EXPERIMENTAL STUDY AND MATHEMATICAL MODELING OF ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL USING GLUCOSE AS THE DOMINANT SUBSTRATE

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Division of Environmental Engineering University of Saskatchewan Saskatoon

By
Niandong Wang
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ABSTRACT

Two parallel sequential batch reactors (SBR) were set up and operated in order to investigate the metabolism of glucose in an enhanced biological phosphorus removal (EBPR) process, and compare the mechanisms of phosphorus removal when using either acetate or glucose as the dominant organic substrate.

Initial results indicated that feeding glucose as the dominant substrate caused poor and unstable EBPR performance. After many variations, the operating procedures for the glucose system were modified to longer anaerobic reaction time, higher glucose concentration in the influent, and shorter aerobic reaction time with a limited DO level. It was also found important to control the pH level near neutral during the reaction. The application of these modified procedures successfully established a stable EBPR performance in the glucose system, which proves that short chain fatty acids (SCFAs) are not the only kind of substrates required for a successful EBPR process.

Measurements of several important intracellular reserves and other compounds from the SBR experiments also revealed that in the glucose system, glycogen has a higher chance to replace the energy role of polyphosphate during the anaerobic reaction, thereby causing the breakdown of EBPR performance. Compared with the acetate system, it was found that during the anaerobic condition, less PO$_4$-P was released into the medium, a lower level of poly-β-hydroxyalkanoate (PHA) was accumulated, and the accumulated PHA was mainly in the form of 3-hydroxyvalerate (3-HV) rather than 3-hydroxybutyrate (3-HB) in the glucose system. Lactate was also found to be released into the medium during the anaerobic condition in the glucose system.
Other experimental results indicated that the bacteria could potentially perform denitrification under anoxic conditions in the glucose system. Microorganism identification indicated similar bacterial compositions with *Aeromonas hydrophilia* as the predominant species in both EBPR systems.

Applying fundamental biochemistry knowledge to the experimental results, a new biochemical model was hypothesized to explain the metabolism of an EBPR system using glucose as the single substrate. Based on this theoretical model, a mathematical model was developed which simulated successfully the dynamics of the key metabolic components in the EBPR system using glucose as the single substrate.
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LIST OF ABBREVIATIONS AND SYMBOLS

ADP  Adenosine diphosphate
AMP  Adenosine monophosphate
ATP  Adenosine triphosphate
BNR  Biological nutrients removal
Bio-P bacteria  Biological phosphorus accumulating bacteria
C    Carbon
C_{g}  The concentration of glucose in the medium (C-mmol/L)
C_{gl}  The concentration of glycogen (C-mmol/L)
C_{p}  The concentration of PO_4-P in the medium (P-mmol/L)
C_{phv}  The concentration of PHV (C-mmol/L)
C_{pp}  The concentration of polyphosphate (P-mmol/L)
C_{x}  The concentration of active biomass (C-mmol/L)
COD  Chemical Oxygen Demand
DO  Dissolved Oxygen
EBPR  Enhanced biological phosphorus removal
ED  The Entner-Douderoff pathway
EMP  The Embden-Meyerhof-Parnas pathway
f_{gl}  The glycogen fraction of the active biomass, f_{gl} = C_{gl}/C_{x}
f_{gl}^{\text{max}}  The maximum glycogen fraction of the active biomass
f_{gl}^{\text{min}}  The minimum glycogen fraction of the active biomass
f_{phv}  The PHV fraction of the active biomass, f_{phv} = C_{phv}/C_{x}
f_{pp}  The polyphosphate fraction of the active biomass, f_{pp} = C_{pp}/C_{x}
f_{pp}^{\text{max}}  The maximum polyphosphate fraction of the active biomass
FAD  Flavin adenine dinucleotide
FADH₂  Reduced flavin adenine dinucleotide
GC/MS  Gas Chromatograph/Mass Spectrophotometer
GTP    Guanosine-5'-triphosphate
GDP    Guanosine-5'-diphosphate
Hac    Acetic Acid
3-HB   3-hydroxybutyrate (the monometric unit of PHB)
3-HV   3-hydroxyvalerate (the monometric unit of PHV)
K      The amount of ATP needed for the formation of 1 C-mol of active biomass
       (ATP-mol/C-mol)
K_{gs} The half saturation constant of glycogen accumulation (C-mmol/L)
{k_{gi}}v The rate constant of PHV formation from glycogen (h⁻¹)
{k_{gi}} The rate constant of aerobic glycogen synthesis (h⁻¹)
K_{gs} The half saturation constant of PHV formation from glucose direct
       conversion (C-mmol/L)
{k_{pp}} max The maximum specific rate of polyphosphate synthesis
             (P-mmol/C-mmol · h)
K_{ps} The half saturation constant of polyphosphate synthesis rate (P-mmol/L)
k_x    The first order constant of active biomass growth rate (h⁻¹)
m_{aer} The specific aerobic maintenance rate (C-mmol/C-mmol · h)
m_{an}  The specific anaerobic maintenance rate (P-mmol/C-mmol · h)
m_{atp} The specific ATP consumption due to biomass maintenance
        (ATP-mol/C-mol · h)
MLSS  Mixed liquor suspended solids
MLVSS Mixed liquor volatile suspended solids, or abbreviated as VSS
N      Nitrogen
NAD    Nicotinamide adenine dinucleotide
NADH₂ Reduced nicotinamide adenine dinucleotide
NADP   Nicotinamide adenine dinucleotide phosphate
NADPH₂ Reduced nicotinamide adenine dinucleotide phosphate
NMR    Nuclear magnetic resonance
NO₃-N  Nitrate in the medium
P Phosphorus
Pi Inorganic phosphate
P_{\text{in, out}} Intracellular phosphate; phosphate in the bulk liquid
\Delta P Proton motive force (kJ/mol)
pH_{\text{in, out}} Intracellular pH value; pH value of the bulk liquid
PHA Poly-\beta-hydroxyalkanoate
PHB Poly-\beta-hydroxybutyrate
PHV Poly-\beta-hydroxyvalerate
PO_{4-P} Orthophosphate in the medium
q^{\text{aer}} The matrix of the aerobic specific reaction rates
q^{\text{an}} The matrix of the anaerobic specific reaction rates
q_{gl} The specific glycogen accumulation rate (C-mmol/C-mmol \cdot h)
q_{gl}^{\text{max}} The maximum glycogen accumulation rate (C-mmol/C-mmol \cdot h)
q_{gV} The specific rate of PHV formation from glucose direct conversion (C-mmol/C-mmol \cdot h)
q_{gV}^{\text{max}} The maximum specific rate of PHV formation from glucose direct conversion (C-mmol/C-mmol \cdot h)
q_{gIV} The specific rate of PHV formation from glycogen (C-mmol/C-mmol \cdot h)
q_{pp} The specific polyphosphate synthesis rate (P-mmol/C-mmol \cdot h)
R Gas constant (kJ/mol \cdot K)
SBR Sequential batch reactor
SCFA Short chain fatty acid
T Temperature (K)
TCA The Tricarboxylic Acid cycle
TOC Total organic carbon
VSS see MLVSS
\alpha^{\text{aer}} The matrix of the aerobic stoichiometric coefficients
\alpha^{\text{an}} The matrix of the anaerobic stoichiometry coefficients
\Delta \psi Electric potential difference of cell membrane (kJ/mol)
\gamma^{\text{aer}} The matrix of the aerobic conversion rates of the key metabolic components
\( \gamma' \)  The matrix of the anaerobic conversion rates of the key metabolic components

\( \gamma^T \)  The transposed aerobic conversion rate matrix of the key metabolic components

\( \gamma_{co} \)  The overall CO\(_2\) conversion rate (mmol CO\(_2\)/L \cdot h)

\( \gamma_{gl} \)  The overall glucose conversion rate (C-mmol/L \cdot h)

\( \gamma_{gl} \)  The overall glycogen conversion rate (C-mmol/L \cdot h)

\( \gamma_{bo} \)  The overall H\(_2\)O conversion rate (mmol H\(_2\)O/L \cdot h)

\( \gamma_{an} \)  The overall aerobic NH\(_3\) conversion rate (mmol NH\(_3\)/L \cdot h)

\( \gamma_{ao} \)  The overall aerobic O\(_2\) conversion rate (mmol O\(_2\)/L \cdot h)

\( \gamma_{po} \)  The overall PO\(_4\)-P conversion rate (P-mmol/L \cdot h)

\( \gamma_{phv} \)  The overall PHV conversion rate (C-mmol/L \cdot h)

\( \gamma_{pp} \)  The overall polyphosphate conversion rate (P-mmol/L \cdot h)

\( \gamma_{ax} \)  The overall aerobic active biomass growth rate (C-mmol/L \cdot h)

\( \delta \)  The amount of ATP produced per electron pair transferred to oxygen (the so-called \( P/O \) ratio, mol-ATP/mol-O)

\( \mu \)  The specific active biomass growth rate (C-mmol/C-mmol \cdot h)

\( \nu^T \)  The transposed aerobic reaction rate matrix
CHAPTER 1 INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Research Background

Enhanced biological phosphorus removal (EBPR) is playing an increasingly important role in wastewater treatment due to its low cost and high efficiency for phosphorus removal. Two strategies have been acknowledged to be critical for a successful EBPR process: cycling anaerobic and aerobic conditions and supplying short chain fatty acids (SCFA) during the anaerobic condition. It is recognized that bacteria which can accumulate a high concentration of polyphosphate will become dominant when applying these two operating strategies in a wastewater treatment plant. The following biochemical model is usually applied to explain an EBPR process (Wentzel et al., 1985, 1986; Comeau et al., 1986, 1987b; Mino et al., 1987; Smolders et al., 1994a, 1994b):

1) Under the anaerobic condition, biological phosphorus accumulating bacteria (bio-P bacteria) uptake SCFAs and convert them to poly-β-hydroxybutyrate (PHB) as an internal carbon reserve. The energy source for the uptake of SCFAs and the conversion to PHB is believed to be from the hydrolysis of stored polyphosphate because it is observed that the dissolved phosphorus level increases significantly during the anaerobic phase.

2) Under the aerobic condition, the PHB reserve is oxidized to provide the carbon and energy requirement for the bio-P bacteria growth, as well as the energy for the re-synthesis of polyphosphate. PO₄-P (orthophosphate in the medium) is excessively absorbed from the liquid phase into the bio-P bacteria for the polyphosphate synthesis, by which PO₄-P is removed from the supernatant.

Current biochemical models on EBPR process all refer to situations where SCFAs (typically acetate) are the only kind of organic substrates that induce the phosphorus removal mechanism. However, data from full-scale EBPR plants often show high variability in PO₄-P removal efficiencies due to the variation of organic substrates in
wastewater. Glucose is believed to have significant impacts on an EBPR process due to its extensive existence in the wastewater and its intimate relationship with glycogen, an important intracellular reserve in the EBPR system. Glucose also plays an essential role in the central biochemical pathways, such as the Embden-Meyerhof-Parnas (EMP) pathway and the Entner-Doudoroff (ED) pathway. Many researchers have incorporated glucose when studying the fate of carbon substrate in biological wastewater treatment.

Contradictory information currently exists regarding the role of glucose in an EBPR process. Many researchers observed the deterioration of EBPR performance when glucose appeared in the influent (Cech and Hartman, 1990, 1993). However, they could not give explicit explanations for this glucose caused deterioration. Some other researchers, on the other hand, observed successful EBPR performance even with glucose as the single added organic substrate (Liu, 1998; Carucci et al., 1994). But no detailed explanations are available for the glucose induced EBPR mechanism.

The effects of organic substrates other than SCFAs in wastewater should be investigated in order to obtain deeper insight into the mechanisms of the EBPR processes, which will help to achieve a more stable and robust EBPR process. Because of the importance of glucose in the study of biological wastewater treatment and the contradictory information of its effect on the EBPR system, it was decided to study the role and metabolic fate of glucose in an EBPR system when glucose is used as the dominant organic substrate.

1.2 Research Objectives

The objectives of this research are:

- To find out why utilization of glucose as the dominant substrate might upset the EBPR process.

- To investigate whether and how glucose can be used as the dominant substrate to induce and maintain successful EBPR performance.
• To compare the metabolic characteristics of the EBPR processes using either SCFAs or glucose as the dominant substrate.

• To develop a metabolic model to explain the mechanism of a glucose induced EBPR process applying fundamental biochemistry knowledge.

• To develop a mathematical model simulating the dynamic performance of an EBPR process using glucose as the single substrate.
CHAPTER 2 LITERATURE REVIEW

2.1 Eutrophication Pollution

2.1.1 Natural Eutrophication

All lakes and reservoirs have a finite life span, sooner or later they will be filled with sediment and replaced by terrestrial communities (Harper, 1992). Naumann, a Swedish botanist (Harper, 1992), was the first to use nutrient status to describe the succession of natural lakes. Young lakes usually have low levels of nutrients and correspondingly low levels of biological activity. Such a lake is referred to as oligotrophic (oligos means little in Greek). Old lakes usually have high levels of nutrients and correspondingly high levels of biological activity. Such a lake is referred to as eutrophic (eu means well in Greek). Mesotrophic is used to describe lakes in a transition state between oligotrophic and eutrophic. The natural process of lake succession usually takes many hundreds of thousands of years to occur and is largely irreversible (Ryding and Rast, 1989). Lakes undergoing such natural eutrophication generally have good water quality and exhibit a diverse biological community throughout much of their existence. The growth of algae and other aquatic plants in a lake is usually minimal and generally in balance with the input of plant nutrients (Harper, 1992).

2.1.2 Cultural Eutrophication

Human settlement and the associated clearing of forests, development of farms and cities, usually change the natural eutrophication process in a dramatic way (Ryding and Rast, 1989). The runoff of contaminated materials from the land surface to the water body is greatly accelerated. An excessively increased input of plant nutrients (primarily phosphorus, nitrogen, and carbon) to a lake can stimulate algal and aquatic plant growths. The excessive "blooms" of algae promoted by these excessive nutrients leads
to the acceleration of lake succession. This accelerated succession of natural water bodies is defined as cultural eutrophication (Porcella, 1974; Ryding and Rast, 1989).

Eutrophication is a problem which became widely recognized by the scientific community in the 1940s and 1950s (Harper, 1992). Excessive algae and aquatic plant growths can interfere significantly with the uses and aesthetic quality of a water body. After algal populations die and sink to the bottom of a water body, their decay by bacteria can reduce oxygen concentrations in the bottom waters to levels which are too low to support fish life (Ryding and Rast, 1989). Such oxygen deficient conditions can also result in excessive levels of iron and manganese in the water, which can interfere with drinking water treatment (Ryding and Rast, 1989). There are also potential health effects, especially in tropical regions, related to such parasitic diseases as schistosomiasis, onchocerciasis and malaria, all of which can be aggravated by cultural eutrophication (Harper, 1992).

2.1.3 Phosphorus Role in Eutrophication Control

Photosynthesis has long been recognized to be the mechanism of algal growth in lakes. According to Stumm and Morgan (1970), this process can be represented by the following equation:

\[
\text{CO}_2 + \text{Nitrogen} + \text{Phosphorous} + \text{H}_2\text{O} + \text{Trace Elements} \rightarrow \text{Sunlight} \rightarrow \text{Algal Protoplast} + \text{O}_2 \quad \text{Eq. 2-1}
\]

Photosynthesis

Eutrophication problems can be controlled by limiting the conditions for photosynthesis. Sunlight and CO₂ are factors that are not manageable by mankind for eutrophication control. Therefore, controlling the input of nitrogen and phosphorous becomes the major approach for eliminating eutrophication in water bodies. It was reported that atmospheric nitrogen could be fixed by blue-green algae (Porcella, 1972). However, Porcella (1972) reported that the only utilizable phosphorus for algal growth is orthophosphate in water. Therefore, the removal of dissolved phosphorus (mainly
orthophosphate) is the most effective procedure for controlling eutrophication in natural waters.

A theoretically and experimentally verified observation (Harper, 1992) states that as the nutrient concentration increases, the algal growth rate increases linearly but eventually the growth rate approaches a constant maximum value. This first order - zero order relationship for algal growth apparently results from the kinetics of uptake of phosphorus and nitrogen. These results imply that in lakes a linear relationship between productivity and nutrient concentration occurs only when the nutrient is well below the saturation level. Thus, if one wishes to decrease algal productivity by decreasing phosphorus concentration, levels of dissolved phosphorus must be reduced to where algal productivity is limited by phosphorus concentration. According to Porcella (1972), phosphorus levels lower than 1 mg/L must be maintained to effectively limit the growth of aquatic plants in natural waters.

2.2 Characterization of Phosphorus

Phosphorus, symbol P, atomic number 15, atomic weight 30.97, belongs to Group V of the periodic table of the elements. P is not found free in nature and almost always occurs in the fully oxidized state as phosphate (PO$_4^{3-}$, Corbridge, 1985). The element is widely distributed in its soluble or particulate forms in soils, rocks, oceans and all living cells. Particulate P commonly includes precipitates and phosphorus existing as part of biomass or inert materials (Jenkins and Hermanowicz, 1991); soluble P includes orthophosphates, organic phosphates and condensed phosphates, the typical concentrations of which in raw domestic wastewater are presented in Table 2.1 (Jenkins and Hermanowicz, 1991).

Compounds containing discrete PO$_4^{3-}$ ions are known as orthophosphates (Corbridge, 1985). Organic phosphates include sugar phosphates, phospholipids, and nucleotides (Snoeyink and Jenkins, 1980). Condensed phosphates are formed by the condensation of two or more orthophosphate units to form a polymer of phosphate linked by phospho-anhydride (P-O-P) bonds (Snoeyink and Jenkins, 1980). Condensed phosphates include
the pyrophosphate (the simplest condensed phosphate), metaphosphates (cyclic groups of condensed phosphates, such as trimetaphosphate) and polyphosphates (linear polymers of phosphate). The number of orthophosphate units in polyphosphate may range from 2 to $10^6$ (Kulaev, 1979). The formation of polyphosphate by bacteria is of particular importance to enhanced biological phosphorus removal from wastewater, which is described in the following section. A few forms of phosphate are shown in Figure 2.1.

**Table 2.1. Typical Phosphorus Concentrations in Raw Domestic Wastewater***

(*) Data Source: Jenkins and Hermanowicz, 1991

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Typical P Concentrations in Raw Domestic Wastewater, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthophosphate (PO₄-P)</td>
<td>3-4</td>
</tr>
<tr>
<td>Condensed phosphate</td>
<td>2-3</td>
</tr>
<tr>
<td>Organic phosphates</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>6-8</td>
</tr>
</tbody>
</table>

2.3 Phosphorus Removal From Wastewater

At present, the major P sources for natural water bodies include point sources such as domestic and industrial wastewater effluents, and non-point sources, such as agricultural drainage, surface irrigation returns, phosphate-containing rocks, and benthic sediment releases (OECD, 1982). It has been established that urban sewage (including domestic and industrial point sources) is the most significant source of P for natural water bodies (OECD, 1982). Modern technologies have been successfully applied to remove P from point sources. These P removal technologies consist mainly of chemical precipitation and enhanced biological P removal in wastewater.
Orthophosphate

Pyrophosphate

Trimetaphosphate
(M – Cation)

Polyphosphate
\( n = 2 \) to \( 1,000,000 \)

Figure 2.1. Several Forms of Phosphate
2.3.1 Chemical Precipitation
The mechanism of P removal by chemical precipitation is fairly simple. It has been used for many years and is well described in standard textbooks (Tchobanoglous and Burton, 1991). It is typically performed by the addition of alum (Al₂(SO₄)₃.18H₂O), sodium aluminate (NaAlO₂), ferric chloride (FeCl₃), ferrous sulfate (FeSO₄), ferrous chloride (FeCl₂), or lime (Ca (OH)₂). The dissolved phosphorus in the wastewater will precipitate after flocculation with the added chemicals. Chemical precipitation for P removal has the advantages of being reliable and simple to apply, however, it suffers the disadvantages of increased chemical and sludge disposal costs.

2.3.2 Enhanced Biological Phosphorus Removal (EBPR)
Biological removal of phosphorus from wastewater occurs as part of normal bacterial growth in biological treatment processes. P is present in microbial cells as a component in various forms, such as nucleic acids, nucleotides, phospholipids, sugar phosphates, orthophosphate and polyphosphate (Corbridge, 1985). The typical %P of bacteria (based on biomass dry weight) is about 3% (Comeau, 1989).

Enhanced biological phosphorus removal (EBPR) can be achieved by modification of the conventional activated sludge process (Barnard, 1992). Essentially, a continuous flow EBPR process uses a bioreactor in which the aerobic zone is preceded by an anaerobic zone. With the addition of wastewater to the anaerobic zone, the orthophosphate (PO₄-P) level in the medium goes up due to the release from the biological phosphorus removal bacteria (bio-P bacteria). In the subsequent aerobic zone, PO₄-P in the medium is taken up by the bio-P bacteria in a large excess of their metabolic requirements. The %P of bio-P bacteria (based on biomass dry weight) can range from 3% to 12% in full-scale plants (Comeau, 1989). Compared to P removal by the chemical method, EBPR is a much more beneficial process due to lower chemical requirements (if any) and lower sludge production (Comeau, 1989).
2.3.2.1 Historical Development of EBPR Process

Grady et al. (1999) noted that the history of the development of enhanced biological phosphorus removal processes is one of the most fascinating ones in environmental engineering. Srinath et al. (1959) were the first few researchers to report the occurrence of biological P removal. They observed that rapid PO₄-P uptake took place when sludge samples were mixed with raw wastewater and aerated. However, they could not explain their observations.

Levin and Shapiro (1965) observed that PO₄-P release took place when sludge from a full-scale plant was kept under anaerobic conditions or aerated for a prolonged period of time. No explanation was offered for the anaerobic PO₄-P release. Later, Shapiro et al. (1967) and Wells (1969) reported that the released PO₄-P could all be subsequently taken up aerobically. However, it was Fuhs and Chen (1975) who first recognized the importance of the anaerobic/aerobic sequence. They observed that when aerobic sludge taken from a full-scale plant was aerated after an anaerobic period that lasted overnight, it contained more polyphosphate than a sludge that had not been aerated. They were also the first to identify the presence of polyphosphate accumulating bacterium, *Acinetobacter*, in the sludge. They suggested that *Acinetobacter* could flourish in such a process because they could grow aerobically on simple carbon compounds produced by fermentative bacteria under anaerobic conditions. Fuhs and Chen (1975) were also the first to report poly-β-hydroxybutyrate (PHB) accumulation inside of *Acinetobacter*. They observed that *Acinetobacter* could metabolize simple substrates but not sugars. Although they could not attribute any specific role to this reserved material, they proposed that the stored PHB could have been used for aerobic polyphosphate accumulation.

Barnard (1974), in developing the Bardenpho process, identified that the presence of nitrates in the anaerobic zone had an adverse effect on the efficiency of P removal. From this observation, he proposed a modification of the Bardenpho process into the Phoredox process. Furthermore, the entrainment of oxygen from air into the anaerobic
zone was identified as detrimental by a rational extension of the effects of nitrates (Paepcke, 1983; Pitman, 1984).

Nicholls and Osborn (1979) first proposed that, under the anaerobic "stressing" condition, simple substrates would be stored as PHB and that, somehow, this mechanism would be linked to PO$_4$-P release from polyphosphate hydrolysis. They also recommended primary sludge digestion as a means of increasing SCFAs to the anaerobic zone to increase the accumulation of PHB.

2.3.2.2 EBPR Process Configurations

A variety of process configurations are used to perform EBPR. They can be broadly divided into "mainstream" and "sidestream" processes (Levin et al. 1972, Arvin, 1985). Mainstream processes include an anaerobic compartment in the main treatment train, while sidestream processes include an anaerobic compartment in a "sidestream". The simplest mainstream EBPR process is the A/O system. It consists of an initial anaerobic (without O$_2$ or NO$_3$-N) basin followed by an aerobic basin in the main wastewater treatment train (Figure 2.2). This configuration is designed primarily for P removal but can also accomplish N removal (Bowker and Stensel, 1990). The average anaerobic hydraulic residence time is typically 1-2 hours (Arvin, 1985). The A/O system is proprietary and is marketed by Air Products and Chemicals of Allentown, PA (Daigger and Polson, 1991).

The A2/O process is a modification of the A/O configuration, in which an initial anaerobic basin is followed by an anoxic (without DO but containing nitrate) basin and then an aerobic basin (Figure 2.2). Besides the enhanced P removal function, substantial N removal can be realized in the anoxic zone of the A2/O process by denitrification. The A2/O process is also proprietary and marketed by Air Products and Chemicals of Allentown, PA (Daigger and Polson, 1991).

Other mainstream processes also include anoxic basins to reduce the introduction of nitrate to the anaerobic zone. These include the modified Bardenpho/Phoredox Process
and the University of Capetown (UCT) Processes (Figure 2.3). In the Modified Bardenpho Process, returned sludge is directed to an initial anaerobic basin (Figure 2.3, Barnard, 1976). To maintain low nitrate concentration in this recycle, an internal recycle is included from the aerated zone to the anoxic zone, where denitrification occurs. A second anoxic basin provides additional denitrification after the initial aeration basin. A second aerated zone is used to prevent anaerobic conditions. In the UCT process, returned sludge is directed to an anoxic zone (where denitrification occurs), and then directed to the initial anaerobic zone.

The most common sidestream process is the Phostrip system, in which the PO₄-P concentration increases in a sidestream from anaerobic biological phosphorus release. The released PO₄-P is then precipitated by chemical addition (Figure 2.4, Levin et al., 1972).
Figure 2.2. A/O and A2/O Processes
Modified Bardenpho/Phoredox Process

UCT Process

Figure 2.3. Modified Bardenpho/Phoredox Process and UCT Process
2.4 Metabolic Characteristics of EBPR

2.4.1 Important Microbial Internal Reserves Involved in EBPR

During the development of the conventional EBPR process, the important roles that three microbial internal reserves play were gradually recognized. The three microbial internal reserves are polyphosphate, glycogen and poly-β-hydroxyalkanoates (PHA) (Comeau et al., 1986, 1987b; Mino et al., 1987, 1998; Jenkins and Tandoi, 1991; Smolders et al., 1994a, 1994b).

2.4.1.1 Polyphosphate

The occurrence of polyphosphate in microorganisms is not uncommon. Dawes and Senior (1973) and Osborn and Nicholls (1978) listed more than 20 genera of microorganisms in which the presence of polyphosphate had been reported.
Harold (1966) recognized two fundamental mechanisms of polyphosphate accumulation: "luxury uptake" and "overplus accumulation". In the "luxury uptake" mechanism, polyphosphate accumulation takes place during the deprivation of another nutrient such as nitrogen or sulfur. Upon addition of the nutrient, growth resumes and polyphosphate reserves are depleted for the synthesis of nucleic acids. In the "overplus accumulation", the deprivation of PO$_4$-P is followed by a sudden exposure of the microorganisms to PO$_4$-P which causes the microorganisms to rapidly accumulate polyphosphate. The accumulated polyphosphate will slowly be degraded afterwards.

The biosynthesis of polyphosphate occurs mainly by the transfer of a phosphate from ATP (Adenosine triphosphate) to a growing chain of polyphosphate via the enzyme polyphosphate kinase. The transfer of one phosphate group from 1,3-diphosphoglycerate (an intermediate of the glycolysis pathway) to polyphosphate has also been reported (Kulaev, 1979).

Polyphosphate degradation usually occurs by simple hydrolysis with the enzyme polyphosphatase and results in the loss of the energy contained in the phosphate bond. At low ATP/ADP ratios (ADP: Adenosine diphosphate), however, polyphosphate has been shown to transfer its energy back to ATP in a reversal action of the enzyme polyphosphate kinase (Kornberg, 1957). A number of other pathways haven been shown in which the high energy phosphate is transferred from AMP (Adenosine monophosphate) to ADP, from NAD (Nicotinamide adenine dinucleotide) to NADP (Nicotinamide adenine dinucleotide phosphate), form glucose to glucose-6-phosphate, and from 3-phosphoglycerate to 1,3-diphosphoglycerate (Kulaev and Vagabov, 1983).

Polyphosphate is found in various locations in the cells and responds differently to different physiological states of the cell (Kulaev and Vagabov, 1983). Polyphosphate forms a useful reserve of activated phosphate that is used in particular, in the metabolism of nucleic acids and carbohydrates. Lipmann (1965) has advanced the suggestion that the earliest organisms used polyphosphate or pyrophosphate as their
prime energy carrier, the role for ATP as the universal energy carrier in contemporary organisms that has arisen from the course of evolution.

The presence of polyphosphate in bio-P bacteria has long been established by staining methods (with methylene blue or toluidine blue) (Levin and Shapiro, 1965; Fuhs and Chen, 1975; Lotter, 1985); by electron dispersive X-ray analysis (EDAX) (Buchan, 1983); by $^{31}$P-nuclear magnetic resonance (NMR) (Florentz and Granger, 1983; Florentz et al., 1984b; Halvorson et al., 1986); and by chemical fractionation method (Mino et al., 1984). The high percentage of phosphorus content of the bio-P bacteria, 3 to 12% compared to a normal sludge 2 to 3% dry weight content also gives an indication of the amount of polyphosphate accumulation in bio-P bacteria.

2.4.1.2 Glycogen

Glycogen is a storage material in both procaryotes (bacteria) and eucaryotes (Dawes and Senior, 1973). This stored polysaccharide is enzymatically synthesized from glucose by a repetitive process in which a single glucose molecule is attached to the end of a glycogen chain (Lehninger, 1993). Glycogen, with the empirical formula $(C_{6}H_{10}O_{5})_{n}$, is highly branched, with branch lengths of approximately 12 residues (Chao and Bowen, 1971). The molecular structure of glycogen is shown in Figure 2.5. The presence of glycogen can be detected by light microscopy when cells are treated with dilute iodine because of the glycogen-iodine reaction that turns the cells red-brown (Chao and Bowen, 1971).

According to Dawes and Senior (1973), microorganisms that accumulate glycogen generally do so under nutrient limitations (most commonly N) and it may be utilized upon later carbon starvation. In general, glycogen synthesis is stimulated by a high adenylate energy charge (similar to a high ATP/ADP ratio) whereas a low adenylate energy charge promotes glycogen degradation and inhibits its synthesis (Dawes and Senior, 1973). The metabolic pathways of glycogen synthesis and degradation are presented in Appendix B-II.
In EBPR studies, reports of glycogen involvement are not in agreement. Nicholls and Osborn (1979) reported that no glycogen accumulation could be detected in a full-scale bio-P treatment plant treating municipal wastewater. Comeau (1989) could not detect significant glycogen storage taking place during a pilot plant EBPR research. However, the presence of glycogen has been reported by many other researchers, especially when glucose is added in the influent (Mino et al., 1987; Arun et al, 1988; Smolders et al., 1994a; Liu et al., 1994; Satoh et al., 1992).

![Glycogen Structure](image)

Figure 2.5. Structure of Glycogen

2.4.1.3 Poly-β-hydroxyalkanoate (PHA)

PHA is a microbial lipid polymer. The most common form of PHA and the most studied is poly-β-hydroxybutyrate (PHB). The role of PHB is more of an energy source than of a carbon source for bacteria (Dawes and Senior, 1973). Deposits of PHB are readily
visible with the light microscope, occurring as dark granules of variable size, scattered throughout the cell.

The major pathway of PHB synthesis is presented in Appendix B-III. The synthesis of PHB involves the condensation of acetyl CoA(s) and reducing power NADH$_2$ (reduced Nicotinamide adenine dinucleotide), the universal electron carrier of microorganisms. The formation of PHB is particularly useful under conditions of oxygen limitation, which supplied the sink of reducing powers. Therefore, PHB reserves may accumulate when cells are limited in oxygen but still have a carbon source available (Dawes and Senior, 1973).

The degradation of PHB results in the production of two acetyl CoA(s) and one NADH$_2$. This degradation occurs when the internal concentration of NAD and free CoA increases while the concentration of acetyl-CoA is low. For example, PHB is degraded in the presence of oxygen when the external carbon sources are limited. However, if both oxygen and an external carbon source are present, PHB should not be degraded (Dawes and Senior, 1973).

The presence of PHB accumulation in the bio-P bacteria was first reported by Fuhs and Chen (1975). Comeau (1989) was the first to confirm that another form of PHA was also accumulated by the bio-P bacteria. Comeau found that, besides PHB, poly-β-hydroxyvalerate (PHV) was also accumulated when SCFAs were fed into the EBPR processes. Furthermore, he also found that, among the substrates he utilized, those substrates made up of an odd number of carbons generally favored PHV formation whereas substrates made up of an even number of carbons favored PHB formation. Another two forms of PHA, poly-β-hydroxy-2-methylbutyrate and poly-β-hydroxy-2-methylvalerate, were reported by Satoh et al. (1992) in his EBPR experiments. The structures and precursors of the detected PHA are presented in Table 2.2.
<table>
<thead>
<tr>
<th>Poly-β-hydroxyalkanoate (PHA)</th>
<th>Unit</th>
<th>Structure</th>
<th>Precursors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-β-hydroxybutyrate (PHB)</td>
<td>3-</td>
<td>CH₃</td>
<td>2 Acetyl-CoA</td>
</tr>
<tr>
<td>hydroxybutyrate</td>
<td></td>
<td>O</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-O-CH-CH₂-C-</td>
<td></td>
</tr>
<tr>
<td>Poly-β-hydroxyvalerate (PHV)</td>
<td>3-</td>
<td>CH₃</td>
<td>Acetyl-CoA</td>
</tr>
<tr>
<td>hydroxyvalerate</td>
<td></td>
<td>CH₂</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O</td>
<td>Propionyl-CoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-O-CH-CH₂-C-</td>
<td></td>
</tr>
<tr>
<td>Poly-β-hydroxy-2-methylbutyrate</td>
<td>3-hydroxy-2-methylbutyrate</td>
<td>CH₃ CH₃ O</td>
<td>Acetyl-CoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-O-CH-CH-C-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Propionyl-CoA</td>
</tr>
<tr>
<td>Poly-β-hydroxy-2-methylvalerate</td>
<td>3-hydroxy-2-methylvalerate</td>
<td>CH₃</td>
<td>2 Propionyl-CoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH₂ CH₃ O</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-O-CH-CH-C-</td>
<td></td>
</tr>
</tbody>
</table>

### 2.4.2 Early Models of EBPR

The practical application of the EBPR process preceded the understanding of its mechanism. The EBPR process has been widely applied in wastewater treatment plants for about thirty years. However, the understanding of the EBPR mechanism is still in dispute and under development (Mino et al., 1998). Early EBPR research (late 1970s to early 1980s) focused on the development of simple mechanistic models to explain the observed phenomena from EBPR applications.

