Polyhydroxyalkanoate Production within a Novel WRRF Configuration

A Thesis

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by

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Authorization to Submit Thesis

This thesis of Derek L. Probst, submitted for the degree of Master of Science with a Major in Civil Engineering and titled "Polyhydroxyalkanoate Production within a Novel WRRF Configuration," has been reviewed in final form. Permission, as indicated by the signatures and dates below, in now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

The perception of wastewater has evolved from being simply described as a waste product to a recoverable resource. Wastewater contains sources of soluble carbon that have the potential of being transformed to polyhydroxyalkanoates (PHA) and marketed as a consumer product. Polyhydroxyalkanoates are a biologically produced biodegradable thermoplastic with similar properties to polypropylene and polyethylene. This study investigates the potential of PHA production within the context of a novel post-anoxic wastewater treatment configuration (referred to as the BIOPHO-PX process, trademark under development by Dr. Erik Coats of the University of Idaho). The goal is to investigate the effects of treatment environments (anaerobic, aerobic, anoxic) on PHA production along with varying loading rates of volatile fatty acids (VFAs). In conclusion that biomass drawn from anaerobic environments and subjected to lower (e.g. Food:Microorganism ratio < 0.30 mgCOD/mgVSS) VFA loading rates maximize PHA accumulation, while maintaining a high (>0.75 Cmmol PHA/Cmmol VFA) PHA carbon yield.

Acknowledgements

Foremost I would like to acknowledge my major professor, Dr. Erik R. Coats, whose constant support and guidance made this thesis possible. I would also like to acknowledge my committee members Kevin Chang Ph.D. and Armando G. McDonald Ph.D. for their assistance in constructing this thesis and their analytical expertise.

I would also like to acknowledge Jason Mellin, Trevor Woodland, Regan Hansen, and Dmitriy Shimberg for their assistance in maintaining the BIOPHO-PX system and sample collection during the long PHA production assessments.

Dedication

This thesis is dedicated to my son, Ryan Probst, who patiently waited for his daddy to finish college; as well as my late grandmother, Judy Probst, who always encouraged me to expand my education.

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Chapter 1: Introduction

Wastewater is increasingly being viewed as a resource, not simply a "waste" product that must be disposed of. Indeed, the traditional name for a facility that collects, treats, and discharges municipal and industrial wastewater, Wastewater Treatment Plant (WWTP), has even been re-identified (by the Water Environment Federation) under a new title, Water Resource Recovery Facility (WRRF). As the name change implies, wastewater is no longer seen as an "end product" that needs to be simply "treated," but as a "resource" that can be "recovered" and used in the production of new and sustainable products. The WRRF can now be seen as a public facility with the ability to provide new services to the community that it serves. The primary service of collecting, treating, and discharging municipal and industrial wastewater still exists, but new and emerging services focus on the ability to return the collected resources from wastewater to the community in the forms of valuable commodities (e.g., reclaimed water, struvite (fertilizer), land applicable biosolids (fertilizer), and biodegradable biopolymers that share the same properties as common household petroleum based plastics). These new services provide, or will provide, commodities with the potential to concurrently reduce environmental impacts and offset WRRF operational costs.

The research conducted under this Thesis focused on advancing the WRRF concept for municipal wastewater. Specifically, this Thesis builds upon a new energy-saving nutrient removal process configuration (hereinafter referred to as the BIOPHO-PX system), with a specific emphasis on integrating resource recovery to produce biodegradable biopolymers. The BIOPHO-PX configuration is a post-anoxic biological nutrient removal process designed to achieve near-complete nitrogen and phosphorus removal. As opposed to conventional biological nutrient removal processes, the process targets aerobic nitritation, post-anoxic denitritation, and the enrichment of carbon storing organisms as a means to achieve energy savings and sustainability.

Investigations presented and discussed herein focused on leveraging the carbon storing organisms that would be enriched in the BIOPHO-PX process, with a specific

emphasis on establishing their capacity and ability to store excess carbon outside the confines of BIOPHO-PX (i.e., in a sidestream operational mode). Previous research has demonstrated that the BIOPHO-PX process enriches for the target carbon-storing microorganisms [*Appel*, 2015; *E. R. Coats et al.*, 2011; *Winkler et al.*, 2011]; these microbes have the potential to be utilized in the production of marketable consumer products. Specifically, these carbon storing organisms store soluble carbon as polymerized granules intracellularly. Biologically these granules, known as polyhydroxyalkanoates (PHA), serve as carbon and energy storage to increase the organism's competitive chances for survival against non-carbon storing organisms (a central tenet of the underlying enhanced biological phosphorus removal (EBPR) process within the BIOPHO-PX process). Industrially these granules, once extracted and purified, exhibit properties similar to many petroleum-based plastics. Biologically derived PHA provides two prominent benefits: (1) bioplastics reduce the demand for the conventional non-renewable petroleum and (2) PHA is completely biodegradable.

The capacity and ability of these EBPR organisms to store excess carbon as PHA was investigated further in this Thesis; to-date such investigations have not been considered. Indeed, the target microorganisms are enriched under starkly differing conditions in the EBPR configuration than would be applied to maximize PHA synthesis. Successful WRRF operations require the regular removal of biomass in order to maintain treatment process stability and resiliency. The removal of biomass maintains a specified solids retention time (SRT) (e.g., sludge age, mean cell retention time (MCRT)). In this research, organisms were transferred from the BIOPHO-PX process, following the SRT principle, into a tertiary process referred to as a PHA production reactor (PPR). PHA production occurs within the PPR by imposing transient substrate conditions in an operational mode referred to as aerobic dynamic feeding (ADF). Sustained PHA production occurs through extended duration ADF operations to accomplish maximum PHA accumulation. Recognizing the early stages of this resource recovery concept, research was conducted to address the following research question.

1.1 Research Questions

1. Is polyhydroxyalkanoate (PHA) production, achieved via the application of Aerobic Dynamic Feeding principles, affected by the treatment environmental condition within the BIOPHO-PX WRRF from which the inocula is withdrawn?
2. How does the Food-to-Microorganism (F:M) ratio, calculated as the quantity of volatile fatty acids (VFAs) fed to a microbial culture divided by the active biomass inocula, affect polyhydroxyalkanoate production using biomass obtained from the BIOPHO-PX WRRF?
3. Can PHA production within the BIOPHO-PX WRRF realize high (>60% d.c.w.) quantities of polyhydroxyalkanoates? Alternately, what quantity of PHA can be produced on inocula obtained from the BIOPHO-PX WRRF configuration?

Chapter 2: A Novel Approach to Wastewater Treatment

The conceptual evolution of WWTPs into WRRFs requires more than simple optimization of current WWTP operations. Indeed, novel, new approaches to wastewater treatment are required to achieve energy savings and resource recovery. In this regard, Chapter 2 introduces and describes the BIOPHO-PX WRRF configuration, with an emphasis on the ability to produce polyhydroxyalkanoates (PHA) bioplastics.

2.1 The Novel WRRF Configuration

To expand the economic potential of a WRRF, a novel post-anoxic biological nutrient removal (BNR) process was investigated that exhibits the potential to achieve reduced operational cost and increased nutrient removal efficiency while concurrently achieving bioplastic (i.e., PHA) production as a new resource recovery process. The core BNR process, referred to as BIOPHO-PX, has been previously investigated for its ability to remove nitrogen and phosphorus while achieving cost and energy savings when compared to conventional approaches. The BIOPHO-PX process (trademark in process) has been developed by the Coats environmental engineering lab group at the University of Idaho [*Appel*, 2015; *E. R. Coats et al.*, 2011; *Winkler et al.*, 2011]. Figure 2.1 details the BIOPHO-PX nutrient removal process and illustrates how bioplastic production will be integrated therein.

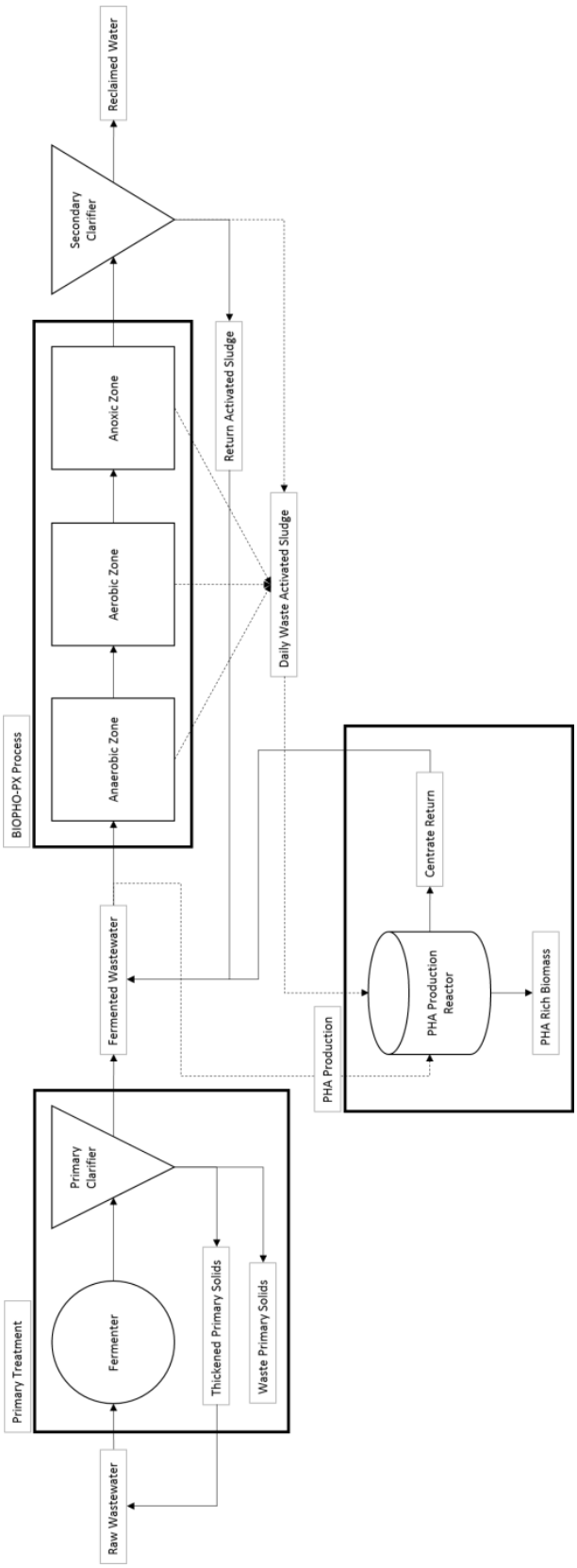

Figure 2.1: WRRF Process Diagram

The novel, integrated WRRF configuration is composed of three key core components: conventional Primary Treatment, Secondary Treatment – the BIOPHO-PX Process, and polyhydroxyalkanoate (PHA) Production. All three components are essential for the successful operations of this novel post-anoxic treatment process; however, as described, the focus of this Thesis is on PHA production. Nevertheless, to contextualize this Thesis, each of these process components is discussed in some detail below.

2.1.1 Primary Treatment

The purpose of primary treatment, within the context of the WRRF configuration (Figure 2.1), is to produce a volatile fatty acid (VFA) rich waste stream essential for both the BIOPHO-PX Process and PHA Production. Historically the objective of primary treatment was to remove readily settleable solids and floating material found in wastewater, thus reducing the suspended solids and organic load on the downstream secondary treatment process and helping achieve overall wastewater treatment. Efficiently designed and operated systems may remove 50 – 70 percent of suspended solids and 25 – 40 percent of the biochemical oxygen demand (BOD) [*Tchobanoglous et al.*, 2014]. Settleable solids represents a large portion of the organic material found in the wastewater. As contrasted with soluble nutrients, this organic material is typically slow to biodegrade and requires longer periods of digestion. Settleable organic material, when subjected to additional treatment (e.g., some form of solids treatment via digestion), is most often land applied as biosolids.

With the goal of resource recovery, organic-rich settleable solids can instead be recovered and utilized internally to enhance treatment processes and for PHA production. Thus, as contrasted with historical approaches, the end goal of the primary treatment system in this WRRF configuration is the transformation of the complex, settleable organic solid material to VFAs through anaerobic fermentation. VFAs represent a soluble and more readily biodegradable carbon source recovered from the slowly biodegradable particulate organic material and are produced within a specific basin called a fermenter.

Anaerobic fermentation involves three sets of reactions: particulate hydrolysis,

fermentation, and anaerobic oxidation. Particulate hydrolysis involves the breakdown of complex, settleable organics into smaller more biodegradable molecules (e.g., carbohydrate to simple sugar); hydrolysis is accomplished via extracellular enzymes excreted by hydrolytic microorganisms. The transformation of hydrolyzed compounds, via fermentation and anaerobic oxidation, produces long chain fatty acids, acetic acid, and soluble hydrogen (H_2). The microbial-based stoichiometry and reactions of fermentation and anaerobic oxidation are vast and complex, relying on syntrophic associations to occur. The products of these biochemical processes include acetate, propionate, and butyrate as the most commonly produced and stable of fermentation products [*Grady et al.*, 2011]. A simplification of the integrated biological processes is illustrated in Figure 2.2. These fermented products are essential for driving the phenomena of soluble phosphorus removal (central to the BIOPHO-PX process) and as a substrate for PHA production (described later in detail).

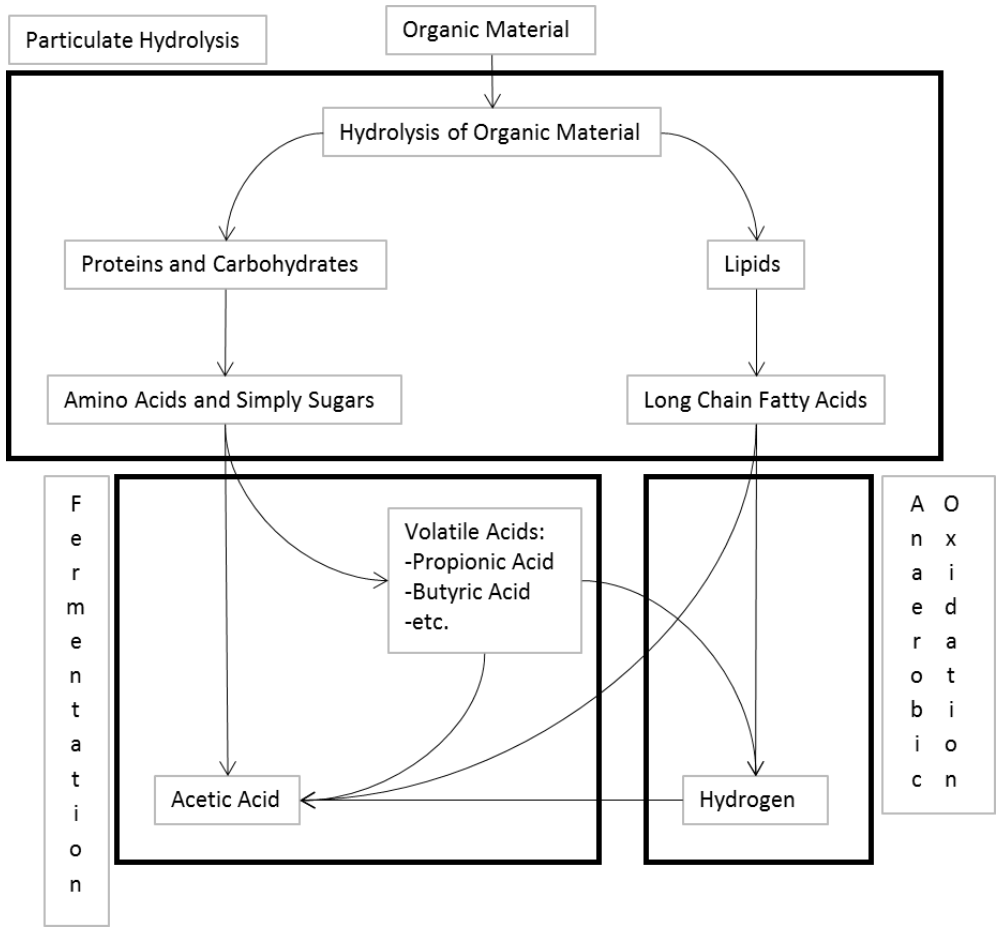

Figure 2.2: Fermentation Process [Grady et al., 2011]

2.1.2 Secondary Treatment – The BIOPHO-PX Process

The BIOPHO-PX process is the largest component of the novel WRRF configuration (Figure 2.1), and will accomplish the majority of the required biological nutrient removal (BNR), including nitrogen and phosphorus removal. The BIOPHO-PX process is a post-anoxic EBPR (Enhanced Biological Phosphorus Removal – process described in more detail below) based treatment configuration [*Appel*, 2015; *E. R. Coats et al.*, 2011; *Winkler et al.*, 2011]. Contrasted with more conventional EBPR processes (which focus exclusively on nutrient removal), the goal of BIOPHO-PX is to reduce WRRF operational costs (via aerobic nitritation) while concurrently maximizing N and P removal (via EBPR and post-anoxic denitritation), coupled with sustaining an enrichment of microorganisms that exhibit the potential to synthesize PHA. The concept of nitritation/denitritation coupled with post-anoxic operations represent the novel features of the BIOPHO-PX process.

2.1.2.1 Conventional BNR vs. BIOPHO-PX

Although no two municipalities or WRRFs are exactly alike, the industry has put its faith in a few basic treatment configurations. A 2007 fact sheet prepared by the United States Environmental Protection Agency (EPA) investigated the performance of different treatment processes and their relative costs [*EPA*, 2007]. The EPA found that the most common treatment processes in operation, with respect to achieving nitrogen and phosphorus removal, are the A^2O (Figure 2.3) and the Modified Bardenpho process (Figure 2.4). These processes are popular due to their efficiency in regard to reliably achieving nitrogen and phosphorus removal. Each process has distinct advantages and limitations (Table 2.1).

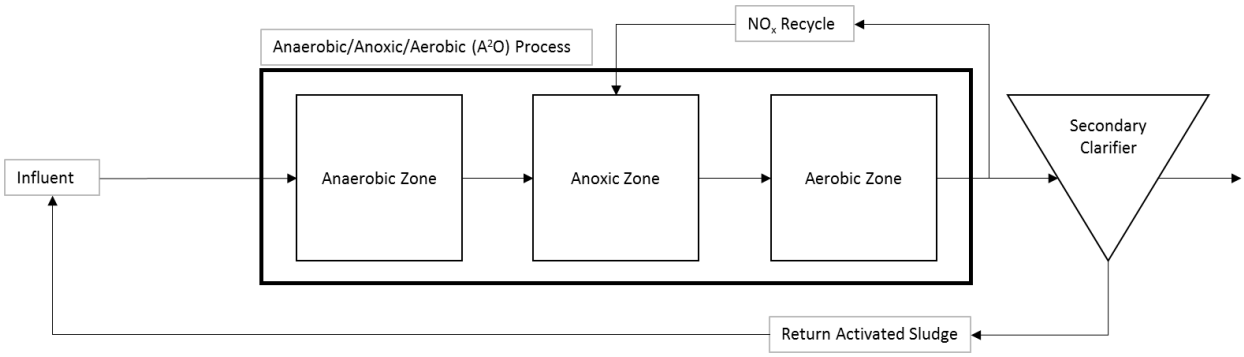

Figure 2.3: A^2O Process Diagram

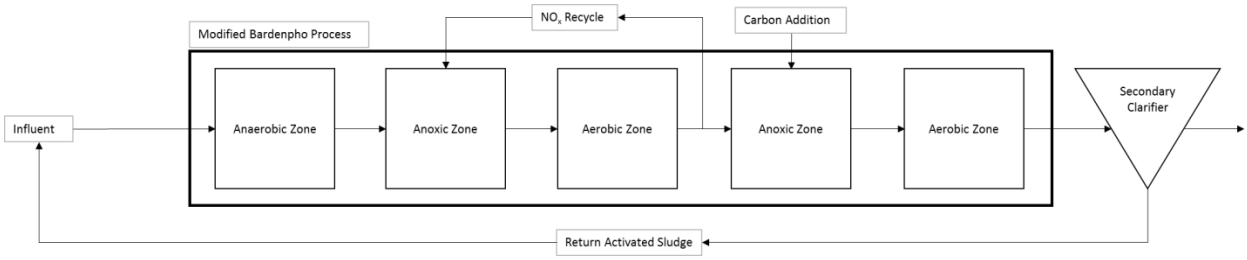

Figure 2.4: Modified Bardenpho Process Diagram

Table 2.1: Process Advantages and Limitations [Tchobanoglous et al., 2014]

Process	Advantages	Limitations
A^2O	• Recovers alkalinity for nitrification • Produces good settling sludge • Operation is relatively simple	• RAS containing nitrate is recycled to anaerobic zone thus adversely impacting phosphorus removal capability • Total nitrogen removal is limited by internal recycle ratio
Modified Bardenpho	• Can achieve very low nitrogen in effluent • Produces good settling sludge • Less nitrate fed to anaerobic zone than for A^2O process	• Less efficient phosphorus removal due to longer SRT • Requires larger tank volumes

As a contrast to these more conventional processes, the BIOPHO-PX process (Figure 2.5) seeks to improve process operations by reducing the energy footprint concurrently with achieving lower effluent total nitrogen. One key operational aspect that separates the BIOPHO-PX process from traditional BNR facilities is its post-anoxic configuration. Placing the anoxic zone after the aerobic zone, as compared to traditional BNR facilities where it is located before the aerobic zone (i.e., pre-anoxic), allows for potential advantages including energy and materials cost savings, and achieves better overall nitrogen removal.

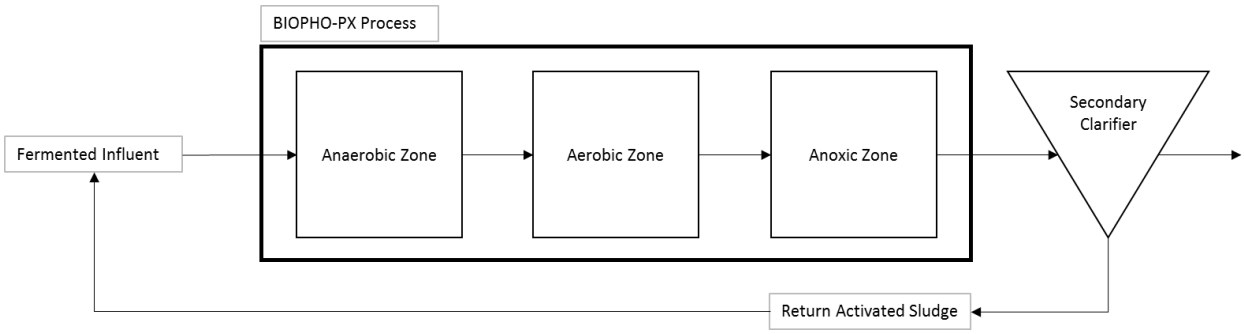

Figure 2.5: BIOPHO-PX Process

The advantages of the BIOPHO-PX process are summarized in Table 2.2 and detailed elsewhere [*Appel*, 2015; *E. R. Coats et al.*, 2011; *Winkler et al.*, 2011]. In brief, the three advantages are (1) eliminating NO_x (oxidized nitrogen) internal recycle, (2) operating under post-anoxic denitrification conditions, and (3) enriching for a carbon storing biomass. Each of these advantages are discussed in more detail below.

Eliminating the need for NO_x internal recycle decreases WRRF capital and operational costs. Traditional BNR facilities (e.g., A^2O and Modified Bardenpho) rely on pumping NO_x from the aerobic basins upstream to the pre-anoxic basins in order to achieve denitrification. These pumps, referred to as mixed liquor return (MLR) pumps, are often very large and typically have capacities to internally recycle 300 – 500% of influent flow. Achieving denitrification without the necessity of internal NO_x recycle relieves the WRRF of capital costs associated with purchasing and installing large capacity MLR pumps, MLR piping and valving, and MLR maintenance. The absence of NO_x internal recycle also decreases WRRF energy requirements associated with MLR daily operations.

