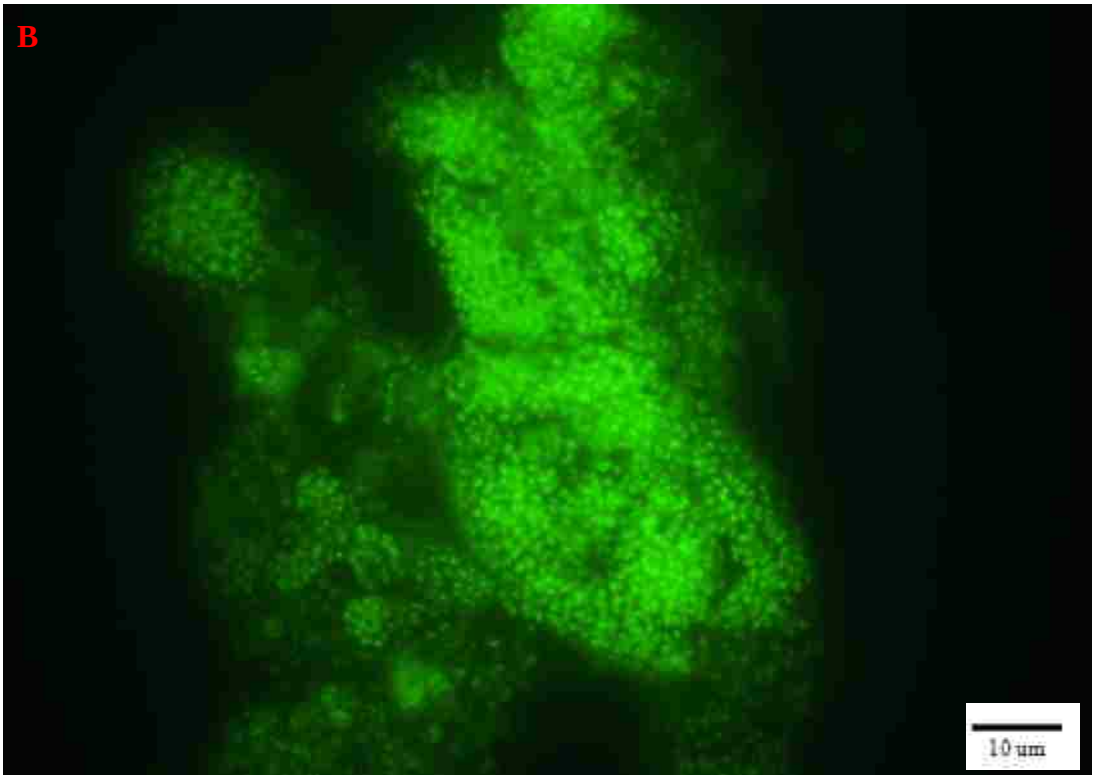


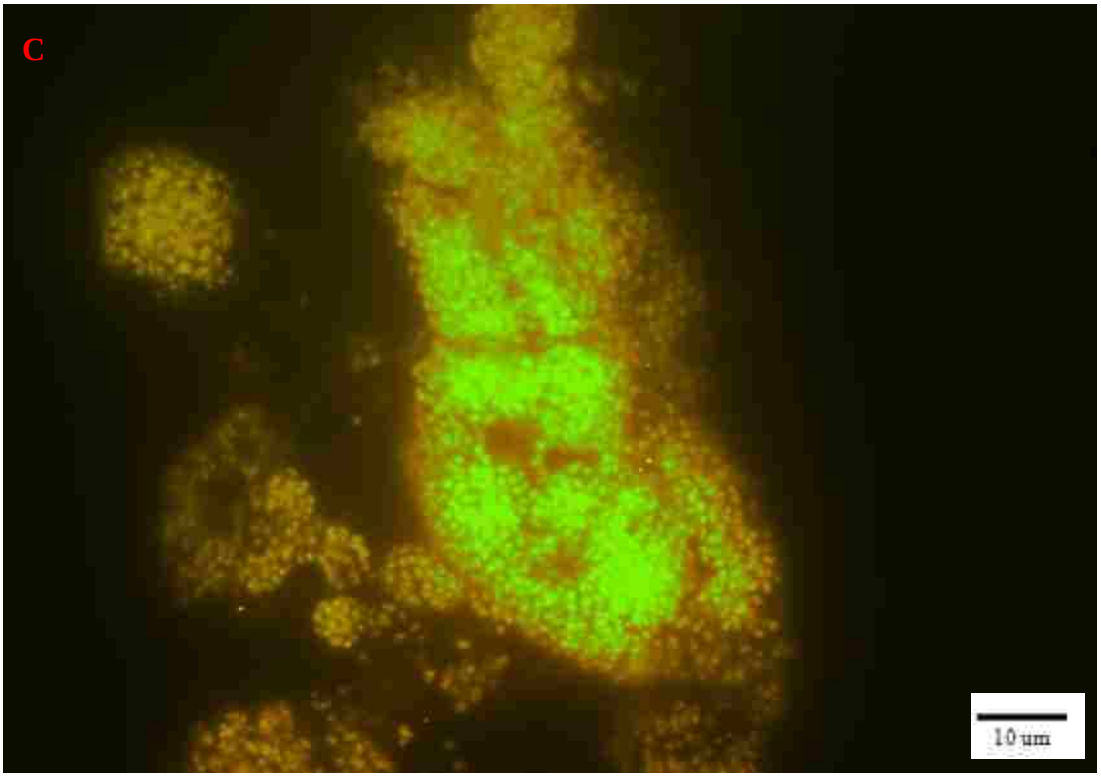
Figure 5.5 Acetate and phosphorus profiles throughout anaerobic and aerobic phases with slow acetate addition (Sample taken on day 567)

During the first 60 days of Stage 3, the Prel/Ac ratios and biomass phosphorus contents gradually increased, suggesting that the PAOs returned to dominate the reactor (Figure 5.2). The Prel/Ac ratio increased steadily from 0.02 (Day 474) to 0.48 (Day 523) mol/C-mol (Figure 5.2B), with an average value of 0.55± 0.04 from Day 523 until the end of Stage 3 (Table 5.1). Similarly, the biomass phosphorus content (Pns/VSS) increased from 0.02 to 0.26 mg/mg, Pns/TSS increased from 0.02 to 0.16 mg/mg over this time range (Figure 5.2A), with the average values of 0.28 ± 0.02mg/mg (Pns/VSS) and 0.16 ± 0.01mg/mg (Pns/TSS) from Day 523 until the end of Stage 3.

Microscopy confirmed that the PAO populations recovered during Stage 3. Neisser staining indicated the culture was once again dominated by PAOs by the end of this Stage (Figure 5.6A). Similarly, FISH analyses indicated 77 ±11% of the bacteria were *Rhodocyclusspp.* targeted by the PAOmix probe set (Figures 5.6B and C). *Competibacter spp.* and cluster 1 *Defluviicoccus spp.* were not detected. Cluster 2 *Defluviicoccus spp.* were around 5 ±1% of the bacteria. The results were consistent with the FISH results from Stage 1.







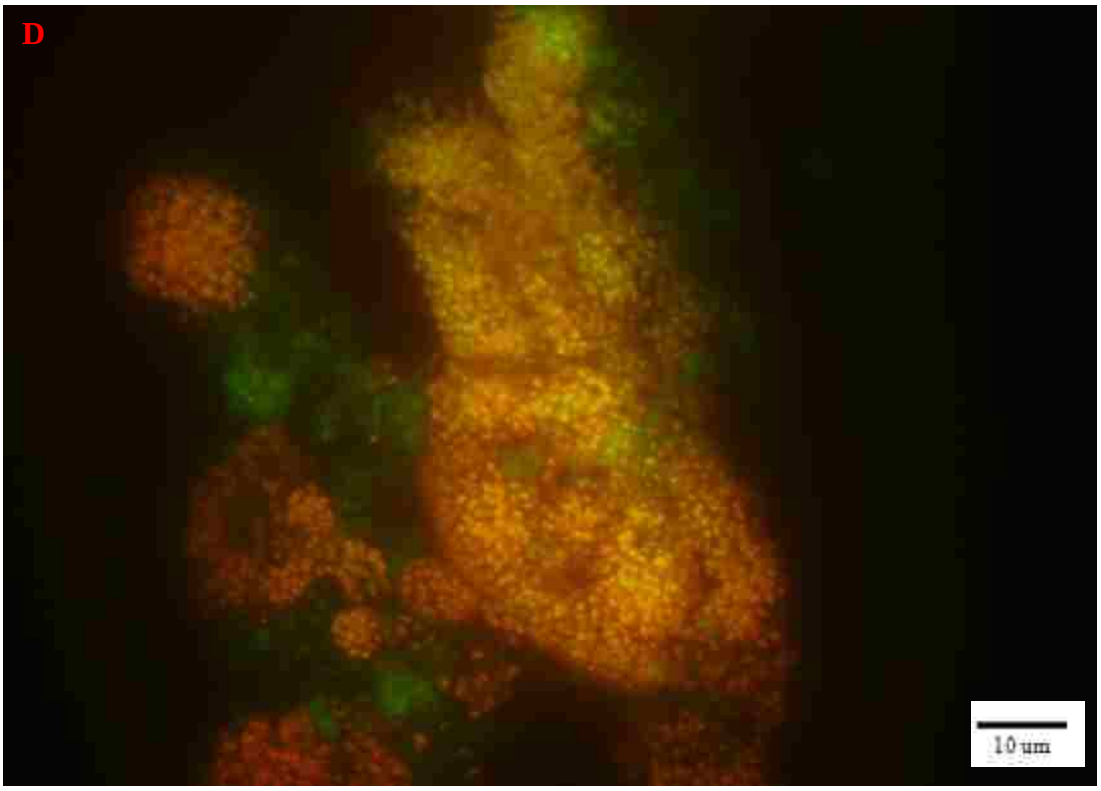


Figure 5.6 Neisser Staining and FISH results on activated sludge samples taken from Stage 3 (Day 540). Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser staining image, dark cells were PAOs, suggesting the accumulation of polyphosphate storage, (B) EUBMIX (FAM) probe targeting most Bacteria (green), (C) FISH with PAOMIX (Cy3) specific for *Accumulibacter spp*. (orange), and (D) Overlay (B) with (C), *Accumulibacter spp*. appear orange.

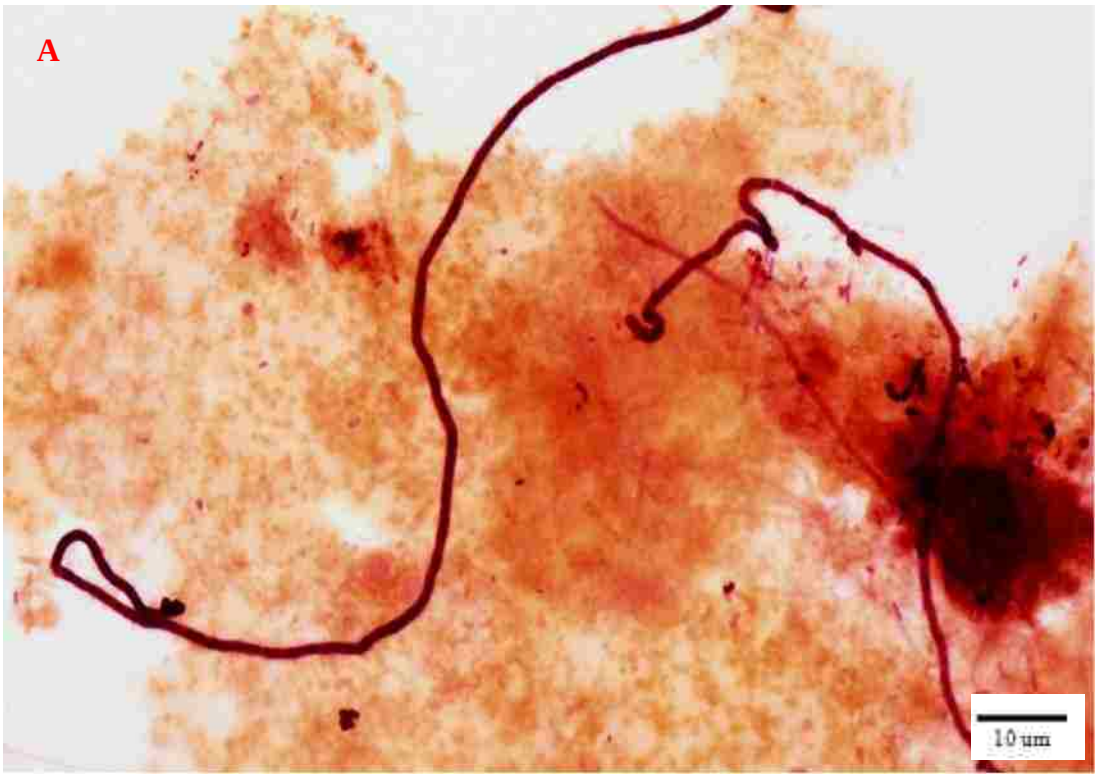
Although PAOs dominated both Stages 1 and 3, the Prel/Ac ratio in Stage 3 was lower than in Stage 1, which was consistent with previous work in batch tests showing that this ratio decreases with decreasing pH. This was the first study able induce EBPR at the lower pH range (Stage 3), however, and the Pns/VSS ratio in Stage 3 (0.28 $\pm$0.02 mg/mg) was higher than in Stage 1 (0.20 $\pm$0.016 mg/mg), indictaing that PAOs accumulated more polyphosphate at the lower pH, low acetate concentration condition than at the high pH, high acetate concentration condition. This suggests that lower acetate concentrations may be advantageous to process performance, as higher quantities polyphosphate storage indicate better P removal.

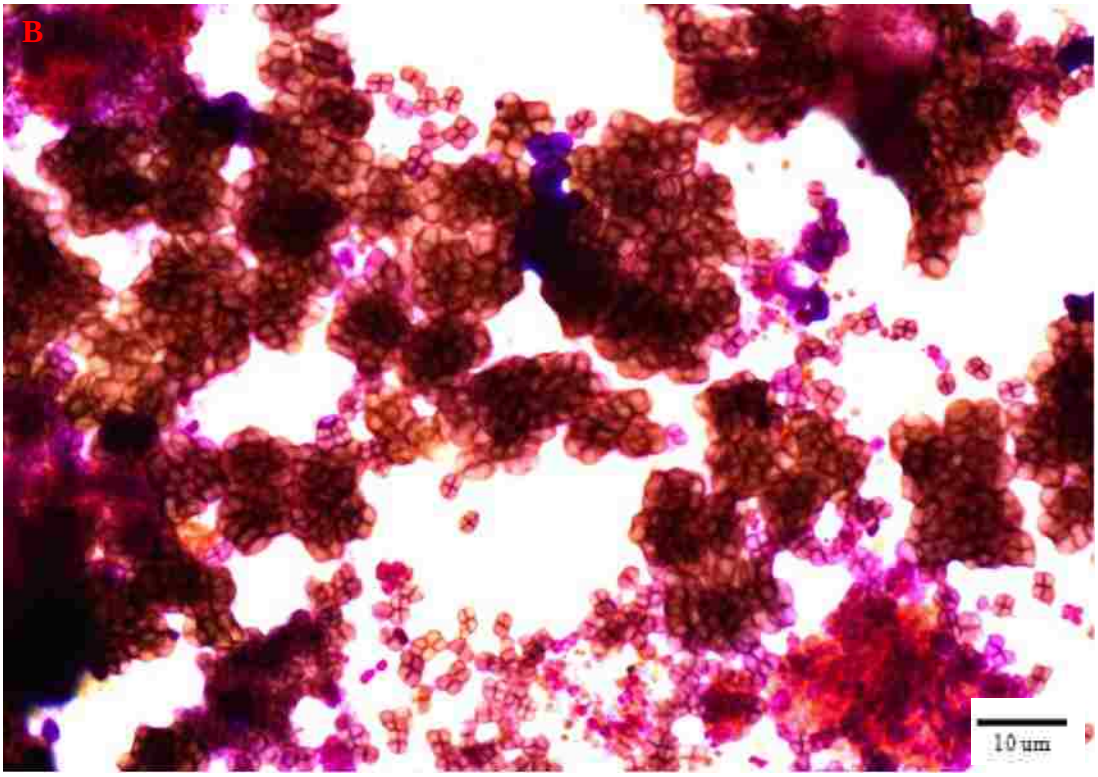
5.4 Stage 4: Low pH, rapid acetate addition

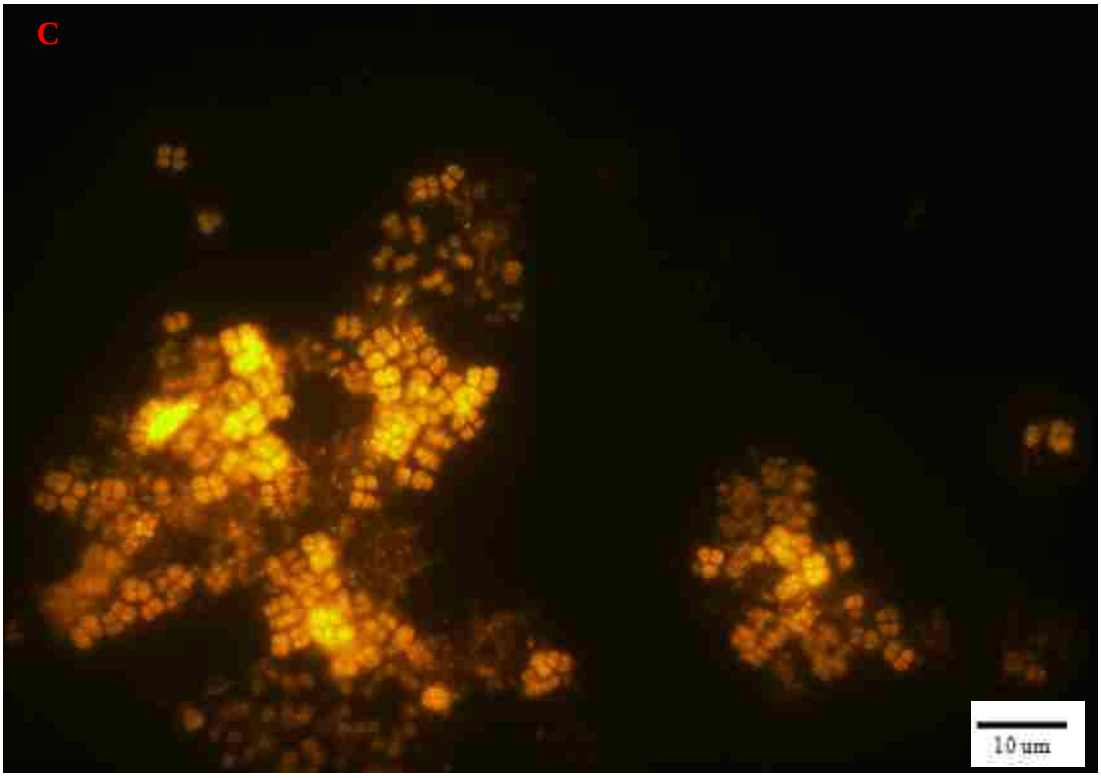
In Stage 4 the reactor was returned to Stage 2 conditions by increasing the acetate addition rate (200mL in 10 min.), while maintaining the lower pH range (6.4-7.0) (Table 5.1). EBPR failed (PAO activity decreased) once again, as indicated by rapidly decreased biomass P content and Prel/Ac uptake ratios (Figure 5.2), similar to the system response when pH was decreased to begin Stage 2. The Pns/VSS, Pns/TSS and Prel/Ac uptake values decreased to approximately 0.03 ± 0.001 mg/mg, 0.02 ± 0.001 mg/mg, and 0.02 ± 0.005 mol/C-mol, respectively, within 18 days after the beginning of Stage 4 (Figure 5.2).

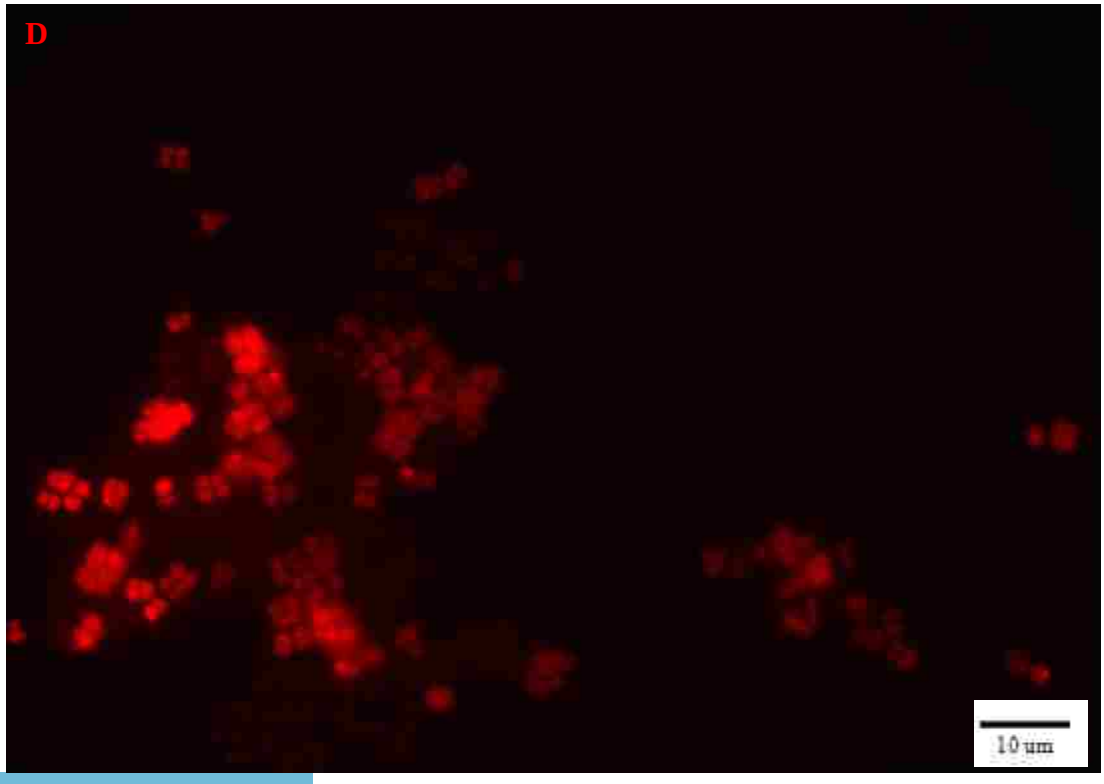
Anaerobic acetate uptake also decreased immediately after the beginning of Stage 4, as indicated by the immediate increase in the end anaerobic phase concentrations, similar to the response when Stage 2 was begun (Figure 5.2B). Again, this suggested that not only had EBPR activity decreased, but that GAO activity had not yet increased sufficiently to fill this niche, and so it appears that competition from GAOs for acetate was not the immediate cause of the PAO decline. By Day 601, 17 days after Stage 4 began, anaerobic acetate uptake was once again complete, but Pns/TSS and Prel/Ac values remained low through the rest of the experiment (Figure 5.2B), suggesting that GAOs once again dominated the reactor population.

Neisser staining results were consistent with this performance data. Neisser-positive cells declined rapidly in Stage 4, indicating few PAOs, and tetrad-forming bacteria, attributed to GAOs such as *Defluvicoccus spp.* were not initially present either. Bacteria with tetrad morphology only appeared in late Stage 4 (Figure 5.7A and 5.7B), when it appeared that GAOs again dominated the culture as described above. FISH analyses showed that cluster 2 *Defluviicoccus spp.* represented 32 ± 7% of the bacteria (Figure 5.7C and 5.7D), Cluster 1 *Defluviicoccus spp*. comprised about of 3.3 ± 0.4% the bacteria. *Competibacter spp*. and *Accumulibacter spp.* were absent.









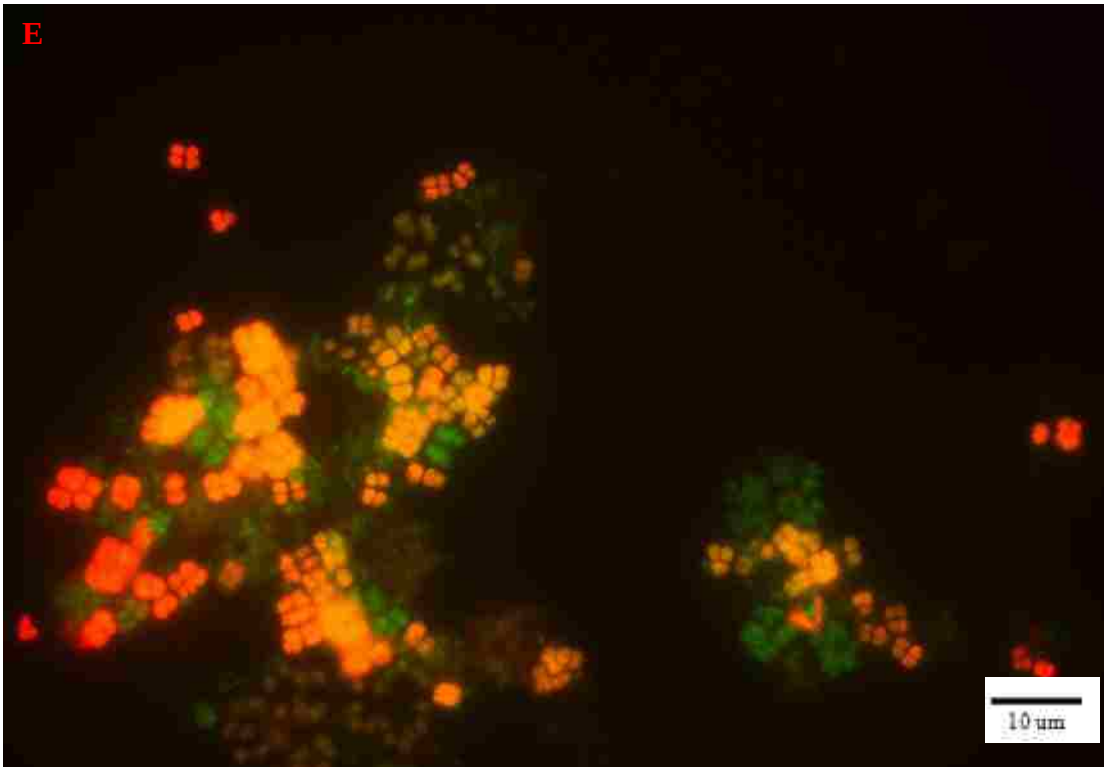
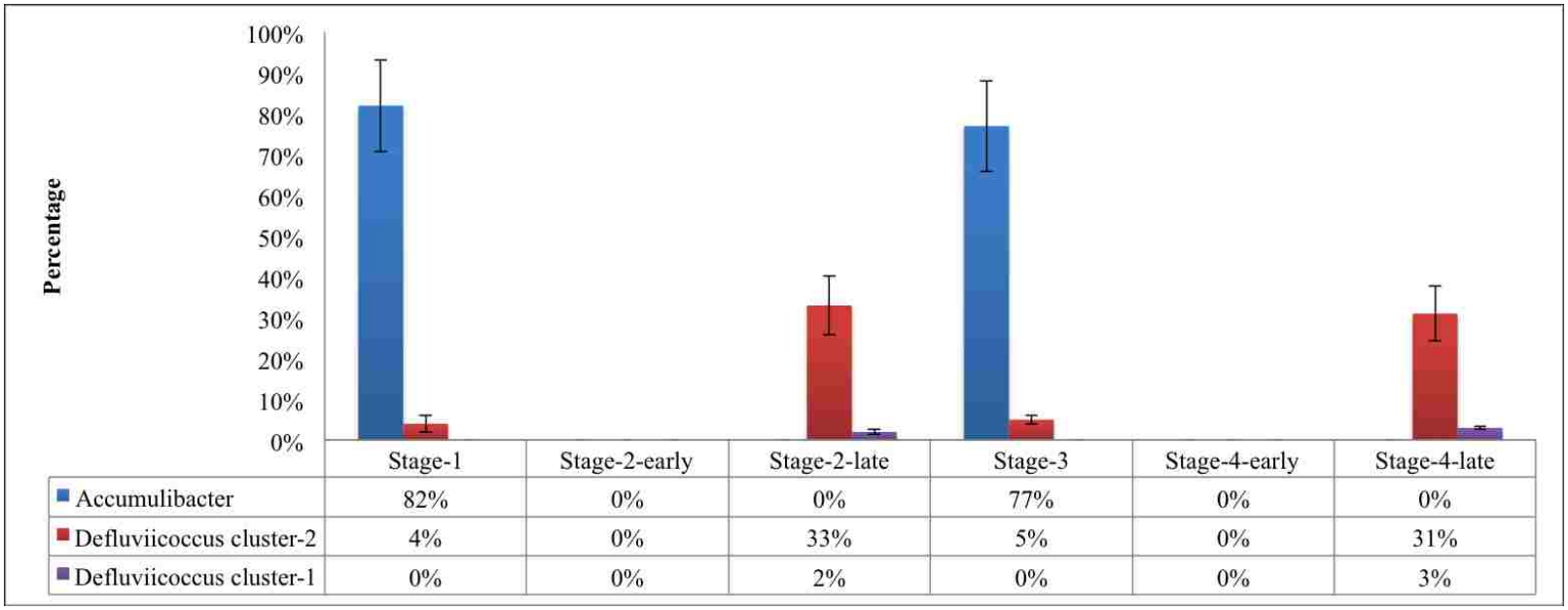


Figure 5.7 Neisser Staining and FISH results on activated sludge samples taken from Stage 4. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser staining image for the period that anaerobic acetate uptake was not complete, no positive results (day 591), (B) Neisser stain image for the period that anaerobic acetate uptake was complete, the typical tetrad cells were most of GAOs (day 613), (C) *In situ* hybridization with probes EUBMIX (TAMRA) specific for many but not all Bacteria (orange), (D) *In situ* hybridization with probes DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) (Cy5) specific for cluster 2 *Defluviicoccus spp.* (red) (day 613), and (E) Overlay (C) with (D), *Defluviicoccus spp*. appear orange.

The FISH data results (Figure 5.8) demonstrated that Stages 1 and 2 were dominated by PAOs (*Accumulibacter spp.*), no PAOs and GAOs were detected in the earlier periods of phase 2 and phase 4, and late periods of phase 2 and phase 4 were dominated by GAOs (*Defluviicoccus spp.* cluster-2).