The major proposals from Nicholls and Osborn (1979), Rensink et al. (1981), Banard (1974, 1976), Fuhs and Chen (1975) can be summarized as follows:

- SCFAs (typically acetate) serve as the single required substrate for bio-P bacteria.
- The continual cycling between the anaerobic and aerobic zones give the bio-P bacteria a competitive advantage over ordinary heterotrophic bacteria, because
without the capability to make and use polyphosphate, the ordinary heterotrophs are not able to take up organic matter in the anaerobic zone.

- In the anaerobic zone, the SCFAs are taken up and stored as PHB by bio-P bacteria. This process requires energy. The energy requirements are supplied by the breakdown of polyphosphate with PO₄-P being released into the bulk solution.

- In the aerobic zone, the stored PHB is used as carbon and energy source for cell functions and growth, as well as an energy source for polyphosphate formation which gives rise to PO₄-P excessive uptake.

- *Acinetobacter spp.* is the principal organism responsible for EBPR.

Marais et al. (1983) introduced a biochemical model by putting forward biochemical pathways for the synthesis of PHB under anaerobic conditions. However, with acetate as substrate, they found it is not possible to identify pathways for PHB synthesis. Sources of reducing power (NADH₂) to reduce acetoacetate for PHB synthesis were not discovered in their work. They were unable, therefore, to find an explanation for PHB storage under anaerobic conditions with acetate as substrate.

Early theoretical models provided lots of important explanations for phenomena observed from EBPR researches. They related the anaerobic PO₄-P release to polyphosphate function, anaerobic PHB accumulation to substrate conversion, aerobic PO₄-P decrease to polyphosphate regeneration and aerobic PHB utilization to aerobic carbon and energy sources. These models also noted the important roles of the anaerobic condition to help the bio-P bacteria compete for substrates and ferment substrates to SCFAs. These models all accepted *Acinetobacter spp* as the principal bacteria responsible for the EBPR mechanism. However, early theoretical models could not provide the rational biochemical pathways to explain the EBPR metabolism. It was not until the late of 1980s that several biochemical models were proposed to explain the metabolism of EBPR. The Comeau (Comeau et al., 1986)/Wentzel (Wentzel et al., 1986) model, the Mino model (Mino et al., 1987) and the adapted Mino model (Wentzel et al., 1991) are the three most recognized conceptual models providing the framework for explaining the anaerobic/aerobic metabolism of an EBPR process.
2.4.3 Three Important Conceptual Models of EBPR

**Comeau/Wentzel Model.** Florentz *et al.* (1983, 1984a) noted the activity of the Tricarboxylic Acid (TCA) cycle enzymes under anaerobic conditions. Based on this, Comeau *et al.* (1986) proposed that the reducing power (NADH₂) for PHB synthesis could be supplied from the operation of the TCA cycle under anaerobic conditions. Comeau *et al.* (1986) also identified the importance of maintaining the proton motive force of bio-P bacteria under anaerobic conditions and developed a conceptual biochemical model. Wentzel *et al.* (1986) accepted the proposal of Comeau *et al.* (1986) that under anaerobic conditions the TCA cycle is invoked and the proton motive force must be maintained, and accepted *Acinetobacter* spp. as the typical bio-P bacteria mediating EBPR. They proposed detailed biochemical pathways for *Acinetobacter* spp. in anaerobic and aerobic reactors, and presented descriptions of how these pathways are regulated by the ATP/ADP and NADH₂/NAD ratios. Since the two biochemical models proposed by Comeau *et al.* and Wentzel *et al.* are essentially the same, they are termed jointly as the Comeau/Wentzel model. The Comeau/Wentzel model is illustrated by Figure 2.6 (for the anaerobic mechanism) and Figure 2.7 (for the aerobic mechanism).

The Comeau/Wentzel model assumes that SCFA (typically acetate) is the principal type of substrate that is utilized by bio-P bacteria. As shown in Figure 2.6, acetate is transported across the cell membrane and activated to acetyl-CoA coupled by ATP hydrolysis, to form ADP. The cell responds to the decreasing ATP/ADP ratio by stimulating ATP re-synthesis by the hydrolysis of the stored polyphosphate. A portion of the acetyl-CoA is metabolized through the TCA cycle to provide the reducing power (NADH₂) required for the synthesis of PHB. The remainder of the acetyl-CoA is converted into PHB, with about 90% of the acetate carbon being conserved in that storage polymer. The hydrolysis of the polyphosphate to form ATP increases the intracellular concentration of PO₄-P, which is released into the bulk liquid.

When the wastewater and associated biomass enter the aerobic zone, the wastewater is very low in soluble organic substrates, but the bio-P bacteria contain high PHB reserves. Furthermore, the wastewater is rich in PO₄-P, while the bio-P bacteria have low
polyphosphate levels. Because oxygen is available as an electron acceptor in the aerobic zone, the accumulated PHB can be oxidized through the TCA cycle. The produced NADH₂ from the TCA cycle can be used to generate high amounts of ATP through oxidative phosphorylation by transferring the electrons to O₂. Furthermore, as the ATP/ADP ratio increases, polyphosphate synthesis is stimulated, thereby removing PO₄-P from the solution and regenerating the reserved polyphosphate of the cells.
Because of the large amount of energy provided by the aerobic metabolism of the stored PHB, the bio-P bacteria are able to take up all of the PO₄-P released in the anaerobic zone plus the PO₄-P originally present in the wastewater.

**Mino Model.** The Mino model (Mino *et al.*, 1987, Arun *et al.*, 1988) illustrated in Figure 2.8 (for the anaerobic metabolism) and Figure 2.7 (for the aerobic metabolism) is very similar to the Comeau/Wentzel model. The major difference is related to the role of glycogen. Mino and his coworkers (Mino *et al.*, 1987, Arun *et al.* 1988) considered that the TCA cycle generally doesn't function under anaerobic conditions. This means that the bio-P bacteria are not able to metabolize part of the acetate through the TCA cycle for reducing power production. However, their measurements on laboratory-scale cultures fed acetate, propionate, glucose, peptone, and yeast extract as carbon sources showed that stored carbohydrate decreased during the anaerobic phase and increased during the aerobic phase. Further support for the involvement of glycogen in the EBPR metabolism was provided by Smolders *et al.* (1994a) who found that anaerobic PHB synthesis was in excess of that predicted by the functioning of the TCA cycle alone as a source of reducing equivalents. In the Mino model, it is proposed that glycogen is metabolized through the Embden-Meyerhof-Parnas (EMP) pathway, from which extra electrons, ATP and pyruvate are produced. Then, pyruvate is assumed to be converted to acetyl-CoA. ATP is used to convert acetate to acetyl-CoA. Electrons are consumed during the formation of PHB from acetyl-CoA.

**Adapted Mino Model.** The adapted Mino model (Figure 2.9 for the anaerobic metabolism and Figure 2.7 for the aerobic metabolism) is similar to the Mino model except that the Entner-Doudoroff (ED) pathway is assumed instead of the EMP pathway to generate reducing power (Wentzel *et al.*, 1991). During the early 1990s, it has been extensively accepted that *Acinetobacter* plays an important role in the EBPR processes. Wentzel *et al.* (1991) proposed the adapted Mino model since Juni (1978) reported that *Acinetobacter* does not possess the EMP pathway.
The three important conceptual models differ mainly in the mechanism assumed to generate the necessary reducing power (NADH₂) for PHB formation. In Figures 2.6 to 2.8 the variable α₁ represents the amount of energy, expressed as ATP required to transport 1 C-mol acetate (1C-mol acetate = 0.5 mole acetate because there are 2 moles
carbon per mole acetate). Factors that affect $\alpha_1$ will be discussed in the next section. Additional ATP is required to activate acetate to acetyl-CoA. It is evident from Figures 2.6 to 2.8 that different amounts of PHB are formed per acetate taken up for the three models. The Comeau/Wentzel model predicts that 0.89 C-mol PHB (1 C-mol PHB = 0.25 mole PHB because there are 4 moles carbon per mole of monometric unit of PHB) is formed per C-mol acetate taken in. Also, 0.11 C-mol CO$_2$ (1 C-mol CO$_2$ = 1 mole CO$_2$ because there is 1 mole carbon per mole CO$_2$) is formed as some acetyl-CoA is fed through the TCA cycle. For the Mino and the Adapted Mino models, 1.33 C-mol PHB is formed per C-mol acetate taken in. At the same time, 0.5 C-mol glycogen is used to produce the reducing power necessary for PHB production.

Different amounts of PO$_4$-P are released per acetate taken in for all three models. The Comeau/Wentzel model predicts that (0.5 + $\alpha_1$) P-mol (1 P-mol phosphate = 1 mole phosphate) of phosphorus is released per C-mol acetate; the Mino model leads to (0.25 + $\alpha_1$) P-mol released per C-mol acetate taken in; and the Adapted Mino model gives a ratio of PO$_4$-P release to acetate uptake to be (0.33 + $\alpha_1$) P-mol/C-mol.

Variations in the glycogen and PHB content of cells during the anaerobic uptake of acetate have been measured (Arun et al., 1988, and Smolders et al. 1994a). Smolders et al. also determined the amount of carbon dioxide released per acetate taken in. The observed stoichiometry relations were close to that predicted by the Mino and the Adapted Mino models, and deviated significantly from the Comeau/Wentzel model.

2.4.4 pH Effect on EBPR

In comparing the Comeau/Wentzel model, the Mino model, and the Adapted Mino model, it is evident that the ratio of PO$_4$-P released per acetate taken up (P/Hac ratio) differs. Smolders et al. (1994a) demonstrated that $\alpha_1$ is a strong function of the medium pH. A wide range of P/Hac ratios has been reported in the literature (see Table 2.3). In a literature survey, Smolders et al. (1994a) found values ranging from 0.5 to 1.5 P-mol/C-mol. Smolders et al. (1994a) found that the PO$_4$-P release rate increased linearly with pH in the range 5.5-8.2, while acetate uptake rate remained unchanged.
Table 2.3. Theoretical and Experimental Values of the Anaerobic PO₄-P Release/Hac Uptake Ratio (* calculated with an ATP requirement for acetate transport of 0.5 P-mol ATP / C-mol acetate uptake, based on Smolders et al. 1994a, at pH = 7)

<table>
<thead>
<tr>
<th>Sources</th>
<th>Anaerobic PO₄-P/Hac uptake (P-mol/C-mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Theoretical Values</strong></td>
<td></td>
</tr>
<tr>
<td>Comeau/Wentzel Model</td>
<td>1</td>
</tr>
<tr>
<td>Mino Model</td>
<td>0.75</td>
</tr>
<tr>
<td>Adapted Mino Model</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Experimental Values</strong></td>
<td></td>
</tr>
<tr>
<td>Wentzel et al. (1985)</td>
<td>0.48</td>
</tr>
<tr>
<td>Mino et al. (1987)</td>
<td>0.3-0.8</td>
</tr>
<tr>
<td>Wentzel et al. (1989)</td>
<td>1.1-1.8</td>
</tr>
<tr>
<td>Cech and Hartman (1990)</td>
<td>0-0.7</td>
</tr>
<tr>
<td>Satoh et al. (1994)</td>
<td>0.04</td>
</tr>
<tr>
<td>Smolders et al. (1994a)</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>(varying pH)</td>
<td></td>
</tr>
<tr>
<td>Liu et al. (1996b)</td>
<td>0.05-1.7</td>
</tr>
<tr>
<td>(varying PO₄-P/MLSS)</td>
<td></td>
</tr>
</tbody>
</table>

Liu et al. (1996b) found that between pH 5 to 6.5, the acetate uptake rate increases linearly with pH from 0 to about 50 (mg C/g MLVSS/h) and the PO₄-P release rate from 20 to 50 (mg P/g MLVSS/h). For the pH range of 6.5-8, acetate uptake rate is constant, but the PO₄-P release rate continues to increase. When pH > 8, acetate uptake rate and PO₄-P release rate decrease. Liu et al. (1996b) also reported that when the pH is between 5.5 to 6.5, the ratio of PO₄-P/Hac is 0.7-1 and varies between 1.5 to 1.7 when the pH is between 6.6 to 8.5. They concluded that the acidic pH inactivated the acetate metabolism, while the basic pH stimulated too much PO₄-P release which resulted in an increase in energy consumption for acetate uptake. They recommended that the optimum pH for EBPR process was pH = 6.8 ± 0.7.
Both Smolders et al. (1994a) and Liu et al. (1996b) proposed the same thermal-dynamic model to explain the pH dependence of the anaerobic PO₄-P release and/or the acetate uptake. Harold (1986) indicated that most substrate transport through the cell membrane occurs against an electron-chemical potential gradient, thus requiring energy coupling. This energy is provided by ATP. Accordingly, Smolders et al. (1994a) indicated that the transport energy ($\Delta G^{\circ'}$) required to move one mole of acetate against the electric potential difference ($\Delta \psi$) across the membrane and against its concentration gradient ($C_{in}/C_{out}$) can be represented by:

$$\Delta G^{\circ'} = n \Delta \psi + 2.3 \, n \, R \, T \, \log \left( \frac{C_{in}}{C_{out}} \right)$$

Eq. 2-2

where $\Delta G^{\circ'}$: acetate transport energy (kJ/mol),
$\Delta \psi$: electric potential difference of cell membrane (kJ/mol),
n: the charge of the transported acetate per mole,
$C_{in}$: the internal concentration of acetate (mol/L),
$C_{out}$: the bulk concentration of acetate (mol/L),
R: gas constant (kJ/mol · K),
T: temperature (K).

The basic bioenergetics equation that describes the contribution of the membrane potential and the difference between the pH inside the cell (pH$_{in}$) and the pH of the medium (pH$_{out}$) to the proton motive forces is as follows (Smolders et al., 1994a, Liu et al., 1996b):

$$\Delta \psi = \Delta P + 2.3 \, R \, T \, (\text{pH}_{in} - \text{pH}_{out})$$

Eq. 2-3

where $\Delta P$ = proton motive force (kJ/mol),
pH$_{in/out}$: intracellular pH value; pH value of the bulk liquid.

Smolders et al. (1994a) and Liu et al. (1996b) concluded the acetate uptake by combining Eqs. 2-2 and 2-3, which yields:
\[ \Delta G^0 = n (\Delta P + 2.3 R T \Delta pH) + 2.3 n R T \log \frac{C_{in}}{C_{out}} \]  

Eq. 2-4

With the assumption that pH\text{in} and ΔpH of a cell are kept constant (Harold, 1986), Eq. 2-4 supports the experimental results of Smolders et al. (1994a) that there is an increase in the PO\textsubscript{4}-P/Hac ratio with pH in the range 5.5-8.5. As the external pH increases, the contribution of the ΔpH (pH\textsubscript{in} − pH\textsubscript{out}) term to the proton motive force decreases. To compensate for this effect, the membrane potential (Δψ) must increase at the same time. Therefore, the energetic cost for the transport of acetate across cell membrane increases.

However, the results of Liu et al. (1996b) showed that pH dependence for the PO\textsubscript{4}-P/Hac ratio occurred in the range 6.5-8.5. In the pH range 5.5-6.4, the PO\textsubscript{4}-P/Hac stayed at a constant level and below pH 5.5, the ratio greatly increased. Liu et al. (1996b) explained that bio-P bacteria could not maintain constant Δp and P\textsubscript{in} beyond a certain pH range.

2.4.5 Denitrification in EBPR

Grady et al. (1999) noted four basic conditions for the growth of all microorganisms: (1) carbon, (2) inorganic nutrients, (3) energy, and (4) reducing power. Microorganisms can derive energy and reducing power from oxidation reactions, which involve the removal of electrons from the substrate with their ultimate transfer to the terminal electron acceptors. In wastewater treatment processes, two kinds of terminal electron acceptors, O\textsubscript{2} and NO\textsubscript{3}-N, are generally utilized by microorganisms for energy derivation purposes. When NO\textsubscript{3}-N works as the electron acceptor, the treatment environment is called an anoxic condition and the treatment process is called denitrification.

Conflicting evidence exists in the literature regarding the fate of nitrate in the EBPR system. Wentzel et al. (1989a, 1989b) reported very low nitrate removal in the continuous flow systems (Bardenpho and UCT configurations) comprised mainly of bio-P bacteria. Their batch tests simulated the anoxic conditions with bio-P cultures and indicated the rate of denitrification was much lower compared to the denitrification rate.
by other heterotrophic organisms from mixed culture systems treating municipal wastewater. Wentzel et al. (1989a, 1989b) concluded that only a small fraction of the bio-P bacteria have the capacity for denitrification and therefore denitrification was excluded from their EBPR mathematical model. Other researchers reported different observations from Wentzel et al. (1989a, 1989b). Malnou et al. (1984) operated a laboratory scale EBPR system. They observed PO₄-P uptake in the anoxic zone. Also, when the nitrogen content of the raw wastewater was high, nitrate was recycled with the settled sludge to the anaerobic zone and effective reduction of NO₃-N occurred.

Vlekke et al. (1988) used a SBR to investigate the possibility of using nitrate as the sole electron acceptor by bio-P bacteria. The results indicated that the bio-P bacteria could oxidize PHA with NO₃-N as the electron acceptor. Vlekke et al. (1988) also noted that when the PO₄-P uptake in both systems was approximately the same, the total amount of PHA consumed was about 50% larger in the nitrate system than in the system supplied with oxygen. The difference led them to surmise that nitrate possibly is not as efficient as oxygen for PO₄-P uptake since more of the stored carbon was utilized for a given amount of P in the anoxic condition.

Kuba et al. (1993, 1994) studied EBPR in anaerobic-aerobic and anaerobic-anoxic SBR systems. Both systems achieved virtually complete phosphorus removal. However, they noted that when nitrate was the electron acceptor, the amount of P taken up per mole of electrons transferred was 20-30% lower than with oxygen as the electron acceptor. Comeau et al. (1987a) observed the release of PO₄-P was proportional to the amount of acetate added by the end of the anaerobic period in their SBR experiments, as well as the accumulation of PHB. With the addition of nitrate, uptake of P was observed until NO₃-N completely disappeared in the system. A decrease of PHB was also observed. Gerber et al. (1986), in their anaerobic batch experiments, observed that PO₄-P release in the presence of nitrate occurred upon the addition of acetate and propionate. The addition of the substrates resulted in a rapid release of PO₄-P and a simultaneous rapid
consumption of nitrate and substrate; the rates of release dropped abruptly once the substrate was consumed. In another study, Gerber et al. (1987) compared PO₄-P uptake/release patterns in aerobic and anoxic batch experiments that received acetate at varying times. The results indicated that the addition of acetate leads to P release while acetate is present regardless of whether oxygen or nitrate is available.

Jesperesen and Henze (1993) proposed that bio-P bacteria could be divided into two groups, one group only utilizing oxygen as oxidant, and another group capable of utilizing both oxygen and nitrate as oxidant. P uptake was more rapid under aerobic than anoxic conditions, because both bacterial groups worked in the aerobic condition.

Chuang et al. (1996) investigated the behavior of simultaneous P release and denitrification under anoxic conditions using P enriched sludge. They reported that the available organic substrate determines the kinetic behavior of P release/Hac uptake and denitrification. The simultaneous P release and denitrification demonstrated a kinetic competition under anoxic conditions. When substrate was abundant, bio-P sludge would release P; nitrate appearance could inhibit P release if substrate was limited.

Barker and Dold (1996) suggested that bio-P bacteria are capable of denitrification. However, they thought that not all bio-P bacteria have this ability. Nitrate may not be as efficient as oxygen for P uptake; P-Uptake/PHB-Oxidation appeared to occur simultaneously with P-Release/PHB-Storage when SCFAs are available under anoxic conditions.

### 2.4.6 Temperature Effect on EBPR

One of the most influential factors affecting microbial growth is temperature (Gaudy, 1978). Microorganisms possess no means of controlling internal temperature. The temperature within the cell is determined by the external environment. Each type of microorganism is able to grow only within a specific range of temperatures. Microorganisms only capable of growing over a restricted temperature range are called *stenothermal* (<10 °C); those capable of growing over a wide range (30-40 °C) are
called *eurythermal* (Gaudy, 1978). The optimum temperatures are defined as the temperatures at which a specific kind of microorganism can most rapidly proliferate itself. Based on the optimum growth temperature, microorganisms are also classified as *psychrophiles* (<20 °C), *mesophiles* (20-45 °C), or *thermophiles* (>45 °C). Since the EBPR process in wastewater treatment plants may experience a sewage temperature as low as 5 °C, or as high as 30 °C, it is vital to study the impact of temperature on EBPR performance.

There are several publications reporting the effect of temperature on the efficiency of EBPR systems with inconsistent results. Shapiro et al. (1967) reported that anaerobic PO₄-P release rates decreased by 2.1 to 2.6 times for every 10 °C temperature decrease in the range of 10 to 30 °C. Boughton et al. (1971) found 24 to 37 °C to be the optimum range for aerobic PO₄-P uptake. Spatzierer et al. (1985) observed a decrease of P removal efficiency in a full scale EBPR plant at temperatures below 10 °C. Sell et al. (1981) reported that P removal efficiency was 40% higher at 5 °C than at 15 °C. Barnard (1985) and Kang (1985) reported excellent (90%) P removal at 9 °C in EBPR plants at Kelowna, Canada, and Pontiac, United States.

Brdjanovic et al. (1997) studied the short-term temperature effect on the stoichiometry and kinetics of the EBPR system. Bio-P bacteria enriched in a SBR at 20 °C were used to study the biological phosphorus removal at 5, 10, 20, and 30 °C in separate batch tests. They found that the stoichiometry of the anaerobic processes was insensitive to temperature change. However, temperature had a strong influence on the kinetics of the processes under anaerobic as well as aerobic conditions. The anaerobic PO₄-P release/aerobic PO₄-P uptake ratio showed a maximum at 20 °C. A continuous increase in kinetic rates was observed in the interval of 5 to 30 °C under aerobic conditions.

Recently Baetens (1999) studied the long-term temperature changes on the stoichiometry and kinetics of different reactions involved in the EBPR process. At 5 °C, breakthrough of acetate into the aerobic period occurred. It was shown that the stoichiometry of the anaerobic processes was insensitive to long-term temperature
changes, whereas the kinetics of both the aerobic and anaerobic processes was clearly affected. The aerobic phosphorus uptake rate showed a maximum in the interval between 15 to 20 °C. All other anaerobic and aerobic conversion rates increased with increasing temperature.

Choi (1998) studied the temperature effects on the whole BNR system. They reported that 90% of nitrification was possible at temperature as low as 8 °C; PO₄-P removal was not affected in the BNR reactor, however, denitrification failed at this temperature.

Helmer and Kunst (1998) studied the temperature effect with a continuous flow pilot plant. They reported that at temperatures between 15 to 20 °C, the uptake of P in the aerobic reactor was correlated directly with the quantity of PO₄-P release in the anaerobic zone. A drop of temperature to 10 °C and below had no significant influence on the efficiency of the EBPR.

2.4.7 EBPR Microorganisms
One of the biggest mysteries of enhanced biological phosphorus removal is the identification of the microorganisms most responsible for the P removal. The correct identification of these microorganisms is still to be achieved. Due to the obvious behaviors of the EBPR system (anaerobic PO₄-P release and PHA accumulation from absorbed acetate; aerobic PO₄-P removal and PHA utilization as an internal substrate), efforts have been made to isolate pure cultures which can demonstrate EBPR mechanisms under anaerobic/aerobic cycling systems.

Fuhs and Chen (1975) first identified *Acinetobacter spp.* as the bacterium most responsible for EBPR because it was observed that *Acinetobacter spp.* could uptake PO₄-P and accumulate PHB under the aerobic condition. The presence of *Acinetobacter spp.* in significant concentrations was positively established subsequently by many other researchers who reported its predominance in the EBPR processes based on culture dependent identification methods, such as the Analytical Profile Index method (API method, Buchan, 1983; Lotter, 1985; Wentzel *et al.*, 1988). Lotter *et al.* (1986)
identified isolates from three lab-scale activated sludge systems. 40% of the isolates from a single-stage aerobic system were *Acinetobacter spp.*; 60% of the isolates from an A/O system were *Acinetobacter spp.*; and 90% of the isolates from a Modified Bardenpho system were *Acinetobacter spp.* Species of the genus *Acinetobacter* have been widely studied and have been advanced as the primary microorganisms most responsible for EBPR. *Acinetobacter* is in the *Moraxella* group, which is generally gram negative, nonmotile, catalase positive, non-flagellated short rods or cocci (Juni, 1978; Stanier et al., 1986).

However, Jenkins and Tandoi (1991) reported that the characteristic anaerobic PO$_4$-P release/acetate uptake associated with the EBPR metabolism has never been observed in a pure culture of *Acinetobacter spp.* Auling et al. (1991) failed to detect *Acinetobacter spp.* in an EBPR plant using a polyamine, diaminopropane (DAP) biomarker for *Acinetobacter* identification.

A fluorescent antibody staining technique for *Acinetobacter* (Cloete and Steyn, 1987) revealed that the number of *Acinetobacter* in the EBPR processes studied was less than 10% of the total bacteria. Also, application of a 16s-rRNA targeted oligonucleotide probe specific for *Acinetobacter spp.* showed that *Acinetobacter* was less than 10% of the total bacteria and was not dominant in the EBPR processes (Wagner et al., 1994; Bond et al., 1995; Kampfer et al., 1996; Mino et al., 1998). Mino et al. (1998) proposed that *Acinetobacter spp.* should not be considered as the principle organisms responsible for the EBPR process.

Many researchers have tried to find other potential bacteria responsible for EBPR. Many pure cultures have been isolated from the EBPR processes, such as *Pseudomonas* and *Aeromononas* (Brodisch and Joyner, 1983; Buchan, 1983), *Citrobacter* (Brodisch and Joyner, 1983; Brodish, 1985; Florentz and Hartemann, 1984a), *Flavobacterium* (Cloete et al., 1985; Meganck, et al., 1985), *Xanthobacter* and *Moraxella* (Streichan et al., 1990). But none of them have exhibited all the characteristics which the EBPR sludge should posses (Mino et al., 1998).
The brochure of Saskatoon Wastewater Treatment Plant Introduction reported that amoeboids were the first microorganisms appeared in the system after the BNR process started up, then flagellates replaced the amoeboids as the dominant protozoa. Along with the increase in bacterial numbers, free-swimming ciliates appeared. Finally, stalked ciliates appeared, indicating a stable process. It is also indicated that if the number of stalked ciliates significantly drops, the process performance deteriorates.

Nakamura et al. (1991, 1995) isolated a polyphosphate accumulating bacterium from a lab-scale EBPR process and named it *Microlumatus phosphovorus* strain NM-1. This bacterium followed most of the EBPR mechanisms, except for the requirement of glucose and casamino acids instead of acetate under the anaerobic condition.

Stante et al. (1996) successfully isolated a PHB storage strain from a SBR system and identified it as *Lampropedia*, which has the capability to take up acetate and store it as PHA under the anaerobic condition. Even though this bacterium functionally behaved as bio-P bacteria, Mino et al. (1998) did not concur with its importance because morphologically this bacterium has a very unique sheet like cell arrangement which is not commonly observed in the EBPR process.

In this thesis, the non-restrictive term bio-P bacteria will be used to describe the unknown bacteria responsible for polyphosphate accumulation in the EBPR process.

2.5 Mathematical Models of EBPR

Computer modeling of a system provides an obvious means for predicting behavior patterns. In recent years, modeling has had a significant impact on the development, design and operational procedures for the activated sludge system. In full-scale plant operations, mathematical modeling is also used as an optimization tool, for assessing the effects of changes in waste flows and loads, changes in operational procedures (such as in recycle flow), and proposed design modifications to the plant (Barker and Dold, 1997a).
There are many metabolic reactions involved into an EBPR process, which is not completely understood even today. The design of the EBPR process relies more on empirical experience obtained from the early years of EBPR development. After extensive research on EBPR processes, many theoretical models (including the three most recognized conceptual models discussed in Section 2.3.2) have been proposed to explain the observed phenomena from EBPR systems. These models only gave general outlines of the EBPR internal mechanisms, the quantification of the mass conversion rate and stoichiometric relations were still unresolved. However, the demand for designing more reliable and robust EBPR processes has propelled researchers to develop quantitative models of the EBPR process. Many mathematical models have been published for EBPR systems since the late of 1980s. Among all published works, there are three widely recognized models (Fillipe and Daigger, 1998): the Wentzel model (Wentzel et al., 1988, 1989a, 1989b), the Activated Sludge Model No.2 (ASM2, by IAWPRC Task Group, Henze, et al., 1995), and the Smolders model (Smolders et al., 1994a, 1994b, 1995).

2.5.1 The Wentzel Mechanistic Model

Wentzel et al. (1988, 1989a, 1989b) developed a mechanistic model that describes a mixed culture biological treatment system, which is categorized into three groups of organisms: heterotrophic organisms able to accumulate polyphosphate, bio-P bacteria; heterotrophic organisms unable to accumulate polyphosphate, non-bio-P bacteria; and autotrophic organisms mediating nitrification, autotrophs. Wentzel et al recognized that the development of an activated sludge kinetic model would require inclusion of all three-microorganism groups and their interactions. With regards to non bio-P and autotrophic organisms, they accepted the general model described earlier by Dold et al. (1980) and Henze et al. (1987). The Wentzel model was extended to incorporate bio-P bacteria behavior in order to enable the model to predict the EBPR mechanism, explained by the proposed Comeau/Wentzel conceptual model. From attempts to obtain information on the kinetic and stoichiometric characteristics of the bio-P bacteria using mixed liquors from the activated sludge system, Wentzel et al. (1988) concluded that the non-bio-P bacteria behavior tends to dominate and mask the bio-P bacteria behavior.
Accordingly, they endeavored to isolate the bio-P bacteria characteristics by developing an enhanced bio-P culture (a culture in which the growth of bio-P bacteria is favored to the extent that they become the dominant primary organism and their behavior dominates the system response). The procedures they applied to develop enhanced bio-P culture were the selection of acetate as the major substrate and the cycling of anaerobic/aerobic processes to create the conditions most favorable for the growth of bio-P bacteria (Wentzel et al., 1988).

In the Wentzel model, twelve compounds associated with thirteen processes are identified as essential to describe an activated sludge system with EBPR. This model assumes that acetates like SCFAs are the dominant substrates that can be used by bio-P bacteria. SCFA generation is assumed to be from the fermentation conversion of other readily biodegradable COD by non-bio-P bacteria in the anaerobic condition. Beyond this relation, the Wentzel model assumes that bio-P bacteria and non-bio-P bacteria act independently. The model of Wentzel et al. (1988, 1989a, 1989b) has been validated using only the measurements of acetate uptake, phosphate release and uptake and oxygen consumption. The dynamics of the storage compounds and the active biomass have not been part of their experimental investigation.

2.5.2 The ASM2 Mechanistic Model
Activated sludge systems become more complex as their function is expanded from carbonaceous removal alone to include nitrification, denitrification and biological phosphorus removal. The number of biological reactions and the number of compounds involved in the process also increase correspondingly. The organization of IAWPRC (Henze et al., 1994) upgraded its Activated Sludge Model 1(ASM1) to incorporate the EBPR process. Three major kinds of processes are described: the carbonaceous removal by non-bio-P bacteria, the enhanced P removal by bio-P bacteria and the nitrogen removal by autotrophic nitrifiers. This model also predicts the substrate hydrolysis processes under different environmental conditions.
All the components in the ASM2 model are divided into two groups: nine soluble components and eight particulate components. Seventeen processes associated with seventeen rate expressions are utilized to express the biological nutrients (C, N, P) removal under different design conditions. The ASM2 model is a mechanistic model, which requires the derivation of all the stoichiometric and kinetic information from each different application. As with the Wentzel model, the ASM2 model only includes PHB and polyphosphate as the internal reserves, which mediates the EBPR process.

2.5.3 The Smolders Metabolic Model

Of all three models, the greatest promise for accurate predictions is offered by the Smolders metabolic model. This is because the stoichiometry for this model was based entirely on fundamental biochemical principles (Fillipe and Daigger, 1998). The Smolders model (Smolders, et al., 1994a, 1994b, 1995), based on the Mino conceptual model, handles the dynamics of important internal reserves applying major biochemical pathways such as the EMP pathway, the ED pathway, the TCA cycle, and the glyoxylate cycle. It is expected that process stoichiometry calculations using such fundamental principles can be applied to a wider set of operating conditions compared to “empirically” derived stoichiometry.

The Smolders metabolic model assumes that glycogen is the source of reduction power. It also considers the pH effect on the energy requirement for the transport of acetate. From the results published (Smolders et al., 1994a, 1994b), this model satisfactorily explains the stoichiometry of PO₄-P, PHB, CO₂, glycogen, polyphosphate, acetate and/or O₂ during the anaerobic/aerobic conditions of the EBPR process. Due to the metabolic characteristics of the Smolders model, the processes and kinetic expressions are much more concise compared with other mechanistic models. This model includes ten compounds in a series of four aerobic and two anaerobic “reactions”. The Smolders model relies heavily on the direct sampling of the key metabolic components such as PHB, glycogen and CO₂ during the anaerobic and aerobic conditions to derive its kinetic parameters and to verify its stoichiometric and kinetic results.
Table 2.4 summarizes the important features of the above three EBPR mathematical models.

### Table 2.4. Comparison of the Three Important EBPR Mathematical Models

<table>
<thead>
<tr>
<th>Model Categorization</th>
<th>The Wentzel Model</th>
<th>The ASM2 Model</th>
<th>The Smolders Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical Basis</td>
<td>The Comeau/Wentzel Model</td>
<td>The Comeau/Wentzel Model</td>
<td>The Mino Model</td>
</tr>
<tr>
<td>Application Level</td>
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<td>Full-scale Continuous</td>
<td>Bench-scale SBR</td>
</tr>
<tr>
<td>Modeling</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Organic Substrate Removal by Non-bio-P Bacteria</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nitrification</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Denitrification by Bio-P Bacteria</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Derivation of the Stoichiometric Coefficients</td>
<td>Individual Experiment</td>
<td>Individual Experiment</td>
<td>Biochemical Theory</td>
</tr>
<tr>
<td>Internal Reserves Predicted</td>
<td>PHB, Polyphosphate</td>
<td>PHB, Polyphosphate</td>
<td>PHB, glycogen, polyphosphate</td>
</tr>
<tr>
<td>Total Kinetic Processes Used in Model</td>
<td>13</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Total Compounds Predicted</td>
<td>12</td>
<td>17</td>
<td>10</td>
</tr>
</tbody>
</table>

### 2.5.4 Other EBPR Mathematical Models

Based on the above three important EBPR mathematical models, many other EBPR mathematical models have been developed during more recent years. Barker and Dold (1997a, 1997b) presented a general model which they applied to simulate the metabolism of different bacterial populations in full-scale continuous systems. A single
set of kinetic and stoichiometric parameters were used under different operating scenarios. This model includes the simulation of the denitrification process by assuming that a fraction of the bio-P bacteria conduct denitrification.

