Assessment of Operationally Effected Metabolic Conditions to Achieve Enhanced Polyhydroxyalkanoate Production on Fermented Dairy Manure

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Master of Science

with a

Major in Civil Engineering

in the

College of Graduate Studies

University of Idaho

by

Cody Andrew Sprague

Major Professor: Erik R. Coats, P.E., Ph.D.

Committee Members: Patricia J. S. Colberg, Ph.D., P.E.; James Moberly, Ph.D.

Department Administrator: Patricia J. S. Colberg, Ph.D., P.E.

May 2018

AUTHORIZATION TO SUBMIT THESIS

This thesis of Cody Andrew Sprague, submitted for the degree of Master of Science with a Major in Civil Engineering and titled "Assessment of Operationally Effected Metabolic Conditions to Achieve Enhanced Polyhydroxyalkanoate Production on Fermented Dairy Manure," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date:_____

Erik R. Coats, P.E., Ph.D.

Committee Members: _____ Date:_____

Patricia J. S. Colberg, Ph.D., P.E.

_____ Date:_____

James Moberly, Ph.D.

Department

Administrator: _____ Date:_____

Patricia J. S. Colberg, Ph.D., P.E.

ABSTRACT

Polyhydroxyalkanoates (PHA) are intracellular storage molecules that can be utilized as biodegradable thermoplastic polymers and possess properties similar to common petrochemical plastics (i.e., HDPE). Commercial production currently utilizes pure bacterial cultures grown under macronutrient limitation with a pure feedstock (i.e., glucose and propionic acid), making synthesis of fossil fuel-based plastics more cost-effective. An alternative method, known as aerobic dynamic feeding (ADF), utilizes mixed microbial consortia fed waste organic carbon, which offers the potential to significantly reduce production costs; however, operational parameters must first be established. Research presented herein focused on advancing a three-stage ADF process, which successfully produced PHA-rich biomass on fermented dairy manure, by assessing the effects of sedimentation and solids retention time (SRT) on the consortium enrichment stage. Introduction of sedimentation resulted in lower average maximum intracellular PHA content, 31.7% and 33.6% total solids (w/w), respectively. Increasing enrichment stage SRT increased intracellular PHA and improved operational process control.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my major professor, Dr. Erik R. Coats, P.E. for providing me with the opportunity to obtain a master's degree in civil engineering from the University of Idaho. His unrelenting support drove me to complete this work, but also extended beyond academics, and he became my friend throughout the process.

I would also like to thank my committee members, Dr. James Moberly and Dr. Patricia J. S. Colberg, for their provided intellectual guidance in preparing this thesis. I would also like to express my appreciation to Dr. Armando McDonald for his assistance with the analytical equipment and data, and Donald Parks for his hard work in building the lab and reactors, without which none of this research would be possible.

I would like to thank Cynthia Brinkman for her assistance and instruction of laboratory procedures; my fellow lab members: Casey Bryant, Alex Crozes, Katie Dillon, Kirsten Dolph, Karina Eyre, Nick Guho, Jason Mellin, Taylor Romenesko, and Nicole Tompkins for their assistance with maintaining and sample collection of the Dairy systems. In addition, Dr. Simon Smith was always available to discuss the research being performed. Their advice, assistance, and humor have made the past two years a memorable experience.

DEDICATION

To my parents, Steve and Mary Kay Sprague for all of their love, support, and guidance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

ADF ..... Aerobic Dynamic Feeding

ATP ..... Adenosine Triphosphate

CoASH ..... Coenzyme-A

CL ..... Cycle Length

COD ..... Chemical Oxygen Demand

CSTR ..... Continuous Stirred Tank Reactor

DFL ..... Dairy Fermenter Liquor

DO ..... Dissolved Oxygen

EBPR ..... Enhance Biological Phosphorus Removal

ER ..... Enrichment Reactor

ERS ..... Settling Enrichment Reactor

F-F ..... Feast-Famine

F:M ..... Food to Microorganism Ratio

GAO ..... Glycogen Accumulating Organisms

HRT ..... Hydraulic Retention Time

MLSS ..... Mixed Liquor Suspended Solids

MLVSS ..... Mixed Liquor Volatile Suspended Solids

MMC ..... Mixed Microbial Consortium

N ..... Nitrogen

OHO ..... Ordinary Heterotrophic Organisms

OLR ..... Organic Loading Rate

P ..... Phosphorus

PAO ..... Phosphorus Accumulating Organism

PHA ..... Polyhydroxyalkanoates

PHB ..... Polyhydroxybutyrate

PHBV ..... Polyhydroxybutyrate-co-Hydroxyvalerate

PHV ..... Polyhydroxyvalerate

PMF ..... Proton Motive Force

PPP ..... Pentose Phosphate Pathway

PR ..... Production Reactor

PRS ..... Production Reactor – Settling Enrichment Reactor

qPHA ..... Specific PHA Synthesis Rate

qVFA ..... Specific Carboxylate Uptake Rate

RCRA ..... Resource Conservation and Recovery Act

RDS ..... Readily Degradable Substrate

rPHA ..... Overall PHA Synthesis Rate

RQ ..... Research Question

rVFA ..... Overall Carboxylate Uptake Rate

SBR ..... Sequencing Batch Reactor

SDS ..... Slowly Degradable Substrate

SRT ..... Solids Retention Time

TCA ..... Tricarboxylic Acid

TS ..... Total Solids

TSS ..... Total Suspended Solids

VFA ..... Volatile Fatty Acids

VS ..... Volatile Solids

VSS ..... Volatile Suspended Solids

1. INTRODUCTION

Microorganisms, including bacteria, are increasingly being leveraged to produce or recover products of value; indeed, microorganisms are a central component in the ongoing efforts to shift from a fossil fuel-based economy to one founded on biological materials [1, 2]. In this regard, multiple species of bacteria are capable of storing carbon as polyhydroxyalkanoates (PHA), an internal storage molecule [3]. Beyond physiological importance, PHA is also a biodegradable thermoplastic. Recognizing the intrinsic value of a biologically produced, biodegradable plastic, investigators have for many years evaluated opportunities to commercialize PHA production [4, 5]. Current commercial PHA production methods utilize pure microbial culture based reactors that are fed agriculturally-derived carbohydrate substrates (e.g., refined corn sugar); however, maintaining requisite axenic conditions, coupled with obtaining synthetic feedstocks and required reagents for PHA extraction and purification, results in a product cost approaching three times that of conventional fossil fuel derived plastics [6] with similar negative environmental attributes [7, 8]. The most expensive components of current pure culture production include the costs of creating a purified substrate and the sterilization required to sustain a pure culture.

In achieving economically sustainable PHA production, one potential remedy to carbon substrate cost involves the use of non-carbohydrate sources of carbon. Specifically, PHA can be readily produced from organic carbon precursors (i.e., acetic acid and volatile fatty acids, VFAs; referred to as carboxylates) generated via bacterial fermentation of lipids and carbohydrates. One approach to reduce the expense of PHA production is to utilize organic-rich waste streams derived from industrial and agricultural processes [9]. Fermentation of waste organic substrate increases the biodegradability of the carbon available by breaking down the solids and complex soluble substrates into simple monomers (carboxylates), yielding a substrate more suitable for the production of PHA. For example, an ideal candidate waste stream would be dairy manure. Over 9 million dairy cows generate more than 226 billion kg of wet manure annually in the U.S. [10]. Not only does degradation of this waste create greenhouse gas emissions (prominently methane), but current manure management strategies also create nutrient issues in the environment associated with inadequate management of the waste via land application; as such the U.S. EPA has strengthened dairy operation rules. Moreover, a 2015

legal settlement in Washington State, based on a federal court finding that dairy manure is a waste to be regulated under the Resource Conservation and Recovery Act (RCRA), portends significant future challenges to U.S. dairy operations [11]. As U.S. dairies look to the future, it will be critical to remedy environmental emissions in order to remain competitive. Production of high-value commodities from dairy manure – including PHA – will help facilitate such a necessary transition [12].

Using organic-rich waste substrate for PHA production introduces another mechanism to potentially reduce overall polymer production costs and the associated environmental footprint; that is, utilizing a mixed microbial consortia (MMC) for PHA synthesis rather than relying on pure bacterial cultures that demand axenic conditions [9]. To elicit a PHA response using MMC-fed carboxylates, bioreactors must be engineered to induce a metabolic 'feast-famine' (F-F) response. A conceptual illustration of the F-F response is shown in Figure 1.1; as shown, peak intracellular PHA occurs concurrently with depletion of readily biodegradable organic carbon (carboxylates). This metabolic pathway is selected by imposing aerobic dynamic feeding (ADF) conditions, where the MMC is exposed to an environment where substrates in bulk solution are either available or unavailable (i.e., the F-F metabolic response). The F-F metabolic response ultimately selects for PHA-producing bacteria and minimizes or eliminates the ordinary heterotrophic organisms (OHOs) whose usual forms of energy are unavailable and are unable to store PHA.

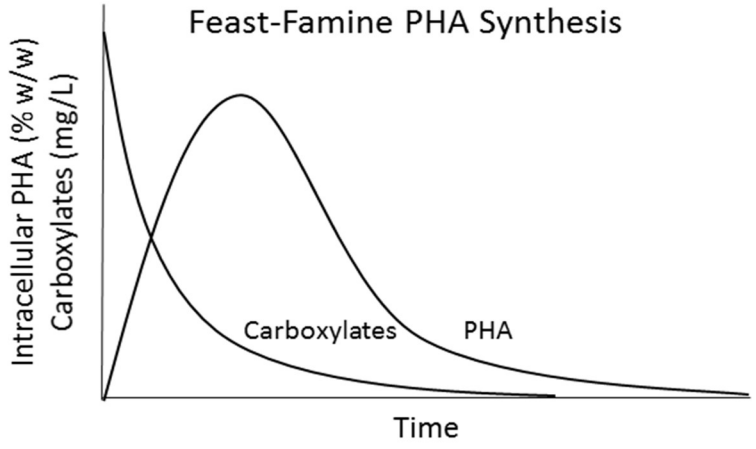

Figure 1.1: Conceptual Feast-Famine Response

To achieve full scale PHA production through engineered ADF conditions using MMC fed fermented organic waste, three-stage processes have become widely used, with slight operational differences within the individual stages [13]. Figure 1.2 illustrates a schematic of a three-stage process utilized for dairy manure.

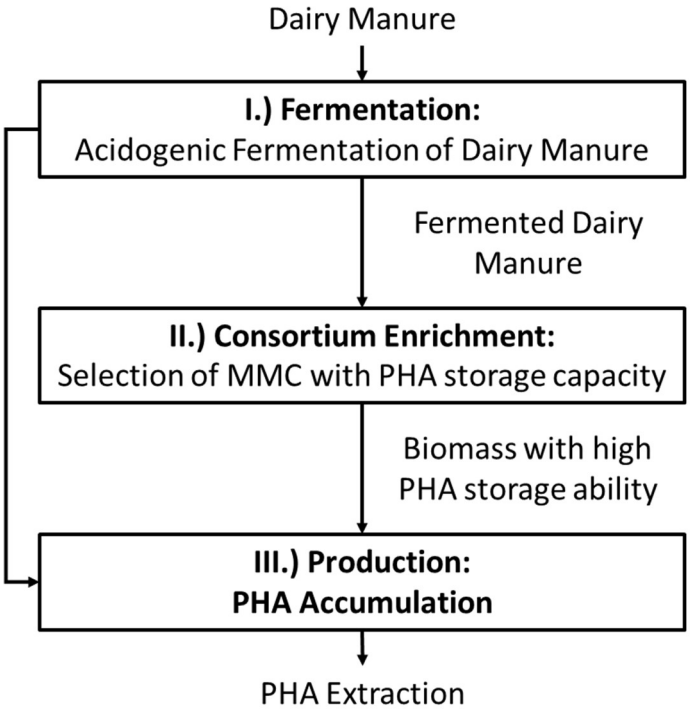

Figure 1.2: Three-Stage PHA Production Process from Dairy Manure

Fermentation occurs in stage one, where organic waste (e.g., dairy manure) is anaerobically converted to a readily-biodegradable substrate – carboxylates – suitable for PHA synthesis. As noted, carboxylates are fundamental for PHA synthesis by MMC. Majone et al. [14] revolutionized ADF-driven PHA production by suggesting the separation of the reactor in which the consortium is enriched or cultured from the reactor where the commodity is produced, thus allowing for independent optimization of both stages. The enrichment stage utilizes an engineered ADF environment to induce the F-F response to select for a consortium that has a high content of PHA storing/producing microbes; this stage is operated as a fed-batch sequencing batch reactor (SBR), pulse-fed periodically (e.g., once per day) with the carboxylate-rich substrate to elicit a PHA storage response. Once the substrate has been consumed and stored (‘feast’) by the MMC, the reactor enters a period of bacterial ‘famine’ in

which organisms that did not store PHA will be eliminated, thus enriching the consortium to increase PHA production. In the final stage, the enriched MMC from the second stage is fed a constant stream of the carboxylate-rich substrate from the fermentation stage, thereby eliminating famine conditions. This provides the MMC the ability to continually uptake carboxylates for PHA storage and creating conditions for maximum PHA production. Key challenges in maximizing PHA production in this third stage include i.) minimizing use of carboxylates for bacterial growth while ii.) ensuring that the PHA synthesis response, concurrent with carboxylate consumption, is maximized.