	Stage-1	Stage-2-early	Stage-2-late	Stage-3	Stage-4-early	Stage-4-late
Accumulibacter	82%	0%	0%	77%	0%	0%
Defluviicoccus cluster-2	4%	0%	33%	5%	0%	31%
Defluviicoccus cluster-1	0%	0%	2%	0%	0%	3%

Figure 5.8 FISH data summary

5.5 Pyrosequencing results for Stages 3 and 4

16s rRNA gene-based pyrosequencing was conducted on samples during stages 3 and 4. The results for a tage 3 sample indicated that the majority (64%) of the sequences were most similar to *Rhodocyclus*, close to FISH results (Figure 5.9) (Table 5.2). Another PAO species, *Tetrasphaera*, comprised 3% of the sequences, and *Dechloromonas*, a close relative *Rhodocyclus*, was 16%. The GAO species, *Defluviicoccus* showed 5% in the entire sample. The results show a good EBPR, which was consistent with our previous results in Stage 3.

Table 5.2 FISH and Pyrosequencing results comparison

	PAOs		GAOs	
Method	FISH (Sample taken on day 540)	Pyrosequencing (Sample taken on 565)	FISH (Sample taken on day 613)	Pyrosequencing (Sample taken on 613)
Percentage	77%	64%	31%	25%

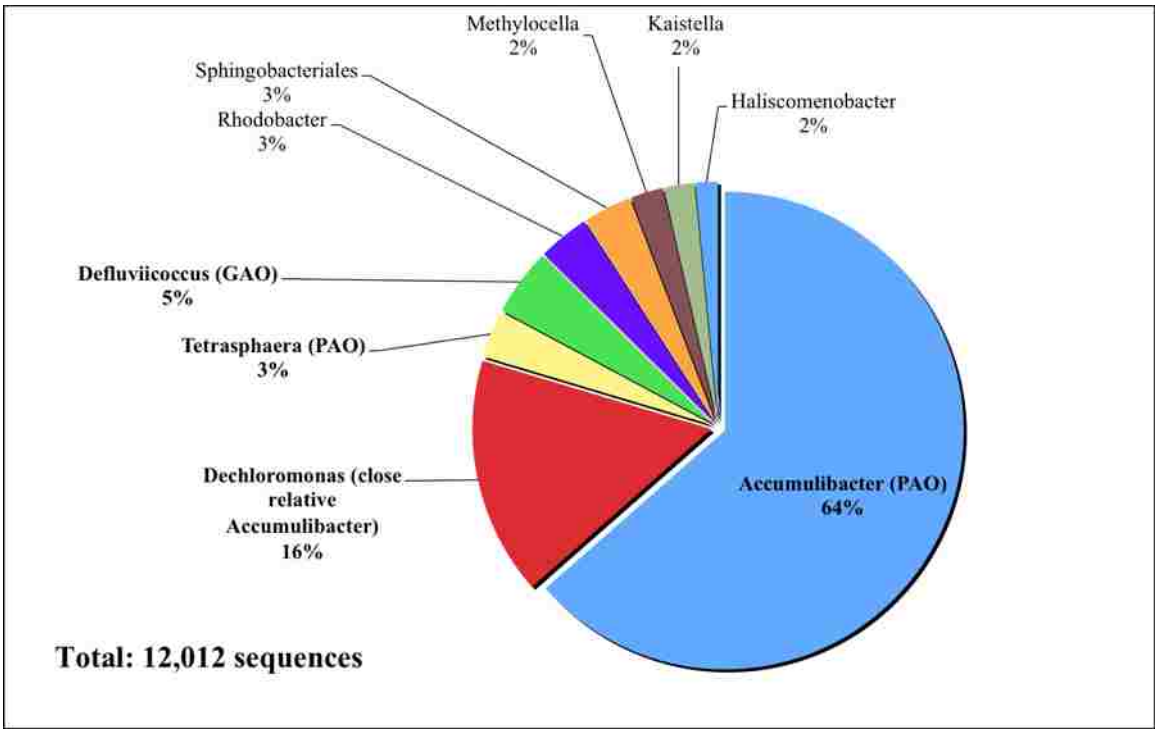


Figure 5.9 Good EBPR samples from Stage 3

Figure 5.10 shows the pyrosequencing results across multiple dates, along with reactor performance, during Stages 3 and 4. This figure shows that bacteria most similar to *Accumulibacter spp.* were most common in Stage 3, while *Defluvicoccus* spp. were relatively low. After the rate of acetate addition was increased to begin Stage 4, PAOs decreased rapidly, simultaneous with a decrease in the biomass P content (Pns/VSS) and an increase in the end-anaerobic acetate concentration. Anaerobic acetate uptake and *Defluvicoccus* spp. content did not begin increasing until after a delay in Stage 3, again suggesting the GAOs may have been opportunists that only increased after acetate became available in the anaerobic phase due to PAO failure. After Day 600, anaerobic acetate was once again complete and *Defluvicoccus* spp. concentrations once again increased (Table 5.2).

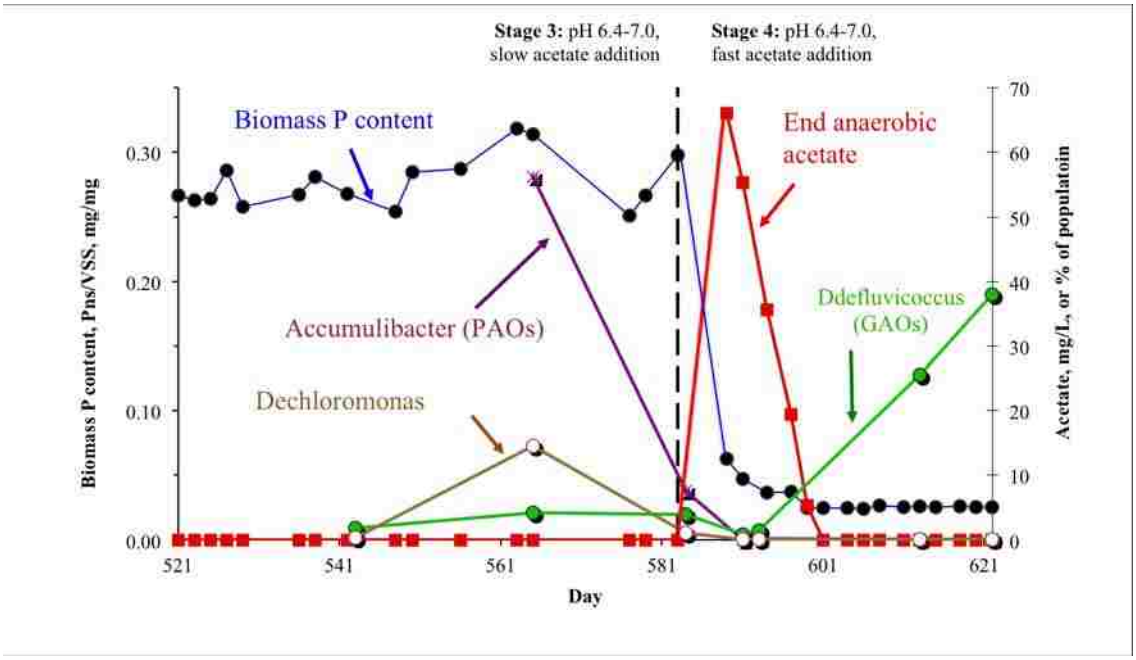


Figure 5.10 Pyrosequencing results in the Stage 3 and Stage 4

The results from Stages 2, 3, and 4 suggested that low pH combined with high acetate concentrations may be inhibitory of PAO metabolism, which led to EBPR failure, rather than direct competition from GAOs at the lower pH values. The reason for this is not known. One hypothesis to explain this is related to the fact that the acetic acid (HAC) form of the acetic acid/acetate acid/base pair a known uncoupler of PMF (Craig Baker-Austin and Dopson 2007), increases as pH decreases (the relative concentration of acetate acid increases approximately 10-fold as pH decreases from 7.5 to 6.5). It is therefore possible that the higher relative concentrations of the acetic acid form at lower pH values is inhibitory to PAOs when combined with relatively high acetate concentrations occurring when acetate is added rapidly in an SBR, which caused the failure of PAOs at the beginning of Stages 2 and 4. According to this hypothesis, the eventual dominance of *Defluvicoccus* spp. during these Stages would suggest that this GAO is more tolerant of the low pH, high acetic acid condition than is *Accumulibacter spp.*.

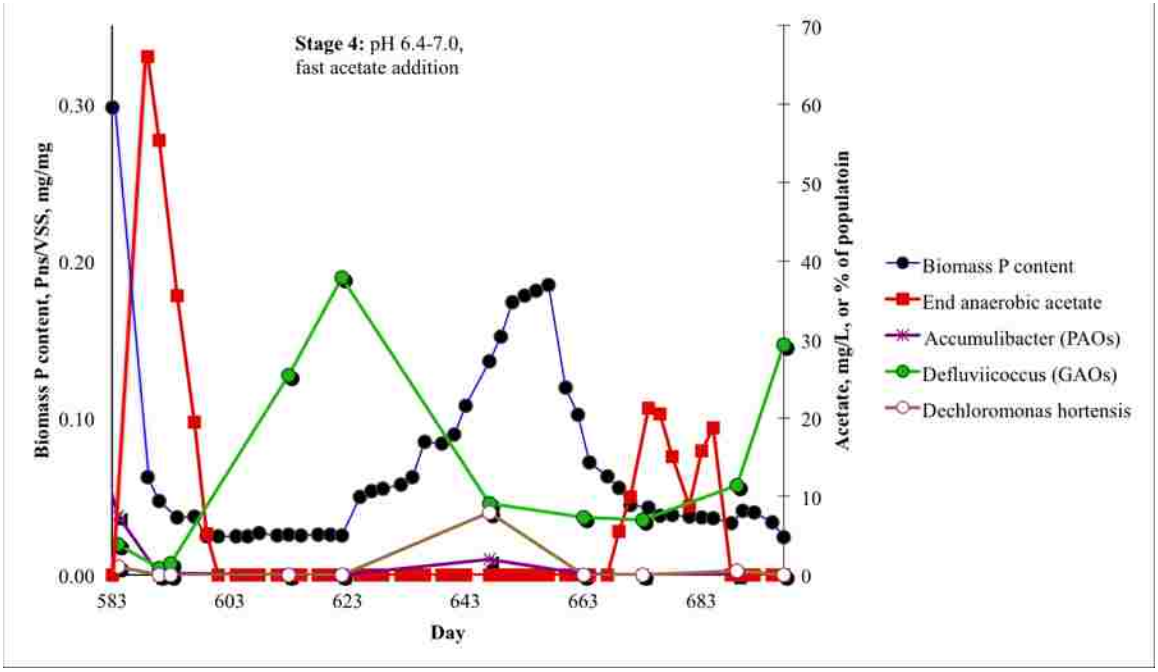


Figure 5.11 Pyrosequencing results in very late Stage 4

The *Dechloromonas spp* was found again in the period for very late stage 4 (around day 647) (Figure 5.11), which is a close relative *Accumulibacter* spp. bacteria. It has been previously reported in an EBPR system (Martin et al. 2006; Ahn et al. 2007), they suggested that *Dechloromonas spp.* was GAOs, since the bacteria didn't accumulate PP during the cycles. However, based on our results, we found the biomass content was increased right after the *Dechloromonas* spp. appeared, and at the same time, there were almost no PAOs (*Accumulibacter* spp.), which suggested that the *Accumulibacter* spp. didn't contribute the increase of biomass content. The GAOs (*Defluviicoccus* ssp.) were also decreased, which provided a clue that there might be other group bacteria competing with the GAOs (since the acetate uptake was still complete), from the figure 5.11, obviously the bacteria was *Dechloromonas spp.* So our results suggested that *Dechloromonas spp.* might be PAOs, however our late DAPI tests showed there was still no PP accumulated in the cell, further research is needed.

5.6 Effect of Pns on Solids composition

In all 4 stages, the difference between mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) was correlated with Pns content (Figure 5.12). The higher Pns content induced higher difference, which caused the

deference between MLSS and MLVSS in the PAOs dominated phase (phase 1 and phase 3) was always much higher than the GAO-enriched culture (late phase 2 and phase 4) (Figure 5.13). The reason is that PAOs take up the soluble phosphorus to recover the polyphosphate level, more P is taken up, more Pns exits in the PAOs, which causes the non-VSS amount to increase. While, GAOs never accumulate P, so there is no Pns included in the GAOs, which means the non-VSS amount is not changed, so in this case, the MLSS and MLVSS are close. The results were consistent with our precious conclusions that Pns was an important indicator to determine whether PAOs dominate in the biomass.

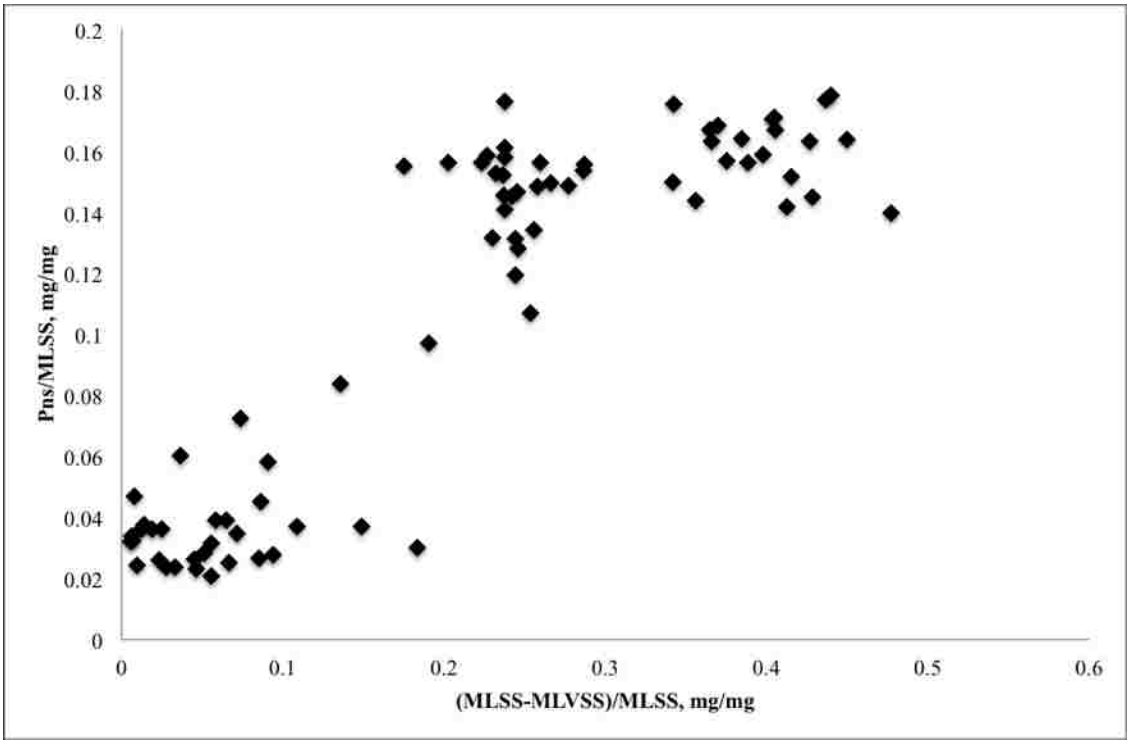


Figure 5.12 The relationship between Pns/TSS and NVSS/TSS

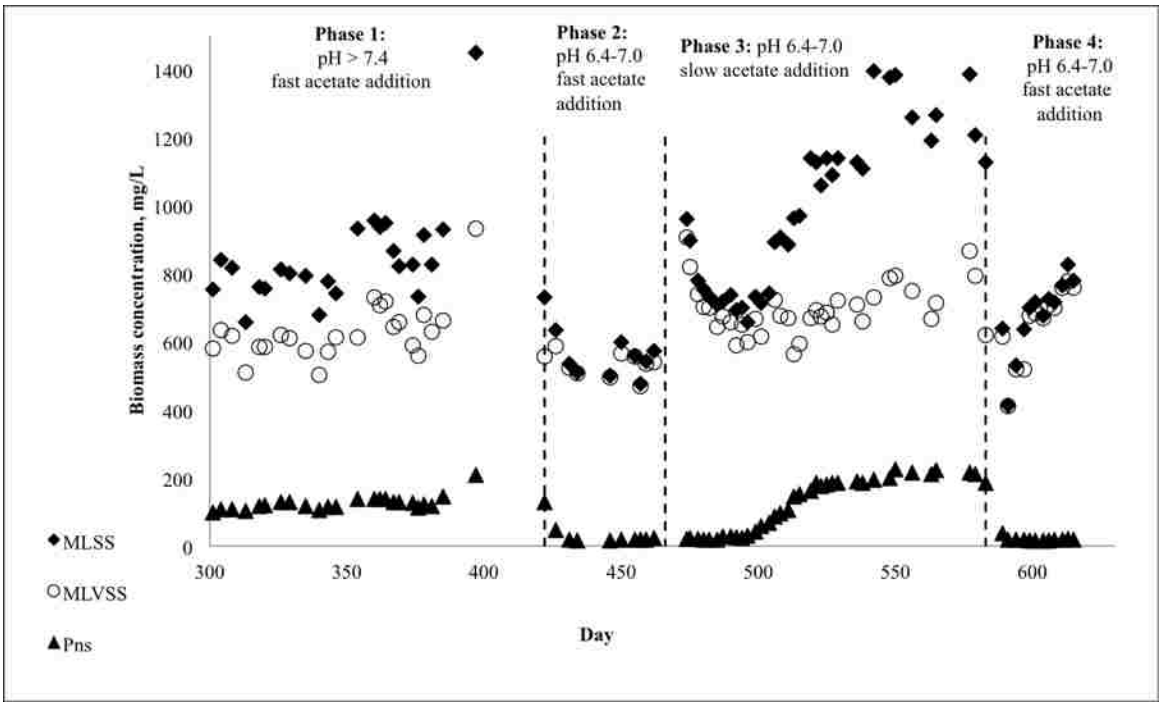


Figure 5.13 MLSS, MLVSS and Pns during four phases of operation with variable pH and rates of acetate addition in the reactor

CHAPTER 6. METABOLISMS FOR ACETATE AND PHOSPHATE TRANSPORT IN EBPR AND BIOKINETICS FOR PAOS AND GAOS

The acetate concentration effects on the PAOs and GAOs cultures suggested that PAOs and GAOs may use different acetate uptake mechanisms. Batch testing of PAO- and GAO-dominated cultures with chemical inhibitors were performed to better elucidate the behaviors of these mechanisms under different acetate concentrations and at different pH values.

6.1 Anaerobic-aerobic profile in Stage 1 PAO-dominated culture

Inhibition tests were carried out with from the Stage 1 (for PAOs) and Stage 2 (for GAOs) in the Reactor 1. The *Accumulibacter spp.* enrichment culture took up acetate (100 mg/L) over the initial 60 min, at a nearly constant rate during the first 30 min (Figure 5.1). Similarly, the P release was nearly linear over the initial 30 min in the anaerobic phase. As noted, the ratio between anaerobic P release to acetate uptake was 0.65 ± 0.09 mol/C-mol (Figure 6.1), consistent with a PAO-dominated culture (Schuler and Jenkins 2003a).

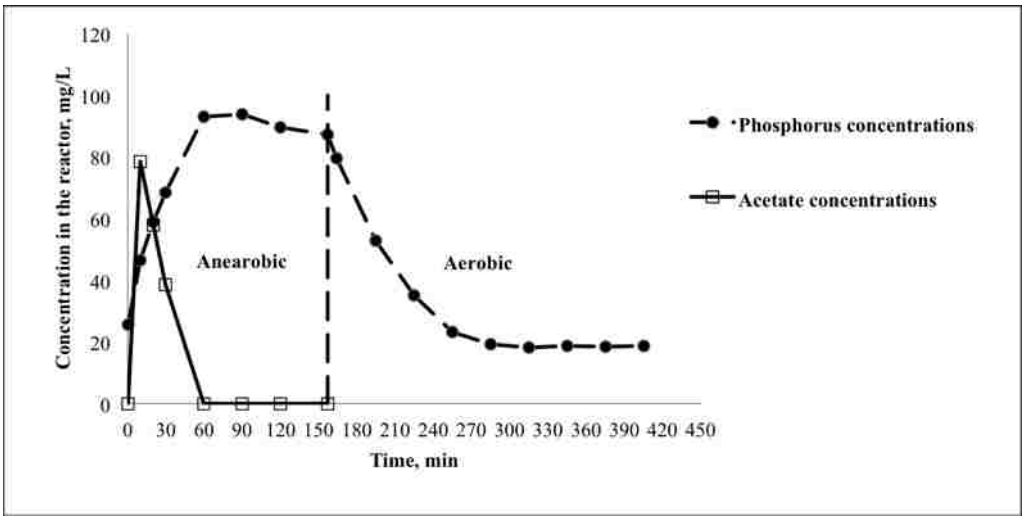


Figure 6.1 Acetate and phosphorus profiles throughout anaerobic and aerobic phases with rapid acetate addition (Sample taken on day 315)

6.2 Effects of inhibitors on PAO-dominated cultures in batch tests

In the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (which diffuses the cellular PMF by allowing protons to cross the cell membrane) a PAO-dominated (Stage 1, day 403 and day 409) culture, the acetate uptake rate decreased by 52% and 90% in batch tests with rapid (pulse) and slow acetate addition, respectively, relative to tests with no inhibitor added (Table 6.1) (Figure 6.2). This suggested that acetate uptake by *Accumulibacter spp.* was sensitive to PMF uncoupling. CCCP also strongly reduced P release rates (Table 6.2), which indicated a strong link between acetate influx and Pi efflux in *Accumulibacter spp.* The results were consistent with the hypothesis that *Accumulibacter spp.* generate the PMF using the phosphate inorganic transport (Pit) system in which *Accumulibacter spp.* transports protons out of the cell coupled with inorganic phosphate (Figure 6.3), which was consistent with previous studies (Saunders et al. 2007; Burow et al. 2008), and the constant ratio between P release and acetate uptake observed in the *Accumulibacter spp.* enrichment (0.65 ± 0.09 P-mol C-mol^{-1}), supports this suggestion (Figure 6.1)

In addition, the slow acetate addition test yielded higher inhibition with CCCP than did the pulse test (Table 6.1). This suggested that acetate uptake was more dependent on the cellular PMF at lower acetate concentrations than at higher acetate concentrations.

However, the slow acetate addition tests presented with CCCP actually had fairly high acetate concentration, since the uptake was significantly inhibited. So additional slow acetate tests controlled at extremely low acetate concentrations with CCCP was operated. The general operation process was the same as method section (Chapter 4), but the sampling time was at 1min, 3 min, 5 min, 10 min, 15 min, 30 min (since the GC detective limitation, there was nothing detected at 1min and 3 min). Based on the results showed in figure 6.4, the additional low acetate concentration test supported our previous slow acetate addition tests results, the acetate uptake inhibition was extremely high presented with CCCP at low acetate concentration.

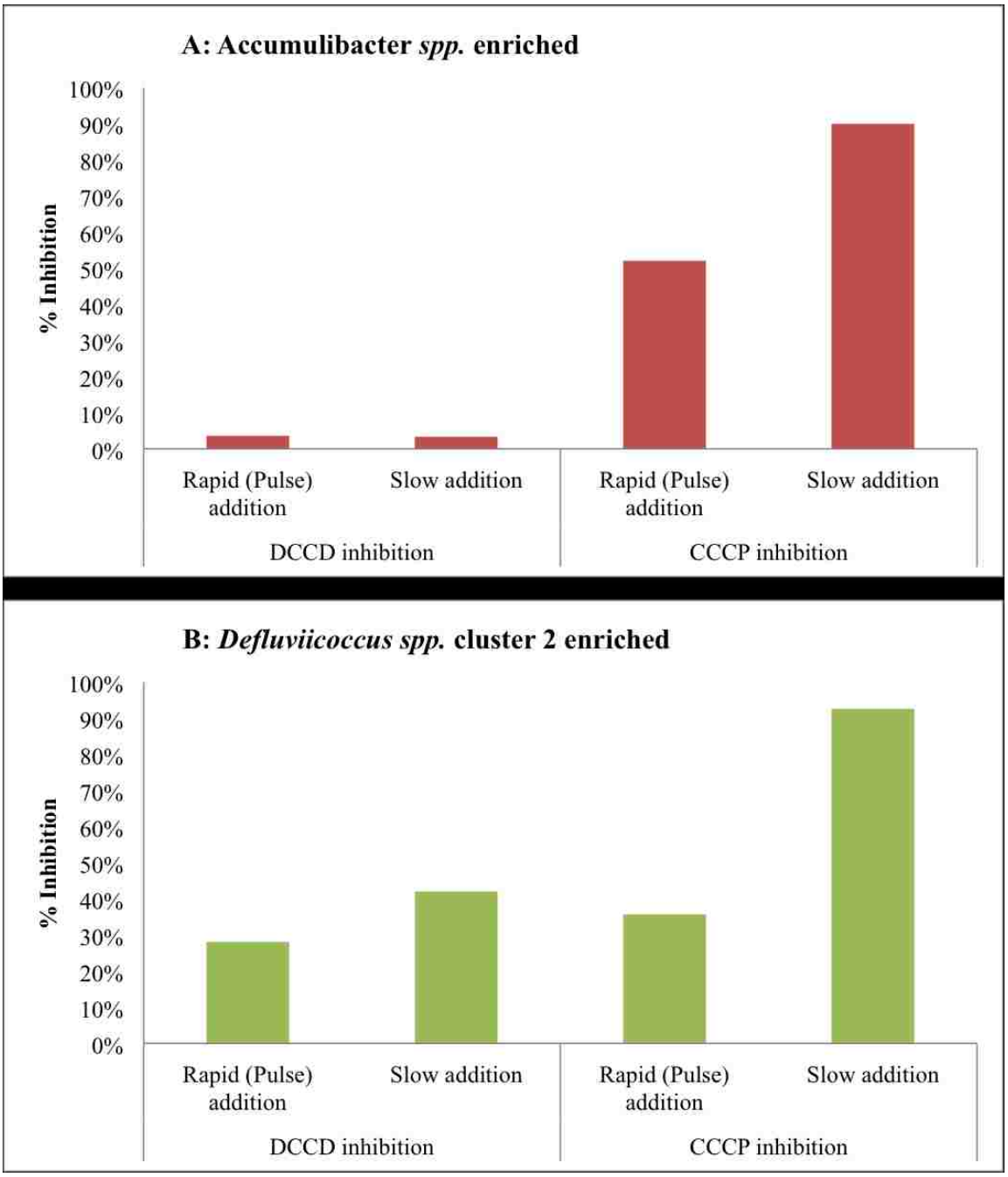


Figure 6.2 Effect of chemical inhibitors on anaerobic acetate uptake rates (A) *Accumulibacter spp.* enriched and (B) *Defluviicoccus spp.* cluster 2 enriched cultures for rapid (pulse) and slow acetate addition rates

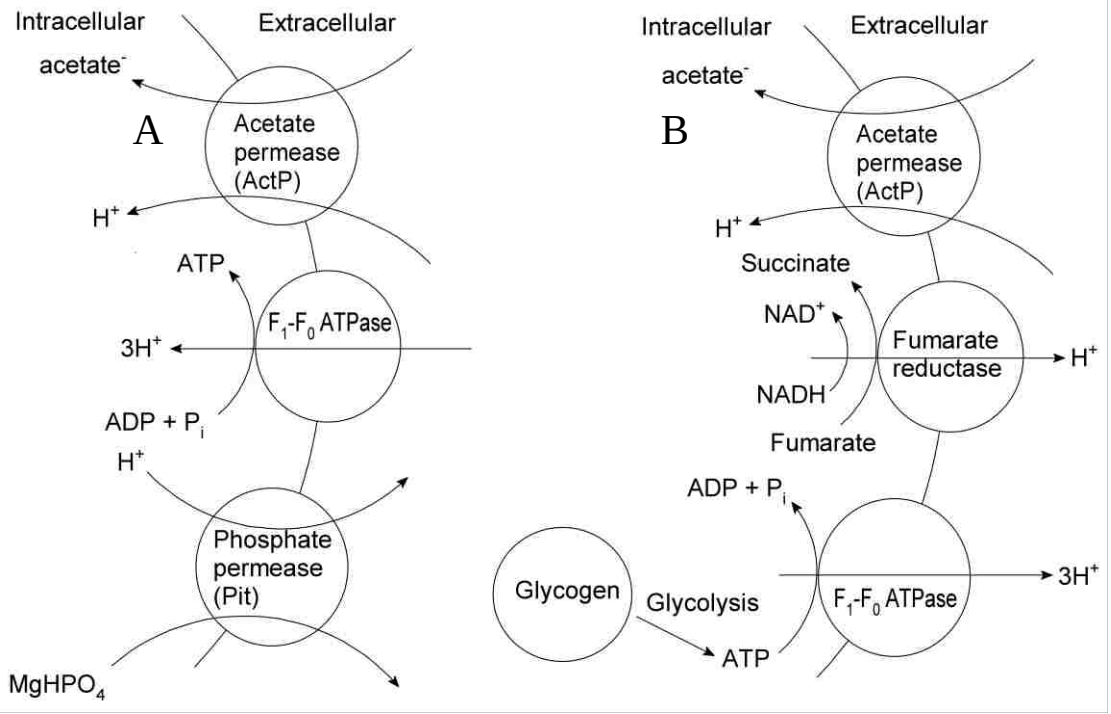


Figure 6.3 Proposed mechanisms of acetate uptake by (A) PAO and (B) GAO (Saunders et al. 2007; Burow et al. 2008)

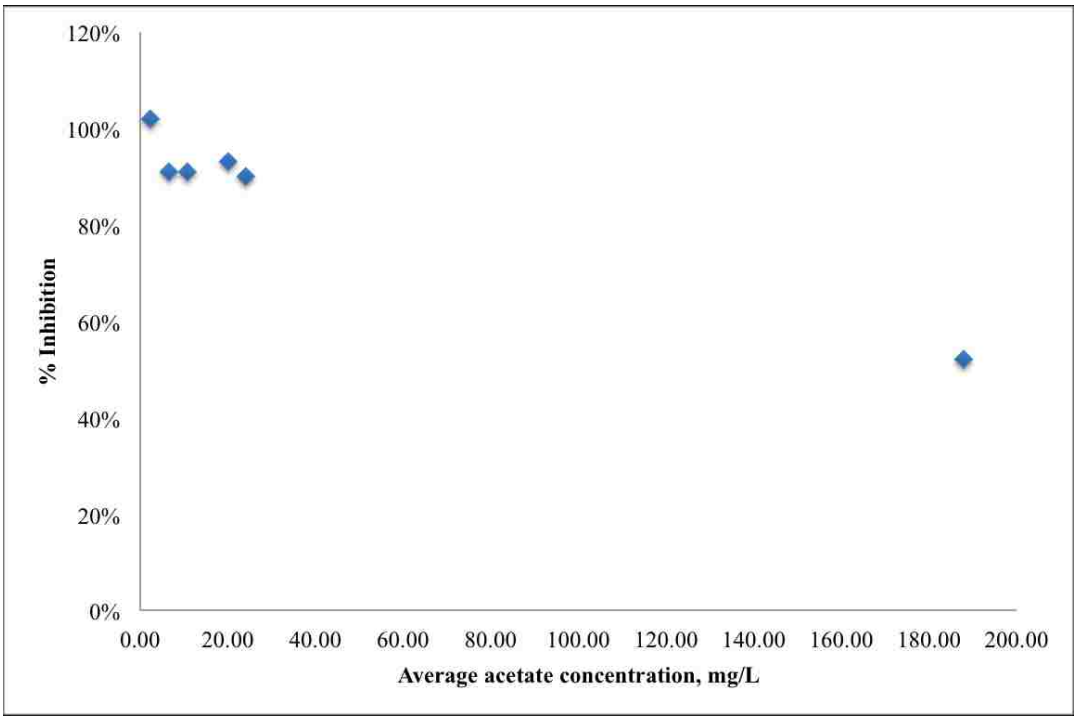


Figure 6.4 Effect of CCCP on anaerobic acetate uptake rates for *Accumulibacter spp.* enriched culture under different acetate concentrations

Acetate uptake by *Accumulibacter spp.* with and without inhibition by *N-N'*-dicyclohexylcarboiimide (DCCD), was also investigated. DCCD is an inhibitor of APTase (Harold et al. 1969). As shown in Tables 6.1, 6.2 and figure 6.2, DCCD had little effect on the acetate uptake or the P release rates in the both pulse and slow tests, which suggests that the F_1F_0-ATPase is not a key source of the PMF for *Accumulibacter spp.*

6.1 Effect of inhibitors on anaerobic acetate uptake in pulse and slow tests for *Accumulibacter spp.* (Reactor-1/Stage-1, day 403 and day 409)

Inhibitor	Anaerobic acetate uptake rate (mg min^{-1} mg^{-1} VSS)					
	Rapid acetate addition (Pulse test)		% Inhibition	Slow acetate addition (Slow test)		% Inhibition
	No inhibitor	Inhibitor		No inhibitor	Inhibitor	
CCCP	2.83	1.36	52	2.28	0.22	90
DCCD	2.62	2.52	3.6	2.40	2.32	3.3

6.2 Effect of inhibitors on anaerobic P release in pulse and slow tests for *Accumulibacter spp.* (Reactor-1/Stage-1, day 403 and day 409)

Inhibitor	Anaerobic P release rate (mg/min/mg VSS)					
	Rapid acetate addition (Pulse test)		% Inhibition	Slow acetate addition (Slow test)		% Inhibition
	No inhibitor	Inhibitor		No inhibitor	Inhibitor	
CCCP	1.82	0.87	52	1.93	0.36	81
DCCD	2.14	1.97	7.9	2.35	2.16	8.2

6.3 Effects of inhibitors on GAO-dominated cultures in batch tests

The inhibitors CCCP and DCCD were also applied to cultures dominated by *Defluviicoccus spp.* (Stage 2, day 455 and day 459). In the presence of CCCP, the acetate uptake rate decreased by 36% and 93% in the pulse and slow tests respectively (Table 6.3 and Figure 6.2), demonstrating that acetate uptake by *Defluviicoccus spp.*, like *Acumulibacter spp.,* inhibited by disruption of the PMF. In contrast to *Accumulibacter spp.*, the acetate uptake rate by *Defluviicoccus spp.* was significantly reduced by DCCD addition in both the pulse and slow tests (Table 6.3), suggesting that the F_1F_0- ATPase may be important to reestablishing the PMF in *Defluviicoccus spp.* This was in disagreement with Burow *et al.* (2008) (Figure 6.5), who reported that DCCD did not inhibited acetate uptake in a *Defluviicoccus spp.* enriched biomass. However, these researchers analyzed culture enriched with *Defluvicoccus* sub-group cluster 1 (as identified by FISH), while the Stage 2 sample was highly enriched with the closely-related *Defluvicoccus* sub-group cluster 2 (also identified by FISH). Saunders (2007) reported DCCD inhibited acetate uptake 81% in a highly enriched *Competibacter* culture (Figure 6.5). They suggested that *Competibacter* derived its PMF through a combined achievement of proton export through the ATPase at the expense of ATP generated by glycolysis and the catalysis of fumarate reductase (Figure 6.3). The finding in this study that *Defluviicoccus spp.* cluster 2 was inhibited by DCCD, could indictate that this group also uses this mechanism for acetate uptake, although further research is needed on this point.