Pramanik et al. (1998), in order to understand how C, energy and redox potential are channeled through the metabolic pathways in each treatment stage, developed a metabolic flux model that contained a complete set of pathways involved in the biomass metabolism. The model accounts for the energy requirements of macromolecule synthesis and of metabolite transport across the cell membrane. The equations for the 163 reversible and 166 irreversible reactions were solved using linear optimization. Data from a lab-scale SBR performing the EBPR process were used as the model inputs.

Fillipe and Daigger (1998) revised the rate expressions of the Smolders model so that they could simulate a full-scale, continuous EBPR system.

Chang (1997) applied Artificial Neural Networks (ANN) for modeling the EBPR process. A simplified EBPR process model and a Neural Network model (residual model) were used for developing a dynamic hybrid model. Both the simplified process model and the residual model receive influent COD, TKN, PO₄-P and NH₄ data and time signals as input. The output of the simplified process model provides a preliminary prediction of the dynamic behavior. The output of the trained neural network model is then used to compensate for the output errors. The final prediction is obtained by summing the results from the two parallel models.

2.6 Role of Glucose in EBPR
All above models refer to situations where SCFAs (typically acetate) are the sole kinds of external substrates utilized by bio-P bacteria. However, readily biodegradable substrates in domestic wastewater usually contain very low concentrations of SCFAs (Carucci et al., 1994). The effects of substrates other than SCFAs on an EBPR process have not been extensively studied before. Data from full-scale EBPR plants often show high variability in PO₄-P removal efficiencies due to the variation of organic substrates
in wastewater. Glucose is believed by many researchers to have a significant impact on
the EBPR process (Satoh et al., 1994; Cech and Hartman, 1990, 1993; Liu et al., 1994;
Tracy and Flammino, 1987; Nakamura et al., 1991, 1995; Liu, 1998, Carucci et al.,
1994, 1995, 1997, 1999). Glucose, C₆H₁₂O₆, is the most important simple carbohydrate
that exists commonly in wastewaters. Glucose plays an essential role in the central
biochemical pathways, such as the EMP and the ED pathways. Therefore, many
researchers have incorporated glucose when studying the fate of carbon substrates in
biological wastewater treatment (Gaudy, 1978). Furthermore, it has been realized,
(Mino et al., 1987), that glycogen is an important internal reserve that acts actively in
bio-P bacteria. Since glucose is the monometric unit of the polymer glycogen, the study
of glucose appearance in the EBPR system becomes more important.

However, contradictory information currently exists regarding the role of glucose in an
EBPR process. Wentzel et al. (1991) reported that bio-P bacteria seemed to be unable to
generate PHB from glucose directly under anaerobic conditions. They proposed that
glucose was converted into SCFAs by the non bio-P bacteria via the Embden-Meyerhof-
Parnas pathway, but without any conclusive confirmation.

Cech and Hartmann (1990, 1993) found that the removal of phosphorus deteriorated
gradually after feeding mixed substrates of acetic acid and glucose into a bench scale
anaerobic-aerobic reactor. Meanwhile, a second reactor fed only with acetic acid
provided excellent PO₄-P removal consistently. Therefore, Cech and Hartmann related
the breakdown of the EBPR process to the appearance of glucose in the influent, which
induced the predominance of other kinds of bacteria, which they named “G” bacteria.

Cech and Hartmann (1993) reported that “G” bacteria usually appear in tetrad or
aggregates like Methanosarcina spp., 2-3 μm in size, non-motile, and Neisser positive
(whole cell, blue). “G” bacteria were found to be unable to accumulate polyphosphate in
the aerobic condition and PHB in the anaerobic condition, but they could accumulate
glycogen upon the uptake of an organic substrate. Mino et al. (1994) and Liu et al.
(1995) termed this kind of bacteria as glycogen accumulating organisms (GAO). Matsuo
(1994) also observed that bio-P sludge from lab-scale anaerobic/aerobic experiments experienced deterioration after glucose was placed in the feed and the system was dominated by two different types of "G" bacteria.

Cech and Hartman (1990, 1993) proposed that, when P removal efficiency deteriorated, the glycogen accumulating bacteria succeeded in the competition for organic substrate against the bio-P bacteria during the anaerobic condition. They suggested that the addition of glucose into a good P removal reactor cultivated with acetate could induce the predominance of glycogen accumulating bacteria.

The microbial metabolism in the activated sludge system of a failed EBPR removal process was investigated by Satoh et al. (1994) to clarify the cause of failure. It was found that the dominant microorganisms consume internal carbohydrates during the uptake process of acetate or propionate under the anaerobic condition. Little anaerobic PO₄-P release was observed (0.04 P-mol/C-mol). The sludge seemed to be dependent on glycogen but not on polyphosphate as the energy source under the anaerobic condition. Satoh et al. explained that polyphosphate is chemically more unstable than glycogen. Glycogen may be preferred to polyphosphate by microorganisms because of its stability. They proposed that polyphosphate is the source of energy and P for bacteria, glycogen is the source of energy and carbon. Occasionally, the lack of available carbon under anaerobic conditions may induce glycogen accumulation if a surplus of carbon exists in the environment. They also surmised that if the influent contains glucose, glycogen would be preferred as the energy source in the system, which caused the deterioration the EBPR process. Liu et al. (1996a) also reported that glucose could be taken up by glycogen accumulating bacteria and converted to PHA without the utilization of polyphosphate.

However, some researchers proposed or observed enhanced phosphorus removal even using glucose as the major or sole substrate in their EBPR experiments. Tracy and Flammino (1987) proposed that PO₄-P could be released by polyphosphate hydrolysis once glucose is added. The absorbed glucose would be used to accumulate glycogen
which is metabolized through the EMP pathway in the anaerobic condition and the TCA cycle in the aerobic condition.

As already discussed, Nakamura et al. (1991, 1994) isolated a novel bacterium, strain NM-1 from the genus Micrococcus, which could accumulate high amounts of polyphosphate when fed only with glucose. Carucci et al. (1994, 1995), after 6 months of process studies, obtained some EBPR performance using a sequential batch reactor (SBR) and glucose as the major substrate. Liu (1998) also observed some EBPR performance in his SBR experiments using glucose as the major organic substrate. But in both Carucci’s and Liu’s experiments, PHB measurements undertaken at the end of each test did not reveal the presence of intracellular granular inclusions. Also, Carucci and Liu could not stabilize the EBPR performance in their SBRs. The PO₄-P removal efficiency gradually diminished even though the operating procedures were not changed during the experiments.
CHAPTER 3 MATERIALS AND METHODS

3.1 Reactor Configurations
The experimental study was carried out in two parallel bench-scale sequential batch reactors (SBR), in which different environmental conditions (anaerobic, anoxic and aerobic) could be obtained in time rather than in space sequence. This unsteady state system allowed investigation of the time profiles of several controlled components in order to acquire an improved understanding of the process.

One SBR was fed with acetate as the dominant substrate and was defined as reactor A; the other SBR was fed with glucose as the dominant substrate and was defined as reactor G. Each of the two bioreactors had a working volume of 1.5 L. The two reactors were equipped with magnetic stirrer, thermometer, DO probe, pH probe and sampling port. Diffusers were installed to transfer air or nitrogen gas into the reactors. The system was completely automated by utilizing six electrical timers with specific settings. The schematic design of one of the SBRs is shown in Figure 3.1.

3.2 Sequential Batch Reactor Operating Procedures
The two bench-scale SBRs were inoculated identically with the mixed cultures collected from the BNR reactors in the City of Saskatoon Wastewater Treatment Plant, Canada. This full-scale BNR reactor exhibits excellent EBPR performance consistently.

The operating conditions of the two bench-scale sequential reactors (reactors A and G) were set initially identical and are summarized in Table 3.1. Both reactors were operated at room temperature (around 25 °C). pH meters were used to monitor the variation of pH values during operation. Any pH fluctuations from neutral values in the reactors were adjusted back to pH neutral (pH 7.0) by adding HCl (0.5 N) or NaOH (0.5 N). 62.5 mL of the mixed liquor suspended solids was discarded at the end of each aerobic reaction
to maintain sludge retention time (SRT) of 8 days and mixed liquid suspended solid level around 2500 mg/L in the reactors. 750 mL of the liquid (including the 62.5 mL of the wasted mixed liquor suspended solids) was withdrawn from the reactor at the end of each cycle of running, which resulted in a hydraulic retention time (HRT) of 16 hours. The anaerobic environment was created by purging nitrogen gas into the reactors 15 minutes before the start of a new cycle until the first 15 minutes of the anaerobic reaction. During the aerobic phase, air was continuously bubbled to maintain the oxygen demand in the reactors. Other operating conditions were as specified in Table 3.1.

Except for the dominant organic substrate supplied in the influents, the compositions of the feeds for reactors A and G were exactly the same, which was adapted from Smolders et al. (1994a) and is shown in Table 3.2. The strength of PO₄-P in the influent was 15 mg/L for both reactors. The same strength of organic carbon (measured as COD) was supplied for both reactor A (500 mg/L of sodium acetate) and reactor G (375 mg/L of glucose).

Figure 3.1. Schematic Design of Bench Scale SBR System
Table 3.1. Operating Conditions of Reactors A and G

<table>
<thead>
<tr>
<th>Temperature</th>
<th>SRT</th>
<th>Stirring Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ± 1 °C</td>
<td>8 d</td>
<td>200 rpm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>HRT</th>
<th>Air/N₂ Supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 ± 0.1</td>
<td>16 hr</td>
<td>0.2 L/min</td>
</tr>
</tbody>
</table>

Table 3.2. Composition of Synthetic Wastewater for Reactors A and G

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/L)</th>
<th>Compound</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose / Acetate</td>
<td>375 / 500</td>
<td>H₃BO₃</td>
<td>0.045</td>
</tr>
<tr>
<td>N (NH₄Cl)</td>
<td>28</td>
<td>Cu (CuSO₄·5H₂O)</td>
<td>0.002</td>
</tr>
<tr>
<td>P (NaH₂PO₄·2H₂O)</td>
<td>15</td>
<td>Mo (NaMoO₄·2H₂O)</td>
<td>0.008</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1</td>
<td>I (KI)</td>
<td>0.04</td>
</tr>
<tr>
<td>Mg (MgSO₄·7H₂O)</td>
<td>9</td>
<td>Mn (MnCl₂·4H₂O)</td>
<td>0.004</td>
</tr>
<tr>
<td>Ca (CaCl₂·2H₂O)</td>
<td>3.8</td>
<td>Zn (ZnSO₄·7H₂O)</td>
<td>0.008</td>
</tr>
<tr>
<td>K (KCl)</td>
<td>19</td>
<td>Co (CoCl₂·6H₂O)</td>
<td>0.01</td>
</tr>
<tr>
<td>EDTA</td>
<td>3</td>
<td>Fe (FeCl₃·6H₂O)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The operating cycles for both SBRs A and G were also set identical at first and are shown in Figure 3.2. Each cycle was eight hours long and consisted of seven phases: influent filling, anaerobic reaction, aerobic reaction, mixed liquor discarding, settling, supernatant withdrawing and idling.

Figure 3.2. Original Operation Cycle for Reactors A and G
3.3 Experimental Methods

Various experiments were conducted to investigate the characteristics of the cultivated bacteria from reactors A and G. During each of these experiments, sampling and chemical analyses were carried out according to different designed procedures. The experimental methods for the conducted experiments are described as follows.

- *The development of EBPR performance in reactors A and G:*
  During the initial phase of the SBR experiments, endeavors were exerted to develop stable EBPR performances in both reactors A and G, especially to explore the optimum operating procedures to maintain stable EBPR performance by applying glucose as the dominant substrate in reactor G. The operating conditions were originally set the same for both reactors A and G except the different organic substrates in the influents. Various operating conditions were tested in order to induce stable EBPR performances after the original operating conditions failed to induce good P removal in reactor G. The PO₄-P level was examined at the end of the anaerobic and aerobic conditions. PHA, glycogen, MLSS/MLVSS were tested during these experiments as well.

- *Batch experiments for the bacterial metabolism in both reactors A and G*
  After stable EBPR performances were achieved in both reactors A and G, various batch experiments were conducted to study the bio-kinetic behavior of the microorganisms from both reactors. Four types of batch experiments were designed for three different purposes.

  The first type of batch experiment was designed to compare the enhanced P removal mechanisms when either acetate or glucose was added as the single organic substrate into either reactor A or G. Replicate batch experiments were conducted using the normal operating procedures for stable EBPR performances in both reactors A and G. The initial PO₄-P concentration was 7.5 mg/L (the PO₄-P concentration in the medium of reactor A or G at the start of a batch reaction from the added NaH₂PO₄·2H₂O). For both reactors A and G, the initial organic substrate concentration was 250 mg/L (the acetate or glucose concentration in the medium of reactor A or G at the start of a batch reaction.
from the added sodium acetate or glucose). Other nutrients were prepared according to Table 3.2.

The second type of batch experiment was conducted to investigate the system responses when different levels of PO₄₋P appeared in the influent and the maximum PO₄₋P removal potential of reactor G. Other operating conditions were kept exactly the same as the first type of batch experiments, except for the varying initial PO₄₋P concentrations applied in reactor G. Five levels of initial PO₄₋P (0, 2.5, 7.5, 15, 30 mg/L) concentrations were tested while the initial concentration of glucose was kept at 250 mg/L.

The third type of batch experiment was conducted to investigate the system responses when different levels of organic substrate appeared in the influent, and the maximum PO₄₋P release potential under the anaerobic condition of reactor G. The collected data from this type of batch experiment in reactor G were also used for the bio-kinetic study to develop an EBPR mathematical model. The operating conditions were kept exactly the same as in the first and second type of batch experiments, except for the varying initial concentrations of glucose applied in reactor G. Five levels of initial glucose (0, 100, 250, 500, 1000 mg/L) concentrations were tested while the initial strength of PO₄₋P was kept at 7.5 mg/L.

The fourth type of batch experiment was conducted to investigate anaerobic lactic acid release after stable EBPR performance was achieved in reactor G. During the batch experiments, glucose was the single organic substrate added with an initial concentration of 250 mg/L. The lactic acid measurements were conducted at designed intervals throughout the experiments to check if part of the added glucose was fermented to lactic acid during the anaerobic condition. pH, lactic acid, glucose and total organic carbon changes in the medium were examined during these experiments.

All batch experiments were conducted directly in the two sequential batch reactors. The settled sludge from the end of a SBR operation cycle was washed twice with distilled
water to remove the residual chemicals from the previous cycle of operation. Then, in order for an absolute anaerobic environment, the reactor was purged with nitrogen gas for half an hour before the start of the batch experiment to dissipate the remaining external electron acceptors (such as $O_2$) in the medium.

Nutrients and a single organic substrate (sodium acetate or glucose) were immediately added at the start of each of the batch experiments. Phosphate release and absorption, organic substrate in the medium, PHA and glycogen content in the cells, and mixed liquor suspended solids (MLSS)/mixed liquor volatile suspended solids (MLVSS), were analyzed at the designed intervals during each of the batch experiments. Ten minutes before the start of a batch experiment, duplicate mixed liquor suspended solid samples were collected for the evaluation of the initial concentrations of the MLSS/MLVSS, glycogen and PHA. After five minutes of the anaerobic reactions, duplicate mixed liquor samples were taken for the evaluation of the immediate changes of these controlled components after the instant addition of the nutrients. Then, the samples were taken every half hour (or one hour) until the end of a batch experiment. The samples taken for MLSS/MLVSS evaluation were analyzed immediately according to the analytical procedures described in the following section. The samples taken for soluble component evaluations (including PO$_4$-P, glucose, acetate) were filtered through membrane filter paper (Sartorius, pore size 0.45 μm) immediately after the sampling and then stored in a freezer (set at -25 °C) together with other mixed liquor suspended solid samples (for PHA, glycogen analysis) right after the sampling and/or pre-treatment. All of the samples were analyzed within three days after being taken.

- **SBR experiments with controlled pH values in reactor G**

After stable EBPR performance was achieved in reactor G, the effect of different pH values on EBPR performance was studied in reactor G. The normal operating procedures for reactor G were applied for the experiments. Three specific pH values (pH = 6, 7 and 8) were maintained throughout the whole cycles of the batch experiments to investigate the system responses in reactor G. PO$_4$-P changes were monitored throughout the cycles of the batch reactions in this series of experiments.
• **Denitrification potential study in reactor G**

After stable EBPR was achieved in reactor G, the potential denitrification capability was studied for the bacteria cultured in reactor G. During this SBR experiment, all other operating conditions were kept as normal operating conditions for reactor G, except Nitrate (in the form of KNO₃) was added during the middle of the anaerobic condition in reactor G to create a potential anoxic environment. This experiment was performed to investigate if the bio-P bacteria in reactor G can reduce NO₃-N for energy derivation.

• **Microorganism identifications**

After both reactors A and G demonstrated good EBPR performance, mixed liquor suspended solids were collected and sent to Norwest Lab (in Edmonton, Canada) to identify the predominant microorganisms from both reactors A and G. The mixed liquors collected from reactors A and G (about 50 mL) were stored in two sample bottles and properly sealed. The bottles were then properly marked of their corresponding sources (reactor A or G) and placed in a compact cooler with ice. This sample package was promptly delivered to Norwest Lab for examination.

• **PHA identification test**

After good EBPR performance was achieved in reactor G, GC-MS was used to separate and identify the major components of PHA extracted from the cells collected from reactor G.

### 3.4 Analytical Methods

#### 3.4.1 Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS)

The determinations of MLSS and MLVSS were based on the Standard Methods for the Examination of Water and Wastewater (A.P.H.A., 1992). In this test, glass fiber filter paper (Whatman, 934-AH™, circle 47 mm φ, Cat. No. 1827 047, purchased from VWR, Canada), was first pretreated. The glass fiber filter paper (with wrinkled side up) is inserted into a filtration apparatus. A vacuum is applied and the filter paper is washed with distilled water. The filter paper is then removed from the filtration apparatus and
transferred to a holder. The holder with the filter paper is then dried in an oven for 1 hour at 105 °C, cooled in a desiccator to reach the room temperature. The cooled holder with glass fiber filter paper is weighed. The glass fiber filter paper is reinstalled on the filtration apparatus. 10 mL of the completely mixed suspended solid liquor from a reactor is slowly placed onto the filter paper and the vacuum is applied for about 1-2 minutes. The filter paper is then carefully removed from the filtration apparatus and transferred to the same holder and is dried for at least 1 hour at 105 °C. The cooled holder and the filter paper are then weighed again. Finally, the holder and the filter paper are transferred to a furnace at 550 °C for 15 minutes. After cooling down to room temperature, the holder with the residual ash is weighed once more.

The MLSS and MLVSS of the sample are calculated according to the following equations:

\[
MLSS (mg/L) = \frac{1000mL \cdot (A - B)}{1L \cdot V_{sample}} = \frac{1000mL \cdot (A - B)}{1L \cdot 10mL}
\]

Eq. 3-1

\[
MLVSS (mg/L) = \frac{1000mL \cdot (A - C)}{1L \cdot V_{sample}} = \frac{1000mL \cdot (A - C)}{1L \cdot 10mL}
\]

Eq. 3-2

Where, 
A (mg) = weight of fiber filter paper + holder before ignition at 550 °C,
B (mg) = initial weight of fiber filter paper + holder,
C (mg) = weight of ash + holder after the ignition at 550 °C,
\( V_{sample} \) (mL) = the volume of mixed liquor sample taken (10 mL).

3.4.2 PO₄-P in Medium and Total Phosphorus Content of Bacteria

PO₄-P in medium and total phosphorus of bacteria were also determined by Stannous Chloride method based on the Standard Methods for the Examination of Water and Wastewater (A.P.H.A., 1992).

The determination of PO₄-P starts with the preliminary preparation of membrane filter papers (Sartorius, pore size 0.45 μm, circle 47 mm \( \phi \), Charge No. 082 2000674251 from
VWR, Canada). The membrane filter papers are washed by soaking them in distilled water overnight before use to avoid contamination of phosphorus from the membrane filters during the filtration process. 5 mL of mixed liquor suspended solids from a reactor is taken and filtered through the pretreated membrane filter paper using the filtration apparatus. A certain amount of the filtered sample is transferred to an Erlenmeyer flask and diluted to 100 mL with distilled water. 4.0 mL ammonium molybdate reagent and 0.5 mL stannous chloride reagent are then added, with thorough mixing after each addition. 10 minutes are allowed for the color development. The developed blue color is measured using a spectrophotometer (Spectronic 20D+, Spectronic Instruments) and the transmittance value of the sample is recorded at 690 nm. The result is compared with a calibration curve to get the matched phosphorus concentration value. The PO$_4$-P concentration in the reactor is calculated by multiplying the dilution ratio (100 mL divided by the applied filtered sample volume).

Total phosphorus content in the bacteria is analyzed by pipetting 5 mL mixed liquor suspended solids into a centrifuge tube. Then, 10 mL 0.85% (w/v) NaCl solution is added to the tube to wash the bacteria. The solids and the liquid are separated after centrifugation (10,000×g for 15 minutes at 4 °C) and the supernatant is decanted. Another 10 mL of 0.85 % (w/v) NaCl solution is added into the tube and the sample is centrifuged again. The supernatant is decanted and the bacteria are re-suspended with distilled water. The re-suspended bacteria are stirred with a magnetic stirrer to mix the sample thoroughly. The volume of the re-suspended bacteria is adjusted to 5 mL with distilled water. 1 mL of the re-suspended bacteria is pipetted into an Erlenmeyer flask and the sample volume is adjusted to 50 mL with distilled water. 1 drop of phenolphthalein indicator solution is added. If a red color develops, H$_2$SO$_4$ solution is added drop wise to just remove the color. Then 1 mL H$_2$SO$_4$ (30%, v/w) solution and 0.4 g solid (NH$_4$)$_2$S$_2$O$_8$ are added and the sample is boiled gently on a preheated hot plate for 45 minutes. The sample is then cooled down to room temperature. 1 drop of phenolphthalein indicator solution is then added into the sample which is neutralize to a faint pink color with 1.5 N NaOH. The sample volume is adjust to 100 mL again and is determined for the dissolved PO$_4$-P by the Stannous Chloride method described above.
The resulting PO₄-P values correspond to the total phosphorus content from the mixed liquor suspended solid sample.

3.4.3 Acetate in Medium

Acetate in medium was analyzed by diethyl ether extraction and gas chromatography (GC) (Manni, 1995). A mixed liquor suspended solid sample is filtered through 0.45 μm membrane filters. 2 mL of the filtrate and 2 mL of diethyl ether are then mixed in a sealed vial; 100 μL of 75% H₂SO₄ is then added into the vial to acidify the sample. The sample is then shaken for 10 minutes and placed into an ice-water bath for 10 minutes to separate ether and water phases by settling. The supernatant ether phase (containing acetate) is used for analysis using a Varian 3400 GC, a capillary column (DB-FFAP from J & W Sci., 15 m length, 0.53 mm ID, and 1 μm thickness), and a FID detector. The temperature program for the GC was: initial temperature at 70 °C for 3 minutes; ramp to 120 °C at 10 °C/min; then to 180 °C at 35 °C/min; remaining at this final temperature for 1 minute. The detector and injector temperatures were set at 250 °C and 210 °C, respectively.

3.4.4 PHA of Bacteria

The analytical method for poly-β-hydroxyalkanoate (PHA) was adapted from Braunegg (1978) and Gerhardt (1994). 5 mL of the mixed liquor suspended solid sample is collected and washed by adding 10 mL of 0.85% NaCl solution. This solution is then centrifuged at 10,000×g for 15 minutes at 4 °C. The supernatant is then decanted and replaced with 5 mL of 5.25% sodium hypochlorite. The sample is incubated at 37 °C for 1 hour and then is centrifuged again at 10,000×g for another 15 minutes at 4 °C. After discarding the supernatant, the precipitate at the bottom of the centrifuge tube is suspended by adding 2 mL of acidified methanol (3% H₂SO₄) and 2 mL chloroform. The mixture is transferred into a boiling tube and sealed tightly. The tube is heated for 3.5 hours at 100 °C, cooled to room temperature, and 1 mL of distilled water is added. After sealing, the tube is shaken for 10 minutes, followed by 10 minute settling to allow complete separation of the water phase from the chloroform phase. The bottom chloroform phase (containing hydrolyzed PHA) is used for the GC test. Two kinds of
PHA were identified in this research: poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were calibrated by DL-β-hydroxybutyric acid (SIGMA) and 2-hydroxyvaleric acid (SIGMA) respectively in the GC analysis. The same GC, capillary column and FID detector were applied as for the acetate analysis. The temperature program was: initial temperature at 60 °C for 1 minute and ramp to 105 °C at 8 °C/min and then to 180 °C at 35 °C/min; hold for 1 minute.

3.4.5 Glucose in Medium
Glucose in the medium was analyzed by the phenol method (Gerhardt, 1994). The sample is filtered through 0.45 μm membrane filters and 1 mL of the filtrate is placed into a vial. 1 mL of phenol (5% w/v) and 5 mL of concentrated H₂SO₄ are then added into the vial. The treated sample was measured for its absorbency (Spectronic 20D+, Spectronic Instruments) at 488 nm after the vial returned to room temperature.

3.4.6 Glycogen of Bacteria
Glycogen was analyzed by an alkali and ethanol extraction method adapted from Gerhardt (1994). 5 mL of the mixed liquor suspended solid sample is collected and washed by adding 10 mL of 0.85% NaCl solution. The mixture is then centrifuged at 10,000×g for 15 minutes at 4 °C. The supernatant is then decanted and 2 mL of 50% KOH is added into the vial, which is held at 100 °C for 1 hour. After cooling, 10 mL of 60% ethanol (stored at 4 °C) is added. The sample is centrifuged again at 10,000×g for 15 minutes at 4 °C and the supernatant is decanted. 2 mL of HCl (6M) is added into the vial which is held at 100 °C for 3 hours to hydrolyze glycogen to glucose. The hydrolyzed sample is then analyzed for glucose by the phenol method.

3.4.7 Lactic Acid in Medium
Lactic acid in medium was analyzed by applying the Lactate Diagnostic Reagent (SIGMA, 735-10). In this test, lactic acid is converted to pyruvate and hydrogen peroxide (H₂O₂) by lactate oxidase. In the presence of the H₂O₂ formed, peroxidase catalyzes the oxidative condensation of chromogen precursors to produce a colored dye with absorption maximum at 540 nm. The increase in absorbance at 540 nm is directly
proportional to the lactate concentration in a sample. The mixed liquor suspended solid sample is filtered through 0.45 μm membrane fibre filter and pipette 30 μL of the filtrate solution into a test tube; add 3 mL of the Lactate Diagnostic Reagent into the test tube. The tube is incubated for 7 minutes and then the absorbance by the spectrophotometer (Spectronic 20D+, Spectronic Instruments) at 540 nm is recorded.

3.4.8 Total Organic Carbon (TOC) in Medium
TOC in medium was analyzed using a TOC Analyzer (Beckman, Model 915) which can provide rapid analysis of a micro-sample of aqueous solution for determination of total organic carbon. The analysis is based on the conversion of the sample carbon into carbon dioxide which is measured by a non-dispersive infrared analyzer.

3.4.9 Nitrate in Medium
Nitrate analysis was adapted from the Standard Methods for the Examination of Water and Wastewater (A.P.H.A., 1992). This method relies on the conversion of nitrate to nitrite by hydrazine in an alkaline solution. Nitrite is then measured through the formation of a reddish purple azo dye produced at pH 2.0 by coupling diazotized sulfanilamide with N-(1-naphthyl)-ethylenediamine dihydrochloride. Measure the absorbency of this sample by the spectrophotometer (Spectronic 20D+, Spectronic Instruments) at 543 nm.
CHAPTER 4 EXPERIMENTAL RESULTS AND DISCUSSION

4.1 The Development of the EBPR Performance Using Acetate or Glucose as the Dominant Substrate

The bacterial seed material collected from the City of Saskatoon Wastewater Treatment Plant was dispensed evenly into reactors A and G. Efforts were first exerted to investigate the feasibility and swiftness of developing EBPR performance in reactors A and G individually during this stage of the research. Significant differences were observed between reactors A and G during the development of the EBPR performance, which is described as follows and shown in Figure 4.1.

![Graph showing the development of EBPR performance over time](image)

Figure 4.1. Development of EBPR Performances in Reactors A and G
• **First Stage**

At the start, identical operating conditions were applied for the operations of reactors A and G. For the first 5 to 7 days, reactors A and G both exhibited very poor PO₄-P removal efficiency. The influents contained 15 mg/L PO₄-P for both reactors. However, PO₄-P levels in the effluents were as high as 11-12 mg/L, which resulted in a removal efficiency of only 20-25% in both reactors. The low removal efficiency could be attributed to the slow bacterial acclimation to the new nutrient source and operational environment. The little PO₄-P removals during the aerobic conditions from the media were probably due to the basic metabolic requirements for bacterial growth, not the production of polyphosphate.

• **Second Stage**

In the second stage, it was considered to cut the strength of the organic substrates and the PO₄-P in the influents for both reactors A and G to help the bacteria adapt to the new operation environments. Therefore, the influents for both reactors were diluted to half of their original strength while all other operating procedures were kept the same. Reactors A and G behaved differently in removing PO₄-P after only two cycles of operation. The effluent PO₄-P in reactor A decreased to 1-2 mg/L with removal efficiency up to 90%. However, reactor G still showed little EBPR with a PO₄-P removal efficiency of only 20-30%.

• **Third Stage**

After the second stage, the nutrient strength for both reactors was returned to the levels used in the first stage. Reactor A maintained excellent EBPR performance with PO₄-P removal efficiency approaching almost 100%. Reactor G still exhibited poor PO₄-P removal. Because successful EBPR was consistently performed by reactor A since then, the operating conditions for reactor A were maintained afterwards. It was further considered that there might be some nutrient deficiency prohibiting the EBPR performance in reactor G. 50 mg/L of peptone was added into the influent. Concentrations of K⁺, Mg⁺⁺ and Ca⁺⁺ were also increased to 26, 15, and 15 mg/L, respectively, to provide sufficient metal cations in reactor G. From Figure 4.1 it can be
seen that the PO$_4$-P removal efficiency really improved from 40% to 50% in reactor G after these changes of nutrients, which proved the effectiveness of those newly added nutrients. However, the enhanced P removal as that performed by reactor A still could not be established in reactor G.

- **Fourth Stage**
Since acetate had proven to be a more effective organic substrate to induce EBPR than glucose, it was decided to begin feeding acetate instead of glucose into reactor G to induce EBPR performance, and then to increase gradually the glucose level in the influent. After switching the substrate from glucose to acetate, reactor G immediately produced the same level of PO$_4$-P removal as reactor A, which indicated that significant bio-P bacteria were already present in reactor G. A special feeding plan was then applied for reactor G: increasing gradually the glucose content (10% as COD) and decreasing the acetate content (10% as COD) in the influent every two cycles. The system maintained good PO$_4$-P removal efficiency until glucose accounted for more than 50% of the carbon substrate (as COD) in the influent. Effluent PO$_4$-P level went up to 7-9 mg/L with PO$_4$-P removal efficiency still only around 50% in reactor G.

- **Fifth Stage**
To further explore the feasibility of inducing EBPR behavior by glucose, the glucose level in the feed was changed back to the original strength for reactor G (375 mg/L of glucose in the influents). The system continued to exhibit only a medium level of PO$_4$-P removal efficiency (50-60%). However, during one cycle of the continuous SBR operation, more than 200 mL of the mixed liquor instead of 62.5 mL was wasted from the reactor at the end of the aerobic condition. Surprisingly, the PO$_4$-P level at the end of the next cycle of operation dropped to 0.3 mg/L in the effluent, which exhibited excellent enhanced phosphorus removal by reactor G. The system maintained this high level of PO$_4$-P removal for about one week.
4.2 Deterioration and Recovery of EBPR in Reactor G

The initial phase of experiment confirmed the feasibility of the induction of EBPR performance applying glucose as the dominant substrate, even though the development was much slower and more complex than the system using acetate. However, excellent PO₄-P removal lasted only for one week in reactor G before PO₄-P level in the effluent gradually increased back to 5-7 mg/L even though the operating procedures were maintained the same. Efforts were then turned to understanding the reasons of the quick deterioration of the EBPR performance and how the successful EBPR performance could be recovered and maintained in reactor G.

During the many investigations it was observed that poor EBPR performance was always associated with poor PO₄-P release during the anaerobically condition. These results are concluded from Figure 4.2 which shows the relation between anaerobic released PO₄-P and PO₄-P removal efficiency in reactor G. The anaerobic release of PO₄-P was calculated from the difference between the initial PO₄-P concentration and the maximum PO₄-P concentration during the anaerobic condition. It is evident that high PO₄-P removal efficiency corresponds closely to high anaerobic PO₄-P release. The data from Figure 4.2 indicate that 90% PO₄-P removal efficiency (15 mg/L of PO₄-P in the influent) is associated with at least about 13 to 15 mg/L of the anaerobic PO₄-P release. If the anaerobic PO₄-P release fell below 10 mg/L, the enhanced biological removal of PO₄-P deteriorated.