In conjunction with eliminating NO_x internal recycle, operating the WRRF in a post-anoxic configuration provides near-complete nitrogen removal. Traditional pre-anoxic BNR configurations rely on endogenous decay and readily biodegradable soluble carbon to drive denitrification. Pre-anoxic configurations, by design, do not possess the capability of achieving complete denitrification as compared to a post-anoxic configuration. Rearranging the terms used to calculate internal recycle ratios for pre-anoxic configurations, Equation 2.1 [*Tchobanoglous et al.*, 2014], illustrates how pre-anoxic NO_x removal is a function of the MLR flowrate. Under the assumption of ideal denitrification and typical return activated sludge (RAS) flows, Equation 2.1 illustrates how complete denitrification (i.e. effluent NO_x = 0) is impossible.

Equation 2.1: Internal Recycle Mass Balance eq. 8-62 [Tchobanoglous et al., 2014]

$$Effluent\ NO_x = \frac{NH_3\ Oxidized}{\frac{MLR\ Flow}{Influent\ Flow} + \frac{RAS\ Flow}{Influent\ Flow} + 1.0}$$

Eliminating the need for internally recycling NO_x upstream (i.e., Equation 2.1 no longer applies) and performing denitrification downstream of the aerobic basin allows for the near-complete nitrogen removal associated with the BIOPHO-PX configuration.

While accomplishing post-anoxic denitrification, along with EBPR (described later), the BIOPHO-PX configuration enriches for the growth of carbon-storing microorganisms. Carbon-storing microorganisms utilize VFAs produced in the primary treatment process, store carbon internally within the anaerobic zone, and later utilize it within the post-anoxic zone to accomplish denitritation/denitrification. These carbon-storing organisms, combined with microbial endogenous decay, do not require the addition of external carbon. Eliminating this external carbon addition provides an additional WRRF cost savings, while achieving near-complete nitrogen and phosphorus removal. Beyond the intrinsic advantages within the BIOPHO-PX process, and to further pursue the resource recovery concept, biomass enriched with carbon-storing microorganisms possess the potential to accumulate large quantities of PHA within a sidestream reactor.

Table 2.2: BIOPHO-PX Advantages and Benefits

BIOPHO-PX Advantages and Benefits	
• No need for NO_x recycle pumps	• Capital cost and energy savings when compared to A^2O and Modified Bardenpho • Minimized risk of nitrate being recycled back to anaerobic basins
• Post-Anoxic nitrogen removal	• Nitrogen removal is not limited by an internal recycle ratio • Can achieve very low total nitrogen in effluent
• Enrichment for carbon storing microorganisms	• No need for anoxic carbon addition • Potential for sidestream resource recovery via PHA production

2.1.2.2 Enhanced Biological Phosphorus Removal (EBPR)

To effectively control advanced eutrophication of a surface water body (principally fresh water), soluble phosphorus (PO_4^{3-}) is the targeted nutrient for removal from wastewater. Phosphorus is the limiting nutrient for excess algal growth in most freshwater systems, and thus its reduction/near elimination in reclaimed water can control advanced eutrophication. Phosphorus can be removed from waste streams using chemical reagents, biological activity, or a combination of both. Typical effluent values required for phosphorus discharge range from 0.1 – 1.0 mg/L [*Tchobanoglous et al.*, 2014]. In the inland Pacific NW, permit levels are 0.1 mg/L or less. Life cycle assessment research has demonstrated that biological means should be the first line of defense in phosphorus removal [*E.R. Coats et al.*, 2011].

The BIOPHO-PX process uses biological activity to achieve phosphorus removal. Moreover, the method for phosphorus removal in the BIOPHO-PX process does not differ

from other phosphorus removing treatment configurations such as A^2O and Modified Bardenpho. WRRF operations to achieve phosphorus removal involve encouraging the growth of Phosphorus Accumulating Organisms (PAOs). PAOs have the ability to store excess PO_4^{3-} within their cells, therefore accomplishing removal of, and concentrating, excess PO_4^{3-} from the waste stream. Moreover, and perhaps more importantly, PAOs are carbon-storage microbes (a feature critical to the research presented herein). To encourage the growth of PAOs, two conditions must be met. First, the influent substrate must contain soluble VFAs (e.g., primary effluent). Typical magnitudes for design recommend greater than eight fold of influent PO_4^{3-} [*Tchobanoglous et al.*, 2014]. Empirical data, Figure 2.6, from the Coats' lab (unpublished) suggest greater than 15 fold of influent PO_4^{3-}.

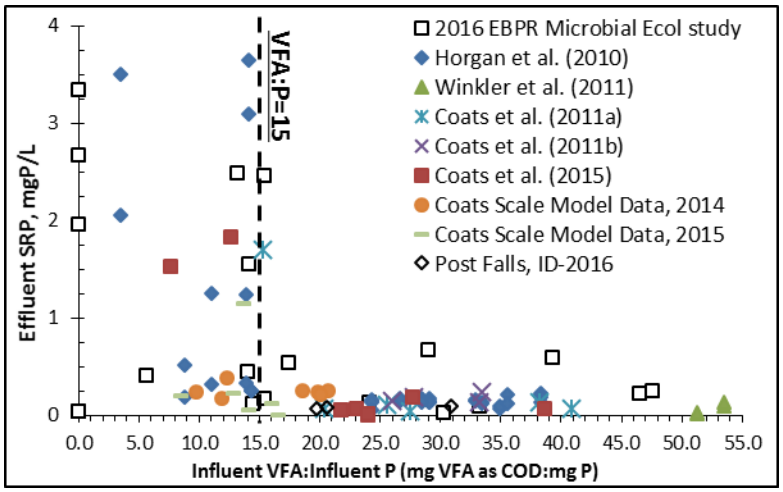

Figure 2.6: Effluent SRP vs. Influent VFA:P

Second, along with VFAs, PAOs need specific environmental conditions in order to grow and achieve PO_4^{3-} removal. Specifically, the PAOs must be exposed to an engineered environment known as electron acceptor cycling. Electron acceptor cycling can be explained, in a simplified manner (Figure 2.7), as follows. First, the PAOs must be exposed to an environment containing VFAs and void of all external electron acceptors (O_2, NO_2^-, NO_3^-; i.e., anaerobic conditions). Under this environmental condition, the PAOs consume VFAs, storing the carbon as PHA. The transformation of VFAs to PHA is an active process and requires energy. The energy comes from hydrolyzing intracellular polyphosphate (Poly P) polymer chains within the PAO cell; coupled with energy generation for the cell, this

reaction releases the hydrolyzed PO_4^{3-} outside the cell and into bulk solution. The requisite EPBR-induced reaction is traditionally accomplished within an anaerobic selector basin at the beginning of the treatment process. Subsequent to the anaerobic environment, the PAOs must be exposed to an environment containing an external electron acceptor (e.g., oxygen, nitrate). The presence of an external electron acceptor allows the PAOs to produce substantial quantities of energy and thus grow using PHA as a source of organic carbon. This growth, accompanied with an external electron acceptor for energy, allows for Poly P to be restored using the expelled PO_4^{3-} from the previous anaerobic environment. PAOs reclaim all of the expelled PO_4^{3-} and accumulate excess (influent) phosphorus through growth, thereby accomplishing excess, or enhanced, PO_4^{3-} removal.

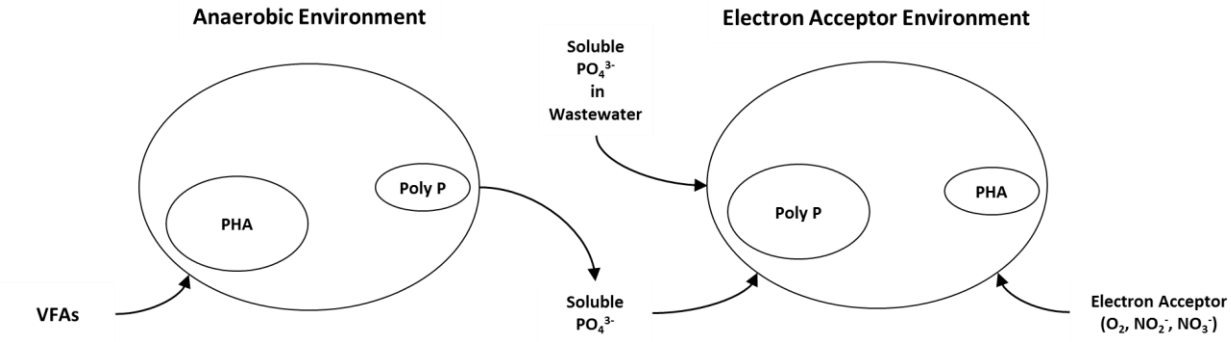

Figure 2.7: Simplified EBPR Cell Diagram

Traditionally aerated basins are placed downstream of an anaerobic basin to provide the external electron acceptor, O_2. Basins containing NO_2^- and NO_3^- are able to replace O_2 as the external electron acceptor and maintain the growth of PAOs [*E. R. Coats et al.*, 2011; *Grady et al.*, 2011; *Tchobanoglous et al.*, 2014; *Winkler et al.*, 2011]. These PAOs are commonly given a special designation as denitrifying PAOs (dnPAOs). Figure 2.7 illustrates the basics of the EBPR process described above.

The BIOPHO-PX process configuration, which incorporates the EBPR configuration described above, achieves two crucial outcomes: (1) encourages the growth of carbon-storing organisms and (2) produces effluent in compliance with phosphorus permit standards. Growth of carbon storing organisms includes, but is not limited to, the PAOs described above. Other carbon-storing organisms, such as glycogen accumulating organisms

(GAOs), exist within the WRRF biomass. GAOs competes anaerobically for available VFAs, yet do not aerobically accumulate excess phosphorus. Subsequently, if the microorganism populations are dominated by GAOs, phosphorus removal can be impaired and lead to phosphorus permit violations. To maintain a sufficient PAO population, thus ensuring phosphorus removal occurs, longer chain VFAs (propionate and butyrate) need to be provided anaerobically. Lab scale reactors investigating primary treatment (data not shown) confirm that primary treatment within the novel WRRF configuration will supply sufficient longer chain VFAs, thus minimizing the presence of GAOs.

2.1.2.3 Aerobic Nitritation

An additional advantage of the BIOPHO-PX process is the focus on inorganic nitrogen removal (NH_4^+) via nitritation (vs. traditional nitrification). Conventional WRRF configuration, such as the A^2O and Modified Bardenpho, target traditional nitrification Figure 2.8, which oxidizes ammonia completely to nitrate (Equation 2.2).

Equation 2.2: Nitrification Stoichiometry

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$$

However, it is well understood that nitrification is a two-step process requiring two separate groups of organisms. The first step involves the oxidation of inorganic nitrogen within an aerobic environment to nitrite (NO_2^-); this process is referred to as nitritation (Equation 2.3).

Equation 2.3: Nitritation Stoichiometry

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$$

Ammonia-Oxidizing Bacteria (AOB) is the general classification given to microorganisms that accomplish nitritation. The second step involves the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-); this process is referred to as nitrite oxidation (Equation 2.4).

Equation 2.4: Nitrite Oxidation Stoichiometry

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$

Nitrite-Oxidizing Bacteria (NOB) is the general classification given to microorganisms that

accomplish nitrite oxidation (Equation 2.4). Combined, AOBs and NOBs form the microbial consortium that has the ability to oxidize inorganic nitrogen to nitrate (again, referred to as "traditional nitrification").

AOBs and NOBs possess unique individual kinetic characteristics that control the level of nitrification. Table 2.3 shows nitrification kinetic values (μ_{max}, K); μ_{max} represents the maximum growth rate of the microorganism, while K represents the half-saturation coefficient (i.e., half-velocity constant) of the microorganism or consortium. The half-velocity constant represents the residual concentration of substrate required for the microorganism to achieve 50% of its maximum growth rate. It can be seen (Table 2.3) that NOBs have a higher growth rate and a lower half-saturation coefficient as compared to AOBs; thus, NOBs are more dominant in terms of growth kinetics. While it appears that NOBs should outgrow and outcompete AOBs for available nitrogen substrate, NOBs are limited to grow on the products created by AOBs. In other words, the reaction illustrated in Equation 2.3 must occur before the reaction illustrated in Equation 2.4 can occur. This does not hinder NOB growth, but offers some explanation to why nitrification, utilizing both AOBs and NOBs synergistically, is often termed an "all or nothing" process. In other words, with low K values (e.g., less than 1.0 g/m^3), AOBs and NOBs are growing at or near their maximum growth rates when nitrogen substrates are present.

Table 2.3: Nitrification Kinetics [Tchobanoglous et al., 2014]

Nitrification Kinetics	μ_{max}	K (Substrate)
AOB	0.30 – 0.95 day^{-1}	0.30 – 0.70 g/m^3
NOB	0.70 – 1.8 day^{-1}	0.05 – 0.30 g/m^3

The BIOPHO-PX process seeks to establish conditions to encourage the growth of only AOBs and thus accomplish only nitritation. As noted, the "all or nothing" kinetics of nitrification cannot be selected against using typical controlling parameters such as SRT or hydraulic retention time (HRT). Therefore, a different approach must be used in order to select for AOBs and accomplish nitritation. Research has demonstrated that the BIOPHO-PX

process can accomplish partial nitritation by controlling the bioreactor residual dissolved oxygen (DO) and the aerobic contact time. The DO kinetic values for AOBs and NOBs differ as compared to their nitrogen substrate kinetic values. Specifically, it has been observed that NOB kinetics are inhibited at low DO concentrations. The dissolved oxygen half saturation coefficient, K_{DO} value, affects the nitrification kinetics of both AOBs and NOBs and ranges from 0.10 – 1.0 g/m^3 [*Tchobanoglous et al.*, 2014], with the K_{DO} value for NOBs often higher as compared to AOBs. Energetically accomplishing nitritation requires 25% less oxygen as compared to nitrification [*Daigger*, 2014]. Nitrite is in a lower oxidation state (+3) as opposed to nitrate (+5). This may provide the opportunity, using controlled aeration and low DO concentrations, for AOBs to out compete NOBs for available DO resulting in an AOB

2.1.2.4 Post-Anoxic Denitritation

Traditional WRRFs target nitrification/denitrification for nitrogen removal. Figure 2.8 illustrates the microbially facilitated process of nitrate reduction (denitrification), performed by facultative heterotrophic microorganisms, to evolve nitrogen gas from solution and achieve nitrogen removal. This process, while reliable, is dependent on sufficient carbon present in the anoxic zone. Conventional BNR facilities often lack the necessary influent carbon and must add an external source of carbon (e.g., methanol) when accomplishing post-anoxic denitrification (Figure 2.4, Section 2.1.2.1).

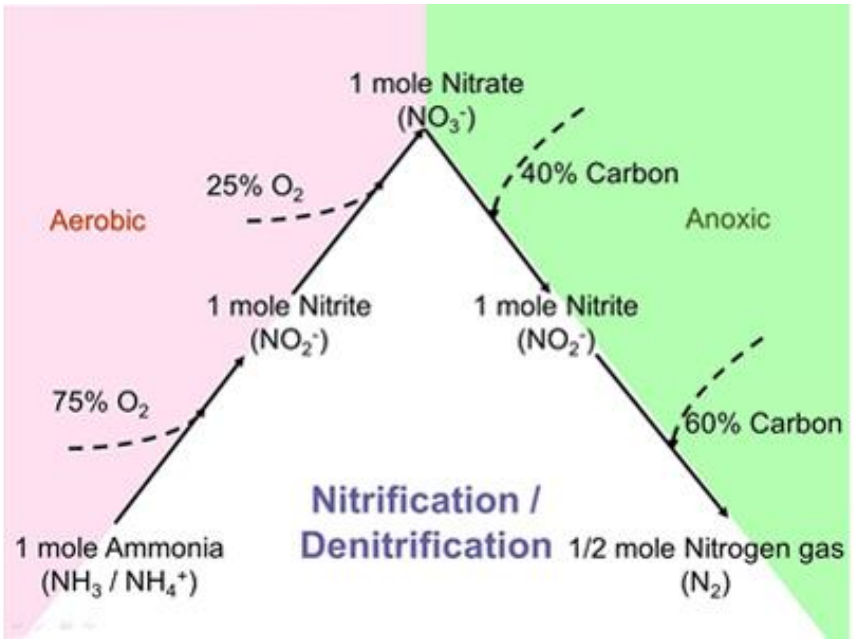

Figure 2.8: Nitrification/Denitrification [Higgins, 2014]

In contrast, the BIOPHO-PX process focuses on aerobic nitritation (described in previous section) and anoxic denitritation, illustrated in Figure 2.9. The same facultative heterotrophic are present, yet by avoiding the oxidation of nitrite to nitrate an estimated theoretical carbon savings of 40% can be achieved during denitritation as compared to traditional denitrification [*Daigger*, 2014; *Higgins*, 2014]. This carbon savings is due to nitrite reduction being more thermodynamically favorable (Table 2.4) as compared to nitrate reduction.

Table 2.4: Nitrite/Nitrate Reduction Half-reactions [Tchobanoglous et al., 2014]

Biological Half-Reactions	ΔG° (kJ per electron equivalent)
$\frac{1}{3}NO_2^- + \frac{4}{3}H^+ + e^- = \frac{1}{6}N_2 + \frac{2}{3}H_2O$	-93.23
$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O$	-71.67

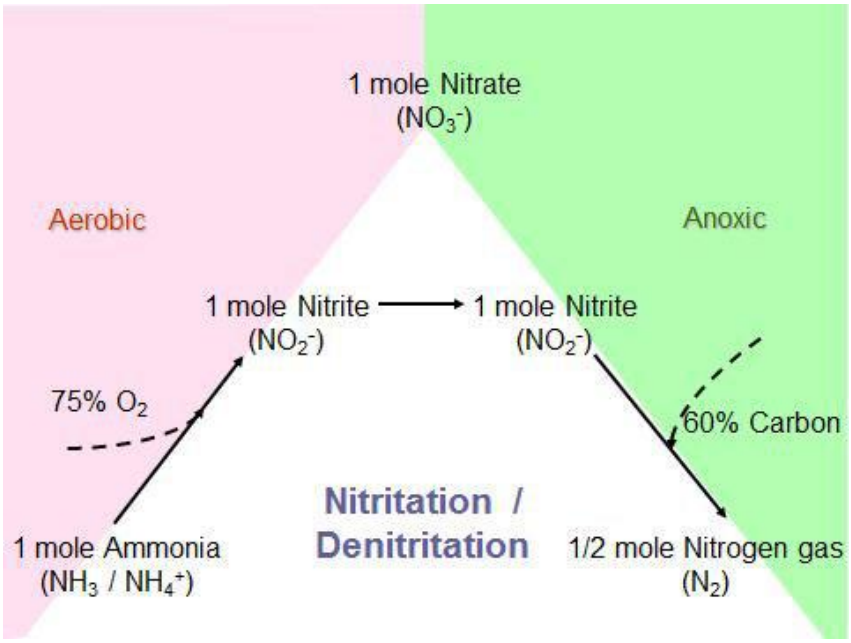

Figure 2.9: Nitritation/Denitritation [Higgins, 2014]

Conventional BNR configurations would view the 40% carbon savings as a potential issue that could generate possible permit violation on effluent carbon (measured as BOD). Since less carbon is consumed within the anoxic environment, additional oxygen would be required downstream, thereby negating the benefits of aerobic nitritation. However, in the envisioned PHA-producing BIOPHO-PX WRRF configuration, this conserved carbon will be instead captured for PHA production. To achieve PHA production, as shown (Figure 2.1), the WRRF configuration will employ a sidestream process that leverages the carbon savings potential for sidestream processes such as PHA production (more detail follows).

2.1.3 PHA Production

PHA production is a proposed tertiary process within the WRRF configuration (Figure 2.1) that utilizes VFA-rich fermented wastewater from the primary treatment (i.e., carbon savings realized through BIOPHO-PX nitritation-denitritation) and carbon storing biomass from the BIOPHO-PX process (enriched through intrinsic EBPR conditions). The narrative that follows provides a detailed literature review on PHA. Chapter 3 of this document will provide more details in regard to the unique features of this process configuration, specifically addressing the operational strategies and associated impacts on maximizing the

amount of PHA.

2.2 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHAs) are intracellular carbon polymers synthesized by bacteria. In terms of cellular use, these polymers serve as a stored source of carbon and energy. In terms of non-microbial use, these polymers are classified as biodegradable thermoplastics, exhibiting similar material properties of polypropylene and capable of being produced from renewable resources (e.g., sugars and fatty acids) [*Reis et al.*, 2003]. Poly(3-hydroxybutyrate) (PHB) is the most common and abundant form of PHA, discovered in 1926 by Lemoigne [*Lemoigne*, 1926]. PHB was originally believed to be the only active PHA monomer in activated sludge, until the discovery of other heteropolymers in chloroform extracts of activated sludge in 1974 [*Wallen and Rohwedde.Wk*, 1974]. Subsequently, Wallen and Rohwedder noted the presence of PHA copolymers composed primarily of β-hydroxybutyric acids and β-hydroxyvaleric acids, along with minor amounts of other longer carbon chain hydroxyacids; a general PHA molecular formula is shown in Figure 2.10. With this discovery that PHAs, containing differing β-hydroxyacid subunits, are synthesized by bacteria in WRRF environments. WRRF bacteria show the potential to synthesize different PHA polymers with different material and chemical properties.

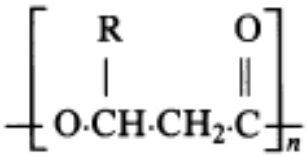

Figure 2.10: 3-hydroxyacid General Formula [Anderson and Dawes, 1990]

2.2.1 PHA Properties

PHAs are divided into two major groups: short-chain-length PHAs (scl-PHAs) that contain monomer units with 3 – 5 carbon atoms, and medium-chain-length PHAs (mcl-PHAs) that contain monomer units of 6 – 18 carbon atoms. The most common PHAs found in WRRF systems are the scl-PHA poly(3-hydroxybutyrate) (P3HB) and scl-PHA poly(3-

hydroxybutyrate-co-3-hydroxyvalerate); often the two are co-polymerized into P(3HB-co-3HV). P3HB and P(3HB-co3HV) are most commonly synthesized on waste organic-based substrate due to the associated VFA speciation within the WRRF system influent. Previous work has shown that high concentrations of acetate and propionate drive the synthesis of P3HB and P(3HB-co-3HV) [*Anderson and Dawes*, 1990; *Wallen and Rohwedde.Wk*, 1974]. P3HB and P(3HB-co-3HV), illustrated in Figure 2.11, are short-chain-length PHAs with mechanical properties that are comparable to polypropylene and polyethylene [*Laycock et al.*, 2014].

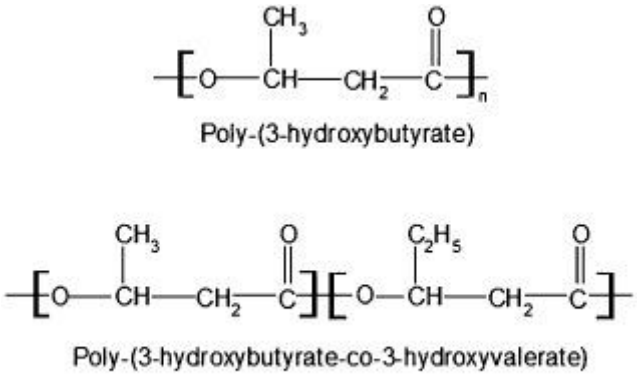

Figure 2.11: P3HB and P(3HB-co-3HV)

The mechanical properties of PHA, while similar to polypropylene and polyethylene, are dependent on the amount of β-hydroxyvaleric acid present within the polymer (3-hydroxyvaleric (3HV) acid being the most common speciation of biosynthesized β-hydroxyvaleric acids). It is well known that PHAs consisting of primarily P3HB are stiff and brittle, with poor impact strength, due to their high crystallinity and significant secondary crystallization that occurs post processing [*Laycock et al.*, 2014]. Therefore, in order to produce a more stable product, 3HV must be present within the polymer. Figure 2.12 illustrates the typical tensile strength and Young's modulus of a P(3HB-co-3HV) compound as a function of percent 3HV content. It can be seen that as the 3HV fraction increases the tensile strength and Young's modulus decreases. Pure P3HB would appear to be desirable, with its high tensile strength (Figure 2.12), yet the polymer is brittle, possesses limited applications, and would have narrow marketability. Polymers exhibiting high tensile

strengths (i.e., being composed of pure P3HB) are thus not desirable for all polymer applications, especially flexibly malleable products (sheeting, films, covers, etc.). Anderson and Dawes noted that the key to producing a marketable and versatile product is maintaining the ability to produce bioplastics containing various amount of 3HV (i.e., P(3HB-co-3HV)). It was also noted that the composition of P(3HB-co-3HV) has been shown to be a function of the VFA speciation supplied during PHA synthesis [*Anderson and Dawes*, 1990].