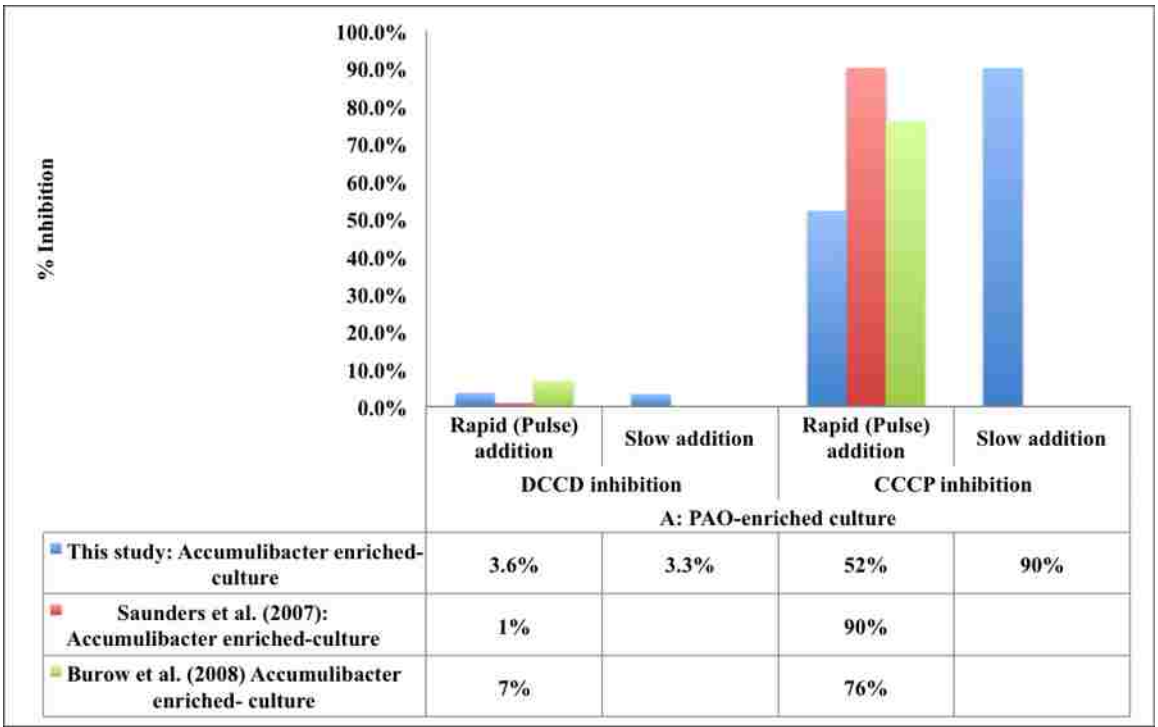
In summary, the inhibition test results (Figure 6.2) indicated that acetate uptake by both the *Accumulibacter spp.*-enriched and *Defluviicoccus spp.* cluster 2-enriched cultures were inhibited by CCCP, suggesting that both groups of organisms required a PMF for acetate uptake. DCCD, on the other hand, only inhibited the *Defluviicoccus spp.* cluster 2 enriched culture, suggesting that *Defluviicoccus spp.* relied on the F_1F_0 ATPase for generation of the PMF. The P release tests for *Accumulibacter spp.* suggested that they use an active process for the uptake of acetate that is driven by the PMF generated via P efflux. The results not only confirmed previous studies (Saunders et al. 2007; Burow et al. 2008), but also expanded the research range, since we used the new GAO species- *Defluvicoccus* sub-group cluster 2 in these inhibition tests.

In addition, the higher rates of inhibition found when acetate was added slowly relative to when it was added as a pulse (Table 6.3 and Figure 6.2) suggested that PMF-dependent transport may play a more important role at lower concentrations (slow acetate addition) than at higher concentrations (fast acetate addition) in both PAOs and GAOs. This may be because higher acetate concentrations create a greater acetate concentration gradient across the cell membrane, which could help drive acetate transport when the PMF is disrupted.

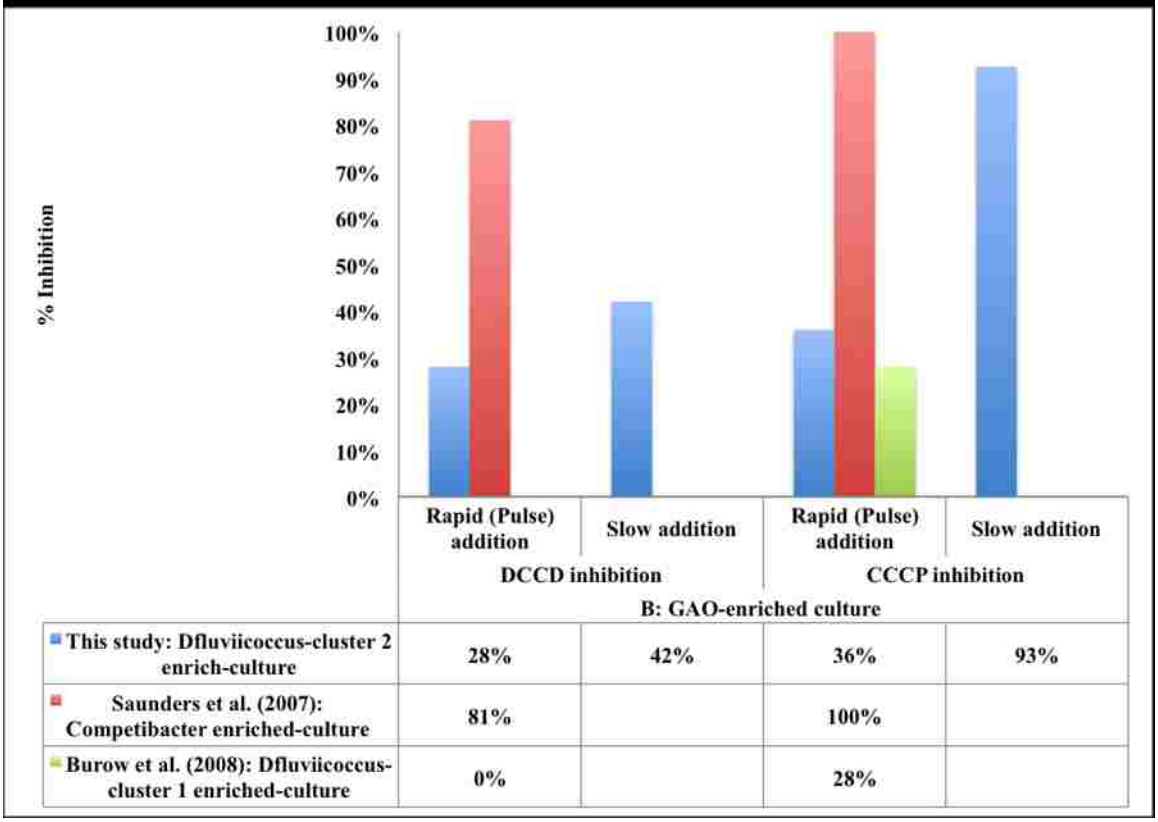
Acetate uptake in the *Defluviicoccus* spp. cluster 2-enrichment was less sensitive to the PMF disruptor (CCCP, 36% inhibition in pulse tests), compared with the *Accumulibacter spp.* enrichment (CCCP, 52% inhibition in pulse tests). The *Defluviicoccus* spp.-enriched culture also had a lower acetate uptake rate (1.74 ± 0.08mg/min/mg VSS) compared with the *Accumulibacter spp.*-enriched culture (2.7 ± 0.15 mg/min/mg VSS). These results were consistent with Burow et al. (2008), and demonstrated that PMF-driven transport may contribute more to acetate uptake in *Accumulibacter spp.* than in *Defluviicoccus spp.*, which supports the hypothesis that PAOs may have a competitive advantage over GAOs under the low substrate conditions, such as those likely to occur in in full-scale EBPR plants.

6.3 Effect of inhibitors on anaerobic acetate uptake in pulse and slow tests for *Defluviicoccus spp.* (Reactor-1/Stage-2, day 455 and day 459)

Inhibitor	Anaerobic acetate uptake rate (mg/min/mg/VSS)					
	Rapid acetate addition (Pulse test)		% Inhibition	Slow acetate addition (Slow test)		% Inhibition
	No inhibitor	Inhibitor		No inhibitor	Inhibitor	
CCCP	1.68	1.08	36	1.62	0.12	93
DCCD	1.79	1.29	28	1.58	0.94	42



This study: Accumulibacter enriched-culture	3.6%	3.3%	52%	90%
Saunders et al. (2007): Accumulibacter enriched-culture	1%		90%	
Burow et al. (2008) Accumulibacter enriched- culture	7%		76%	



This study: Dfluviicoccus-cluster 2 enrich-culture	28%	42%	36%	93%
Saunders et al. (2007): Competibacter enriched-culture	81%		100%	
Burow et al. (2008): Dfluviicoccus-cluster 1 enriched-culture	0%		28%	

Figure 6.5 Effect of chemical inhibitors on anaerobic acetate uptake rates comparison (A) PAO enriched and (B) GAO enriched cultures for rapid (pulse) and slow acetate addition rates.

6.3 Acetate uptake rates under anaerobic conditions by PAOs at Different pH values

Acetate uptake rate tests for PAOs were carried out with PAO-dominated biomass from the Stage 3 in the Reactor 1 To obtain a better understanding of the effect of acetate concentration on the anaerobic metabolism of PAOs in conjunction with pH a series of batch tests were at a range of acetate concentrations and at 2 different pH values (6.5 and 7.5). In addition to focusing on acetate concentration effects, to our knowledge this was the first such analyses of a PAO system acclimated to a low pH range (6.4 to 7.0), since prior to this study such a system did not exist.

As shown in Figure 6.6, the specific acetate uptake rate for PAOs appeared to be independent of pH for the values tested. The acetate uptake rate was dependent on the acetate concentration and saturated at high acetate concentrations, but did not following the Monod relationship (poor fit with Eadie-Hofstee plot), which was contrast to the findings of previous studies (Smolders et al. 1995; Kuba et al. 1996; Murnleitner et al. 1997) (Figure 6.7).

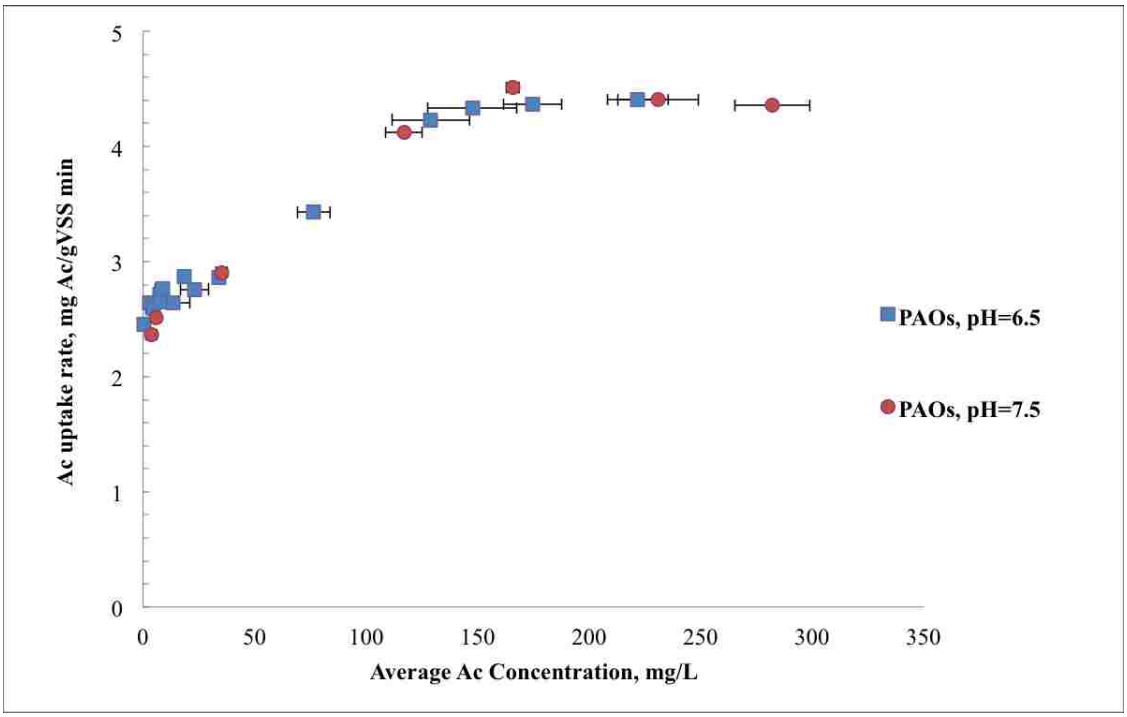


Figure 6.6 Acetate uptake rates in *Accumulibacter spp.* enriched culture under different pH values

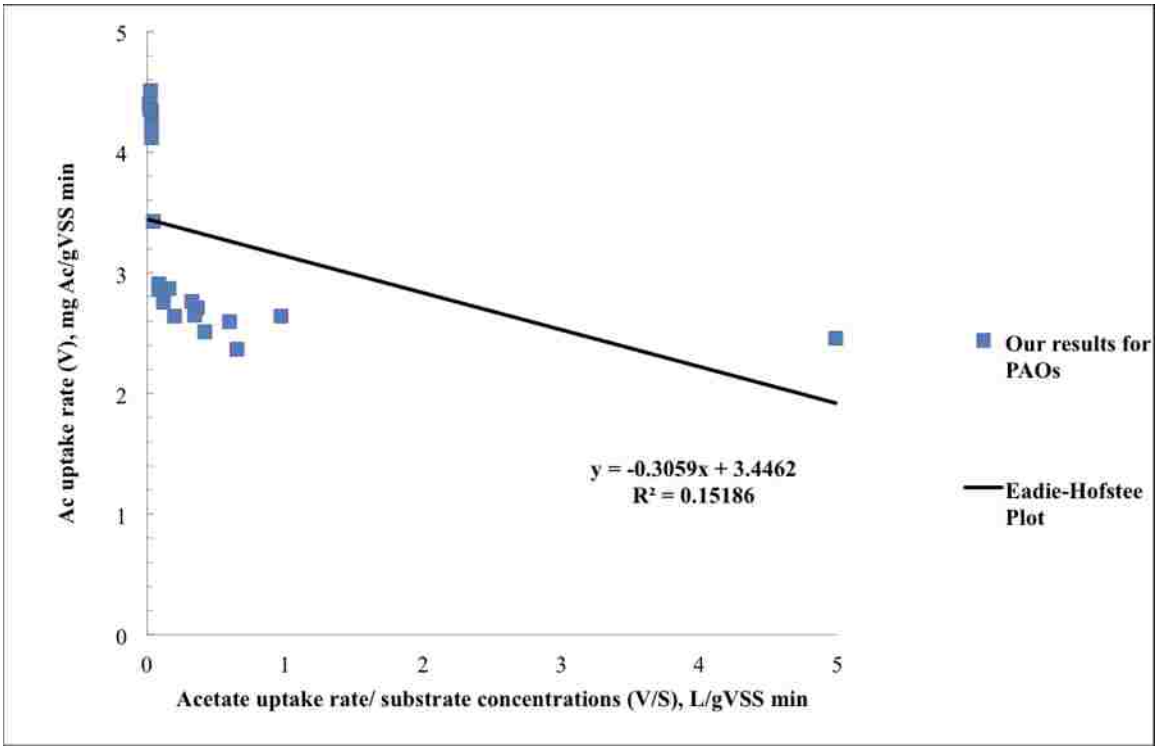


Figure 6.6 Acetate uptake rates in *Accumulibacter spp.* were fitted poorly with Eadie-Hofstee Plot

6.4 Acetate uptake rates under anaerobic conditions by GAOs at different pH values

In contrast to the results found for the PAO-enriched culture, pH had a strong effect on acetate uptake rates in the GAO (*Defluvicoccus* cluster 2)-enriched culture from Stage 4 (Day 611-620) (Figure 6.8) At the lower pH (pH=6.5) condition, the acetate uptake rate was more or less independent of the acetate concentration, and the average acetate rate was 2.37 ± 0.12mg/gVSS*min. At pH 7.5, the acetate uptake rate was somewhat lower than at pH 6.5 for the lower range of acetate concentrations (1.53 ± 0.22 mg/gVSS*min for acetate concentrations from 0 to 90 mg/L). At acetate concentrations greater than approximately 100 mg/L, the pH 7.5 acetate uptake rates were much lower at pH 7.5 (0.28 ± 0.04 mg/gVSS*min) than they were at pH 6.5. This suggested that the GAOs were strongly inhibited under the high acetate concentration (the high acetate concentration in this group batch tests was much higher than Reactor 1/Stage 4, the maximum acetate concentration in stage 4 was below 97mg/L, and might be around the transition to very low rates of acetate uptake) and high pH condition.

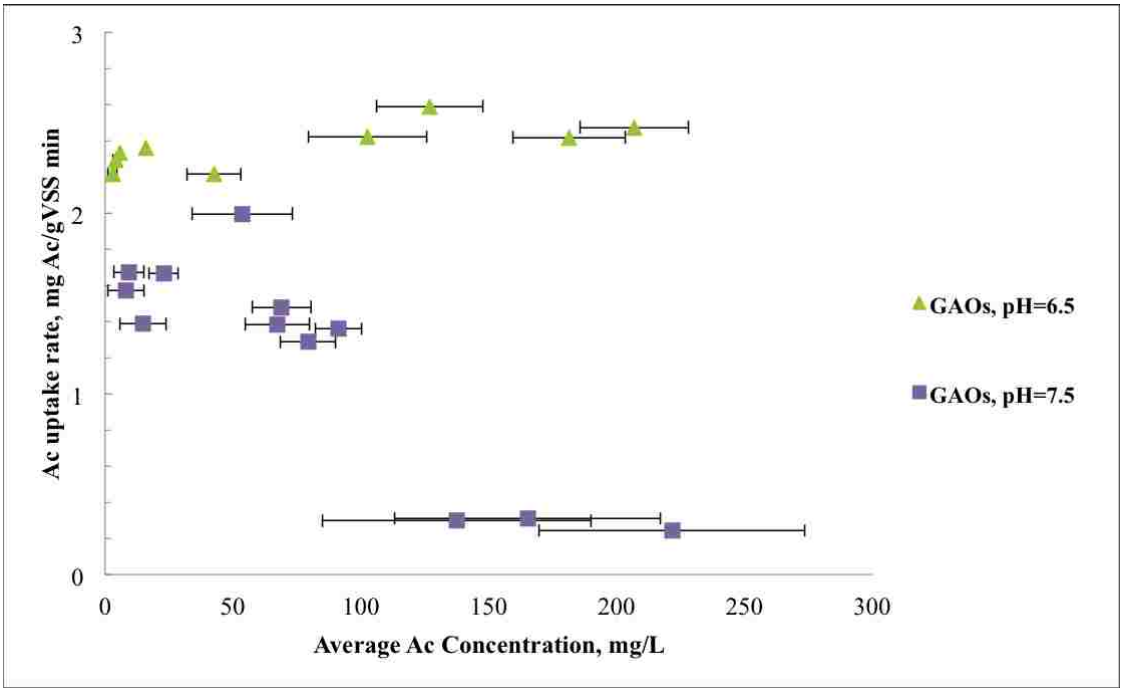


Figure 6.8 Acetate uptake rates in *Defluvvicoccus spp.* enriched culture under different pH values.

6.5 Comparison of PAO and GAO acetate uptake rates

The data from Figures 6.6 and 6.8 are combined in Figure 6.9 to facilitate comparison of the two cultures. Under the higher pH condition, the acetate uptake rate by PAOs was higher than GAOs across all acetate concentrations. Both cultures were from systems acclimated to pH 6.5 (Stages 3 and 4). At pH 6.5 and low acetate concentrations, the acetate uptake rates by the two cultures were close (approximately 2.2 mg/gVSS*min), while at high acetate concentrations, the PAO acetate uptake rates were higher than those of GAOs (approximately 4.51 mg/gVSS*min vs 2.59 mg/gVSS*min) as the rates of acetate uptake in the PAO dominated culture increased with increasing acetate concentration, while they were more or less constant in the GAO-dominated culture.

The results show in Figure 6.9 can be compared to the experimental results shown in Figure 5.3. First, the results are consistent with PAO dominance in Stage 1, as it appears that PAOs may take up acetate more rapidly in the Stage 1 conditions (higher acetate concentrations and pH ~ 7.5). This conclusion is somewhat qualified by observation that the cultures tested to produce the results shown in Figure 6.9 were both

acclimated to the lower pH condition (6.5). Second, the results are consistent with PAO dominance in Stage 3 (pH 6.5, slow acetate addition yielding very low acetate concentrations), as PAOs maintained a small advantage in their acetate uptake rate even at the lowest acetate concentrations. It is also noted that the minimum acetate concentration tested for the PAOs was 4.36 mg/L, and the minimum for the GAOs was 2.86 mg/L (Figure 6.9) due to challenges in maintaining low acetate concentrations during the batch tests. The acetate concentrations in the reactors during the slow rate of acetate addition after PAO dominance (late Stage 3) were somewhat lower at less than 2 mg/L (below the detect limitation), and so it is possible that PAOs may have had a greater advantage over GAOs at the very low acetate concentrations occurring in the reactors than indicated in the Figure 6.9. The reason for why the PAOs still took up acetate faster than GAOs under lower pH and higher acetate concentration was our tests were short-time tests, which only last 30 min, if applying the tests to the long-term, based on our previous long-term study, the PAOs would be crashed under lower pH condition, and then the GAOs took the advantage in the culture as opportunists.

However, the reason why GAOs were able to dominate in Stages 2 and 4 (pH ~6.5, rapid acetate concentration) is apparently not due the superior rates of acetate uptake, as PAOs had a clear advantage in this regard at higher acetate concentrations (Figure 6.9). This was consistent with the observation that PAOs appeared to fail at the beginnings of Stages 2 and 4, after which GAOs were able to increase in concentration (Chapter 5), and supports the hypothesis that GAOs may have been opportunists rather than being the direct cause of PAO failure. Some other factor associated with low pH and high acetate concentrations therefore appears to have been the cause of the PAO failure, as discussed in Chapter 5. Also the rate of acetate uptake by PAOs was independent of the external pH, while the acetate uptake rate of GAOs was inhibited by high pH, particularly at higher acetate concentrations, which was consistent with previous suggestions that that higher pH may improve EBPR (Schuler and Jenkins 2002).

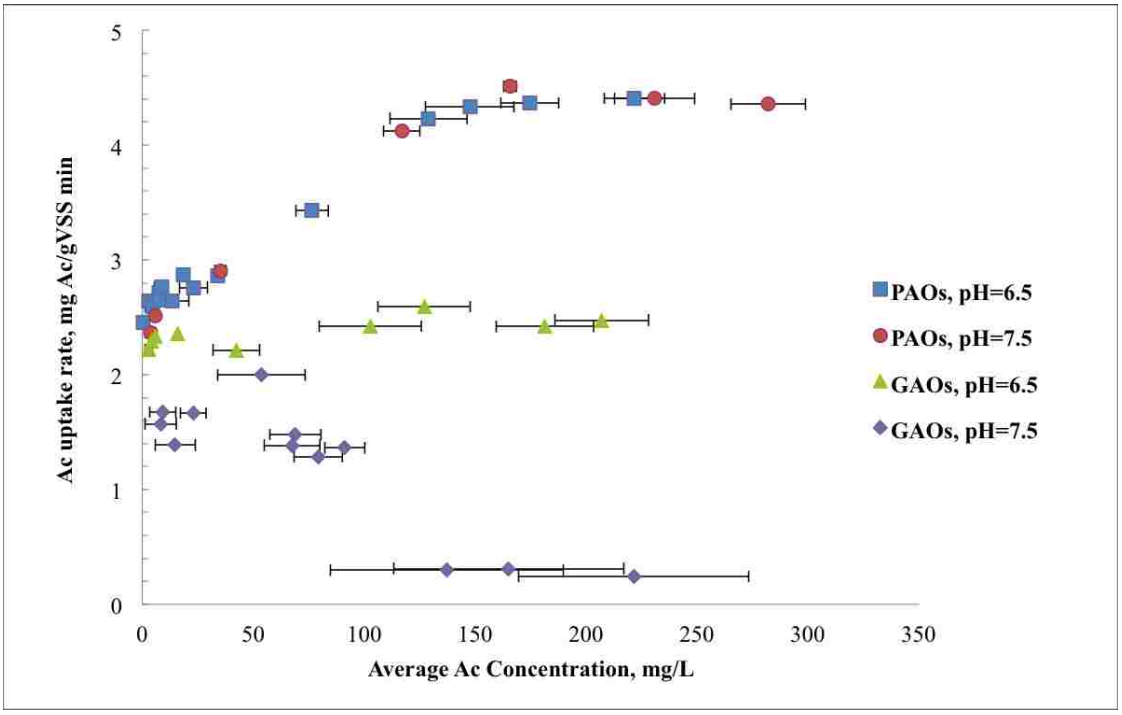


Figure 6.9 Acetate uptake rates in *Accumulibacter spp.* and *Defluvvicoccus spp.* enriched culture under different pH values.

CHAPTER 7. TEMPERATURE EFFECT ON THE COMPETITION BETWEEN PAOS AND GAOS IN EBPR SYSTEMS

As discussed in Chapter 2, temperature has been previously demonstrated an important factor in the competition between PAOs and GAOs and the related stability of EBPR systems, with higher temperatures reported to inhibit EBPR and possibly to favor GAOs (Whang and Park 2002; Panswad et al. 2003). In this section temperature and rates of acetate addition were varied in an SBR (Reactor 2) to verify that GAOs have an advantage over PAOs at higher temperatures, to evaluate whether low acetate concentrations (through slow rates of addition in the SBR) may increase EBPR activity at elevated temperatures, and to test other strategies for improving EBPR at elevated temperatures. Reactor was operated in the following conditions for this portion of the research (Table 7.1):

Table 7.1A Reactor 2 operational characteristics over 5 stages

Stage	Rate and duration of concentrated acetate feed addition/pH	Relative acetate concentration in anaerobic phase	Temperature	Cycle length (hour)	Day range (duration)
1	20 ml/min; 10 min/ 7.4-8.4	high	22 ±1 ^{o} C	8	20 to 39 (19)
2	20 ml/min; 10 min/ 7.4-8.4	high	29 ±1 ^{o} C	8	40 to 95 (55)
3	1.7 ml/min; 120 min/ 7.4-8.4	low	29 ±1 ^{o} C	8	96 to 112 (16)
4	1.7 ml/min; 120 min/ 7.4-8.4	low	29 ±1 ^{o} C	4	113 to 133 (20)

Table 7.1BReactor 2 performance characteristics over 4 stages

Stage	Pns/TSS (mg/mg)	Pns/VSS (mg/mg)	Anaerobic P release/acetate uptake (mol/C-mol)	Day range (duration)
1	0.15 ±0.003	0.21 ±0.008	0.60 ±0.007	20 to 39 (19)
2	0.027 ±0.002*	0.028 ±0.002*	0.016 ±0.003*	40 to 95 (55)
3	0.026 ±0.002*	0.027 ±0.002*	0.014 ±0.003*	96 to 112 (16)
4	0.027 ±0.002*	0.028 ±0.002*	0.023 ±0.006*	113 to 133 (20)

* Average value after stage reached to a new steady state

7.1 Stage 1: High pH, rapid acetate addition and low temperature

The conventional SBR operation in Reactor 2, with rapid acetate addition, relatively high pH (7.4-8.4), and relatively low temperature (22 ± 1 ^{o} C), during Stage 1 resulted in acetate concentrations at the end of the 10 minute acetate addition period of approximately 77 mg/L, which was less than the total 97.9 mg of acetate/L of reactor volume, due to acetate uptake during acetate feed addition. Acetate uptake was complete by end of the anaerobic phase (Figure 7.1 and 7.2B).