Examinations of the PHA level during the deterioration of PO₄-P removal efficiency show that less PHA was accumulated in the anaerobic phases than when the system demonstrating good EBPR performance in reactor G even with the same level of glucose in the influents. It has been proposed that PHA is likely the only available energy source for the bio-P bacteria to synthesize polyphosphate during the aerobic condition, therefore, the deterioration of EBPR in reactor G is probably also associated with the reduced anaerobic accumulation of PHA.
Good PO₄-P release and PHA accumulation during the anaerobic phase seemed to be critical for the success of enhanced PO₄-P removal during the aerobic condition. In order to stimulate higher PO₄-P release and PHA accumulation, three new operating procedures were applied for the SBR operation of reactor G: longer anaerobic reaction time (from 2 to 2.5 hours), higher glucose in the influent (from 375 to 500 mg/L) and shorter aerobic reaction time (from 4 to 3.5 hours) with limited DO level (dissolved oxygen in medium < 3 mg/L). Extended anaerobic reaction time was expected to create a longer stressful environment to stimulate more PO₄-P release from the bacteria; higher glucose content in the influent was also expected to induce more PO₄-P release through the glucose uptake and provide more organic carbon for the synthesis of PHA; and the shortened aerobic condition and lower DO level were expected to reduce the consumption of PHA during the aerobic condition. Successful EBPR performance was immediately established after the application of all these modified operating procedures (Table 4.1). The system continued to exhibit very stable EBPR performance under these new operating conditions. The modified operating procedures came to be the normal operating procedures for reactor G to maintain successful EBPR performance for the subsequent batch experiments.

**Figure 4.2. Anaerobic Released PO₄-P and PO₄-P Removal Efficiency during the Development of EBPR in Reactor G**
Table 4.1. The Original and Modified Operating Procedures for Reactor G

<table>
<thead>
<tr>
<th></th>
<th>Anaerobic Reaction Time (hr)</th>
<th>Aerobic Reaction Time (hr)</th>
<th>Aerobic DO level (mg/L)</th>
<th>Nutrients in the Influent (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Original</strong></td>
<td>2</td>
<td>4</td>
<td>5-6</td>
<td>Glucose 375 K⁺ 19 Mg²⁺ 9 Ca²⁺ 3.8 Peptone 0</td>
</tr>
<tr>
<td><strong>Modified</strong></td>
<td>2.5</td>
<td>3.5</td>
<td>2-3</td>
<td>500 K⁺ 26 Mg²⁺ 15 Ca²⁺ 15 Peptone 50</td>
</tr>
</tbody>
</table>

4.3 Metabolic Characteristics of Reactors A and G

In order to compare the metabolic characteristics of the EBPRs produced by reactors A and G, batch experiments were conducted after stable EBPR behaviors had been established in both reactors A and G.

4.3.1 Batch Experimental Results from Reactor A with Stable EBPR Performance

Batch experiments in reactor A were conducted applying the normal SBR operating procedures, except the nutrients were added immediately at the start of the reaction. The typical results from these batch experiments in reactor A are presented in Figure 4.3. From Figure 4.3 it can be seen that sodium acetate was rapidly taken into the biomass after the anaerobic reaction started. The concentration of sodium acetate decreased from 73 C-mg/L (milligram carbon of acetate per liter) down to less than 10 C-mg/L within half an hour of the anaerobic reaction. At the same time, significant quantities of PO₄-P were released into the liquid phase, from 7.5 mg/L up to more than 50 mg/L. The maximum PO₄-P in the liquid phase was around 68 mg/L which appeared at the end of the anaerobic reaction. It is also evident that after the depletion of sodium acetate in the medium, the release of PO₄-P slowed down gradually.

During the anaerobic phase, considerable changes of two intracellular reserves - glycogen and PHA were detected. After the addition of sodium acetate, the glycogen content of the bacteria was found to decrease; however, the content of PHA in the bio-P
Figure 4.3. Profiles of (a): Sodium Acetate/PO\text{4}-P, and (b): Glycogen/PHA from Batch Experiments in Reactor A with Stable EBPR Performance (Initial concentrations of sodium acetate and PO\text{4}-P were 250 mg/L and 7.5 mg/L; error bars in this figure are based on 1 standard deviation)
bacteria was found to increase significantly at the same time. The rates of glycogen reduction and PHA accumulation seem to be related to the sodium acetate uptake rate. Before the depletion of sodium acetate, PHA was quickly accumulated. The accumulation of PHA slowed down after sodium acetate diminished in the system. The maximum PHA accumulation was around 33 C-mg/g MLSS which appeared at the end of the anaerobic condition. The bacteria seem to continuously consume glycogen, whose content was reduced from about 60 C-mg/g MLSS down to around 34 C-mg/g MLSS during the anaerobic condition.

In the aerobic phase, it is assumed that very little extracellular organic carbon existed in the system since almost all of the sodium acetate was found to be consumed before the end of the anaerobic condition. The PHA content reduced from around 33 to less than 3 C-mg/g MLSS during the aerobic reaction, which indicates that the bacteria utilized the internal reserve of PHA for the oxidative-phosphorylation reaction to supply ATP and carbon for the growth and other metabolic requirements. It was also found that the concentration of PO₄-P decreased continuously along with the consumption of PHA. At the end of the aerobic condition, it was found that almost all the PO₄-P in the medium was removed by the bacteria. The glycogen content of the bacteria was re-synthesized to its original level during the aerobic phase (around 60 C-mg/g MLSS). It was also detected by GC that the accumulated PHA was composed principally of PHB and PHV, with PHB accounting for the majority of the accumulated PHA. For example, at the end of the anaerobic condition, PHB accounted for more than 80% of the total accumulated PHA. The dynamic change of the PHA composition during the batch experiments is shown in Figure 4.4.
Figure 4.4. Profile of PHA Composition from Batch Experiments in Reactor A with Stable EBPR Performance (Initial concentrations of sodium acetate and PO₄-P were 250 mg/L and 7.5 mg/L; error bars in this figure are based on 1 standard deviation)

4.3.2 Batch Experimental Results from Reactor G with Stable EBPR Performance

After stable EBPR performance was established in reactor G, batch experiments were conducted applying the modified SBR operating procedures (also is applied as the normal operating procedures, see Table 4.1), except the nutrients were added instantly at the start of the reaction. The typical batch experimental results for reactor G are presented in Figures 4.5. From Figure 4.5(a) it can be seen that during the anaerobic phase, the PO₄-P level increased rapidly after the addition of glucose. Glucose was immediately taken up by the bacteria after its instant addition. A rapid increase of glycogen in bio-P bacteria occurred at the beginning of the anaerobic reaction, along with the instant removal of glucose. After glucose diminished in the solution, the glycogen level gradually decreased during the rest of the anaerobic reaction. The pattern for PHA change in reactor G was similar to that in reactor A. PHA increased gradually from 2 C-mg/g MLSS to 33 C-mg/g MLSS during the anaerobic condition.
Figure 4.5. Profiles of (a): Glucose/PO$_4$-P, and (b): Glycogen/PHA from Batch Experiments in Reactor G with Stable EBPR Performance (Initial concentrations of glucose and PO$_4$-P were 250 mg/L and 7.5 mg/L; error bars in this figure are based on 1 standard deviation)
However, the examination of the PHA composition by GC/MS revealed that most of the PHA accumulated in reactor G was PHV instead of PHB (Figure 4.6). It was found that PHV accounted for around 83% of the total PHA accumulated at the end of the anaerobic reaction. During the aerobic phase, there was negligible extracellular glucose for the bio-P bacteria. The PHA content decreased along with efficient PO₄-P removal from the medium and glycogen was re-synthesized in the bio-P bacteria in the aerobic condition.

![Figure 4.6. Profile of PHA Composition from Batch Experiments in Reactor G with Stable EBPR Performance](image)

Initial concentrations of glucose and PO₄-P were 250 mg/L and 7.5 mg/L; error bars in this figure are based on 1 standard deviation)

4.4 Role of Glucose in an EBPR Process

Wentzel et al. (1991) proposed that in the EBPR process, glucose was fermented and converted to SCFAs for the bio-P bacteria. However, in this experimental work, glucose was found to be rapidly taken up by bio-P bacteria after its addition and the intracellular reserves of PHA and glycogen both increased significantly at the same time. This rapid glucose uptake and the simultaneous increase of intracellular reserves imply that glucose
is transported into the bio-P bacteria and primarily stored as organic reserves after its addition. Some fermentation of glucose still might happen, because it was found that the pH value dropped after the addition of glucose. However, the immediate uptake of glucose associated with the rapid build-up of bacterial internal reserves indicates that direct uptake and storage of glucose seem to be the predominant metabolic fate of glucose under the anaerobic condition.

There is a sound biochemical principle governing the metabolism of microorganism: organisms seldom engage in metabolic activities without a specific purpose (Tracy and Flammino, 1987). In the EBPR process, PO$_4$-P is released significantly during the anaerobic reaction. This increase of PO$_4$-P is assumed to be from the hydrolysis of stored polyphosphate inside of the bio-P bacteria. The energy generated from the hydrolysis of polyphosphate is used by bio-P bacteria for different metabolic requirements during the anaerobic condition (Comeau et al., 1986; Mino et al., 1987; Smolders et al., 1994a). In order to maintain this metabolic role for polyphosphate, bio-P bacteria have to synthesize polyphosphate by taking up PO$_4$-P excessively in the aerobic phase. Therefore, high release of PO$_4$-P in the anaerobic condition usually is indicative of an excellent EBPR process.

From these experiments, it can be seen that the mechanism of PO$_4$-P release and uptake was quickly established using acetate as the dominant organic substrate in reactor A. However, it was very slow and difficult to induce this mechanism using glucose as the dominant organic substrate in reactor G. Liu (1998) found that during the deterioration of the EBPR process, the total carbohydrate content in the bacteria was much higher than when the process performed good P removal. In Mino's bio-chemical model (Mino et al., 1987), glycogen is metabolized to supply the reducing power (NADH$_2$) for the formation of PHA, as well as part of the energy requirement for other metabolic activities during the anaerobic reaction. Satoh et al. (1992) and Cech and Hartman (1993) found that the bacteria utilized glycogen instead of polyphosphate as the energy source when the system lost EBPR performance. Satoh et al.(1994) investigated the cause of a failed EBPR process and found that the dominant microorganisms consumed
internal carbohydrates during the uptake process of acetate/propionate under the anaerobic condition. They assumed that the sludge was dependent on glycogen instead of polyphosphate as the energy source under the anaerobic condition. Satoh et al. also pointed out that polyphosphate is chemically more unstable than glycogen. Therefore, glycogen appears to be able to replace the metabolic role of polyphosphate for the bacteria during the anaerobic condition. Since glucose is a monomer of glycogen, it is readily converted to glycogen when high concentration of glucose appears in the influent. It is assumed that the utilization of glucose as the major substrate has a higher probability to induce the replacement of the energy role of polyphosphate by glycogen and upset the EBPR performance. The long time poor EBPR behavior in reactor G during the development of EBPR performance agrees with this assumption. Cech and Hartman (1990, 1993) also proposed that the deterioration of EBPR performance would occur whenever glucose appeared in the influent. In addition, based on the studies of Satoh et al. (1992) and Liu et al. (1994), the replacement of the energy role of polyphosphate by glycogen could happen even in the EBPR system fed with acetate as the dominant substrate.

However, the experimental results in this research showed that the metabolic role of polyphosphate and the EBPR performance could still be induced and stabilized by using glucose as the major substrate under the modified operating procedures. Longer anaerobic reaction time, higher glucose in the influent and shorter aerobic reaction time with limited DO level proved to be effective operating procedures to induce and maintain the EBPR performance when using glucose as the major substrate.

The stress from the longer anaerobic reaction seems to be able to force the bacteria to use the energy from polyphosphate hydrolysis for metabolic requirements, which helps to enhance the energy role of polyphosphate. Liu (1998) reported that the deterioration of EBPR process was always associated with the continuous increment of glycogen level in the bacteria, which resulted in the suppression of the polyphosphate energy role. In order to prevent the replacement of polyphosphate energy role by glycogen, glycogen
content should be limited within a certain level. Extended anaerobic conditions could consume excessive glycogen and stabilize the energy role of polyphosphate.

Nakamura et al. (1991) found that the logarithmic growth phase was the best time when bacteria accumulate phosphorus from the medium. It is known that bacteria can maintain logarithmic growth when they get acclimated to the environment and enough food in the medium are available for the requirements of their growth. The application of a low DO level during the aerobic condition was applied to control the bacteria growth and maintain optimum food/biomass ratio, so that the bio-P bacteria can grow under the logarithmic phase. During the 5th stage of the EBPR development in this work (Figure 4.1), it was also found that EBPR was induced from the excessive wastage of the mixed liquor suspended solids. It is possible that the bacteria in reactor G were brought back to the logarithmic growth phase when more of the bacteria in the system were wasted, since the food/biomass ratio was improved. Another possibility of the sudden appearance of the enhanced P removal after excessive wastage of bacteria was that less glycogen in the whole system was available for the bacteria to metabolize the same level of glucose as previous cycles, and the bacteria were forced to rely on the energy role of polyphosphate.

Maintaining a high level of PHA accumulation under the anaerobic condition is another critical strategy to ensure the stability of the EBPR process. It was found that the gradual deterioration of the EBPR process in reactor G was always associated with the decrease of PHA accumulation in the anaerobic condition. Long aerobic conditions with high DO level might result in the over consumption of PHA, which could not be recovered in the subsequent anaerobic cycle (it is not clear what alternative internal reserves the bacteria might accumulate when less PHA was accumulated during the anaerobic condition). Therefore, a shorter aerobic reaction time with lower DO level may help to avoid the loss of PHA accumulation in the anaerobic condition. Meanwhile, supplying more glucose in the influent provides more carbon for the anaerobic PHA accumulation, which can lead to a more stable EBPR process. The metabolism of
increased amounts of glucose would also induce more polyphosphate hydrolysis, which may further strengthen the EBPR process.

4.5 Metabolic Differences of Enhanced Biological Phosphorus Removals in Reactors A and G

According to the batch experimental results reported above, the metabolic characteristics of the EBPR process induced by glucose are very similar to that induced by acetate, which indicates that the widely accepted biochemical model for an EBPR process induced by acetate can basically be used to explain the metabolic mechanism of an EBPR process induced by glucose. However, there are also some significant differences between the two systems. Table 4.2 shows the stoichiometric relations of PO₄-P release per absorbed carbon substrate and PHA accumulation per absorbed carbon substrate calculated from typical batch experimental results. For every C-mg of absorbed organic carbon, the bio-P bacteria released 4 times as much PO₄-P in reactor A as in reactor G. As already discussed, the release of PO₄-P in the anaerobic condition indicates the energy function of polyphosphate. It is evident that bio-P bacteria in reactor A spend much more energy derived from polyphosphate hydrolysis than the bio-P bacteria in reactor G. Part of the energy utilized in reactor G is probably derived from the glycolysis of glycogen during the anaerobic condition.

The ratio of PHA accumulated to substrate consumed also shows that, for every C-mg of absorbed carbon substrate, more carbon was accumulated as PHA in reactor A than in reactor G. The ratio of accumulated PHA to consumed substrate in reactor A is more than 1 C-mg/C-mg, which implies that some other form of carbon source has been converted to PHA also. This carbon source is probably glycogen. Mino et al. (1987) assumed that the glycolysis of glycogen in the anaerobic condition supplied the reducing power (NADH₂) and part of the required energy for the synthesis of PHA. But, this ratio also suggests that part of the glycogen may be converted to PHA during the anaerobic condition. The ratio of PHA accumulated to glucose absorbed in reactor G is only 0.75 C-mg/C-mg. This implies that not all of the carbon in glucose is used for PHA accumulation. Figure 4.5 shows a rapid glycogen increase after the addition of glucose.
in reactor G, which indicates that a large amount of glucose was converted to glycogen at the beginning of the anaerobic reaction. After the depletion of glucose, the glycogen level decreased gradually. Glycogen is probably used to supply the reducing power and carbon for the PHA accumulation during the rest of the anaerobic reaction in reactor G.

The development of EBPR in both reactors A and G indicates that the polyphosphate hydrolysis and PHA accumulation under the anaerobic condition are the critical metabolic activities the bacteria must demonstrate in order to establish a successful EBPR performance. While feeding glucose, the bio-P bacteria undergo less polyphosphate hydrolysis and accumulate less PHA than when acetate is fed as the substrate. This explains why the EBPR behavior in reactor G is much more vulnerable than in reactor A and supports that acetate is a more effective organic substrate to induce and maintain successful EBPR processes. Therefore, special operational procedures (see Table 4.1) should be carefully applied in order to sustain a successful EBPR performance when glucose is used as the major substrate.

Table 4.2. PO₄-P Release and PHA Accumulation during Anaerobic Conditions in Reactors A and G (Calculated from typical batch experiments with stable EBPR performances, initial concentrations of acetate and PO₄-P were 250 mg/L and 7.5 mg/L for reactor A; initial concentrations of glucose and PO₄-P were 250 mg/L and 7.5 mg/L for reactor G; MLSS was kept at 2500 mg/L during the batch reactions in both reactors)

<table>
<thead>
<tr>
<th></th>
<th>Reactor A</th>
<th>Reactor G</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PO₄-P Released</strong></td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
</tr>
<tr>
<td><strong>External Organic</strong></td>
<td>73</td>
<td>100</td>
</tr>
<tr>
<td>Carbon Absorbed</td>
<td>(Acetate, C-mg/L)</td>
<td>(Glucose, C-mg/L)</td>
</tr>
<tr>
<td><strong>Ratio of Released PO₄</strong></td>
<td>0.82</td>
<td>0.2</td>
</tr>
<tr>
<td>to Consumed Substrate</td>
<td>(P-mg/C-mg)</td>
<td>(P-mg/C-mg)</td>
</tr>
<tr>
<td><strong>PHA Accumulated</strong></td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>(C-mg/g MLSS)</td>
<td>(C-mg/g MLSS)</td>
<td></td>
</tr>
<tr>
<td><strong>Ratio of Accumulated</strong></td>
<td>1.1</td>
<td>0.75</td>
</tr>
<tr>
<td>PHA to Consumed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>(C-mg/C-mg)</td>
<td>(C-mg/C-mg)</td>
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It was also detected that, different from reactor A, the accumulated PHA was composed mainly of PHV instead of PHB when glucose was used as the major substrate in reactor G. Figure 4.6 shows that PHV accounted for around 83% of the total PHA accumulated at the end of the anaerobic stage in reactor G. It is known that the monometric unit of PHB is 3-hydroxybutyrate (3-HB) and the monometric unit of PHV is 3-hydroxyvalerate (shown in Table 2.2). A 3-HB unit is formed by combining two acetyl-CoA molecules, while a 3-HV unit of PHA is formed by combining one propionyl-CoA molecule and one acetyl-CoA molecule (shown in Table 2.2). Therefore, PHV enriched PHA suggests that the major metabolic pathway produces propionyl-CoA in addition to acetyl-CoA inside of the bio-P bacteria. Satoh et al. (1992) and Liu et al. (1994) assumed partial conversion of pyruvate to propionyl-CoA for PHV formation. There are two pathways known for this conversion in certain anaerobic bacteria: the succinate-propionate pathway and acrylic acid pathway (Gottschalk, 1986). It is likely that the former is functioning in the EBPR process, since the intermediates located in the succinate-propionate pathway can be readily metabolized by bio-P bacteria (Mino et al., 1998).

Satoh et al. (1992) proposed an oxidation-reduction theory to explain the accumulation of PHV during the anaerobic condition. Satoh et al. considered that the uptake of substrate and conversion to organic reserve should not disturb the redox balance in the bio-P bacteria. Mino et al. (1987) proposed that glycogen degradation through the Embden-Meyerhof-Parnas pathway would produce the reducing power (NADH₂) under the anaerobic condition. When acetate is supplied as the major substrate in an EBPR process, reducing power produced from the glycolysis of glycogen would be consumed for the synthesis of PHB enriched PHA. However, when glucose is fed as the major organic substrate in an EBPR process, extra reducing power might be generated from the glycolysis of glucose. The total reducing powers produced from the glycolysis of both glucose and glycogen exceed the requirement for PHA synthesis. These extra NADH₂ must be oxidized before the bio-P bacteria can further metabolize glucose/glycogen and accumulate PHA. Therefore, the formation of PHV is probably used to regulate the redox balance inside of the bio-P bacteria. The succinate-propionate
pathway proposed by Satoh et al. is likely used for propionyl-CoA formation, during which extra electrons generated from glucose/glycogen glycolysis are consumed to balance the internal redox.

4.6 Batch Experiments with Varying Initial Glucose Concentrations in Reactor G
In order to further investigate the mechanism of the EBPR process using glucose as the single organic substrate, a series of batch experiments were conducted in reactor G with stable EBPR performance. The operating conditions of these batch experiments were kept exactly the same as the normal operating procedures except that varying amounts of glucose were instantly added into reactor G as the single organic substrate. Five runs of batch experiments were conducted with initial glucose levels equal to 0 mg/L, 100 mg/L, 250 mg/L, 500 mg/L and 1000 mg/L, respectively. The initial concentration of PO₄-P for each of the five runs was kept at the 7.5 mg/L. The results from this series of batch tests are presented in Figures 4.7, 4.8, and 4.9.

![Graph](image_url)

**Figure 4.7. Profiles of PHA from Batch Experiments with Varying Initial Glucose Concentrations in Reactor G**

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Figure 4.8. Profiles of Glycogen from Batch Experiments with Varying Initial Glucose Concentrations in Reactor G

Figure 4.9. Profiles of PO₄-P from Batch Experiments with Varying Initial Glucose Concentrations in Reactor G
Glucose was taken up immediately after its addition into the reactor. Even with an initial glucose concentration of 1000 mg/L, the glucose was mostly taken up during the first 15 minutes of the anaerobic reaction. For the rest of the reaction time, there was almost no glucose in the medium.

Figure 4.7 shows that the PHA concentrations also exhibited rapid increases after the additions of glucose. The PHA concentrations continued to increase but the rate of increase declined until the end of the anaerobic phase. Higher concentrations of initial glucose also resulted in more accumulation of PHA inside of bio-P bacteria. After the start of the aerobic condition, PHA contents were found to be quickly utilized and were almost depleted even with an initial glucose concentration of 1000 mg/L. A small PHA increase was detected even when the initial glucose concentration was zero, probably from the conversion of the internal reserve glycogen. Figure 4.7 suggests that PHA is most likely the ultimate internal organic reserve and glycogen is converted to PHA gradually under the anaerobic phase. This is supported by the fact that the glycogen decrease in Figure 4.8 was directly associated with PHA accumulation shown in Figure 4.7.

Corresponding to the rapid removal of glucose, Figure 4.8 shows that the glycogen contents of the bio-P bacteria increased immediately after the additions of glucose. Since glucose was added instantaneously at the beginning of the anaerobic reaction of the batch experiments, the rapid increase of glycogen was directly related to the uptake of glucose by the bacteria. Figure 4.8 also shows that higher amount of added glucose resulted in more initial glycogen increase. After the immediate increase, glycogen was consumed during the rest of the anaerobic phase. Higher initial glucose concentrations resulted in more glycogen consumption as well. During the aerobic phases glycogen contents were re-synthesized. At 0 mg/L of initial glucose addition, a small consumption of glycogen was detected. However, little glycogen was re-synthesized during the following aerobic condition at this level of initial glucose addition.
The immediate increase and subsequent decrease of glycogen implies that it plays an important metabolic role in the anaerobic phase. Glucose is a monomer of glycogen, which implies that bio-P bacteria can readily take up glucose and convert it to glycogen. It seems that glycogen may not be a preferred form of organic carbon storage, since after the immediate increase, the glycogen contents were found to decrease gradually during the rest of the anaerobic reactions.

Figure 4.9 shows that PO₄-P was released into the medium during the anaerobic condition. The rate of release was faster at the beginning than in the later stage of the anaerobic phase. Higher concentration of added glucose resulted in more release of PO₄-P into the medium. At 0 mg/L of initial glucose, only 5-7 mg/L of PO₄-P was released into the medium. However, 40-45 mg/L of PO₄-P was released into the medium when the initial glucose concentration went up to 1000 mg/L. The PO₄-P levels in the medium were reduced in the following aerobic phase. Excellent removals of PO₄-P were achieved at all levels of initial glucose additions except for 0 mg/L of initial glucose in the medium. The profiles shown in Figure 4.9 suggest that PO₄-P release was closely related to glucose uptake and PHA accumulation in the anaerobic condition. Since PO₄-P release is assumed to result from polyphosphate hydrolysis for energy derivation, it can be inferred that the required energy for glucose uptake and PHA synthesis was partly supplied from polyphosphate hydrolysis. The energy role of polyphosphate is also supported by the fact that higher PO₄-P release happened when more glucose was added at the beginning of the reaction. The PO₄-P release at zero initial glucose concentration must result from energy requirements for PHA formation from glycogen.

4.7 Batch Experiments with Varying Initial Phosphorus Concentrations in Reactor G

In order to investigate the system responses when different P/C ratios appeared in the influents of reactor G with stable EBPR performance, a series of batch experiments were conducted in reactor G applying the normal operating procedures except that varying amounts of PO₄-P were added at the start of the reaction. Another five runs of batch experiments were conducted with initial PO₄-P levels equal to 0 mg/L, 2.5 mg/L,
7.5 mg/L, 15 mg/L and 30 mg/L respectively. The initial concentration of glucose (as the single organic substrate) for each of the 5 runs was kept at 250 mg/L. The results from this series of batch tests are presented in Figure 4.10 and Figure 4.11.

It can be seen from Figure 4.10 that there were significant PO₄-P releases even with 0 mg/L initial PO₄-P addition under the anaerobic conditions. The PO₄-P levels reached the maximum at the end of the anaerobic condition from every run of the batch experiments. Reductions of PO₄-P happened during the aerobic phases. PO₄-P removals were successfully achieved from the medium only in the reactions with initial PO₄-P levels from 0 to 7.5. However, when initial PO₄-P levels increased up to 15-30 mg/L, large amounts of PO₄-P still remained in the medium at the end of the operational cycles. PHA profiles shown in Figure 4.11 also indicate that the failure to remove all the remaining PO₄-P was probably due to the depletion of PHA during the aerobic conditions when initial PO₄-P was higher than 15 mg/L.

![Figure 4.10. Profiles of PO₄-P from Batch Experiments with Varying Initial PO₄-P Concentrations in Reactor G](image)
Figure 4.11. Profiles of PHA from Batch Experiments with Varying Initial PO₄-P Concentrations in Reactor G

Another finding from Figure 4.10 is that the levels of anaerobic PO₄-P release were very close from these batch experiments with different initial PO₄-P concentration. The anaerobic PO₄-P releases were all between 15 to 18 mg/L. It seems that the anaerobic PO₄-P release is determined by the initial strength of glucose instead of the initial strength of PO₄-P. The maximum PO₄-P removal ability at this level of initial organic carbon condition (with 250 mg/L initial glucose concentration in the medium) seems to be less than 10 mg/L net PO₄-P reduction from the system.

Figure 4.11 indicates that PHA profiles are very similar among the five batch experiments with different initial PO₄-P concentrations in the medium. The variations are within the ranges of experimental errors from PHA analysis. About the same amounts of PHA was accumulated during the anaerobic conditions of the five batch experiments. This implies that different strengths of PO₄-P in the influents do not affect the metabolism of PHA accumulation if the initial glucose strength is kept the same.
4.8 Denitrification Study in Reactor G

After stable EBPR performance was established in reactor G, the potential capability of denitrification by the bio-P bacteria in reactor G was investigated through inserting an anoxic phase in the original anaerobic phase of the SBR operation in reactor G. During the sequential batch reaction in reactor G, 50 mg/L of NO$_3$-N was added after 1.5 hours of the anaerobic reaction, which made an external electron acceptor (NO$_3$-N) available during the rest of the anaerobic reaction. Other operating conditions were kept the same as the normal operating conditions for reactor G. The initial glucose concentration and PO$_4$-P concentration were kept at 250 mg/L and 7.5 mg/L, respectively. The results are given in Figure 4.12.

From Figure 4.12 it can be seen that reactor G followed typical EBPR characteristics during the first 1.5 hours of the anaerobic reaction, in that the glycogen content went up after the glucose addition and was then gradually consumed, PHA content was accumulated, as well as a significant amount of PO$_4$-P was released into the medium. However, after the addition of NO$_3$-N during the middle of the anaerobic reaction, the PHA level of the bacteria and PO$_4$-P level of the medium started to decrease. During the 1.5 hours of the anoxic reaction, the NO$_3$-N level in the medium was significantly reduced from 50 mg/L at the start to about 20 mg/L at the end of the anoxic condition. However, glycogen was still consumed during the anoxic condition. After the aerobic condition started, the NO$_3$-N level increased gradually from about 20 mg/L to about 38 mg/L in the medium at the end of the aerobic condition, probably from the nitrification by the bacteria. The glycogen content was still consumed during the first half an hour of the aerobic condition and then it was re-synthesized during the rest of the aerobic condition. It was also found that PHA was almost completely depleted by oxidation during the aerobic condition. However, PO$_4$-P was not completely removed from the medium at the end of the aerobic reaction.
Figure 4.12. SBR System Responses with NO$_3$-N Addition during the Anaerobic Condition in Reactor G” (the NO$_3$-N change in this figure includes NO$_2$-N and NO$_3$-N)

From the above observations, we can see that bio-P bacteria from reactor G have the potential capability of denitrification. After the addition of NO$_3$-N during the anaerobic phase, the PHA level of the bacteria and PO$_4$-P level of the medium dropped simultaneously along with the reduction of added NO$_3$-N. This can be explained by the mechanism of the denitrification reaction. The bio-P bacteria in reactor G were probably able to utilize NO$_3$-N as the external electron acceptor to oxidize the internal organic PHA for energy production. At the same time, PO$_4$-P removal was probably due to the bio-P bacteria growth and the synthesis of polyphosphate. The significant decrease of NO$_3$-N probably resulted from the reduction of NO$_3$-N to N$_2$ gas which would be discharged by the system. However, another organic reserve, glycogen, was not found to be re-synthesized during the anoxic condition. Instead, the glycogen content decreased throughout the anoxic reaction. This is probably because glycogen could not be formed with the absence of O$_2$ as the electron acceptor.
During the following aerobic condition, it was found that PO$_4$-P in the medium was not as completely removed as when NO$_3$-N had not been added in the anaerobic condition, even though all the other operating conditions were exactly the same. Many other researchers (see Section 2.4.5) reported that NO$_3$-N was not as efficient as O$_2$ for enhanced biological phosphorus removal. This is because, during the anoxic condition, more PHA content was consumed for the amount of PO$_4$-P removal compared to the case when O$_2$ appeared as the sole external electron acceptor. This resulted in less PHA being available for enhanced P removal during the following aerobic condition, and much of the PO$_4$-P still remained in the medium at the end of the SBR cycle.

4.9 Lactate Release Study in Reactor G

During the SBR operation, it was always observed that after the addition of glucose in reactor G, the pH value dropped to between 5 and 6 from an initial value of 7. It was suspected that some organic acids were generated from the glucose fermentation. Total organic carbon (TOC) and glucose changes in the medium were monitored during one cycle of SBR operation in reactor G with glucose as the single added organic substrate. Figure 4.13 shows that there is a surplus of TOC over the organic carbon from glucose in the medium after the addition of glucose during the anaerobic reaction, which indicates that some other form(s) of organic carbon existed in the medium. The TOC surplus over glucose gradually diminished during the rest of the anaerobic reaction. However, GC analysis did not find any significant amounts of acetate in the medium during the anaerobic condition. Lactate analysis was then conducted to detect if any lactic acid was present when feeding glucose into the reactor. Figure 4.14 shows that lactic acid was released into the medium after the addition of glucose and was taken up by the bacteria during the rest of the anaerobic reaction. Lactate detection from the medium during the anaerobic condition revealed that, besides the direct glucose conversion to the internal organic reserves, part of the glucose was fermented to lactic acid at the same time. However, it was also found that the loss of glucose from the fermentation was soon recovered during the rest of the anaerobic reaction through the uptake of the fermentation product lactate by the bacteria. It is likely that the absorbed lactate is also used for PHA formation by bio-P bacteria afterwards.
Figure 4.13. Profiles of TOC and Glucose from SBR Operation in Reactor G with Initial Glucose Concentration = 250 mg/L

Figure 4.14. Profiles of Lactic Acid and pH in the Medium during the SBR Operation in Reactor G with Initial Glucose Concentration = 250 mg/L
4.10 pH Effect on the EBPR Process in Reactor G

The pH effect on the EBPR performance in reactor G was investigated by conducting three SBR experiments with different maintained pH values throughout the complete cycles of the reaction. Except for the predetermined pH value, all other operating conditions were the normal SBR operating conditions for reactor G. Three constant pH levels (pH = 6.0, 7.0, or 8.0) were maintained for each of the three experiments by adding HCl (0.5 N) or NaOH (0.5 N) into the reactor during these reactions.

From Figure 4.15 it can be seen that there was a significant effect of pH on the EBPR process in reactor G. Higher pH caused more anaerobic PO₄-P release (around 32 mg/L at pH = 8); while, lower pH caused less anaerobic PO₄-P release (around 12 mg/L at pH = 6). However, it is also evident that the best PO₄-P removal happened when the pH value is around 7 during its aerobic condition. pH values at either 6 or 8 resulted in incomplete PO₄-P removal during the aerobic reaction phases.

![Figure 4.15. Profiles of PO₄-P from the Batch Experiments with Different Maintained pH values during the Reactions in Reactor G (Initial glucose and PO₄-P concentrations were 250 mg/L and 7.5 mg/L)](image)
The observed phenomena corresponded well with the pH effect studied by Smolders et al. (1994a) and Liu et al. (1996b) in acetate induced EBPR systems. Smolders et al. (1994a) found that the PO₄-P release rate increased linearly with pH in the range 5.5 to 8.2. Both Smolders et al. and Liu et al. applied bioenergetics theory to explain this pH dependence of anaerobic PO₄-P release which has been discussed in Section 2.4.4. According to Smolders et al. and Liu et al., bacteria need to spend energy to maintain the constant proton motive force during the transport of organic substrate across the cell membrane. The energy source for organic substrate uptake is assumed to be from the polyphosphate hydrolysis. At higher pH, more energy needs to be spent to maintain the constant proton motive force for the transport of organic substrate. Extra PO₄-P is therefore released from polyphosphate degradation at higher pH values.

Figure 4.15 shows that excellent P removal was only realized at pH around 7, which suggests the importance of maintaining pH at 7 during the EBPR reaction in reactor G. The lower pH value caused by glucose fermentation to lactate is especially detrimental to the EBPR process, because it was found in the early experiments that a pH lower than 7 could result in the breakdown of the EBPR performance in reactor G, which was not recovered after many cycles of operation. This is probably because the bio-P bacteria lost the polyphosphate energy role in the acidic environment due to the reduced energy requirement from polyphosphate hydrolysis. The basic environment can induce more polyphosphate utilization, however, it seems that there is not enough energy for the bio-P bacteria to uptake all the excessively released PO₄-P under the aerobic condition. Therefore, the most appropriate pH environment for EBPR process using glucose as the major substrate is near pH 7.