While the ADF process has received significant research attention [13, 15-27] since the original work of Majone et al. [14], much remains to be understood about process fundamentals before commercialization can be realized. For example, the metabolic pathways controlling the ADF-induced F-F response that yield maximum PHA storage within the cells remain poorly understood [15]; in particular, the feast metabolism linked to reactor carbon loading must be better understood, both at a macro and micro scales. Related, solids residence time (SRT) and organic loading rate (OLR) are the two most important controllable parameters in the second stage of the PHA synthesis system that might be leveraged to effect 'feast,' yet little work has been performed to better understand these factors and their impact on system performance. Coupled with reactor operational parameters, for MMC ADF PHA production, the effects of excess macronutrients (i.e., nitrogen, phosphorus) in the waste substrate versus utilizing a nutrient-poor waste substrate, have not been fully explored; recognizing that some industrial waste streams could be carboxylate rich and nutrient poor (e.g., sugar beet wastewater), commingling of substrates might be reasonable. Finally, the potential effects of slowly biodegradable substrate (i.e., non-carboxylates) on the MMC in the enrichment reactor is not well understood. These substrates are available to the MMC throughout an operational cycle, thus potentially enriching against PHA producing bacteria by providing a means of bacterial growth during the famine stage. Collectively, an enhanced understanding of these fundamentals – all substrate centric – is needed to ensure commercial viability of the process.

Building from real opportunities for achieving PHA production using MMC cultured on the plentiful quantities of organic-rich wastes, while recognizing needs for additional process knowledge, research presented and discussed in this thesis was ultimately focused on

wastewater carbon effects – coupled with SRT – related to ADF metabolic pathways and kinetics, with the aim to further enhance process output and operation. The process researched herein specifically provides an alternative method of addressing growing dairy manure challenges, while simultaneously providing an opportunity to derive a commercially viable commodity for this critical industry.

This thesis was driven by the following research questions (RQs), hypotheses, and objectives:

RQ 1: How will implementation of a biomass sedimentation phase, and withdrawal of wastewater, at the end of the feast phase increase the enrichment of PHA-producing bacteria in the MMC and commensurately improve overall PHA production?

Hypothesis 1: Removing residual wastewater at the end of the feast phase will remove slowly biodegradable substrates from the reactor; therefore, increasing enrichment of a PHA-producing MMC, while concurrently enhancing the requisite famine metabolism. Consequently, the MMC should exhibit the ability to convert and store more carboxylates as PHA, vs. growth on carboxylates by non-PHA OHOs. When utilized in a PHA Production mode, the MMC should then generate more PHA.

Objective 1: Evaluate and assess the effects of slowly biodegradable substrate on PHA production.

RQ 2: How does enrichment reactor SRT impact PHA production and the associated parameters?

Hypothesis 2: A shorter SRT will enrich for a MMC capable of storing increased quantities of carboxylates as PHA.

Objective 2: Examine the effect of enrichment reactor SRT on PHA production in both ER and PR.

2. LITERATURE REVIEW

2.1. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are intracellular carbon and energy storage molecules produced by many species of bacteria. PHA is biologically synthesized under conditions of stress, similar to how and why bacteria synthesize other storage macromolecules such as glycogen and polyphosphate [9]. What makes PHA unique, however, is that when the polymer is extracted from the cell, it becomes a useful thermoplastic alternative to synthetic petroleum-based plastics.

2.1.1. PHA Composition

PHA belongs to a class of polymers composed of hydroxyalkanoate monomers. These monomers are composed of a chain of carbon molecules with an alcohol and carboxylic acid group on each end. The general form of PHA is the result of dehydration of the monomers during polymerization. It is estimated there are between 125 and 150 different monomers of PHA [1, 28]; however, only two homopolymers are consistently produced within mixed microbial culture (MMC) systems: poly-3-hydroxybutryate (PHB) and poly-3-hydroxyvalerate (PHV). The PHB homopolymer is foundationally synthesized from 3HB monomers (4-carbon molecule), while PHV is synthesized from both 3HB and 3HV, yielding a 5-carbon molecule. It has been observed that when these two monomers are present, bacteria will randomly polymerize the monomers and form the co-polymer polyhydroxybutyrate-co-hydroxyvalerate (PHBV) [29].

Bacterial cultures can utilize many different forms of simple carbon sources for PHA production; however, carboxylic acids (i.e., acetate and VFAs) are the most direct precursors to PHA synthesis. Volatile Fatty Acids are short chain (3-6 carbon) carboxylic acids. In general, even-numbered carboxylates will yield PHB monomers, while both even- and odd- numbered carboxylates will result in PHV synthesis. It is theorized that the co-polymer synthesized will contain a ratio of even-to-odd carbon molecules similar to that of the carboxylate source [30]; therefore, a mixed ratio of even-to-odd carbon chain carboxylate substrates can be utilized to produce co-polymers of a similar ratio.

2.1.2. PHA Material Properties

PHA exists in the bacterial cell as an amorphous granule; the structure is a spiral chain of repeating monomers. The structures of common PHA monomers are illustrated in Figure 2.1. The physical properties of PHA is dependent on the amount and composition of the constituent monomers. PHB homopolymer is a very brittle and stiff material, but the addition of PHV monomers generates PHBV, which greatly improves its mechanical properties [31]. The chemical structure of PHBV is included in Figure 2.1. Utilization of PHA as a thermoplastic source has the engineering and economic advantages of being completely polymerized within the bacterial cell, allowing for the removal of the external polymerization process required in the production of other synthetic-based polymers.

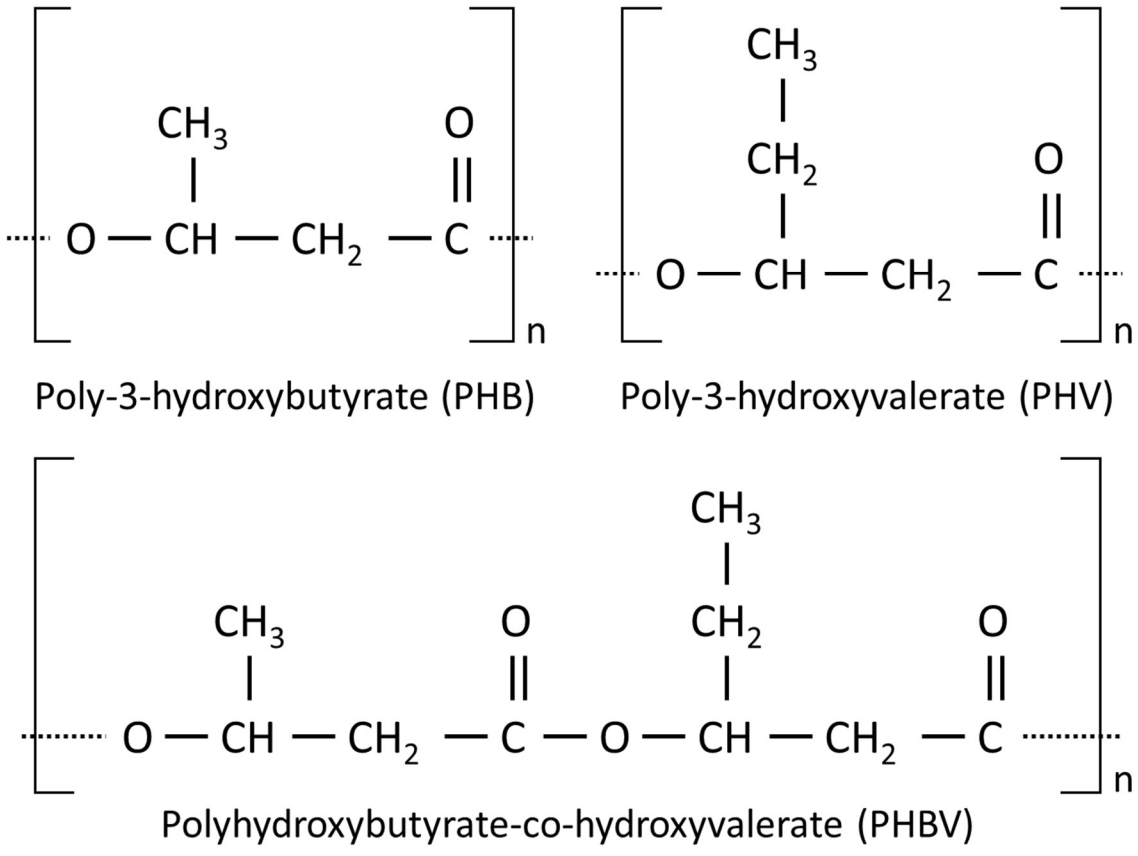

Figure 2.1: Common Polyhydroxyalkanoate Monomers

Another advantage of using PHA polymers for plastics is their biodegradability. Being a biologically synthesized storage molecule, microbes are capable of producing enzymes to break

down the polymers and utilize them for energy and growth. While monomers for petro-plastics polypropylene, polyethylene, and polyethylene terephthalate can be produced biologically, the polymerization stage occurs through synthetic means, making these materials recalcitrant to microbial degradation [1]. Bacteria are able to degrade PHA completely due to intracellular polymerization of the granule. While PHA degrades readily in active microbial environments, degradation rates can be controlled if the material is stored or used in a dry, cool environment. Figure 2.2 illustrates the ability of aerobic sludge to degrade PHA [32].

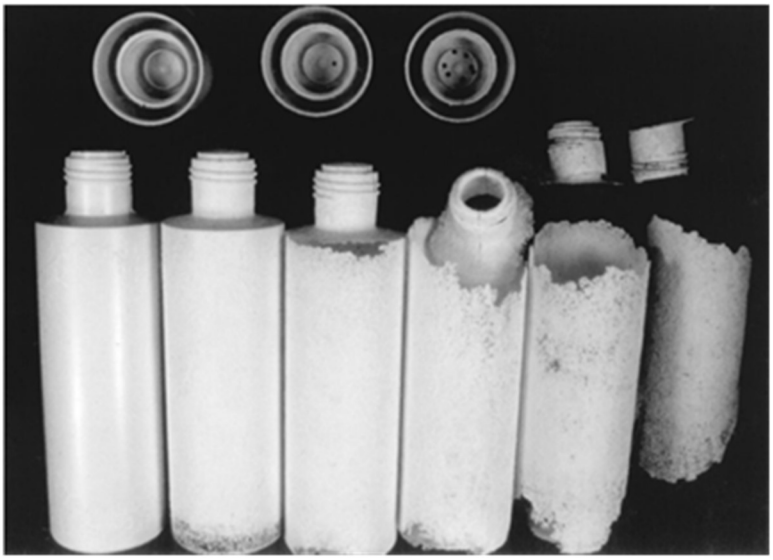

Figure 2.2: PHA Degradation in Aerobic Sludge After 0, 2, 4, 6, 8, and 10 Weeks [32]. Reproduced with Permission.

2.2. Achieving PHA Production

Microbes store carbon as PHA when exposed to an environment that induces stress. Conditions elucidating this response include deprivation of essential growth nutrients or exposure to an oversupply excess of organic carbon. The PHA accumulation response has been observed in both pure-cultures and mixed microbial consortia (MMC), but different stresses must be induced to achieve PHA synthesis using pure-cultures vs. MMC. Three engineered techniques have been developed to induce microbial stress and elicit the PHA accumulation response: imposing anaerobic conditions (deprivation of an external terminal electron acceptor), controlled-growth feeding (nutrient deprivation), and aerobic dynamic feeding (carbon oversupply). Each is discussed in more detail below.

2.2.1. Anaerobic PHA Synthesis

PHA storage by MMC within an engineered environment was first observed in an enhanced biological phosphorus removal (EBPR) system [33]. In such systems, biomass is cycled through aerobic, anaerobic, and anoxic zones depending on the treatment goal of the system [34]. Enrichment for a culture that utilizes phosphorus in solution – stored as polyphosphate – is essential for the EBPR process. PHA-synthesizing microorganisms that utilize polyphosphate and glycogen to facilitate PHA storage are called phosphorus-accumulating organisms (PAOs), and those that utilize glycogen only for secondary storage reserves – but also store PHA – are called glycogen-accumulating organisms (GAOs). Populations of GAOs can inhibit or even shut down the EBPR process; however, GAOs have been shown to facilitate higher PHA yields than PAOs [16, 34].

In EBPR systems, the MMC is first blended with wastewater in an anaerobic zone, ensuring the highest concentration of readily biodegradable carbon (carboxylates). Under anaerobic conditions, the microbes are deprived of molecular oxygen, nitrate, or nitrite as terminal electron acceptors, which in turn inhibits ordinary cellular functions (e.g., oxidative phosphorylation) and growth that would otherwise demand carbon and electrons from the carboxylates. Cells capable of storing carboxylates as PHA are provided an advantage to survive under these conditions. Once the culture enters the following aerobic stage and the readily degradable substrate is depleted, the stored PHA is used for growth and for restoring secondary storage reserves [9].