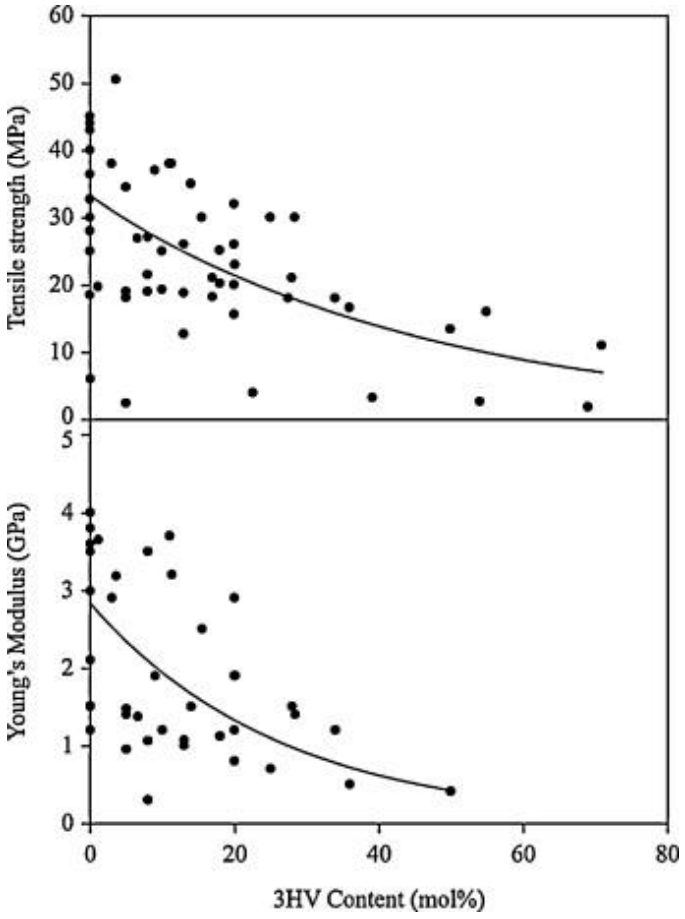

Figure 2.12: Tensile Strength and Young's Modulus of P(3HB-co-3HV) [Laycock et al., 2014]

2.2.2 PHA Biosynthesis

Inducing the biosynthesis of PHA is complex, requiring expression of multiple genes that encode a variety of enzymes that are directly or indirectly involved. Figure 2.13 illustrates all known pathways of PHA synthesis using a variety of substrates. The multiple described pathways available to microorganisms to synthesize PHA are regulated and/or

induced at four levels [*Laycock et al.*, 2014], including: (1) Activation of PHA gene expression due to specific environmental signals, such as nutrient starvation; (2) Activation of the PHA synthetic enzymes by specific cell components or metabolic intermediates; (3) Inhibition of metabolic enzymes of competition pathways and therefore enrichment of required intermediates for PHA synthesis; or (4) A combination of the above three. These mechanisms of induction are important as related to understanding the potential for achieving WRRF PHA production (discussed in section 2.2.3).

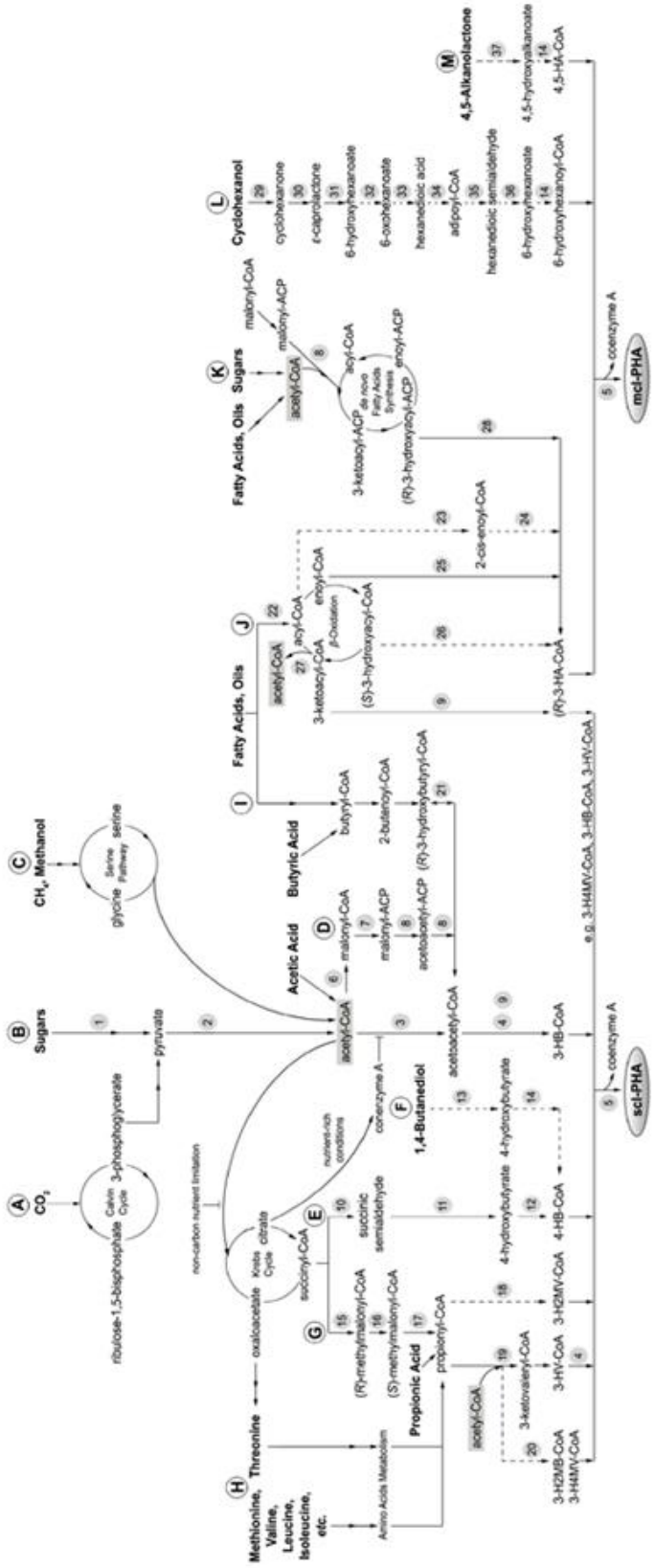

Figure 2.13: Metabolic pathways of PHA Synthesis [Tan et al., 2014]

At a molecular level, as PHA is synthesized, the PHA monomers polymerize into amorphous granules within the cell wall (Figure 2.14). The granules are surrounded by/coated with proteins: PHA Synthases, PHA depolymerases, regulatory proteins, and phasins. These proteins are produced during PHA accumulation and are essential in PHA synthesis and ultimate degradation. Phasins, being the most abundant of the proteins, may cover 14 – 54% of the granule surface; phasins are only produced during PHA accumulation, and control the amount, size, and number of granules synthesized. In the absence of phasins, cells only accumulate one single large granule [*Koller et al.*, 2013]. Ultimately, the biochemistry of PHA production is quite complex, and elements remain poorly understood. This research and associated Thesis – being focused on PHA production using mixed microbial consortia and waste-based substrate – will emphasize principally on the applied, engineering principles of PHA production, and not the molecular-level aspects of the metabolism.

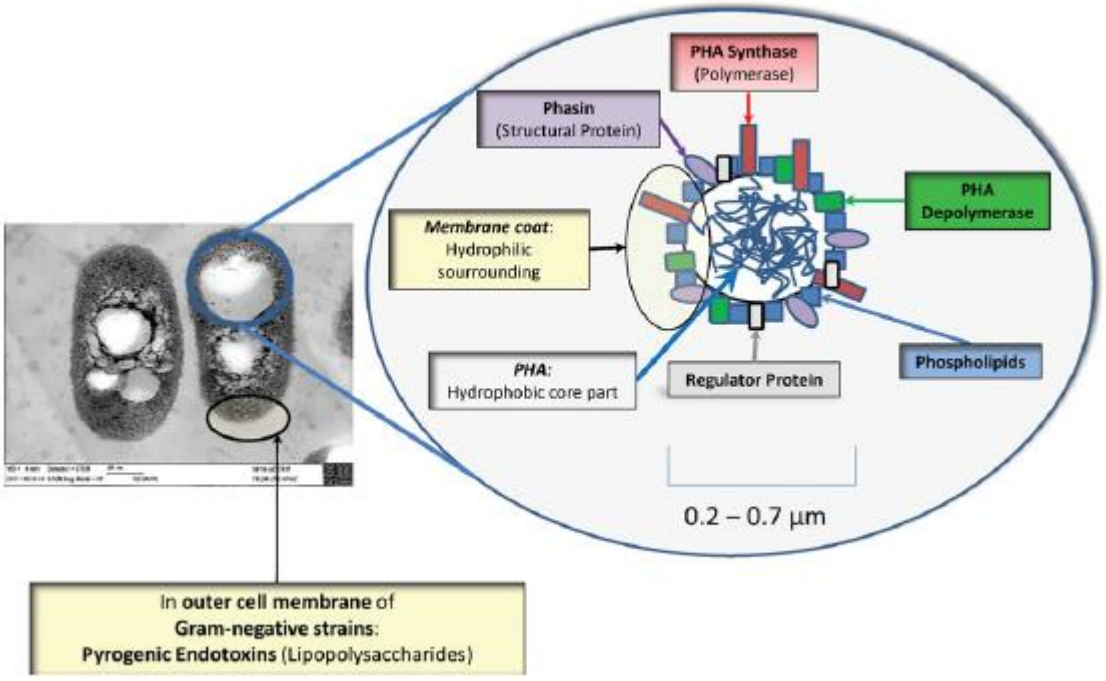

Figure 2.14: Intracellular PHA Granule [Koller et al., 2013]

It is well known that activated sludge submitted to transient conditions, mainly

caused by discontinuous feeding and variations in electron acceptor presence (i.e., EBPR), exhibits the ability to store large amounts of PHA [*Reis et al.*, 2003]. A typical WRRF may accumulate 1 – 5% of cell dry mass as PHA during typical treatment operations achieving nutrient removal [*E. R. Coats et al.*, 2011; *Li et al.*, 2014; *Liu et al.*, 2013; *Winkler et al.*, 2011]; such intracellular concentrations are nowhere near the 80 – 90% of cell dry mass reported by [*Laycock et al.*, 2014], nor are they near a minimum commercial level. However, typical WRRFs are not operated with PHA storage as the end goal, and accumulations realized within the EBPR-based process are sufficient to accommodate treatment requirements. Nevertheless, this research investigated that discrepancy. In order to better understand the potential to pragmatically realize higher intracellular PHA concentrations in mixed microbial cultures with WRRF-based environments, it is important to first understand – at an applied level – how PHA is synthesized by MMC.

2.2.3 Engineered Environments for the Synthesis of PHA

The PHA synthesis potential of the mixed microbial consortia (MMC) within a WRRF system is a product of the environmental conditions imposed. Moreover, it is understood that not all organisms are capable of storing carbon internally as PHA. There exists competition within the MMC between the PHA storing organisms and the non-PHA storing organisms for the available nutrients, namely carbon. The goal, then, is to impose environmental conditions to maximize enrichment of PHA storing organisms vs. non-PHA storing organisms.

Two engineering methods are typically applied to achieve selection for PHA storing organisms within mixed microbial cultures grown on waste carbon sources: (1) Electron Acceptor Cycling (EAC), and (2) Aerobic Dynamic Feeding (ADF). However, only one method exhibits the metabolic potential to produce commercial quantities of PHA. EAC is applied to accomplish nutrient removal with EBPR, with mixed cultures typically sustaining PHA concentrations of 1 – 5%. Conversely, ADF can be imposed to induce feast/famine PHA synthesis, and is the primary applied engineering method for realizing large quantities of PHA [*Dias et al.*, 2008; *Tamis et al.*, 2014]. While conventional research on PHA production

focuses on ADF, in BNR configurations accomplishing EBPR the EAC mechanisms will predominate. A challenge of this research was to integrate the two concepts to realize PHA production concurrent with wastewater treatment; to-date such integration has not been investigated, nor achieved, and thus this research is quite novel and potentially ground-breaking. Both EAC and ADF are discussed in more detail below.

2.2.3.1 Anaerobic PHA Synthesis (Electron Acceptor Cycling)

Anaerobic PHA synthesis is a well-documented occurrence and a necessary driving force for phosphorus removal within EPBR. Groups of organisms are known to consume VFAs and store PHA within an environment lacking an external electron acceptor. These organisms are known as either PAOs (Polyphosphate Accumulating Organisms) or GAOs (Glycogen Accumulating Organisms). Under applied EAC conditions, both groups of organisms can uptake VFAs under anaerobic conditions (at the expense of energy (ATP)), transport them over the cell membrane, and activate them (in the case of acetate) to Acetyl-CoA. Acetyl-CoA then is transformed to PHA [*Reis et al.*, 2003]. For PAOs, the TCA cycle is active in this environment, but PAOs rely more on the hydrolysis of polyphosphate to produce energy (ATP); glycogen catabolism also generates energy for the cell. Conversely, for GAOs the hydrolysis of glycogen through glycolysis is the sole source of energy (ATP), with no polyphosphate accumulation (or, of course, phosphorus removal). Figure 2.15 illustrates the metabolic pathways induced during electron acceptor cycling allowing for anaerobic PHA production.

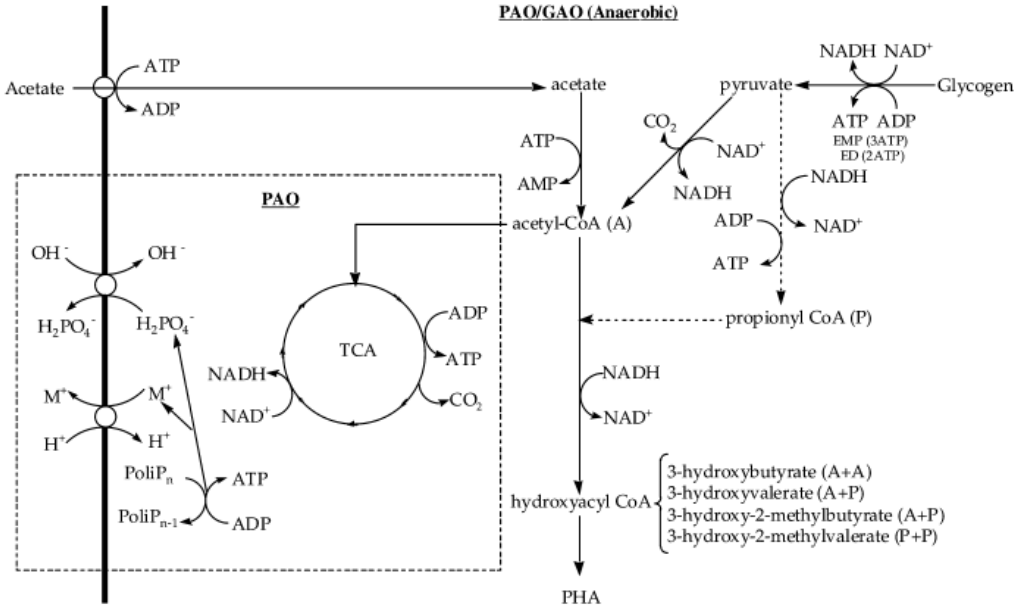

Figure 2.15: Anaerobic PHA Metabolism (Reis et al., 2003)

During anaerobic VFA uptake, the energy and intermediates produced during polyphosphate hydrolysis and glycogen hydrolysis are essential for PHA production, but not sufficient to sustain the growth rates required by PAOs and GAOs. In order for PAOs and GAOs to compete with other organisms within the biomass, PHA is synthesized anaerobically and later hydrolyzed under aerobic (or anoxic) conditions where oxidative phosphorylation can occur to produce the necessary energy (ATP) to sustain growth. In summary, these microorganisms gain a competitive edge by sequestering soluble available substrate within environments that are unfavorable for growth of non-PHA storing organisms (i.e., anaerobic conditions); these microbes then use the PHA under respiratory conditions (anoxic, aerobic) to grow. While this engineered induced mechanism results in PHA synthesis, MMC do not accumulate large quantities of PHA; instead, this engineered environment facilitates excess phosphorus removal through enrichment of PAOs.

2.2.3.2 Aerobic PHA Synthesis (Aerobic Dynamic Feeding)

When compared to anaerobic PHA synthesis and the associated limited kinetics, aerobic PHA synthesis presents an opportunity to produce much larger quantities of PHA. Aerobic PHA synthesis is associated with an engineered environmental condition known as

aerobic dynamic feeding (ADF). ADF is characterized by exposing biomass to a transient excess carbon supply while also maintaining an external electron acceptor, such as oxygen, in excess; conventionally VFAs are provided as the carbon substrate and PHA precursors. Specifically, under ADF conditions microorganisms are submitted to periods of carbon excess (feast) and deficit (famine), generating unbalanced growth conditions that induce a suite of metabolisms referred to as a feast/famine response [*Reis et al.*, 2003]. These conditions are not growth limiting, when compared to EAC PHA synthesis; growth and storage occur simultaneously during the feast period, with only growth occurring during the famine. Under ADF conditions, PHA storing organisms with induced feast/famine metabolisms have an advantage over non-PHA storing organisms, and thus become overly enriched within the MMC. Figure 2.16 illustrates the metabolic pathways induced during ADF allowing for aerobic PHA production.

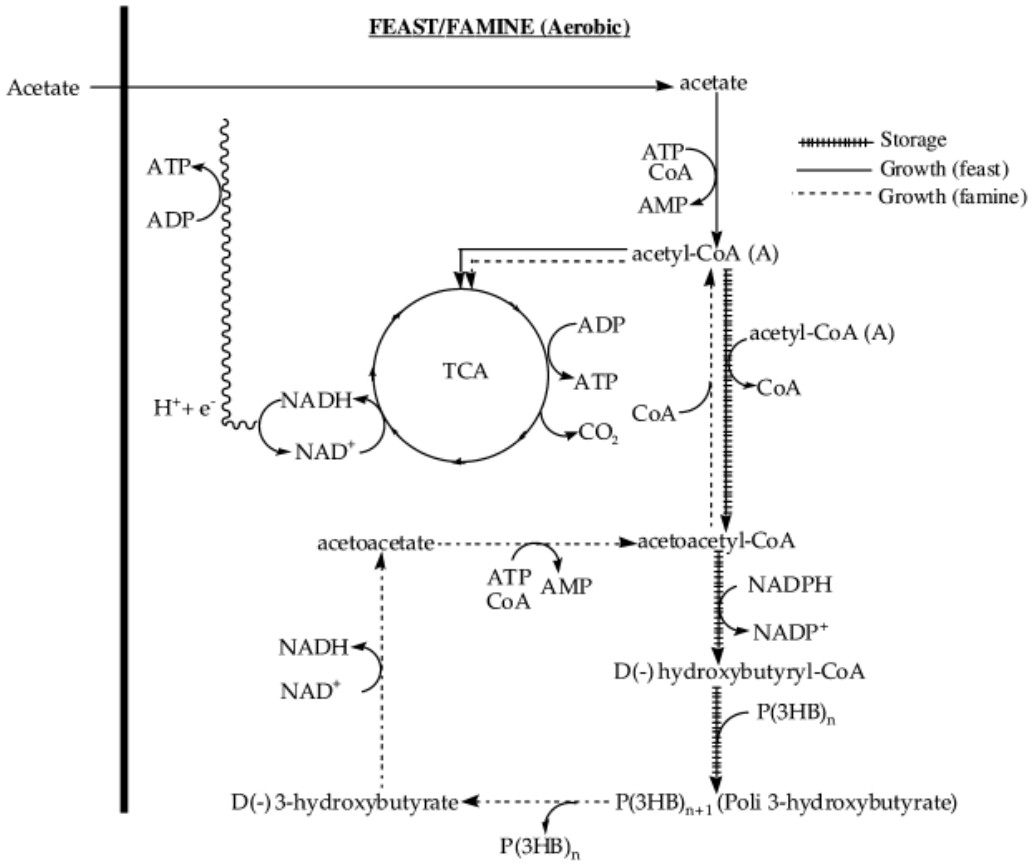

Figure 2.16: Aerobic PHA Metabolism [Reis et al., 2003]

ADF conditions, while able to accumulate larger quantities of PHA, do not sustain the ability to achieve nutrient removal. Organisms such as PAOs and denitrifiers do not efficiently grow under ADF environmental conditions. PAOs are responsible for phosphorus removal and require EAC (i.e., anaerobic-aerobic/anoxic conditions). Denitrifiers, which accomplish nitrogen removal, require anoxic conditions (i.e, conditions containing oxidized nitrogen and no oxygen). Within ADF environmental operations these necessary environments do not exist. PAOs and denitrifiers that do inhabit an ADF system are unable to compete with the other carbon-storing organisms, or simply do not express the specific nutrient removal phenomenon, because they are unnecessary for cell growth.

To feasibly place an ADF system within a WRRF configuration and produce large quantities of PHA, while simultaneously removing phosphorus and nitrogen, the ADF system would have to be tertiary and independent from the main nutrient removal process. Therefore, the WRRF may maintain its targeted nutrient removal and produce larger commercial quantities of PHA.

Chapter 3: Producing PHA

The production of PHA involves exposing the enriched biomass obtained from the BIOPHO-PX process to a VFA rich substrate within a proper environment engineered and operated for maximum PHA production (i.e., ADF conditions). Building upon the foundational theory described in Chapter 2, several strategies for introducing the VFA rich substrate exist. These strategies are described in detail below.

3.1 Feeding Strategies

Foundationally, ADF conditions are necessary to achieve commercial-scale quantities of PHA in MMC. However, currently the literature does not detail a single preferred operational method for the production of large quantities of PHA using mixed microbial consortia (MMC) cultured within a WRRF. Indeed, there is no single "recipe" for producing commercial quantities of PHA. Rather, the methods used in literature vary greatly in operational strategies, feedstocks available, and end results. Nevertheless, what is commonly agreed upon in the literature is the ideology of how PHA production is to be accomplished. The ideology, in broad terms, states that a microbial consortium enriched within a WRRF system must be subjected to transient conditions that will allow for the "hyperstorage" of carbon and the "overproduction" of PHA. To achieve the "hyperstorage" effect, literature does prescribe a preferred method for the production of PHA. Specifically, environmental conditions that induce a cyclical feast/famine response via an applied engineered ADF regime is the consensus method of choice. Figure 3.1 illustrates a typical feast/famine response during ADF operations. As shown, this response is characterized by a rapid increase in intracellular PHA (PHBV in this illustration) as soluble VFAs are consumed, followed by a decrease in intracellular PHA as the microbes consume stored carbon for energy and growth. "Hyperstorage" would be realized through an extended feast condition and prevention of the famine response.