4.11 Identifications of the Predominant Microorganism for EBPR in Reactors A and G

Bacteria samples were collected when both reactors A and G achieved excellent EBPR performance. Two mixed liquor samples (50 mL) collected from reactors A and G were sent to Norwest Lab (Edmonton, Canada) for analysis immediately after the samplings.
The results presented in Table 4.3 reveal that the compositions of the predominant bacteria from both reactors are similar. *Aeromonas hydrophilia* is the species most predominant in both reactor A (80%) and reactor G (90%). *Acinetobacter calcoaceticus* is the second predominant species in both reactors.

As discussed in Section 2.4.7, *Acinetobacter* used to be considered the species most responsible for the EBPR process after its first identification by Fuhs and Chen (1975). However, its role in the EBPR process has been questioned by many other researchers (discussed in Section 2.4.7) since 1990. The microorganism identification in this work identified *Aeromonas hydrophilia* to be the most predominant species in both reactors A and G. *Acinetobacter calcoaceticus* was found to be the second largest group of bacteria in both reactors.

*Aeromonas hydrophilia*, according to Bergey’s Manual of Determinative Bacteriology (Robert, et al., 1957), is rod shaped, gram negative, occurring singly and in chains. It is aerobic and facultative with optimum growth temperature at around 37 °C. *Aeromonas hydrophilia* is also pathogenic for frogs, fish and human beings. *Aeromonas hydrophilia* has also been identified by many researchers (Brodisch and Joyner, 1983; Buchan, 1983; Meganck, 1987) from different EBPR processes. However, it is hard to ascertain it as the primary bacteria most responsible for the EBPR mechanism, because the pure culture of *Aeromonas hydrophilia* did not possess all the typical EBPR characteristics observed from stable EBPR processes (Mino et al., 1998). It is reasoned that the EBPR mechanism in reactors A and G is not performed by one kind of bacteria. It is probable that a few groups of bacteria work together to fulfill the enhanced removal of PO_4-P in the medium and *Aeromonas hydrophilia* is the group that plays more important function than other groups of the bacteria in an EBPR process.

The most important finding from the microorganism identification study is that similar bacterial compositions were detected in both reactors, even though different dominant organic substrates were fed into the reactors for about three months after the start of SBR operations. It seems that, in an EBPR system, the composition of primary
microorganism(s) might not be greatly affected by the organic substrate whenever the EBPR performance is established.

Table 4.3. Predominant Bacteria Identification Report (by Norwest Lab)

<table>
<thead>
<tr>
<th>Lab #</th>
<th>Date</th>
<th>Sample Description</th>
<th>Results: (Genus Level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>235716-1</td>
<td>Sept. 15 1999</td>
<td>G Bacteria Collected from Tank Fed Glucose</td>
<td>1) <em>Aeromonas hydrophilia</em> (90%) 2) <em>Acinetobacter calcoaceticus</em></td>
</tr>
<tr>
<td>235716-2</td>
<td>Sept. 15 1999</td>
<td>A Bacteria Collected from Tank Fed Acetate</td>
<td>1) <em>Aeromonas hydrophilia</em> (80%) 2) <em>Acinetobacter calcoaceticus</em> 3) <em>Pseudomonas maltophilia</em></td>
</tr>
</tbody>
</table>

4.12 Deterioration of EBPR in Reactor G

This series of experiments were conducted during the deterioration of the EBPR performance in reactor G. The purposes are to compare the different characteristics of the bacteria when they demonstrate good EBPR performance and when they lose their EBPR performance, and to grasp the internal mechanism inducing the deterioration of the EBPR in reactor G. Figures 4.16 to 4.18 show the results from SBR experiments during both the poor and good EBPR conditions in reactor G.

From Figure 4.16 it can be seen that, glucose was still immediately removed from the medium after its addition under anaerobic condition even when the system lost the EBPR performance. However, the PO₄-P release was much weaker during the poor EBPR condition than the PO₄-P release during good EBPR performance in reactor G. Instead of 20-22 mg/L of anaerobic PO₄-P release during the good EBPR condition in reactor G, the maximum anaerobic PO₄-P release only reached 7-8 mg/L under the poor EBPR performance.

From Figure 4.17 and Figure 4.18 it can be seen that the internal reserves of PHA and glycogen have different characteristics between the processes with EBPR and without EBPR performances. The anaerobic accumulation of PHA was 70% lower in the poor
EBPR process than in the good EBPR process. Bacteria seem to contain higher levels of glycogen during the poor EBPR process than during the good EBPR process. As in the process with good EBPR, glycogen was first synthesized after the addition of glucose and then its level decreased during the anaerobic condition with poor EBPR in reactor G. However, it is not known what other organic reserve(s) glycogen was converted to during the anaerobic condition, since not as much PHA was accumulated during the anaerobic condition in reactor G with poor EBPR performance.

It can be inferred from these observations that when reactor G lost EBPR ability, the bacteria were still able to metabolize glucose effectively. During the anaerobic condition, the absorbed glucose seems to be converted to glycogen as with good EBPR performance. However, the mechanisms of polyphosphate hydrolysis and PHA accumulation from glycogen are not very effective anymore. Much less anaerobic PHA accumulation and lower PO₄-P release are probably the reasons causing the deterioration of the EBPR performance. It also seems that higher levels of glycogen can be accumulated by the bacteria. During the poor EBPR condition in reactor G, bacteria seem to utilize more glycogen instead of polyphosphate as the energy source under the anaerobic condition. However, there are still many uncertainties about the bacterial mechanisms of reactor G during the poor EBPR performance, such as the mechanisms of anaerobic glycogen conversion and aerobic glycogen re-synthesis, which require further investigation.
Figure 4.16. Profiles of Glucose and $\text{PO}_4$-$P$ from Batch Experiments in Reactor G with/without EBPR Performance (Initial concentrations of glucose and $\text{PO}_4$-$P = 250 \text{ mg/L}$ and $7.5 \text{ mg/L}$)

Figure 4.17. Profiles of Glycogen from SBR Experiments in Reactor G with/without EBPR Performance (Initial Glucose and $\text{PO}_4$-$P$ Concentrations = 250 $\text{ mg/L}$ and 7.5 $\text{ mg/L}$)
4.13 Experimental Uncertainty Analysis

It is recognized that many chemical analyses are compromised by errors no matter how well they are planned and conducted. There are many factors affecting the accuracy and precision of chemical analysis, which includes the instrument conditions, skills of personnel, ambient conditions, imperfections of the sampling procedures, etc. In this research, the uncertainty of the analytical procedures was evaluated by conducting replicate independent analyses of the controlled components. Samples were taken from a SBR using the same procedures as for the formal batch experiments. To check the reproductivity of the analytical results, 5 replicate samples were analyzed by the same analytical methods described in Section 3.4. For the soluble components (including acetate, glucose, PO₄-P and lactic acid), the five replicate samples were obtained from the filtered solutions sampled from a SBR. For the insoluble components (including glycogen, PHB, PHV, total phosphorus, MLSS/MLVSS), the five replicate samples were sampled directly from a SBR at the same moment. To further evaluate the extraction methods of the internal reserves PHA and glycogen, standard DL-β-
hydroxybutyric acid (H-6501, ordered from SIGMA), 2-hydroxyvaleric acid (21-998-3, ordered from ALDRICH), and glycogen (G-8876, ordered from SIGMA) were prepared and analyzed by the same analytical procedures for them extracted from biomass.

The evaluation of the uncertainty of the analytical experiments is presented in Table 4.4.

From the results it can be seen that, the analysis of the soluble compounds (such as PO₄-P, acetate and glucose) produces lower uncertainty than the analysis of the insoluble compounds (such as PHB, PHV, and glycogen). The analysis of glycogen had the highest uncertainty in this research. Therefore, more reliable procedures for the analysis of these internal reserves in the EBPR process is expected to improve the analytical results.
Table 4.4 Uncertainty Analysis of the Analytical Methods Applied in the Research

(PO₄-P is in the unit of 3-hydroxybutyrate (HB) mg/L; PHV is in the unit of 3-hydroxyvalerate (HV) mg/L; Glycogen is in the unit of glucose mg/L)

<table>
<thead>
<tr>
<th></th>
<th>Results from 5 Replicate Experiments (mg/L)</th>
<th>Average (mg/L)</th>
<th>Standard Deviation (mg/L)</th>
<th>Coefficient of Variation (%)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PO₄-P</strong></td>
<td>30.00 31.00 30.00 31.75 31.75</td>
<td>31.75</td>
<td>0.88</td>
<td>2.84</td>
<td>29.81 - 31.99</td>
</tr>
<tr>
<td><strong>Acetate</strong></td>
<td>31.50 32.60 34.30 34.00 36.50</td>
<td>33.78</td>
<td>1.89</td>
<td>5.59</td>
<td>31.43 - 36.13</td>
</tr>
<tr>
<td><strong>Lactic Acid</strong></td>
<td>36.39 34.85 33.90 34.85 38.61</td>
<td>35.72</td>
<td>1.85</td>
<td>5.18</td>
<td>33.42 - 38.02</td>
</tr>
<tr>
<td><strong>NO₃-N</strong></td>
<td>14.11 13.20 10.70 11.97 12.76</td>
<td>12.76</td>
<td>1.29</td>
<td>10.28</td>
<td>10.95 - 14.15</td>
</tr>
<tr>
<td><strong>MLSS</strong></td>
<td>2630 2651 2572 2741 2468</td>
<td>2612</td>
<td>101.0</td>
<td>3.87</td>
<td>2487 - 2737</td>
</tr>
<tr>
<td><strong>MLVSS</strong></td>
<td>1867 1802 1955 2055 1752</td>
<td>1886</td>
<td>121</td>
<td>6.42</td>
<td>1736 - 2036</td>
</tr>
<tr>
<td><strong>Total P</strong></td>
<td>197.5 167.5 180.0 205.0 162.6</td>
<td>182.5</td>
<td>18.43</td>
<td>10.10</td>
<td>159.6 - 205.4</td>
</tr>
<tr>
<td><strong>Glycogen</strong></td>
<td>487.0 388.7 491.3 355.1 426.3</td>
<td>429.7</td>
<td>59.86</td>
<td>13.93</td>
<td>355.4 - 504.0</td>
</tr>
<tr>
<td><strong>PHB</strong></td>
<td>133.6 168.7 141.5 148.4 160.2</td>
<td>150.6</td>
<td>14.1</td>
<td>9.38</td>
<td>133.1 - 168.1</td>
</tr>
<tr>
<td><strong>PHV</strong></td>
<td>77.2 94.9 90.4 68.7 75.9</td>
<td>81.42</td>
<td>10.88</td>
<td>13.35</td>
<td>67.9 - 94.9</td>
</tr>
<tr>
<td><strong>Glycogen</strong>*</td>
<td>240.6 234.1 296.2 256.3 247.8</td>
<td>255.2</td>
<td>24.47</td>
<td>9.59</td>
<td>224.8 - 285.5</td>
</tr>
<tr>
<td><strong>PHB</strong>*</td>
<td>184.8 211.6 179.3 201.6 205.4</td>
<td>196.5</td>
<td>13.84</td>
<td>7.04</td>
<td>179.4 - 213.7</td>
</tr>
<tr>
<td><strong>PHV</strong>*</td>
<td>278.2 262.3 242.2 273.1 233.9</td>
<td>257.9</td>
<td>19.27</td>
<td>7.47</td>
<td>234.0 - 281.9</td>
</tr>
</tbody>
</table>

(*Glycogen, PHB and PHV are measured using the standard samples with 250 glucose mg/L, 200 HB mg/L, and 250 HV mg/L respectively)
CHAPTER 5 MATHEMATICAL MODELING

5.1 Metabolic Model of the EBPR Process Using Glucose as the Single Substrate

To study the glucose metabolism in the EBPR process, a metabolic model was first hypothesized based on biochemical theories and experimental results. Since it is not technically available to separate the bio-P bacteria from the mixed bacterial community in the system, the observed experimental results are assumed to be produced by the bio-P bacteria alone. This assumption should not be far away from the truth, since with the specific SBR operating configurations and the specific dominant organic substrate, bio-P bacteria should become the dominant community over any other non-bio-P communities in the system.

Therefore, this metabolic model is established to reveal the major metabolic activities by the bio-P bacteria when glucose, instead of acetate, is added as the single substrate into the EBPR system.

5.1.1 Anaerobic Metabolic Model

Enhanced biological phosphorus removal is realized by cycling an anaerobic reaction with an aerobic reaction. The anaerobic reaction plays a more important role for the whole EBPR process because the anaerobic mechanism determines the success of enhanced phosphorus removal. It is assumed that all of the external substrate is utilized for the synthesis of internal carbon reserves and no biomass growth happens under the anaerobic condition. The major metabolic reactions during the anaerobic phase are described in the following sections and shown in Figure 5.1.
5.1.1.1 Glucose Uptake
Glucose was fed into the bioreactor at the start of the anaerobic reaction. The cytoplasmic membrane of bio-P bacteria is not readily permeable to glucose. However, after acclimating to glucose, bio-P bacteria could develop an active transport mechanism to "recognize" and "pick up" glucose from the medium and release it at the cytoplasmic side of the membrane (Gottschalk, 1986). The rapid removal of glucose upon the addition into the SBR supports this assumption. According to Gottschalk (1986), glucose is phosphorylated during the transport into the cytoplasm at the expense of ATP:

\[
\text{ATP + glucose} \rightarrow \text{glucose-6-phosphate + ADP} \quad \text{Eq. 5-1}
\]

5.1.1.2 Glycogen Accumulation and Degradation
With the addition of glucose into the SBR at the beginning of the anaerobic condition, an immediate increase of glycogen content of the cells was detected; after this

![Hypothesized Metabolic Pathways of the Glucose Induced EBPR System during the Anaerobic Condition](image)

Figure 5.1. Hypothesized Metabolic Pathways of the Glucose Induced EBPR System during the Anaerobic Condition
immediate increase of glycogen, it was found subsequently that the glycogen content of
the bio-P bacteria decreased during the rest of the anaerobic reaction. Based on these
observations, it is therefore assumed that an immediate increase of glycogen content
results from the conversion of phosphorylated glucose. The subsequent decrease of the
glycogen content arises from the continuous conversion of glycogen to PHA during the
anaerobic phase. It is therefore assumed that glycogen is hydrolyzed back to glucose-6-
phosphate which is then utilized to synthesize PHA. The metabolic pathway of glycogen
synthesis proposed by Dawes and Senior (1973, see Appendix B-II) is summarized in
Eq. 5-2:

\[ \text{ATP} + \text{glucose-6-phosphate} + (C_6H_{10}O_5)_n + H_2O \rightarrow \]
\[ (C_6H_{10}O_5)_{n+1} + \text{ADP} + 2H_3PO_4 \]
\[ \text{Glycogen} \quad \text{Eq. 5-2} \]

The degradation of glycogen to glucose-6-phosphate (Dawes et al., 1973, see Appendix
B-II) is summarized in Eq. 5-3:

\[ (C_6H_{10}O_5)_{n+1} + H_3PO_4 \rightarrow \text{glucose-6-phosphate} + (C_6H_{10}O_5)_n \]
\[ \text{Glycogen} \quad \text{Eq. 5-3} \]

5.1.1.3 Polyphosphate Hydrolysis for Energy Production
During anaerobic conditions, PO₄-P was found to be released into the medium. The
release of PO₄-P is assumed to be from the hydrolysis of intracellular polyphosphate for
energy production. Actually, polyphosphate in some microorganisms can fulfil the
function of ATP (Dawes and Senior, 1973). Lipmann (1965) has advanced the
suggestion that the earliest organisms used polyphosphate or pyrophosphate as their
prime energy carrier, the role for ATP as the universal energy carrier in contemporary
organisms.

Polyphosphate thermodynamically is a high energy phosphate compound. Thus the free
energy of hydrolysis of the anhydride linkage yields approximate 9 kcal per phosphate
bond (Dawes and Senior, 1973):
(Polyphosphate)$_n$ + H$_2$O $\rightarrow$ (Polyphosphate)$_{n-1}$ + H$_3$PO$_4$ \[ \Delta G^{\circ} = -9 \text{ kcal/mole} \]  

Eq. 5-4

However, based on Gaudy (1978), the free energy released from ATP hydrolysis is approximately 7 kcal per mole of ATP consumed.

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4 \quad \Delta G^{\circ} = -7 \text{ kcal/mole} 
\]

Eq. 5-5

Therefore, based on the amount of free energy released from polyphosphate hydrolysis and ATP hydrolysis, 1 mole of polyphosphate is equivalent to 1.29 mole of ATP from the view of energy production.

The composition of polyphosphate, based on Smolders et al., (1994a), is Mg$_{1/3}$K$_{1/3}$PO$_3$. Because the elements magnesium and potassium are not considered here, the phosphorus group of polyphosphate is made electroneutral with a proton and polyphosphate is represented by (HPO$_3$)$_n$. No energy is required for the export of phosphate by the cells (Smolders et al., 1994a). The reaction of polyphosphate hydrolysis for energy production is represented by Eq. 5-6:

\[
(\text{HPO}_3)_n + \text{H}_2\text{O} \rightarrow (\text{HPO}_3)_{n-1} + \text{H}_3\text{PO}_4
\]

Polyphosphate  

Eq. 5-6

5.1.1.4 Glycolysis

After transport into the cytoplasm, there are two possible pathways for the glycolysis reaction of the phosphorylated glucose: the Embden-Meyerhof-Parnas (EMP) pathway or the Entnner-Douderoff (ED) pathway (Mino et al., 1998). The EMP pathway is the most important pathway for glucose metabolism (see Appendix B-I) and is summarized in Eq. 5-7:

\[
3\text{ADP} + \text{glucose-6-phosphate} + 2\text{NAD} + 2\text{H}_3\text{PO}_4 \rightarrow 2\text{CH}_3\text{COCOOH} + 3\text{ATP} + 2\text{NADH}_2 + 2\text{H}_2\text{O}
\]

Pyruvate  

Eq. 5-7

95
From Eq. 5-7 it can be seen that 3 moles of ATP and 2 moles of reducing equivalent NADH$_2$ are produced per mole of glucose-6-phosphate consumed.

However, bio-P bacteria might use the ED pathway (See Appendix B-V) for glucose metabolism, which is summarized in Eq. 5-8:

$$2\text{ADP} + \text{glucose-6-phosphate} + 2\text{NAD} + \text{H}_3\text{PO}_4 \longrightarrow 2\text{CH}_3\text{COCOOH} + 2\text{ATP} + 2\text{NADH}_2 + \text{H}_2\text{O} \quad \text{Eq. 5-8}$$  

Pyruvate

From Eq. 5-8, it can be seen that compared with the EMP pathway, one mole less of ATP is produced per mole of the reacted glucose-6-phosphate from the ED pathway. However, the same amount of reducing equivalents is produced from the ED pathway as from the EMP pathway.

5.1.1.5 Release and Uptake of Lactic Acid

Lactic acid was detected in the medium after the addition of glucose during the anaerobic phase. Lactic acid is assumed to be a fermentation product from the added glucose. It is assumed that lactic acid was converted from pyruvate, the end product of glycolysis. The subsequent disappearance of lactic acid indicated that lactic acid was taken up and converted back to pyruvate by bio-P bacteria. The formation of lactic acid from pyruvate is summarized in the reversible Eq. 5-9:

$$\text{CH}_3\text{COCOOH} + \text{NADH}_2 \underset{\text{Pyruvate}}{\overset{\text{Lactic Acid}}{\longrightarrow}} \text{CH}_3\text{CHOHCOOH} + \text{NAD} \quad \text{Eq. 5-9}$$

Satoh et al. (1992) detected that when lactate was fed as external substrate, PHA was also produced under the anaerobic condition, therefore, it is assumed that after lactic acid was converted back to pyruvic acid, it was used to accumulate PHA by bio-P bacteria.
5.1.1.6 PHA Formation from Pyruvate

I. PHB Formation from Pyruvate

It was found that the PHB level of the cells increased during the anaerobic condition. It is assumed that part of the end product of glycolysis, pyruvate, was utilized for PHB synthesis. The precursors for PHB formation are 2 acetyl-CoA. The pathway of PHB synthesis from pyruvate (Dawes and Senior, 1973, Appendix B-III) is summarized in Eq 5-10:

\[
2\text{CH}_3\text{COCOOH} + \text{NAD} + (\text{C}_4\text{H}_6\text{O}_2)_n \rightarrow (\text{C}_4\text{H}_6\text{O}_2)_{n+1} + 2\text{CO}_2 + \text{NADH}_2 \quad \text{Eq. 5-10}
\]

Pyruvate

PHB

II. PHV Formation from Pyruvate

The experimental results show that PHV was the predominant form of PHA that was accumulated during the anaerobic condition. PHV formation requires two precursors in equal amounts: acetyl-CoA and propionyl-CoA. It is assumed that the formation of propionyl-CoA is through the succinate-propionate pathway (Satoh et al., 1992, see Appendix B-IV) and will consume the excessive NADH\_2 to enable a redox balance. The formation of propionyl-CoA through the succinate-propionate pathway is summarized in Eq 5-11:

\[
\text{CH}_3\text{COCOOH} + 2\text{NADH}_2 + \text{CoASH} + \text{ATP} \rightarrow \text{CH}_3\text{CH}_2\text{COSCoA} + \text{H}_2\text{O} + 2\text{NAD} + \text{ADP} + \text{H}_3\text{PO}_4 \quad \text{Eq. 5-11}
\]

Propionyl-CoA

The formation of acetyl-CoA from pyruvate is summarized in Eq. 5-12 according to Gaudy (1978):

\[
\text{CH}_3\text{COCOOH} + \text{CoASH} + \text{NAD} \rightarrow \text{CH}_3\text{COSCoA} + \text{CO}_2 + \text{NADH}_2 \quad \text{Eq. 5-12}
\]

Acetyl-CoA
Since acetyl-CoA and propionyl-CoA are the precursors for PHV formation, the equation for PHV formation can be derived by coupling Eq. 5-11 and Eq. 5-12:

$$2\text{CH}_3\text{COCOOH} + 2\text{NADH}_2 + \text{ATP} + (\text{C}_5\text{H}_9\text{O}_2)_n \rightarrow (\text{C}_5\text{H}_9\text{O}_2)_{n+1} + \text{H}_2\text{O} + 2\text{NAD} + \text{CO}_2 + \text{ADP} + \text{H}_3\text{PO}_4$$  \hspace{1cm} \text{Eq. 5-13}

Eq. 5-13 shows that for every mole of unit PHV formation from pyruvate, two moles of reducing power (NADH$_2$) are consumed.

### 5.1.2 NADH$_2$ and ATP Balance in the Hypothesized Anaerobic Metabolic Model

Based on the analysis above, the anaerobic metabolic model is composed of 6 major types of reactions (described in Sections 5.1.1.1 to 5.1.1.6). Two important metabolic principles are considered to judge the hypothesized model for the anaerobic glucose metabolism. The first principle is that bacteria have to maintain redox balances during the various metabolic reactions. It is known that an organic compound is oxidized biochemically by losing electrons in the form of hydrogen atoms. Consequently, oxidation is synonymous with dehydrogenation (Bailey, 1986). Figure 5.2 shows the production and consumption of electrons during the anaerobic metabolism of glucose. Since lactic acid was found to be completely taken up after its initial release, it is assumed that all the lactic acid produced from pyruvate was converted back to pyruvate afterwards. Therefore, the effect of lactic acid on the reducing power balance is ignored.

From Figure 5.2 it can be seen that, the production of pyruvate through the EMP or the ED pathway produces 4 moles of electrons per mole of glucose-6-phosphate consumed. The production of PHB from pyruvate will also generate 2 moles of electrons per mole of 3-HB (3-hydroxybutyrate, the monometric unit of PHB) formed. The generated electrons must be re-oxidized in order to maintain the redox balance of the bio-P bacteria. However, under the anaerobic condition, there are no external ultimate electron acceptors (i.e., O$_2$, NO$_3$-N). Therefore, the extra electrons must be consumed by an internal mechanism. Figure 5.2 indicates that the formation of PHV can serve as a
significant sink of the extra reducing power. Four moles of electrons will be consumed for the formation of 1 mole of 3-HV (3-hydroxyvalerate, the monometric unit of PHV) from pyruvate. It can be inferred from Figure 4.12 that in order to balance the electrons produced from the EMP pathway, all of the generated pyruvate should be utilized for the synthesis of PHV. In our batch experiments, it was detected that 3-HV was the predominant unit of accumulated PHA (accounted for over 80% of the total accumulated PHA under the anaerobic condition). However, small amounts of PHB synthesis during the anaerobic condition were also detected, which indicates that some other electron sink must also exist to balance the extra electrons produced from the synthesis of PHB. However, in this work, the mechanism of balancing the reducing power generated from the PHB synthesis was not found.

![Figure 5.2. ATP and Reducing Power Balances in the Hypothesized Metabolic Model during the Anaerobic Condition](image)

Figure 5.2. ATP and Reducing Power Balances in the Hypothesized Metabolic Model during the Anaerobic Condition (The number before each compound in this figure indicates the stoichiometric relation between the corresponding reactant and product; the negative sign means the consumption of the compound; the positive sign means the production of the compound).

Since PHV is the predominant PHA accumulated under the anaerobic condition and the mechanism for balancing the reducing equivalents from PHB production is unknown, PHV is assumed to be the only form of PHA accumulated during the anaerobic
condition to simplify the hypothesized metabolic model. The observed small increment of PHB is replaced with the equivalent portion of PHV and combined with all the other PHV to form the total PHA accumulated during the anaerobic phase (similar simplification was adopted by Smolders et al., 1994a, 1994b, 1995, who selected PHB as the only form of PHA in the metabolic model using acetate as the single substrate).

The second principle dictating metabolic reactions is that microorganisms seldom engage in metabolic activities without a specific purpose. Figure 5.2 shows the ATP balance during the anaerobic condition. The transport of glucose across the membrane of bio-P bacteria results in the phosphorylation of glucose, which requires 1 mole of ATP to transport 1 mole of glucose. The phosphorylated glucose has then two alternative pathways: (1): entering the glycolysis pathway by which 3 moles of ATP (through the EMP pathway) or 2 moles of ATP (through the ED pathway) are generated per mole of glucose-6-phosphate consumed; or (2): synthesizing glycogen where 1 mole of ATP is consumed per mole of unit glycogen synthesized. There is no ATP requirement for PHB formation from pyruvate. However, one mole of ATP is required for the formation of 1 unit of PHV from pyruvate. It can be observed that the cell could maintain the ATP requirements if glucose were metabolized through the EMP pathway, since the 3 moles of ATP generated from the glycolysis of 1 mole of glucose-6-phosphate could cover the energy requirements for all other anaerobic metabolisms. According to the second principle, the microorganisms would not seek energy from another source if they could derive enough energy from metabolizing glucose alone. Under this situation, polyphosphate would not be utilized as an energy source for other anaerobic reactions. The decline of polyphosphate utilization as an energy source would minimize the phosphate removal ability by the bio-P bacteria during the aerobic reaction. These deductions agree with the experimental facts that PO₄-P removal efficiency deteriorates easily when using glucose as the major substrate and poor PO₄-P removal efficiency was always accompanied by the poor anaerobic PO₄-P release in reactor G.
However, in this research, glucose was successfully utilized as the dominant substrate to induce and maintain excellent EBPR performance under the modified operating procedures. PO₄-P was released into the medium in significant levels (indicating polyphosphate was used for energy production) by proper manipulation of the EBPR operating procedures. It is reasoned that, when the system demonstrated good EBPR performance, another important glycolysis pathway, the Entner-Douderoff (ED) pathway is likely used by the bio-P bacteria to metabolize glucose. Results of $^{13}$C-NMR measurements after feeding $^{13}$C labeled acetate found that glycogen was degraded via the ED pathway (Hesselmann, 2000), which confirms the involvement of the ED pathway in EBPR processes. According to the second principle, the higher level of energy derivation from polyphosphate hydrolysis can strengthen the enhanced phosphorus removal during the aerobic condition. Since the ED pathway produces one mole less of ATP than the EMP pathway for every mole of glucose metabolized, polyphosphate could be involved in the anaerobic reactions as an energy source based on the ATP balance in Figure 5.2.

From Figure 5.2 it can also be seen that extra ATP is required for glycogen synthesis from glucose-6-phosphate and for PHV formation from pyruvate. Therefore, the pathway of transforming more glucose first to glycogen and then from glycogen to pyruvate will consume more internal energy, which will help to enhance the energy derivation from polyphosphate hydrolysis. On the other hand, if glucose is directly metabolized without converting to glycogen, no extra energy requirement for glycogen synthesis is necessary and polyphosphate will not be used for energy derivation. In addition, the predominance of PHV enriched PHA not only helps the bio-P bacteria maintain the internal redox balance, but also induces extra energy consumption, which increases the energy demand from polyphosphate hydrolysis. Finally, our experimental results prove that a longer anaerobic condition will enhance the stability of an EBPR process using glucose as the major substrate. This can be explained by the extra energy that would be required to meet the maintenance requirement for the bio-P bacteria under the longer anaerobic condition, which further necessitates the energy derivation from polyphosphate hydrolysis.
5.1.3 Aerobic Metabolic Model

The aerobic phase is also an important phase because the enhanced removal of phosphorus takes place during this phase. The major metabolic activities taking place during the aerobic phase are described as follows and are shown in Figure 5.3.

![Diagram of metabolic pathways](image)

**Figure 5.3. Hypothesized Metabolic Pathways of the Glucose Induced EBPR System during the Aerobic Condition** ("\(\rightarrow\)": indicate the conversion or transport of compounds; "\(\longrightarrow\)": indicate the transport of energy ATP)

### 5.1.3.1 PHV Oxidation

It was found in the batch experiments that the PHA levels diminished after the start of the aerobic condition. Under the aerobic condition, there is no external substrate in the system. It is assumed that PHA, the organic internal reserve, is utilized by the bio-P bacteria for different metabolic purposes including the enhanced uptake of phosphorus from the medium. The oxidation of PHV is formulated to represent all PHA oxidation during the aerobic phase.
During the oxidation, PHV is assumed to be first de-polymerized to its unit 3-HV by Eq. 5-14:

\[
(C_{2n}H_{2n}O_2)_n \xrightarrow{PHV} (C_{2n-2}H_{2n-2}O_2)_{n-1} + C_5H_8O_2 \quad \text{Eq. 5-14}
\]

3-HV is then degraded to acetyl-CoA and propionyl-CoA by Eq. 5-15:

\[
C_5H_8O_2 + NAD + 2CoASH \xrightarrow{3-HV} CH_3COSCoA + CH_3CH_2COSCoA + NADH_2 \quad \text{Acetyl-CoA Propionyl-CoA Eq. 5-15}
\]

The pathway of propionyl-CoA oxidation to pyruvate is assumed to be through the Methylcitrate Cycle (Susanne, 1997, see Appendix B-VI) and is summarized in Eq. 5-16 (assuming FADH2 and FAD are equivalent to NADH2 and NAD, respectively):

\[
CH_3CH_2COSCoA + 2H_2O + 2NAD \rightarrow CH_3COCOOH + 2NADH_2 + CoASH \quad \text{Eq. 5-16}
\]

Pyruvate is then oxidized to acetyl-CoA according to Gaudy (1978):

\[
CH_3COCOOH + CoASH + NAD \rightarrow CH_3COSCoA + NADH_2 + CO_2 \quad \text{Eq. 5-17}
\]

The general equation of 3-HV oxidation to acetyl-CoA can then be derived by combining Eq. 5-15, Eq. 5-16 and Eq. 5-17:

\[
C_5H_8O_2 + 4NAD + 2H_2O + 2CoASH \rightarrow 2CH_3COSCoA + 4NADH_2 + CO_2 \quad \text{Eq. 5-18}
\]

Acetyl-CoA is oxidized through the TCA pathway based on Gaudy (1978, see Appendix B-VII) under aerobic condition, which is summarized in Eq. 5-19 (assuming NADP and FAD are equivalent to NAD; NADPH2 and FADH2 are equivalent to NADH2):
\[2\text{CH}_3\text{COSCoA} + 4\text{H}_2\text{O} + 8\text{NAD} + 2\text{H}_3\text{PO}_4^{\text{in}} + 2\text{ADP} \rightarrow 8\text{NADH}_2 + 2\text{ATP} + 2\text{CoASH} + 4\text{CO}_2 \quad \text{Eq. 5-19}\]

Where $\text{H}_3\text{PO}_4^{\text{in}}$ is the phosphate inside of biomass (it is assumed that bio-P bacteria can only utilize the PO$_4$-P after its being transported into the cytoplasm).

The general equation of PHV oxidation to produce NADH$_2$ can be derived by coupling Eq. 5-14, Eq. 5-18, and Eq. 5-19 as shown in Eq. 5-20:

\[\text{C}_2\text{H}_8\text{O}_2 + 12\text{NAD} + 6\text{H}_2\text{O} + 2\text{H}_3\text{PO}_4^{\text{in}} + 2\text{ADP} \rightarrow 12\text{NADH}_2 + 5\text{CO}_2 + 2\text{ATP} \quad \text{Eq. 5-20}\]

### 5.1.3.2 Oxidative Phosphorylation

Under aerobic conditions, oxygen is available as an ultimate electron acceptor for the NADH$_2$ produced from the oxidation of PHV. The amount of ATP produced per electron pair transferred to oxygen is represented by δ, the so-called $P/O$ ratio, which resembles the efficiency of the oxidative phosphorylation. This process can be stoichiometrically represented by Eq. 5-21 (Smolders et al., 1994b):

\[\text{NADH}_2 + 0.5\text{O}_2 + \delta\text{ADP} + \delta\text{H}_3\text{PO}_4^{\text{in}} \rightarrow \text{NAD} + (1+\delta)\text{H}_2\text{O} + \delta\text{ATP} \quad \text{Eq. 5-21}\]

### 5.1.3.3 Active Biomass Synthesis from PHV

During the aerobic condition, biomass growth occurs with the carbon source from PHV degradation and the energy source from PHV oxidative phosphorylation. In order to investigate the dynamics of the important internal reserves, Smolders et al. (1994b) categorized the biomass into different compositions. They proposed an active biomass component to differentiate it from the important internal reserves of bio-P bacteria. In their model, the measured mixed liquor suspended solids (MLSS) includes two parts: VSS (volatile suspended solids) and Ash. VSS is composed of PHA, glycogen, and the volatile part of the biomass; Ash is mainly composed of polyphosphate and the non-
volatile part of the active biomass. Therefore, active biomass is the part of MLSS not including PHA, glycogen, and polyphosphate (See Figure 5.4).