Cycling between anaerobic and aerobic conditions enriches for PHA-accumulating organisms; however, PHA accumulation is at a level associated only with the wastewater carboxylate concentration. Maximum storage is observed when removing the biomass to an accumulation reactor where it is exposed to an extended period of carboxylate exposure [35]. The accumulation reactor may be operated anaerobically or aerobically [35]; however, PHA accumulation associated with anaerobic operation is limited by the amount of secondary storage mechanisms present within the culture and by the lack of respiration required to generate the needed cellular energy for continued carboxylate uptake. Conversely, aerobic operation of the accumulation reactor would induce the PHA storage response through carbon oversupply; however, such a metabolic response contradicts the anaerobic mechanisms used for the

enrichment of the culture [35]. Aerobic operation of the accumulation reactor has seen more success, but with only moderate results, causing research to be performed on other accumulation technologies [9, 36]. Figure 2.3 illustrates a proposed process diagram which would allow for an optimized process to be implemented at existing treatment facilities [37]. Research is ongoing, conducted by others, to test the viability of this method for PHA accumulation [38-40].

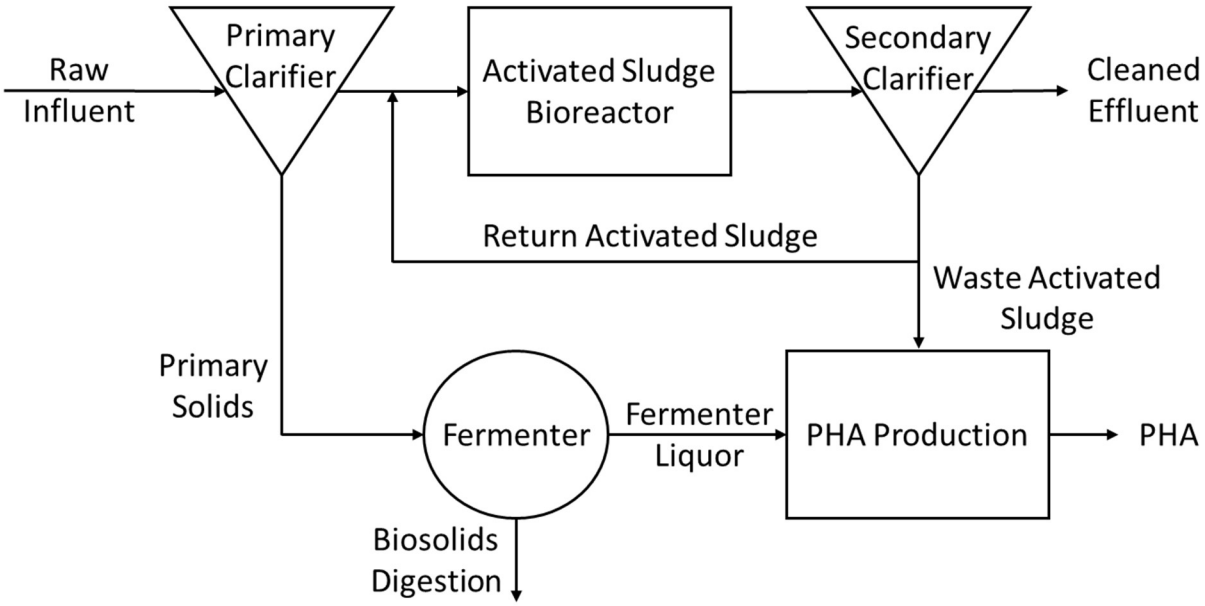

Figure 2.3: Proposed PHA Production Process Diagram at Wastewater Facilities. Based on Original Drawing by Coats et. al. [37].

2.2.2. Controlled-Growth Feeding PHA Synthesis

Discovery of PHA within activated sludge ultimately led to research to identify the genera and species of PHA accumulators [16]. Once PHA-accumulating microbes began to be identified, it was theorized that a pure culture of a known accumulator could be easily manipulated to produce high quantities of PHA not otherwise obtainable with a MMC [5, 41]. Both pure-cultures and recombinant bacterial species have been extensively evaluated for PHA production potential [42].

Production of PHA using controlled-growth feeding and pure-cultures is typically performed using a two-stage process [41]. The first stage consists of the pure culture with known high PHA productivity characteristics being grown under fully aerobic conditions on a substrate free

of impurities [9]. The culture is then transferred to the second stage where it continues to grow until achieving critical mass; thereafter, a nutrient limitation – i.e., a microbial stress, typically induced through deprivation of nitrogen or phosphorus – is applied to force the substrate to be used for storage instead of growth. Without applying the nutrient limitation, the stress response would not occur, allowing the substrate to be continuously used for growth without PHA accumulation. This method of PHA accumulation has been well studied and is capable of consistently obtaining intracellular PHA content of 90% by weight [32].

While this method has been shown to produce high levels of PHA, the approach does not yet possess a large enough economic advantage over production of synthetic plastics due to the high costs required for maintaining both a pure culture and an impurity-free substrate [41]. Moreover, due to the necessity for a sterile substrate, this method is not compatible for the utilization of organic-rich waste streams.

2.2.3. Aerobic Dynamic Feeding PHA Synthesis

Through investigations to reduce the high cost of PHA production using pure-cultures, research has been performed on the use of a MMC, which can reduce costs associated with the purity of both the culture and the substrate. Discoveries showed that a MMC-based system exposed to alternating states of carboxylate excess and carboxylate depletion under fully aerobic conditions selects for floc-forming, PHA-accumulating biomass and against filamentous bulking organisms [43]; however, some filamentous organisms (e.g., Meganema) are able to produce PHA and may be enriched in the ADF process [15, 24]. This so-called ‘feast-famine’ metabolic response is associated with imposition of an engineered ADF environment [13]. In the other known methods of PHA storage (described above), accumulation is caused through deprivation of an essential growth condition, but ADF instead creates a condition of excess external substrate associated with pulse feeding at the beginning of an operational cycle [44]. The discovery of this metabolic response has generated an entirely new field of study on PHA production, separate from the original activated sludge studies of the PHA accumulation [16].

The F-F microbial response under applied ADF conditions causes most of the carbon (carboxylates) to be stored as PHA rather than be used for growth, despite the excess availability of requisite macro- and micro-nutrients. The current hypothesis rationalizing this behavior is that after the famine phase the bacteria have a reduced metabolic capacity, rendering them

unable to grow at their maximum rate. Then in the feast phase, when excess carbon is rapidly introduced to the system, the cells are metabolically unable to optimally utilize the substrate for growth; instead, they store the carbon as PHA. This metabolic response has been referred to as an 'overflow metabolism' that induces 'energy spilling' [45-47]. As external carbon becomes depleted from the system, microbes utilize internal PHA stores for growth and maintenance. The ability to store PHA allows select microbes to survive the famine phase. Figure 2.4 displays a typical F-F response [17].

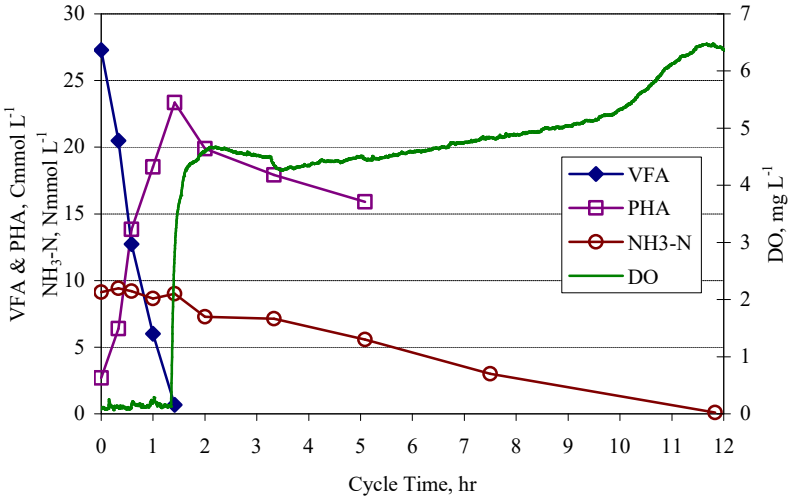

Figure 2.4: Typical Feast-Famine Response [17].
Reproduced with permission.

Carboxylates are required under ADF operations, because they are a direct precursor to PHA and can be consumed and utilized readily [48]; other forms of carbon (e.g., carbohydrates, alcohols) require the induction of additional metabolic pathways that ultimately do not facilitate PHA storage in a MMC under applied ADF conditions. Populations of PHA-accumulators will continue to grow during the famine stage through utilization of stored intracellular carbon, while non-storing, so-called 'flanking' populations – generally referred to as Ordinary Heterotrophs (OHOs) – are minimized or incapacitated from consuming a significant amount of substrate in the upcoming feast period. Engineered ADF conditions are typically obtained with SBRs to better create conditions of feast and famine, thereby resulting in a superior enrichment of the culture for PHA accumulation. An ADF configuration can theoretically be paired with any organic carbon-rich waste stream.

2.3. PHA Synthesis by a Mixed Microbial Consortium

Current commercial production of PHA is performed using the controlled-growth mechanism described above, with pure culture systems fed synthetic substrate – typically a corn sugar-based carbon source [6]. While these systems are capable of producing high intracellular yields of PHA, the costs associated with refining the substrate and maintaining axenic conditions are much higher than the production costs associated with producing polypropylene [6, 9]. After discovering that intracellular PHA accumulation greater than 65% could be achieved using a MMC under ADF conditions [18], the production of PHA using MMC systems has created a potential means of significantly decreasing PHA production costs [9, 49]; however, research on PHA production by MMC has identified potential productivity limitations, including low intracellular yields and low mixed liquor concentrations of PHA producers.

Current pure culture-synthetic substrate commercial production facilities consistently achieve intracellular accumulations greater than 80% [6], while reported accumulation for ADF-based MMC systems are highly variable ranging from 8% [50] to 89% [26]; the latter being the highest reported accumulation to-date. The inefficiencies associated with use of substrate for growth vs. PHA production and MMC enrichment of OHOs capable of surviving on the slowly degradable substrate ultimately require a larger amount of chemical and economic input to extract the PHA stores, potentially making some methods of production less or infeasible. For example, increasing intracellular PHA accumulation from 50% to 88% has been estimated to reduce production costs by $3.88 per kilogram [51]; therefore, maximizing intracellular PHA content in MMC systems will be essential to reduce production costs and make them more competitive with pure-culture systems.

A second limitation to PHA productivity in MMC systems is achieving a high enough concentration of biomass. Current commercial operations typically maintain biomass concentrations greater than 100 gTSS/L [51], while typical municipal activated sludge systems – which generally represent the biomass accumulation potential for a MMC system – operate between 3 and 10 gTSS/L [52]. In current commercial processes, PHA is produced in batches, allowing the culture to grow to a desired concentration before being harvested, resulting in large biomass concentrations being reported. It has been suggested that MMC systems must approach the same concentrations of biomass as pure-culture systems to become commercially viable.

While operation of MMC systems should prioritize higher biomass concentrations to reduce PHA polymer production costs, most MMC systems are being designed to generate PHA on a continuous basis; this means that maintaining large biomass concentrations may not be required for commercialization.

Increasing biomass concentrations within the production system can be achieved by increasing the average amount of time the microbes spend in the system (i.e., SRT). An increase in the SRT will cause the number of microbes within the system to increase, thereby increasing the concentration of solids in the system. A larger SRT will also create a potentially more resilient system, but can also decrease the overall metabolic activity and PHA production capability of the culture [52, 53]. Moreover, as the SRT increases, the relative fraction of fixed suspended solids (largely minerals) increases, and thus the viable biomass fraction – and potential PHA fraction – of the TSS decreases.

2.4. Metabolic Pathways of PHA Synthesis

Understanding the metabolic pathways involved in PHA synthesis is essential for optimizing the accumulation potential of a culture. Most of the known metabolic pathways have been described using pure-cultures fed synthetic substrates, including the three-step pathway presented in Figure 2.5. Due to a limited amount of research using MMC, it is assumed that the specific pathways approximately follow the pathways described using pure-cultures [54]. This assumption is beneficial to understand the interaction between the metabolic pathways utilized and the resultant effects on bulk solution behavior. Metabolic pathways are separated into four processes: carboxylate uptake and conversion, PHA synthesis, intracellular PHA degradation, and the catabolic oxidation of metabolic intermediates.

2.4.1. Carboxylate Uptake and Conversion

Carboxylate uptake is presumed to occur through free diffusion across the cell membrane due to the relative size and lipid solubility of the undissociated form [55]. Intracellular acid-base equilibrium causes carboxylate dissociation within the cell, as intracellular pH is typically higher than organic acid pKa (4.7 to 4.9), resulting in an increase in intracellular free protons [56]. The cell is then required to remove these excess protons, using Adenosine Triphosphate (ATP), to sustain the proton motive force (PMF) [57]; ATP cost is dependent on the pH gradient

across the cell membrane, extracellular carboxylate concentration, carboxylate equilibrium constant, and the carboxylate permeability.