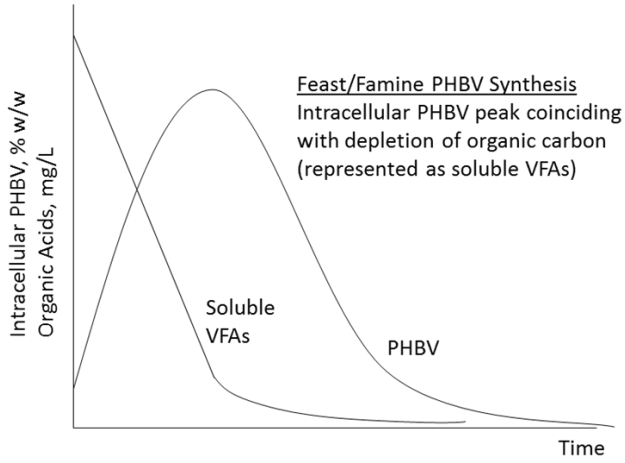

Figure 3.1: Feast/Famine PHBV Synthesis [Erik R. Coats et al., 2011]

As described, PHA production is the common term used when achieving the "hyperstorage" affect. Illustrated in Figure 3.2, PHA production targets the feast regime of the feast/famine response; a focus on the feast aspect is characterized by the consumption of soluble VFAs and the synthesis of PHA. Achieving PHA "hyperstorage" involves transferring MMC from a WRRF into a tertiary environment engineered and operated to sustain continuous PHA intracellular storage. Sufficient soluble VFAs (e.g., acetate, propionate, etc.) are added to the tertiary environment to induce the synthesis, and optimally saturation, of the MMC with PHA copolymers (i.e., P(3HB-co3HV)). Once all soluble VFAs are consumed and the MMC is believed to be saturated with PHA, the MMC will be recovered for PHA purification.

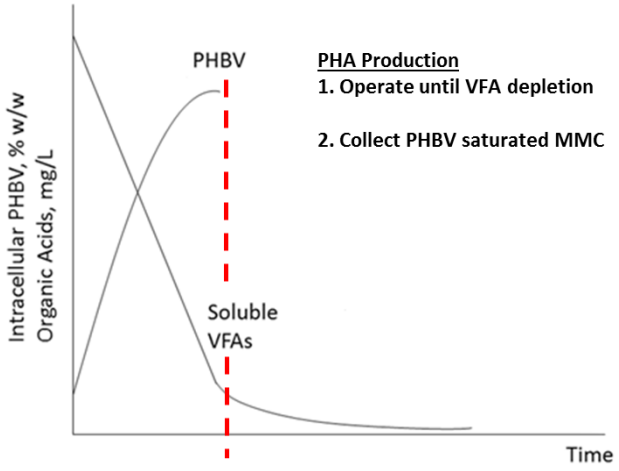

Figure 3.2: PHA Production Diagram

Three engineered strategies exist to maximize "hyperstorage" and achieve successful PHA production. These strategies target a prolonged feast period coupled with sufficient VFA addition. The strategies (described below) include Single Pulse Addition, Continuous Addition, and Multiple Pulse Addition.

3.1.1 Single Pulse Feeding

Single pulse feeding involves, as the name implies, feeding a single pulse of carbon substrate to a PHA production reactor to achieve excess PHA accumulation. This method seems to be the preferred method in several investigations [*Erik R. Coats et al.*, 2011; *Coats et al.*, 2007; *Valentino et al.*, 2014]. It allows for simple operations and "black box" predictive capabilities. With known VFA content of feedstock, PHA yield of the MMC, and correct operation, an estimation of the mass of potential PHA can be calculated before the production begins. While this is a simple method of producing PHA, two crucial disadvantages exist that can affect the efficiency of any PHA production assessment.

3.1.1.1 pH effects

To achieve large quantities of PHA, sufficient amounts of VFAs are required within the single substrate pulse feeding. Loading rates may be in excess of 1 gram VFA to 1 gram MMC (on a VSS basis). Depending on alkalinity of the substrate, adding feedstock

concentrated with VFAs can have an adverse effect on the reactor in which PHA production is occurring. VFAs are weak acids that have the ability to add additional protons into solution, thus lowering the pH. Lower pH values have an adverse effect on neutrophilic bacteria by slowing down metabolic processes, or even causing bacteria death. Typical pH values of BNR configurations, including the novel WRRF configuration, range from 6.8 – 8.4 during daily operations (data not shown). These MMC organisms, grown within the BIOPHO-PX system, are classified as neutrophiles and thrive in environments of pH 6.0 – 8.0 [*Madigan and Martinko*, 2006]. The addition of a VFA concentrated feedstock may shift the pH below 6.0, thus harming the MCC and associated PHA synthesis. To counteract the lowering of pH, alkalinity such as sodium bicarbonate, sodium hydroxide, lime (CaO), or soda ash (Na_2CO_3) would need to be added to the feedstock. Beyond the hazards of chemical management, such an operation increases process cost that could be detrimental at a commercial scale.

3.1.1.2 Substrate Inhibition

Coinciding with pH effects increased VFA loading rates may also inhibit microbial metabolisms. Known as substrate inhibition, an excess of VFA substrate will saturate current microbial metabolisms within the MMC forcing the synthesis of additional enzymes to compensate. The synthesis of these additional enzymes will alter microbial activity, thus affecting PHA accumulation and PHA transformation efficiencies. Empirical data has suggested that MMC display decreases in PHA production at VFA loading rates above 2.0 gVFA as COD/gMMC as VSS [*Erik R. Coats et al.*, 2011]. With the goal of maximizing PHA production other feeding strategies, such as adding multiple pulse addition of lower strength feedstock, will deter the potential for substrate inhibition.

3.1.2 Continuous Feeding

To avoid stockpiling sufficient quantities of feedstock for PHA production, WRRFs may employ a method to achieve PHA production by continuously feeding smaller quantities of VFA rich wastewater over the course of PHA production. The purpose of this substrate addition method is provide the MMC with a continual addition of PHA precursors

in order to maintain a sufficient residual VFA concentration within the PHA production reactor, thus sustaining feast metabolisms and the desired extended feast response. Successful experiments have been able to achieve PHA production using this method [*Marang et al.*, 2014; *Takabatake et al.*, 2002], yet required significant operational monitoring and control. This control involves constant pH monitoring (with potential alkalinity addition), VFA monitoring (to avoid famine conditions), and dissolved oxygen monitoring. Continuous feeding is also hindered by the same challenges faced by single pulse feeding (pH changes and large reactor volumes) dependent on feedstock VFA concentrations.

3.1.3 Multiple Pulse Feeding

An alternative, and more productive, approach to single pulse feeding and continuous feeding involves pulse feeding a reduced quantity of substrate multiple times to a MMC within a single production run. Known as multiple pulse feeding or simply pulse feeding, this method is more favorable, yet more complex [*Anterrieu et al.*, 2014; *Morgan-Sagastume et al.*, 2010; *Morgan-Sagastume et al.*, 2015]. Multiple pulse feeding PHA production reactors are operated similar to a sequencing batch reactor (SBR), allowing for VFA depleted feedstock to be decanted and replaced with new VFA rich feedstock. The challenges realized during multiple pulse feeding include: determining sufficient amounts of VFAs per pulse feeding, how frequently during an extended feast (i.e., PHA accumulation) period to pulse feed, and how many pulses of feed are required to realize maximum PHA concentrations and yields.

The challenge of determining sufficient VFA concentrations per each individual pulse is essential to achieving the desired PHA accumulation and avoiding the issues associated with single pulse feeding. Contrary to single pulse feeding, distributing the total mass of VFAs over multiple pulses provides better process control. The single pulse feeding issues are alleviated by requiring less feedstock volume per pulse feeding, equating not only to smaller PHA production reactor volumes but also lower VFA concentration realized by the MMC. Lower realized VFA concentrations will also help to mitigate pH changes by dilution

effects and lessen external alkalinity additions.

Coupled with determining VFA mass loading, engineers must also address the challenge of determining the elapsed time between pulse additions. Using multiple pulse feeding to maintain the extended feast period requires insight into the PHA storage capacity and VFA uptake rates. While VFA consumption rates decrease at elevated VFA concentrations (i.e., bulk VFA concentration greater than 2 fold of MMC concentrations) [*Erik R. Coats et al.*, 2011; *Majone et al.*, 1996], sufficient VFAs must be provided to account for slight changes in uptake rates, leading to early VFA depletion. The challenge is to apply information regarding VFA uptake rates in determining the necessary reaction time between each pulse. A balance between allowing the MMC time to consume VFAs and avoiding complete VFA consumption (e.g., onset of a famine period) in the PHA production reactor is essential in maintaining optimum PHA accumulation.

Engineers can utilize feedstock VFA concentrations and the time required between each pulse feeding to address the final challenge: determining the number of pulse feedings. The challenge of determining the number of pulse additions is dependent on PHA yields and the targeted PHA amount. Sufficient pulse additions must be provided in order to reach the targeted PHA amount, while avoiding MMC adaptation to growth (and thus PHA depletion). MMC exposed to ADF conditions for extended periods of time will begin to physiologically adapt and cease PHA accumulation [*Reis et al.*, 2003]. Once the carbon-storing organisms within the MMC reach PHA saturation or otherwise produce sufficient proteins for growth, metabolisms will shift from PHA accumulation to PHA utilization (e.g., growth). Abrupt changes in PHA yields and VFA uptake are signs of MMC adaptation; corresponding to a ceasing of PHA accumulation.

3.2 F:M (Food/Microorganism) Operational Parameter

With the selection of an ADF feeding strategy, operational parameters of the PHA production reactor are another crucial factor in achieving large marketable quantities of PHA. Currently the literature does not agree on which operational parameters maximizes PHA production. The literature varies greatly among synthetic feedstocks, raw wastewaters,

industrial waste streams, pure cultures, mixed microbial cultures, and imposing nutrient (nitrogen, phosphorus) limitations. In the context of the WRRF configuration, several parameters were considered inaccessible for further investigation. These parameters relate to synthetic feedstocks, industrial waste streams, pure cultures, and imposing nutrient limitations. The WRRF configuration will be a mixed microbial system using raw fermented wastewater. The wastewater will contain VFAs produced via primary treatment methods, and will not contain any chemical additions to increase VFA concentrations or impose nutrient limitations. The goal is to operate and model investigations as if they were operated at a full scale WRRF.

The F:M ratio is a commonly measured parameter used to understand the effect of transient loading conditions imposed upon a MMC. It represents the mass of organic carbon or "food" available to a mass of microorganisms. Given that growth rates are dependent on cellular history (i.e., contact time with substrate and SRT) and, during ADF conditions, substrate uptake rates are independent from the growth rate [*Reis et al.*, 2003], excess substrate that enters the cell must be metabolized by the microorganism to increase the microorganism's chances for survival. Importantly, PHA synthesis is the buffer between these two independent rates, providing certain microorganisms a better chance at survival. Following this methodology, increasing the available substrate (e.g., F:M ratio) should facilitate the production of PHA saturated biomass; this thesis further investigates this concept.

Literature F:M values (Table 3.1) favor a range of 1.0 – 2.0 gCOD/gVSS when attempting to accumulate quantities of PHA. VFA speciation, used to calculate literature F:M values, is predominantly acetate and propionate, which matches the results of WRRF primary treatment. The typical approach has been to feed PHA production reactors the same volume of feedstock used to feed the parent or treatment reactor. To push beyond observed PHA values, more feedstock is required, resulting in a higher F:M. The goal is to leverage the buffering capacity explained above in an attempt at higher PHA quantities.

Table 3.1: Literature F:M Values

Source	F:M	PHA (w/w)
[*Coats et al.*, 2007]	1.5 mLFermentate / mLMicrobial Seed	53%[a]
[*Morgan-Sagastume et al.*, 2010]	0.12 – 0.22 gCOD/gVSS	8 – 23%[a]
[*Erik R. Coats et al.*, 2011]	1.0 – 2.0 gCOD/gVSS	12.5 – 28.7%[b]
[*Valentino et al.*, 2014]	2.0 gCOD/gVSS	14 – 50.6%[a]
[*Morgan-Sagastume et al.*, 2015]	2.2 – 3.3 gCOD/gTSS	17 – 39%[b]

[a] Inoclua grown under ADF conditions

[b] Inocula grown under typical (e.g. EAC) conditions

Chapter 4: Materials and Methods

4.1 BIOPHO-PX Reactor Set Up

Two 2L laboratory-scale sequencing batch reactors (SBRs, Figure 4.1) were operated at room temperature without pH control (the pH varied between 7.2 and 8.2). Both SBRs (BIOPHO-PX 2 and BIOPHO-PX 3) received traditional EBPR substrate of raw wastewater and VFA-rich fermenter liquor consisting of 90% raw wastewater augmented with 10% primary effluent (volumetric basis). Raw wastewater was obtained regularly from the Moscow, ID EBPR WRRF (stored at 4^{o}C until use), while primary effluent was generated using a laboratory primary solids fermenter (4d SRT; chemostat; primary solids obtained regularly from the Pullman, WA WRRF). The BIOPHO-PX 2 and BIOPHO-PX 3 reactors were selected from a previous study [*Appel*, 2015].

Each 6-hr BIOPHO-PX SBR cycle included the following periods: feed (5 min), anaerobic (1 hr), aerobic (1.5 hr) for BIOPHO-PX 2 and (2.0 hr) for BIOPHO-PX 3, anoxic (2.75 hr) for BIOPHO-PX 2 and (2.25 hr) for BIOPHO-PX 3, settle (30 min), and decant (10 min). A programmable logic controller was used to control operations. Effluent was decanted each cycle and replaced with an equal volume of substrate to maintain an 18 hour hydraulic residence time (HRT). The solids residence time (SRT) was controlled at 20 days by automatically wasting 25 mL of mixed liquor at the end of each aerobic period (i.e., Garrett wasting mode). DO concentrations were controlled using Hach sc100^{TM} controllers using Hach LDO sensors. Air was introduced via JW Pet Company Fusion Air model 600 pumps through stone diffusers to create aerobic conditions. Each pump was relay controlled using a Hach sc100^{TM} to maintain a desired DO range. Target BIOPHO-PX 2 DO ranged from 1.30 – 1.50 mg/L and BIOPHO-PX 3 DO ranged from 0.60 – 0.80 mg/L. Reactors were completely mixed with magnetic stir bars. All pumping was performed using peristaltic pumps (Watson Marlow Bredel, Wilmington, MA, USA).

4.2 PHA Production Assessment (PPR) Experimental Setup

To simulate the operations of a PHA Production Reactor (PPR) at full scale, an automated lab scale system was assembled and used. This PPR consisted of a 1L beaker,

aeration via a stainless steel air stone, and two Watson-Marlow 323 peristaltic pumps. The pumps were controlled by three INTERMATIC® Model ST01 Wall Switch Timers; these timers controlled feeding, decanting, mixing, and aeration. The timer control followed that of a typical sequencing batch reactor (Figure 4.1).

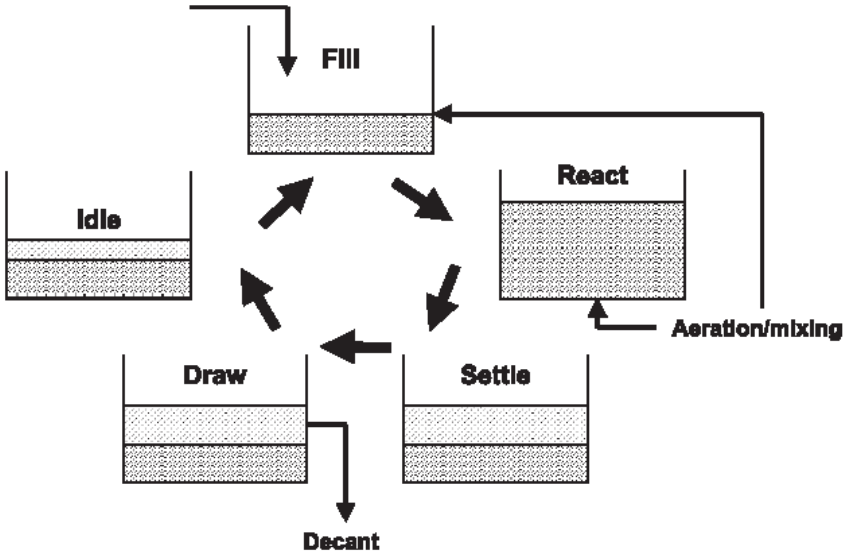

Figure 4.1: Sequencing Batch Reactor (SBR)

4.3 Analytical Technique

Samples were collected to monitor pH, DO, volatile fatty acids (VFAs), mixed-liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and PHA. For soluble constituents, samples were first centrifuged to remove biomass and then filtered through a 0.22 µm syringe filter (Millipore Corp., Billerica, MA, USA) prior to testing.

MLSS and MLVSS were measured in accordance with Standard Methods 2540 D and 2540 E [*Eaton et al.*, 1998], respectively. Measurement of pH was accomplished with a Thermo-Fisher Scientific Accumet AP85 Waterproof pH/Conductivity Meter. DO measurements were collected using a Hach HQ30d Meter with a LDO101 DO Probe.

VFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids) were quantified using a Hewlett-Packard 6890 series gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame-ionization detector and a Hewlett-Packard

7679 series injector. The system was interfaced with the Hewlett-Packard GC ChemStation software version A.06.01. VFA separation was achieved using a capillary column (Heliflex® AT™-AquaWax-DA , 30 m x 0.25 mm ID, W. R. Grace & Co., Deerfield, IL, USA) which was ramped from an initial 50°C to 200°C in three steps (following 2 min at 50°C, ramp to 95°C at 30°C min^{-1} then to 150°C at 10°C min^{-1} and hold for 3 min; finally, ramp to 200°C at 25°C min^{-1} and hold for 12 min) with helium as the carrier gas (1.2 mL min^{-1}). The split/splitless injector and detector were operated isothermally at 210 and 300°C, respectively. Prior to analysis, samples were acidified to a pH of 2 using HCl. 0.5 µL of each sample was injected in 20:1 split mode. VFA concentrations were determined through retention time matching with known standards (Sigma-Aldrich Co., St. Louis, MO, USA; Thermo Fisher Scientific Inc., Waltham, MA, USA) and linear standard curves (R^2>0.99).

Biomass PHA content was determined by gas chromatography/mass spectrometry (GC-MS) as described in [*Braunegg et al.*, 1978]. Dried biomass samples were digested at 100°C in 2 mL of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added as an internal standard to the chloroform at 0.25 mg/mL. After digestion, 2 mL of deionized water was added and vortexed to separate into chloroform and water phases. The chloroform phase was extracted and filtered through sodium sulfate anhydrous to remove excess moisture and particulates. GC-MS was performed on a Thermofinnigan PolarixQ iontrap GC-MS instrument (Thermo Electron Corporation). The sample was introduced using split injection. Separation was achieved on a ZB1 (15 m, 0.25 mm ID) capillary column (Phenomenex, Torrance, California, USA) with helium as the carrier gas (1.2 mL min^{-1}) and an initial temperature of 40°C (2 min) ramped to 200°C at 5°C min^{-1}. The compounds were confirmed by retention time and mass spectral matching with known PHA standards (PHB and PHB-co-HV: Sigma Aldrich; NaHB: Alfa Aeser; Tianan) as methyl ester derivatives, and quantified based on the internal standard. The Xcalibur software program (Thermo Electron Corporation) was used to facilitate PHA quantification, and the m/z 103 ion was chosen for PHA quantification was relative to methyl benzoate. PHB eluted at approximately 5.4-5.6 min, and PHV eluted at approximately 7.9-8.4 min. The benzoic acid standard eluted at 11.9-12.1 min. Total intracellular PHA content was determined on a

percent dry weight basis (mass PHA per mass TSS, w/w) and a percent cell weight basis (mass PHA per mass VSS, w/w).

Chapter 5: Results and Discussion

Research was conducted to gain an understanding on the potential to achieve PHA production within the context of the BIOPHO-PX based WRRF configuration. Resource recovery from wastewater is a necessary direction that must be pursued to realize more sustainable infrastructure, and PHA is but one potential example of a high-value resource that can be generated from wastewater. SBRs were operated in accordance with the BIOPHO-PX treatment process and fed raw wastewater collected from the Moscow WWTP. The raw wastewater was augmented with fermenter liquor, produced from the fermentation of thickened primary solids collected from the Pullman WWTP, to provide the necessary VFAs to drive the necessary BIOPHO-PX biochemical reactions (principally EBPR reactions). These BIOPHO-PX reactors were maintained throughout the entirety of the conducted research and monitored regularly to ensure proper performance was maintained.

The conducted research advanced a concept that has not yet been investigated. Specifically, to the author's best understanding, achieving ADF-based PHA production using a mixed microbial inocula enriched under EBPR environmental conditions (i.e., electron acceptor-based PHA production) has briefly been investigated [*Chua et al.*, 2003; *Erik R. Coats et al.*, 2011; *Takabatake et al.*, 2002]. As described, EAC-based PHA synthesis is energetically, and likely metabolically, quite different from ADF PHA synthesis. Results presented and discussed in this chapter are structured to align with each research question: investigation of PHA production potential using BIOPHO-PX WAS as inocula; investigation on the impact of VFA loading on PHA production; and establishing maximum PHA production potential.

5.1 Investigation of PHA Production Potential Using BIOPHO-PX WAS as Inocula

The initial stages of this research utilized two BIOPHO-PX reactors identified as BIOPHO-PX 2 and BIOPHO-PX 3. Biomass was drawn from each treatment environment (anaerobic, aerobic, anoxic; see also Figure 2.1) of each reactor and subjected to PHA production analysis to establish corresponding PHA production potential. Table 5.1 displays

the results of individual PHA production assessments followed by further details into the observed PHA carbon yields. As a reminder, each PHA production test (Table 5.1) was operated by pulsing the inocula with six pulse additions containing 90% wastewater and 10% VFA rich fermenter liquor.

PHA accumulation ranged 7.8 – 21.8% d.c.w. (gPHA/gVSS) during this investigation. The data shows BIOPHO-PX 2 accumulating more PHA than BIOPHO-PX 3 during nearly every PHA production assessment with the exception of the 9/12/2015 aerobic PHA production assessment. Anaerobic inocula produced 9.9 – 21.8% d.c.w, aerobic inocula produced 7.8 – 20.1% d.c.w, and anoxic inocula produced 9.1 – 16.6% d.c.w. This suggests that anaerobic inocula has the potential for producing larger quantities of PHA, when compared to the other environments in which the inocula was drawn. It should be noted that anaerobic PHA production was corrected by subtracting any initial amounts of PHA that may have been present at the start of a PHA production assessment. In accordance with EBPR theory (Section 2.1.2.2) anaerobic inocula is expected to contain an initial amount of PHA. The initial amounts observed within this study were minuscule (i.e., less than 1.0% d.c.w).

Further investigation into the accumulated intracellular PHA showed a significant amount of HV (hydroxyvalerate). Recalling from Section 2.2.1, HV is desired within the intracellular PHA copolymer. HV content ranged 50 – 71% (molHV/molPHA) during this investigation; suggesting the production of highly malleable bioplastic. Anaerobic inocula produced 50 – 60% HV, aerobic inocula produced 51 – 67% HV, and anoxic inocula produced 52 – 71% HV. There appears to be little difference in HV contentment between the individual environmental inocula. This aligns well with literature [*Anderson and Dawes*, 1990]; suggesting that differing environmental conditions do not have an impact on the distribution of HV with the intracellular PHA copolymer.

Table 5.1: Preliminary PHA Carbon Yield Study of BIOPHO-PX. PHA Production Data using inocula from anaerobic, aerobic, and anoxic treatment environments.