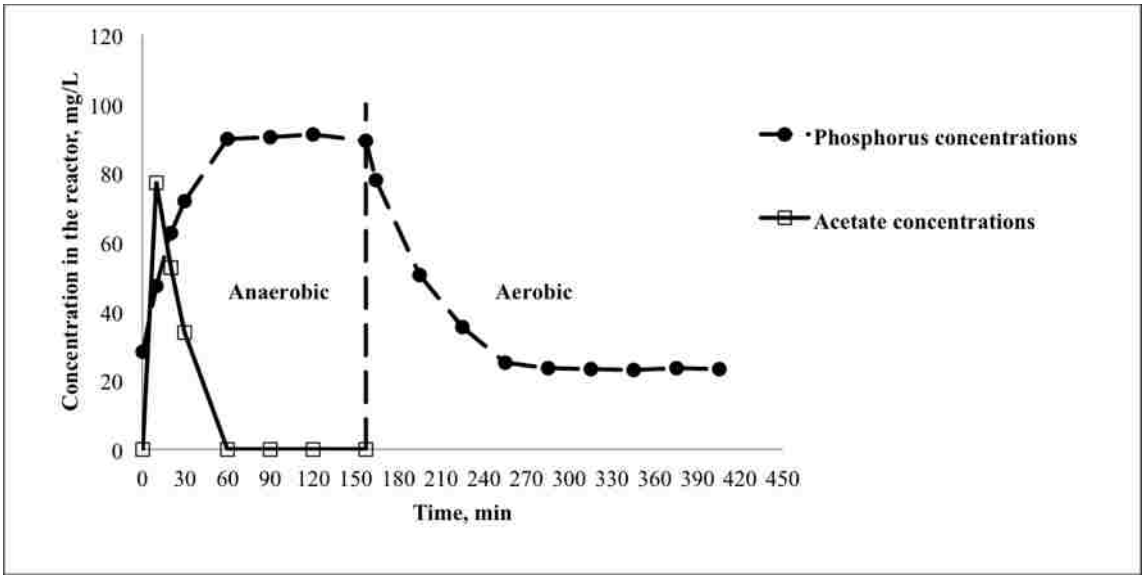


Figure 7.1 Acetate and phosphorus profiles throughout anaerobic and aerobic phases at stage 1 in the Reactor 2 (Samples taken on day 20)

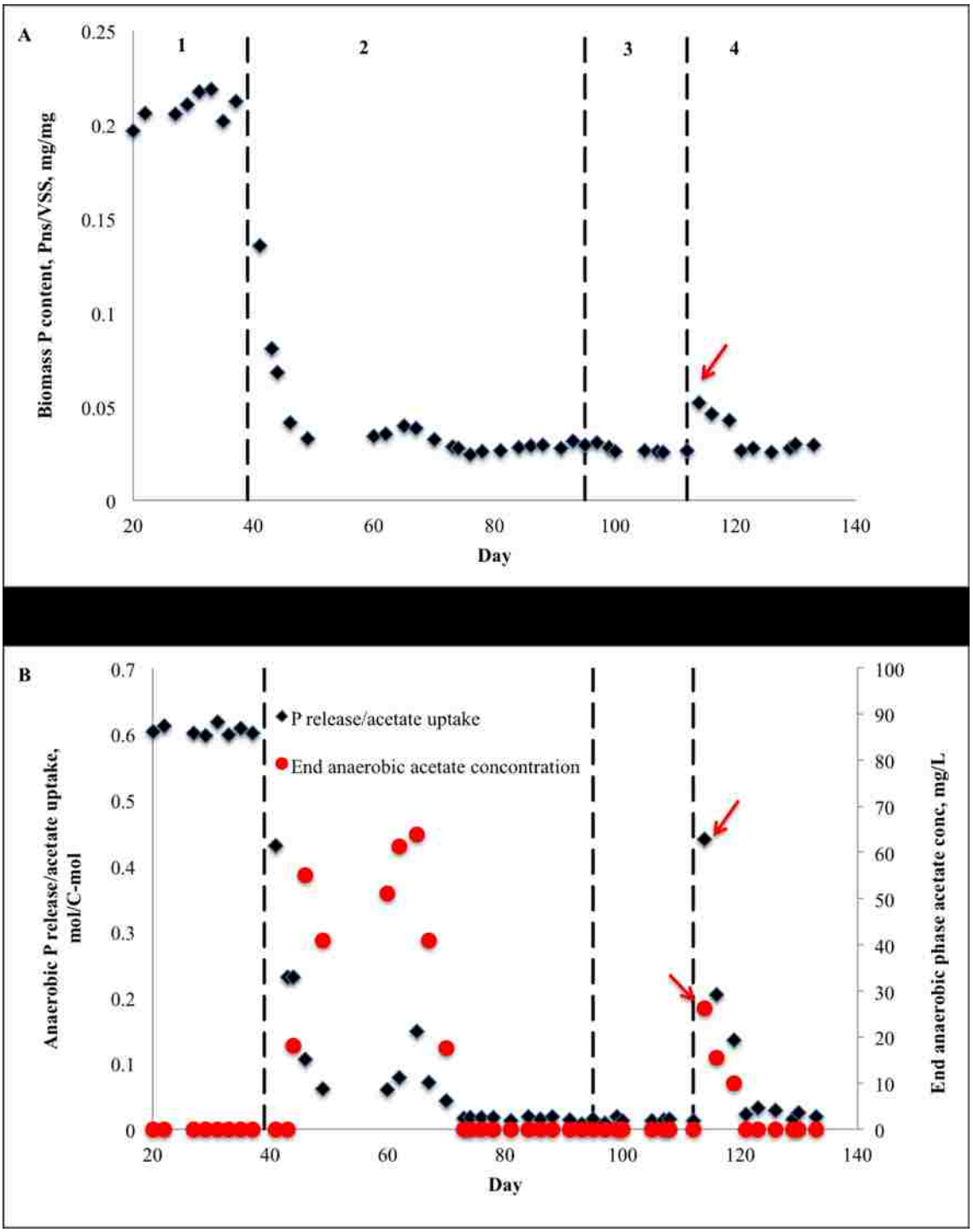
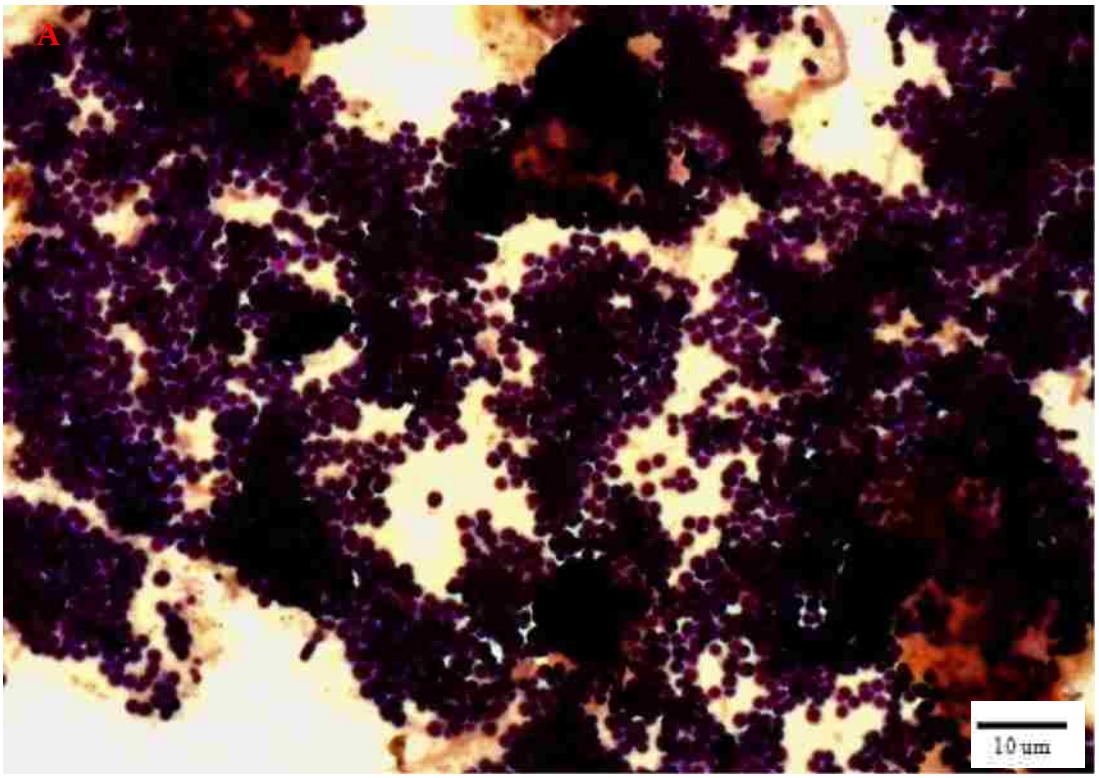
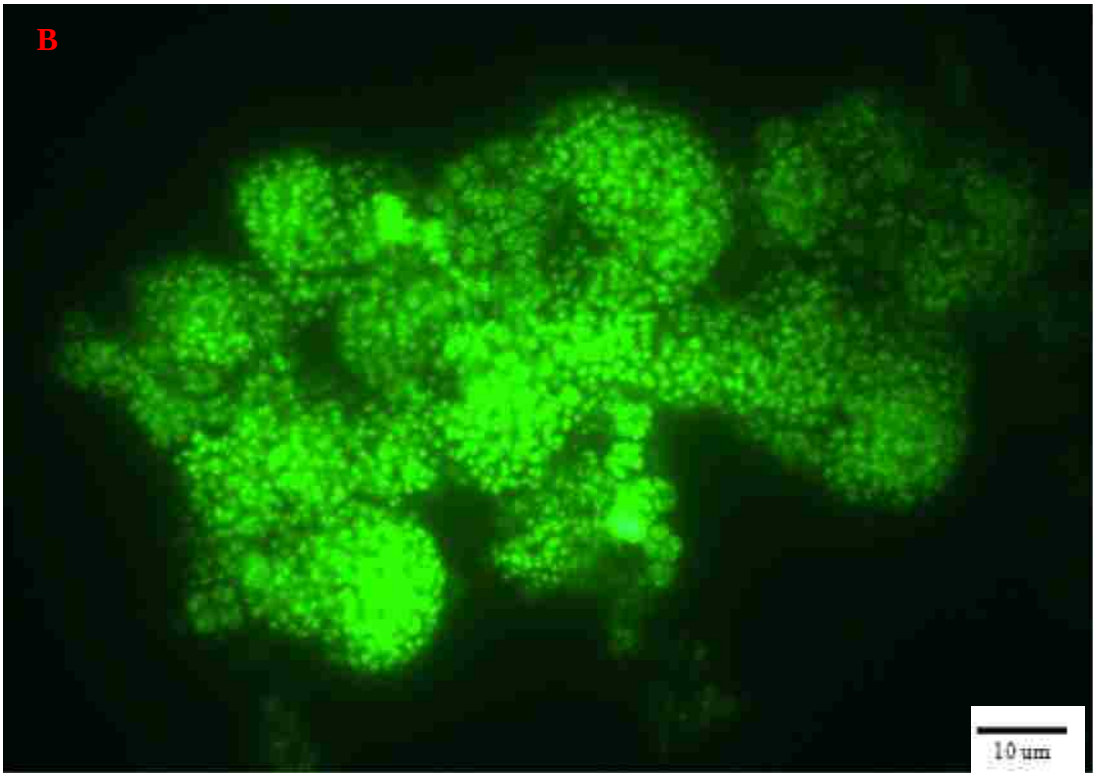


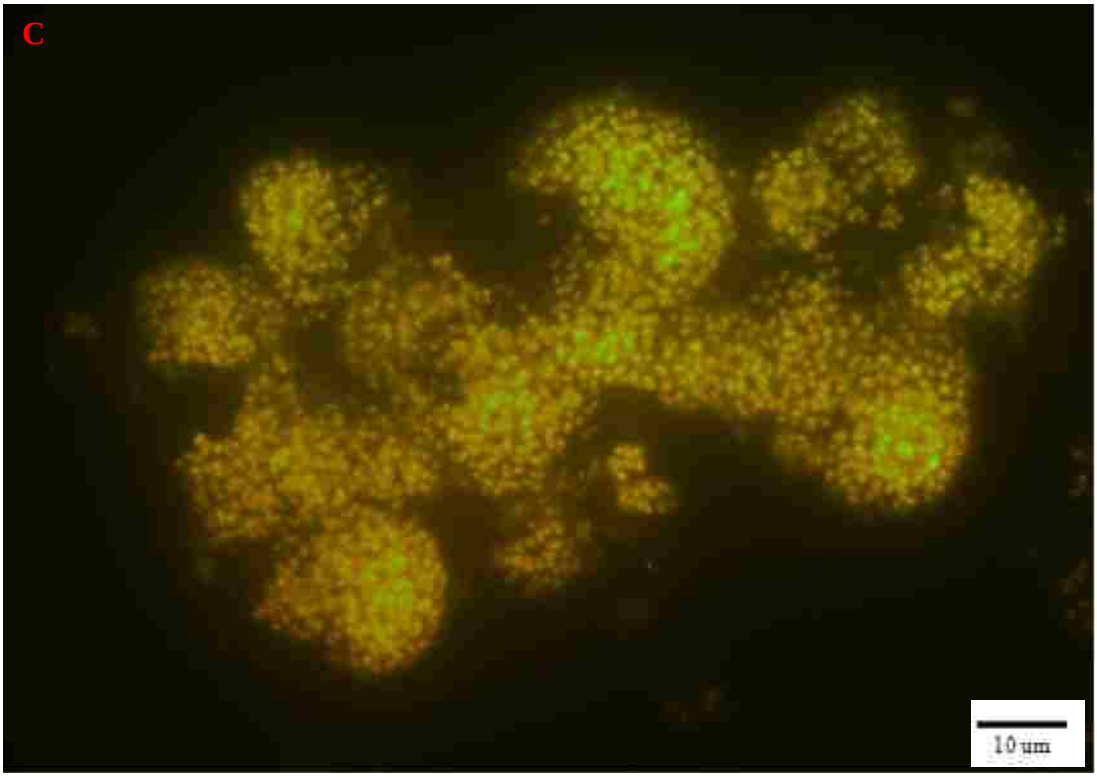
Figure 7.2 Reactor performance with respect to (A) phosphorus content of the biomass (Pns/VSS), and (B) anaerobic P release/acetate uptake and end anaerobic acetate concentrations during four Stages of operation with variable temperatures, rates of acetate addition, cycle length and magnesium concentrations.
Stage 1: Temperature controlled at 22 ± 1 ^{o} C; Stage 2: Temperature controlled at 29 ± 1 ^{o} C; Stage 3: Acetate addition rate changed to slow addition; Stage 4: SBR operation cycle changed to short cycle; Red arrow indicates seeding 200 ml PAO-enriched EBPR sludge on day 114.

Several measurements indicated that the Stage 1 culture was highly enriched with PAOs, similar to Stage 1 in the Reactor 1. The biomass had a relatively high phosphorus content (non-soluble P/TSS [Pns/TSS] of 0.15 ±0.003 mg/mg, non-soluble P/VSS [Pns/VSS] of 0.21 ±0.008 mg/mg, Table 7.1B), and Prel/Ac was 0.60 ±0.007 mol/C-mol, which were evidence for high amounts of polyphosphate storage.

In addition, microscopic examination confirmed that the Reactor 2 Stage 1 conditions enriched for a highly PAO-dominated culture. Neisser staining indicated a large quantity of polyphosphate-containing cocci, which is consistent with the PAO phenotype (Figure 7.3A). FISH analyses indicated 76 ±9% of the bacteria were positive for the PAOmix probe targeting *Accumulibacter spp.* (Figures 7.3B and 7.3C) in samples taken during Stage 1. *Competibacter spp.* and cluster 1 *Defluviicoccus spp.* were not detected. Cluster 2 *Defluviicoccus spp.* were around 4 ±3% of the bacteria. (Figure 7.4).







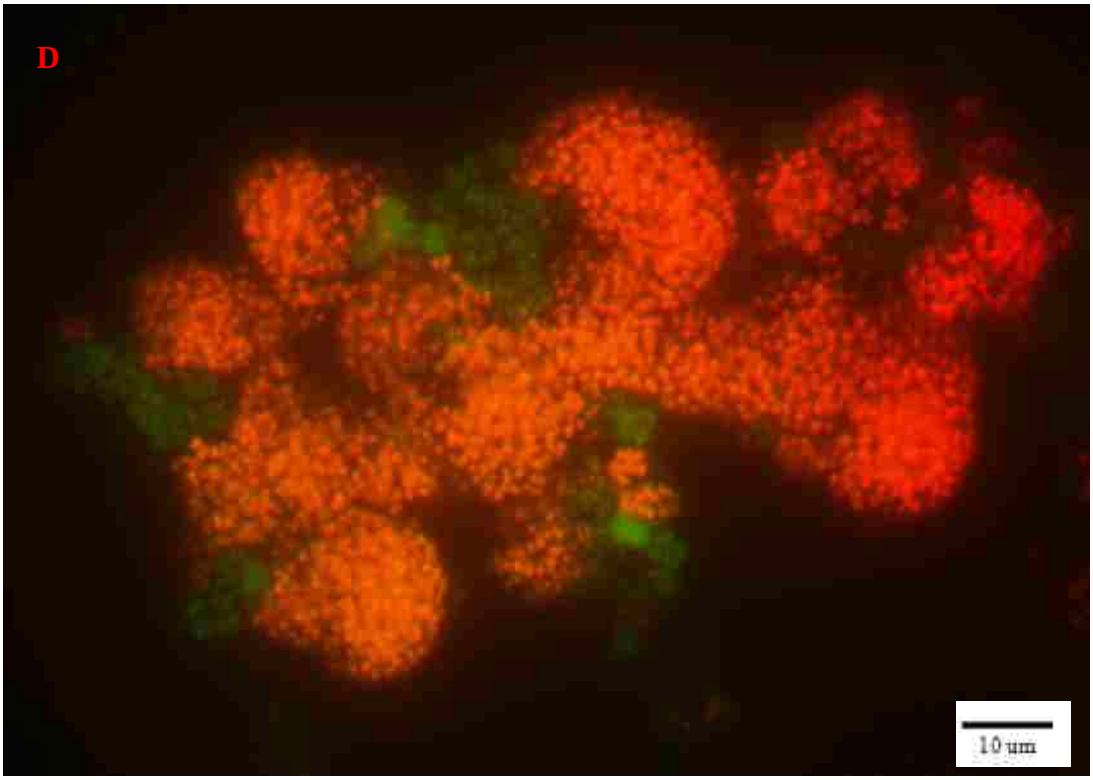


Figure 7.3 Neisser Staining and FISH results on reactor samples taken Stage 1 in the reactor 2. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser stain, dark cells were PAOs, suggesting the accumulation of polyphosphate storage (day 29), (B*) In situ* hybridization with probes EUBMIX (FAM) specific for many but not all Bacteria (green), (C) *In situ* hybridization with probes PAOMIX (Cy3) specific for *Accumulibacter spp.* (orange) (day 20), and (D) Overlay (B) with (C), *Accumulibacter spp*. appear orange.

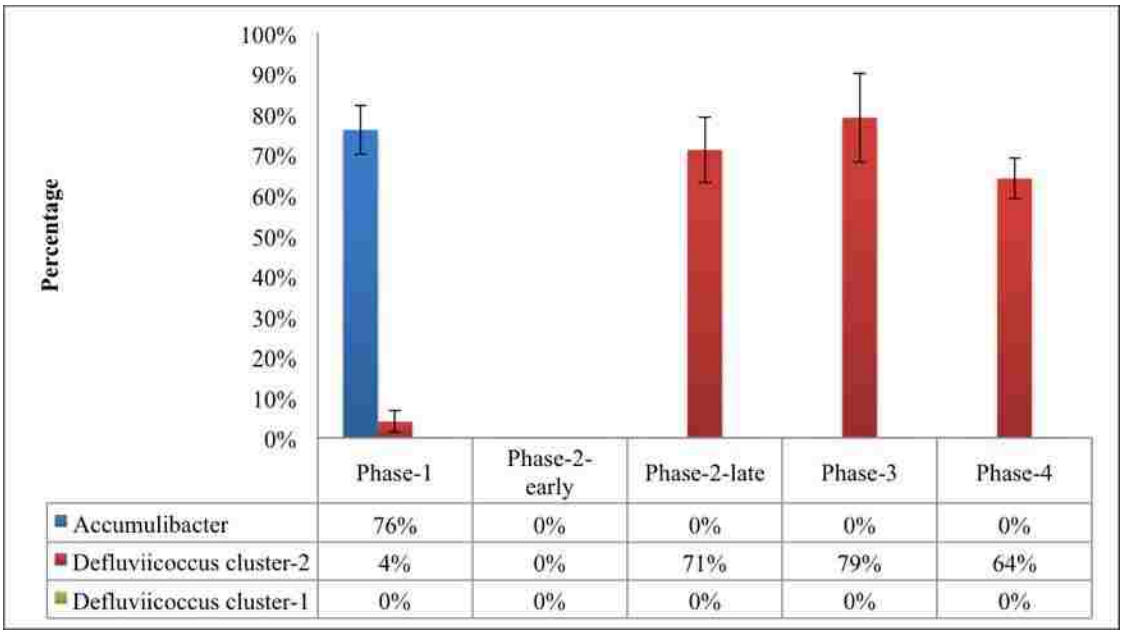


Figure 7.4 FISH Data summary

7.2 Stage 2: High pH, rapid acetate addition and high temperature

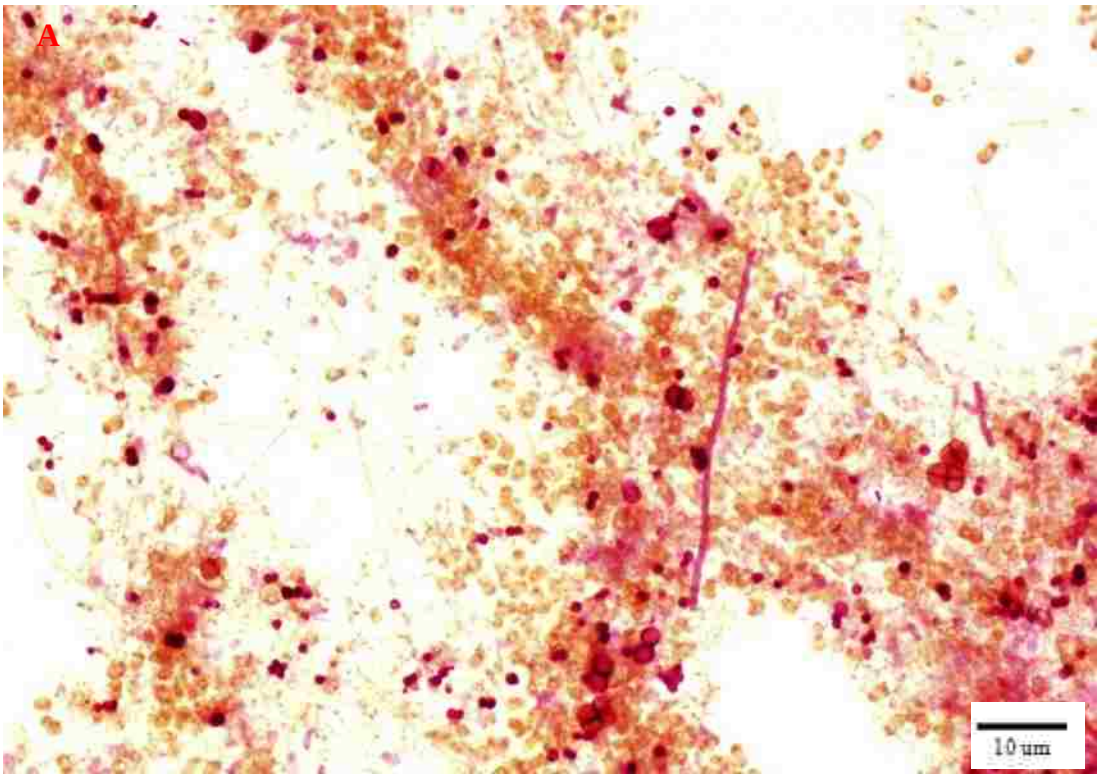
Stage 2 was initiated by increasing the reactor temperature to 29 ± 1 ^{o} C on Day 40, while maintaining the other operations the same (Table 7.1). The increased temperature change led to a decline in PAO activity, as indicated by a drop in the Pns/VSS and Prel/Ac values after the change was made (Figure 7.2). The biomass phosphorus content decreased from 0.21 ± 0.008 (based on MLVSS) at the end of Stage 1 to 0.027 ± 0.002 (based on MLVSS) by day 95. The low biomass phosphorus content and absence of significant phosphorus release after day 49 indicated that EBPR had ceased. Another indication that PAO activity decreased was that acetate began to "leak" into the aerobic phase after the temperature was increased, with end anaerobic acetate concentrations increasing from zero to a maximum of 67 mg/L 20 days after the temperature change (Figure 7.2B). It took approximately 40 days for the reactor to reach a new steady-state with respect to Pns/VSS, Prel/Ac ratio, and return to complete acetate uptake during the anaerobic phase (Figure 7.2).

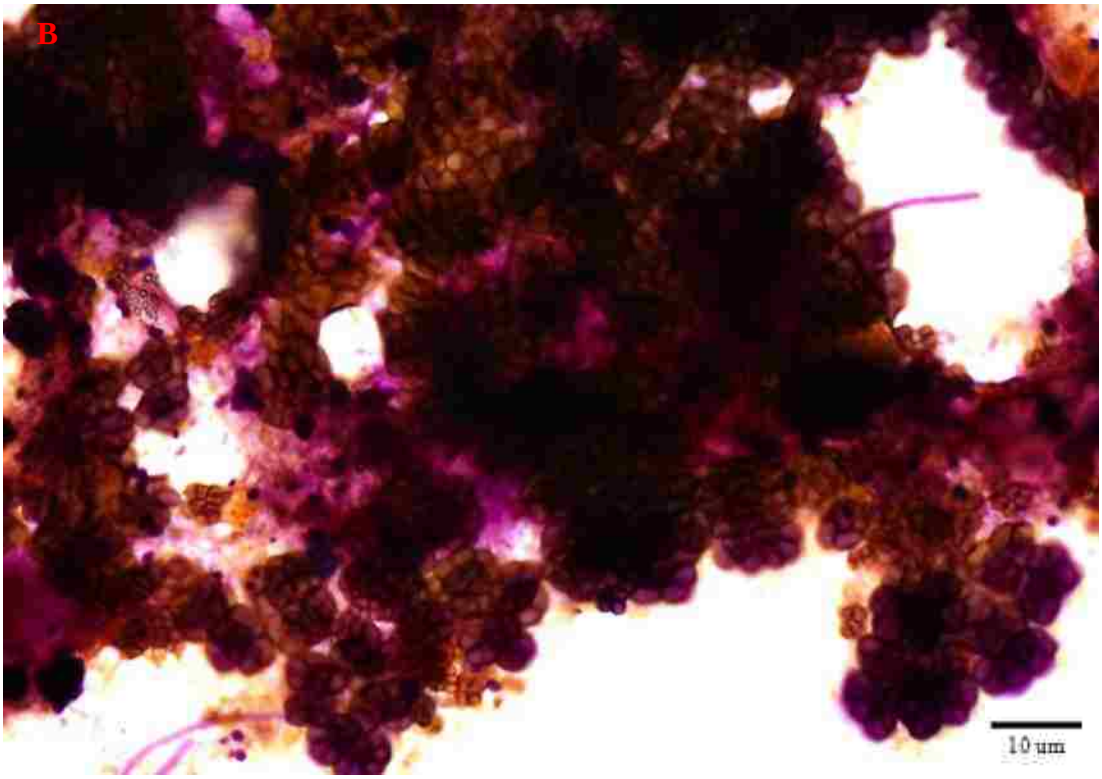
The presence of acetate in the reactor at the end of the anaerobic phase after the temperature increase (Figure 7.2B) indicated that increased competition from GAOs for this substrate was not the cause of the PAO decline, which, consistent with the results

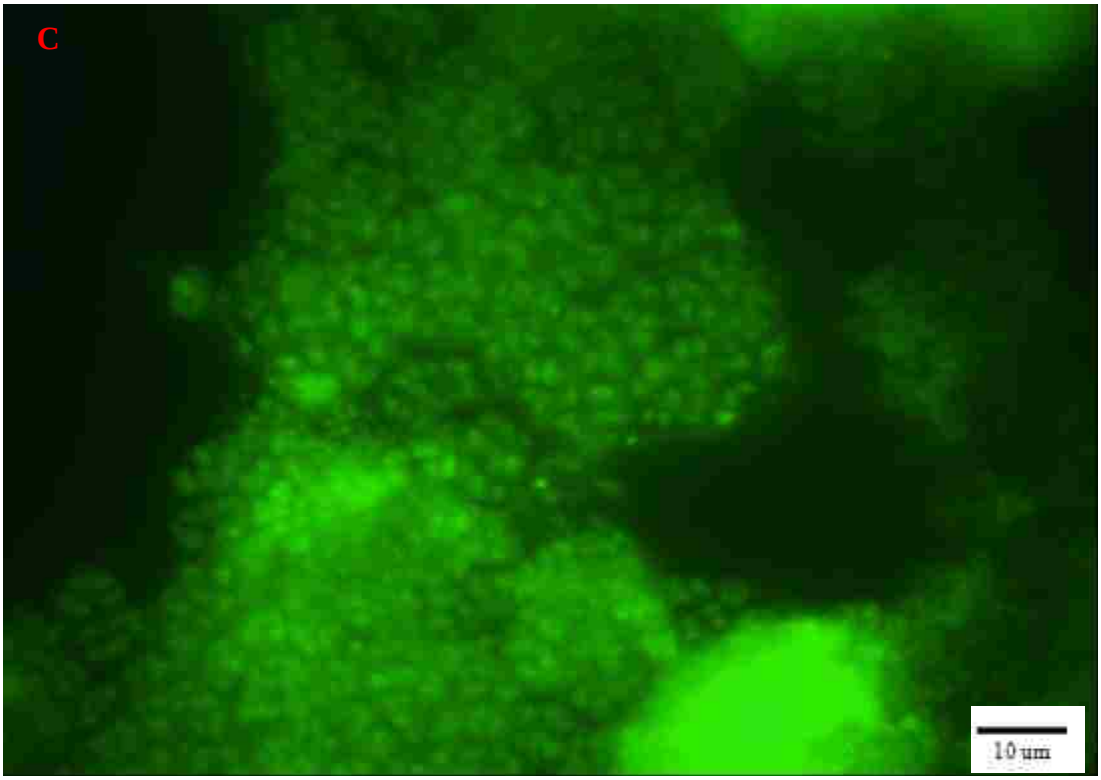
from decreased pH in Chapter 5, suggested that the GAOs may have been opportunists that were able to thrive in the system only after anaerobic uptake by PAOs had decreased, as discussed in Chapter 5. A

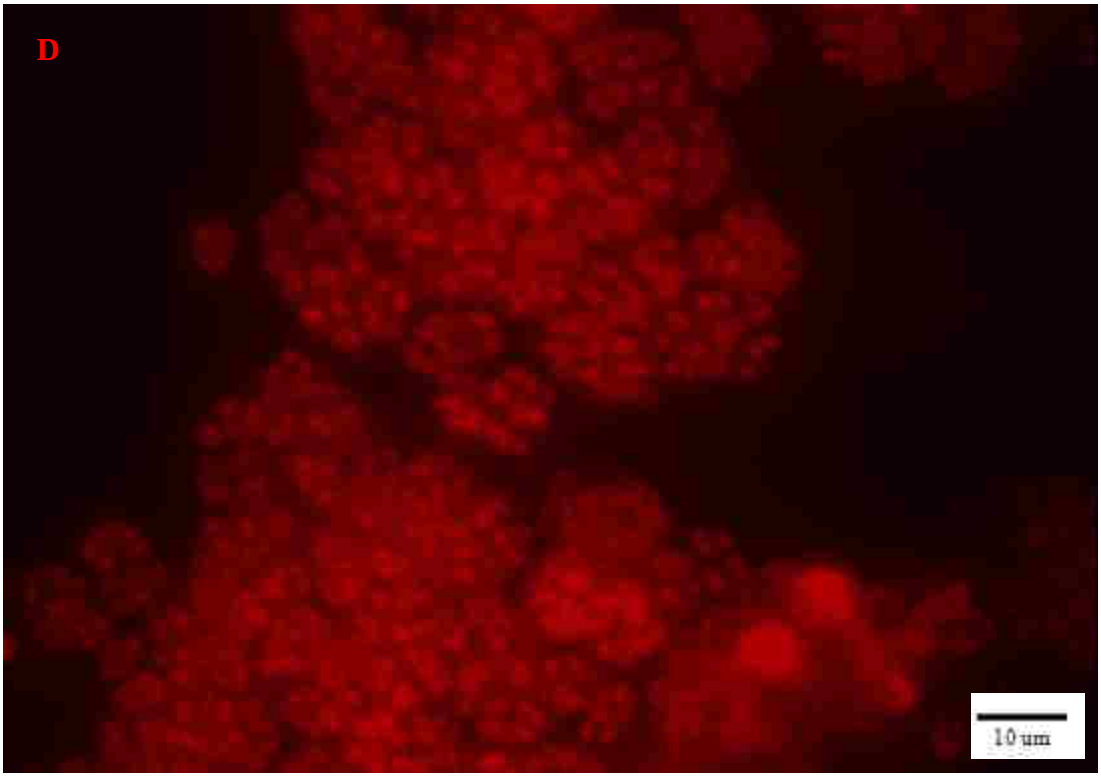
Figure 7.5 showed the characteristic morphology of the dominant microorganisms in the SBR. Neisser staining demonstrated that the bacteria arranged in distinctive tetrads, which are very specific morphology of the GAOs dominated the culture in late Stage 2 (Figure 7.5A and 7.5B). Quantitative FISH showed that cluster 2 *Defluviicoccus spp.* represented 71 ±8% of the bacteria, Cluster 1 *Defluviicoccus spp.*, *Competibacter spp.* and *Accumulibacter spp.* were all absent (Figure 7.4, Figure 7.5C and 7.5D).