<table>
<thead>
<tr>
<th>MLSS</th>
<th></th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>Glycogen</td>
<td>Polyphosphate</td>
</tr>
</tbody>
</table>

**Figure 5.4. Composition of Biomass and Relations with MLSS, VSS, and Ash Content**

For the synthesis of active biomass, Smolders et al. (1994b) found that 0.27 mole of CO₂ is produced per C-mol of biomass synthesized. The amount of ATP needed for the formation of 1 C-mol of biomass is represented by K. According to Smolders et al. (1994b), the expected value of K = 1.5 mol ATP / C-mol active biomass. The maintenance term, \( m_{\text{arp}} \), is the specific ATP consumption (mol-ATP/C-mol / h) due to the maintenance requirement of biomass. Smolders et al. (1994b) applied CHON analysis to determine the active biomass composition with the correction for polyphosphate, PHA and glycogen contents. The composition of active biomass from his finding is CH₂₂.₉₉O₇.₅₄N₀.₅₄P₀.₀₁₅. From the bacterial identification experiments of this research, it was found that the compositions of the microorganisms were very similar between reactors A and G. Therefore, the active biomass composition Smolders et al. detected from the acetate fed EBPR system is applied in this model. The growth of 1 C-mol of active biomass is represented by Eq. 5-22:

\[
0.254(C_3H_6O_2)_n + 0.015H_3PO_4^{in} + 0.512H_2O + 0.8055NAD + (K+m_{\text{arp}/\mu})H_2O + (K+m_{\text{arp}/\mu})ATP + 0.2 \text{NH}_3 \rightarrow
\]

\[
0.254(C_3H_6O_2)_{n-1} + \text{CH}_2.09\text{O}_{0.54}\text{N}_{0.2}\text{P}_{0.015} + 0.27\text{CO}_2 + 0.8055\text{NADH}_2 + (K+m_{\text{arp}/\mu})\text{ADP} + (K+m_{\text{arp}/\mu})H_3\text{PO}_4^{in}
\]

Eq. 5-22

where \( \mu \) is the specific active biomass growth rate \((h^{-1})\); \( m_{\text{arp}/\mu} \) is the amount of ATP consumed for the biomass maintenance purpose.

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5.1.3.4 Phosphate Transport into the Bio-P Bacteria

The enhanced phosphate removal takes place in the aerobic phase. Bio-P bacteria are able to synthesize polyphosphate by uptaking significant amounts of phosphate from the medium. The transport of phosphate across the cell membrane is a process which requires energy (Smolders et al., 1994b). Phosphate is a negatively charged ion and has to be taken up against the electric potential difference over the cell membrane. Positive ions required for the polyphosphate synthesis (Mg$^{2+}$ and K$^+$) are taken up without energy costs. The energy used for the transport of phosphate is generated from the oxidative phosphorylation. Assuming a certain amount of phosphate, $\xi$, can be transported for each consumed NADH$_2$ during oxidative phosphorylation. The transportation of external phosphate into the biomass is summarized in Eq. 5-23:

$$H_3PO_4^{\text{out}} + (1/2\xi)NADH_2 + (1/2\xi)O_2 \rightarrow H_3PO_4^{\text{in}} + (1/\xi)H_2O + (1/\xi)NAD$$ Eq. 5-23

where $H_3PO_4^{\text{out}}$ is the phosphate in the medium

5.1.3.5 Polyphosphate Synthesis

Besides being used for the active biomass growth, the majority of the transported phosphate is for the purpose of polyphosphate synthesis. The mechanism of polyphosphate synthesis based on Smolders et al. (1994b) is represented by Eq. 5-24:

$$(HPO_3)_n + H_3PO_4^{\text{in}} + ATP \rightarrow (HPO_3)_{n+2} + H_2O + ADP$$ Eq. 5-24

5.1.3.6 Glycogen Synthesis from PHV

Glycogen content of the bio-P bacteria was found to increase during the aerobic phase. This is explained by the conversion of PHV to synthesize glycogen. PHV is assumed to be first degraded to acetyl-CoA by Eq. 5-25:

$$(C_5H_8O_2)_n + 4NAD + 2H_2O + 2CoASH \rightarrow 2CH_3COSCoA + (C_5H_8O_2)_{n-1} + 4NADH_2 + CO_2$$ Eq. 5-25

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Then acetyl-CoA is converted to oxaloacetate through the Glyoxylate Pathway according to Smolder et al. (1994b) by Eq. 5-26 (See Appendix B-VIII):

$$\text{2CH}_3\text{COSCoA} + 3\text{H}_2\text{O} + 3\text{NAD} \rightarrow \text{COOHCOCH}_2\text{COOH} + 3\text{NADH}_2 + 2\text{CoASH}$$  \hspace{1cm} \text{Eq. 5-26}

Oxaloacetate

The general equation for PHV conversion to oxaloacetate can be derived by coupling Eq. 5-25 and Eq. 5-26:

$$\text{(C}_5\text{H}_6\text{O}_2)_n + 7\text{NAD} + 5\text{H}_2\text{O} \rightarrow \text{COOHCOCH}_2\text{COOH} + (\text{C}_5\text{H}_6\text{O}_2)_{n-1} + 7\text{NADH}_2 + \text{CO}_2$$  \hspace{1cm} \text{Eq. 5-27}

Oxaloacetate  \hspace{1cm} \text{PHV}

Based on Smolder et al. (1994b), oxaloacetate is then converted to glucose-1-phosphate through Gluconeogenesis (See Appendix B-IX), which is summarized in Eq. 5-28:

$$2\text{Oxaloacetate} + 4\text{ATP} + 3\text{H}_2\text{O} + 2\text{NADH}_2 \rightarrow \text{Glucose-1-phosphate} + 2\text{CO}_2 + 4\text{ADP} + 2\text{NAD} + 3\text{H}_3\text{PO}_4^{\text{in}}$$  \hspace{1cm} \text{Eq. 5-28}

Glucose-1-phosphate is then converted to glycogen based on Dawes and Senior (1973, see Appendix B-II):

$$\text{Glucose-1-phosphate} + \text{ATP} + \text{H}_2\text{O} + (\text{C}_6\text{H}_{10}\text{O}_5)_n \rightarrow (\text{C}_6\text{H}_{10}\text{O}_5)_{n+1} + \text{ADP} + 2\text{H}_3\text{PO}_4^{\text{in}}$$  \hspace{1cm} \text{Eq. 5-29}

Glycogen

By combining 5-27, 5-28 and 5-29, a general equation describing the synthesis of glycogen from PHV can be summarized in Eq. 5-30:

$$\text{(C}_5\text{H}_8\text{O}_2)_n + (\text{C}_6\text{H}_{10}\text{O}_5)_n + 12\text{NAD} + 14\text{H}_2\text{O} + 5\text{ATP} \rightarrow (\text{C}_5\text{H}_8\text{O}_2)_{n-2} + (\text{C}_6\text{H}_{10}\text{O}_5)_{n+1} + 12\text{NADH}_2 + 4\text{CO}_2 + 5\text{ADP} + 5\text{H}_3\text{PO}_4^{\text{in}}$$  \hspace{1cm} \text{Eq. 5-30}
5.2 Stoichiometric and Kinetic Study

The hypothesized theoretical model for the glucose induced EBPR process is based on fundamental biochemical knowledge and the SBR experimental results. This model reveals the internal mechanisms of enhanced biological phosphorus removal with glucose as the single substrate. However, this hypothesized model only gives a theoretical framework of the major biochemical reactions under the anaerobic and aerobic conditions. Based on the hypothesized model and the experiment results, a stoichiometric and kinetic study was conducted to develop a mathematical model simulating the dynamics of a glucose induced EBPR system.

5.2.1 Anaerobic Mathematical Model

As already discussed, it is the anaerobic stage when the bio-P bacteria establish the mechanism of enhanced phosphorus removal. The hypothesized metabolic model consists of 8 reactions reflecting the anaerobic metabolism. It has been assumed that lactic acid, after its release into the medium, is completely taken up by the bio-P bacteria again. The conversion reactions between lactate and pyruvate are therefore eliminated from the mathematical model. In addition, since PHV was detected to be the predominant form of PHA in reactor G, it was decided to choose PHV as the only accumulated form of PHA. The PHB accumulations are converted to the equivalent amount of PHV. The maintenance energy is assumed to be supplied by the polyphosphate hydrolysis. It is also assumed that glucose is metabolized through the ED pathway as explained in Section 5.1.2.

For the convenience of computation, all substrates and polymeric components, such as active biomass, PHV, polyphosphate, and glycogen, are expressed as per mole of carbon or per mole of phosphorus in their elemental formulas, which is also a standard procedure for structuring biochemical model (Roles, 1983).

Based on the above assumptions and simplifications, the metabolic equations under the anaerobic conditions are written as below:
• Glucose Transport:
Based on Eq. 5-1, the reaction of glucose transport can be expressed as:

\[-\frac{1}{6} \text{ATP} \cdot \text{CH}_2\text{O} + \text{CH}_{13/6}\text{O}(\text{PO}_3)_{1/6} + \frac{1}{6} \text{ADP} = 0\]

Glucose Glucose-6-phosphate

Eq. 5-31

• Glycogen Accumulation:
Based on Eq. 5-2, the reaction of glycogen accumulation can be expressed as:

\[-\text{CH}_{13/6}\text{O}(\text{PO}_3)_{1/6} - \frac{1}{6} \text{ATP} - \frac{1}{6} \text{H}_2\text{O} + \text{CH}_{5/6}\text{O}_{5/6} + \frac{1}{6} \text{ADP} + \frac{1}{3} \text{H}_3\text{PO}_4 = 0\]

Glucose-6-phosphate Glycogen

Eq. 5-32

• Glycogen Degradation:
Based on Eq. 5-3, the reaction of glycogen degradation can be expressed as:

\[-\text{CH}_{5/3}\text{O}_{5/6} - \frac{1}{6} \text{H}_3\text{PO}_4 + \text{CH}_{13/6}\text{O}(\text{PO}_3)_{1/6} = 0\]

Glycogen Glucose-6-phosphate

Eq. 5-33

• Polyphosphate Hydrolysis:
Based on Eq. 5-6, the reaction of polyphosphate hydrolysis can be expressed as:

\[-\text{HPO}_4 - \text{H}_2\text{O} + \text{H}_3\text{PO}_4 = 0\]

Polyphosphate

Eq. 5-34

• Glycolysis (ED):
Based on Eq. 5-8, the glycolysis by the ED pathway can be expressed as:

\[-\text{CH}_{13/6}\text{O}(\text{PO}_3)_{1/6} - \frac{1}{3} \text{ADP} - \frac{1}{6} \text{H}_3\text{PO}_4 - \frac{1}{3} \text{NAD} + \text{CH}_4\text{O} + \frac{1}{3} \text{ATP} + \frac{1}{3} \text{NADH}_2 + \frac{1}{6} \text{H}_2\text{O} = 0\]

Glucose-6-phosphate Pyruvate

Eq. 5-35

• PHV Synthesis:
Based on Eq. 5-13, the reaction of PHV synthesis can be expressed as:
- 1.2 CH$_{4}$O - 2/5 NADH$_{2}$ - 1/5 ATP

Pyrurate

+ CH$_{8}$/O$_{2}$/5 + 1/5 H$_{2}$O + 2/5 NAD + 1/5CO$_{2}$ + 1/5ADP + 1/5H$_{3}$PO$_{4}$ = 0  Eq. 5-36

PHV

### 5.2.1.1 Anaerobic Stoichiometric Coefficients

It has been assumed that, during the anaerobic condition, all organic carbon is taken up and converted to internal organic reserves. PHV is considered to be the ultimate organic reserve for absorbed glucose during the anaerobic condition. It is clear that there are two alternative pathways for PHV synthesis after glucose is transported into the bacteria (see Figure 5.5). The first pathway is that glucose is converted to glycogen which is then utilized for PHV synthesis; the second pathway assumes that glucose is directly converted to PHV.

![Figure 5.5. Two Pathways for PHV Formation in the Glucose Induced EBPR System ("\(\cdots\)"): Pathway 1; "\(\rightarrow\)": Pathway 2)

- **Pathway 1**: Glucose → Glycogen → PHV

By coupling Eq. 5-31 (Glucose Transport) and Eq. 5-32 (Glycogen Accumulation), the equation of glycogen formation from glucose can be expressed as:

- 1/3 ATP - CH$_{2}$O - 1/6 H$_{2}$O + 1/3 ADP + CH$_{5}$/O$_{4}$/6 + 1/3H$_{3}$PO$_{4}$ = 0  Eq. 5-37

Glucose  Glycogen

From Eq. 5-37 it can be seen that, for every mole of glycogen synthesis from glucose, 1/3 mole of ATP needs to be supplied to complete this reaction. This energy requirement can be partially satisfied by the subsequent conversion of glycogen to PHV. By combining Eq. 5-33 (glycogen degradation), Eq. 5-35(glycolysis-ED) and Eq. 5-36
(PHV synthesis), a general equation for PHV formation from glycogen can be derived as follow:

\[
- \text{CH}_3\text{O}_5\text{S}_6 - \frac{1}{6} \text{H}_3\text{PO}_4 - \frac{1}{6} \text{ADP} + \frac{5}{6} \text{CH}_8\text{O}_2\text{S}_5 + \frac{1}{6} \text{ATP} + \frac{1}{3} \text{H}_2\text{O} + \frac{1}{6} \text{CO}_2 = 0
\]

\text{Glycogen} \quad \text{PHV} \quad \text{Eq. 5-38}

From Eq. 5-38, it can be seen that for 1 C-mol of glycogen degradation, \(5/6\) C-mol of PHV is formed and \(1/6\) mole of ATP is produced. By coupling Eq. 5-37 and Eq. 5-38, a general equation can be derived for PHV formation through Pathway 1:

\[
- \text{CH}_2\text{O} - \frac{1}{6} \text{ATP} + \frac{5}{6} \text{CH}_8\text{O}_2\text{S}_5 + \frac{1}{6} \text{ADP} + \frac{1}{6} \text{H}_3\text{PO}_4 + \frac{1}{6} \text{H}_2\text{O} + \frac{1}{6} \text{CO}_2 = 0
\]

\text{Glucose} \quad \text{PHV} \quad \text{Eq. 5-39}

From Eq. 5-39 it can be seen that, \(1/6\) mole of ATP is required for 1 C-mol of glucose conversion to PHV through Pathway 1. It is assumed that polyphosphate degradation (Eq. 5-34) balances the remaining energy requirement for PHV synthesis. It has been discussed that 1 P-mol of polyphosphate is equivalent to 1.29 mole of ATP from the view of energy production. Therefore, the required ATP in Pathway 1 can be replaced by 0.129 P-mol of polyphosphate (equivalent to \(1/6\) mole ATP) for 1 C-mol of glucose conversion to PHV. Multiplying Eq. 5-34 by 0.129 yields:

\[
-0.129 \text{HPO}_3 = -0.129 \text{H}_2\text{O} + 0.129 \text{H}_3\text{PO}_4 = 0
\]

\text{Polyphosphate} \quad \text{Eq. 5-40}

The ATP consumption in Eq. 5-39 can be replaced by the equivalent polyphosphate hydrolysis in Eq. 5-40. Rewriting Eq. 5-39 using polyphosphate hydrolysis instead of ATP hydrolysis, the general equation for PHV formation through Pathway 1 is re-written as:

\[
- \text{CH}_2\text{O} - 0.129 \text{HPO}_3 + \frac{5}{6} \text{CH}_8\text{O}_2\text{S}_5 + 0.129 \text{H}_3\text{PO}_4 + 0.204 \text{H}_2\text{O} + \frac{1}{6} \text{CO}_2 = 0
\]

\text{Glucose} \quad \text{Polyphosphate} \quad \text{PHV} \quad \text{Eq. 5-41}

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However, Eq. 5-41 cannot reflect the dynamics of glycogen during the formation of PHV. Therefore, Eq. 5-41 is split into two equations: one describing carbon conversion from glucose to glycogen with polyphosphate hydrolysis balancing the energy requirement and the other describing carbon conversion from glycogen to PHV:

**Glucose – Glycogen:**

\[-\text{CH}_2\text{O} - 0.129 \text{HPO}_3 + 0.0377 \text{H}_2\text{O} + \text{CH}_{5/3}\text{O}_{5/6} + 0.129 \text{H}_3\text{PO}_4 = 0 \quad \text{Eq. 5-42}\]

**Glycogen – PHV:**

\[-\text{CH}_{5/3}\text{O}_{5/6} + 5/6\text{CH}_8\text{O}_{2/5} + 1/6\text{CO}_2 + 1/6\text{H}_2\text{O} = 0 \quad \text{Eq. 5-43}\]

- **Pathway 2: Glucose \rightarrow PHV**

The other pathway of PHV formation is through the direct conversion of glucose to PHV. The general equation of PHV formation through pathway 2 can be derived by combining Eq. 5-31, Eq. 5-35 and Eq. 5-36:

**Glucose – PHV:**

\[-\text{CH}_2\text{O} + 5/6 \text{CH}_8\text{O}_{2/5} + 1/3 \text{H}_2\text{O} + 1/6 \text{CO}_2 = 0 \quad \text{Eq. 5-44}\]

From the above analysis it can be seen that, PHV formation by Pathway 1 cannot derive enough energy from glycolysis reaction and the degradation of polyphosphate is necessary to compensate for the outstanding energy requirement. PHV formation by pathway 2, however, can just derive enough energy from its glycolysis and no extra energy is required from polyphosphate degradation. Therefore, it is more beneficial for the EBPR process if more glucose is metabolized through Pathway 1 for PHV formation, since more energy would be derived from the polyphosphate hydrolysis.

Besides the metabolism of carbon flow, bio-P bacteria also consume a certain amount of energy for maintenance requirement. The maintenance energy is assumed to be derived from polyphosphate hydrolysis:
• **Maintenance:**

\[- \text{HPO}_3 - \text{H}_2\text{O} + \text{H}_3\text{PO}_4 = 0\]  

Eq. 5-45

Therefore, the total anaerobic metabolism is encompassed by four equations: (1) Eq. 5-42: glycogen formation from transported glucose with polyphosphate hydrolysis supplying the energy; (2) Eq. 5-43: glycogen degradation for PHV formation; (3) Eq. 5-44: PHV formation from glucose direction conversion; and (4) Eq. 5-45: biomass maintenance from polyphosphate hydrolysis. The overall stoichiometric coefficients of the key metabolic compounds are expressed in these equations.

**5.2.1.2 Anaerobic Kinetic Modeling**

From the above metabolic analysis, it can be concluded that under the anaerobic conditions, the ultimate fate of glucose is to be transported into the bio-P bacteria and converted to PHV. The bio-P bacteria also require a certain amount energy for maintenance. Eq. 5-42 to Eq. 5-45 describe the overall stoichiometric coefficients of the key metabolic compounds during the anaerobic metabolism. These reactions are termed "internal reactions" which cannot be observed directly. However, the internal reaction rates can be related to the observable conversion rates outside the cell.

The conversion rates of the compounds can be expressed as a function of the internal reaction rates with parameters composed of the stoichiometric coefficients of the internal reactions (Roels, 1983). Assume \( q_{gb} \), \( q_{glv} \), \( q_{gv} \) and \( m_{an} \) to be the active biomass specific rates of the reaction of glycogen accumulation from glucose (Eq. 5-42), the reaction of PHV formation from glycogen degradation (Eq. 5-43), the reaction of PHV formation from glucose direction conversion (Eq. 5-44) and the reaction of maintenance (Eq. 5-45), respectively. These specific reaction rates can be expressed in the matrix \( q^{an} \):

\[
q^{an} = \begin{pmatrix}
q_{gl} \\
q_{glv} \\
q_{gv} \\
m_{an}
\end{pmatrix}
\]

Eq. 5-46
The conversion rates of each of the compounds are determined by the overall effect of these four internal reactions. A metabolic stoichiometry matrix ($\alpha^{an}$) can be written such that each element in $\alpha^{an}$ matches the stoichiometric coefficient for a specific compound in a corresponding reaction. All the elements in the stoichiometry matrix $\alpha^{an}$ are derived from the biochemical equations (Eq. 5-42 to Eq. 5-45) as discussed above.

If the anaerobic compound conversion rates are represented by the vector $\gamma^{an}$, and the active biomass specific rates of the reactions by the vector $q^{an}$, then the conversion rates of the key metabolic compounds in the anaerobic reactions can be calculated according to Eq. 5-47a (Roels, 1983):

$$\gamma^{an} = \alpha^{an} \cdot q^{an} \cdot C_x$$  \hspace{1cm} \text{Eq. 5-47a}$$

$C_x$ in Eq. 5-47a is the concentration of active biomass (C-mmol/L), which is the component of the MLSS excluding glycogen, PHA, and polyphosphate (see Figure 5.4). During the anaerobic reaction, $C_x$ is a constant since it is assumed that there is no bacterial growth and the maintenance energy comes from polyphosphate hydrolysis during the anaerobic phase. The structured anaerobic kinetic calculation by Eq. 5-47a can then be expressed as follows:

$$q^{an} = q_{gl} \quad q_{glv} \quad q_{gv} \quad m_{an}$$

$$\begin{bmatrix}
\gamma_{pbv} \\
\gamma_{pp} \\
\gamma_{gli} \\
\gamma_g \\
\gamma_p \\
\gamma_{co} \\
\gamma_{bo}
\end{bmatrix} = \begin{bmatrix}
\alpha_{g}^{an} \\
\alpha_{p}^{an} \\
\alpha_{co}^{an} \\
\alpha_{bo}^{an}
\end{bmatrix} \times \begin{bmatrix}
0 & +5/6 & +5/6 & 0 \\
0 & 0 & 0 & -1 \\
-0.129 & 0 & 0 & 0 \\
+1 & -1 & 0 & 0 \\
-1 & 0 & -1 & 0 \\
+0.129 & 0 & 0 & +1 \\
0 & +1/6 & +1/6 & 0 \\
+0.0377 & +1/6 & +1/3 & -1
\end{bmatrix} \times C_x$$  \hspace{1cm} \text{Eq. 5-47b}$$
where $\gamma^{a}$ is the matrix of the anaerobic conversion rates of the key metabolic compounds,

$\alpha^{a}$ is the matrix of the stoichiometric coefficients of the key metabolic compounds,

$q^{an}$ is the matrix of the anaerobic specific reaction rates,

$C_x$ is the concentration of active biomass (C-mmol/L),

$q_{gl}$ is the specific glycogen accumulation rate (C-mmol/C-mmol · h),

$q_{glv}$ is the specific rate of PHV formation from glycogen (C-mmol/C-mmol · h),

$q_{gv}$ is the specific rate of PHV formation rate from glucose direct conversion (C-mmol/C-mmol · h),

$m_{an}$ is the specific anaerobic maintenance rate (P-mmol/C-mmol · h),

$\gamma_{phv}$ is the overall PHV conversion rate (C-mmol/L · h),

$\gamma_{pp}$ is the overall polyphosphate conversion rate (P-mmol/L · h),

$\gamma_{gg}$ is the overall glycogen conversion rate (C-mmol/L · h),

$\gamma_{gg}$ is the overall glucose conversion rate (C-mmol/L · h),

$\gamma_{o}$ is the overall PO4-P conversion rate (P-mmol/L · h),

$\gamma_{co}$ is the overall CO2 conversion rate (mmol CO2/L · h),

$\gamma_{ho}$ is the overall H2O conversion rate (mmol H2O/L · h).

In Eq. 5-47b, the conversion rates in $\gamma^{a}$ are determined by the overall effects from the four specific reaction rates multiplied by the relevant stoichiometric coefficients and the active biomass concentration. For example, the anaerobic conversion rates of PHV ($\gamma_{phv}$) and glycogen ($\gamma_{gg}$) can be expressed as:

$$
\gamma_{phv} = \gamma_{gl} \cdot C_x + \frac{5}{6} \cdot q_{glv} \cdot C_x + \frac{5}{6} \cdot q_{gv} \cdot C_x + 0 \cdot m_{an} \cdot C_x
$$

$$
= \frac{5}{6} \cdot q_{glv} \cdot C_x + \frac{5}{6} \cdot q_{gv} \cdot C_x
$$

Eq. 5-47c

$$
\gamma_{gl} = \gamma_{gl} \cdot C_x - 1 \cdot q_{glv} \cdot C_x + 0 \cdot q_{gv} \cdot C_x + 0 \cdot m_{an} \cdot C_x = q_{gl} \cdot C_x - q_{glv} \cdot C_x
$$

Eq. 5-47d

From the above two examples, it can be seen that for the calculation of the conversion rates of the controlled compounds, the stoichiometric coefficients are already known. It
is also assumed that the active biomass concentration \( (C_a) \) and the specific anaerobic maintenance rate \( (m_{an}) \) are constants. The only variables in the equations are these specific reaction rates which need to somehow be determined in order to solve the dynamic conversion rates of the key metabolic components.

The specific reaction rate of glycogen accumulation from glucose, \( q_{gl} \), is assumed to be dependent on the glucose concentration in the medium, which is expressed using the conventional Monod-type relation. It is also assumed that the production of glycogen is limited by some maximum glycogen content in the biomass (Smolders et al., 1994a). In the kinetic description of the glycogen production, this maximum glycogen content is represented by \( f_{gl}^{max} \). When the fraction of glycogen in the biomass \( (f_{gl}) \) approaches the maximum glycogen fraction \( f_{gl}^{max} \), a linear decrease of \( q_{gl} \) with increasing \( f_{gl} \) is assumed to occur. When the fraction of glycogen in the biomass \( (f_{gl}) \) equals to the maximum glycogen fraction of the biomass, the specific rate of glycogen accumulation from glucose will be zero. The specific glycogen accumulation rate is therefore given as:

\[
q_{gl} = q_{gl}^{max} \cdot \frac{C_g}{K_{gl} + C_g} (1 - \frac{f_{gl}'}{f_{gl}^{max}}) \tag{Eq. 5-48}
\]

where \( q_{gl} \) is the specific glycogen accumulation rate \((\text{C-mmol/C-mmol} \cdot \text{h})\),
\( q_{gl}^{max} \) is the maximum glycogen accumulation rate \((\text{C-mmol/C-mmol} \cdot \text{h})\),
\( K_{gl} \) is the half saturation constant of glycogen accumulation \((\text{C-mmol/L})\),
\( C_g \) is the concentration of glucose \((\text{C-mmol/L})\),
\( f_{gl} \) is the glycogen fraction of the active biomass, \( f_{gl}' = C_{gl} / C_s \),
\( C_{gl} \) is the concentration of glycogen \((\text{C-mmol/L})\),
\( f_{gl}^{max} \) is the maximum glycogen fraction of the active biomass.

The PHV formation from glycogen degradation is determined by the glycogen content of the bio-P bacteria. The specific reaction rate of glycogen degradation for PHV formation \( (q_{gh}) \) is assumed to be dependent linearly on the difference between the fraction of glycogen in the biomass \( (f_{gl}) \) and the minimum fraction of glycogen content
in the biomass. When $f_g$ approaches to the minimum glycogen fraction of bio-P bacteria, the specific rate $q_{glv}$ gradually decreases according to the following relation:

$$q_{glv} = k_{glv} \cdot \left(1 - \frac{f_g^{\text{min}}}{f_g}\right)$$  
Eq. 5-49

where $q_{glv}$ is the specific rate of PHV formation from glycogen (C-mmol/C-mmol · h),

$k_{glv}$ is the rate constant of PHV formation from glycogen (h$^{-1}$),

$f_g^{\text{min}}$ is the minimum glycogen fraction of the active biomass.

The formation of PHV from direct glucose conversion is also determined by the glucose concentration. This specific reaction rate ($q_g$) is assumed to be dependent on the concentration of glucose in the medium by a Monod-type relation:

$$q_g = q_g^{\text{max}} \cdot \frac{C_g}{K_g^{*} + C_g}$$  
Eq. 5-50

where $q_g$ is the specific rate of PHV formation from glucose direct conversion (C-mmol/C-mmol · h),

$q_g^{\text{max}}$ is the maximum specific rate of PHV formation from glucose direct conversion (C-mmol/C-mmol · h),

$K_g^{*}$ is the half saturation constant of PHV formation from glucose direct conversion (C-mmol/L).

It is assumed that there is a constant requirement of polyphosphate per unit of active biomass for maintenance purpose. $m_{an}$ is the specific anaerobic maintenance rate and is assumed to be constant (P-mmol/C-mmol · h).

We can now incorporate the expressions of the four specific reaction rates ($q_{glb}$, $q_{glv}$, $q_g$, and $m_{an}$) into the conversion rate of the key metabolic compounds based on Eq. 5-47b. The overall conversion rate of PHV ($\gamma_{phv}$) becomes:
\[
\gamma_{phv} = \frac{5}{6} q_{gh} C_x + \frac{5}{6} q_{gr} C_x = \frac{5}{6} k_{gh} \left(1 - \frac{f_{gl}^{\text{min}}}{f_{gl}} \right) C_x + \frac{5}{6} q_{gr}^{\text{max}} \frac{C_x}{K_{gr} + C_x} C_x 
\]
Eq. 5-51

The overall conversion rate of glycogen (\(\gamma_{gl}\)) becomes:

\[
\gamma_{gl} = q_{gl} C_x - q_{gh} C_x = q_{gl}^{\text{max}} \frac{C_x}{K_{gl} + C_x} \left(1 - \frac{f_{gl}}{f_{gl}^{\text{max}}} \right) C_x - k_{gh} \left(1 - \frac{f_{gl}^{\text{min}}}{f_{gl}} \right) C_x 
\]
Eq. 5-52

The overall conversion rate of PO₄-P (\(\gamma_p\)) becomes:

\[
\gamma_p = 0.129 q_{gl} C_x + m_{an} C_x = 0.129 q_{gl}^{\text{max}} \frac{C_x}{K_{gl} + C_x} \left(1 - \frac{f_{gl}}{f_{gl}^{\text{max}}} \right) C_x + m_{an} C_x 
\]
Eq. 5-53

The overall conversion rate of glucose (\(\gamma_g\)) becomes:

\[
\gamma_g = -q_{gl} C_x - q_{gr} C_x = -q_{gl}^{\text{max}} \frac{C_x}{K_{gl} + C_x} \left(1 - \frac{f_{gl}}{f_{gl}^{\text{max}}} \right) C_x - q_{gr}^{\text{max}} \frac{C_x}{K_{gr} + C_x} C_x 
\]
Eq. 5-54

### 5.2.2 Aerobic Mathematical Model

During the aerobic condition, there is no external organic substrate in the system. Therefore, the oxidation of PHV supplies the energy and carbon for different metabolic activities and the maintenance requirement of the bio-P bacteria. Based on the hypothesized aerobic metabolic model, six equations describing the aerobic reactions can be written with all the substrates and polymeric components expressed as per mole of carbon or per mole of phosphorus in their elemental formulas.

- **PHV Oxidation:**

Based on Eq. 5-20, the reaction of PHV oxidation can be expressed as:
-CH8\textsubscript{5}O2\textsubscript{5} -12/5\textit{NAD} - 6\textit{H}_2\textit{O} - 2/5\textit{H}_3\textit{PO}_4\textsuperscript{in} - 2/5\textit{ADP} + 12/5\textit{NADH}_2 + \textit{CO}_2 + 2/5\textit{ATP} \\
PHV

\[ = 0 \quad \text{Eq. 5-55} \]

- **Oxidative Phosphorylation:**
Based on the research of Smolders et al. (1994b), the amount of ATP produced per electron pair (\(\delta-\text{P/O}\)) is assumed to be 1.85 (mol-ATP/mol-O) during the oxidative phosphorylation. According to Eq. 5-21, the reaction of oxidative phosphorylation can then be expressed as:

\[-\text{NADH}_2 - 0.5\textit{O}_2 -1.85\textit{ADP} -1.85\textit{H}_3\textit{PO}_4\textsuperscript{in} + \textit{NAD} + 2.85 \textit{H}_2\textit{O} + 1.85\textit{ATP} = 0 \quad \text{Eq. 5-56} \]

- **Biomass Synthesis from PHV:**
The ATP requirement for active biomass synthesis, the K value, is assumed to be 1.5 mol ATP per C-mol active biomass (Smolders et al., 1994b). Based on Eq. 5-22, the equation of biomass synthesis from PHV is expressed as:

\[-1.27\text{CH}_{8.5}\text{O}_{2.5} - 0.2\textit{NH}_3 - (2.012 + m_{\text{arp}/\mu}) \textit{H}_2\textit{O} - 0.8055\textit{NAD} - (1.5 + m_{\text{arp}/\mu})\textit{ATP} \]

\[+ \text{CH}_{2.09}\text{O}_{0.54}\text{N}_{0.2}\text{P}_{0.015} + 0.27\textit{CO}_2 + 0.8055\textit{NADH}_2 + (1.5 + m_{\text{arp}/\mu})\textit{ADP} \]

\[+ (1.485 + m_{\text{arp}/\mu}) \textit{H}_3\textit{PO}_4\textsuperscript{in} = 0 \quad \text{Eq. 5-57} \]

- **Phosphate Transport into the bio-P bacteria:**
Again, based on Smolders et al. (1994b), the amount of phosphate, \(\xi\), that can be transported for each mole of consumed \(\textit{NADH}_2\) is 7 P-mol/\(\textit{NADH}_2\)-mol; and based on Eq. 5-23, the reaction of phosphate transport into the bio-P bacteria is expressed as:

\[-\text{H}_3\textit{PO}_4\textsuperscript{out} - (1/7)\textit{NADH}_2 - (1/14)\textit{O}_2 + \text{H}_3\textit{PO}_4\textsuperscript{in} + (1/7)\textit{H}_2\textit{O} + (1/7)\textit{NAD} = 0 \quad \text{Eq. 5-58} \]

- **Polyphosphate Synthesis:**
Based on Eq. 5-24, the reaction of polyphosphate synthesis can be expressed as:
-H$_3$PO$_4^{in}$ - ATP + 2HPO$_3$ + H$_2$O + ADP = 0

polyphosphate

\[ \text{Eq. 5-59} \]

- **Glycogen Synthesis from PHV:**

Based on Eq. 5-30, the reaction of glycogen synthesis from PHV can be expressed as:

\[ \begin{array}{c}
-5/3\text{CH}_8\text{O}_5\text{O}_{25} \text{PHV} - 2\text{NAD} - 7/3\text{H}_2\text{O} - 5/6\text{ATP} + \text{CH}_8\text{O}_5\text{O}_{56} + 2\text{NADH} + 2/3\text{CO}_2 + 5/6\text{H}_3\text{PO}_4^{in} \\
\text{Glycogen} + 5/6\text{ADP} = 0 \end{array} \]

\[ \text{Eq. 5-60} \]

**5.2.2.1 Aerobic Stoichiometric Coefficients**

Similar to the anaerobic mathematical model, the conversion rates of the key aerobic metabolic compounds can be expressed as a function of the internal reaction rates by a set of linear equations with parameters composed of the stoichiometric coefficients of the internal reactions (Roles, 1983). The reaction rates of Eq. 5-55 to Eq. 5-60 are assumed to be v1 to v6 and expressed with the transposed reaction rate matrix $\mathbf{v}^T$:

\[ \mathbf{v}^T = (v1, v2, v3, v4, v5, v6) \]  

\[ \text{Eq. 5-61} \]

There are 14 compounds appearing in the above 6 reactions; however, ATP/ADP and NADH$_2$/NAD are paired compounds, ADP and NAD are eliminated to simplify the derivation. The overall conversion rates of the remaining 12 compounds consist of the transposed reaction rate matrix $\gamma^T$:

\[ \gamma^T = (\gamma_{\text{phv}}, \gamma_{gl}, \gamma_{pp}, \gamma_x, \gamma_n, \gamma_{pin}, \gamma_{pout}, \gamma_{co}, \gamma_{ho}, \gamma_{\text{NADH}}, \gamma_{\text{ATP}}, \gamma_o) \]  

\[ \text{Eq. 5-62} \]

where $\gamma$ is the overall conversion rate of the key metabolic compounds, and the subscripts stand for: phv = PHV, gl = glycogen, pp = polyphosphate, x = active biomass, n = NH$_3$, pin = internal phosphate, pout = external phosphate, co = CO$_2$, ho = H$_2$O, NADH = NADH$_2$, ATP = ATP, o = O$_2$. 