Date	Inocula	MLVSS (mgVSS/L)	VFA Feed (mgCOD/L)	F:M	Maximum PHA (% d.c.w)	% HV (mol/mol)	Yield (Cmmol PHA /Cmmol VFA)
BIOPHO-PX 2							
5/21/15	Anaerobic	2800	188.30	0.10	21.8%	58%	0.678
8/14/15	Anaerobic	3040	95.49	0.05	16.1%	65%	1.127
10/10/15	Anaerobic	2740	132.23	0.07	10.0%	55%	0.608
5/28/15	Aerobic	2700	172.18	0.10	20.1%	60%	0.600
9/12/15	Aerobic	3560	65.62	0.03	12.5%	63%	1.045
10/10/15	Aerobic	2860	132.23	0.07	9.5%	51%	0.601
6/11/15	Anoxic	2440	129.23	0.08	16.6%	70%	0.478
9/13/15	Anoxic	3080	93.62	0.05	13.2%	66%	0.790
10/10/15	Anoxic	2940	132.23	0.07	9.9%	52%	0.485
BIOPHO-PX 3							
5/21/15	Anaerobic	3860	188.30	0.07	12.1%	50%	0.468
8/14/15	Anaerobic	3440	95.49	0.04	12.3%	66%	1.185
10/11/15	Anaerobic	3120	93.03	0.04	9.9%	54%	0.799
5/28/15	Aerobic	3460	172.18	0.07	17.6%	56%	0.721
9/12/15	Aerobic	3360	65.62	0.03	14.0%	67%	1.107
10/11/15	Aerobic	3260	93.03	0.04	7.8%	57%	0.495
6/11/15	Anoxic	3200	129.23	0.06	13.9%	64%	0.467
9/13/15	Anoxic	2760	93.62	0.05	11.6%	71%	0.656
10/11/15	Anoxic	3600	93.03	0.04	9.1%	54%	0.832

5.1.1 PHA Carbon Yield from Each WRRF Environment

As one assessment on the impact of BIOPHO-PX treatment environments on PHA production, PHA carbon yield values under the applied (hyper accumulating PHA) ADF conditions were calculated at the end of each pulse addition. PHA carbon yield provides an assessment of PHA production potential by reporting the fraction of VFA carbon transformed, between pulse additions, into intracellular PHA. Ideally an efficient PHA carbon yield should be greater than 1.0 Cmmol PHA/Cmmol VFA. Values approaching 1.0 Cmmol PHA/Cmmol VFA suggest two possible characteristics of the inocula MMC. First, there exists a sufficient population of carbon storing microorganisms and second, this population maintains the ability to efficiently transform bulk solution VFAs to intracellular PHA. Previous work has suggested that activated sludge has a low, less than 0.50 Cmmol PHA/Cmmol VFA, PHA carbon yield [*Morgan-Sagastume et al.*, 2010; *Morgan-Sagastume et al.*, 2015; *Takabatake et al.*, 2002]; in other words, it has been suggested that conventional WRRF treatment processes do not sufficiently enrich for the correct populations of carbon storing microorganisms.

Triplicate PHA production investigations were conducted per BIOPHO-PX reactor, with inocula from each treatment environment (i.e., anaerobic, aerobic, anoxic; thus 9 investigations per BIOPHO-PX reactor). Each PHA production investigation was operated in an ADF engineered environment and followed the multiple pulse feeding strategy. The PHA production reactors were inoculated with 200mL of biomass from the parent BIOPHO-PX reactor and operated with six pulse additions. Individual pulse additions lasted one hour: 1-minute fill, 49-minute react time, 8-minute settling, 1-minute draw and decanting, and 1-minute idle. Pulse additions contained 300mL of 90% wastewater and 10% municipal fermenter liquor. The 90:10 ratio was the same ratio used during daily operations of the BIOPHO-PX parent reactors. While this mixture contained the necessary nutrients to facilitate growth, sufficient PHA accumulation was noticed throughout the production run.

Cumulative PHA synthesized by the end of each pulse feeding was related to the cumulative total VFAs consumed for each new pulse feeding of substrate. Table 5.1

summarizes total overall yield values for each of the production assessments, while Table 5.2 summarizes the average PHA carbon yields at the end of each pulse feeding and an overall average of associated with the PHA production evaluation (average of the three production assessments). All average PHA carbon yields were greater than 0.50 Cmmol PHA/Cmmol VFA, suggesting that enough carbon storing organisms were present within the MMC to efficiently transform VFA carbon to PHA; these results were very encouraging. Experimental values observed at the end of each individual pulse feeding ranged between a low of 0.512 Cmmol PHA/Cmmol and a high 0.950 Cmmol PHA/Cmmol VFA.

Table 5.2: Time series PHA Carbon Yields (Cmmol PHA/Cmmol VFA) of inocula from anaerobic, aerobic, and anoxic treatment environments.

Inocula	Pulse Additions						Overall
	1	**2**	**3**	**4**	**5**	**6**	Average
BIOPHO-PX Reactor 2							
Anaerobic	0.889	0.853	0.904	0.781	0.713	0.728	0.811
Aerobic	0.573	0.835	0.734	0.803	0.785	0.762	0.749
Anoxic	0.512	0.556	0.610	0.639	0.523	0.571	0.568
BIOPHO-PX Reactor 3							
Anaerobic	0.905	0.874	0.932	0.869	0.727	0.597	0.817
Aerobic	0.610	0.676	0.717	0.903	0.950	0.792	0.775
Anoxic	0.570	0.721	0.657	0.787	0.603	0.573	0.652

Examining the data in more detail, MMC inocula from the anoxic environment displayed the lowest average PHA carbon yield, whereas MMC from the anaerobic environment displayed the highest average PHA carbon yield. While the explanation for this difference in PHA carbon yield was not the primary goal of this investigation, one possible reason may be due to hysteresis and associated shifts in intracellular machinery and enzymatic activity. More specifically, MMC cultures removed from anoxic conditions had been conditioned for the degradation of stored carbon (glycogen, PHA) and also growth.

This conditioning induced the genetic expression of enzymes and associated metabolisms that break down carbon and enable growth. Indeed, recent proteomic-level interrogation of a MMC performing ADF PHA synthesis demonstrated the intensified expression of PHA degradation related proteins [*Hanson et al.*, 2016-accepted]. In contrast, MMC cultures removed from anaerobic conditions have been conditioned specifically for PHA accumulation, expressing associated enzymes and metabolisms. This suggests that, when comparing anaerobic inocula to anoxic inocula, the treatment environment from which the inocula is withdrawn can impact PHA production.

When comparing anaerobic inocula to aerobic inocula, the same conclusion is not as clear. Statistical analysis (Student's t test) suggests that overall average PHA carbon yields are not significantly different statistically. P-values were 0.25 for BIOPHO-PX 2 and 0.56 for BIOPHO-PX 3. Comparing all six individual pulse additions shows that anaerobic inocula displayed higher PHA carbon yields during the first three pulse additions, while aerobic inocula displayed higher PHA carbon yields during the last three pulse additions. Overall averages showed anaerobic inocula having the highest PHA carbon yields (0.811 and 0.817 Cmmol PHA/Cmmol VFA) during a six pulse PHA production assessment, but this observation only pertains to PHA production assessments of six pulse additions. Certainly the same issue expressed in the AN/AX comparison (i.e., enzymes and metabolisms) would also be the case for the aerobic inocula; however, the anoxic inocula had been exposed to a longer "starvation" condition in the BIOPHO-PX reactor compared to the aerobic inocula, which may have affected overall metabolic potential as related to PHA synthesis. Further investigation into the shift in PHA carbon yields is described below.

5.1.2 The Shift in PHA Carbon Yield

As mentioned above, a shift in PHA carbon yield over the course of a production evaluation, between anaerobic and aerobic inocula, was observed. Figure 5.1 and Figure 5.2 illustrate the tabulated information from Table 5.2 as each PHA production investigation commenced. The aforementioned PHA carbon yield shift occurred after three pulse additions. This shift, seen in all PHA production assessments, is an indicator that possible

metabolic changes occurred.

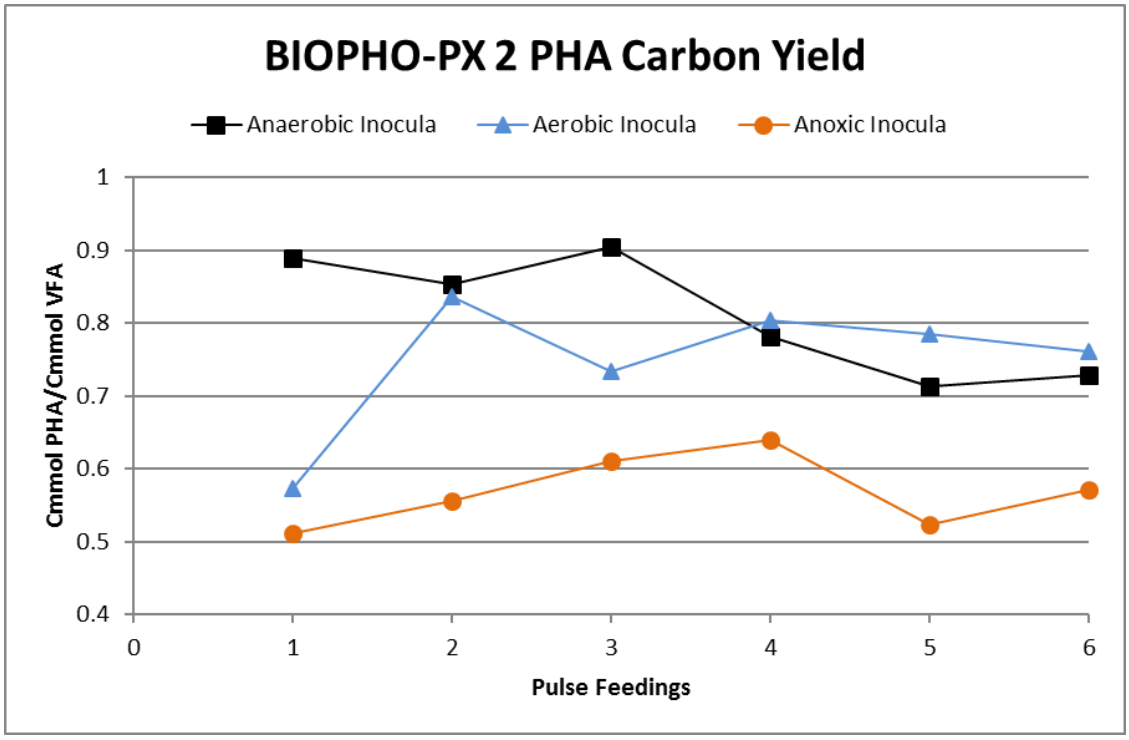

Figure 5.1: Time series PHA Carbon Yields of anaerobic, aerobic, and anoxic inocula from BIOPHO-PX 2

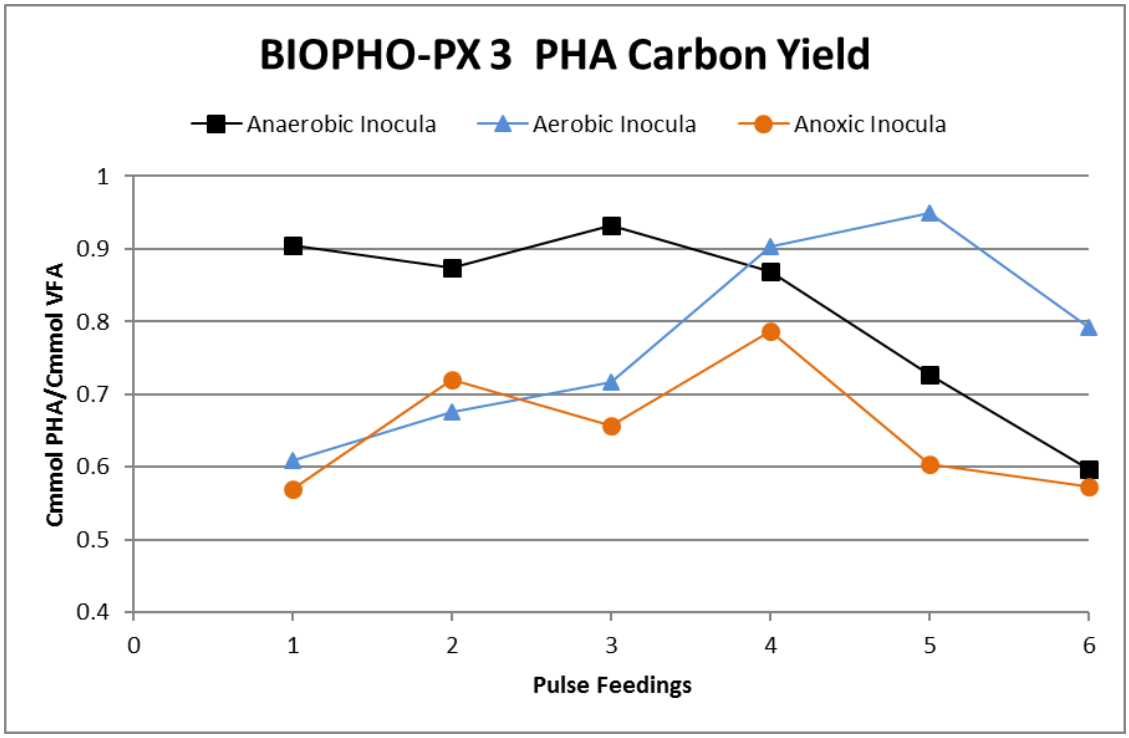

Figure 5.2: Time series PHA Carbon Yields of anaerobic, aerobic, and anoxic inocula from BIOPHO-PX 3

The presumed change in metabolic activity may have been caused by brief onsets of famine (i.e., depletion of VFAs) or potentially PHA saturation. Operational data over the respective production cycles (Figure 5.3, Figure 5.4, Figure 5.5) show bulk solution VFA profiles. As shown, for the latter two operational dates VFAs approach zero immediately preceding a new pulse addition of substrate; as such, the respective microbial consortium may have realized a corresponding brief period of famine. Conversely, for the first operational date, sufficient VFAs remained to avoid the onset of a PHA "famine" condition, and the data suggest that the MMC continued producing PHA. Lacking real-time VFA, monitoring the exact occurrence and duration of these famine periods remains unknown. The observed periods of famine in the latter two operational tests likely triggered metabolic changes within the MMC and altered metabolisms from PHA accumulation to PHA degradation; it is well understood that PHA degradation enzymes (depolymerases) are co-synthesized with PHA polymerases, and also reside on the PHA granule [*Hanson et al.*, 2016-accepted], thereby allowing for rapid onset of PHA consumption. The data also shows, for anaerobic and anoxic MMC, continual PHA accumulation coupled with a decrease in PHA carbon yield during the latter three pulse additions. While this data suggests that the anaerobic and anoxic MMC were approaching their full carbon storing potential (e.g., PHA saturation), it could alternately represent a metabolic shift to PHA consumption caused by the continual onsets of famine periods. Also, after realizing three famine periods in succession, PHA accumulating metabolisms may have begun to shut down. Conversely, aerobic MMC realized an increase in PHA carbon yield following the first three pulse additions of substrate. Thus, PHA accumulating metabolisms within the aerobic MMC may require priming before reaching their peak ability to transform the consumed VFAs into PHA.

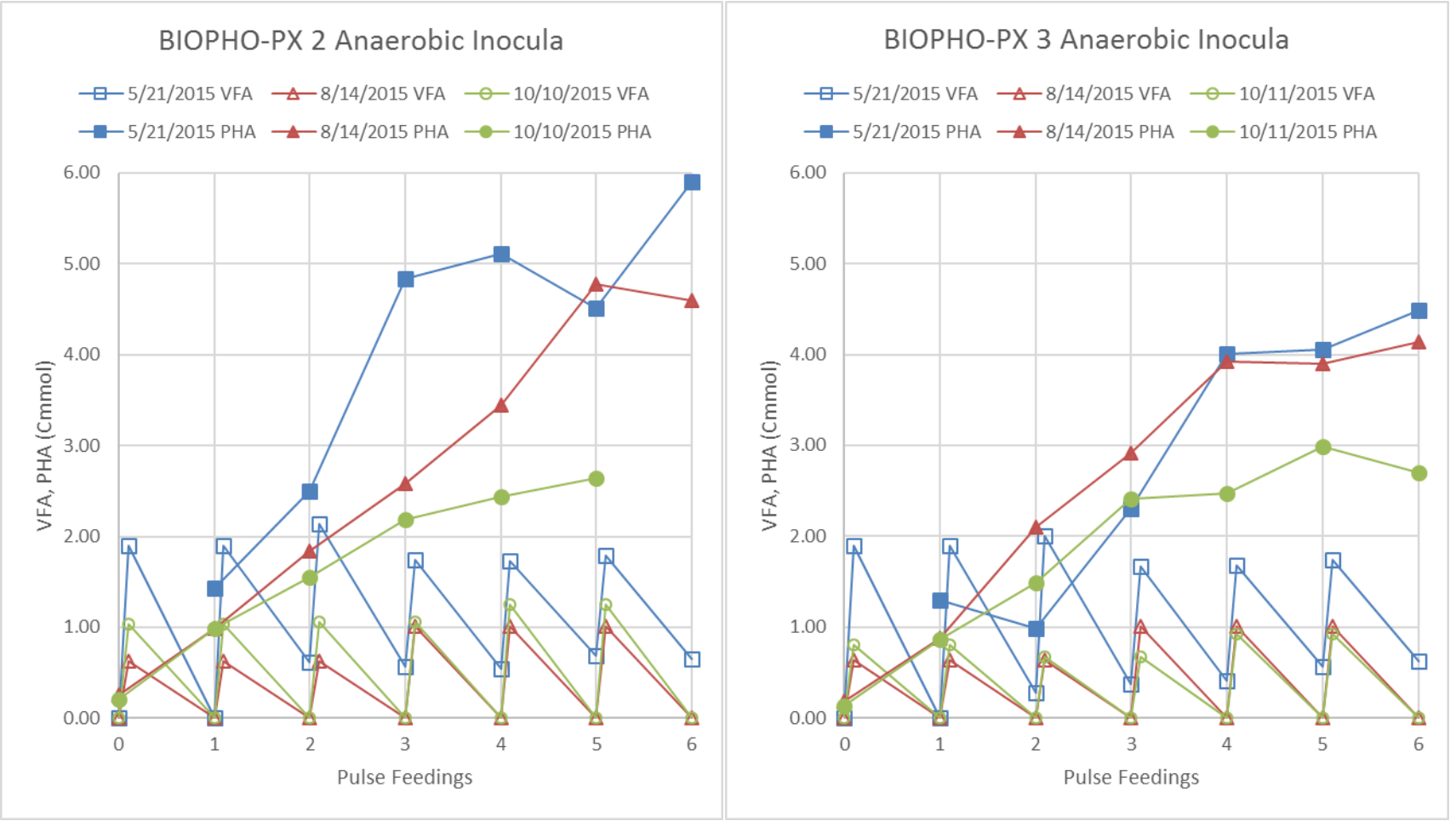

Figure 5.3: Time series VFA consumption and PHA production using Anaerobic inocula from BIOPHO-PX 2, BIOPHO-PX 3

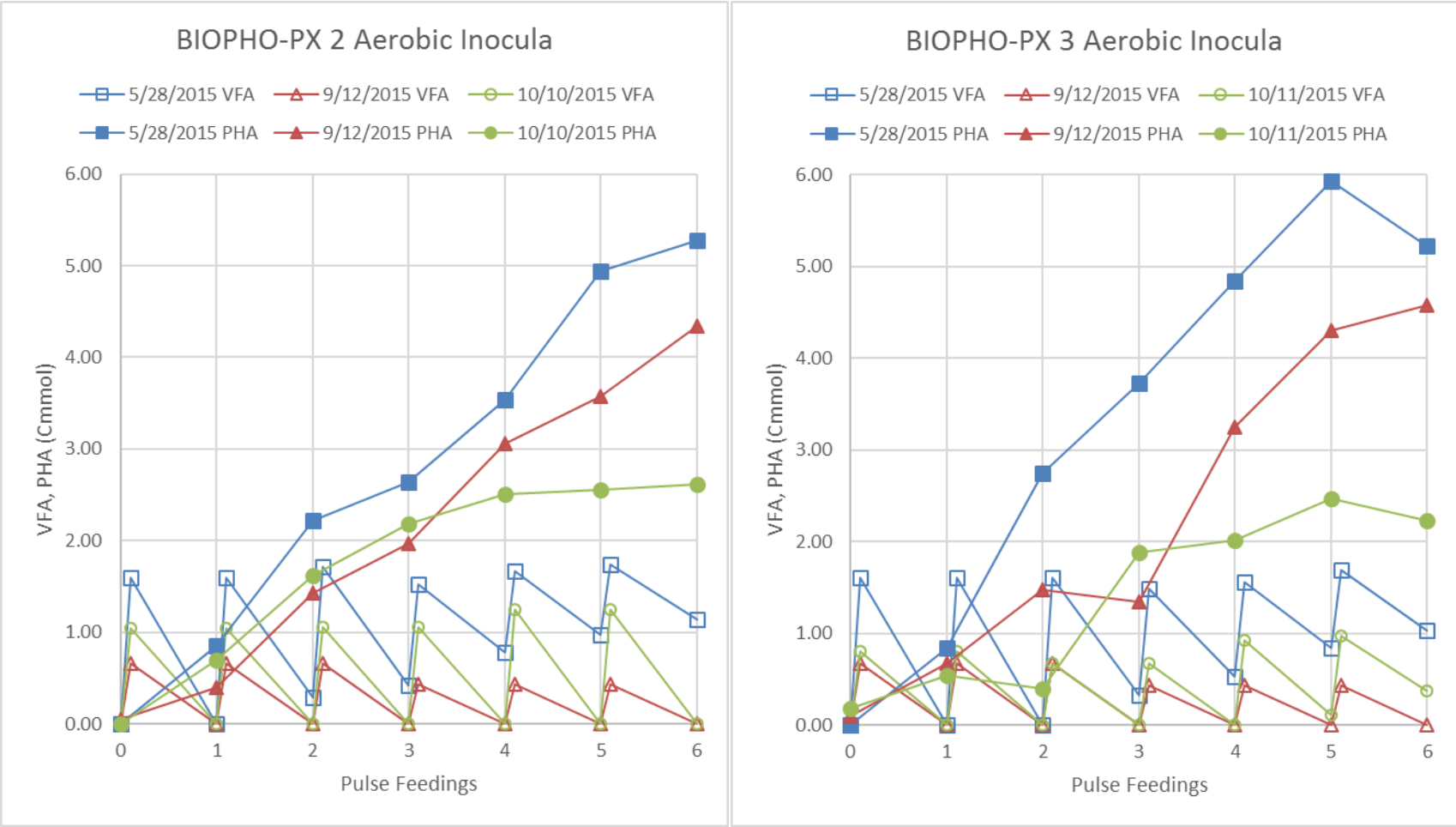

Figure 5.4: Time series VFA consumption and PHA production using aerobic inocula from BIOPHO-PX 2, BIOPHO-PX 3

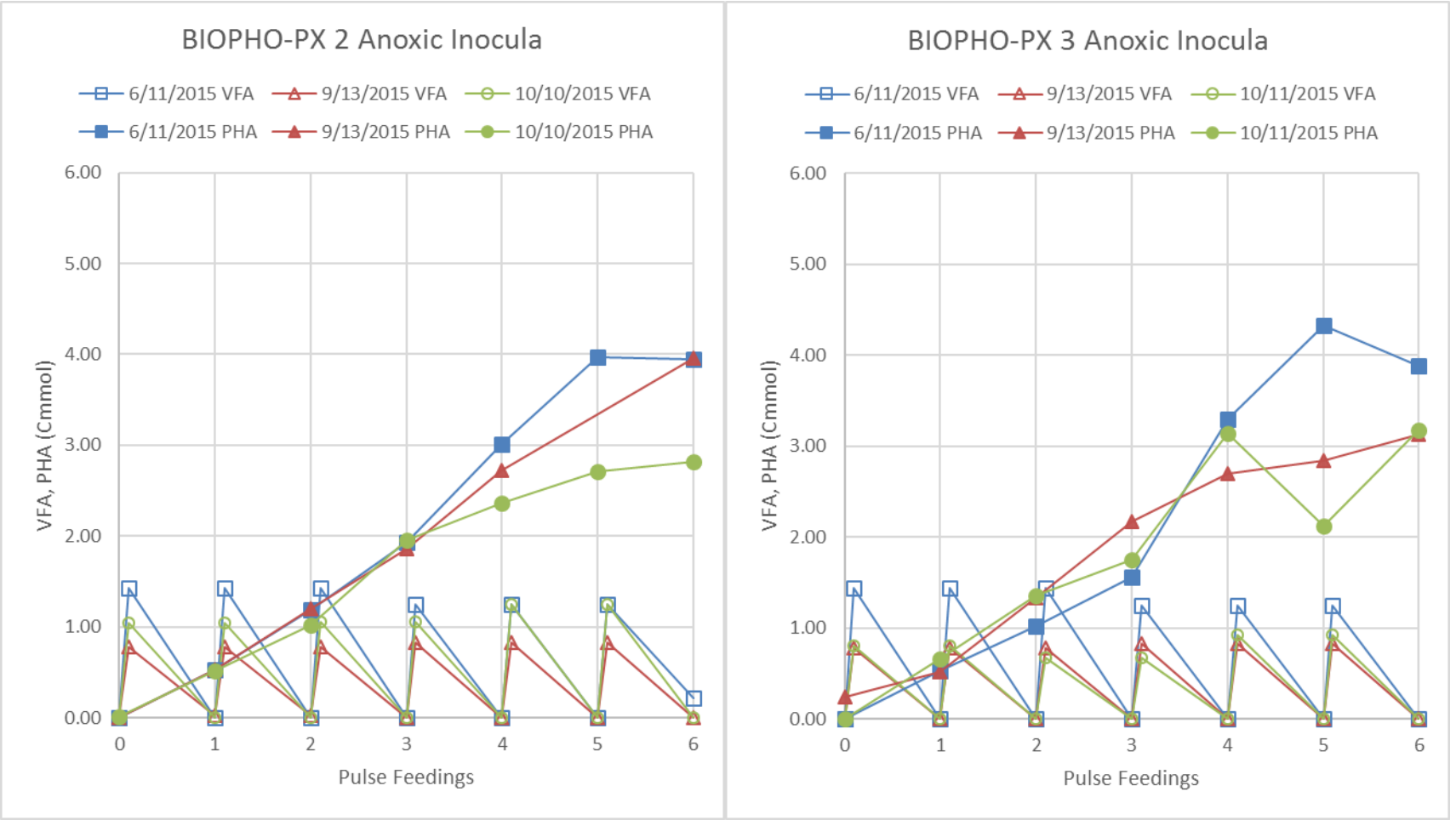

Figure 5.5: Time series VFA consumption and PHA production using anoxic inocula from BIOPHO-PX 2, BIOPHO-PX 3

In conclusion, the data acquired in this phase of the study provided preliminary answers to the first research question (Is polyhydroxyalkanoate (PHA) production within the BIOPHO-PX WRRF, achieved via the application of Aerobic Dynamic Feeding principles, affected by the treatment environmental conditions from which the inocula is withdrawn?). The data suggests that inocula for sidestream PHA production should be drawn from the anaerobic period of the BIOPHO-PX process. This inocula displayed the highest PHA carbon yield; therefore, it is most suited for efficient transformation of consumed VFAs to PHA.