The variations of P release/Ac uptake and biomass phosphorus content shown in Figure 7.2 confirmed that increased temperature can inhibit EBPR, in agreement with the results of previous long-term studies (Whang and Park 2002). They also operated the SBRs at 20 ^{o} C and 30 ^{o} C respectively, and found the Pns/VSS was about 18% (20% for our research) at 20 ^{o} C, however the Pns/VSS at 30 ^{o} C was decreased to less than 1% (0.028% for our research).









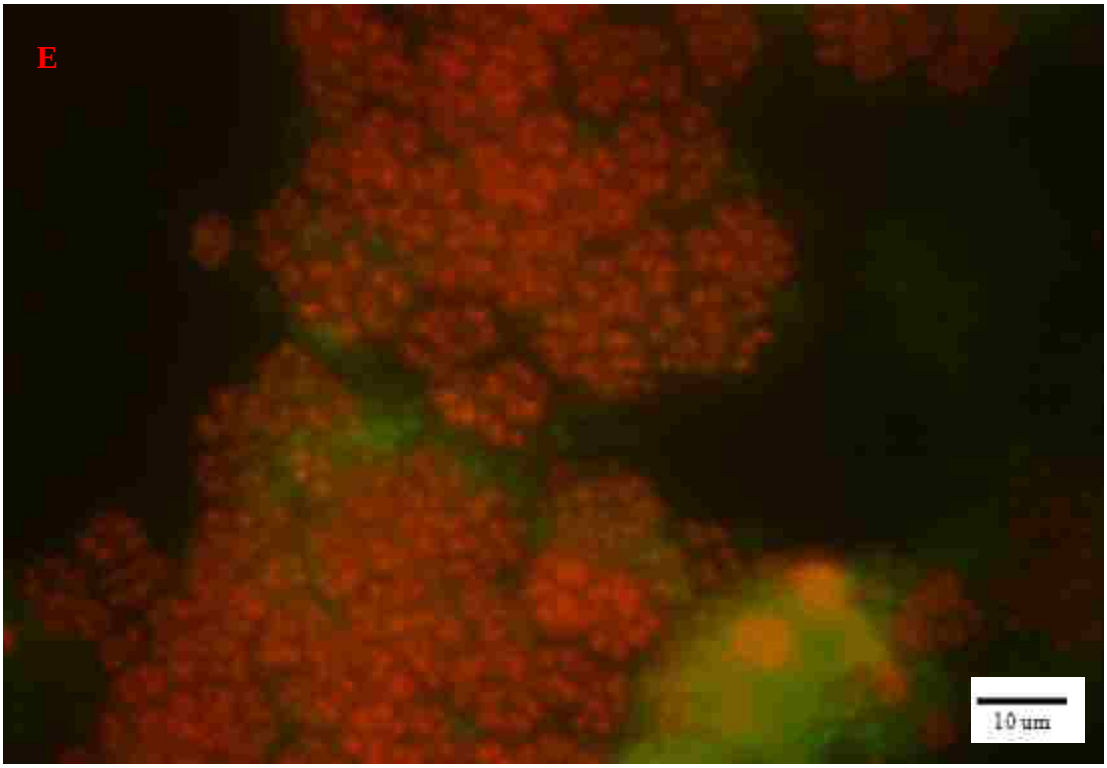


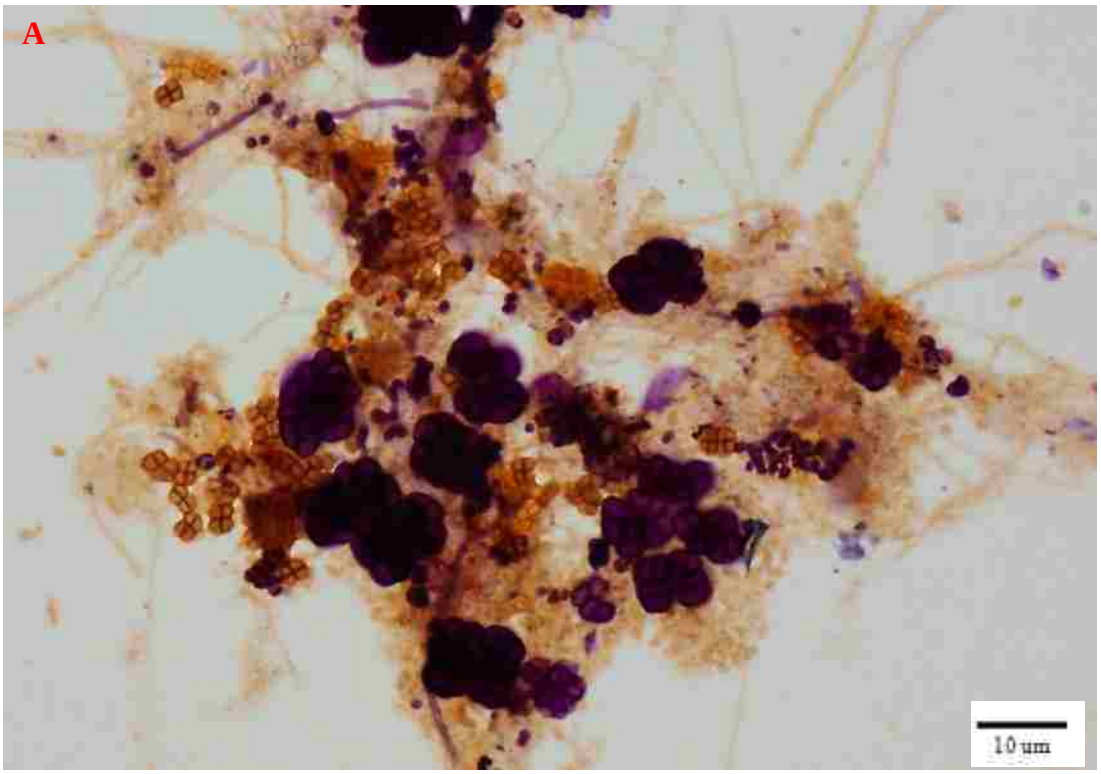
Figure 7.5 Neisser Staining and FISH results on activated sludge samples taken stgage-2 in the reactor 2. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser staining image for the period that anaerobic acetate uptake was not complete, almost no positive results (day 61), (B) Neisser stain image for the period that anaerobic acetate uptake was complete, the typical tetrad cells were most of GAOs (day 92), (C) *In situ* hybridization with probes EUBMIX (TAMRA) specific for many but not all Bacteria (orange), (D) *In situ* hybridization with probes DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) (Cy5) specific for cluster 2 *Defluviicoccus spp*. (red) day (91)**,** and (E) Overlay (C) with (D), *Defluviicoccus spp*. appear orange.

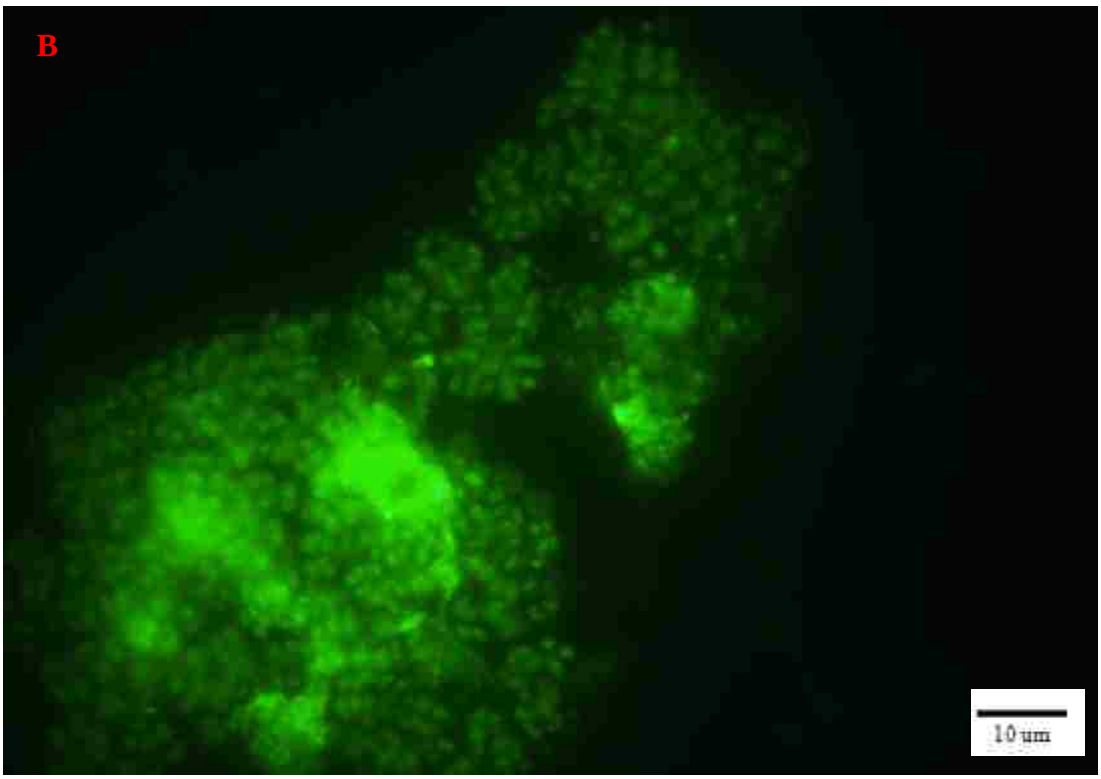
7.3 Stage 3: Slow acetate addition with high temperature

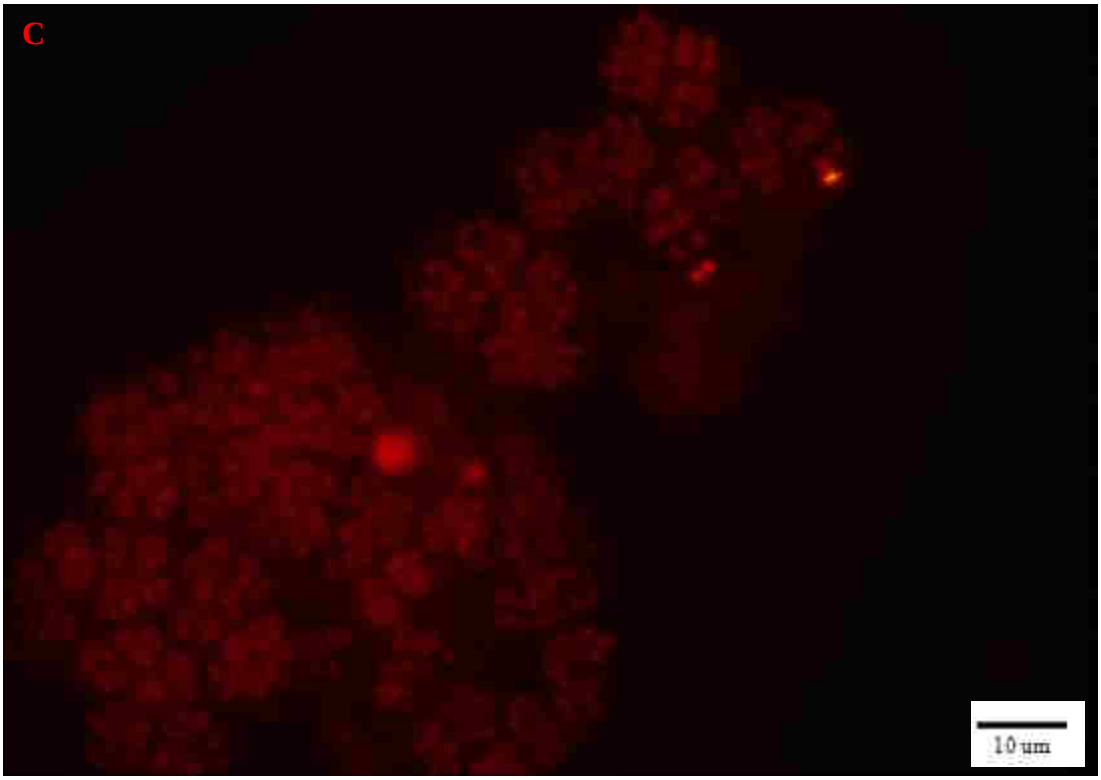
Because low acetate concentrations obtained by slowing down the rates of acetate feed addition were found to increase PAO populations in Reactor 1 (Chapter 5), this strategy was also applied to the high temperature (30 ^{o} C) condition to see if this would improve EBPR in this condition as well. In Stage 3, the acetate feed flow rate was decreased so that it was added over the course of 112 minutes, rather than over 10 minutes as in Stages 1 and 2, with the other operations maintained in the same as Stage 2.

Throughout Stage 3, the Prel/Ac ratios and biomass phosphorus contents remained low (Figure 7.2) suggesting that the PAOs didn't return to dominate the reactor. The acetate uptake in the end of anaerobic phase was also complete throughout this Stage (Figure 7.2B), demonstrating that GAOs didn't fail in the reactor. The Prel/Ac ratio was 0.014 ± 0.003, and the biomass phosphorus content (Pns/VSS) was 0.027± 0.002 mg/mg over this Stage (Table 7.1B).

Microscopy confirmed that the GAO populations were still enriched in the culture during Stage 3. Neisser staining indicated the culture was dominated by tetrads morphology GAOs in this Stage (Figure 7.6A). Similarly, FISH analyses indicated 79 ± 11% of the bacteria were cluster 2 *Defluviicoccus spp.* targeted by the DF2mix probe set (Figures 7.6B and C). Cluster 1 *Defluviicoccus spp*., *Competibacter spp*. and *Accumulibacter spp.* were all absent (Figure 7.4).







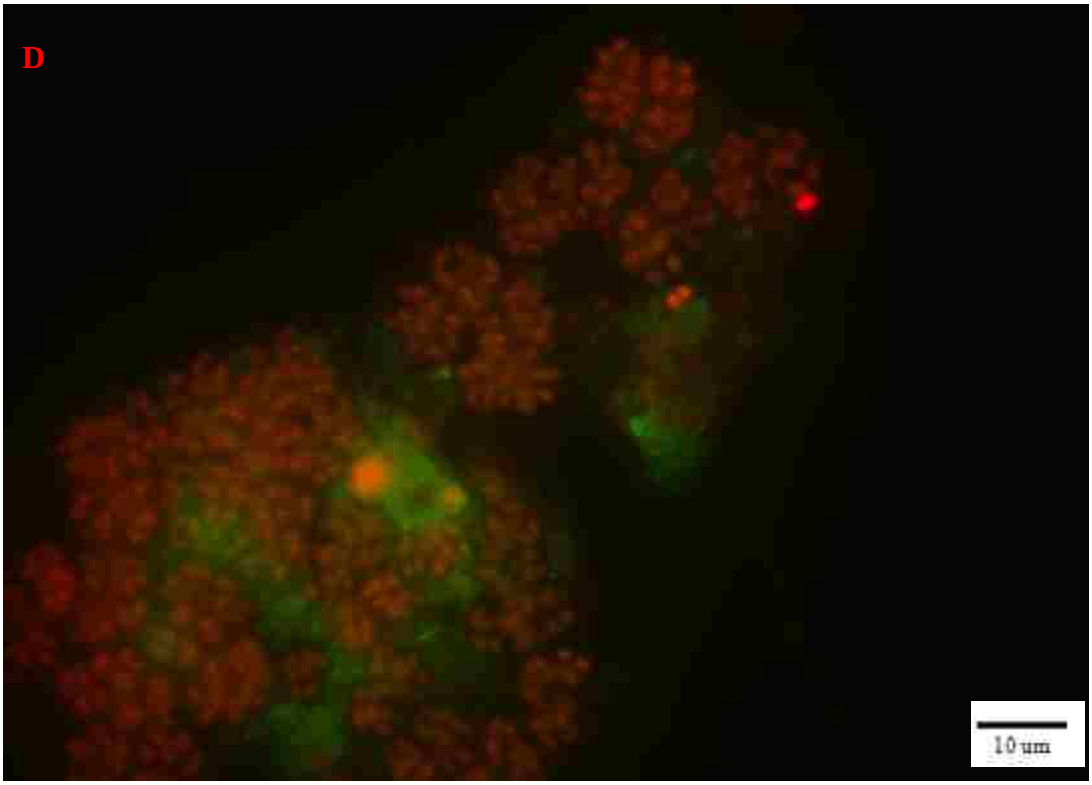


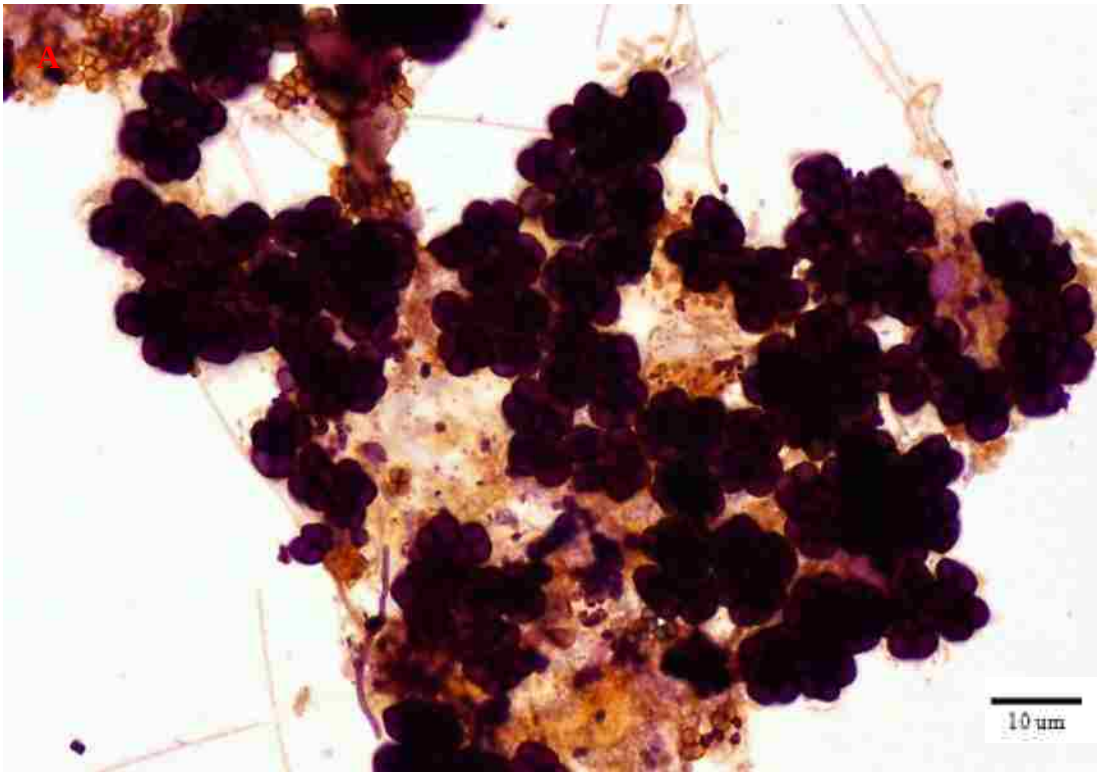
Figure 7.6 Neisser Staining and FISH results on activated sludge samples taken stgage-3 in the reactor 2. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser stain image for the period that anaerobic acetate uptake was complete, the typical tetrad cells were most of GAOs (day 98), (B) *In situ* hybridization with probes EUBMIX (TAMRA) specific for many but not all Bacteria (orange), (C) *In situ* hybridization with probes DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) (Cy5) specific for cluster 2 *Defluviicoccus spp*. (red) (day 109), and (D) Overlay (B) with (C), *Defluviicoccus spp*. appear orange.

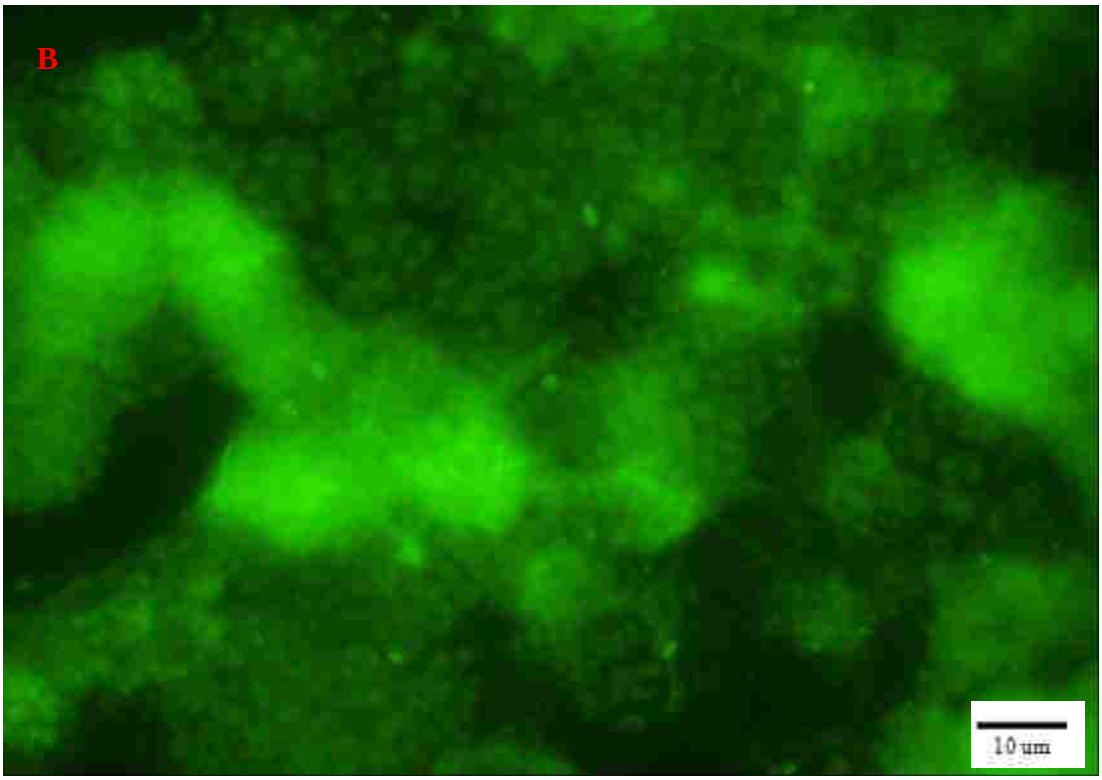
7.4 Stage 4: Reduce the cycle from 8 hours to 4 hours

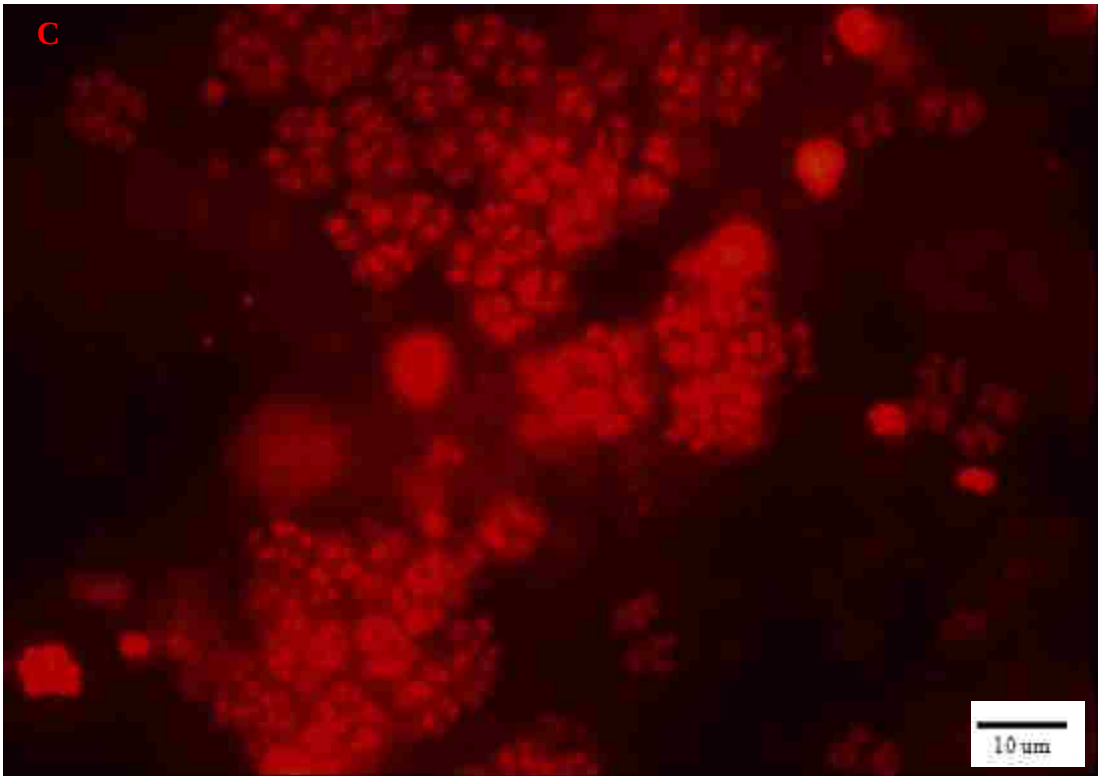
It was hypothesized that one reason for failed EBPR at higher temperatures may have been linked to increased rates of maintenance energy consumption (such as endogenous resperation) linked to higher temperatures, which could lead to depletion of internal storage products (Lopez-Vazquez et al. 2007; Lopez-Vazquez et al. 2008; Lopez-Vazquez et al. 2009b). A sub-hypothesis was that shortening the anaerobic and aerobic phase lengths could be a feasible remedy to this problem, as this would decrease the time in which the bacteria were consuming energy for maintenance while "waiting" for the next phase to start. In Stage 4, the reactor was operated with a 4 h cycle and seeded with 200 ml reactor 1 sludge (PAO-enriched sludge), the other operations were the same as Stage 3 (Table 7.1).

The result of these changes was that EBPR failed once again, as indicated by low biomass P content and Prel/Ac uptake ratios (Figure 7.2). The increase of biomass P content and Prel/Ac uptake ratios in the beginning of Stage 4, which was due to the seeding with fresh PAO-enriched sludge (Figure 7.2). The Pns/VSS and Prel/Ac uptake values were approximately the same as Stages 2 and 3, at 0.028 ± 0.002 mg/mg and 0.023 ± 0.006 mol/C-mol respectively, within 6 days after the beginning of Stage 4 (Figure 7.2). cetate began to "leak" into the aerobic phase after the cycle was decreased, but it was completely taken up in the anaerobic phase after 6 days, which suggested that the GAOs required this time to adapt to the shortened cycle length (Figure 7.2B).