Inside the bacterial cell, the carboxylates are esterified with free Coenzyme-A (CoASH), resulting in a corresponding alkyl-CoA [58], allowing the molecule to enter multiple central metabolic pathways. For larger carboxylates (four carbons or larger), β-oxidation can break down the substrate into shorter alkyl-CoAs and reducing equivalents. This process continues until two carbon atoms are separated as acetyl-CoA for even numbered carbon carboxylates or as one acetyl-CoA and one propionyl-CoA for odd numbered carbon carboxylates. Activation of carboxylates requires CoA and ATP, while β-oxidation also consumes CoA but generates reducing equivalents. Figure 2.5 and Figure 2.6 illustrate key aspects of these metabolism pathways.

Reducing equivalents function as intracellular metabolites that catalyze the transfer of electrons from donors to acceptors [59]. The most relevant reducing equivalents for the ADF process are NADH and NADPH [16]. NADH is used to produce energy through the transfer of electrons from the substrate to the electron acceptor. NADPH is responsible for macromolecule synthesis (PHA) by transferring electrons to acetyl-CoA. To store PHA, the cell must convert some NADH to NADPH via transhydrogenation or generate it through another pathway such as the pentose phosphate pathway (PPP) [16].

2.4.2. Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle is the primary pathway utilized by aerobic bacteria to generate energy (i.e., ATP). As shown in Figure 2.6, the TCA cycle oxidizes acetyl-CoA to CO_2 and reducing equivalents, phosphorylates ADP to synthesize ATP, and generates key cellular precursors [58]. The chemical energy stored within the acetyl-CoA molecule is obtained through the removal of electrons from the chemical structure and oxidizing it to carbon dioxide. In the presence of an external electron acceptor (e.g., oxygen; nitrate; nitrite), the reducing equivalents are oxidized by the electron acceptor which phosphorylates ADP to ATP, capturing energy for the cell [59]. Unless inhibited, the cycle will continuously generate carbon dioxide and reducing equivalents.

Propionyl-CoA can also be oxidized via the TCA cycle, entering as succinyl-CoA [60, 61]. This pathway follows three steps and requires ATP [61]. This, along with four other possible pathways, provides the means for some propionyl-CoA to be transformed into acetyl-CoA, as is known to occur when propionic and valeric acids are used as substrates [30].

2.4.3. PHA Synthesis

Intracellular PHA synthesis from carboxylates incorporates a network of metabolic pathways. Figure 2.5 and Figure 2.6 illustrate the potential pathways for the metabolism of common carboxylates. The process begins with the activation of the carboxylate, as described above, requiring CoA and ATP. Then, multiple alkyl-CoA precursors are condensed using β-ketothiolase to form a 3-ketoacyl-CoA compound, liberating CoASH [62]. The resulting 3-ketoacyl-CoA molecules are reduced using NADPH dependent acetoacetyl-CoA reductase to produce the corresponding 3-hydroxyacyl-CoA [63]; PHA synthase then incorporates the 3-hydroxyacyl-CoA into the polymer, liberating the remaining CoA [64].

Acetic acid, the most common carboxylic acid, is activated directly to acetyl-CoA, two of which are condensed by β-ketothiolase to generate the 3HB precursor acetoacetyl-CoA [65]. Cultures grown on acetate as the sole carbon source primarily generate 3HB, with cellular concentrations dependent upon concentration of acetate in bulk solution [66]. Propionic acid is activated to propionyl-CoA [9], which can also then be converted to acetyl-CoA, making the generation of both 3HV and 3HB possible [66]. Butyric acid can either generate the direct 3HB precursor acetoacetyl-CoA or two acetyl-CoA molecules through β-oxidation. Cultures fed only butyric acid generate only PHB [67]. Valeric acid can be the direct precursor for 3HV or can be catabolized into one acetyl-CoA and one propionyl-CoA using β-oxidation [67]. Like propionic acid, a fraction of the available valeric acid is oxidized to acetyl-CoA and propionyl-CoA, resulting in PHBV. Caproic acid is the direct precursor for 3-hydroxyhexanate but, through subsequent β-oxidations, can generate 3HB directly along with one acetyl-CoA or three acetyl-CoA molecules. Additional organic acids common to fermentation include isobutyric and isovaleric, which are structural isomers of butyric and valeric acids, respectively. Typically these acids exist in relatively low concentrations, resulting in little impact on polymer composition.

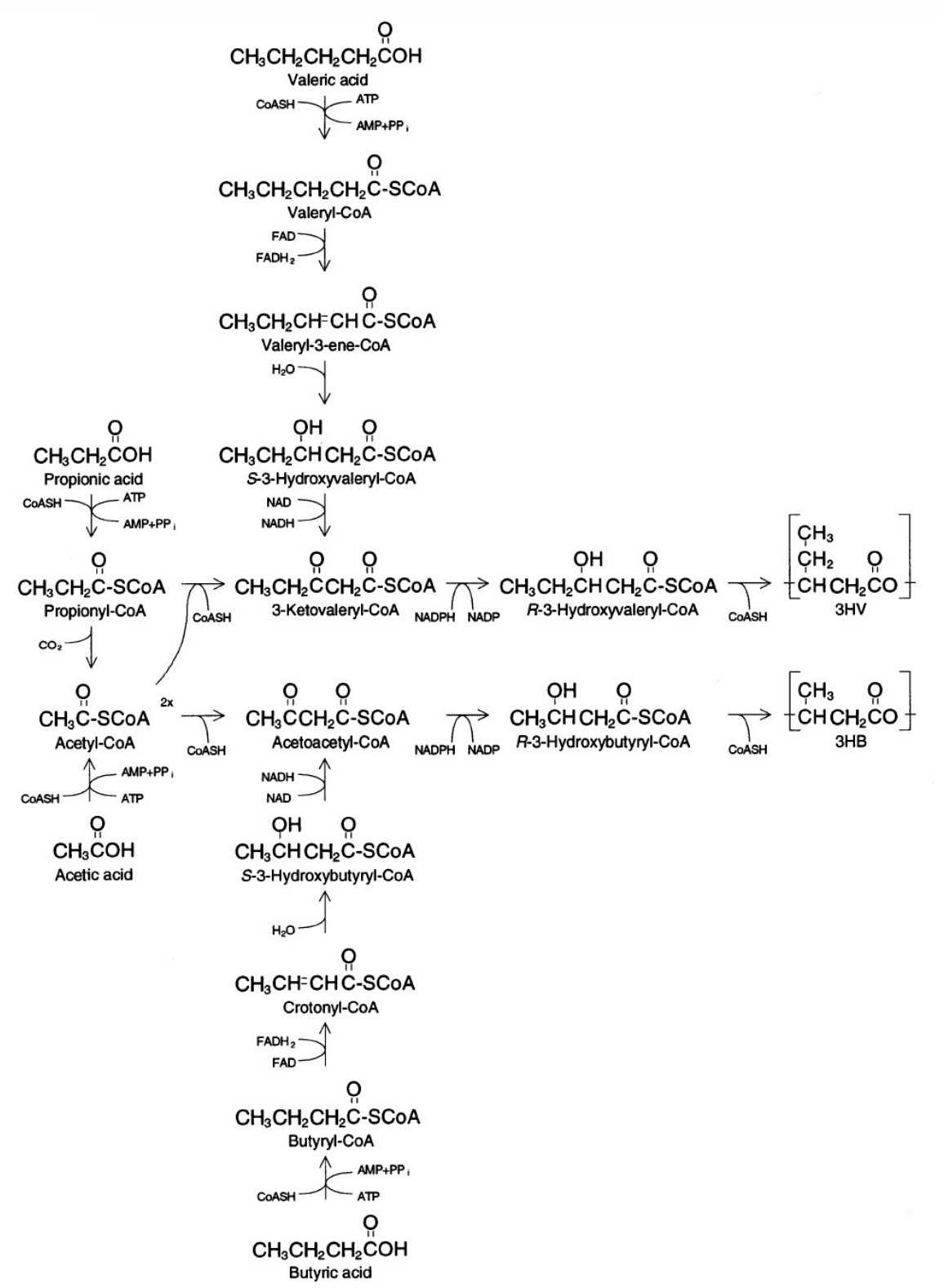

Figure 2.5: PHA Synthesis from Common Carboxylates.

Synthesized from Braunegg et. al. [68]

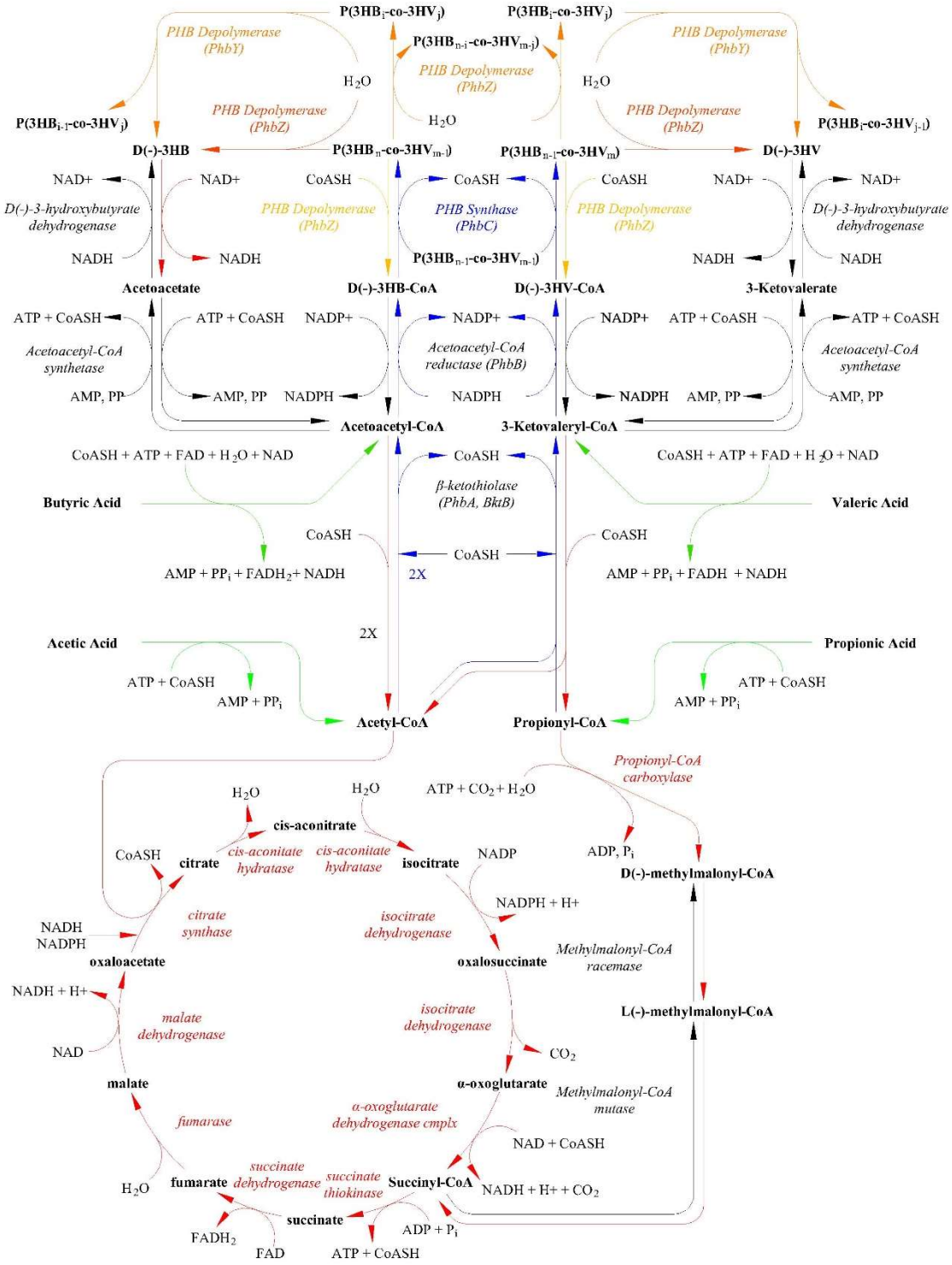

Figure 2.6: PHA Metabolisms and Regulation in Ralstonia eutropha.

Synthesized from Multiple Sources [54, 58, 61, 63, 68-71].

2.4.4. Intracellular PHA Degradation

Intracellular PHA degradation has not received as much attention as extracellular PHA depolymerization, principally due to the difficult nature of isolating the PHA granule bound proteins [72]. As shown in Figure 2.6, however, three pathways have been elucidated for the intracellular degradation of PHA in *Ralstonia eutropha*.

In the original pathway, shown in red-orange, the polymer is first hydrolyzed by the PHA depolymerase, PhaZ, causing a release of 3HB. The 3HB is then oxidized to acetoacetate by D(-)-3-hydroxybutyrate dehydrogenase, reducing NAD^+ to NADH. Acetoacetate is then thiolyzed with CoASH; thiolysis occurs by either Acetoacetyl-CoA synthase, requiring ATP, or by a transferase [73], wherein the CoASH is transferred from another molecule, not requiring ATP [71]. Energy is required to complete the activation process, either at the thiolysis reaction or one preceding.