5.2 Investigation on the Impact of VFA Loading on PHA Production

With the source of inocula for PHA production established (anaerobic zone), and associated PHA carbon yields determined, PHA production analyses continued only using inocula from BIOPHO-PX 3. Biomass was henceforth subjected to PHA production analysis consisting of three pulse additions. These operating parameters and conditions were chosen for future experiments in an attempt to continue realizing the observed PHA accumulation and efficiency (i.e., preliminary data revealed a maximal response with three pulses). In addition, investigations were conducted with increased VFA loading rates in an attempt to realize larger quantities of PHA. The pre-screening inocula-focused investigations utilized the same substrate conditions as realized by said inocula in the parent BIOPHO-PX reactor. However, PHA production and maximal intracellular PHA concentration requires dosing larger quantities of substrate to the inocula biomass.

To better understand the potential impact of increasing the VFA loading on PHA production reactor operations (more VFAs could extend the associated feast period, thereby effecting the time periods for substrate addition), substrate uptake rates were first evaluated. Once VFA uptake rate-VFA loading relationships were established, experiments were designed and operated with higher VFA loading rates. Further details involving this phase of the investigations are provided below.

5.2.1 VFA Substrate Uptake Rates

The investigation of VFA uptake rates provided critical insight into design and

operations of a successful PHA production reactor. In targeting maximal PHA accumulation, periods of extended feast must be maintained in order for PHA synthesis to continue. If periods of famine occur between pulse additions of substrate, as seen in the preliminary PHA carbon study, biomass metabolic shifts may occur and PHA synthesis may cease concurrent with PHA utilization (while also further mobilizing PHA degradation enzymes (which are maintained on the PHA granule [*Koller et al.*, 2013]), thereby impairing the target operations). Conversely, if VFAs are not sufficiently consumed between pulse additions, a buildup of VFAs can occur, leading to possible substrate inhibition [*Erik R. Coats et al.*, 2011].

The kinetic parameter of specific VFA uptake describes the mass of VFAs consumed per mass (gVSS) of MMC per unit time; the specific VFA uptake rate is often written as q_{VFA}, with units mgCOD VFA/(gVSS*minute). Applying the specific VFA uptake rate, in combination with a known mass of VFAs in the substrate, will help establish the amount of time needed to deplete all available VFAs between pulse additions. With this knowledge, the operations of the PHA production reactor can be adjusted accordingly to allow for desired extended feast periods while maintaining a minimum/maximum bulk solution VFA concentration.

A total of 15 specific VFA uptake experiments were conducted with inocula from the BIOPHO-PX 3 reactor. The BIOPHO-PX 3 reactor was chosen because said inocula displayed the highest PHA carbon yield in the preliminary investigations, and thus was anticipated to accumulate the maximum amount of PHA. Each experiment was inoculated with 150 mL of inocula drawn from the anaerobic period and operated in an ADF engineering environment with a single pulse of substrate. Wastewater feedstock composition varied from 10% municipal fermenter liquor (MFL) to 100% MFL (volumetric basis), which thus provided a range of different VFA concentrations (and thus varying F:M ratios). It has been noted that F:M is the main parameter which establishes if growth or PHA accumulation occurs [*Majone et al.*, 1996]. Majone showed that increasing the F:M increases the amount of PHA stored per VFAs removed (specifically PHB/Acetate), while simultaneously observing a decrease in VFA uptake as loading rates became “very high”. Specific details regarding the VFA uptake

rates as a function of F:M for BIOPHO-PX MMC can be found in Table 5.3.

Table 5.3: VFA Uptake Rate Results for design of F:M Experiments

% MFL	VSS (mg)	Acetate F:M (mgCOD/mgVSS)	Propionate F:M (mgCOD/mgVSS)	Total F:M (mgCOD/mgVSS)	% Propionate (mgCOD/mgCOD)	Specific VFA Uptake (mgCOD/gVSS*minute)
10%	457.5	0.039	0.067	0.106	62.95	1.19
10%	457.5	0.055	0.070	0.125	55.97	0.98
10%	457.5	0.058	0.076	0.134	56.93	1.08
25%	420.0	0.129	0.153	0.283	54.25	0.97
25%	420.0	0.099	0.186	0.285	65.36	0.96
50%	432.0	0.206	0.328	0.534	61.36	0.81
60%	385.0	0.166	0.441	0.607	72.65	0.98
50%	432.5	0.322	0.405	0.727	55.71	0.94
60%	385.0	0.262	0.567	0.829	68.41	1.50
80%	385.0	0.352	0.777	1.130	68.80	1.24
90%	385.0	0.329	0.948	1.278	74.24	1.33
80%	385.0	0.398	0.887	1.284	69.03	1.10
100%	385.0	0.356	1.071	1.427	75.05	1.31
100%	375.0	0.740	1.103	1.843	59.87	2.10
100%	375.0	0.776	1.134	1.911	59.37	2.17
				0.33		1.83
[*Frison et al.*, 2015b]				0.45		3.28
				0.20		3.98
				0.10		1.88
[*Frison et al.*, 2015a]				0.10		2.11
				0.17		2.11
				0.17		2.35
				0.17		7.28
[*Morgan-Sagastume et al.*, 2010]				0.17		3.29
				0.17		3.76

The observed specific VFA uptake rate as a function of the F:M ratio is illustrated in Figure 5.6. The VFAs within the feedstock were dominated by acetate and propionate, with minimal (if any) longer chain VFAs. It can be seen that q_{VFA} ranged from approximately 0.8 to above 2.0, with a noticeable increase occurring around an F:M of 0.75. The dotted line plotted in Figure 5.6 shows the general trend in q_{VFA} as F:M increases. While the exact cause of the shift is unknown, one possible explanation may involve the concentration dependent kinetics and associated energetics of VFA metabolisms. Specifically, less energy (as ATP) is required to uptake and metabolize HPr vs. HAc [*Filipe et al.*, 2001; *Oehmen et al.*, 2005; *Zhang et al.*, 2008]. In comparison to literature values (Table 5.3), observed q_{VFA} is approximately 100 – 200% lower at the respective F:M. However, as F:M values increase it can be seen that observed specific VFA uptake begins to reach the values observed in literature.

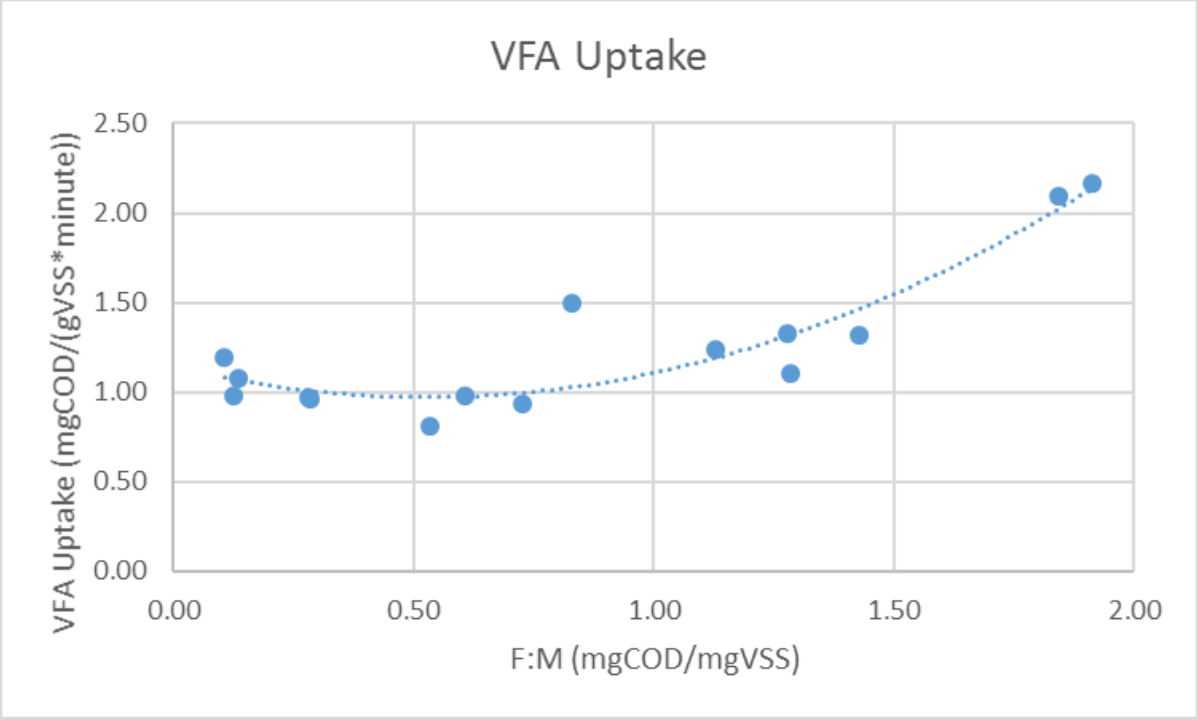

Figure 5.6: VFA Uptake Rates as a function of F:M for design of F:M experiments.

These q_{VFA} vs. F:M investigations provide a crucial design parameter for further development of PHA production with the context of the WRRF configuration. This

foundation provides key insight into the design of future PHA production with a goal of achieving sufficient VFA consumption while simultaneously avoiding the onset of famine.

5.2.2 F:M as an Applied Operating Parameter for PHA Production

Building upon the foundational q_{VFA} vs. F:M investigations discussed above, seven subsequent PHA production investigations were conducted. The first set of F:M experiments (referred to as F:M Experiment 1) consisted of four individual PHA production tests (operated concurrently, with the same inocula). Three of the PHA production tests (R1, R2, R3) were designed with an F:M ratio of 0.52 (per pulse, not for the full PHA production assessment; based on gVSS inocula) and followed the multiple pulse feeding strategy. A F:M of 0.52 represented an approximate 10-fold increase over the F:M of the preliminary work (Section 5.1). Increasing the F:M substantially (10-fold) will allow for insight into the potential impacts of F:M upon PHA production, while remaining within empirically derived ranges (Section 3.2) and avoiding possible substrate inhibition (Section 3.1.1.2). Due to increased VFA concentrations within the feedstock (associated with the increased F:M), sodium bicarbonate was added to neutralize the low pH. Feedstock pH values initially ranged from 4.50 – 5.30; the pH was increased to a value of approximately 6.60. Individual PHA production reactors received three pulse additions of VFA-rich substrate, which equated to an accumulated F:M of 1.56 over the full production assessment. Using the established VFA uptake rate data (Figure 5.6), the time between each pulse substrate addition was established at approximately six hours, equating to a total PHA production time of 18 hours. For comparison (with both the triplicate 3-pulse PHA production reactors, and with the original q_{VFA} vs. F:M data), a separate reactor (Single Pulse) was operated at an F:M of 1.60 following the single pulse feeding strategy.

Each discrete PHA production assessment was evaluated for observed VFA uptake, PHA carbon yield, and maximum intracellular PHA dried cell weight. Average values (Table 5.4) collected show little difference between individual PHA production assessments in terms of PHA carbon yield and maximum intracellular PHA (inocula gVSS basis). Average PHA carbon yield ranged between 0.28 – 0.31 Cmmol PHA/Cmmol VFA and maximum PHA

ranged between 13.20 – 15.03% dried cell weight (d.c.w.). Time series PHA accumulation (Figure 5.7) showed similar accumulation trends within and across all assessments. The similarities between R1, R2, and R3 were expected, as each was based on the same inocula and exposed to the same operating conditions. The single pulse reactor, while containing the same inocula, realized similar intracellular PHA content throughout its single pulse addition corresponding with the 1st and 2nd pulse addition of R1, R2, and R3. This suggests that the high F:M realized by the single pulse reactor encouraged a similar response to the accumulation of PHA. Observations at the end of the PHA production assessment showed a decrease in intracellular PHA accumulation, for all PHA production assessments, indicating that PHA had begun to be degraded.

Table 5.4: F:M Experiment 1 Results

Feeding Strategy	F:M (mgCOD/mgVSS)	VFA Uptake (mgCOD/(gVSS*min)	PHA Carbon Yield (Cmmol PHA/Cmmol VFA)	Maximum PHA (% d.c.w.)	%HV (mol/mol)
R1 3-Pulse	0.52	2.34 (±0.16)	0.28 (±0.05)	13.60%	68%
R2 3-Pulse	0.52	2.28 (±0.24)	0.31 (±0.09)	13.20%	68%
R2 3-Pulse	0.52	2.35 (±0.38)	0.30 (±0.11)	15.03%	68%
Single Pulse	1.60	1.89 (±0.33)	0.29 (±0.19)	14.59%	67%

Considering VFA kinetics, a notable difference was observed in specific VFA Uptake Rate between the three pulse and single pulse PHA production reactors. The three pulse fed reactors (R1, R2, R3) averaged a higher specific VFA uptake, ranging from 2.28 – 2.35 mgCOD/(gVSS*min); when compared to the single pulse reactor, which averaged at 1.89 mgCOD/(gVSS*min). Since all PHA production assessments contained the same inocula, this difference suggests that operational conditions, multiple pulse feeding vs. single pulse feeding, impact specific VFA uptake. While the total mass of VFAs provided to the inocula was similar, the single pulse feeding strategy exposed the inocula to a higher VFA substrate loading at the start of the PHA production assessment. The data suggests that this initial exposer of larger quantities of VFAs inhibited specific VFA uptake.

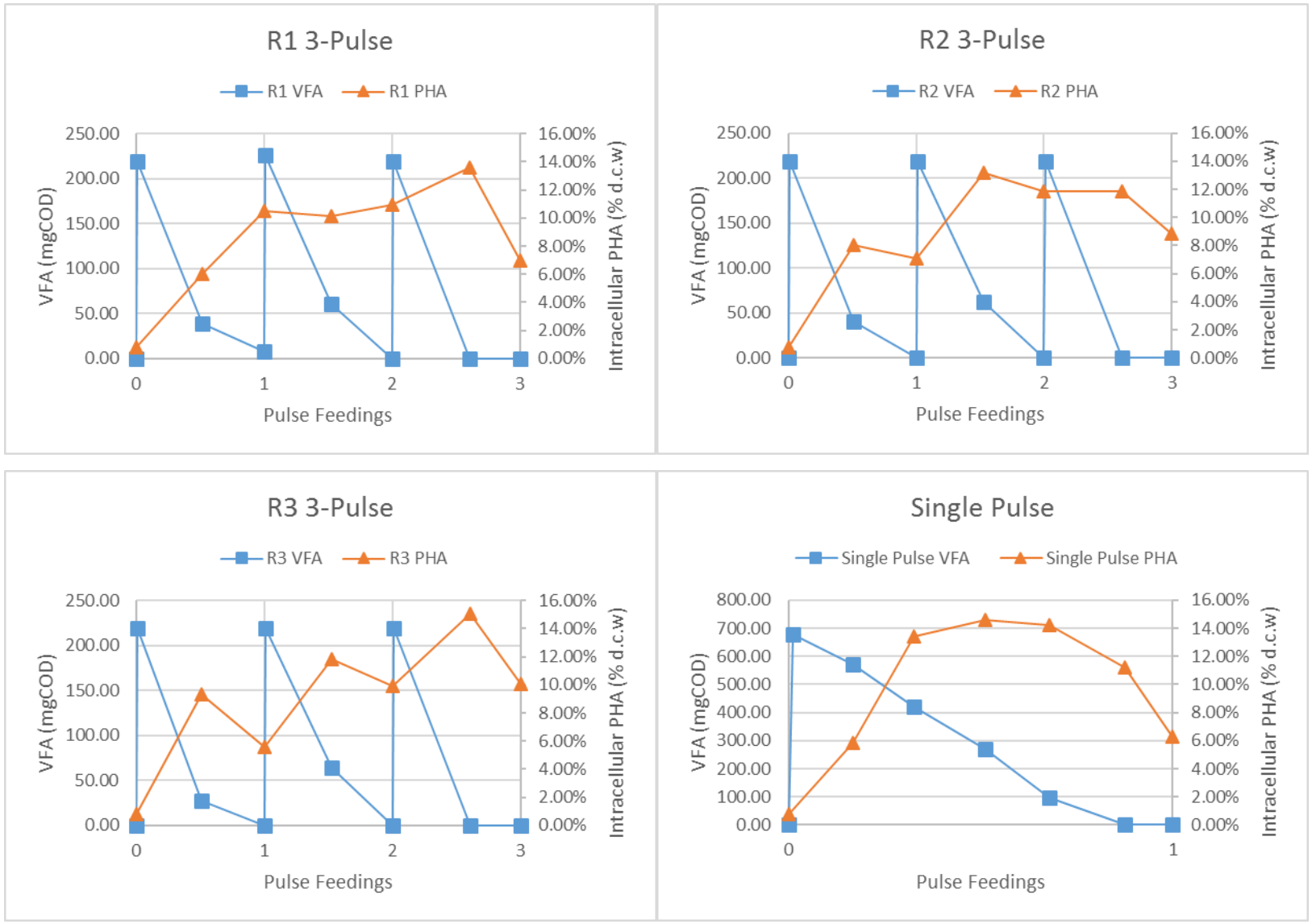

Figure 5.7: Time series VFA consumption and PHA production of F:M Experiment 1. R1, R2, and R3 operated with a F:M of 0.52; Single Pulse operated with a F:M of 1.60.

When comparing these results to the preliminary work (Figure 5.6), two major differences were observed. First, the observed specific VFA uptake differed substantially from the initial VFA substrate uptake rates investigation used for experimental design; Figure 5.8 illustrates the specific VFA uptake observed during F:M experiment 1. As shown, the specific VFA uptake at a F:M of 0.52 was approximately a 230% increase of the original value used for experimental design. Conversely, The VFA uptake for a F:M of 1.60 approximately fit the value used for experimental design. The result of this increase in VFA uptake was an early onset of famine during individual PHA production assessments. VFA profiles, Figure 5.7, show VFAs being depleted before the next pulse feeding, indicating that periods of famine had occurred. The onset of these famine periods during individual PHA production assessments may offer explanation to why observed maximum intracellular PHA percentages remained low (i.e., < 20% d.c.w.).

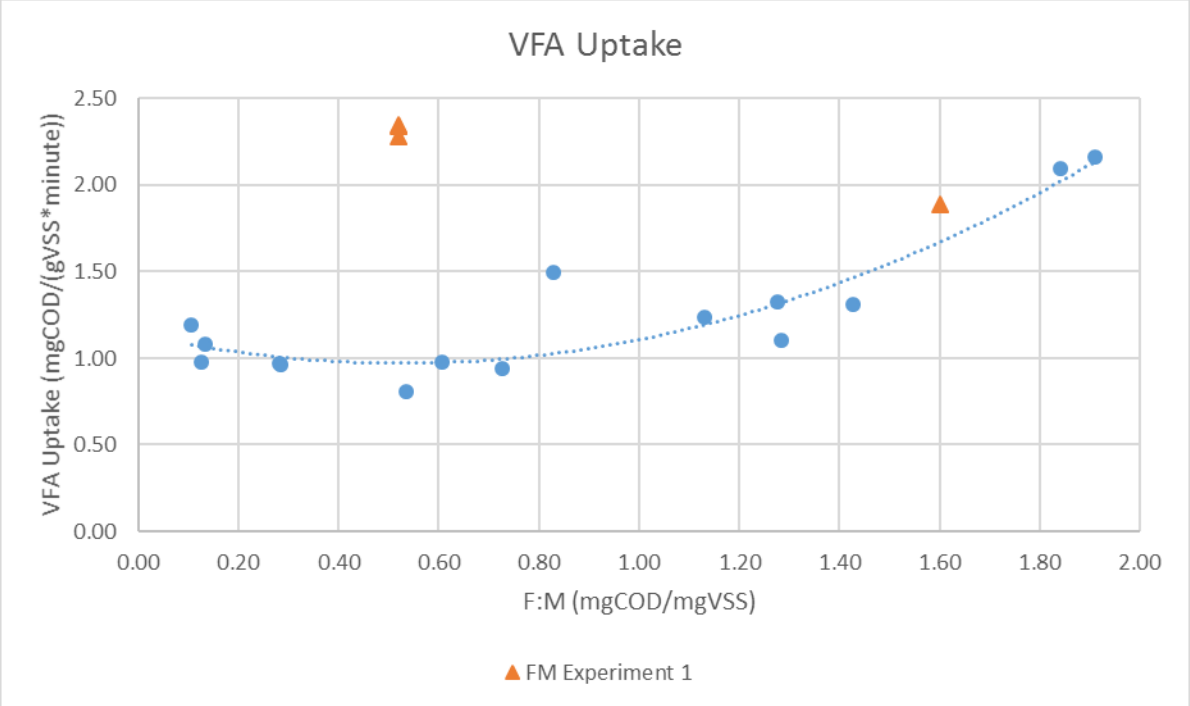

Figure 5.8: Comparison of F:M Experiment 1 VFA uptake with original design VFA uptake.

Second, PHA carbon yield measurements were substantially lower from those measured in the preliminary work (Section 5.1; Table 5.1). Specifically, over the course of the PHA production assessment, PHA carbon yield values ranged 0.09 – 0.54 Cmmol PHA/Cmmol

VFA (Figure 5.9), with all assessments realizing an average of approximately 0.30 Cmmol PHA/Cmmol VFA (Table 5.4). Low PHA carbon yields (e.g., < 0.50) may have occurred for two reasons. Periods of famine may have been experienced, thus shifting metabolisms towards degrading PHA for cellular growth instead of accumulating it, or increased F:M ratios may have induced some level of inhibition on the ability of the MMC to store VFAs as PHA. Further investigation was warranted to determine if famine periods are inhibiting PHA production at increased F:M ratios.