Microscopy also indicated that the GAO populations were enriched in the culture during Stage 4. Neisser staining showed the tetrad morphology GAOs (Figure 7.7A). FISH analyses showed that cluster 2 *Defluviicoccus spp.* represented 64 ± 5% of the bacteria. Cluster 1 *Defluviicoccus spp*., *Competibacter spp*. and *Accumulibacter spp.* were all absent (Figure 7.4, Figures 7.7B and 7.7C).







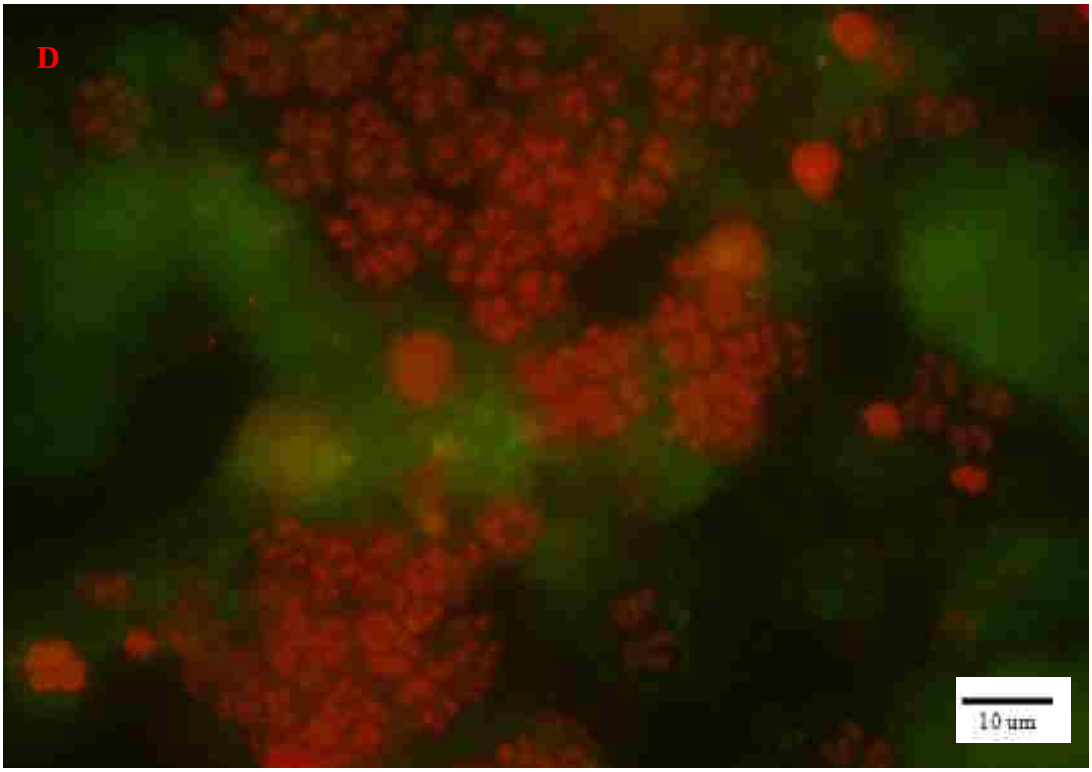


Figure 7.7 Neisser Staining and FISH results on activated sludge samples taken stgage-4 in the reactor 2. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser stain image for the period that anaerobic acetate uptake was complete, the typical tetrad cells were most of GAOs (day 126), (B) *In situ* hybridization with probes EUBMIX (TAMRA) specific for many but not all Bacteria (orange), (C) *In situ* hybridization with probes DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) (Cy5) specific for cluster 2 *Defluviicoccus spp*. (red) (day 130)**,** and (D) Overlay (B) with (C), *Defluviicoccus spp*. appear orange.

These results confirmed that PAO are better suited to cooler temperatures (around 20 ^{o} C), while they failed at 30 ^{o} C and GAOs were enriched. Strategies to improve EBPR at the higher temperature, including (decrease the acetate addition rate and reduce the cycle length), failed. Further research is necessary to determine satisfactory approaches to improve EBPR at warmer temperatures.

CHAPTER 8. EFFECTS OF PH, ACETATE FEED RATE, AND CYCLE LENGTH ON PNS/VSS AND PREL/AC VALUES

The results presented in Chapter 5 demonstrated that PAOs were enriched in the Stage 1 (rapid acetate addition, pH = 7.4 to 8.4) and the Stage 3 (slow acetate addition, pH = 6.4 to 7.0) conditions, and that these conditions produced biomasses with different phosphorus contents (Pns/VSS) and anaerobic P release/acetate uptake values (Figure 5.3). Because both pH and the rate of acetate addition varied between these conditions, it was not known which of these variables were the primary cause of these changes, or whether both of them contributed. Additional experiments were conducted with slow acetate addition and high pH values to resolve this question. In addition, it was hypothesized that the SBR cycle length may affect phosphorus storage in the biomass, as longer cycle lengths are associated with less frequent acetate addition and more acetate added each anaerobic phase. This in turn could require more polyphosphate degradation each cycle to accommodate this acetate uptake, and could be associated with increased polyphosphate storage. Because high Pns/VSS values are desirable for P removal from wastewater, these results could help to improve the EBPR process. The additional experiments including operation of Reactor 3 with a shorter cycle length (6 h) than used in Reactor 1 (8 h) and these conditions described in Table 8.1:

Table 8.1 Reactor characteristics over 4 stages

Stage	Rate and duration of concentrated acetate feed addition/pH	Relative acetate concentration in anaerobic phase	Pns/TSS (mg/mg)	Pns/VSS (mg/mg)	Anaerobic P release/acetate uptake (mol/C-mol)	Day range (duration)
1	20 ml/min; 10 min/7.4-8.4	High	0.14 ± 0.004	0.19 ± 0.004	0.62 ± 0.013	22 to 40(19)
2	1.7 ml/min; 120 min/7.4-8.4	Low	0.20 ± 0.005*	0.35 ± 0.015*	0.77 ± 0.012*	41 to 92 (52)

* Average value after stage reached to a new steady state

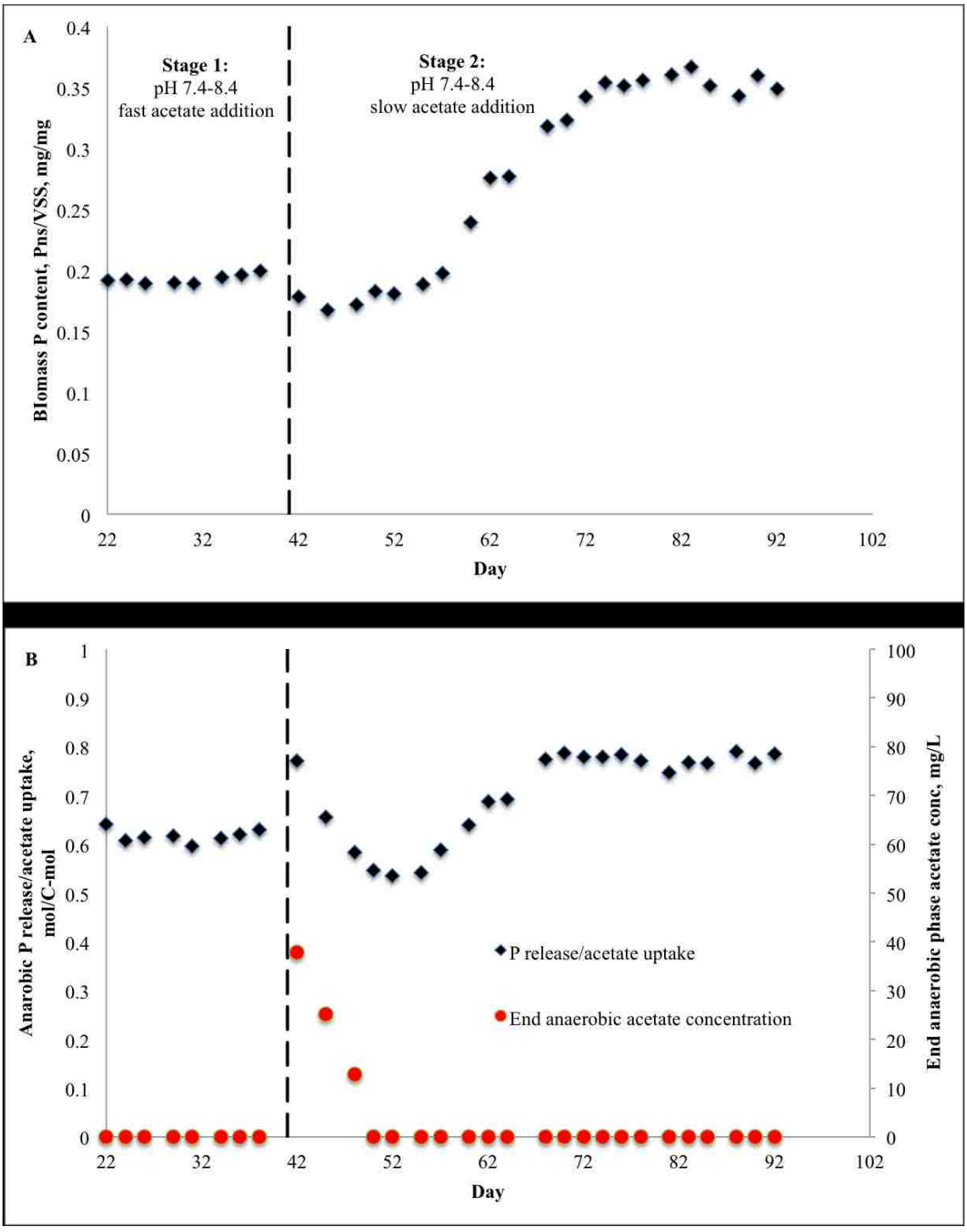


Figure 8.1 Reactor 3 performance with respect to (A) phosphorus content of the biomass (Pns/VSS), and (B) anaerobic P release/acetate uptake and end anaerobic acetate concentrations during Stages 1 (rapid acetate addition) and 2 (slow acetate addition)

8.1 Stage 1: Rapid acetate addition and higher pH

As noted, Reactor 3 was operated with a shorter cycle length (6 h) than Reactor 1 (8 h), to test the effect of cycle length on EBPR behavior. Reactor 3 Stage 1 conditions were similar to conventional SBR operation, with rapid acetate addition and pH = 7.4 to 8.4. The phases within the cycle are described (Table 8.1)

The results from a typical anaerobic/aerobic profile are shown in Figure 8.2. These are similar to those found for Reactor 1, Stage 1 (Figure 5.1), with acetate concentrations at the end of the 10 minute acetate addition period of approximately 79 mg/Land complete acetate uptake by end of the anaerobic phase.

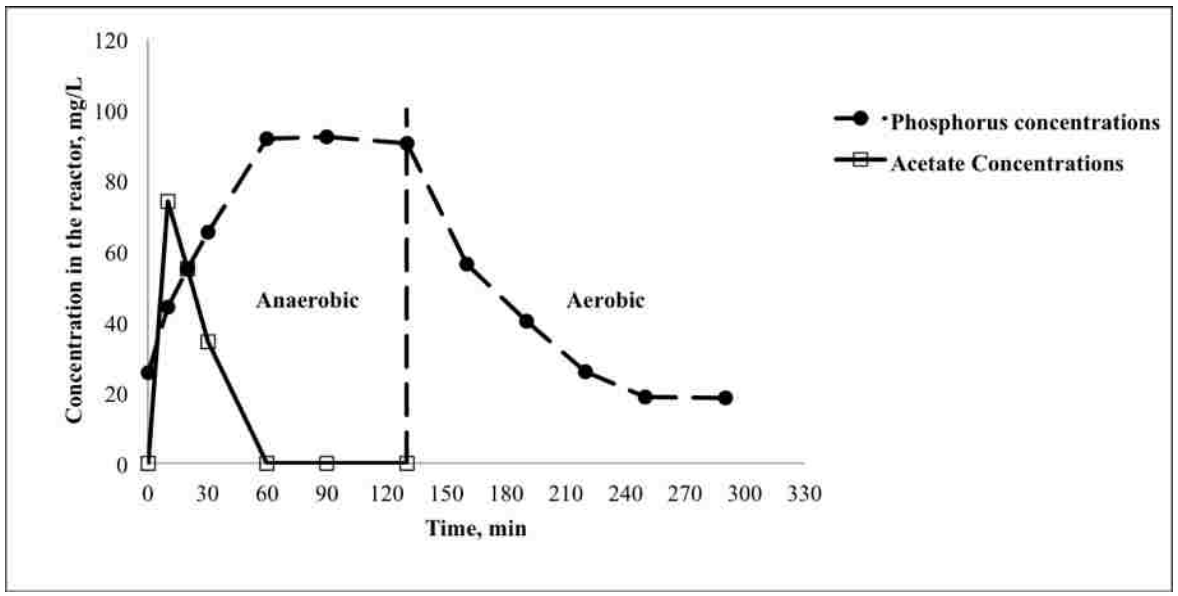
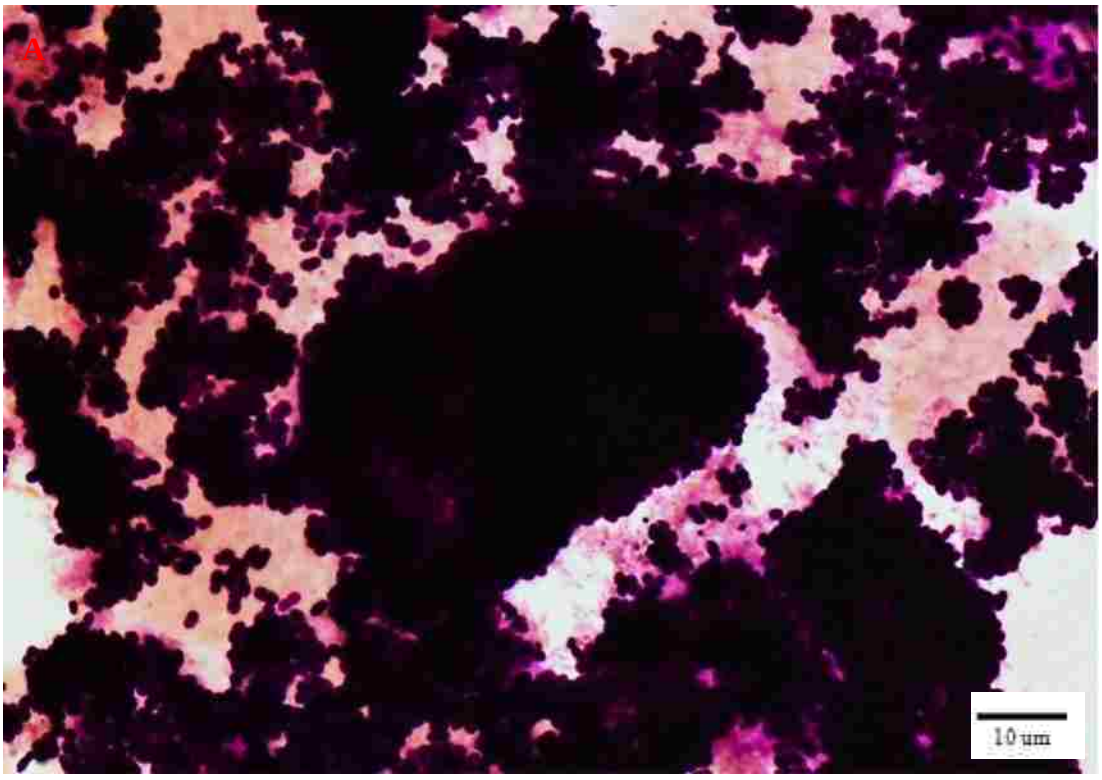


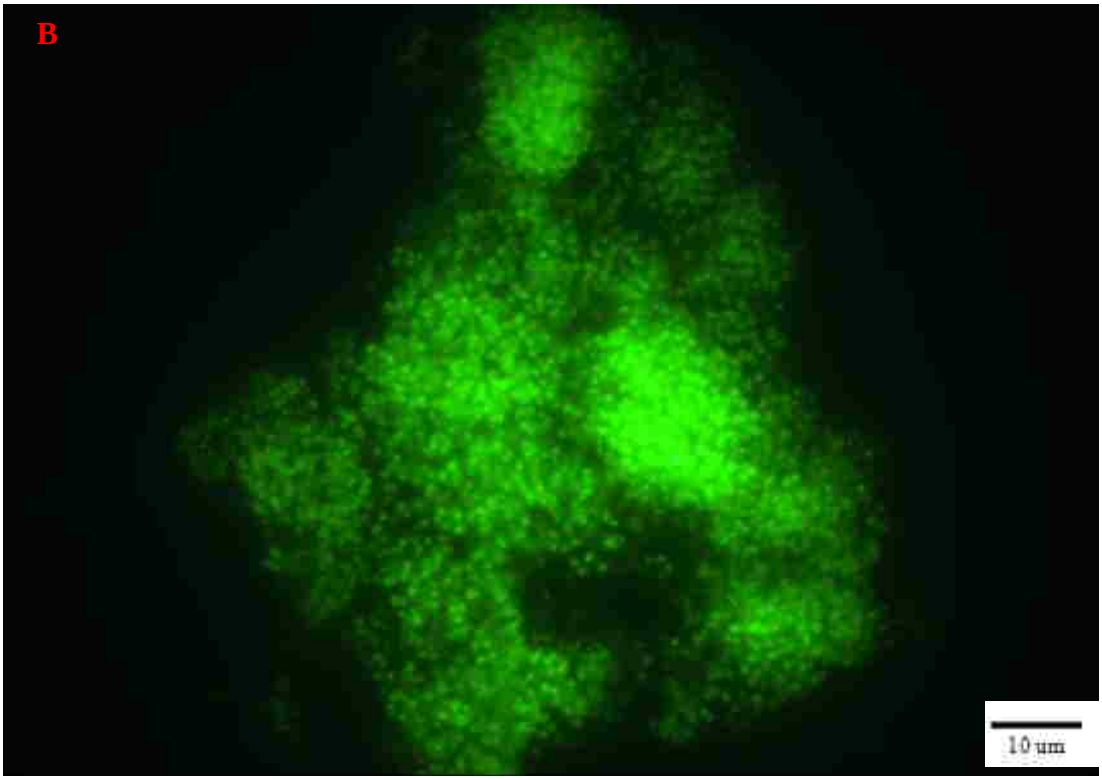
Figure 8.2 Reactor 3, Stage 1: Acetate and phosphorus profiles throughout anaerobic and aerobic phases with rapid acetate addition, pH = 7.4-8.4, 6 h cycle (Sample taken on day 24)

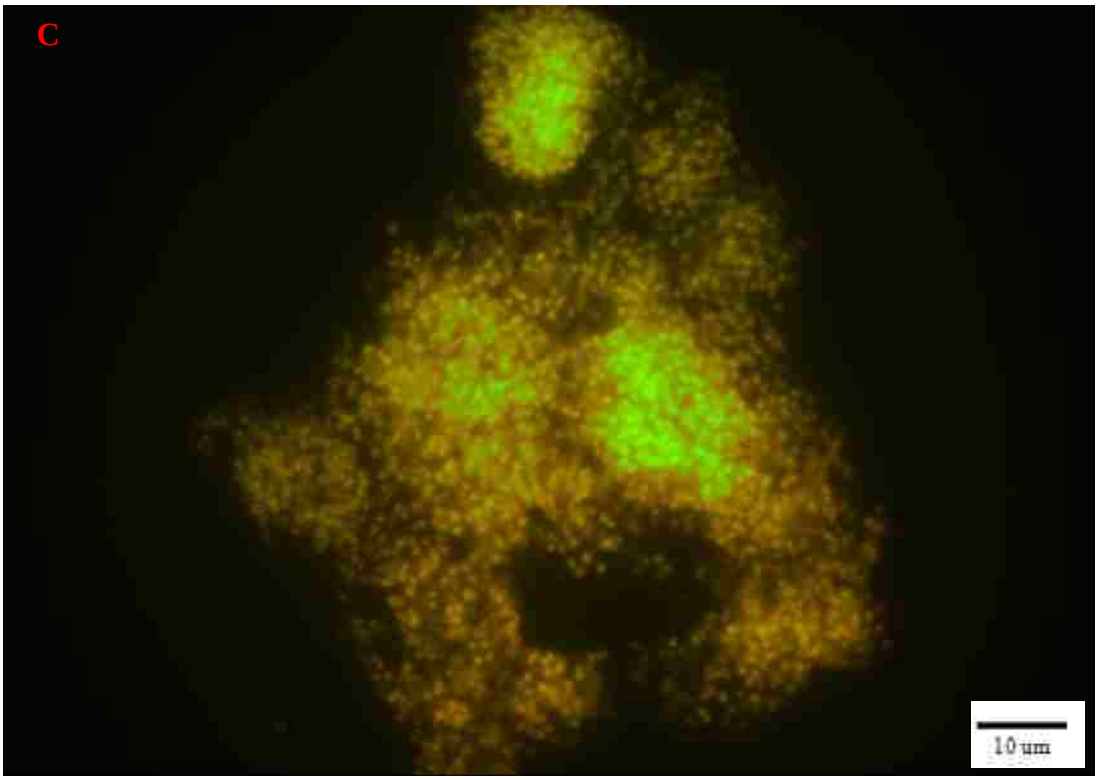
Stage 1 culture was highly enriched with PAOs, based on the phosphorus content in the biomass (non-soluble P/VSS [Pns/VSS]) was 0.19 ±0.004 mg/mg, Table 8.1), as well as the anaerobic P release/acetate uptake (Prel/Ac) ratio was 0.62 ±0.013 mol/C-mol (Table 8.1) (Figure 8.1). These results are compared with the Reactor 1 results below.

Microscopic examination also indicated that the Stage 1 conditions enriched for a highly PAO-dominated culture. Neisser staining indicated a large quantity of polyphosphate-containing granules (FIgure 8.3A). FISH analyses demonstrated that 88 ±

9% of the bacteria were positive for the PAOmix probe targeting *Accumulibacter spp.* (Figures 8.2B and 8.2C) in samples taken during Stage 1. *Competibacter spp.* and cluster 1 *Defluviicoccus spp.* were absent. Cluster 2 *Defluviicoccus spp.* were around 6 ± 3% of the bacteria.







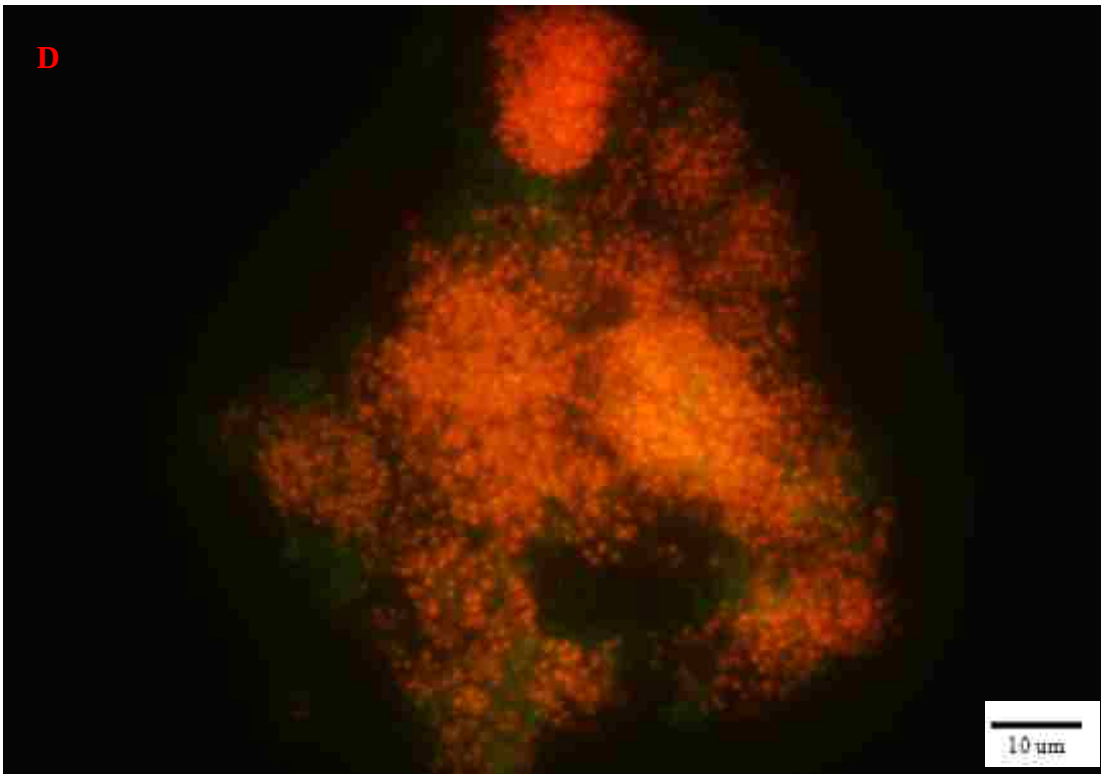


Figure 8.4 Neisser Staining and FISH results on activated sludge samples taken Stage 1. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser staining image, dark cells were PAOs, suggesting the accumulation of polyphosphate storage (day 29), (B*) In situ* hybridization with probes EUBMIX (FAM) specific for many but not all Bacteria (green), (C) *In situ* hybridization with probes PAOMIX (Cy3) specific for *Accumulibacter spp.* (orange) (day 27), and (D) Overlay (B) with (C), *Accumulibacter spp*. appear orange.

8.2 Stage 2: High pH, slow acetate addition

In Stage 2, the acetate feed flow rate was decreased so that it was added over the course of 112 minutes, rather than over 10 minutes as in Stage 1, with the pH maintained in the same range as Stage 1 (7.4-8.4). The decreased rate of acetate feed addition accomplished the goal of maintaining low acetate concentrations during the anaerobic phase, with acetate concentrations less than the detection limit during the anaerobic and aerobic phases. Furthermore, acetate was never detected at the end of the anaerobic phase, indicating that anaerobic acetate uptake was complete through Stage 2 (Figure 8.4).

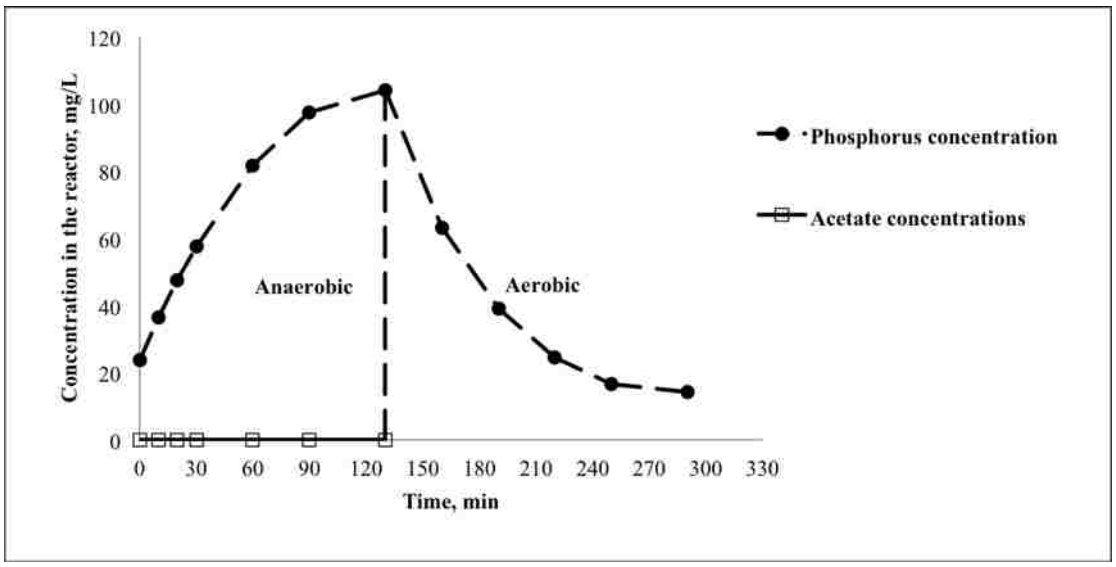
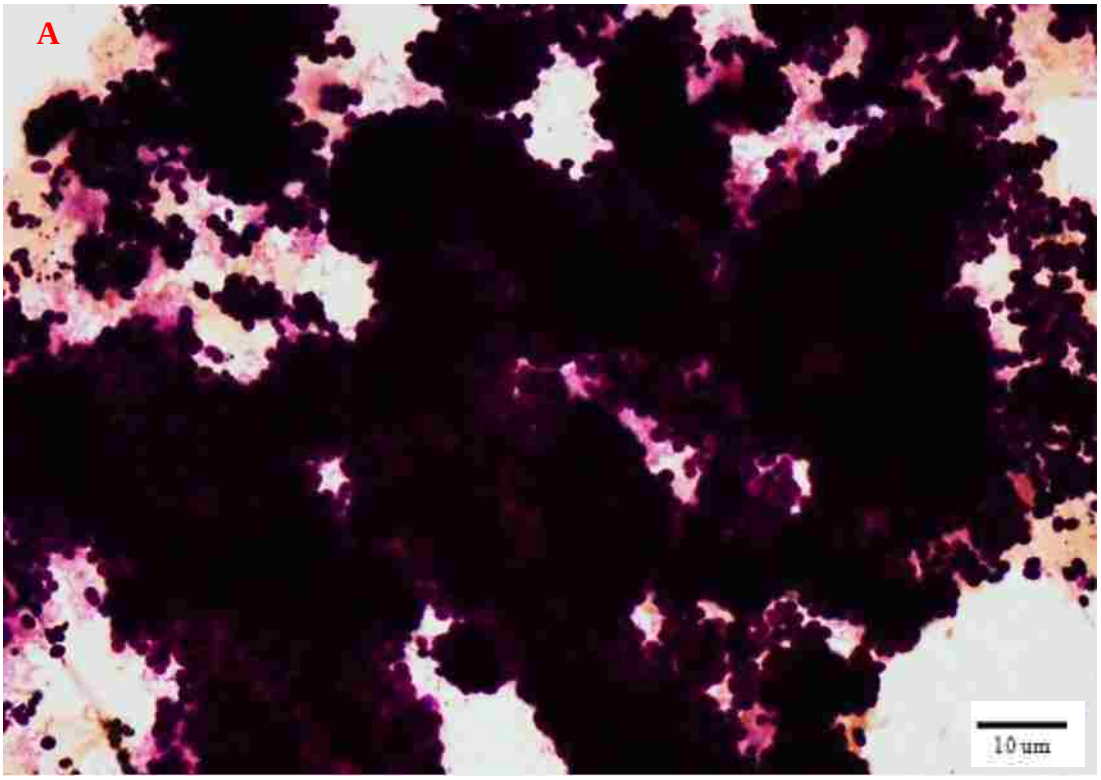
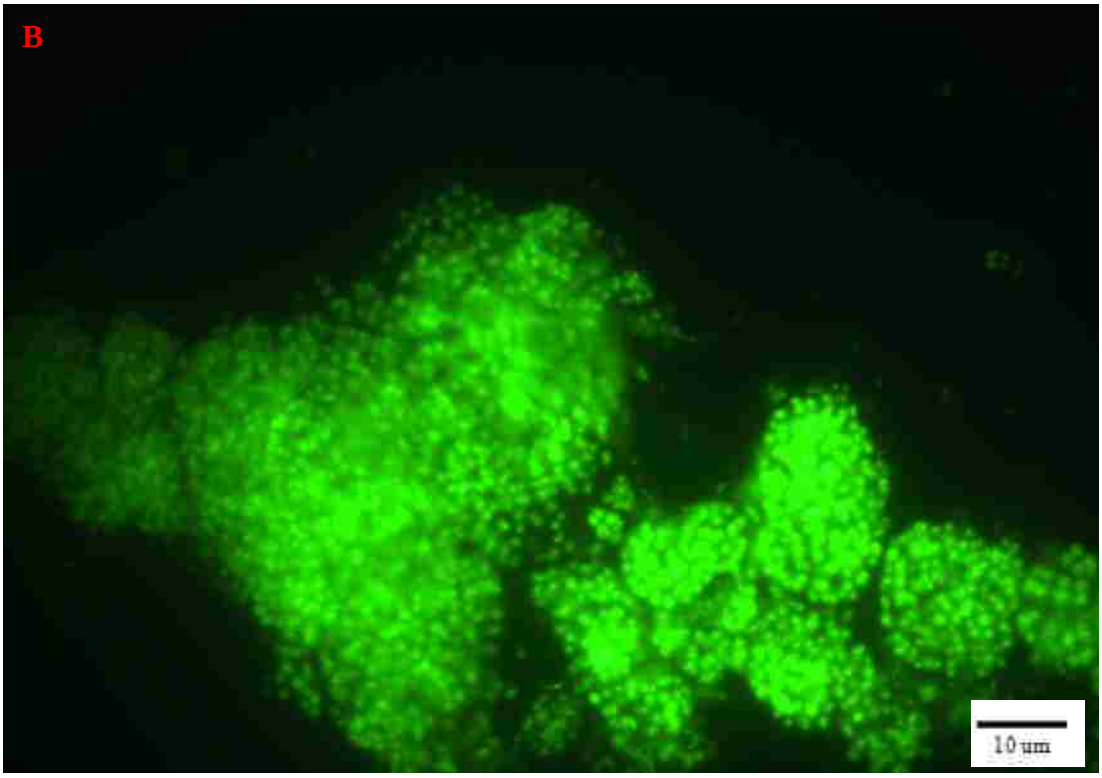