A second pathway, shown in orange, utilizes another PHA depolymerase, PhaY. Two forms of the PhaY enzyme have been isolated with one version capable of acting in solution and being bound to the PHA granule [74] and the other acting weakly on the granule [75]. As in the original pathway, 3HB is produced, which must be subsequently reactivated to acetoacetyl-CoA for metabolism.

PHB granules, in the presence of CoASH, can be thiolyzed to release 3HB-CoA [76]. Research performed on the enzyme PhaZa1 demonstrated that it was capable of catalyzing the thiolysis of the polymer with CoASH. This third pathway, shown in gold, conserves the energy of the initial activation, making it the desired pathway for PHA degradation [77]. Unfortunately, hydrolysis of both PHA monomers and polymers occurs, allowing the potential for all three pathways to occur simultaneously. Under PHA-accumulating conditions, the activity of depolymerase, and hence the fraction of the carbon liberated, is thought to be approximately 10 times slower than PHA synthesis [69], resulting in net PHA accumulation.

2.5. Feast-Famine Metabolic Response

Excess PHA accumulation realized through applied ADF operations has received growing attention as a means of utilizing a MMC to commercially produce PHA at a lower cost than conventional pure culture-based processes. While research has progressed with ADF operations

[13, 15-27], metabolic pathways within the F-F response are not universally established [17], which has led to variable operating parameters and associated guidance. Indeed, it can be argued that much of the ADF research to date is not sufficiently based on theoretical considerations, but more from the perspective of phenomenological operations. The success of maximizing enrichment of PHA producers in an ADF regime is currently based on the high biomass specific uptake rate of PHA-producing microbes compared to microorganisms that use the substrate directly for growth [78]; however, many organisms, when subjected to ADF conditions, maximize substrate uptake rate, while growing at a balanced rate [19].

The ADF regime requires a period of rapid substrate addition and associated uptake, followed by a sufficiently long period without providing an exogenous, readily-biodegradable substrate; the goal is to select for PHA-producing bacteria over non-PHA storing organisms (OHOs), which are unable to survive sustained carbon deficiencies. The balance between the lengths of the feast and famine periods and, more critically, the length of the feast period, is an important parameter that can be used to assess and potentially increase the PHA-storage biomass capacities. This operational parameter is known as the F-F ratio and is measured as the ratio of the length of the feast period to the length of the famine period (Equation 3.1). A low F-F ratio ($\leq$0.25), indicating a short feast period and long famine period, has been suggested as a target to select against non PHA-accumulating biomass [79]. Such organisms cannot be sustained due to the long absence of an external carbon source, while PHA-accumulating organisms are able to survive the famine by using the stored PHA for metabolic purposes [20, 21].

2.6. Readily and Slowly Biodegradable Substrate

Success in enriching for PHA-accumulating organisms in an ADF system is dependent on the substrate utilized. Bacterial populations within activated sludge systems – both a source of inocula for ADF PHA systems but also a representative model system for the study of MMC that might perform F-F synthesis – specialize based on the type of substrate consumed. A portion of the population consumes readily degradable substrates (RDS), while other fractions consume slowly degradable substrates (SDS) [80]. The RDS represent the fraction of the substrate that is consumed most rapidly by the biomass and consists of soluble substances such as sugars, alcohols, and carboxylates; the SDS consist of large organic molecules and

particulate or insoluble substrates that must first be hydrolyzed by the cell and cannot be degraded rapidly [52].

Knowing that RDS (i.e., carboxylates) are required for PHA production and that PHA-accumulating organisms exhibit higher substrate uptake rates than non-accumulators, suggests that PHA-producing organisms do not require SDS. While PHA production has not been shown to be reduced in the presence of SDS substrates, the presence of SDS-accumulating organisms has been shown to reduce PHA content of the resulting biomass [81]. This response is likely associated with growth of non-PHA-accumulating populations that survive on the presence of SDS. Removal of SDS from ADF PHA production systems could serve to further hinder the ability of non-producing organisms to survive through the famine period. SDS removal could also have the effect of amplifying the stress induced to accumulating microbes, therefore increasing PHA output by potentially increasing enrichment of the target organisms.

2.7. Phase Separation and SDS Removal

One strategy for accomplishing SDS removal is the inclusion of a settling stage – phase separation – directly after depletion of PHA precursors (i.e., carboxylates) [81]. A settling stage induces a solid-liquid separation within the reactor; the settling stage could also remove OHOs, while concurrently selecting for PHA-producing bacteria based on cell specific gravity. Once biomass is concentrated, a stage of decanting the supernatant can be applied to remove any remaining exogenous substrate (SDS) from the system. Implementation of such an operational stage increases the potential for selective removal of substrates that are not utilized for PHA production, thus disadvantaging 'flanking' non-PHA producing microbial populations.

In the settling stage, suspended particles that are heavier than water are separated from solution through gravitational sedimentation. Implementation of settling and supernatant removal phases to a MMC ADF system has been shown to increase maximum PHA storage capacity of the biomass from 48% to 70% on a weight basis [82]. Due to the reported density of PHB (1.19 and 1.25 g/cm^3), it is expected that PHA-containing cells have a higher density than 'empty' cells [83]; ADF operations not only select for PHA-producing microbes (and desirable floc-formers), but also select against filamentous bulking organisms, increasing the ability of an enriched culture to settle. With PHA accumulators typically being suited for settling, the decanting of a supernatant would not only remove substrates unfit for PHA production, but also

potentially remove a fraction of flanking populations, leading to a dramatic increase in the number PHA producers in solution. As stated earlier, however, some filamentous genera (e.g., Meganema) are capable of producing PHA and have been shown to be enriched for using a dairy manure substrate within the ADF process [15, 24]. Therefore the application of a settling mechanism with this configuration may select for an altered PHA-producing microbial population.

2.8. Three-Phase ADF Operations

ADF research at the laboratory scale has often been performed using synthetic carboxylates, with essential nutrients added to sustain necessary metabolic processes [84]. While these studies have helped to enhance the understanding of ADF operations, the ultimate goal is to obtain full-scale production using complex waste streams. Employing a three-stage ADF process has become the typical means of production when using more complex substrates [13]. The stages consist of acidogenic fermentation, consortium enrichment, and PHA production. A schematic diagram of a three-stage process utilizing a dairy manure waste source is shown in Figure 2.7; the process configuration for ADF would be similar regardless of the substrate used.

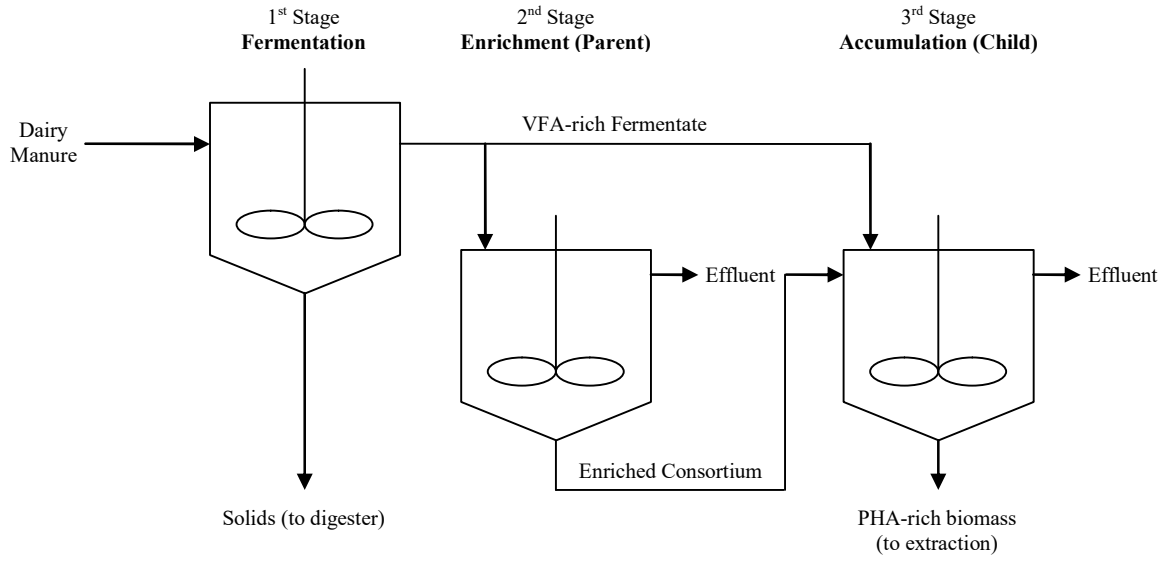

Figure 2.7: Simplified Three-Stage PHA Production Train from Dairy Manure

Fermentation of the substrate in the first stage of the process converts complex biodegradable carbon and particulate matter in the substrate to carboxylates. Methanogenic bacteria present in this stage are restricted from carboxylate uptake and growth by maintaining a low (<4 days)

SRT [13, 85]. While successful fermentation is essential to the remaining stages, optimization of this stage has been thoroughly researched [53]. Operation of this stage will, therefore, be considered simply as a means to an end as the source of carboxylates and readily degradable carbon.

The second stage of the process is consortium enrichment. It is in this stage that the ADF mechanism is applied to the MMC, eliciting the F-F response to select for organisms capable of PHA synthesis. An SBR with rapid feed and decant periods is the preferred operational format for culture enrichment [25, 26, 44, 86, 87], but the F-F response has also been achieved through utilization of a two-stage continuous stirred tank reactor (CSTR) system [22, 85]. In the CSTR configuration, the process is adjusted so that the feast occurs in the first enrichment reactor and the famine in the second, with the requisite selective pressure applied via hydraulic retention time (HRT).

Production of PHA occurs in the third and final stage of the process. This stage consists of the enriched biomass from the previous stage being fed an excess of external carbon substrate until maximum PHA levels are obtained. Observed intracellular PHA concentrations are larger in the production stage, where the culture has a constant supply of substrate vs. the single batch-fed enrichment stage. In this production stage, PHA production has been shown to be inhibited when high concentrations of carboxylates are present [18], so substrate feeding needs to be closely monitored. Feeding has typically been accomplished through a series of pulses, with substrate concentration decreasing to low levels between each pulse [44, 48], but can also be accomplished through a continuous feed to the reactor [16]. Biomass will continue to synthesize and store PHA until the storage metabolism is affected by unknown factors, as long as the carboxylate rich substrate and terminal electron acceptor are continuously available. After reaching maximum PHA production, the biomass is recovered, and the PHA is extracted [88]; alternately, high-value products may be produced using PHA-rich biomass [89].

Three-stage ADF production is widely recognized as the primary means of PHA production using MMC and waste substrate, due in part to the advantage of optimizing each process independently of the others. Alternatively, production may be limited due to difficulties in determining optimal operating conditions in each stage. Development of ideal operational parameters has yet to be defined, leading to a lack of understanding of optimal PHA production

in MMC systems. Increasing ADF research into development of criteria for optimal reactor performance would help elucidate an optimal production scheme and improve full-scale operations to be economically viable.

Primary operational parameters under ADF conditions in an SBR that need to be studied as a basis for design include: SRT, OLR, F-F ratio, terminal electron acceptor levels, and MMC kinetics. Minor operational parameters observed alongside primary studies include: pH, temperature, and essential nutrient concentrations. Research into the optimal combination of ADF operational parameters for PHA production remains a critical hurdle to process commercialization.

2.8.1. Solids Retention Time and Cycle Length

Solids retention time impacts the MMC concentrations within the biological reactor. Generally, SRT is defined as the quantity of biomass in a system divided by the biomass removed daily. A larger SRT will increase the amount of biomass within the system by decreasing the amount of biomass removed each day. Batch reactor SRT is inversely proportional to bacterial specific growth rate; thus, a shorter SRT generally results in a MMC with higher growth rates and, therefore, lower PHA yields, as more carbon is required to sustain rapid growth [90]. Longer SRTs create systems that retain similar populations each cycle, yielding a more stable and robust operation. Carta et al. [91] reported that as SRT increases (>2 days), the ratio of substrate uptake to PHA synthesis becomes a nearly constant value. While longer SRTs create a more stable environment, there appears to be an upper limit for optimal operations; for example, research by Chua et al. report a 3-day SRT performing approximately 10% better than a 10-day system [92].

The ratio between reactor SRT (days) and cycle length (CL; days) has been identified as a potential parameter for improved ADF system operations and for increasing intracellular PHA concentrations during the feast period [86]. In general, CL to SRT ratios of 0.04 to 0.5 have been investigated for ADF systems [13, 18, 25, 26, 79, 85, 87, 93, 94]. A CL to SRT ratio of 0.5 was used to achieve an 89% PHA weight percent, the highest reported value for an MMC system [26]. This CL to SRT ratio was approximately five times higher than those used in previous studies, suggesting that the CL to SRT ratio could be useful in optimizing operational conditions.