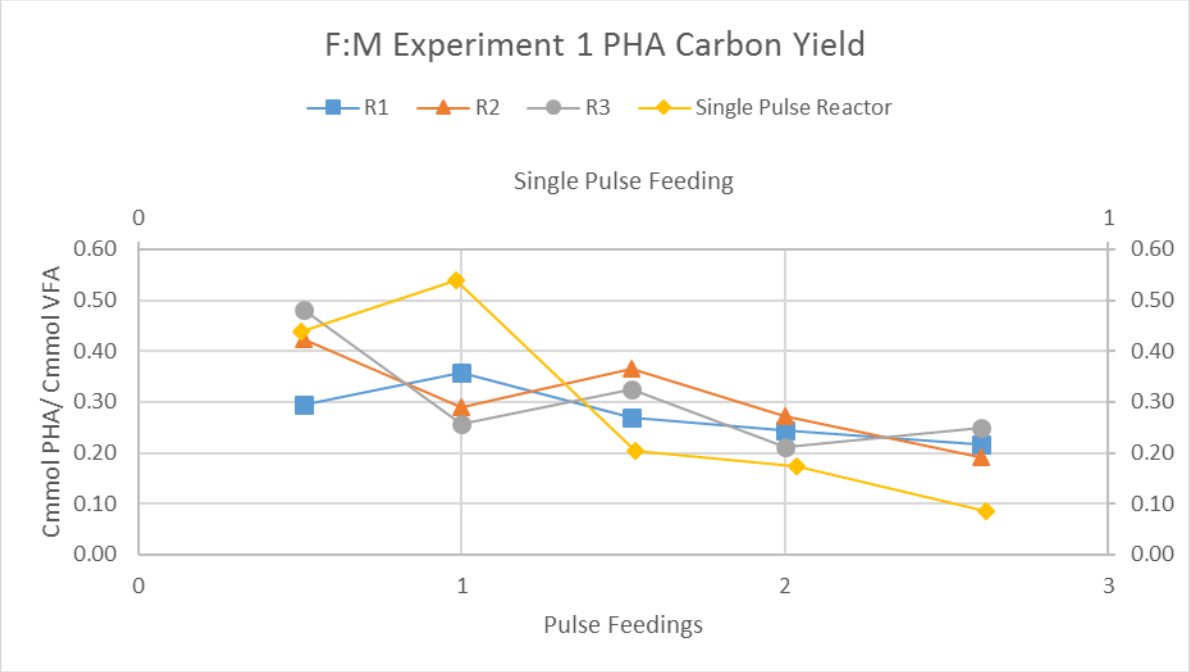

Figure 5.9: Time series PHA carbon yield of F:M Experiment 1. R1, R2, and R3 operated with a F:M of 0.52; Single Pulse operated with a F:M of 1.60.

The second set of F:M experiments (F:M Experiment 2) consisted of three individual PHA production reactors, each operated concurrently (i.e., same inocula). Similar to the operational state of F:M experiment 1, these PHA production reactors (R4, R5, R6) followed the multiple pulse feeding strategy, but each was operated under different F:M ratios (Table 5.5). Each reactor received three pulse additions of pH-neutralized feedstock with time periods between substrate feeding corrected for the increased VFA uptake observed in F:M experiment 1. The experiments were based on the observed specific VFA uptake of 2.50 mgCOD/(gVSS*min) combined with maintaining a minimum bulk solution VFA

concentration equal to 50% of VFAs added; this equated to time increments between substrate feeding of 1 hr 45 min for R4, 2 hr 45 min for R5, and 3 hr 30 min for R6. During the third substrate feeding, feedstock was switched between PHA production reactors R5 and R6. In other words, R6's feedstock was fed to R5 provide additional VFAs, while R5's feedstock was fed to R6 in an attempt to avoid any possible substrate inhibition. The goal of this investigation was to eliminate the onset of a famine period between substrate pulses while concurrently avoiding VFA inhibition, allowing for enhanced insight into the impact of F:M on PHA production.

Indeed, F:M experiment 2 successfully avoided famine periods during operations (Figure 5.10). The VFA profiles reveal that VFA concentrations avoided the onset of famine periods during PHA production, which was expected as a result of the 50% VFA concentration "floor" applied in the experimental design. VFA concentrations observed throughout the PHA production assessment did not decrease below 50% of the previous VFA addition. Data collected (Table 5.5) show average specific VFA Uptake ranging 2.09 – 3.90 mgCOD/(gVSS*min), which generally aligned with the experimental design value of 2.50 mgCOD/(gVSS*min). In comparison to the F:M experiment 1 multiple pulse reactors (R1, R2, R3), F:M experiment 2 specific VFA uptake showed similarities to the values previously observed (2.28 – 2.35 mgCOD/(gVSS*min)), but experienced a larger range overall. For direct comparison, experiment R5, which experienced an F:M similar to that of R1, R2, and R3 (total applied F:M of 0.52 over three substrate pulses), realized a specific VFA uptake of 2.27 mgCOD/(gVSS*min). Amongst the three PHA production reactors, average specific VFA Uptake (Table 5.5) appeared to increase slightly with an increase in F:M. Further investigations using statistical analysis (Student's t test) result in p-values of 0.31 for R4 and R5, 0.69 for R5 and R6, and 0.15 for R4 and R6, which suggests that specific VFA uptake between PHA production reactors was not statistically different.

Table 5.5: F:M Experiment 2 Results

Feeding Strategy	F:M (mgCOD/mgVSS)	VFA Uptake (mgCOD/(gVSS*min)	PHA Carbon Yield (Cmmol PHA/Cmmol VFA)	Maximum PHA (% d.c.w.)	%HV (mol/mol)
R4 3-Pulse	0.33	2.09 (±0.52)	0.71 (±0.12)	12.53%	67%
R5 3-Pulse	0.50[1]	2.27 (±0.63)	0.59 (±0.20)	10.66%	69%
	0.96[2]	3.00 (±1.94)	0.16 (±0.02)		
R6 3-Pulse	0.96[1]	2.46 (±1.08)	0.22 (±0.07)	13.33%	69%
	0.50[2]	3.90 (±2.20)	0.14 (±0.02)		

[1]First two pulses of substrate; [2]Last substrate pulse.

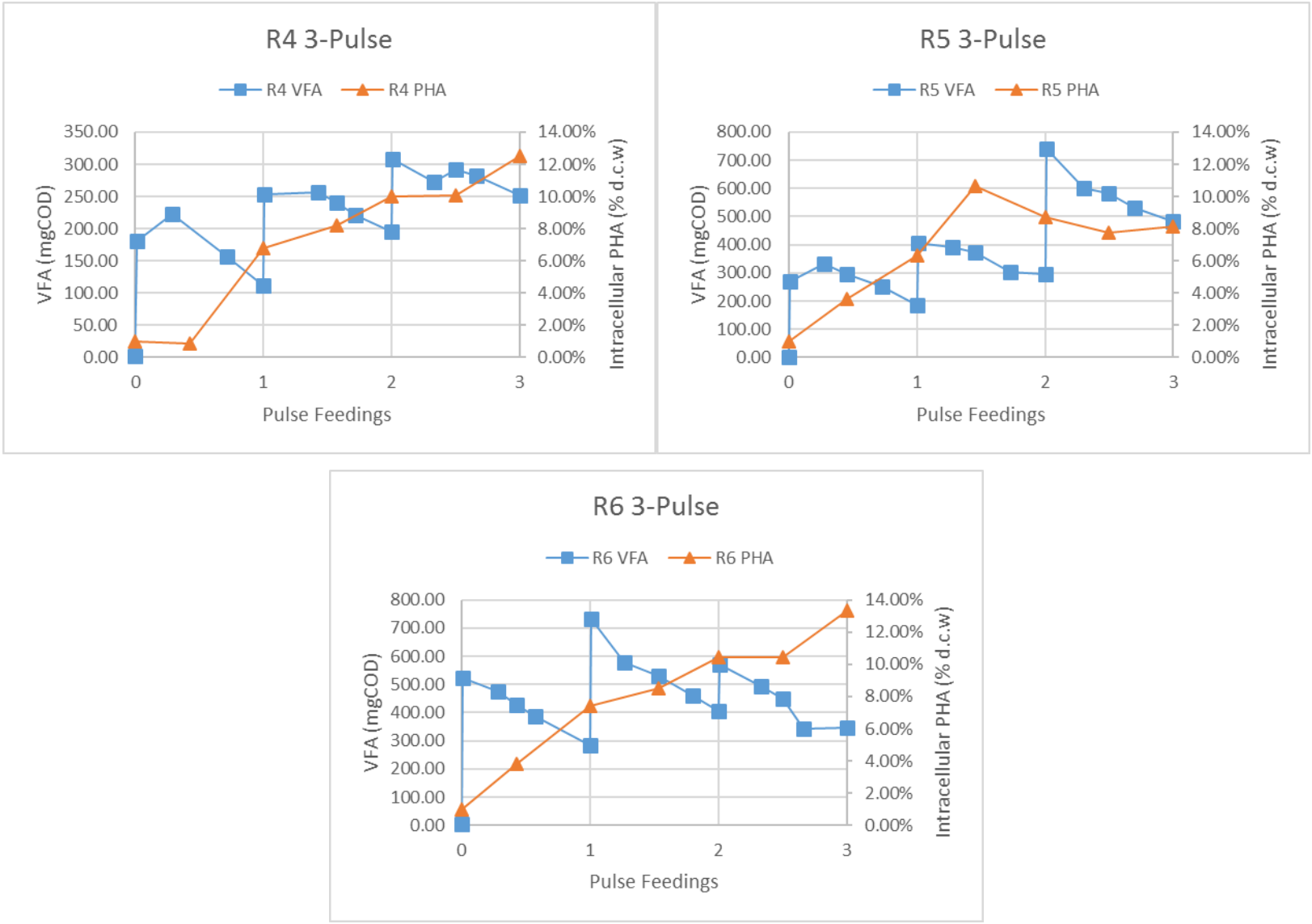

Figure 5.10 Time series VFA consumption and PHA production of F:M Experiment 2. R4 operated at a F:M of 0.33, R5 at a F:M of 0.50, and R6 at a F:M of 0.96.

Data from F:M experiment 2 was compared against F:M experiment 1 and the earlier VFA Substrate Uptake Rates investigation (Figure 5.11). Similar to F:M experiment 1, specific VFA uptake increased, realizing an approximately 200 – 400% increase over the original experimental design values illustrated by the dotted line. Interestingly, the third pulse addition of R5 and R6 resulted in larger specific VFA uptake rates compared to the first two pulse additions. Specific VFA uptake increased 32% and 59% respectively to 3.0 and 3.9 mgCOD/(gVSS*min).

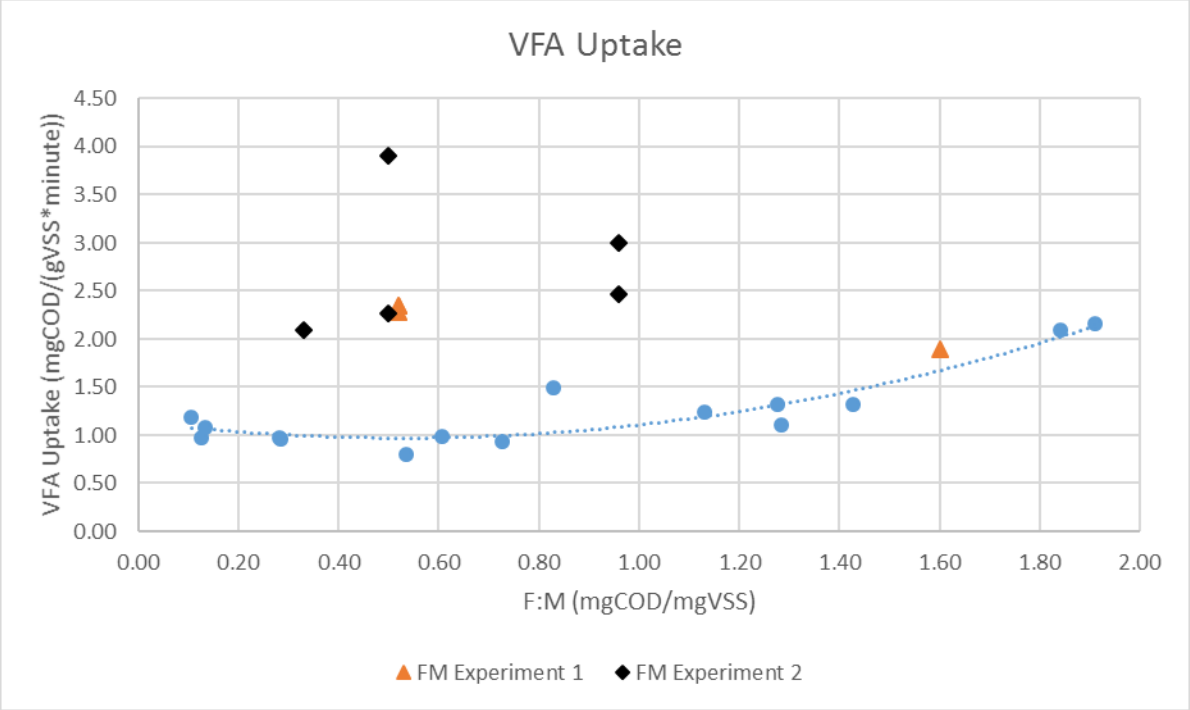

Figure 5.11: Comparison of F:M Experiment 2 VFA uptake with F:M Experiment 1 and original design VFA uptake.

Maximum intracellular PHA accumulation for F:M experiment 2 ranged from 10.66 – 13.33% d.c.w. (Table 5.5). In comparison, maximum intracellular PHA accumulation did not reach the values realized by F:M experiment 1 (13.20 – 15.03%). This result was unexpected, as the operational conditions for F:M experiment 2 were similar to those of F:M experiment 1, and F:M experiment 2 successfully avoiding the onset of famine conditions. However, a closer look at the time series intracellular PHA concentration, Figure 5.10, shows upwards trends for R4 and R6 as the PHA production assessments commenced; these results are a stark contrast to that observed in F:M experiment 1, wherein

intracellular concentrations leveled off and then decreased with time. All three reactors (R4, R5, R6) generally showed similar increases in PHA accumulation, despite each reactor experiencing a different design F:M ratio. However, reactor R5 exhibited a general decrease in intracellular PHA at pulse feeding 2 and thereafter, which corresponded with a significant increase in F:M.

PHA carbon yields measured within F:M experiment 2 show a better fit with those measured in the preliminary work (Section 5.1), and an improvement over F:M experiment 1. Figure 5.12 illustrates the observed time series PHA carbon yields for F:M experiment 2. PHA carbon yield values ranged 0.60 – 0.86 Cmmol PHA/Cmmol VFA for R4, 0.15 – 0.78 Cmmol PHA/Cmmol VFA for R5, and 0.13 – 0.19 Cmmol PHA/Cmmol VFA for R6. It was observed that R4 and R5 PHA carbon yields were highest within the first two pulse additions (lower F:M) and decreased during the third, while PHA carbon yields for R6 remained relatively constant for the duration of operations (higher initial F:M). The F:M of R5 matched that of R1, R2, and R3, yet realized an increase in PHA carbon yield. During F:M experiment 1, R1, R2, and R3 experienced an average PHA carbon yield of 0.28 – 0.31 Cmmol PHA/Cmmol VFA. Comparatively, during F:M experiment 2, R5 experienced an average PHA carbon yield of 0.59 Cmmol PHA/Cmmol VFA. However, the q_{VFA} was similar across R1-3, 5. Considering that these reactors principally differed operationally to avoid the onset of famine (R5 avoided; R1-3 did not), the data suggests that the onset of famine (experience in R1, R2, and R3) did negatively impact PHA carbon yields. Further investigation into R4, R5, and R6 showed that PHA carbon yield decreases as F:M increased, which suggests that after eliminating the onset of famine, PHA carbon yields nonetheless remain negatively impacted by elevated F:M ratios.

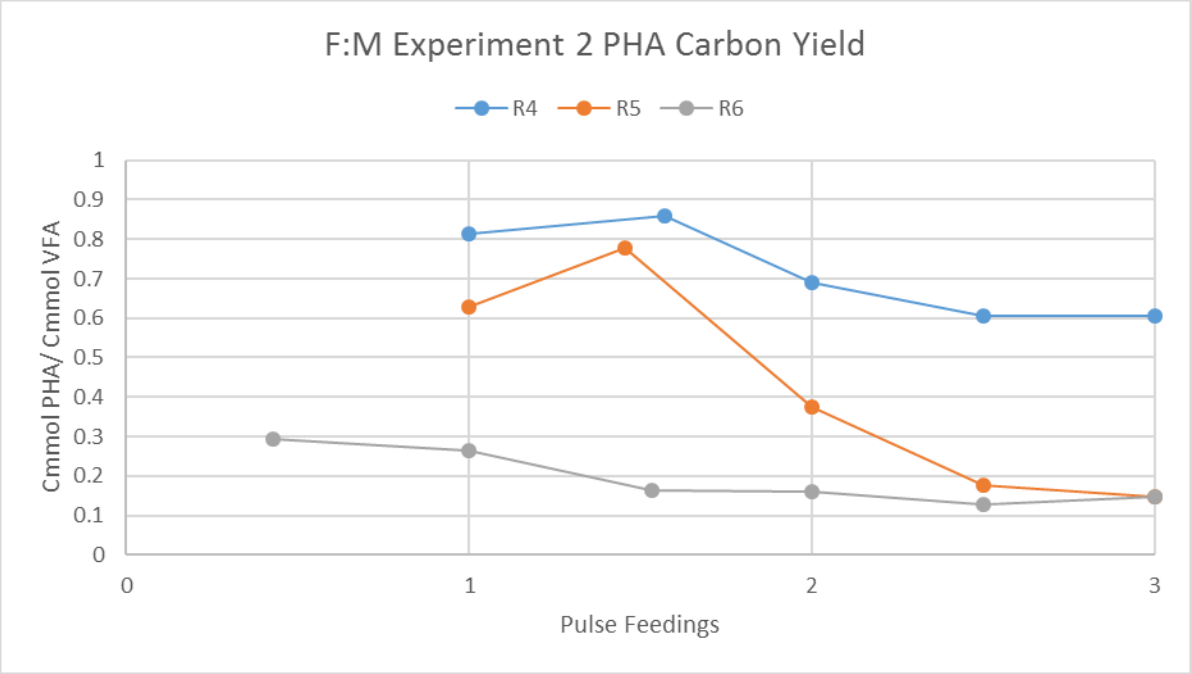

Figure 5.12: Time series PHA carbon yield of F:M Experiment 2. R4 operated at a F:M of 0.33, R5 at a F:M of 0.50, and R6 at a F:M of 0.96.

The data acquired and discussed herein provided preliminary answers to the second research question (What effect does the ratio between volatile fatty acids (VFAs) and active biomass, measured and expressed as the Food-to-Microorganism (F:M) ratio, have on polyhydroxyalkanoate production?). Specifically, the data suggests increased F:M ratios do not lead to increased PHA production. While specific VFA uptake rates appeared unaffected, increasing the F:M realized by the PHA production MMC resulted in lower PHA carbon yields and no consequential increase in intracellular PHA. Therefore, lower F:M ratios should be utilized during sidestream PHA production. Results presented herein suggest a F:M ratio less than 0.30 mgCOD/mgVSS.

5.3 Establishing Maximum PHA Production Potential

Maximum realized PHA production was investigated amongst all three sets of PHA production assessments (Preliminary PHA Carbon Yield Study, F:M Experiment 1, F:M Experiment 2). PHA production capacities were assessed by relating the maximum mass of PHA accumulated to the mass of inocula (gPHA/gVSS). This relationship represents the percent of the dried cell weight (% d.c.w) containing PHA and is the standard approach for

presenting PHA production quantities. To economically extract and purify PHA from its associated MMC, higher intracellular concentrations are ideal [*Koller et al.*, 2013]. Higher percentages (e.g., 60% d.c.w or greater) are typically realized using MMC, which have been conditioned for PHA accumulation and pure VFA feedstocks [*Albuquerque et al.*, 2011; *Anterrieu et al.*, 2014; *Valentino et al.*, 2014; *Valentino et al.*, 2015]. In contrast, MMC cultures conditioned for nutrient removal (i.e., EBPR) using feedstock that resemble that of a conventional WRRF have shown PHA production capacities ranging from 0 – 35% d.c.w [*Erik R. Coats et al.*, 2011; *Morgan-Sagastume et al.*, 2010; *Morgan-Sagastume et al.*, 2015; *Takabatake et al.*, 2000; 2002]. Experimental design for the three sets of PHA production assessments targeted specific intracellular PHA accumulation via the proportion between mass of VFAs added and mass of MMC with in the inocula. A desired intracellular PHA content was chosen (i.e., % d.c.w.) combined with the known MMC concentration (i.e., dried cell weight) and used to determine the sufficient mass of VFAs required. Maximum PHA production illustrated in Figure 5.13 display the results of this work compared with previous studies. As shown, maximum PHA production is divided into three attainable regions: 5 – 15% d.c.w, 15 – 25% d.c.w, and 25 – 35% d.c.w. These segregations allow for a "low, medium, high" type of presentation illustrating 5 – 15% being "low", 15 – 25% being "medium", and 25 – 35% being "high".

Maximum PHA production within the preliminary PHA carbon yield study primarily fit within the 5 – 15% d.c.w section. Thirteen of the 18 total PHA production assessments produced maximum intracellular PHA within this range, while the remaining five PHA production assessment resulted in PHA intracellular concentrations of 15 – 25% d.c.w. These results were expected as the results matched the total mass of VFAs added during preliminary PHA carbon yield study proportional to the mass of MMC inocula. The preliminary study targeted maximum intracellular PHA contents ranging from 20 – 30% d.c.w. The same cannot be said for F:M experiment 1 and 2. Specifically, three of the four F:M experiment 1 PHA production assessments fit within the 5 – 15% d.c.w section, while only one fit with in the 15 – 25% d.c.w. section. For F:M experiment 2, all PHA production assessments fit within the 5 – 15% d.c.w. section. Lower quantities of PHA in the targeted

F:M experiments was unexpected, considering the original presumptions that larger VFA loading would lead to higher PHA accumulation. The experimental design for F:M experiment 1, for the proportion of VFA added to MMC inocula, targeted 60% d.c.w.; however, the experiment itself realized maximum PHA contents of 13 – 15% d.c.w. For F:M experiment 2, the experiments were designed to achieve 25% d.c.w for R4, 25% d.c.w for R5, and 35% d.c.w. for R6. The observed results are 12% d.c.w., 10% d.c.w., and 13% d.c.w. respectively. The data suggest that perhaps simply increasing the carbon loading rates does not equate to more PHA production within the context of the BIOPHO-PX environment.

5.4 PHA Production Limitations Associated with the Mixed Microbial Consortium

As presented and discussed in Sections 5.1 – 5.3, ultimate PHA yields peaked at a maximum of approximately 15% d.c.w. One potential explanation for this outcome lies in the characteristics of the mixed microbial consortium. Specifically, the ability to realize maximum intracellular PHA accumulation will ultimately be limited by the fraction of the MMC capable of synthesizing PHA. To better understand the potential for PHA synthesis, quantitative polymerase chain reaction (qPCR) was applied to estimate the fraction of PHA producers in the MMC. qPCR was applied to target PAOs and GAOs, both of which can produce PHA and are intrinsic to the EBPR process (source of inocula for the PHA production reactor). qPCR data from F:M Experiment 1 and F:M Experiment 2 show PAO and GAO populations approximately 6 – 12% of the MMC inocula. Assuming that all PAOs and GAOs become fully saturated with PHA and a specific gravity of approximately 1.3 for PHA this qPCR data offers insight into why the PHA production MMC peaked at a maximum of approximately 15% d.c.w. PHA. Increasing the PAO and GAO populations, via the optimization of operating conditions, potentially may increase maximum intracellular PHA.