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From the above 6 aerobic reactions, an aerobic stoichiometry matrix \( \alpha^{\text{aer}} \) can be expressed with each element in \( \alpha^{\text{aer}} \) equal to the stoichiometric coefficient for a specific compound in the corresponding reaction.

\[
\alpha^{\text{aer}} = \begin{pmatrix}
v1 & v2 & v3 & v4 & v5 & v6 \\
\gamma_{\text{PHV}} & -1 & 0 & -1.27 & 0 & 0 & -5/3 \\
\gamma_{\text{Gl}} & 0 & 0 & 0 & 0 & 0 & +1 \\
\gamma_{\text{Gp}} & 0 & 0 & 0 & 0 & +2 & 0 \\
\gamma_{\text{x}} & 0 & 0 & 1 & 0 & 0 & 0 \\
\gamma_{\text{n}} & 0 & 0 & -0.2 & 0 & 0 & 0 \\
\gamma_{\text{pin}} & -2/5 & -1.85 & 1.485 + m_{\text{ATP}}/\mu & +1 & -1 & +5/6 \\
\gamma_{\text{dout}} & 0 & 0 & 0 & -1 & 0 & 0 \\
\gamma_{\text{co}} & +1 & 0 & +0.27 & 0 & 0 & +2/3 \\
\gamma_{\text{ho}} & -6/5 & +2.85 & -2.012 - m_{\text{ATP}}/\mu & +1/7 & +1 & -7/3 \\
\gamma_{\text{o}} & 0 & -0.5 & 0 & -1/14 & 0 & 0 \\
\gamma_{\text{NADH}} & +12/5 & -1 & +0.8055 & -1/7 & 0 & +2 \\
\gamma_{\text{ATP}} & +2/5 & +1.85 & -1.5 - m_{\text{ATP}}/\mu & 0 & -1 & -5/6
\end{pmatrix}
\]

Twelve linear relations can then be obtained by writing out the compound balances for each of the twelve compounds (PHV to ATP).

\[
\gamma^T = \alpha^{\text{aer}} \cdot v
\]

There are twelve linear equations above including six reaction rates \((v_1 - v_6)\) and twelve compound conversion rates, resulting in eighteen unknowns. Based on known biochemical regulations, several assumptions are made to simplify the problem:

- The change of reducing power is assumed to be 0, no net increase or decrease of NADH during the metabolic reaction, i.e., \( \gamma_{\text{NADH}} = 0 \);
- The change of ATP is assumed to be 0, no net increase or decrease of ATP during the metabolic reaction, i.e., \( \gamma_{\text{ATP}} = 0 \);
- The change of internal PO4-P is assumed to be 0, no net increase or decrease of internal PO4-P during the metabolic reaction, i.e., \( \gamma_{\text{pin}} = 0 \);
The system can now be simplified with twelve linear equations and fifteen unknowns, which means three degrees of freedom. With the measurements of $15-12 = 3$ unknown rates, all the remaining conversion rates in the system can be described. The three overall conversion rates, $\gamma_x$, $\gamma_{pp}$, $\gamma_{gl}$, are selected to solve the expressions of other unknowns in the twelve linear equations. During the aerobic condition, $\gamma_x$, $\gamma_{pp}$, $\gamma_{gl}$ are the independent synthesis rates of active biomass, polyphosphate and glycogen. The solutions of other conversion rates are presented as follows:

$$\gamma_{phv} = 1.27 \gamma_x + (0.211 m_{sp}/\mu) \gamma_x + 0.158 \gamma_{pp} + 1.072 \gamma_{gl}$$  \hspace{1cm} \text{Eq. 5-65}

$$\gamma_0 = 0.409 \gamma_x + (0.247 m_{sp}/\mu) \gamma_x + 0.29 \gamma_{pp} + 0.189 \gamma_{pp}$$  \hspace{1cm} \text{Eq. 5-66}

$$\gamma_{pout} = \gamma_{pp} + 0.015 \gamma_x$$  \hspace{1cm} \text{Eq. 5-67}

$$\gamma_n = 0.2 \gamma_x$$  \hspace{1cm} \text{Eq. 5-68}

$$\gamma_{ho} = 0.299 \gamma_x + (0.168 m_{sp}/\mu) \gamma_x + 1.126 \gamma_{pp} + 0.031 \gamma_{gl}$$  \hspace{1cm} \text{Eq. 5-69}

$$\gamma_{co} = 0.27 \gamma_x + (0.211 m_{sp}/\mu) \gamma_x + 0.158 \gamma_{pp} + 0.072 \gamma_{gl}$$  \hspace{1cm} \text{Eq. 5-70}

Therefore, all other conversion rates of the key metabolic components are expressed by $\gamma_x$, $\gamma_{pp}$, $\gamma_{gl}$ in Eqs. 5-65 to 5-70. Eq. 5-65 indicates that the aerobic PHV conversion rate is associated with four factors (on the right side of the equation): the active biomass growth ($\gamma_x$), the maintenance ($\gamma_x$ with $m_{sp}/\mu$), the polyphosphate synthesis ($\gamma_{pp}$) and the glycogen synthesis ($\gamma_{gl}$). The ratio of $\gamma_{phv}$ to the conversion rate of one of the factors specifies their relevant stoichiometric relations. For example, it can be inferred, from the ratio of $\gamma_{phv}$ to $\gamma_x$ in Eq. 5-65, $1.27$ C-mol of PHV will be consumed (negative sign) for the production of 1 C-mol of active biomass and $(0.211 m_{sp}/\mu)$ C-mol of PHV will be consumed for the maintenance of 1 C-mol of active biomass; from the ratio of $\gamma_{phv}$ to $\gamma_{pp}$ in Eq. 5-65, $0.158$ C-mol of PHV will be oxidized for the synthesis of 1 P-mol of polyphosphate; and from the ratio of $\gamma_{phv}$ to $\gamma_{gl}$ in Eq. 5-65, $1.072$ C-mol of PHV will be consumed for the production of 1 C-mol of glycogen. Similarly, Eqs. 5-66 to 5-70 express the aerobic conversion rates of $O_2$, $P_{out}$, $NH_3$, $H_2O$ and $CO_2$ by several or all of these four factors.
From Eqs. 5-65 to 5-70 it can be seen that during the aerobic condition, PHV is oxidized for four metabolic activities: active biomass growth, polyphosphate synthesis, glycogen synthesis and aerobic maintenance. These four metabolic activities are the governing aerobic reactions that can be built with the components and the relevant stoichiometric relations in Eqs. 5-65 to 5-70. The four governing aerobic reactions are described individually below:

- **Active Biomass Growth:**

  This aerobic reaction describes active biomass growth by utilizing PHV as both the energy and the carbon sources. In Eqs. 5-65 to 5-70, if any compound by the rate expression (at the left side of the equations) is associated with $\gamma_x$ (not related with $m_{ap/\mu}$), it is used to build the active biomass growth reaction with the relevant stoichiometry. For example, in Eq. 5-65, PHV is found to be associated with $\gamma_x$ with a stoichiometric relation of $-1.27$ C-mol PHV/C-mol active biomass; in Eq. 5-66, $O_2$ is found to be associated with $\gamma_x$ with a stoichiometric relation of $-0.409$ mol $O_2$/C-mol of active biomass. Similarly, $NH_3$, $H_2O$ and $CO_2$ are also found to be associated with $\gamma_x$ in Eqs. 5-67 to 5-70 respectively with the relevant stoichiometric relations. The reaction of active biomass growth can be expressed as:

$$-1.27 \text{CH}_ {0.95}\text{O}_ {0.25} - 0.409 \text{O}_2 - 0.015 \text{H}_3\text{PO}_4 - 0.2 \text{NH}_3 + \text{CH}_ {2.09}\text{O}_ {0.34}\text{N}_ {0.2}\text{P}_ {0.015} + 0.27 \text{CO}_2 + 0.299 \text{H}_2\text{O} = 0$$

Eq. 5-71

- **Polyphosphate Synthesis**

  The energy for the synthesis of polyphosphate during the aerobic condition is also derived from the PHV oxidation. The components and stoichiometric relations in this equation are found in Eqs. 5-65 to 5-70 for the synthesis of 1 P-mol of polyphosphate, which is expressed as:

$$-0.158\text{CH}_ {8.5}\text{O}_ {2.5} - 0.189\text{O}_2 - \text{H}_3\text{PO}_4 + \text{HPO}_3 + 0.158\text{CO}_2 + 1.126\text{H}_2\text{O} = 0$$

Eq. 5-72
• **Glycogen Synthesis:**
The energy and the carbon source for the synthesis of glycogen are also derived from the oxidation of the internal reserve of PHV. The components and stoichiometric relations in this equation can also be found in Eqs. 5-65 to 5-70 for the synthesis of 1 C-mol of glycogen, which is expressed as:

\[-1.072 \text{CH}_8\text{S}_5\text{O}_{2/5} - 0.29 \text{O}_2 + \text{CH}_5\text{S}_3\text{O}_{5/6} + 0.072 \text{CO}_2 + 0.031 \text{H}_2\text{O} = 0\]  
Eq. 5-73

• **Maintenance:**
It is assumed that the aerobic maintenance energy is derived directly from the oxidation of PHV. In Eqs. 5-65 to 5-70, if any compound by the rate expression (at the left side of the equations) is associated with $\gamma_x$ (related with $m_{\text{arp}}/\mu$), it is used to build the aerobic maintenance reaction with the relevant stoichiometry. The aerobic reaction for the maintenance of 1 C-mol of active biomass is expressed as:

\[-(0.211m_{\text{arp}}/\mu)\text{CH}_8\text{S}_5\text{O}_{2/5} - (0.247m_{\text{arp}}/\mu)\text{O}_2 + (0.211m_{\text{arp}}/\mu)\text{CO}_2 + (0.168m_{\text{arp}}/\mu)\text{H}_2\text{O} = 0\]  
Eq. 5-74a

Eq. 5-74a is simplified by divided by $(0.211m_{\text{arp}}/\mu)$:

\[-\text{CH}_8\text{S}_5\text{O}_{2/5} - 1.171\text{O}_2 + \text{CO}_2 + 0.796\text{H}_2\text{O} = 0\]  
Eq. 5-74b

The aerobic metabolism is now encompassed by these four internal equations: (1) Eq. 5-71 for active biomass growth; (2) Eq. 5-72 for polyphosphate synthesis; (3) Eq. 5-73 for glycogen synthesis; and (4) Eq. 5-74b for maintenance reaction.

**5.2.2.2 Aerobic Kinetic Modeling**
The matrix of the stoichiometric coefficients from the aerobic reactions ($\alpha^{\text{arp}}$) can now be revised to be composed of the coefficients from the newly built Eqs. 5-71 to 5-73, and 5-74b. Assuming $\mu$ is the specific biomass growth rate (Eq. 5-70), $q_{pp}$ is the specific rate of polyphosphate synthesis (Eq. 5-71), $q_{gl}$ is the specific rate of glycogen synthesis.
(Eq. 5-72), and \( m_{aer} \) is the specific rate for maintenance (Eq. 5-74b). The specific aerobic reaction rates can be expressed by the matrix \( q^{aer} \):

\[
q^{aer} = \begin{pmatrix}
\mu \\
q_{pp} \\
q_{gl} \\
m_{aer}
\end{pmatrix}
\]

Eq. 5-75

The aerobic conversion rate of the key metabolic components (\( \gamma^{aer} \)) can then be calculated according to Eq. 5-76a (Roels, 1983):

\[
\gamma^{aer} = \alpha^{aer} \cdot q^{aer} \cdot C_x
\]

Eq. 5-76a

The structured aerobic kinetics by Eq. 5-76a is expressed as:

\[
\begin{array}{cccc}
\mu & q_{gl} & q_{pp} & m_{aer} \\
\hline
+1 & 0 & 0 & 0 \\
-1.27 & -1.072 & -0.158 & -1 \\
0 & 0 & +1 & 0 \\
0 & +1 & 0 & 0 \\
\end{array}
\]

\[
\times
\begin{array}{cccccc}
\gamma_x & \gamma_{phv} & \gamma_{pp} & \gamma_{gl} & \gamma_p & \gamma_o & \gamma_{co} & \gamma_{ho} \\
\hline
1 & 1 & -1 & 1 & -1.27 & -0.409 & +0.27 & +0.299 \\
0 & -1 & 0 & 1 & 0.015 & -0.2 & +0.072 & +0.031 \\
0 & 0 & 1 & -1 & -1 & 0 & +0.158 & +1.126 \\
0 & 0 & 0 & 0 & 0 & 0 & 0.1 & +0.796 \\
\end{array}
\times C_x
\]

Eq. 5-76b

where \( \gamma^{aer} \) is the matrix of the aerobic conversion rates of the key metabolic compounds, \( \alpha^{aer} \) is the matrix of the aerobic stoichiometric coefficients,
\( q_{aer} \) is the matrix of the aerobic specific reaction rates,
\( \mu \) is the specific active biomass growth rate (C-mmol/C-mmol \cdot h),
\( q_{gl} \) is the specific glycogen synthesis rate (C-mmol/C-mmol \cdot h),
\( q_{pp} \) is the specific polyphosphate synthesis rate (P-mmol/C-mmol \cdot h),
\( m_{aer} \) is the specific aerobic maintenance rate (C-mmol/C-mmol \cdot h),
\( \gamma_x \) is the overall aerobic active biomass growth rate (C-mmol/L \cdot h),
\( \gamma_n \) is the overall aerobic NH\( _3 \) conversion rate (mmol NH\( _3 \)/L \cdot h),
\( \gamma_o \) is the overall aerobic O\( _2 \) conversion rate (mmol O\( _2 \)/L \cdot h).

In Eq. 5-76b, the conversion rates in \( \gamma_{aer} \) are determined by the overall effects from the four specific reaction rates multiplied by the relevant stoichiometric coefficients and the active biomass concentration. For example, the aerobic conversion rates of PHV (\( \gamma_{phv} \)) and glycogen are expressed as:

\[
\gamma_{phv} = -1.27 \cdot \mu \cdot C_x - 1.072 \cdot q_{gl} \cdot C_x - 0.158 \cdot q_{pp} \cdot C_x - m_{aer} \cdot C_x \quad \text{Eq. 5-76c}
\]

\[
\gamma_{gl} = 0 \cdot \mu \cdot C_x + 1 \cdot q_{gl} \cdot C_x + 0 \cdot q_{pp} \cdot C_x + 0 \cdot m_{aer} \cdot C_x = q_{gl} \cdot C_x \quad \text{Eq. 5-76d}
\]

From the above two examples, it can be seen that for the calculation of the conversion rate of the key metabolic components, the stoichiometric coefficients are known from the biochemical analyses. The specific aerobic maintenance rate (\( m_{aer} \)) is also assumed to be constant, in other words a constant amount of PHV is oxidized for the maintenance of a unit amount of active biomass per unit time. The unknown variables in the equations are the other three specific reaction rates (\( \mu, q_{gl}, q_{pp} \)) and the active biomass concentration that need to somehow be determined in order to solve the conversion rates of the key metabolic components.

The growth of the bio-P bacteria in the aerobic phase is primarily determined by the PHV content of the cells. The specific active biomass growth rate \( \mu \) (in C-mmol active
biomass produced per C-mmol active biomass per hour) is assumed to rely solely on the internal reserve PHV, which is described using first-order kinetics:

\[ \mu = k_x f_{phv} \quad \text{Eq. 5-77} \]

where \( \mu \) is the specific active biomass growth rate (C-mmol/C-mmol·h),
\( k_x \) is the first order constant of the active biomass growth rate (h\(^{-1}\)),
\( f_{phv} \) is the PHV fraction of the active biomass, \( f_{phv} = C_{phv} / C_x \),
\( C_{phv} \) is the concentration of PHV (C-mmol/L).

The specific synthesis rate of polyphosphate, \( q_{pp} \), is assumed to be determined by three factors: the external phosphate concentration (\( C_p \)), the PHV content (\( f_{phv} \)), and the polyphosphate content (\( f_{pp} \)) of the cells. If the external phosphate concentration is too high, the synthesis rate of polyphosphate becomes saturated. If the external phosphate concentration is negligible, the synthesis rate of polyphosphate becomes zero. The dependence of the specific polyphosphate synthesis rate on the external phosphate concentration is expressed by a conventional Monod-type relation. The specific polyphosphate synthesis rate is further assumed to depend on the PHV content of the cells. If the PHV fraction in the cell decreases, the rate of polyphosphate synthesis decreases also, and when the PHV fraction is exhausted, the P uptake and polyphosphate synthesis will stop. It is assumed that \( q_{pp} \) depends linearly on the PHV content of the active biomass. Finally, the polypshosphate synthesis rate depends on the fraction of polyphosphate stored in the cells: \( f_{pp} \). The phosphate uptake capacity of the bio-P bacteria is limited to a certain extent, and therefore when the maximum polyphosphate content of the cells (\( f_{pp\,\text{max}} \)) is reached, the uptake rate of phosphate will become zero. A linear decrease of \( q_{pp} \) with the increase of \( f_{pp} \) is assumed. The specific polyphosphate synthesis rate (\( q_{pp} \)) is expressed as:

\[ q_{pp} = k_{pp\,\text{max}} \frac{C_p}{K_p + C_p} (1 - \frac{f_{pp}}{f_{pp\,\text{max}}}) f_{phv} \quad \text{Eq. 5-78} \]
where \( q_{pp} \) is the specific polyphosphate synthesis rate (P-mmol/C-mmol \cdot h),
\( k_{pp}^{\text{max}} \) is the maximum specific rate of polyphosphate synthesis (P-mmol/C-mmol \cdot h),
\( K_{ps} \) is the half saturation constant of polyphosphate synthesis rate (P-mmol/L),
\( C_p \) is the concentration of \( \text{PO}_4 \)-P in the medium (P-mmol/L),
\( f_{pp} \) is the polyphosphate fraction of the active biomass, \( f_{pp} = C_{pp} / C_x \),
\( C_{pp} \) is the concentration of polyphosphate (P-mmol/L),
\( f_{pp}^{\text{max}} \) is the maximum polyphosphate fraction of the active biomass.

The specific synthesis rate of glycogen is assumed to be determined by both the content of PHV and glycogen of the cell. It is assumed that \( q_{gl} \) depends linearly on the PHV content of the active biomass. The glycogen synthesis rate also depends linearly on the fraction of glycogen stored in the cells, \( f_{gl} \). When the maximum glycogen content inside the cells \( (f_{gl}^{\text{max}}) \) is reached, the synthesis of glycogen will become zero. \( q_{gl} \) is expressed as:

\[
q_{gl} = k_{gl} f_{phv} \left( 1 - \frac{f_{gl}}{f_{gl}^{\text{max}}} \right) \tag{Eq. 5-79}
\]

where \( q_{gl} \) is the specific glycogen synthesis rate (C-mmol/C-mmol \cdot h),
\( k_{gl} \) is the rate constant of aerobic glycogen synthesis (h\(^{-1}\)).

\( m_{aer} \), as already discussed, is assumed to be the constant specific aerobic maintenance rate (C-mmol/C-mmol \cdot h).

We can now write the aerobic conversion rates of the key metabolic compounds by expanding Eq. 5-46b with these specific reaction rate expressions \((\mu, q_{pp}, q_{gl}, m_{aer})\):

\[
\gamma_x = \mu C_x = k_x f_{phv} C_x \tag{Eq. 5-80}
\]

\[
\gamma_{gl} = q_{gl} C_x = k_{gl} f_{phv} \left( 1 - \frac{f_{gl}}{f_{gl}^{\text{max}}} \right) C_x \tag{Eq. 5-81}
\]
\[
\gamma_{pp} = q_{pp} C_x = k_{pp}^{\max} \frac{C_p}{K_{ps} + C_p} (1 - \frac{f_{pp}}{f_{pp}^{\max}}) f_{phv} C_x 
\]
Eq. 5-82

\[
\gamma_{p} = -0.015 q_{pp} C_x = -0.015 k_x f_{phv} C_x - k_{pp}^{\max} \frac{C_p}{K_{ps} + C_p} (1 - \frac{f_{pp}}{f_{pp}^{\max}}) f_{phv} C_x 
\]
Eq. 5-83

\[
\gamma_{phv} = -1.27 q_{pp} C_x - 1.072 k_{x} f_{phv} C_x - 0.158 q_{pp} C_x - m_{aer} C_x 
\]
\[= -1.27 k_{x} f_{phv} C_x - 1.072 k_{x} f_{phv} (1 - \frac{f_{gl}}{f_{gl}^{\max}}) C_x - 0.158 k_{pp}^{\max} \frac{C_p}{K_{ps} + C_p} (1 - \frac{f_{pp}}{f_{pp}^{\max}}) f_{phv} C_x - m_{aer} C_x \]
Eq. 5-84

5.3 Optimum Parameter Estimation
Since the experiments were conducted in a sequential batch reactor, the conversion rates of the controlled components can be expressed using the following equation:

\[
\gamma = \frac{dC}{dt} 
\]
Eq. 5-85

where \(\gamma\) is the conversion rate of the key metabolic component (mmol/L \cdot h),

\(C\) is the concentration of the key metabolic component (mmol/L).

With the two sets of kinetic equations: Eq. 5-51 to Eq. 5-54 for the anaerobic conversion rates and Eq. 5-80 to Eq. 5-84 for the aerobic conversion rates, the concentration changes of the controlled components during both the anaerobic and aerobic phases of the SBR can be calculated by the integration of Eq. 5-85 over the length of anaerobic or aerobic reaction time (0 – t). However, the kinetic constants \((k_{glv}, K_{gs}, q_{glv}^{\max}, q_{gl}^{\max}, K_{glv}, m_{an}, f_{gl}^{\min}, f_{gl}^{\max})\) from the anaerobic equations and the kinetic constants \((k_o, k_{gl}, k_{pp}^{\max}, K_{ps}, f_{pp}^{\max}, m_{aer})\) from the aerobic reactions are unknown parameters that need to be somehow determined before the kinetic calculations can be conducted.

For the parameter estimation purposes, five independent sets of batch experiments were conducted with different initial glucose levels (0, 100, 250, 500, 1000 mg/L) added into reactor G at the start of the experiments. The glucose and phosphate levels in the medium, the glycogen and PHA contents of the biomass and the MLSS/MLVSS levels
were monitored at planned intervals throughout the whole anaerobic and aerobic reactions. The data sets derived in this way were used to fit the kinetic parameters by the least square, nonlinear regression technique. This is done through the calculation of the concentration profiles of all components during the anaerobic and aerobic phase and adjusting the parameters to minimize the squares of the differences between the experimental results and the simulated results from the kinetic model.

An EXCEL spreadsheet procedure was applied here to conduct this nonlinear least square calculation. The ordinary differential equations were solved for the concentration profiles of the controlled compounds applying Runge-Kuta, 4th order numerical procedure in the spreadsheet of EXCEL. The time step for this simulation was selected to be 10 seconds to guarantee calculation convergence. The least squares of the differences between the experimental results and the simulated results were solved by the SOLVER function in the EXCEL program to find out the optimum kinetic constants.

5.3.1 Anaerobic Parameter Estimation

During the anaerobic phase, glucose uptake, PHA accumulation, glycogen conversion, and PO4-P release were monitored during the five sets of batch experiments. These results were used for least square methods to find out the optimum anaerobic kinetic constants. The eight unknown kinetic constants ($k_{glv}, K_{gp}, q_{glv}^{max}, q_{gl}^{max}, K_{glis}, m_{an}, f_{gl}^{min},$ and $f_{gl}^{max}$) were estimated by the nonlinear least square method. The optimum constants obtained through this procedure are listed in Table 5.1

Figure 5.6 shows the simulation results using these optimum kinetic constants in Table 5.1 for the case of 250 mg/L of initial glucose concentration. Figure 5.7 compares all the anaerobic simulation results with all the experimental results at differential initial glucose concentrations.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Rate Constant of PHV Formation from Glycogen</td>
<td>$k_{glv}$</td>
<td>0.074</td>
<td>1/h</td>
</tr>
<tr>
<td>The Half Saturation Constant of PHV Formation from Glucose</td>
<td>$K_{gs}$</td>
<td>1.95</td>
<td>C-mmol/L</td>
</tr>
<tr>
<td>The Maximum Specific PHV Formation Rate from Glucose</td>
<td>$q_{glv}^{max}$</td>
<td>14.10</td>
<td>C-mmol/C-mmol·h</td>
</tr>
<tr>
<td>The Maximum Glycogen Accumulation Rate</td>
<td>$q_{gl}^{max}$</td>
<td>699.7</td>
<td>C-mmol/C-mmol·h</td>
</tr>
<tr>
<td>The Half Saturation Constant of Glycogen Accumulation</td>
<td>$K_{glu}$</td>
<td>104.1</td>
<td>C-mmol/L</td>
</tr>
<tr>
<td>The Specific Anaerobic Maintenance Rate</td>
<td>$M_{an}$</td>
<td>0.0013</td>
<td>P-mmol/C-mmol·h</td>
</tr>
<tr>
<td>The Minimum Glycogen Fraction of the Active Biomass</td>
<td>$f_{gl}^{min}$</td>
<td>0.100</td>
<td>C-mmol/C-mmol</td>
</tr>
<tr>
<td>The Maximum Glycogen Fraction of the Active Biomass</td>
<td>$f_{gl}^{max}$</td>
<td>0.34</td>
<td>C-mmol/C-mmol</td>
</tr>
</tbody>
</table>
Figure 5.6. Kinetic Simulation of the Glucose Induced EBPR Metabolism during the Anaerobic Condition (a): PHV Simulation; (b): Glycogen Simulation; (Initial concentrations of glucose and PO$_4$-P were 250 mg/L and 7.5 mg/L)
Figure 5.6. (Cont.) Kinetic Simulation of the Glucose Induced EBPR Metabolism during the Anaerobic Condition (c): PO₄-P Simulation; (d): Glucose Simulation (Initial concentrations of glucose and PO₄-P were 250 mg/L and 7.5 mg/L)
5.3.2 Aerobic Parameter Estimation

During the aerobic phase, PO₄-P uptake, PHA degradation, and glycogen synthesis were monitored during the batch experiments. There are six unknown kinetic constants ($k_o$, $k_g$, $k_{pp}^{max}$, $K_{pa}$, $f_{pp}^{max}$, $m_{aer}$) which need to be estimated by the nonlinear least square method. The five sets of aerobic batch experiments were conducted directly after the anaerobic reactions in reactor G. During these experiments, the content of active biomass and polyphosphate were not measured, but their values are required for the simulation of the aerobic dynamics of PHV, glycogen and PO₄-P. In order to simulate the dynamics of active biomass and polyphosphate, their initial values at the start of the aerobic condition must be available. The initial value of active biomass for the
simulation was estimated by two steps: firstly, the volatile part of the active biomass was estimated from the measured VSS value after the corrections for the measured glycogen and the measured PHV at the start of the aerobic reaction (based on the MLSS categorization described in Figure 5.4); secondly, the non-volatile part of the active biomass is assumed to be phosphorus that accounts for 1.8% of the total active biomass (based on the active biomass formula: CH\textsubscript{2.09}O\textsubscript{0.54}N\textsubscript{0.02}P\textsubscript{0.015}), therefore, the total active biomass equals to the estimated volatile part of the active biomass divided by 0.982. The initial value of polyphosphate for the simulation was evaluated from the measured MLSS after the correction of the estimated initial active biomass value and the measured initial PHV value and the measured initial glycogen value (also based on the relationships of internal reserves and active biomass in Figure 5.4).

Based on these estimated initial values, the simulations of active biomass and polyphosphate can be conducted applying Eq. 5-80 and Eq. 5-82. Because the experimental results were not available, the simulated active biomass and polyphosphate were not used for the solution of the optimum aerobic parameters. The optimum aerobic kinetic parameters were solved by finding the least squares of the differences between the experimental results and the simulated results of PHV, glycogen and PO\textsubscript{4}-P. The optimum aerobic kinetic parameters are listed in Table 5.2.

Table 5.2. Kinetic Parameters for Aerobic Reactions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Rate Constant of Active Biomass Growth</td>
<td>(k_x)</td>
<td>0.147</td>
<td>(1/h)</td>
</tr>
<tr>
<td>The Rate Constant of Aerobic Glycogen Synthesis</td>
<td>(k_{gl})</td>
<td>1.27</td>
<td>(1/h)</td>
</tr>
<tr>
<td>The Maximum Specific Rate of Polyphosphate Synthesis</td>
<td>(k_{pp}^{\text{max}})</td>
<td>0.200</td>
<td>P-mmol/C-mmol (\cdot) (\text{h})</td>
</tr>
<tr>
<td>The Half Saturation Constant of Polyphosphate Synthesis</td>
<td>(K_{ps})</td>
<td>0.0032</td>
<td>P-mmol/L</td>
</tr>
<tr>
<td>The Maximum Polyphosphate Fraction of The Active Biomass</td>
<td>(f_{pp}^{\text{max}})</td>
<td>0.30</td>
<td>P-mmol/C-mmol</td>
</tr>
<tr>
<td>The Specific Aerobic Maintenance Rate</td>
<td>(m_{aer})</td>
<td>0.004</td>
<td>C-mmol/C-mmol (\cdot) (\text{h}).</td>
</tr>
</tbody>
</table>

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Figure 5.8 shows the simulation results using these optimum kinetic constants in Table 5.2 for the case of 250 mg/L of initial glucose concentration. Figure 5.9 compares all the aerobic simulation results with all the batch experimental results at different initial glucose concentrations.

(a)

Figure 5.8. Kinetic Simulation of the Glucose Induced EBPR Metabolism during the Aerobic Condition (a): PHV Simulation (Initial glucose and PO₄-P concentrations were 250 mg/L and 7.5 mg/L)
Figure 5.8. (Cont.) Kinetic Simulation of the Glucose Induced EBPR Metabolism during the Aerobic Condition (b): Glycogen Simulation; (c): PO$_4$-P Simulation (Initial glucose and PO$_4$-P concentrations were 250 mg/L and 7.5 mg/L).
Figure 5.9. Comparison of the Simulation and the Experimental Results at Several Initial Glucose Concentrations during the Aerobic Condition (a): PHV; (b): Glycogen; (c): PO$_4$-P
5.4 Kinetic Parameter Uncertainty Analysis

Monte Carlo simulation is utilized to analyze the sensitivity of the proposed mathematical model. The Monte Carlo method consists of "sampling" to create many artificial data sets that are analyzed statistically to learn how well a model performs. The evaluation of the mathematical model with Monte Carlo "sampling" is carried out by the following procedure.

- Monitoring the changes of the four components (PHA, glycogen, PO_{4}P and glucose) during the normal SBR operations in reactor G using glucose as the single substrate.
- Repeating such experiment 5 times.
- Calculating the means and the average percentage differences of the measured results for each of the components at each specified measuring time from the 5 replicate experiments.

The mean for a controlled component at a specific measuring time is calculated by Eq.5-56:

\[
x = \frac{\sum_{i=1}^{5} x_i}{5}
\]

Eq. 5-86

Where \(x_i\) is the \(i\)th measurement for a controlled component;

The average percentage difference for a controlled component at a specific measuring time is calculated by Eq. 5-87:

\[
\text{Diff.}(\%) = \frac{\sum_{i=1}^{5} \frac{|x_i - \bar{x}|}{\bar{x}}}{5}
\]

Eq. 5-87

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• Carrying out Monte Carlo “Sampling” to produce “artificial experimental results” using computer generated random numbers with a uniform distribution (EXCEL).

The “artificial experimental results” are generated according to Eq. 5-88 in EXCEL:

\[
\text{Simulated Result} = (1-\text{Diff.(%)}) \cdot \bar{x} + 2 \cdot \text{Diff.(%)} \cdot \bar{x} \cdot \text{Rand()} \quad \text{Eq. 5-88}
\]

Where \( \text{Rand()} \) is the uniformly distributed random number between (0,1), generated by EXCEL.

• Repeating the Monte Carlo “Sampling” for 20 times to generate 20 sets of “artificial experimental data”.

• Using the 20 sets of “artificial experimental data” to reproduce the kinetic constants of the anaerobic and aerobic reactions applying the nonlinear least square method.

• Analyzing all the obtained kinetic constants statistically to give the uncertainty range of each of the kinetic constants.

All of the statistical analysis results are presented in Table 5.3.