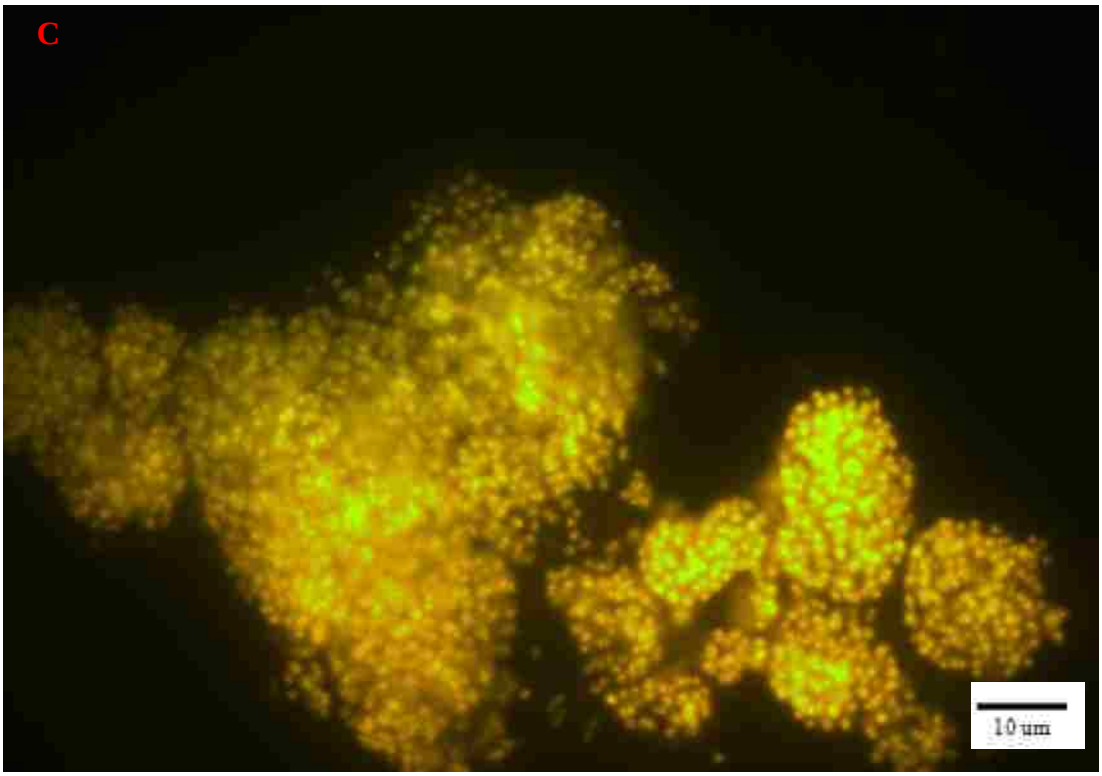
Figure 8.4 Acetate and phosphorus profiles throughout anaerobic and aerobic phases with slow acetate addition (Sample taken on day 78)

Throughout Stage 2, the Prel/Ac ratios and biomass phosphorus contents remained high, suggesting that the PAOs dominated the reactor (Table 8.1). The Prel/Ac ratio was 0.77 ±0.012 mol/C-mol from Day 41 until the end of Stage 2 (Table 8.1). Correspondingly, the biomass phosphorus content (Pns/VSS) was also high, with an average value of 0.35 ±0.015 mg/mg from Day 41 until the end of Stage 2 (Table 8.1) (Figure 8.1).

Microscopy confirmed that the PAO populations still dominated culture during Stage 2. Neisser staining indicated that the culture was enriched by polyphosphate accumulators (Figure 8.5A). Correspondingly, FISH analyses indicated 82 ±8% of the bacteria were *Accumulibacter spp.* targeted by the PAOmix probe set (Figures 8.5B and C). *Competibacter spp.* and cluster 1 *Defluviicoccus spp.* were not found in that stage. Cluster 2 *Defluviicoccus spp.* were around 3 ±1% of the bacteria.







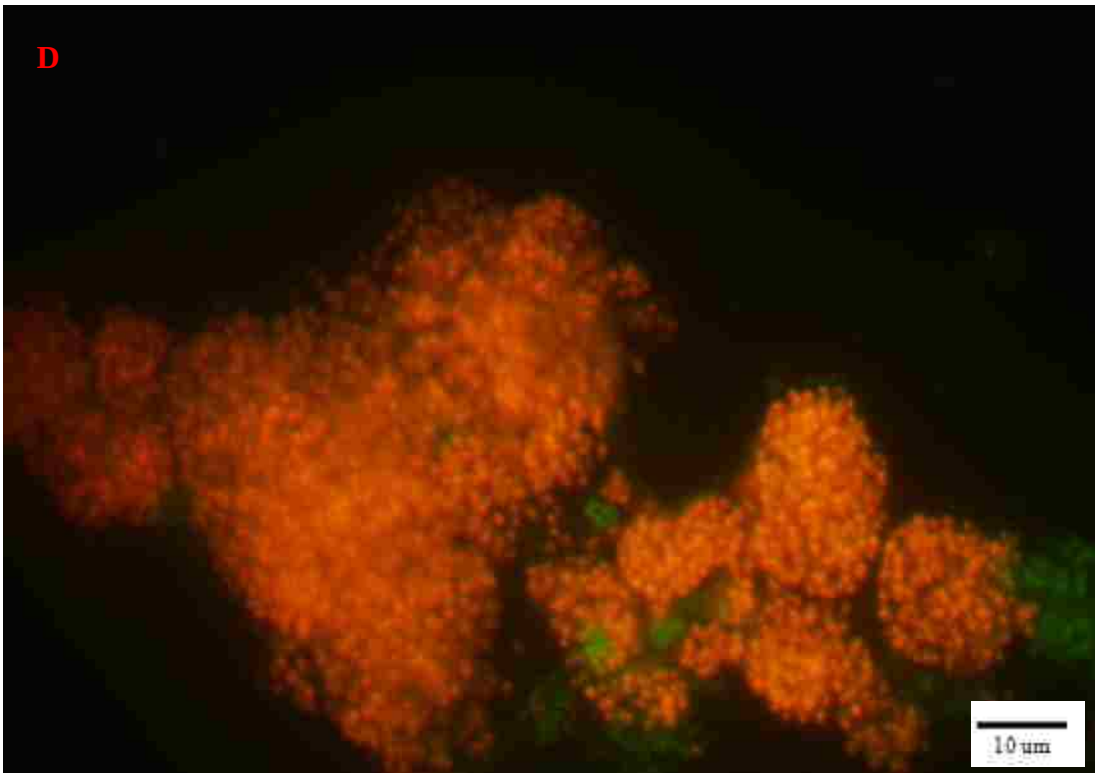


Figure 8.5 Neisser Staining and FISH results on activated sludge samples taken from Stage 3. Images of FISH were collected at two different filter sets depending on the fluorescence label and artificially coloured. (A) Neisser staining image, dark cells were PAOs, suggesting the accumulation of polyphosphate storage (day 90), (B) *In situ* hybridization with probes EUBMIX (FAM) specific for many but not all Bacteria (green), (C) *In situ* hybridization with probes PAOMIX (Cy3) specific for *Accumulibacter spp.* (orange) (day 82)**,** and (D) Overlay (B) with (C), *Accumulibacter spp*. appear orange.

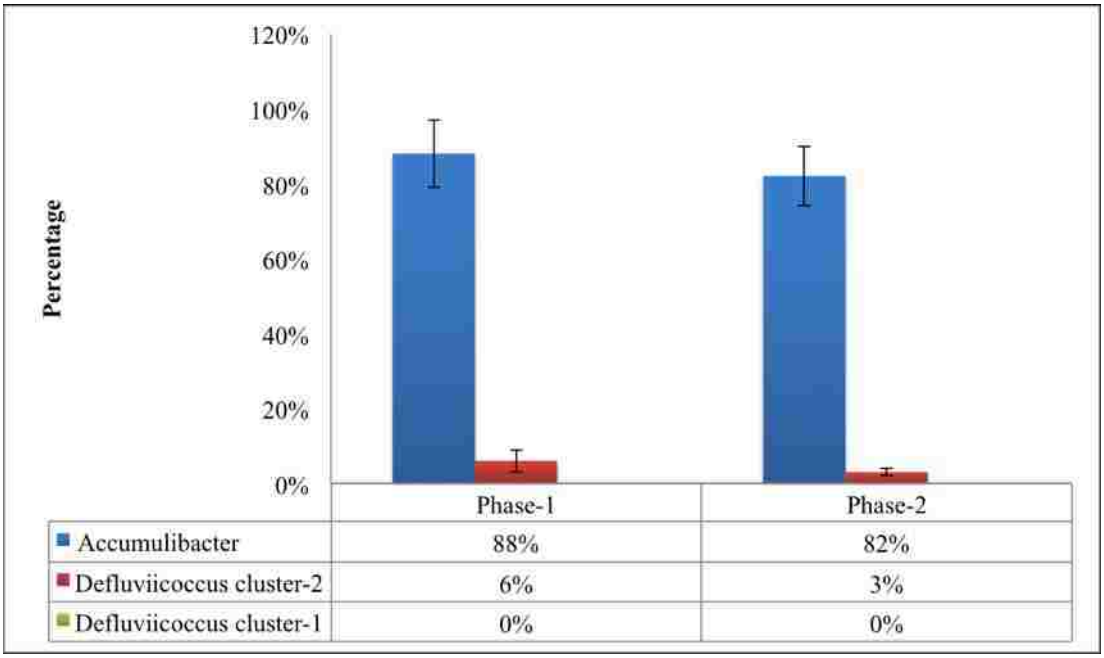


Figure 8.6 FISH Data summary

8.3 Conclusions

Although PAOs dominated both Stages 1 and 2, the Prel/Ac ratio in Stage 2 (0.77 ±0.012 mol/C-mol) was significantly ($p < 0.05$) higher than in Stage 1 (0.62 ±0.013 mol/C-mol), and the Pns/VSS ratio in Stage 2 (0.35±0.015 mg/mg) was significantly higher than in Stage 1 (0.19 ±0.004 mg/mg) (Table 8.3 and Table 8.4). This suggested that PAOs accumulated more polyphosphate at the low acetate concentration condition than at the high acetate concentration condition when operated at pH 7.4 to 8.4 (Figure 8.1). This was consistent to the results Reactor 1 Stages 1 and 3 (Table 8.2) (Figure 8.7), although in that case Reactor 1 Stage 3 was operated at a lower pH range (6.4 to 7.0) than Reactor 1 Stage 1 (7.4 to 8.4). These results therefore suggest that it was the decreased acetate feed rate that led to the increased biomass phosphorus contents in Reactor 1 Stage 3 relative to Reactor 1 Stage 1, and not the change in pH. Which was consistent with previous calculations of energy available for active transport of acetate (Schuler and Jenkins 2003b), and may be linked, therefore, to the ability of PAOs to scavenge low acetate concentrations through their particular transport mechanism. Because previous laboratory scale EBPR studies have most commonly utilized SBRs, which under conventional operation (with rapid feed addition) inherently provide relatively high

substrate concentrations, while full scale systems generally have complete mixed characteristics, this result suggests that competition from GAOs may have been historically overstated.

A comparison of the Reactor 1 Stage 1 and Reactor 3 Stage 1, which were both operated with the higher pH condition and rapid acetate addition, results suggests that cycle length (8 h and 6 h, respectively) had little effect on the biomass P content and the Prel/Ac ratio (Table 8.2) (Figure 8.7). Although the statistic significant differences were still high ($P < 0.05$) (Table 8.3 and Table 8.4) between these values in the two stages, the percentages of difference were extremely low, with average Pns/VSS of first stage in Stage 1 of Reactors 1 and 3 both equal to approximately 0.20 mg/mg (Table 8.2) (the difference percentage was 5%), and the Prel/Ac values were 0.65 ± 0.053 in Reactor 1 Stage and 0.62 ± 0.013 (Table 8.2) (the difference percentage was 4.6%), respectively.

Based on a comparison of Reactor 1 Stage 3 and Reactor 3 Stage 2, which were both operated with slow acetate addition, it appears that decreased pH (Reactor 1 Stage 3) led to significantly ($p<0.05$) (Table 8.3 and Table 8.4) less phosphorus storage (Pns/VSS = 0.28 ± 0.02 mg/mg) and lower Prel/Ac ratios (0.55 ± 0.04 mol/Cmol) than obtained at the higher pH values (Reactor 3 Stage 2), which yielded Pns/VSS = 0.35 ± 0.015 mg/mg and Prel/Ac = 0.77 ± 0.012 mol/Cmol (Table 8.2) (Figure 8.7). One reason to explain this is related to the fact that the acetic acid (HAC) form of the acetic acid/acetate acid/base pair a known uncoupler of PMF (Craig Baker-Austin and Dopson 2007).

Previous reports on EBPR behavior in the relative close systems are summarized in Table 8.5. Our results were in the range of these studies, but not in excellent agreement, which probably were due to the different pH control and SRT. Together with Chapter 5 results, it suggests that the lower rate of acetate addition might advantageous to process performance, as higher quantities polyphosphate storage indicates better P removal.

Table 8.2 PAO Comparisons performance in reactor1 and reactor3 comparisons

Reactors/stages		Pns/VSS (mg/mg)	P release/Ac uptake (mol/C-mol)
Reactor-1	Stage 1: fast acetate addition, pH 7.4-8.4, cycle-8 hours	0.20 ±0.016	0.65 ±0.053
	Stage 3: slow acetate addition, pH 6.4-7.0, cycle-8 hours	0.28 ±0.020	0.55 ±0.040
Reactor-3	Stage 1: fast acetate addition, pH 7.4-8.4, cycle-6 hours	0.19 ±0.004	0.62 ±0.013
	Stage 2: slow acetate addition, pH 7.4-8.4, cycle-6 hours	0.35±0.015	0.77 ±0.012

Table 8.3 P-values for P release/Ac uptake of 4 PAOs domination stages in Reactor-1 and Reactor-3

P release/Ac uptake	Reactor-1 /Stage-1	Reactor-1 /Stage-3	Reactor-3 /Stage-1
Reactor-1/Stage-1	_	_	_
Reactor-1/Stage-3	9.99E-10	_	_
Reactor-3/Stage-1	9.23E-05	2.35E-06	_
Reactor-3/Stage-2	6.63E-07	9.49E-14	1.86E-13

Table 8.4 P-values for Pns/VSS of 4 PAOs domination stages in Reactor-1 and Reactor-3

Pns/VSS	Reactor-1 /Stage-1	Reactor-1 /Stage-3	Reactor-3 /Stage-1
Reactor-1/Stage-1	_	_	_
Reactor-1/Stage-3	2.18E-11	_	_
Reactor-3/Stage-1	0.01	8.37E-11	_
Reactor-3/Stage-2	3.02E-18	1.68E-10	3.07E-14

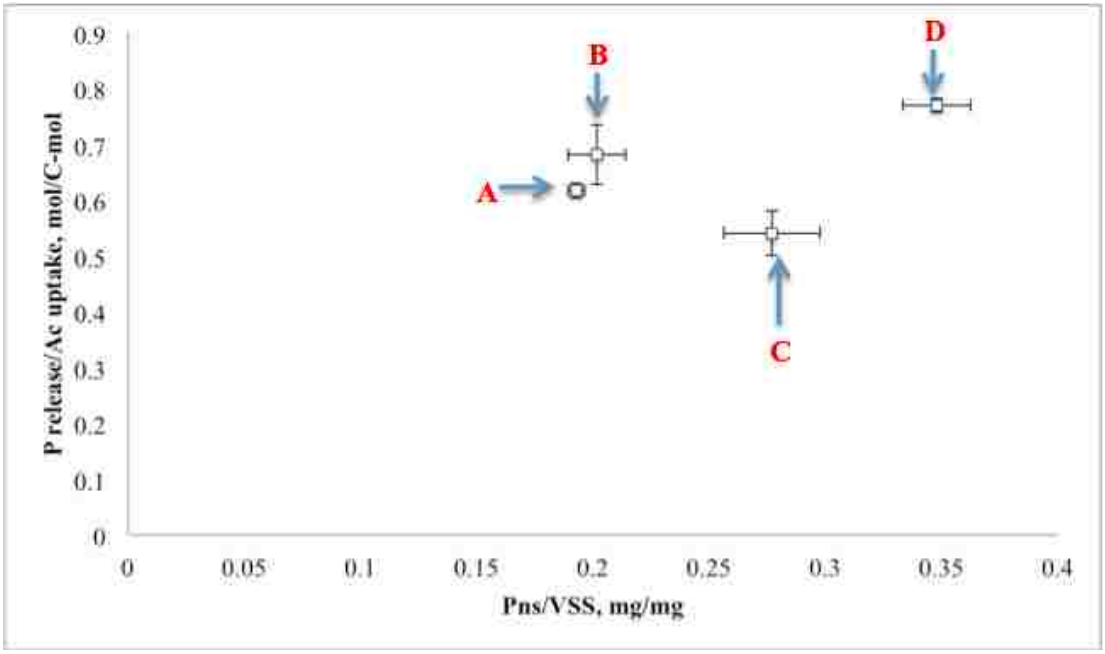


Figure 8.7: Comparison of PAO-enriched stages in the reactor 1 and reactor 3: (A): Reactor-3/Stage-1, (B): Reactor-1/Stage-1, (C): Reactor-1/Stage-3, and (D): Reactor-3/Stage-2

Table 8.5 Stoichiometric values reported by other researchers for EBPR systems* (based on Schuler and Jenkins (2003a)

Pns/TSS (mg/mg)	Anaerobic P release/acetate uptake (mol/C-mol)	pH	SRT (days)	Source
0.11	0.46	7-7.3		(Kuba et al. 1993)
0.08	0.48	7	8.6	(Smolders et al. 1994)
0.12	0.55	7	7.5-8	(Liu et al. 1996)
0.12	0.53	7	8.6	(Brdjanovic et al. 1998d)
0.06	0.37	7.35	7	(Hesselmann et al. 2000)
0.075	0.57	7.4	7	(Filipe et al. 2001c)
0.16	0.73	7.15-7.25	4	(Schuler and Jenkins 2003a)

*Blank values are not reported

CHAPTER 9. CONCLUSIONS AND APPLICATION TO PRACTICE

This chapter is a summary of the conclusions presented at the ends of Chapters 5 to Chapter 8.

9.1 Conclusions

This research provided the first demonstration that substrate (acetate) concentration is an important environmental factor affecting EBPR through studies of SBRs in which acetate concentrations were manipulated by varying the rate of acetate feed addition. Specifically, lower acetate concentrations (slow rates of addition) favored PAOs and provided successful EBPR under low pH conditions (6.4 to 7.0) that otherwise led to EBPR failure in this and many previous studies when combined with high anaerobic acetate concentrations (rapid acetate addition rate). The finding of a competitive advantage of PAOs over GAOs at lower acetate concentrations is consistent with previous calculations of energy available for active transport of acetate (Schuler and Jenkins 2003b), and may be linked, therefore, to the ability of PAOs to scavenge low acetate concentrations through their particular transport mechanism. Because previous laboratory scale EBPR studies have most commonly utilized SBRs, which under conventional operation (with rapid feed addition) inherently provide relatively high substrate concentrations, while full scale systems generally have complete mixed characteristics, this result suggests that competition from GAOs may have been historically overstated.

Furthermore, one surprising finding was that under no conditions did it appear that PAO failure (as at was directly caused by GAO competition, since GAOs appeared to be opportunists that were able to increase in population and eventually dominate the systems only after PAOs had already failed and stopped taking up acetate anaerobically. So the GAOs importance may be have been over-stated in previous lab studies based on the reasons: (1) PAOs are inhibited by high acetate concentrations linked to SBRs; and, (2) GAOs appeared to be opportunists rather than competitors.

Batch testing with chemical inhibitors confirmed some previous research and provided new clues about the PAO and GAO acetate transport. Acetate uptake by both

the *Accumulibacter spp.*-enriched and *Defluviicoccus spp.*-enriched cultures were inhibited by CCCP, suggesting that both groups of organisms required a PMF for acetate uptake, consistent with previous work (Saunders et al. 2007; Burow et al. 2008). Inhibition of the *Accumulibacter*-enriched culture by DCCD was minimal, suggesting that F_1F_0 ATPase was not used for regeneration of the PMF during acetate uptake, and consistent with results by (Saunders et al. 2007; Burow et al. 2008 ENREF 20). A new finding was that DCCD inhibited acetated uptake in the *Defluviicoccus spp.* enriched culture, which suggested reliance on F_1F_0 ATPase during acetate uptake, in contradiction to results by Burow et al. 2008. The reason for this may have been that Burow et al. 2008 tested a *Defluviicoccus* cluster 1 enriched culture, which this study tested a *Defluviicoccus* cluster 2 enriched culture. Further study is necessary to determine whether these two groups of closely-related *Defluviicoccus* strains do indeed utilize different mechanisms, or whether this difference may have been due to some adaptation of the strains to the particular conditions in each study.

Chemical inhibition of P release for *Accumulibacter spp.* suggested that these organisms used an active process for the uptake of acetate that is driven by the PMF generated via P efflux (Saunders et al. 2007).

Higher rates of inhibition were consistently found when acetate was added slowly relative to when it was added as a pulse, suggesting that PAOs may be more dependent on the reestablishing their PMF at lower concentrations (slow acetate addition) than at higher concentrations (fast acetate addition) in both PAOs and GAOs. This may be because higher acetate concentrations create a greater acetate concentration gradient across the cell membrane, which could help drive acetate transport when the PMF is disrupted.

Acetate uptake in the *Defluviicoccus* spp. enrichment was less sensitive to PMF disruptor compared with the *Accumulibacter spp.* enrichment. These results were consistent with Burow's study (2008), and suggested that active acetate transport contributes comparatively more to overall acetate uptake in *Accumulibacter spp.* than in *Defluviicoccus spp.*, which support our hypothesis that PAOs may have a competitive advantage over GAOs under the low substrate conditions often observed in full-scale EBPR plants.

Further batch tests suggested that the acetate uptake by PAOs was independent of the external pH. Under the higher pH (7.5) condition, the acetate uptake rate by PAOs was higher than GAOs across all acetate concentrations, but at pH 6.5 and low acetate concentrations, the acetate uptake rates by the two cultures were similar, but when the pH, while at high acetate concentrations (pH still=6.5), the PAO acetate uptake rates were higher than those of GAOs. The reason for why the PAOs still took up acetate faster than GAOs under lower pH and higher acetate concentration was our tests were short-time tests, which only last 30 min, if applying the tests to the long-term, based on our previous long-term study, the PAOs would be crashed under lower pH condition, and then the GAOs took the advantage in the culture as opportunists. On the contrast, the acetate uptake rate of GAOs was strongly inhibited by high pH (7.5), particularly at extremely high acetate concentration (higher than 130mg/L), so could provide one reason why GAOs are consistently low in EBPR systems operated at these pH values.

Testing of EBPR systems with varying temperature and acetate addition rates confirmed that GAOs were enriched at higher temperatures (30 ^{o} C), consistent with previous studies (Whang and Park 2002) and strategies to improve EBPR, including low acetate concentration (slow rate of addition), and shortened cycle length, did not suppress GAOs proliferation at 30 ^{o} C.

Analysis of Pns/VSS and Prel/Ac uptake values indicated that operation with low acetate concentrations tended to increase phosphorus storage (Pns/VSS), which indicated improved EBPR, and this also led to increased anaerobic Prel/Ac uptake ratios relative to when acetate was added rapidly as in a conventional SBR. Cycle length (6h or 8 h) had little effect on Pns/VSS or the anaerobic Prel/Ac ratio. While for the first time successful EBPR was obtained at relatively low pH values (6.4-7.0), higher Pns/VSS values obtained during operation at higher pH (7.4-8.4).

9.2 Application to practice

The finding of a competitive advantage of PAOs over GAOs at lower acetate concentrations indicates a novel strategy to improve EBPR process which may have application in full-scale wastewater treatment systems. Since one important difference between laboratory and full-scale systems is that laboratory studies of EBPR have

typically utilized SBRs due to their ease of construction and operation, while full scale systems typically include CMFRs. For SBRs, due to their batch nature, have inherently high substrate (acetate or other VFA) concentrations at the beginning of each feed cycle. CMFRs, however, have inherently lower substrate concentration due to their completely mixed and continuous nature, in which case influent is immediately diluted, and is equal to the effluent substrate concentration. So our finding may be important in full-scale systems, the GAOs may be not a problem for EBPR due to the nature low acetate concentrations. For lab research, our operation will supply a better strategy to culture better performance of PAOs.

The pH and temperature tests supported previous studies that PAOs favor high pH (around 7.4-8.4) and low temperature (around 20 ^{o} C), which supplied the strategies to improve EBPR in the full-scale systems, controlling the pH and temperature in the appropriate ranges. Particularly for the plants built in the tropic zone, they need monitor the temperature very carefully for the EBPR process.

CHAPTER 10. RECOMMENDATIONS FOR FUTURE RESEARCH

This study has shown that PAOs had competitive advantage over GAOs under low acetate concentrations, which is probably due to the particular acetate uptake mechanisms. Further research is required to determine whether the [HAC] form, which is a known uncoupler of PMF, increases with the pH decreases, consequently a significant resistant on PMF. While, based on our inhibition test results, PMF is essential for PAO taking up the acetate. And also, PAO failure did not appear to be linked to competition from GAOs. Rather, it appears that high acetate concentrations at low pH are themselves inhibitory to PAO metabolism. A better understanding of the metabolic pathways involved in PAOs and GAOs would also improve the accuracy of the transport systems.

Based on our kinetic results, we found GAOs was significantly inhibited under high pH and high acetate concentration conditions, however the reason was still not clear. The further research is needed to investigate whether it's only caused by different pH adaption of GAOs or other external factors.

The EBPR systems failed under high temperature, and the low acetate concentrations did not give the PAOs advantage, PAOs still failed under this condition. Further research is required to find how to culture PAOs under high temperature, or what's the maximum temperature for PAOs can survive under low acetate concentrations. The high temperature test is very useful for the full-scale wastewater plant built in tropics and high temperature area.

The isolation of EBPR organisms has been studied for long time, however none succeeded. A pure culture EBPR system would greatly facilitate determination of metabolic pathway operation and regulation, carbon and phosphate transport mechanisms, growth rates, inhibitory factors, and nutritional requirements. Both PAOs and GAOs pure culture systems would be useful for improving the understanding of these metabolisms and their interactions.

It was demonstrated that the Pns/VSS value was much higher under low acetate concentration conditions than high acetate concentration conditions after all the stages reached to steady phases. This result approved our hypothesis that PAOs prefer low acetate concentrations from another way, which is important for full-scale wastewater

treatment systems. However it has yet to be determined whether the reason is that PAOs have different behaviors under different acetate concentrations. Further research, such as the measurement of PHB and glycogen is required to determine PAOs' behaviors under different conditions.

CHAPTER 10. REFERENCES

Ahn, J., T. Daidou, S. Tsuneda and A. Hirata (2002). "Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay." Water Research **36**(2): 403-412.

Ahn, J., S. Schroeder, M. Beer, S. McIlroy, R. C. Bayly, J. W. May, G. Vasiliadis and R. J. Seviour (2007). "Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor." Applied and Environmental Microbiology **73**(7): 2257-2270.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "BASIC LOCAL ALIGNMENT SEARCH TOOL." Journal of Molecular Biology **215**(3): 403-410.

Andreotti, R., A. A. P. de Leon, S. E. Dowd, F. D. Guerrero, K. G. Bendele and G. A. Scoles (2011). "Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing." Bmc Microbiology **11**: 11.

APHA, Ed. (1992). Standard Methods for the Examination of Water and Wastewater, 18th Ed. Washington, D.C.

Bailey, M. T., S. E. Dowd, N. M. A. Parry, J. D. Galley, D. B. Schauer and M. Lyte (2010b). "Stressor Exposure Disrupts Commensal Microbial Populations in the Intestines and Leads to Increased Colonization by Citrobacter rodentium." Infection and Immunity **78**(4): 1509-1519.

Bailey, M. T., J. C. Walton, S. E. Dowd, Z. M. Weil and R. J. Nelson (2010a). "Photoperiod modulates gut bacteria composition in male Siberian hamsters (Phodopus sungorus)." Brain Behavior and Immunity **24**(4): 577-584.