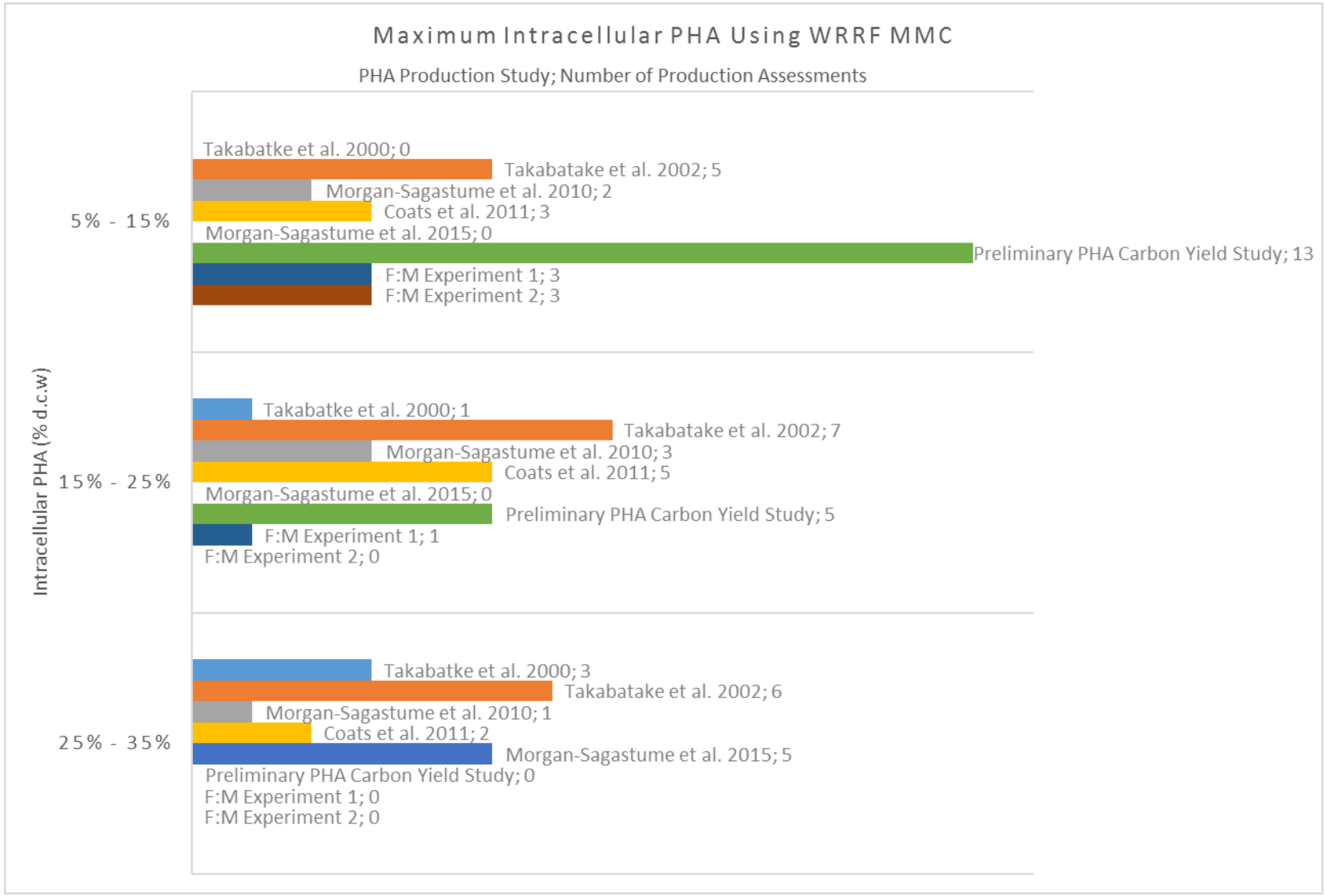

Figure 5.13: Maximum intracellular PHA produced in Preliminary PHA Carbon Yield Study, F:M Experiment 1, F:M Experiment 2, and literature using WRRF biomass as inocula.

Chapter 6: Conclusion

6.1 Operation Recommendations for PHA Production

Considering the investigations presented in this Thesis, operational parameters can be utilized for PHA production within the context of the novel WRRF configuration. In order to realize efficient PHA production, biomass from each treatment environment (anaerobic, aerobic, anoxic) was investigated to determine PHA production potential. Biomass from the anaerobic zone of the BIOPHO-PX process, for use as inocula in PHA production reactors, displayed the highest PHA carbon yields (0.811 and 0.817 Cmmol PHA/Cmmol VFA for BIOPHO-PX 2 and BIOPHO-PX 3, respectively). MMC from this treatment environment displayed the most efficient capabilities in transforming bulk solution VFAs to intracellular PHA. Efficient transformation ensures that a sufficient fraction of the feedstock VFAs fed to the MMC are transformed to PHA, as opposed to catabolic reactions and growth. Moreover, inoculating from the anaerobic zone realizes the intrinsic benefit of pre-synthesized PHA, since PHA synthesis also occurs in the anaerobic zone of the EBPR-configured system.

Using the established PHA production assessment inocula, further investigation into the effect of the food-to-microorganism ratio on PHA synthesis was performed. Preliminary work determined the efficiency and potential of BIOPHO-PX MMC to accumulate PHA, therefore a next logical step was to increase the mass of VFAs fed to individual PHA production reactors in an attempt to increase PHA production. Increased PHA production encompasses higher PHA accumulation, measured in percent dried cell weight (% d.c.w), while maintaining high PHA carbon yields.

F:M experiment 1 was the first attempt to investigate the impact of increased VFA loading rates upon PHA accumulation. Here three reactors (R1, R2, R3) experienced three pulse additions at a F:M of 0.52 mgCOD/mgVSS. These PHA production assessments produced PHA accumulation of 13 – 15% d.c.w. while maintaining a PHA carbon yield of approximately 0.30 Cmmol PHA/ Cmmol VFA. This investigation suggests that increased F:M ratios have negative impacts on PHA accumulation, but the experiment was hindered by the onset of famine periods between pulse additions. The onset of famine periods may have led

to metabolic shifts, therefore reducing the MMC's ability to transform bulk solution VFAs to intracellular PHA.

A second F:M experiment, F:M experiment 2, was conducted with modified operational conditions to avoid the onset of famine periods between pulse additions. F:M experiment 2 consisted of three PHA production reactors (R4, R5, R6) and followed the same PHA production framework as experiment 1, but realized F:M ratios of 0.33, 0.50, and 0.96 mgCOD/mgVSS, respectively. PHA accumulation for R4, R5, and R6 achieved 12.53%, 10.66%, and 13.33% d.c.w., respectively. F:M experiment 2 remained similar to that of F:M experiment 1 in terms of PHA intracellular PHA accumulation (% d.c.w.), suggesting that F:M has little to no impact on PHA accumulation. In contrast, F:M experiment 2 experienced a decrease in PHA carbon yield (Cmmol PHA/Cmmol VFA), as F:M increased. R4, R5, and R6 realized PHA carbon yields of 0.71, 0.59, and 0.22 Cmmol PHA/Cmmol VFA, respectively. This data suggests that increasing the F:M ratio during a PHA production assessment does not achieve increased intracellular PHA accumulation (% d.c.w), while simultaneously decreases the PHA carbon yield (Cmmol PHA/Cmmol VFA).

Recognizing the ultimate plan to integrate PHA production into a full-scale BIOPHO-PX WRRF, a preliminary mass balance assessment was developed utilizing the observed operational parameters in an attempt to understand potential full-scale PHA production. Figure 6.1 illustrates the process's main components; mass balance assumptions made to simulate WRRF and PHA Production operations. Note that the BIOPHO-PX process was modeled, for the purpose of estimating potential WAS quantities to the PHA production reactor, as a single CSTR using the appropriate equations [*Tchobanoglous et al.*, 2014].

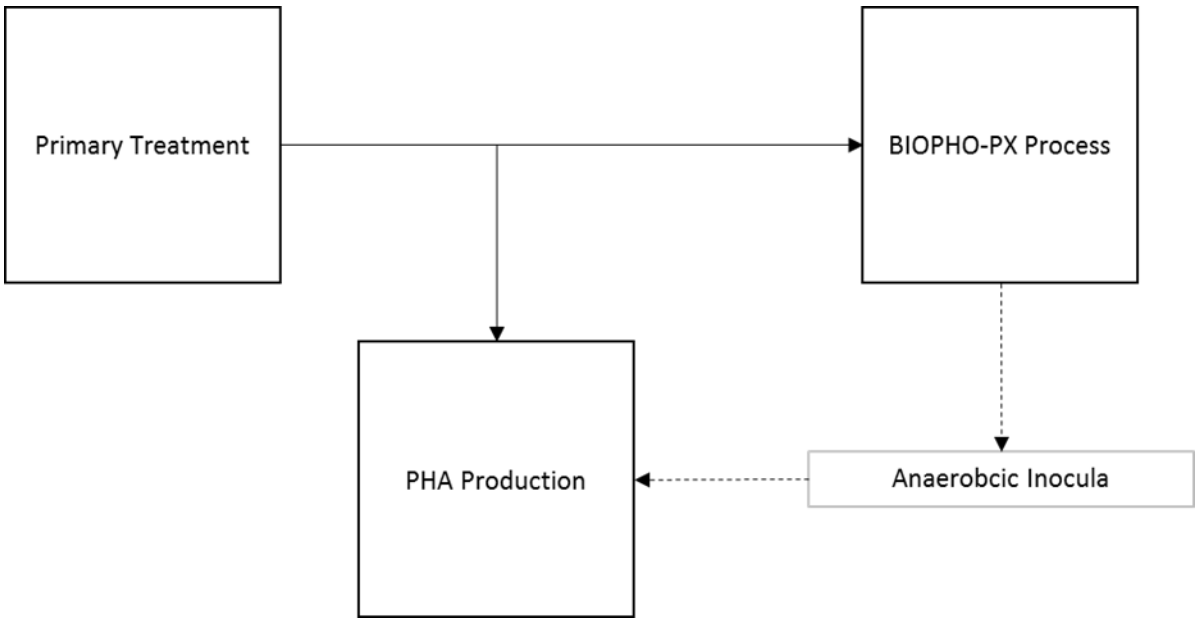

Figure 6.1: PHA production using mass balance of a "hypothetical" WRRF.

- PHA Production inocula is drawn from the anaerobic period of the BIOPHO-PX WRRF
- Primary effluent consisted of 143.4 mg VFAs as sCOD/L and total soluble COD of 350 mg sCOD/L; this VFA concentration was based on data collected from the lab-scale fermenter.
- The influent flow rate was 10 million gallons per day (mgd)
- Growth kinetics for the simplified BIOPHO-PX process: biomass Yield = 0.65 mgVSS/mgCOD; biomass endogenous decay = 0.05 day^{-1}
- SRT of BIOPHO-PX WRRF = 20 days
- Target F:M within PHA Production reactor = 0.30 mgCOD/mgVSS
- PHA production yield = 0.25 mgPHA/mgVFA as sCOD
- Primary effluent flow rates to the PHA Production reactor ranged 0 – 50%

The results of this mass balance (Figure 6.2) illustrate the estimated PHA production capabilities and associated percentage of primary effluent flow to be diverted from the BIOPHO-PX process and to PHA production. Based on these preliminary analyses, diverting 21% of the primary effluent towards PHA production would yield 628 lb/day of PHA while maintaining the target F:M of 0.30 mgCOD/mgVSS. The dashed line on Figure 6.2 represents the theoretical capabilities of the BIOPHO-PX MMC to produce PHA (based on data

developed in this thesis), while the solid line represents the actual PHA produced (based on available VFAs and associated WAS). The inflection point (21% primary effluent) indicates the diversion of primary effluent required for all of the daily BIOPHO-PX biomass growth to realize the target F:M of 0.30 mgCOD/mgVSS. Diverting less than this amount (<21%) results in more soluble VFAs flowing into the BIOPHO-PX process and under-optimized PHA production. Diverting greater than 21% results in excess VFAs provided to the inocula relative to the target F:M (i.e., F:M exceeding the target value), thus hindering PHA production (again, based on the results presented herein). Based on these assumptions, and using a gross value of $2.50 per lbPHA, gross revenue (excluding system O&M; PHA extraction) would be $570,000 per year of operation.

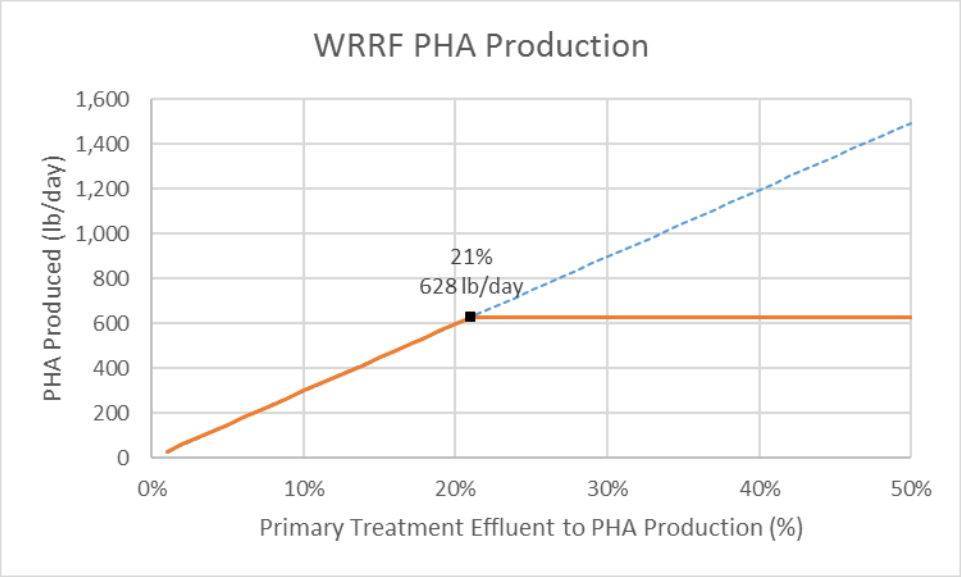

Figure 6.2: PHA production as a function of primary effluent diverted from secondary treatment of "hypothetical" WRRF mass balance.

6.3 Future Investigations

The purpose of this Thesis was to investigate the potential for PHA production within the novel BIOPHO-PX WRRF configuration. Combined investigations, involving the impact of previous treatment environments and increases in the F:M distribution, provided a general framework for how to successfully achieve PHA production. However, while PHA production was accomplished, several instances of famine between pulse additions

occurred that adversely impaired PHA synthesis. Further investigation with more stringent operational control, in an attempt to reduce the onset of famine conditions, should be conducted.

To potentially avoid the possibility of realizing famine conditions within a PHA production assessment, more investigation into the VFA substrate uptake rates for BIOPHO-PX MMC should be conducted. If possible, monitoring devices for real time VFAs should be used to control pulse feeding. If not possible, more investigation into the VFA consumption rates and capabilities should be investigated to ensure proper extended feast conditions.

Another possible investigation is to upscale the WRRF configuration to produce more biomass and more operation control. Lab scale experiment may have been inhibited by the amount of available carbon-storing organisms present and the usage of sequencing batch reactors. Results showed a consistent PHA accumulation of approximately 15% d.c.w. regardless of the PHA production operating changes. Further investigation into operational changes, specific to increasing the population of carbon-storing biomass should be conducted. In conjunction with increasing the population of carbon-storing biomass, more accurate methods of carbon-storing organism quantification should be investigated.

The research group currently operates a 3,000 gallon scale model located at the Moscow, ID WRRF with the capacity to add PHA production within its current configuration. This highly modifiable flow though system may provide insight into the necessary operational changed required to increase the population of carbon-storing organisms, thus increasing PHA accumulation.

References

Albuquerque, M. G. E., V. Martino, E. Pollet, L. Averous, and M. A. M. Reis (2011), Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties, *Journal of Biotechnology*, *151*(1), 66-76.

Anderson, A. J., and E. A. Dawes (1990), OCCURRENCE, METABOLISM, METABOLIC ROLE, AND INDUSTRIAL USES OF BACTERIAL POLYHYDROXYALKANOATES, *Microbiological Reviews*, *54*(4), 450-472.

Anterrieu, S., et al. (2014), Integration of biopolymer production with process water treatment at a sugar factory, *New Biotechnology*, *31*(4), 308-323.

Appel, F. J. (2015), Advancing a Post-Anoxic Biological Nutrient Removal Process Selecting for Nitritation, 75 pp, University of Idaho, Moscow, ID.

Braunegg, G., B. Sonnleitner, and R. M. Lafferty (1978), RAPID GAS-CHROMATOGRAPHIC METHOD FOR DETERMINATION OF POLY-BETA-HYDROXYBUTYRIC ACID IN MICROBIAL BIOMASS, *European Journal of Applied Microbiology and Biotechnology*, *6*(1), 29-37.

Chua, A. S. M., H. Takabatake, H. Satoh, and T. Mino (2003), Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent, *Water Research*, *37*(15), 3602-3611.

Coats, E. R., A. Mockos, and F. J. Loge (2011), Post-anoxic denitrification driven by PHA and glycogen within enhanced biological phosphorus removal, *Bioresource Technology*, *102*(2), 1019-1027.

Coats, E. R., D. L. Watkins, and D. Kranenburg (2011), A comprative environmental life cycle analysis for removing phosphorus from wastewater: Biological versus physical/chemical processes, *Water Environ. Res.*, *83*(8), 750-760.

Coats, E. R., K. E. VandeVoort, J. L. Darby, and F. J. Loge (2011), Toward Polyhydroxyalkanoate Production Concurrent with Municipal Wastewater Treatment in a Sequencing Batch Reactor System, *Journal of Environmental Engineering-Asce*, *137*(1), 46-54.

Coats, E. R., F. J. Loge, M. P. Wolcott, K. Englund, and A. G. McDonald (2007), Synthesis of polyhydroxyalkanoates in municipal wastewater treatment, *Water Environment Research*, *79*(12), 2396-2403.

Daigger, G. T. (2014), Oxygen and Carbon Requirements for Biological Nitrogen Removal Processes Accomplishing Nitrification, Nitritation, and Anammox, *Water Environment Research*, *86*(3), 204-209.

Dias, J. M. L., A. Oehmen, L. S. Serafim, P. C. Lemos, M. A. M. Reis, and R. Oliveira (2008), Metabolic modelling of polyhydroxyalkanoate copolymers production by mixed microbial cultures, *Bmc Systems Biology*, *2*, 21.

Eaton, A. D., L. S. Clesceri, A. E. Greenberg, M. A. H. Franson, A. American Public Health, A. American Water Works, and F. Water Environment (1998), *Standard methods for the examination of water and wastewater*, American Public Health Association, Washington, DC.

EPA, U. S. (2007), Biological Nutrient Removal Processes and Costs, edited by U. S. E. P. Agency, Office of Water, Washington, DC.

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

Frison, N., E. Katsou, S. Malamis, A. Oehmen, and F. Fatone (2015a), Nutrient removal via nitrite from reject water and polyhydroxyalkanoate (PHA) storage during nitrifying conditions, *Journal of Chemical Technology and Biotechnology*, *90*(10), 1802-1810.

Frison, N., E. Katsou, S. Malamis, A. Oehmen, and F. Fatone (2015b), Development of a Novel Process Integrating the Treatment of Sludge Reject Water and the Production of Polyhydroxyalkanoates (PHAs), *Environmental Science & Technology*, *49*(18), 10877-10885.

Grady, C. P. L., G. T. Daigger, N. Love, and C. D. M. Filipe (2011), *Biological Wastewater Treatment*, 3 ed., CRC Press, Boca Raton, FL.

Hanson, A. J., N. M. Guho, A. J. Paszczynski, and E. R. Coats (2016-accepted), Community Proteomics Provides Functional Insight into Polyhydroxyalkanoate Production by a Mixed Microbial Culture Cultivated on Fermented Dairy Manure, *Appl. Microbiol. Biotechnol.*

Higgins, P. (2014), Shortcut the Nitrogen Removal Process in Wastewater with UV Sensors, in *YSI Blog*, edited, YSI Incorporated.

Koller, M., H. Niebelschutz, and G. Braunegg (2013), Strategies for recovery and purification of poly (R)-3-hydroxyalkanoates (PHA) biopolyesters from surrounding biomass, *Engineering in Life Sciences*, *13*(6), 549-562.

Laycock, B., P. Halle, S. Pratt, A. Werker, and P. Lant (2014), The chemomechanical properties of microbial polyhydroxyalkanoates, *Progress in Polymer Science*, *39*(2), 397-442.

Lemoigne, M. (1926), Produit de déshydratation et de polymérisation de l'acide β-oxybutyrique, *Bull Soc Chim Biol*, *8*, 770-782.

Li, Z. M., S. Y. Wang, W. T. Zhang, L. Miao, T. H. Cao, and Y. Z. Peng (2014), Nitrogen removal from medium-age landfill leachate via post-denitrification driven by PHAs and glycogen in a single sequencing batch reactor, *Bioresource Technology*, *169*, 773-777.

Liu, G., X. Y. Xu, L. Zhu, S. Xing, and J. Y. Chen (2013), Biological nutrient removal in a continuous anaerobic-aerobic-anoxic process treating synthetic domestic wastewater, *Chemical Engineering Journal*, *225*, 223-229.

Madigan, M. T., and J. M. Martinko (2006), *Brock Biology of Microorganisms*, 11th ed., 992 pp., Pearson Prentice Hall, Upper Saddle River, NJ.

Majone, M., P. Massanisso, A. Carucci, K. Lindrea, and V. Tandoi (1996), Influence of storage on kinetic selection to control aerobic filamentous bulking, *Water Science and Technology*, *34*(5-6), 223-232.

Marang, L., Y. Jiang, M. C. M. van Loosdrecht, and R. Kleerebezem (2014), Impact of non-storing biomass on PHA production: An enrichment culture on acetate and methanol, *International Journal of Biological Macromolecules*, *71*, 74-80.

Morgan-Sagastume, F., A. Karlsson, P. Johansson, S. Pratt, N. Boon, P. Lant, and A. Werker (2010), Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus, *Water Research*, *44*(18), 5196-5211.

Morgan-Sagastume, F., et al. (2015), Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale, *Bioresource Technology*, *181*, 78-89.

Oehmen, A., R. J. Zeng, Z. Yuan, and J. Keller (2005), Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems, *Biotechnol. Bioeng.*, *91*(1), 45-53.

Reis, M. A. M., L. S. Serafim, P. C. Lemos, A. M. Ramos, F. R. Aguiar, and M. C. M. Van Loosdrecht (2003), Production of polyhydroxyalkanoates by mixed microbial cultures, *Bioprocess and Biosystems Engineering*, *25*(6), 377-385.

Takabatake, H., H. Satoh, T. Mino, and T. Matsuo (2000), Recovery of biodegradable plastics from activated sludge process, *Water Science and Technology*, *42*(3-4), 351-356.

Takabatake, H., H. Satoh, T. Mino, and T. Matsuo (2002), PHA (polyhydroxyalkanoate) production potential of activated sludge treating wastewater, *Water Science and Technology*, *45*(12), 119-126.

Tamis, J., L. Marang, Y. Jiang, M. C. M. van Loosdrecht, and R. Kleerebezem (2014), Modeling PHA-producing microbial enrichment cultures-towards a generalized model with predictive power, *New Biotechnology*, *31*(4), 324-334.

Tan, G.-Y. A., C.-L. Chen, L. Li, L. Ge, L. Wang, I. M. N. Razaad, Y. Li, L. Zhao, Y. Mo, and J.-Y. Wang (2014), Start a research on biopolymer polyhydroxyalkanoate (PHA): a review, *Polymers*, *6*(3), 706-754.

Tchobanoglous, G., H. D. Stensel, R. Tsuchihashi, and F. Burton (2014), *Wastewater Engineering Treatment and Resource Recovery*, 5 ed., 2048 pp., Metcalf & Eddy; McGraw-Hill Education, New York, NY.

Valentino, F., M. Beccari, S. Fraraccio, G. Zanaroli, and M. Majone (2014), Feed frequency in a Sequencing Batch Reactor strongly affects the production of polyhydroxyalkanoates (PAHs) from volatile fatty acids, *New Biotechnology*, *31*(4), 264-275.

Valentino, F., L. Karabegouic, M. Majone, F. Morgan-Sagastume, and A. Werker (2015), Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels, *Water Research*, *77*, 49-63.

Wallen, L. L., and Rohwedde.Wk (1974), POLY-BETA-HYDROXYALKANOATE FROM ACTIVATED-SLUDGE, *Environmental Science & Technology*, *8*(6), 576-579.

Winkler, M., E. R. Coats, and C. K. Brinkman (2011), Advancing post-anoxic denitrification for biological nutrient removal, *Water Research*, *45*(18), 6119-6130.

Zhang, C. Y., C. Yinguang, A. A. Randall, and G. Guowei (2008), Anaerobic metabolic models for phosphorus- and glycogen-accumulating organisms with mixed acetic and propionic acids as carbon sources, *Water Res.*, *42*, 3745-3756.