For similar operational systems, the CL to SRT has been shown to have a significant impact on the PHA-accumulator selection stage of the process [86]. Increasing the CL while maintaining the same SRT will exert a greater stress on the organisms present in the system as the time between feed pulses is increased, resulting in a longer famine phase to remove non-storing microbes. The effects of this ratio on the MMC metabolism that might enhance or impair PHA production are not fully understood, therefore interpretations remain empirical.

2.8.2. Feast-Famine Ratio (F-F Ratio) and Organic Loading Rate (OLR)

The F-F ratio is a proportion of the durations of the feast and famine phases relative to the total CL. The ratio becomes difficult to define because famine has not been clearly characterized. Throughout the literature, the transition to a famine state has been defined as: an increase in dissolved oxygen (DO) concentration after feeding, the exhaustion of carboxylates from the system, or as the complete consumption of carbon substrate (including PHA). Regardless of the definition of famine used in each study, there is consensus that a sufficient famine period is required to induce a PHA accumulation response.

The F-F ratio is ultimately a measured metabolic response by the MMC and is a function of the OLR, CL, and SRT, thereby making it difficult – practically impossible – to exert operational control to yield a target ratio. For sufficiently high CL to SRT and resultant low F-F ratios (e.g., <0.25), the required growth rate by non-accumulating bacteria needed to sustain their presence in the system becomes so large that it effectively culls them out completely, leaving a highly enriched PHA-accumulator population [16]. Due to the ratio being difficult to control directly, it is more of a process indicator; it is also more commonly reported vs. OLR, which can be more tightly controlled. Albuquerque et al. [85] showed that increasing the OLR for a constant CL to SRT ratio nominally increased the F-F ratio, principally due to a population increase associated with the higher substrate concentration. Alternatively, Dias et al. [48] noted that increasing the OLR leads to a decrease in the length of the famine period, thus reducing the selective pressure that favors PHA-accumulating populations.

Minimization of the F-F ratio applies a greater amount of selective pressure for PHA storing microbes [85]. Dionisi et al. [79] confirmed that a significant drop in accumulation potential occurs when the F-F is greater than 0.25. A F-F ratio less than 0.25 is generally accepted as the universal standard, but a ratio less than 0.20 is preferred [86]. Yang et al. [86] tested the effect

of the ratio when conducting a similar study to Johnson et al. [26]; the F-F ratio was reduced by 50%, from 0.10 to 0.05, resulting in PHA production of 82% compared to the original 90%, implying that there is a potential lower bound to the F-F ratio.

2.8.3. Constant Supply of a Terminal Electron Acceptor (Aeration)

Typical operation of ADF systems involves supplying the enrichment and production reactors with excess aeration to ensure that dissolved oxygen levels do not inhibit the capabilities of the mixed-culture [17, 26]; however, reduction of aeration in the enrichment phase can be employed to reduce operating costs without a significant reduction in PHA production [23, 24]. Typical aeration during the enrichment and production stages supplies oxygen as the primary electron acceptor, but facultative heterotrophic bacteria may also utilize nitrate or nitrite as electron acceptors under low residual oxygen concentrations, presenting another potential avenue for cost savings.

Aeration is typically measured using k_La, or a volumetric mass transfer coefficient, although others use DO as a surrogate parameter. Values for k_La are determined through empirical assessment of a given reactor configuration, due to the large variation of mixing characteristics of any reactor. Reactor k_La values are dependent on water quality, tank geometry, mixing characteristics, and aeration equipment used [95]. A reduction of reactor k_La has been shown to increase PHA yield on a carboxylate consumption basis [96], although research using a real waste substrate did not confirm this observation [24]. A reduction in aeration is believed to increase the stress response in the enrichment phase, providing PHA-accumulating organisms an advantage over non-storing microbes; however, PHA production on alternate electron acceptors, such as nitrate and nitrite, might require a more specialized MMC. Research performed by Watson et al. [23, 24] suggested that reduction in aeration on a k_La basis within the enrichment phase did not cause any net negative effects to maximum intracellular PHA levels, PHA to carboxylate yield, or mixed liquor suspended solids. These findings indicate that the PHA accumulation and enrichment processes are not inhibited as long as an adequate supply of a terminal electron acceptor is available in the reactors.

2.8.4. MMC Stoichiometry and Kinetics

Elucidation of microbial kinetics within each discrete PHA production reactor is required to fully understand and optimize ADF operations; indeed, each MMC and the associated substrate is unique, requiring optimization on a case-by-case basis. Understanding MMC kinetics and capabilities within the enrichment stage is paramount to successful and stable ADF operations, as it is the only known indicator of how to appropriately apply substrate to the production phase without producing limiting or inhibiting conditions. Mixed-cultures, however, exhibit complex kinetic behaviors and population dynamics, which makes it difficult to universally estimate and support optimal conditions for PHA production [97].

There are three primary parameters for calculating and estimating culture feast performance: PHA/VFA yield, carboxylate uptake rate, and PHA synthesis rate. The first parameter, PHA/VFA yield, which is best calculated on a Cmmol per Cmmol basis, is used to measure the efficiency of PHA production based on the substrate ('VFAs' represent all carboxylates) applied to the system. This yield parameter helps indicate the efficiency of carboxylate conversion to PHA vs. bacterial growth. Higher yields indicate more substrate was used for PHA during the feast phase, while lower yields indicate more of the substrate was used for growth [23]; the former being the preferred process operational state. While this parameter gives insight into the efficiency of the enrichment of an MMC, it is not fully indicative of what processes may be occurring, as some PHA-accumulating bacteria may be growing instead of storing additional PHA.

Measuring the rate of carboxylate uptake indicates the capacity of the MMC for consuming substrate. In particular, the carboxylate uptake rate exhibited by the MMC in the enrichment reactor represents a potential metric for modifying feeding frequency during the production phase, as it indicates the optimal rate of substrate addition or time between pulses to sustain bulk solution carbon concentrations in the reactor, while not inhibiting PHA production. Carboxylate uptake rates are measured using two primary means: overall carboxylate uptake (rVFA) and specific carboxylate uptake (qVFA). Overall consumption rate is measured as the amount of carboxylates consumed over time and is measured only during the feast period. Conversely, the specific uptake rate is the rate of consumption based on the mixed liquor volatile suspended solids (MLVSS, biomass) present. This metric provides a more accurate

assessment of the PHA production potential; however, because the specific uptake rate is a measure of the concentration of biomass present, it can be highly variable from reactor to reactor and across substrates, making it difficult to universally define the optimum level. Such rates are necessarily MMC- and substrate-specific.

Calculating the rate of PHA synthesis is beneficial for estimating production rates of the MMC. Similar to the carboxylate uptake rate, PHA synthesis is measured using an overall (rPHA) and a specific rate (qPHA). Similar to the carboxylate rates, the PHA rates are measured as the amount of PHA synthesized over time and the amount of PHA synthesized over time with respect to the biomass of the reactor. While the PHA synthesis rates are not used for process control purposes, they present an important estimation of how the process may function at full-scale.

2.8.5. Minor Operational Parameters

Temperature, pH, and nutrients added to the system comprise the less controlled parameters of ADF operation. Controlling these parameters in the reactors tends to overcomplicate the internal processes and can lead to a much higher overall cost of production [87]; however, it is important to monitor and measure these metrics to maintain process control.

Reactor pH is postulated to be a factor that influences PHA production [98]. Under low pH conditions (pH<4.75), carboxylates remain in the undissociated form, allowing for rapid diffusion into cells where they then dissociate and impose an intracellular proton load, lowering the intracellular pH [92]; however, the effect of this potential hydrogen loading on the MMC and on PHA synthesis is unknown. Studies into the effect of pH on ADF PHA production processes with a MMC have been contradictory. A range of optimal pH levels have been suggested including: 6.0 to 7.5 [98] and 7.0, due to more active PHA enzymes at a neutral pH [99], and at higher levels of 8.0 to 9.0 [18, 92, 94, 100]. Differences in results obtained for pH are assumed to be attributed to the different characteristics of the mixed-cultures used and variations in enrichment conditions. Montiel-Jarillo et al. [101] demonstrated that the amount of PHA stored by an enriched biomass was affected by pH, with a pH ≥ 7.5 exhibiting high accumulation; elimination of pH control enriched a MMC to accumulate up to 36% PHA by weight. These studies demonstrate the importance of pH on the performance of the enrichment

process, but removal of pH control still enriches for a high accumulation of PHA, presenting a potential for a reduction in both operational costs and complexity.

Nitrogen (N) and phosphorus (P) concentrations have also been shown to affect PHA production. Literature suggests that under conditions of nutrient excess, substrate is utilized more for growth [50]; conversely, N- or P-limitation have been shown to promote PHA production [27]. In most cases, higher PHA accumulation was found under N-limitation, although PHA specific storage rates and productivities are suggested to improve when operating at non-limiting N levels [25]. It is proposed that N-limitation decreases enzymatic activity, thus restricting the potential for growth [102]. P-limitation conditions are thought to favor PHA accumulation through restricting the TCA cycle [103]. It is further reported that higher PHA levels are achieved through limitation of only one essential nutrient, as it reduces cell metabolism complexity resulting in substrate used for production instead of growth [87, 103].

Within the literature, the order of importance of these parameters when using activated sludge is thought to be: pH > N-concentration > P-concentration > Substrate (on a chemical oxygen demand (COD) basis) [104]; however, most waste substrates are not nutrient limited, and therefore is not applicable to the research presented herein.

3. MATERIALS AND METHODS

3.1. Experimental Set-up

A three-stage PHA process, as illustrated (Figure 2.7), was employed for the duration of this research.

3.1.1. Feedstock and Source of Microorganisms

Dairy manure was collected from the University of Idaho dairy farm approximately every two weeks. The collection site was near the feeding stalls to avoid contaminating the sample with bedding material. Total solids (TS) and volatile solids (VS) measurements were made in triplicate with every new batch of manure collected, with average values (n=36) of 16.6% ± 3.4% and 81.7% ± 4.6%, respectively.

The inocula for the enrichment reactors used in this research was obtained from an existing ADF reactor fed with fermented dairy manure and operated since August 2011 [16]. The original inocula was grown in the laboratory using ADF to select for PHA-accumulators from bacteria that occur naturally in dairy manure.

3.1.2. Bench-Scale Fermenter

A bench-top fermenter was operated with a working volume of 16 L and a SRT/HRT of 4 days. The organic loading rate was maintained at 11.0 gVSS/L*day, with the wet weight of manure calculated from the total solids (TS) and volatile solids (VS) data. The fermenter was operated in SBR mode with 4 L decanted every day and refilled with 4 L (diluted, wet manure) at the same time every day to maintain the target SRT. Mixing was obtained using a 3.75” diameter helical impeller; the impeller was driven by an Oriental Motor (San Jose, CA, USA) USM315-401W 15 W AC speed control motor connected to 3GN35SA reduction gearbox operated at a speed sufficient to provide uniform mixing of the reactor contents. Carboxylates were measured regularly with average values (n=217) of 2348 ± 530 mg/L (acetate), 937 ± 256 mg/L (propionate), 362 ± 111 mg/L (butyrate), 59 ± 42 mg/L (iso-butyrate), 123 ± 42 mg/L (valerate), 107 ± 23 mg/L (iso-valerate), and 30 ± 24 mg/L (caproate). Total carboxylates were on average 5220 ± 860 as mg-COD/L and 149 ± 25 as Cmmol/L.

3.1.3. Consortium Enrichment Reactors

The enrichment reactors were operated as SBRs (identified as ER and ERS) with 3 L working volumes as shown in Figure 3.1. The SRT and HRT were adjusted between 2-, 3-, and 4-days, while maintaining the total cycle length at one day. The reactors were decanted and fed every day at the same time – digital timers were used to maintain constancy – with two Watson Marlow (400 rpm max), model 323 peristaltic pumps. The substrate for the enrichment reactors was obtained from the fermenter effluent (dairy fermenter liquor, DFL), which was centrifuged at 9000 rpm for 60 minutes. Mixing and aeration for the settling enrichment reactor (ERS) was shut off two hours after feeding (assumed end of feast based on past data) for sedimentation of the MMC. The ERS supernatant was decanted after a 20-minute settling period using an additional Watson Marlow (400 rpm max), model 323 peristaltic pump. The enrichment SBR processes are shown in Figure 3.2 and Figure 3.3. Working volume for ERS was 1 L after removal of the supernatant for 2-day SRT testing, and 1.5 L for 3- and 4-day SRT testing, while reactor ER was maintained at a total volume of 3 L. To maintain SRT, proportionate reactor volumes were removed at the end of a 24-hr cycle under complete mixed conditions. While reactor ER operated with HRT=SRT, reactor ERS experienced HRT<SRT. Specifically, under 2-, 3-, and 4-day SRTs, the average HRT for ERS was 11.2, 19.5, and 19.5 hours, respectively. Reactor OLR was set at 25 Cmmol/L-d; fermenter liquor carboxylate concentration was determined for each new batch with feed substrate volumes adjusted accordingly to maintain the OLR. Aeration was controlled by Aera PI-98 Series pressure insensitive mass flow controllers (1000 SCCM max) through stainless steel diffusers, with aeration set at 100% of the maximum flow rate (excess oxygen maintained). The reactors were mixed with Teflon™-coated stir bars and magnetic stir plates. The reactors were covered to prevent excess foaming, but vented to the atmosphere.