Barak, Y. and J. van Rijn (2000). "Atypical polyphosphate accumulation by the denitrifying bacterium Paracoccus denitrificans." Applied and Environmental Microbiology **66**(3): 1209-1212.

Barnard, J. L. (1974). "Cut P and N without chemicals." Water & Wastes Engineering **11**(8): 41-&.

Beer, M., H. M. Stratton, P. C. Griffiths and R. J. Seviour (2006). "Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia?" Journal of Applied Microbiology **100**(2): 233-243.

Beyene, A., W. Legesse, L. Triest and H. Kloos (2009). Urban impact on ecological integrity of nearby rivers in developing countries: the Borkena River in highland Ethiopia. Environmental Monitoring and Assessment. **153:** 461-476.

Bond, P. L., R. Erhart, M. Wagner, J. Keller and L. L. Blackall (1999). "Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems." Applied and Environmental Microbiology **65**(9): 4077-4084.

Bond, P. L., J. Keller and L. L. Blackall (1998). "Characterisation of enhanced biological phosphorus removal activated sludges with dissimilar

phosphorus removal performances." Water Science and Technology **37**(4-5): 567-571.

Bowker, R. P. G. and H. D. Stensel (1990). Phosphorus removal from wastewater-Issue 189 of Pollution technology review, Noyes Data Corp.

Brdjanovic, D., S. Logemann, M. C. M. Van Loosdrecht, C. M. Hooijmans, G. J. Alaerts and J. J. Heijnen (1998c). "Influence of temperature on biological phosphorus removal: Process and molecular ecological studies." Water Research **32**(4): 1035-1048.

Brdjanovic, D., A. Slamet, M. C. M. van Loosdrecht, C. M. Hooijmans, G. J. Alaerts and J. J. Heijnen (1998a). "Impact of excessive aeration on biological phosphorus removal from wastewater." Water Research **32**(1): 200-208.

Brdjanovic, D., M. C. M. van Loosdrecht, C. M. Hooijmans, G. J. Alaerts and J. J. Heijnen (1998b). "Minimal aerobic sludge retention time in biological phosphorus removal systems." Biotechnology and Bioengineering **60**(3): 326-332.

Brdjanovic, D., M. C. M. van Loosdrecht, C. M. Hooijmans, T. Mino, G. J. Alaerts and J. J. Heijnen (1998d). "Bioassay for glycogen determination in biological phosphorus removal systems." Water Science and Technology **37**(4-5): 541-547.

Buchan, L. (1983). "POSSIBLE BIOLOGICAL MECHANISM OF PHOSPHORUS REMOVAL." Water Science and Technology **15**(3-4): 87-103.

Burow, L. C., A. N. Mabbett, A. G. McEwan, P. L. Bond and L. L. Blackall (2008). "Bioenergetic models for acetate and phosphate transport in bacteria important in enhanced biological phosphorus removal." Environmental Microbiology **10**(1): 87-98.

Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff and D. J. Nisbet (2010). "Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing." Journal of Animal Science **88**(12): 3977-3983.

Campbell, L. M., S. B. Wandera, R. J. Thacker, D. G. Dixon and R. E. Hecky (2005). "Trophic niche segregation in the Nilotic ichthyofauna of Lake Albert (Uganda, Africa)." Environmental Biology of Fishes **74**(3-4): 247-260.

Cech, J. S. and P. Hartman (1990). "Glucose-induced break down of enhanced biological phosphate removal." Environmental Technology **11**(7): 651-656.

Cech, J. S. and P. Hartman (1993). "Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems." Water Research **27**(7): 1219-1225.

Christensson, M., L. L. Blackall and T. Welander (1998). "Metabolic transformations and characterisation of the sludge community in an enhanced biological phosphorus removal system." Applied Microbiology and Biotechnology **49**(2): 226-234.

Comeau, Y., K. J. Hall, R. E. W. Hancock and W. K. Oldham (1986). "Biochemical-model for enhanced biological phosphorus removal." Water Research **20**(12): 1511-1521.

Craig Baker-Austin and M. Dopson (2007). "Life in acid: pH homeostasis in acidophiles." TRENDS in Microbiology **15**(4): 165-171.

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

Crocetti, G. R., P. Hugenholtz, P. L. Bond, A. Schuler, J. Keller, D. Jenkins and L. L. Blackall (2000). "Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation." Applied and Environmental Microbiology **66**(3): 1175-1182.

Dai, Y. (2006). Polyhydroxyalkanoate Copolymer Production from Synthetic Carbonaceous Wastewater Using Glycogen Accumulating Organisms, University of Queensland.

Dai, Y., Z. G. Yuan, X. L. Wang, A. Oehmen and J. Keller (2007). "Anaerobic metabolism of Defluviicoccus vanus related glycogen accumulating organisms (GAOs) with acetate and propionate as carbon sources." Water Research **41**(9): 1885-1896.

de-Bashan, L. E. and Y. Bashan (2004). "Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003)." Water Research **38**(19): 4222-4246.

Donnert, D. and M. Salecker (1999). "Elimination of phosphorus from waste water by crystallization." Environmental Technology **20**(7): 735-742.

Eikleboom, D. H. and H. J. J. Van Buijsen (1981). Microscopic Sludge Investigation Manual. Delft, The Netherlands, TNO Research Institute for Environmental Hygiene. Water and Soil Division.

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

Erdal, Z. K. (2002). The biochemistry of enhanced biological phosphorus removal: role of glycogen in biological phosphorus removal and the impact of the operating conditions on the involvement of glycogen. Ph.D. Thesis, Virginia Polytechnic Institute and State University.

Eschenhagen, M., M. Schuppler and I. Roske (2003). "Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents." Water Research **37**(13): 3224-3232.

Filipe, C. D. M., G. T. Daigger and C. P. L. Grady (2001a). "A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: Stoichiometry, kinetics, and the effect of pH." Biotechnology and Bioengineering **76**(1): 17-31.

Filipe, C. D. M., G. T. Daigger and C. P. L. Grady (2001b). "pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms." Water Environment Research **73**(2): 223-232.

Filipe, C. D. M., G. T. Daigger and C. P. L. Grady (2001c). "Stoichiometry and kinetics of acetate uptake under anaerobic conditions by an enriched culture of phosphorus-accumulating organisms at different pHs." Biotechnology and Bioengineering **76**(1): 32-43.

Finegold, S. M., S. E. Dowd, V. Gontcharova, C. Liu, K. E. Henley, R. D. Wolcott, E. Youn, P. H. Summanen, D. Granpeesheh, D. Dixon, M. Liu, D. R. Molitoris and J. A. Green, 3rd (2010). "Pyrosequencing study of fecal microflora of autistic and control children." Anaerobe **16**(4): 444-453.

Fuhs, G. W. and M. Chen (1975). "Microbiological Basis of Phosphate Removal in the Activated Sludge Process for the Treatment of Wastewater." Microbial Ecology **2**(2): 119-138.

Fukase, T., M. Shibata and Y. Miyaji (1985). "The role of an anaerobic stage on biological phosphorus removal." Water Science and Technology **17**(2-3): 69-80.

Gontcharova, V., E. Youn, Y. Sun, R. D. Wolcott and Scot E Dowd1 (2010a). "A Comparison of Bacterial Composition in Diabetic Ulcers and Contralateral Intact Skin." Open Microbiol J. **2010; 4: 8–19.**

Gontcharova, V., E. Youn, R. D. Wolcott, E. B. Hollister, T. J. Gentry and S. E. Dowd (2010b). "Black Box Chimera Check (B2C2): a Windows-Based Software for Batch Depletion of Chimeras from Bacterial 16S rRNA Gene Datasets." Open Microbiol J. **2010; 4: 47–52.**

Handl, S., S. E. Dowd, J. F. Garcia-Mazcorro, J. M. Steiner and J. S. Suchodolski (2011). "Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats." Fems Microbiology Ecology **76**(2): 301-310.

Harold, F. M., J. R. Baarda, C. Baron and A. Abrams (1969). "Inhibition of membrane-bound adenosine triphosphatase and of cation transport in streptococcus faecalis by N,N'-dicyclohexylcarbodiimide." Journal of Biological Chemistry **244**(9): 2261-&.

Hesselmann, R. P. X., R. Von Rummell, S. M. Resnick, R. Hany and A. J. B. Zehnder (2000). "Anaerobic metabolism of bacteria performing enhanced biological phosphate removal." Water Research **34**(14): 3487-3494.

Hesselmann, R. P. X., C. Werlen, D. Hahn, J. R. van der Meer and A. J. B. Zehnder (1999). "Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge." Systematic and Applied Microbiology **22**(3): 454-465.

Huang, X. Q. and A. Madan (1999). "CAP3: A DNA sequence assembly program." Genome Research **9**(9): 868-877.

Ian Woertz1, L. F., Tryg Lundquist1,2* (2009). "Nutrient Removal & Greenhouse Gas Abatement with CO2 Supplemented Algal High Rate Ponds." Environment **2009**(10): 7924-7936.

Ishak, H. D., R. Plowes, R. Sen, K. Kellner, E. Meyer, D. A. Estrada, S. E. Dowd and U. G. Mueller (2011). "Bacterial Diversity in Solenopsis invicta and Solenopsis geminata Ant Colonies Characterized by 16S amplicon 454 Pyrosequencing." Microbial Ecology **61**(4): 821-831.

Jenkins, D. and S. W. Hermanowícz (1991). Principles of Chemical Phosphate Removal. Phosphorus and nitrogen removal from municipal wastewater: principles and practice. T. S. a. D. A. Richard l. Sedlak Technical Director, New York, NY. New York**:** 91-110.

Jenkins, D. and V. Tandoi (1991). "The applied microbiology of enhanced biological phosphate removal - accomplishments and needs." Water Research **25**(12): 1471-1478.

Jeon, C. O., D. S. Lee, M. W. Lee and J. M. Park (2001). "Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: Effect of pH." Water Environment Research **73**(3): 301-306.

Jones, M. and T. Stephenson (1996). "The effects of temperature on enhanced biological phosphate removal." Environmental Technology **17**(9): 965-976.

Kampfer, P., R. Erhart, C. Beimfohr, J. Bohringer, M. Wagner and R. Amann (1996). "Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes." Microbial Ecology **32**(2): 101-121.

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

Kloeke, F. V. and G. G. Geesey (1999). "Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs." Microbial Ecology **38**(3): 201-214.

Kong, Y. H., J. L. Nielsen and P. H. Nielsen (2005). "Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants." Applied and Environmental Microbiology **71**(7): 4076-4085.

Kong, Y. H., S. L. Ong, W. J. Ng and W. T. Liu (2002). "Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes." Environmental Microbiology **4**(11): 753-757.

Kornberg, A., N. N. Rao and D. Ault-Riche (1999). "Inorganic polyphosphate: A molecule of many functions." Annual Review of Biochemistry **68**: 89-125.

Kuba, T., E. Murnleitner, M. C. M. vanLoosdrecht and J. J. Heijnen (1996). "A metabolic model for biological phosphorus removal by denitrifying organisms." Biotechnology and Bioengineering **52**(6): 685-695.

Kuba, T., G. Smolders, M. C. M. Vanloosdrecht and J. J. Heijnen (1993). "BIological phosphorus removal from waste-water by anaerobic-anoxic sequencing batch reactor." Water Science and Technology **27**(5-6): 241-252.

Lee, N., P. H. Nielsen, H. Aspegren, M. Henze, K. H. Schleifer and J. L. Jansen (2003). "Long-term population dynamics and in situ physiology in activated sludge systems with enhanced biological phosphorus removal operated

with and without nitrogen removal." Systematic and Applied Microbiology **26**(2): 211-227.

Levin, G., V. and J. Shapiro (1965). "Metabolic uptake of phosphorus by wastewater organisms." Journal (Water Pollution Control Federation) **37**: 800.

Li, N., N. Q. Ren, X. H. Wang and H. Kang (2010). "Effect of temperature on intracellular phosphorus absorption and extra-cellular phosphorus removal in EBPR process." Bioresource Technology **101**(15): 6265-6268.

Liu, W. T., T. Mino, T. Matsuo and K. Nakamura (1996). "Biological phosphorus removal processes - Effect of pH on anaerobic substrate metabolism." Water Science and Technology **34**(1-2): 25-32.

Liu, W. T., T. Mino, K. Nakamura and T. Matsuo (1994). "Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic - aerobic activated-sludge with a minimized polyphosphate content." Journal of Fermentation and Bioengineering **77**(5): 535-540.

Liu, W. T., K. Nakamura, T. Matsuo and T. Mino (1997). "Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors - Effect of P/C feeding ratio." Water Research **31**(6): 1430-1438.

Liu, Y., Y. G. Chen and Q. Zhou (2007). "Effect of initial pH control on enhanced biological phosphorus removal from wastewater containing acetic and propionic acids." Chemosphere **66**(1): 123-129.

Lopez-Vazquez, C. M., C. M. Hooijmans, D. Brdjanovic, H. J. Gijzen and M. C. M. van Loosdrecht (2009b). "Temperature effects on glycogen accumulating organisms." Water Research **43**(11): 2852-2864.

Lopez-Vazquez, C. M., Y. I. Song, C. M. Hooijmans, D. Brdjanovic, M. S. Moussa, H. J. Gijzen and M. C. M. van Loosdrecht (2008). "Temperature effects on the aerobic metabolism of glycogen-accumulating organisms." Biotechnology and Bioengineering **101**(2): 295-306.

Lopez-Vazquez, C. M., Y. I. Song, C. M. Hooijmans, D. Brdjanovic, M. S. Moussa, H. J. Gijzen and M. M. C. van Loosdrecht (2007). "Short-term temperature effects on the anaerobic metabolism of glycogen accumulating organisms." Biotechnology and Bioengineering **97**(3): 483-495.

Lotter, L. H. (1985). "The role of bacterial phosphate-metabolism in enhanced phosphorus removal from the activated-sludge process." Water Science and Technology **17**(11-1): 127-138.

Mace, S. and J. Mata-Alvarez (2002). "Utilization of SBR technology for wastewater treatment: An overview." Industrial & Engineering Chemistry Research **41**(23): 5539-5553.

Madigan, M. T., J. M. Martinko and J. Paker (1997). Brock Biology of Microorganisms, Prentice-Hall, Upper Saddle River, NJ.

Martin, H. G., N. Ivanova, V. Kunin, F. Warnecke, K. W. Barry, A. C. McHardy, C. Yeates, S. M. He, A. A. Salamov, E. Szeto, E. Dalin, N. H. Putnam, H. J. Shapiro, J. L. Pangilinan, I. Rigoutsos, N. C. Kyrpides, L. L. Blackall, K. D. McMahon and P. Hugenholtz (2006). "Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities." Nature Biotechnology **24**(10): 1263-1269.

Maszenan, A. M., R. J. Seviour, B. K. C. Patel, P. Schumann, J. Burghardt, Y. Tokiwa and H. M. Stratton (2000). "Three isolates of novel polyphosphate-accumulating Gram-positive cocci, obtained from activated sludge, belong to a new genus, Tetrasphaera gen. nov., and description of two new species, Tetrasphaera japonica sp, nov and Tetrasphaera australiensis sp nov." International Journal of Systematic and Evolutionary Microbiology **50**: 593-603.

Maszenan, A. M., R. J. Seviour, B. K. C. Patel, P. Schumann and G. N. Rees (1999). "Tessaracoccus bendigoensis gen. nov., sp. nov., a Gram-positive coccus occurring in regular packages or tetrads, isolated from activated sludge biomass." International Journal of Systematic Bacteriology **49**: 459-468.

Maurer, M., W. Gujer, R. Hany and S. Bachmann (1997). "Intracellular carbon flow in phosphorus accumulating organisms from activated sludge systems." Water Research **31**(4): 907-917.

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

Melasniemi, H., A. Hernesmaa, A. S. L. Pauli, P. Rantanen and M. Salkinoja-Salonen (1998). "Comparative analysis of biological phosphate removal (BPR) and non-BPR activated sludge bacterial communities with particular reference to Acinetobacter." Journal of Industrial Microbiology & Biotechnology **21**(6): 300-306.

Menar, A. B. and D. Jenkins (1970). "Fate of phosphorus in waste treatment process - enhanced removal of phosphate by activated sludge." Environmental Science & Technology **4**(12): 1115-&.

Merzouki, M., J. P. Delgenes, N. Bernet, R. Moletta and M. Benlemlih (1999). "Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors." Current Microbiology **38**(1): 9-17.

Metcalf and Eddy (2002). Wastewater engineering: treatment and reuse, McGraw-Hill Science/Engineering/Math, New York.

Meyer, R. L., A. M. Saunders and L. L. Blackall (2006). "Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing." Microbiology-Sgm **152**: 419-429.

Milbury, W. F., D. McCauley and C. H. Hawthorne (1971). "Operation of conventional activated sludge for maximum phosphorus removal." Journal (Water Pollution Control Federation) **43**: 117.

Mino, T., Arun, V., Tsuzuki, Y., Matsuo, T., Ed. (1987). Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process. Biological Phosphate Removal from Wastewaters, Oxford ; New York : Pergamon Pres.

Mino, T., W. T. Liu, F. Kurisu and T. Matsuo (1995). "Modeling glycogen-storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes." Water Science and Technology **31**(2): 25-34.

Mino, T., M. C. M. Van Loosdrecht and J. J. Heijnen (1998). "Microbiology and biochemistry of the enhanced biological phosphate removal process." Water Research **32**(11): 3193-3207.

Mullan, A., J. P. Quinn and J. W. McGrath (2002). "Enhanced phosphate uptake and polyphosphate accumulation in Burkholderia cepacia grown under low-pH conditions." Microbial Ecology **44**(1): 69-77.

Murnleitner, E., T. Kuba, M. C. M. vanLoosdrecht and J. J. Heijnen (1997). "An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal." Biotechnology and Bioengineering **54**(5): 434-450.

Nakamura, K., A. Hiraishi, Y. Yoshimi, M. Kawaharasaki, K. Masuda and Y. Kamagata (1995). "Microlunatus phosphovorus gen-nov, sp-nov, a new gram-positive polyphosphate-accumulating bacterium isolated from activated-sludge." International Journal of Systematic Bacteriology **45**(1): 17-22.

Nelson, D. L. and M. M. Cox (2008). Lehninger Principles of Biochemistry, W. H. Freeman.

Nelson, D. L., A. L. Lehninger and M. M. Cox (2008). Lehninger principles of biochemistry. New York, W.H. Freeman.

Nielsen, A. T., W. T. Liu, C. Filipe, L. Grady, S. Molin and D. A. Stahl (1999). "Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor." Applied and Environmental Microbiology **65**(3): 1251-1258.

Oberholster, P. J., A. M. Botha and P. J. Ashton (2009). "The influence of a toxic cyanobacterial bloom and water hydrology on algal populations and macroinvertebrate abundance in the upper littoral zone of Lake Krugersdrift, South Africa." Ecotoxicology **18**(1): 34-46.

Oberholster, P. J., A. M. Botha and T. E. Cloete (2008). "Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa." Environmental Pollution **156**(1): 184-192.

OECD (2008). Eutrophication of Waters (OECD): Monitoring, Assessment and Control, Research of the Organization for Economic Co-Operation and Development (OECD)

Oehmen, A., P. C. Lemos, G. Carvalho, Z. G. Yuan, J. Keller, L. L. Blackall and M. A. M. Reis (2007). "Advances in enhanced biological phosphorus removal: From micro to macro scale." Water Research **41**(11): 2271-2300.

Oehmen, A., M. T. Vives, H. B. Lu, Z. G. Yuan and J. Keller (2005a). "The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms." Water Research **39**(15): 3727-3737.

Oehmen, A., Z. G. Yuan, L. L. Blackall and J. Keller (2005b). "Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms." Biotechnology and Bioengineering **91**(2): 162-168.

Olafson, P. U., K. H. Lohmeyer and S. E. Dowd (2010). "Analysis of expressed sequence tags from a significant livestock pest, the stable fly (Stomoxys calcitrans), identifies transcripts with a putative role in

chemosensation and sex determination." Arch Insect Biochem Physiol **74**(3): 179-204.

Padan, E., D. Zilberstein and S. Schuldiner (1981). "PH HOMEOSTASIS IN BACTERIA." Biochimica Et Biophysica Acta **650**(2-3): 151-166.

Panswad, T., A. Doungchai and J. Anotai (2003). "Temperature effect on microbial community of enhanced biological phosphorus removal system." Water Research **37**(2): 409-415.

Penetra, R. G., M. A. P. Reali, E. Foresti and J. R. Campos (1999). "Post-treatment of effluents from anaerobic reactor treating domestic sewage by dissolved-air flotation." Water Science and Technology **40**(8): 137-143.

Pereira, H., P. C. Lemos, M. A. M. Reis, J. Crespo, M. J. T. Carrondo and H. Santos (1996). "Model for carbon metabolism in biological phosphorus removal processes based on in vivo C-13-NMR labelling experiments." Water Research **30**(9): 2128-2138.

Pijuan, M., A. M. Saunders, A. Guisasola, J. A. Baeza, C. Casas and L. L. Blackall (2004). "Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source." Biotechnology and Bioengineering **85**(1): 56-67.

Pitta, D. W., W. E. Pinchak, S. E. Dowd, J. Osterstock, V. Gontcharova, E. Youn, K. Dorton, I. Yoon, B. R. Min, J. D. Fulford, T. A. Wickersham and D. P. Malinowski (2010). "Rumen Bacterial Diversity Dynamics Associated with Changing from Bermudagrass Hay to Grazed Winter Wheat Diets." Microbial Ecology **59**(3): 511-522.

Puig, S., M. Coma, H. Monclus, M. C. M. van Loosdrecht, J. Colprim and M. D. Balaguer (2008). "Selection between alcohols and volatile fatty acids as external carbon sources for EBPR." Water Research **42**(3): 557-566.

Puig, S., L. Corominas, M. D. Balaguer and J. Colprim (2007). "Biological nutrient removal by applying SBR technology in small wastewater treatment plants: carbon source and C/N/P ratio effects." Water Sci. Technol. **55**(7): 135-141.

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

Ren, N. Q., H. Kang, X. H. Wang and N. Li (2011). "Short-term effect of temperature variation on the competition between PAOs and GAOs during acclimation period of an EBPR system." Frontiers of Environmental Science & Engineering in China **5**(2): 277-282.

Saito, T., D. Brdjanovic and M. C. M. van Loosdrecht (2004). "Effect of nitrite on phosphate uptake by phosphate accumulating organisms." Water Research **38**(17): 3760-3768.

Santos, M. M., P. C. Lemos, M. A. M. Reis and H. Santos (1999). "Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium Microlunatus phosphovorus." Applied and Environmental Microbiology **65**(9): 3920-3928.

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

Satoh, H., T. Mino and T. Matsuo (1994). "Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation." Water Science and Technology **30**(6): 203-211.

Satoh, H., W. D. Ramey, F. A. Koch, W. K. Oldham, T. Mino and T. Matsuo (1996). "Anaerobic substrate uptake by the enhanced biological phosphorus removal activated sludge treating real sewage." Water Science and Technology **34**(1-2): 9-16.

Saunders, A. M. (2005). The physiology of microorganisms in enhanced biological phosphorus removal. Ph.D. Thesis, University of Queensland.

Saunders, A. M., A. N. Mabbett, A. G. McEwan and L. L. Blackall (2007). "Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions." Fems Microbiology Letters **274**(2): 245-251.

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

Schuler, A. J. (1998). The effects of Varying Influent Phophate and Acetate Concentrations on Enhanced Biological Removal of Phosphate from Wastewater. Doctor of Philosophy, University of California, Berkeley.

Schuler, A. J. (2005). "Diversity matters: Dynamic simulation of distributed bacterial states in suspended growth biological wastewater treatment systems." Biotechnology and Bioengineering **91**(1): 62-74.

Schuler, A. J. (2006). "Process hydraulics, distributed bacterial states, and biological phosphorus removal from wastewater." Biotechnology and Bioengineering **94**(5): 909-920.

Schuler, A. J. and D. Jenkins (2002). "Effects of pH on enhanced biological phosphorus removal metabolisms." Water Science and Technology **46**(4-5): 171-178.

Schuler, A. J. and D. Jenkins (2003a). "Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part I: Experimental results and comparison with metabolic models." Water Environment Research **75**(6): 485-498.

Schuler, A. J. and D. Jenkins (2003b). "Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part II: Anaerobic adenosine triphosphate utilization and acetate uptake rates." Water Environment Research **75**(6): 499-511.

Schuler, A. J., D. Jenkins and P. Ronen (2001). "Microbial storage products, biomass density, and settling properties of enhanced biological phosphorus removal activated sludge." Water Science and Technology **43**(1): 173-180.

Sedlak, R. (1991). Introduction. Phosphorus and nitrogen removal from municipal wastewater: principles and practice. T. S. a. D. A. Richard l. Sedlak Technical Director, New York, NY. New York**:** 1-2.

Serafim, L. S., P. C. Lemos and M. A. M. Reis (2002). "Effect of pH control on EBPR stability and efficiency." Water Science and Technology **46**(4-5): 179-184.

Smith, D. M., D. E. Snow, E. Rees, A. M. Zischkau, J. D. Hanson, R. D. Wolcott, Y. Sun, J. White, S. Kumar and S. E. Dowd (2010). "Evaluation of the bacterial diversity of Pressure ulcers using bTEFAP pyrosequencing." Bmc Medical Genomics **3**: 12.

Smolders, G. J. F., J. Vandermeij, M. C. M. Vanloosdrecht and J. J. Heijnen (1994). "Model of the anaerobic metabolism of the biological phosphorus removal process-stoichiometry and pH influence." Biotechnology and Bioengineering **43**(6): 461-470.

Smolders, G. J. F., J. Vandermeij, M. C. M. Vanloosdrecht and J. J. Heijnen (1995). "A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process." Biotechnology and Bioengineering **47**(3): 277-287.

Snoeyink, V. L. and D. Jenkins (1980). Water Chemistry. New York, NY, John Wiley & Sons.

Srinath, E. G., C. A. Sastry and S. C. Pillai (1959). "Rapid removal of phosphorus from sewage by activated sludge." Experientia **15**(9): 339-340.

Stante, L., C. M. Cellamare, F. Malaspina, G. Bortone and A. Tilche (1997). "Biological phosphorus removal by pure culture of Lampropedia spp." Water Research **31**(6): 1317-1324.

Stensel, H. D. (1991). Principles of Biological Phosphate Removal. Phosphorus and nitrogen removal from municipal wastewater: principles and practice. T. S. a. D. A. Richard l. Sedlak Technical Director, New York, NY. New York**:** 141-166.

Stephenson, M. F., L. Mfuna, S. E. Dowd, R. D. Wolcott, J. Barbeau, M. Poisson, G. James and M. Desrosiers (2010). "Molecular Characterization of the Polymicrobial Flora in Chronic Rhinosinusitis." Journal of Otolaryngology-Head & Neck Surgery **39**(2): 182-187.

Strom, P. F. (2006). Introduction to phosphorus removal. Wastewater Treatment Operator's Workshop, 91st Annual Meeting, NJWEA. Atlantic City, NJ.

Tchobanoglous, G., F. L. Burton, H. D. Stensel, Metcalf and Eddy (2003). Wastewater engineering : treatment and reuse. Boston, McGraw-Hill.

Thomas, M., P. Wright, L. Blackall, V. Urbain and J. Keller (2003). "Optimisation of Noosa BNR plant to improve performance and reduce operating costs." Water Science and Technology **47**(12): 141-148.

Tsai, C. S. and W. T. Liu (2002). "Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorus removal systems." Water Science and Technology **46**(1-2): 179-184.

Vacker, D., C. H. Connell and W. N. Well (1967). "Phosphate removalthrough municipal wastewater treatment at San Antonio, Texas." Journal (Water Pollution Control Federation) **39**: 750.

van Loosdrecht, M. C. M., C. M. Hooijmans, D. Brdjanovic and J. J. Heijnen (1997). "Biological phosphate removal processes." Applied Microbiology and Biotechnology **48**(3): 289-296.

van Loosdrecht, M. C. M., M. A. Pot and J. J. Heijnen (1997). "Importance of bacterial storage polymers in bioprocesses." Water Science and Technology **35**(1): 41-47.

Wagner, M., R. Amann, H. Lemmer and K. H. Schleifer (1993). "Probing activated-sludge with oligonucleotides specific for proteobacteria - inadequacy of culture-dependent methods for describing microbial community structure." Applied and Environmental Microbiology **59**(5): 1520-1525.

Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi and K. H. Schleifer (1994). "Development of an ribosomal-RNA-targeted oligonucleotide probe specific for the genus acinetobacter and its application for in-situ monitoring in activated-sludge." Applied and Environmental Microbiology **60**(3): 792-800.

Wentzel, M. C., L. H. Lotter, G. A. Ekama, R. E. Loewenthal and G. V. R. Marais (1991). "Evaluation of bochemical-models for biological excess phosphorus removal." Water Science and Technology **23**(4-6): 567-576.

Wentzel, M. C., L. H. Lotter, R. E. Loewenthal and G. V. Marais (1986). "Metabolic behavior of acinetobacter spp in enhanced biological phosphorus removal-a biochemical model." Water Sa **12**(4): 209-224.

Whang, L. M. and J. K. Park (2002). "Competition between polyphosphate- and glycogen-accumulating organisms in biological phosphorus removal systems - effect of temperature." Water Science and Technology **46**(1-2): 191-194.

Whang, L. M. and J. K. Park (2006). "Competition between polyphosphate- and glycogen-accumulating organisms in enhanced-biological-phosphorus-removal systems: Effect of temperature and sludge age." Water Environment Research **78**(1): 4-11.

Wiegand, C. and S. Pflugmacher (2005). "Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review." Toxicology and Applied Pharmacology **203**(3): 201-218.

Williams, W. L., L. O. Tedeschi, P. J. Kononoff, T. R. Callaway, S. E. Dowd, K. Karges and M. L. Gibson (2010). "Evaluation of in vitro gas production and rumen bacterial populations fermenting corn milling (co)products." Journal of Dairy Science **93**(10): 4735-4743.

Wong, M. T., T. Mino, R. J. Seviour, M. Onuki and W. T. Liu (2005). "In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan." Water Research **39**(13): 2901-2914.

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

Zeng, R. J., A. M. Saunders, Z. G. Yuan, L. L. Blackall and J. Keller (2003). "Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms." Biotechnology and Bioengineering **83**(2): 140-148.

Zhang, T., Y. Liu and H. H. P. Fang (2005). "Effect of pH change on the performance and microbial community of enhanced biological phosphate removal process." Biotechnology and Bioengineering **92**(2): 173-182.

Zilles, J. L., J. Peccia, M. W. Kim, C. H. Hung and D. R. Noguera (2002). "Involvement of Rhodocyclus-related organisms in phosphorus removal in full-scale wastewater treatment plants." Applied and Environmental Microbiology **68**(6): 2763-2769.