To further understand the developed mathematical model, a comparison between the kinetic parameters from the acetate induced EBPR model (Smolders et al., 1995) and some of the kinetic parameters from the new glucose induced EBPR model was conducted and is show in Table 5.4.
Table 5.3. Statistical Analysis of the Parameters Estimated from the Monte Carlo “Sampling”

<table>
<thead>
<tr>
<th>Anaerobic Parameter</th>
<th>Unit</th>
<th>Values Applied in Model</th>
<th>Means Calculated from Monte Carlo Samplings</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Limit</td>
</tr>
<tr>
<td>$k_{gh}$</td>
<td>1/h</td>
<td>0.074</td>
<td>0.074</td>
<td>0.070</td>
</tr>
<tr>
<td>$K_{gs}$</td>
<td>C-mmol/L</td>
<td>1.95</td>
<td>1.75</td>
<td>1.53</td>
</tr>
<tr>
<td>$q_{gV}^{max}$</td>
<td>C-mmol /C-mmol·h</td>
<td>14.10</td>
<td>15.70</td>
<td>13.50</td>
</tr>
<tr>
<td>$q_{gl}^{max}$</td>
<td>C-mmol /C-mmol·h</td>
<td>699.7</td>
<td>699.5</td>
<td>699.0</td>
</tr>
<tr>
<td>$K_{gl}$</td>
<td>C-mmol/L</td>
<td>104.1</td>
<td>106.1</td>
<td>104.3</td>
</tr>
<tr>
<td>$m_{an}$</td>
<td>P-mmol/C-mmol·h</td>
<td>0.0013</td>
<td>0.0014</td>
<td>0.0013</td>
</tr>
<tr>
<td>$f_{gl}^{min}$</td>
<td>C-mmol /C-mmol</td>
<td>0.100</td>
<td>0.100</td>
<td>0.099</td>
</tr>
<tr>
<td>$f_{gl}^{max}$</td>
<td>C-mmol /C-mmol</td>
<td>0.340</td>
<td>0.336</td>
<td>0.327</td>
</tr>
<tr>
<td>Aerobic Parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{x}$</td>
<td>1/h</td>
<td>0.147</td>
<td>0.129</td>
<td>0.102</td>
</tr>
<tr>
<td>$k_{gl}$</td>
<td>1/h</td>
<td>1.27</td>
<td>1.32</td>
<td>1.25</td>
</tr>
<tr>
<td>$k_{pp}^{max}$</td>
<td>P-mmol/C-mmol·h</td>
<td>0.200</td>
<td>0.204</td>
<td>0.196</td>
</tr>
<tr>
<td>$K_{ps}$</td>
<td>P-mmol/L</td>
<td>0.0032</td>
<td>0.0030</td>
<td>0.0028</td>
</tr>
<tr>
<td>$f_{pp}^{max}$</td>
<td>P-mmol/C-mmol</td>
<td>0.30</td>
<td>0.29</td>
<td>0.27</td>
</tr>
<tr>
<td>$m_{aer}$</td>
<td>C-mmol/C-mmol·h</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Anaerobic Parameters</td>
<td>Symbol</td>
<td>Value</td>
<td>Symbol</td>
<td>Value</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>The maximum specific substrate conversion rate (C-mmol/C-mmol·h)</td>
<td>$q_s^{\text{max}}$</td>
<td>0.43</td>
<td>$q_{gv}^{\text{max}}$</td>
<td>14.10</td>
</tr>
<tr>
<td>The half saturation constant of substrate conversion (C-mmol/L)</td>
<td>$K_s$</td>
<td>1.60</td>
<td>$K_{gs}$</td>
<td>1.95</td>
</tr>
<tr>
<td>The specific anaerobic maintenance rate (P-mmol/C-mmol·h)</td>
<td>$m_{an}$</td>
<td>0.025</td>
<td>$m_{an}$</td>
<td>0.0013</td>
</tr>
<tr>
<td>Aerobic Parameters</td>
<td>Symbol</td>
<td>Value</td>
<td>Symbol</td>
<td>Value</td>
</tr>
<tr>
<td>The rate constant of active biomass growth (1/h)</td>
<td>$k_x$</td>
<td>0.165</td>
<td>$k_x$</td>
<td>0.147</td>
</tr>
<tr>
<td>The maximum specific rate of polyphosphate synthesis (P-mmol/C-mmol·h)</td>
<td>$k_{pp}^{\text{max}}$</td>
<td>0.55</td>
<td>$k_{pp}^{\text{max}}$</td>
<td>0.20</td>
</tr>
<tr>
<td>The half saturation constant of polyphosphate synthesis (P-mmol/L)</td>
<td>$K_{ps}$</td>
<td>0.1</td>
<td>$K_{ps}$</td>
<td>0.0032</td>
</tr>
<tr>
<td>The maximum polyphosphate fraction of the active biomass (P-mmol/C-mmol)</td>
<td>$f_{pp}^{\text{max}}$</td>
<td>0.30</td>
<td>$f_{pp}^{\text{max}}$</td>
<td>0.30</td>
</tr>
<tr>
<td>The specific aerobic maintenance rate (C-mmol/C-mmol·h)</td>
<td>$m_{aer}$</td>
<td>0.004</td>
<td>$m_{aer}$</td>
<td>0.004</td>
</tr>
<tr>
<td>The maximum glycolgen fraction of the active biomass (C-mmol/C-mmol)</td>
<td>$f_{gl}^{\text{max}}$</td>
<td>0.27</td>
<td>$f_{gl}^{\text{max}}$</td>
<td>0.34</td>
</tr>
</tbody>
</table>

From Table 5.4, it can be seen that even though the metabolisms of the acetate system and the glucose system are different, many of the parameter constants can be compared to reveal the metabolic differences and similarity between the two systems. During the anaerobic condition, the maximum specific substrate conversion rate in the acetate model is related only to the maximum specific rate of acetate conversion to PHB ($q_s^{\text{max}}$), which is 0.43 C-mmol/C-mmol·h. However, the maximum specific substrate
conversion rate in the glucose model is composed of two factors: the maximum specific rates of glucose direct conversion to PHV \( q_{gV}^{\text{max}} \) and glucose conversion to glycogen \( q_{gl}^{\text{max}} \), which are 14.1 and 699.7 C-mmol/C-mmol \cdot h \) respectively. The much higher total glucose conversion rate is agreeable to the experimental observations that even at very high initial glucose concentration (up to 1000 mg/L), glucose was instantly removed from the medium by bio-P bacteria. However, the specific anaerobic maintenance rate is much bigger in the acetate model than in the glucose model.

During the aerobic condition, the acetate model and the glucose model share many similarities. The rate constants of active biomass growth \( (k_a) \) are very close in both models. The maximum specific rate of polyphosphate synthesis \( (k_{pp}^{\text{max}}) \) is much higher in the acetate model \( (0.55 \text{ P-mmol/C-mmol} \cdot \text{h}) \) than it in the glucose model \( (0.2 \text{ P-mmol/C-mmol} \cdot \text{h}) \), which is agreeable to the experimental results that much more \( \text{PO}_4^{3-} \text{P} \) could be removed by reactor A than reactor G during the aerobic condition. The maximum polyphosphate fraction of the active biomass \( (f_{pp}^{\text{max}}) \) and the specific aerobic maintenance rate \( (m_{aer}) \) are exactly the same between the two models. However, the maximum glycogen fraction of the active biomass \( (f_{gl}^{\text{max}}) \) is higher in the glucose model \( (0.34 \text{ C-mmol/C-mmol}) \) than in the acetate model \( (0.27 \text{ C-mmol/C-mmol}) \), which can also be explained by the fact that the bio-P bacteria could accumulate more glycogen when glucose was used as the dominant organic substrate in reactor G.

5.5 Conclusions of the Mathematical Modeling

In this study, a metabolic model has been developed to simulate the dynamics of the enhanced biological phosphorus removal using glucose as the single substrate. Based on the proposed theoretical model, only four anaerobic reactions and four aerobic reactions with fourteen kinetic constants are required to describe all the dynamics of the key metabolic compounds in the glucose induced EBPR system. This model demonstrates more clarity and simplicity than many other complex mathematical models. The development of the stoichiometric coefficients for the reactions was totally based on fundamental biochemical principles, which should allow the resulting model to be applied to a wider range of operating conditions. From the comparison of the simulation
results and the experimental results, it can be seen that the developed model not only can predict the dynamics of the external components (such as glucose and PO₄-P in the medium), it also gives very good predictions of the changes of important internal reserves (such as glycogen and PHA). The successful simulation results support that the hypothesized biochemical model probably agrees with the actual internal mechanisms of the glucose induced EBPR process.

However, from the results, it can also be seen that the model underestimated the resynthesis of glycogen during the aerobic condition, and over-estimated PO₄-P release during the anaerobic condition. These inconsistencies might result from the uncertainty of the measurements or some unresolved mechanisms of the glucose induced EBPR system. For instance, the assumption that all the accumulated PHA is in PHV form is not exactly true. However, due to the limitation of not being able to find the sink to balance the reducing powers produced from PHB production, PHV was selected as the single form of PHA accumulated in the anaerobic condition. Even though this assumption is fairly accurate, it could contribute to the errors observed here. Also, the measurements of PHV and glycogen suffered from high uncertainty, which influences the ability to obtain more accurate kinetic constants for this mathematical model. It is expected that with better experimental procedures, the developed mathematical model could be further improved in the future.
CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions
The key conclusions from the results of this research are as follows:

- Aside from SCFAs, glucose can also be used to develop a successful EBPR performance with an anaerobic/aerobic cycling reaction system. Therefore, SCFAs are not the only kind of substrates that can induce a successful EBPR mechanism.

- Compared with the development of EBPR performance using acetate as the dominant substrate, the development of EBPR performance using glucose as the dominant substrate appeared to be very slow and unstable. Special operating procedures have been developed and successfully applied to induce and sustain a stable EBPR process with glucose as the dominant substrate in the influent. These new procedures are extended anaerobic reaction time, higher glucose concentration in the influent and shorter aerobic reaction time with limited DO level. The reduced DO supply and shorter aerobic reaction time may give the glucose system an advantage of economical power consumption over the acetate system.

- In the EBPR system using glucose, the majority of the organic substrate is directly transported into the bio-P bacteria and converted to PHA and glycogen in the anaerobic condition. Glycogen is consumed gradually during the rest of the anaerobic reaction for the accumulation of PHA. Some fermentation also happens with the release of lactic acid into the medium. Compared with the acetate system, it is clear that during the anaerobic condition in the glucose system, reduced PO₄-P is released into the medium, lower PHA is accumulated for every absorbed organic carbon, and 3-HV enriched PHA, rather than 3-HB enriched PHA, is accumulated.
• Metabolic studies indicate that reduced PO₄-P release in the glucose system is associated with less polyphosphate hydrolysis during the anaerobic condition. The lower PHA accumulation and the reduced utilization of polyphosphate as the energy source are probably the causes of the unstable EBPR performance in the glucose system. Besides, in the anaerobic condition, the accumulation of glycogen after the addition of glucose might also induce the replacement of the energy role from polyphosphate to glycogen, which results in the quick breakdown of the EBPR system.

• The formation of 3-HV predominated PHA in the glucose system is very well explained by the reducing power balance during the metabolism of the microorganisms. From the biochemical pathway, it can be seen that the formation of 3-HV serves as the sink for the electrons generated from the glycolysis of the absorbed glucose.

• Other experimental results indicate that the bacteria can potentially perform denitrification under anoxic conditions in glucose system. The microorganism identifications showed similar bacterial compositions in both the acetate and glucose EBPR systems, and Aeromonas hydrophilia was found to be the predominant species in both systems. It seems that the composition of the primary microorganism(s) might not be affected by the organic substrate whenever the EBPR performance is established in wastewater treatment system. It is also probable that a few groups of bacteria work together to fulfil the EBPR mechanism and Aeromonas hydrophilia is the group that plays the most important role.

• It is confirmed that, in the glucose system, more anaerobic PO₄-P release will happen at higher pH values (above pH 7). The overly released PO₄-P at higher pH levels cannot be completely removed during the aerobic reaction. Lower pH levels (lower that pH 7) result in less polyphosphate hydrolysis and the breakdown of the EBPR process. Therefore, it is very important to maintain neutral pH during the EBPR reaction.

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• Applying fundamental biochemistry knowledge to the experimental results, a new biochemical model is hypothesized to explain the metabolism of an EBPR system using glucose as the single substrate. Based on this theoretical model, a mathematical model is established with only four anaerobic reactions and four aerobic reactions to simulate the metabolisms of the glucose induced EBPR system. The development of the stoichiometric coefficients of the reactions was based on fundamental biochemical principles. The developed metabolic model simulates successfully the dynamics of the important components from the EBPR system using glucose as the single substrate.

6.2 Recommendations

A few recommendations for possible future studies are given as follows:

• One limitation of the proposed metabolic model is that the sink for the reducing power from the anaerobic PHB formation was not found and all of the PHA accumulated was assumed to be PHV. It is expected that finding the reducing power sink for PHB formation can greatly improve the understanding of the glucose induced EBPR system and the proposed metabolic model.

• In this research, it was found that Aeromonas hydrophilia was the predominant species in both acetate and glucose systems. However, it is reasoned that a few groups of bacteria work together to fulfil the EBPR mechanism since the pure culture of Aeromonas hydrophilia did not present the fundamental characteristics of an EBPR process. A future microbial study should be undertaken to investigate how different groups of bacteria work together to fulfil EBPR.

• It is expected to further elucidate the metabolic characteristics of an EBPR process if both glucose and acetate are added together as multi-substrates. Special testing methods should be developed to trace the different metabolic fates of glucose and acetate in the same P removal system. It would also be necessary to develop a new
metabolic model to predict the conversions of important internal reserves and other compounds during the multi-substrate EBPR process.

- In the future, more advanced analytical methods should be used for the measurements of polyphosphate and glycogen inside of bio-P bacteria. With these improved measurements, it would be possible to improve the metabolic model such that it could be used as a useful tool to predict under what operating conditions the EBPR process might fail.

- The mathematical model in this research was developed from the bench-scale SBR experiments and future study should be undertaken to extend this model to simulate the dynamics of full-scale continuous EBPR operation using glucose as the dominant organic substrate.
REFERENCES


Gottschalk, G. 1986. *Bacterial Metabolism*. Springer-Verlag Newyork, INC.


APPENDIX A: EXPERIMENTAL RESULTS

Table A.1. PO₄-P Changes in the Influent and Effluent for Reactors A and G during the Development of EBPR Performance

Table A.2. Anaerobic Released PO₄-P and PO₄-P Removal Efficiency during the Development of EBPR Performance in Reactor G

Table A.3. Batch Experiment Results (5 replicate runs) from Reactor A with Stable EBPR Performance

Table A.4. Batch Experiments Results (5 replicate runs) from Reactor G with Stable EBPR Performance

Table A.5. PHA Compositions from 5 Replicate Batch Experiments (Run 1 to 5) in Both Reactors A and G with Stable EBPR Performances

Table A.6. Batch Experimental Results with Varying Initial Glucose Concentrations in Reactor G with Stable EBPR

Table A.7. Batch Experimental Results with Varying Initial PO₄-P Concentrations in Reactor G with Stable EBPR Performance

Table A.8. Batch Experimental Results with NO₃-N (50 mg/L) Added during the Middle of the Anaerobic Reaction in Reactor G

Table A.9. Batch Experimental Study of Lactic Acid Release during the Anaerobic Condition in Reactor G with Stable EBPR Performance

Table A.10. SBR Experimental Study of pH Effects on PO₄-P Anaerobic Release and Aerobic Uptake in Reactor G with Stable EBPR Performance
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Table A.3. Batch Experiment Results (5 replicate runs) from Reactor A with Stable EBPR Performance (Initial concentrations of sodium acetate and PO₄-P were 250 mg/L and 7.5 mg/L)

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Table A.4. Batch Experiments Results (5 replicate runs) from Reactor G with Stable EBPR Performance (Initial concentrations of glucose and PO4-P were 250 mg/L and 7.5 mg/L)

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Table A.4. (Continue) Batch Experiments Results (5 replicate runs) from Reactor G with Stable EBPR Performance (Initial concentrations of glucose and PO₄-P were 250 mg/L and 7.5 mg/L)

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Table A.5. PHA Compositions from 5 Replicate Batch Experiments (Run 1 to 5) in Both Reactors A and G with Stable EBPR Performances (Initial concentrations of acetate and PO₄-P were 250 mg/L and 7.5 mg/L for reactor A; initial concentrations of glucose and PO₄-P were 250 mg/L and 7.5 mg/L for reactor G)

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<td>4.80</td>
<td>18.20</td>
<td>5.65</td>
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<td>4.85</td>
<td>21.90</td>
<td>4.80</td>
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<td>30.11</td>
<td>4.67</td>
<td>23.18</td>
<td>4.98</td>
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<td>5.24</td>
<td>22.38</td>
<td>5.10</td>
<td>23.90</td>
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<td>31.67</td>
<td>5.48</td>
<td>27.03</td>
<td>5.20</td>
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<td>5.33</td>
<td>25.37</td>
<td>5.45</td>
<td>30.21</td>
</tr>
<tr>
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<td>3.82</td>
<td>15.15</td>
<td>3.15</td>
<td>12.30</td>
<td>3.25</td>
<td>13.35</td>
<td>3.50</td>
<td>13.82</td>
<td>3.10</td>
<td>12.56</td>
</tr>
<tr>
<td>4</td>
<td>1.38</td>
<td>4.87</td>
<td>1.74</td>
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<td>1.70</td>
<td>6.54</td>
<td>1.83</td>
<td>6.14</td>
<td>1.92</td>
<td>5.61</td>
</tr>
<tr>
<td>4.5</td>
<td>0.97</td>
<td>2.57</td>
<td>1.18</td>
<td>2.82</td>
<td>1.22</td>
<td>4.04</td>
<td>1.30</td>
<td>3.14</td>
<td>1.21</td>
<td>3.23</td>
</tr>
<tr>
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<td>1.83</td>
<td>0.85</td>
<td>2.15</td>
<td>0.68</td>
<td>1.63</td>
<td>0.70</td>
<td>1.94</td>
<td>0.72</td>
<td>1.63</td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>1.35</td>
<td>0.83</td>
<td>1.87</td>
<td>0.70</td>
<td>1.60</td>
<td>0.65</td>
<td>1.65</td>
<td>0.94</td>
<td>1.18</td>
</tr>
</tbody>
</table>
Table A.6. Batch Experimental Results with Varying Initial Glucose Concentrations in Reactor G with Stable EBPR (5 runs, with initial glucose concentrations ranging from 0 to 1000 mg/L; initial PO₄-P was kept at 7.5 mg/L)

| Time (hr) | Run1 | Run2 | Run3 | Run4 | Run5 | Run1 | Run2 | Run3 | Run4 | Run5 |
|----------|------|------|------|------|------|------|------|------|------|------|------|
|          | C-mg/g MLSS |      |      |      |      | C-mg/g MLSS |      |      |      |      |      |
| 0        | 1.63 | 1.79 | 2.06 | 2.21 | 1.93 | 50.79 | 58.12 | 39.79 | 59.90 | 54.82 |
| 0.0833   | 3.09 | 11.50 | 15.44 | 45.72 | 55.20 | 48.25 | 61.55 | 59.15 | 79.86 | 122.27 |
| 0.5      | 4.15 | 15.43 | 22.75 | 54.77 | 90.72 | 46.69 | 48.07 | 53.66 | 68.20 | 110.78 |
| 1        | 4.80 | 16.66 | 25.26 | 58.99 | 108.67 | 46.09 | 46.14 | 51.36 | 59.26 | 92.40 |
| 1.5      | 5.00 | 19.75 | 27.76 | 63.80 | 113.14 | 46.09 | 43.79 | 50.70 | 50.72 | 63.82 |
| 2        | 5.75 | 20.62 | 30.02 | 66.58 | 119.49 | 45.09 | 43.30 | 48.14 | 48.07 | 81.33 |
| 2.5      | 5.67 | 22.53 | 33.66 | 75.04 | 119.75 | 44.22 | 39.27 | 32.56 | 46.79 | 66.96 |
| 3        | 1.61 | 8.55 | 16.93 | 40.80 | 69.26 | 46.11 | 50.20 | 48.72 | 59.15 | 74.40 |
| 4        | 1.60 | 7.50 | 7.78 | 20.90 | 35.14 | 47.46 | 51.91 | 49.38 | 61.04 | 79.84 |
| 5        | 1.37 | 3.85 | 4.32 | 16.39 | 22.33 | 47.96 | 56.09 | 51.37 | 64.94 | 90.40 |
| 5.5      | 1.09 | 2.98 | 2.59 | 5.81 | 14.30 | 48.63 | 57.63 | 53.07 | 70.32 | 96.32 |
| 6        | 1.08 | 2.21 | 2.36 | 4.66 | 7.53 | 48.71 | 58.02 | 56.59 | 76.09 | 105.31 |

| Time (hr) | Run1 | Run2 | Run3 | Run4 | Run5 | Run1 | Run2 | Run3 | Run4 | Run5 |
|----------|------|------|------|------|------|------|------|------|------|------|------|
|          | P-mg/L |      |      |      |      | C-mg/L |      |      |      |      |      |
| 0        | 7.50 | 7.50 | 7.50 | 7.50 | 7.50 | 0.00 | 40.00 | 100.00 | 200.00 | 400.00 |
| 0.0833   | 10.00 | 12.00 | 12.50 | 18.91 | 27.807 | 0.00 | 1.62 | 2.31 | 6.84 | 20.96 |
| 0.5      | 11.30 | 16.50 | 21.00 | 26.04 | 35.061 | 0.00 | 1.60 | 2.21 | 6.12 | 5.48 |
| 1        | 12.50 | 17.98 | 27.00 | 30.60 | 38.285 | 0.00 | 1.64 | 1.63 | 5.52 | 4.96 |
| 1.5      | 13.00 | 17.50 | 28.00 | 38.40 | 47.431 | 0.00 | 1.58 | 1.58 | 5.20 | 4.80 |
| 2        | 13.00 | 21.00 | 28.50 | 39.99 | 49.872 | 0.00 | 1.48 | 1.39 | 4.80 | 4.24 |
| 2.5      | 14.00 | 16.00 | 27.20 | 40.38 | 52.7 | 0.00 | 1.30 | 1.34 | 4.67 | 3.64 |
| 3        | 7.75 | 12.00 | 20.00 | 20.00 | 33.5 | 0.00 | 0.89 | 1.16 | 4.12 | 3.64 |
| 4        | 7.74 | 1.00 | 8.99 | 7.66 | 23.5 | 0.00 | 0.65 | 1.11 | 3.01 | 3.36 |
| 5        | 7.13 | 0.00 | 4.40 | 4.00 | 6.4 | 0.00 | 0.60 | 0.97 | 2.91 | 2.88 |
| 5.5      | 7.25 | 0.00 | 0.10 | 0.00 | 0.00 | 0.00 | 0.38 | 0.88 | 2.86 | 2.76 |
| 6        | 7.13 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.38 | 0.33 | 2.66 | 2.52 |
Table A.7. Batch Experimental Results with Varying Initial PO_{4}-P Concentrations in Reactor G with Stable EBPR Performance (5 runs, with initial PO_{4}-P concentrations ranging from 0 to 30 mg/L; initial glucose concentration was kept at 250 mg/L)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>PO_{4}-P P-mg/L</th>
<th>PHA C-mg/g MLSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>0 0.0833</td>
<td>3.50</td>
<td>4.80</td>
</tr>
<tr>
<td>0.5</td>
<td>11.00</td>
<td>13.50</td>
</tr>
<tr>
<td>1</td>
<td>16.00</td>
<td>18.00</td>
</tr>
<tr>
<td>1.5</td>
<td>18.00</td>
<td>20.50</td>
</tr>
<tr>
<td>2</td>
<td>18.40</td>
<td>22.00</td>
</tr>
<tr>
<td>2.5</td>
<td>18.50</td>
<td>23.50</td>
</tr>
<tr>
<td>3</td>
<td>14.50</td>
<td>17.00</td>
</tr>
<tr>
<td>4</td>
<td>2.50</td>
<td>5.34</td>
</tr>
<tr>
<td>5</td>
<td>0.00</td>
<td>2.30</td>
</tr>
<tr>
<td>5.5</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table A.8. Batch Experimental Results with NO_{3}-N (50 mg/L) Added during the Middle of the Anaerobic Reaction in Reactor G (initial glucose and PO_{4}-P concentrations were 250 mg/L and 7.5 mg/L)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>NO_{3}-N mg/L</th>
<th>PO_{4}-P mg/L</th>
<th>Glycogen C-mg/g MLSS</th>
<th>PHA C-mg/g MLSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0.0833</td>
<td>7.50</td>
<td>45.20</td>
<td>3.09</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>12.50</td>
<td>58.80</td>
<td>11.53</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.00</td>
<td>52.80</td>
<td>20.55</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>27.00</td>
<td>51.20</td>
<td>25.50</td>
<td></td>
</tr>
<tr>
<td>1.5833</td>
<td>50.00</td>
<td>50.00</td>
<td>28.05</td>
<td></td>
</tr>
<tr>
<td>1.75</td>
<td>38.80</td>
<td>24.00</td>
<td>25.25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30.49</td>
<td>23.40</td>
<td>17.85</td>
<td></td>
</tr>
<tr>
<td>2.25</td>
<td>25.04</td>
<td>21.00</td>
<td>18.49</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>23.00</td>
<td>20.30</td>
<td>18.00</td>
<td></td>
</tr>
<tr>
<td>2.75</td>
<td>22.50</td>
<td>20.30</td>
<td>14.66</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20.00</td>
<td>21.00</td>
<td>12.75</td>
<td></td>
</tr>
<tr>
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<td>20.20</td>
<td>18.00</td>
<td>13.26</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>25.00</td>
<td>12.00</td>
<td>6.38</td>
<td></td>
</tr>
<tr>
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<td>34.00</td>
<td>9.30</td>
<td>3.83</td>
<td></td>
</tr>
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<td>36.00</td>
<td>7.10</td>
<td>2.93</td>
<td></td>
</tr>
<tr>
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<td>38.00</td>
<td>5.60</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>
Table A.9. Batch Experimental Study of Lactic Acid Release during the Anaerobic Condition in Reactor G with Stable EBPR Performance (initial glucose and PO₄-P concentrations were 250 mg/L and 7.5 mg/L)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Lactate (C-mg/L)</th>
<th>pH</th>
<th>TOC (C-mg/L)</th>
<th>Glucose (C-mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.1792</td>
<td>6.43</td>
<td>27.65</td>
<td>5.56</td>
</tr>
<tr>
<td>0.083</td>
<td>22.4</td>
<td>6.12</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>0.25</td>
<td>16.8</td>
<td>5.87</td>
<td>20.8</td>
<td>4.04</td>
</tr>
<tr>
<td>1</td>
<td>4.48</td>
<td>5.78</td>
<td>9.35</td>
<td>3.852</td>
</tr>
<tr>
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<td>5.75</td>
<td>2.35</td>
<td>3.116</td>
</tr>
<tr>
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<td>3.056</td>
</tr>
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<td>6.23</td>
<td>2.4</td>
<td>2.912</td>
</tr>
<tr>
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<td>2.3</td>
<td>2.008</td>
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<tr>
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<td>0</td>
<td>6.31</td>
<td>2.4</td>
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</table>

Table A.10. SBR Experimental Study of pH Effects on PO₄-P Anaerobic Release and Aerobic Uptake in Reactor G with Stable EBPR Performance (three pH values were maintained during the three experiments: pH = 6, 7 or 8)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>pH=6 PO₄-P (mg/L)</th>
<th>pH=7 PO₄-P (mg/L)</th>
<th>pH=8 PO₄-P (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7.50</td>
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<td>21.00</td>
<td>25.00</td>
</tr>
<tr>
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<td>18.40</td>
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<td>34.80</td>
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<td>18.60</td>
<td>27.00</td>
<td>36.40</td>
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<td>19.50</td>
<td>28.00</td>
<td>38.50</td>
</tr>
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<td>20.20</td>
<td>28.50</td>
<td>40.60</td>
</tr>
<tr>
<td>3</td>
<td>14.50</td>
<td>20.00</td>
<td>34.00</td>
</tr>
<tr>
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<td>13.00</td>
<td>9.00</td>
<td>28.00</td>
</tr>
<tr>
<td>4</td>
<td>11.60</td>
<td>4.40</td>
<td>23.50</td>
</tr>
<tr>
<td>4.5</td>
<td>9.50</td>
<td>1.40</td>
<td>19.00</td>
</tr>
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<td>16.00</td>
</tr>
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<td>5.00</td>
<td>0.03</td>
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APPENDIX B:

IMPORTANT METABOLIC PATHWAYS IN THE EBPR SYSTEM

I. The Embden-Meyerhof-Parnas Pathway

II. The Pathway of Microbial Glycogen Synthesis and Degradation

III. The Metabolic Pathway of PHB Synthesis

IV. The Metabolic Pathway of PHV Synthesis (the Succinate-Propionate Pathway)

V. The Entner-Douderoff Pathway

VI. The Methylcitrate Cycle

VII. The Tricarboxylic Acid (TCA) Cycle

VIII. The Glyoxylate Pathway

IX. The Gluconeogenesis

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I. The Embden-Meyerhof-Parnas Pathway

Glucose → ATP → ADP

Glucose-6-phosphate

Fructose-6-phosphate → ATP → ADP

Fructose-1, 6-diphosphate

Glyceraldehyde-3-phosphate ↔ Dihydroxyacetone-phosphate

2 NAD \rightarrow 2 \text{Pi}

2 NADH

2 1,3-diphosphoglyceric Acid

2 ADP

2 ATP

2 3-phosphoglyceric Acid

2 2-phosphoglyceric Acid

2 H₂O

2 Phosphoenolpyruvic Acid

2 ADP

2 ATP

2 Pyruvic Acid

(Pi: inorganic phosphate)

1 Adapted from Gaudy, 1978.
II. The Pathway of Microbial Glycogen Synthesis and Degradation\textsuperscript{2}

\begin{center}
\begin{tikzpicture}
\node (glycogen) at (0,0) {Glycogen};
\node (adp) at (0,-2) {ADP};
\node (pyrophosphate) at (0,-4) {Pyrophosphate};
\node (atp) at (0,-6) {ATP};
\node (h2o) at (2,-4) {$\text{H}_2\text{O}$};
\node (2pi) at (2,-6) {2 Pi};
\node (adp-glucose) at (0,-3) {ADP-glucose};
\node (glucose-1-phosphate) at (0,-5) {Glucose-1-phosphate};
\node (glucose-6-phosphate) at (0,-7) {Glucose-6-phosphate};
\node (pi) at (-2,-3) {Pi};
\end{center}

\textsuperscript{2} Adapted from Dawes and Senior, 1973.
III. The Metabolic Pathway of PHB Synthesis

2 Pyruvate
2 NAD $\rightarrow$ 2 CoASH
2 NADH$_2$ $\rightarrow$ 2 CO$_2$

2 Acetyl-CoA

CoASH

Acetoacetyl-CoA

NADH$_2$

NAD

3-hydroxybutyryl CoA

CoASH

PHB

---

3 Adapted from Dawes and Senior, 1973.
IV. The Metabolic Pathway of PHV Synthesis (Succinate-Propionate Pathway)  

\[
\begin{align*}
\text{Pyruvate} & \rightarrow \text{ATP} \rightarrow \text{CO}_2 + \text{H}_2\text{O} \\
& \downarrow \text{ADP} + \text{Pi} \\
\text{Oxaloacetate} & \rightarrow \text{NADH}_2 \\
& \downarrow \text{NAD} \\
\text{Malate} & \rightarrow \text{NADH}_2 \\
& \downarrow \text{H}_2\text{O} \\
\text{Fumarate} & \rightarrow \text{NADH}_2 \\
& \downarrow \text{NAD} \\
\text{Succinate} & \rightarrow \text{CoASH} \\
& \downarrow \text{H}_2\text{O} \\
\text{Succinyl-CoA} & \rightarrow \text{ATP} + \text{H}_2\text{O} \\
& \downarrow \text{ADP} + \text{Pi} \\
\text{Acetyl-CoA} & \rightarrow \text{CO}_2 \\
\text{Propionyl-CoA} & \rightarrow \text{NADH}_2 \\
& \downarrow \text{NAD} + 2 \text{CoASH} \\
\text{PHV} &
\end{align*}
\]

---

4 Adapted from Satoh et al., 1992.
V. The Entner-Douderoff Pathway

Glucose

ATP

ADP

Glucose-6-phosphate

H₂O

NAD

NADH₂

6-phosphogluconate

H₂O

2-keto-3-deoxy-6-phosphogluconate

Glyceraldehyde-3-phosphate

Pyruvic Acid

NAD

Pi

NADH₂

1,3-diphosphoglyceric Acid

ADP

ATP

3-phosphoglyceric Acid

2-phosphoglyceric Acid

H₂O

Phosphoenolpyruvic Acid

ADP

ATP

Pyruvic Acid

---

5 Adapted from Gaudy, 1978.
VI. The Methylcitrate Cycle

VI. The Methylcitrate Cycle

Propionyl-CoA

H₂O

CoASH

Oxaloacetate

NADH₂

NAD

Malate

H₂O

Fumarate

FADH₂

FAD

Methylcitrate

2-methylisocitrate

Pyrurate

Succinate

Adapted Susanne, 1997.
VII. The Tricarboxylic Acid (TCA) Cycle\textsuperscript{7}

\textsuperscript{7} Adapted from Gaudy, 1978.
VIII. The Glyoxylate Pathway

(* Acetyl-CoA enters the Glyoxylate pathway)

* Adapted from Gottschalk, 1986.
IX. The GLUCONEOGENESIS

Glucose
   ↑ Pi
Glucose-1-phosphate
   ↓
Glucose-6-phosphate
   ↓
Fructose-6-phosphate
   ↑ Pi
   ↓ H₂O
Fructose-1, 6-diphosphate
   ↓
Triose Phosphates
   ↑ 2 NAD + 2 Pi
   ↓ 2 NADH₂
   ↓ 2 ADP
2 1,3-diphosphoglycerate
   ↓ 2 ATP
2 3-phosphoglycerate
   ↓ 2 H₂O
2 Phosphoenolpyruvate
   ↓ 2 ADP
   ↓ 2 ATP
2 CO₂
2 Oxaloacetate

(Triose phosphates include: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate)

---

9 Adapted from Montgomery et al., 1990.