Figure 3.1: Consortium Enrichment Reactors

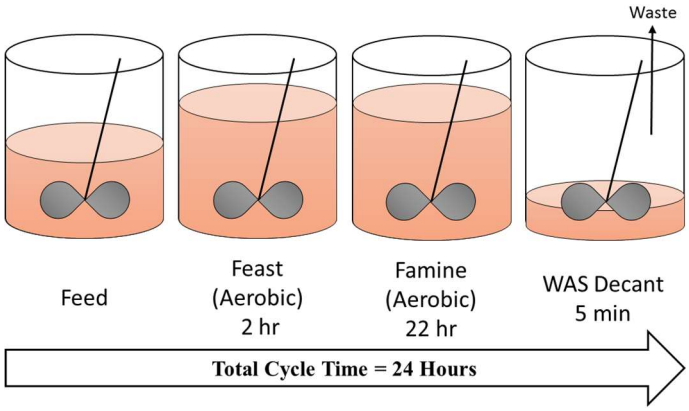

Figure 3.2: ER Cycle Detail

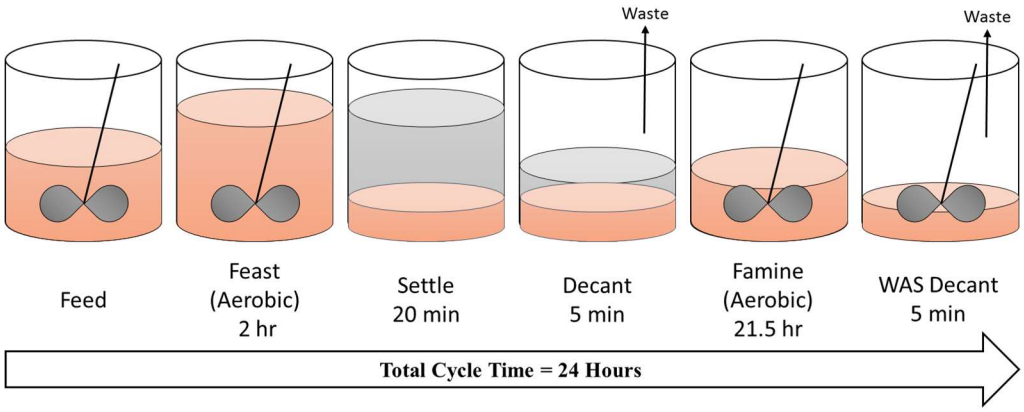

Figure 3.3: ERS Cycle Detail

3.1.4. PHA Production Reactors

Production reactors were operated as batch reactors in constructed acrylic reactors (actual volume varied over time, but was always less than 3 L). All experiments were conducted using a continuous fed operation mode. Dissolved oxygen, pH, carboxylates, PHA, TSS, and VSS were sampled periodically. The reactors were aerated with 6 inch diameter Sanitaire Silver Series II membrane fine bubble disk diffusers. Additional mixing was not required due to the fine bubble diffusers and small reactor diameters. Feed to the reactors was undiluted fermenter effluent centrifuged for 1 hour at 9000 rpm. The feedstock was supplied with Watson Marlow (400 rpm max), model 323 peristaltic pumps.

3.2. Analytical Techniques

Samples were collected to monitor pH, DO, TS, VS, TSS, VSS, COD, soluble nutrients (i.e., ammonia, nitrate, and phosphorus), carboxylates, and PHA. To analyze 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. Measurement of pH was accomplished with a Hach (Loveland, CO, USA) Intellical pHC101 pH Electrode. TS, VS, TSS and VSS were measured in accordance with Standard Methods 2540 D and 2540 E [105], respectively. DO measurements were collected using a Hach (Loveland, CO, USA) HQ40d Meter with a LDO101 DO Probe.

3.2.1. Nutrient Analysis

A Thermo-Fisher Scientific Corp Spectronic® 20 Genesys™ spectrophotometer was utilized to measure the absorbance of the reacted sample at a wavelength of 655 nm for NH_4, 410 nm for NO_3, and 890 nm for PO_4. Soluble NH_4, NO_3, and PO_4 concentrations were determined utilizing standard curves ($R^2 > 0.99$). Testing for soluble NH_4, NO_3, and PO_4 testing followed Hach method 10031, 10020, and 8048 (method equivalent to Standard Methods 4500-PE [105]), respectively.

3.2.2. VFA Analysis

VFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids) and ethanol 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 then to 150°C at 10°C/min and hold for 3 min; finally, ramp to 200°C at 25°C/min and hold for 12 min) with helium as the carrier gas (1.2 mL/min). The split/splitless injector and detector were operated isothermally at 210°C and 300°C, respectively. Prior to analysis, samples were acidified to a pH< 2 using nitric acid. 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 ($R^2 > 0.99$).

3.2.3. Intracellular PHA Analysis

Biomass PHA content was determined by gas chromatography/mass spectrometry (GC-MS) as described by Braunegg et al. [106]. 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) and an initial temperature of 40°C (2 min) ramped to 200°C at 5°C/min. The compounds were confirmed by retention time and mass spectral matching with known PHA standards (PHB and PHBV: 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).

3.3. Calculations

Reactor feast lengths, used to subsequently calculate the F-F ratio (Equation 3.1), were measured as the time of carboxylate depletion. PHA yield was calculated at the point of maximum PHA concentration and used the carboxylates depleted from solution to that point. For the enrichment reactors, this equals the concentration of PHA at the end of the feast divided by the concentration of carboxylates at the start of the cycle. For the production reactor, however, frequent sampling reduces the amount of carboxylates available for consumption and removes PHA from the system. Production reactor influent and effluent must, therefore, be accounted for and incorporated into calculations. Calculation of PHA yield informs the efficiency of the MMC during feast, described as a mass balance (Equation 3.5). Carbon and energy supplied (carboxylates) are either incorporated into the polymer (PHA), anabolized for biomass production (X), or oxidized for energy (O_2). Dividing by carboxylates, the equation is expressed in terms of a sum of yields for each component (i.e., $Y_{PHA/VFA}$, $Y_{X/VFA}$, and $Y_{O2/VFA}$). A higher $Y_{PHA/VFA}$ indicates more of the carbon fed during the feast phase is being converted to PHA.

The specific carboxylate uptake (q_{VFA}) and PHA storage (q_{PHA}) rates were calculated by applying a linear regression to VFA and PHA data with respect to the cycle duration. These

rates provide estimates for carboxylate utilization and polymer production rates for the average microbe in the system. Quantified rates were normalized to the initial biomass concentration, approximated as the VSS at the beginning of the cycle, with a biomass formula of $C_5H_7O_2N$ assumed for the computation of Cmmol/L equivalence of 0.0442 Cmmol/gVSS [107].

PHA and carboxylates were computed in terms of their theoretical chemical oxygen demand (COD); these quantities were also normalized to Cmmol to allow quantities to be compared between operations, independent of individual constituents. PHA values determined on a Cmmol/L basis were calculated as a function of PHB and PHV mass (mg) and the reactor TSS (mg/L). The calculations for the enrichment and production reactors were performed with Microsoft Excel® software. Statistical assessments were performed using Excel and R Statistical software.

3.3.1. Equations

Equation 3.1: Feast-Famine Ratio

$$F/F\ Ratio = \frac{Time_{Feast}}{Time_{Famine}}$$

Equation 3.2: Fermenter Manure Loading

$$M_{Manure} = \frac{OLR * V_{Fermenter}}{1000 * (TS) * (VS)}$$

Equation 3.3: Specific Carboxylate Uptake Rate

$$q_{VFA} = \frac{VFA_{Consumed}}{MLVSS * Time} \quad \left[\frac{Cmmol\ Carboxylates}{g\ \text{MLVSS} * hr}\right]$$

Equation 3.4: Specific PHA Synthesis Rate

$$q_{PHA} = \frac{PHA_{Synthesized}}{MLVSS * Time} \quad \left[\frac{Cmmol\ PHA}{g\ MLVSS * hr}\right]$$

Equation 3.5: PHA Carbon Mass Balance

$$\int_{t_0}^{t_t} VFA(t)dt = \int_{t_0}^{t_t} PHA(t)dt + \int_{t_0}^{t_t} X(t)dt + \int_{t_0}^{t_t} O_2(t)dt$$

Equation 3.6: ERS HRT

$$\frac{V_{decanted} * \frac{Time_{Feast}}{Cycle\ Length} + V_{Post-Decant} * \frac{Cycle\ Length}{Cycle\ Length}}{Q} \quad \left[\frac{L * \frac{hours}{24\ hours}}{L/day}\right]$$

Equation 3.7: Monod Kinetic Relationship

$$q = q_{max}\frac{S}{K_s + S}$$

4. RESULTS AND DISCUSSION

Two Enrichment SBRs were operated and evaluated for a period of 148 days to achieve and maintain an environment enriched for PHA-accumulating bacterial organisms. The primary focus of this research was to analyze enrichment reactor operations under two modes of operation: settling – identified as reactor ERS, and non-settling – identified as reactor ER, while concurrently assessing the associated impacts on PHA output during the production stage. Comprehensive process assessments (i.e., samples collected every 30 minutes over the course of the estimated 2 hour feast length plus 1 hour to capture potentially longer 'feasts') were performed a minimum of three times for each SRT evaluated (2-, 3-, and 4-days). Base-level process monitoring was also regularly performed between sampling runs (i.e., MLVSS, carboxylates). Each SBR was fed the same substrate as described in Section 3.1.3; the resulting MMC response was evaluated through measured Enrichment and Production stage parameters.

To summarize the results obtained, enrichment and production modes for each SRT evaluated are presented independently, followed by a holistic operational assessment integrating all system data – including SRT assessment comparisons – to extract important operational findings. The final section describes the relationships between operating parameters and PHA output to establish a basis for engineering design.

4.1. 2-Day SRT Assessment

4.1.1. Enrichment Reactor Evaluations

Comprehensive sampling for the 2-day SRT enrichment assessments was performed on operational days 21, 70, 79, and 85. Figure 4.1 displays carboxylate uptake and PHA accumulation for each comprehensive 2-day SRT sampling assessment.

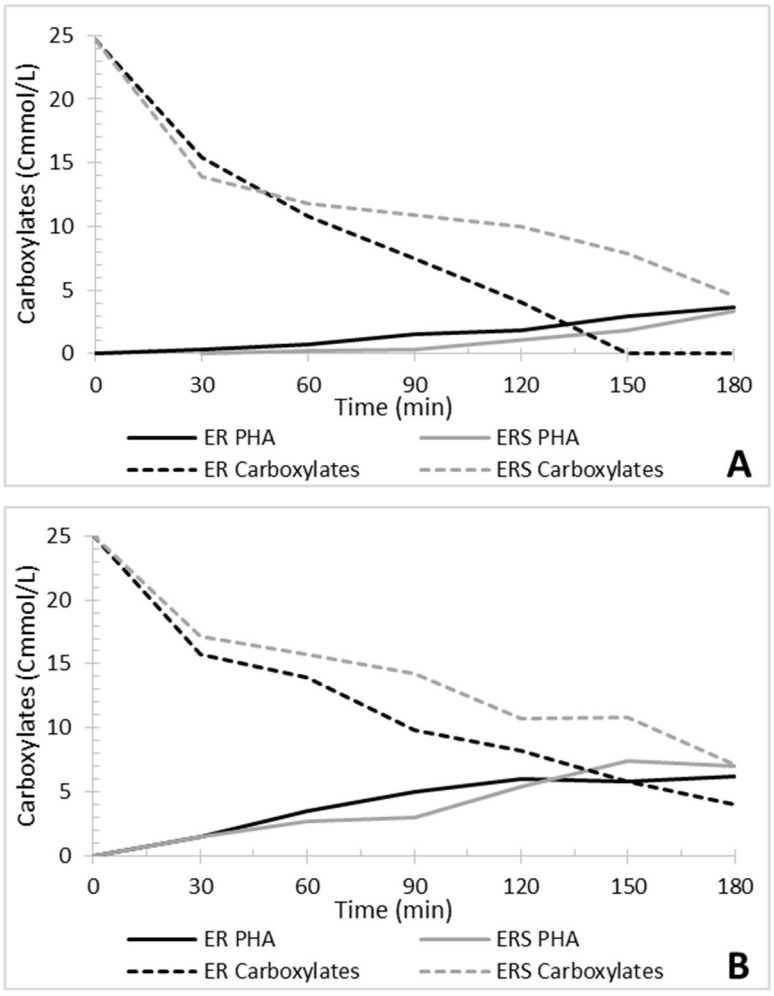

Figure 4.1: 2-Day SRT Enrichment Sampling Assessments
(A and B represent operational days 21 and 70, respectively)

Figure continued on the next page.

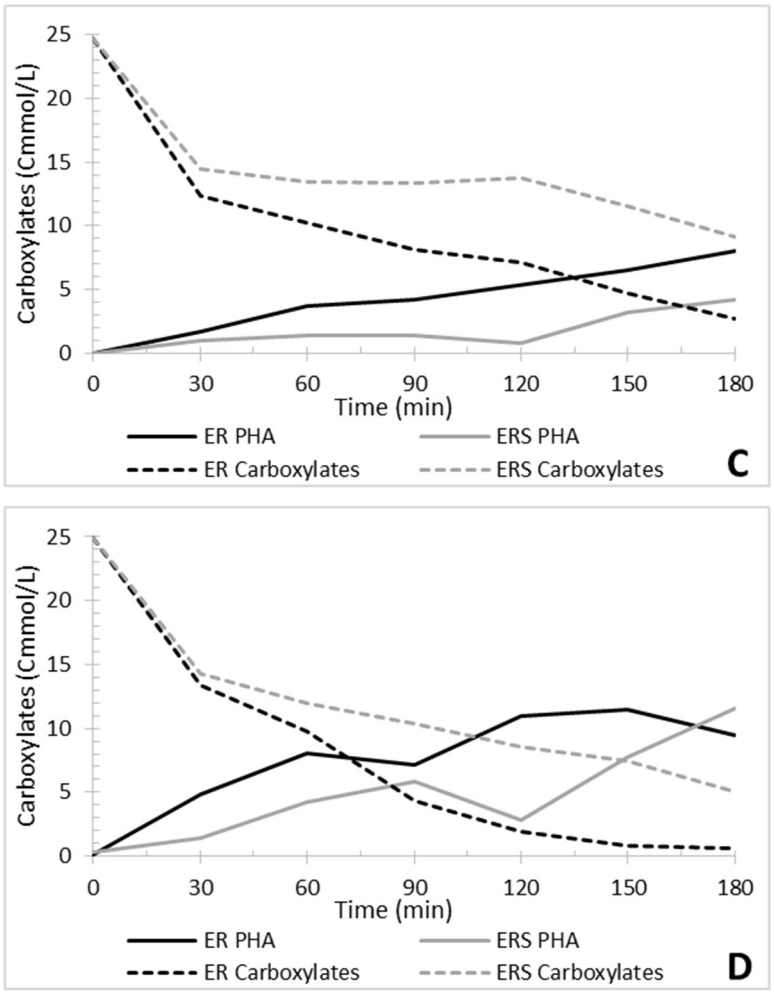

Figure 4.1: 2-Day SRT Enrichment Sampling Assessments
(C and D represent operational days 79 and 85, respectively)

As shown, reactor ER experienced higher rates of carboxylate consumption, resulting in shorter feast lengths than reactor ERS; however, a reduced carboxylate uptake rate did not necessarily result in lower PHA production, as noted with maximum PHA accumulation within each reactor remaining generally similar throughout 2-day SRT evaluations. Regarding feast length, while reactor ER generally exhibited more rapid carboxylate depletion, the feast length was not consistently limited to 3 hours; additionally, the microbial consortium in reactor ERS never was observed to achieve carboxylate depletion within 3 hours and continued to consume carboxylates following the sedimentation and decant phase. To assess ERS decant, t=120 minute samples avoided collecting settled biomass; therefore, decreased solids and PHA values were expected.

Solids concentrations were evaluated at the start of each operational cycle to assess changes over time. Values for reactor WAS represent solids at the end of each operational cycle. Reactor solids at t=0 were determined based on WAS concentrations, assuming no solids added from the substrate. While the DFL did contain solids – despite being centrifuged for 60 minutes at 9000 rpm – this assumption was made to reflect the actual concentration of microbes in the reactor potentially capable of PHA production and assumes DFL solids were largely hydrolyzed and used as substrate. Figure 4.2 represents the mixed liquor solids inventory during each 2-day SRT comprehensive sampling assessment.

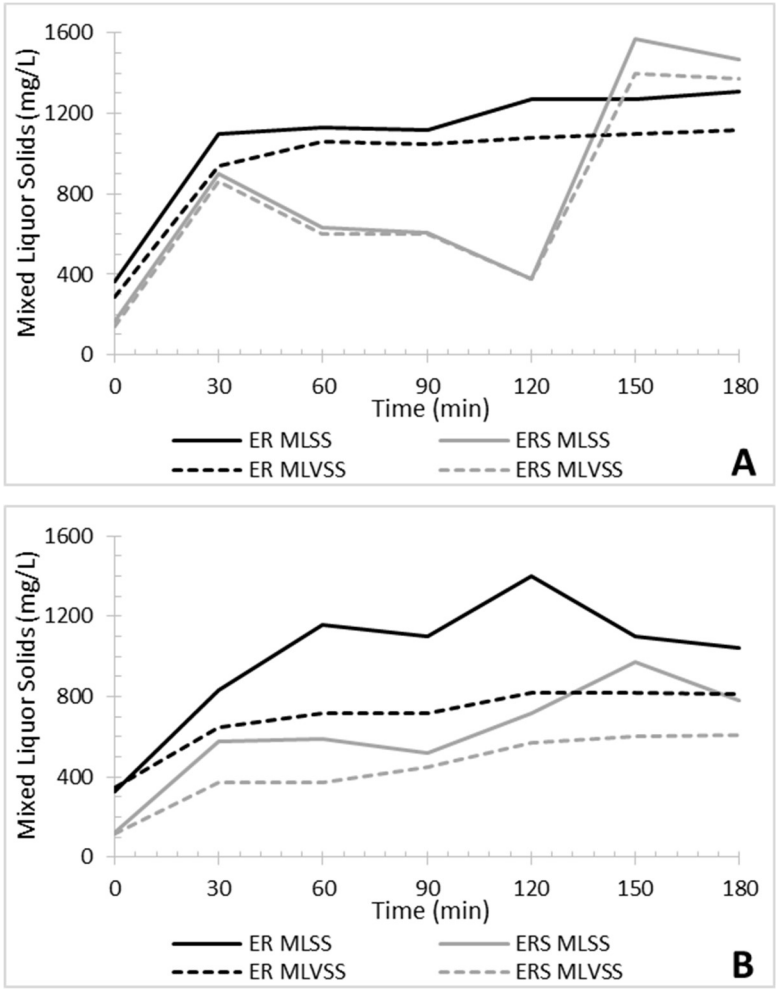

Figure 4.2: 2-Day SRT Enrichment Mixed Liquor Solids Inventory
(A and B represent operational days 21 and 70, respectively)

Figure continued on the next page

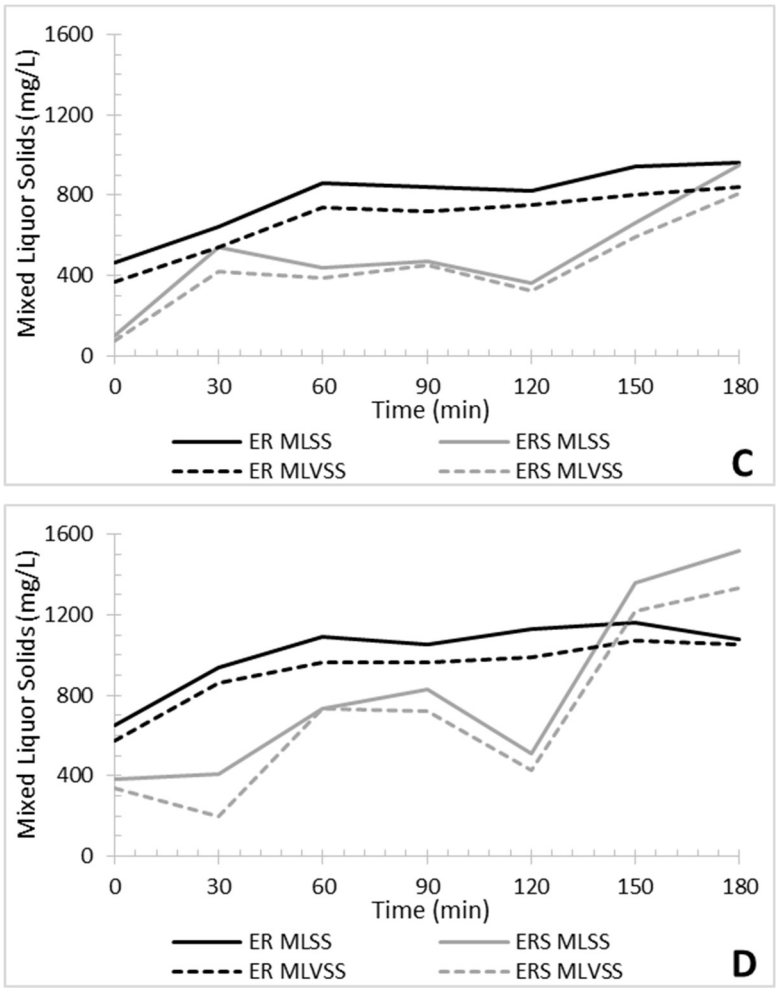

Figure 4.2: 2-Day SRT Enrichment Mixed Liquor Solids Inventory
(C and D represent operational days 79 and 85, respectively)

Average WAS values for ER and ERS were 916 and 1149 mg/L for TSS and 790 and 1007 mg/L for VSS; average VSS ratios were 86.5% and 86.8%. Differences in ER and ERS values are due to the low solids volume decanted for each ERS each cycle, and the fact that the settling/decant stage concentrated the biomass; combined, ERS WAS exhibited a higher solids concentration. Average calculated values for ER and ERS at t=0 were 458 and 192 mg/L for MLSS, and 388 and 160 mg/L for MLVSS, respectively, with average VSS ratios of 84.0% and 84.4%. Larger MLVSS concentrations are desired to increase efficiency of PHA extraction, thus reducing commercial operating costs. As shown, solids concentrations increased over the duration of the feast period for both ER and ERS. The initial solids increase was due to solids added with the DFL; average TSS values were 700 ± 257 mg/L (n= 8) for the DFL. Subsequent

reactor solids increases were the result of growth of the MMC biomass and accumulation of PHA. Average solids values at t=180 for ER and ERS were 1098 and 1180 mg/L for TSS, and 955 and 1030 mg/L for VSS; average VSS ratios were 87.0% and 86.0%. Larger values in ERS than ER after t=120 are due to decanting the low solids content supernatant after settling, thus increasing concentrations in the remaining volume. Table 4.1 summarizes solids data for each ER and ERS 2-day SRT sampling assessment.

Table 4.1: 2-Day SRT Enrichment Reactor Solids Inventory (Avg. ± SD)

Time	Parameter	ER	ERS
WAS (n=4)	***TSS (mg/L)***	906 ± 287	1149 ± 771
	TSS (g)	1.36 ± 0.43	1.72 ± 1.16
	VSS (mg/L)	790 ± 249	1007 ± 693
	VSS (g)	1.19 ± 0.37	1.51 ± 1.04
t=0 (n=4)	***MLSS (mg/L)***	453 ± 143	191 ± 129
	TSS (g)	1.36 ± 0.43	0.57 ± 0.39
	MLVSS (mg/L)	395 ± 124	168 ± 116
	VSS (g)	1.18 ± 0.37	0.50 ± 0.35
t=180 (n=4)	***MLSS (mg/L)***	1098 ± 150	1180 ± 371
	TSS (g)	3.29 ± 0.45	1.18 ± 0.37
	MLVSS (mg/L)	955 ± 153	1030 ± 379
	VSS (g)	2.87 ± 0.46	1.03 ± 0.38

Lower initial biomass concentrations in ERS ultimately resulted in a longer feast length than ER and a commensurately larger F-F ratio, since there were fewer microbes available to consume the substrate added to the reactor each cycle. Average F-F ratios were 0.14 for ER and 0.21 for ERS. Values for ER are less than the recommended maximum ratio of 0.20 [86], while values for ERS are slightly above the recommended threshold. These values suggest adequate famine lengths were achieved in both reactors to induce stress for PHA accumulation.

Lower biomass concentrations also resulted in larger specific rates for carboxylate uptake (q_{VFA}) and PHA accumulation (q_{PHA}) for ERS than ER. Average rates were 0.14 and 0.10 Cmmol/min for carboxylate uptake (r_{VFA}) for ER and ERS, respectively, and 0.04 Cmmol/min for PHA synthesis (r_{PHA}) for both reactors. Average specific rates were 0.36 and 0.74 Cmmol/gVSS*min for q_{VFA} and 0.11 and 0.23 Cmmol/gVSS*min for q_{PHA} for ER and ERS, respectively. Specific rates were calculated based on Equation 3.3 and Equation 3.4 and were evaluated on linearity assuming a zero-order kinetic relationship.

Average observed PHA yields were 0.32 and 0.35 $Cmmol_{PHA}/Cmmol_{VFA}$ for ER and ERS, respectively. These values are lower than observed by others using a similar DFL feedstock (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]); however, these comparative data sets used SRTs greater than 2 days, which appears to affect yield (see subsequent narrative about longer SRTs). A lower yield indicates that more substrate is being utilized for growth than for PHA storage. Yield values were determined at the point of peak PHA (Cmmol/L) for each assessment. The MMC in ERS outperformed ER on a carboxylate-to-PHA conversion basis for nearly all 2-day SRT assessments.

With more substrate being utilized for growth, less can be stored as PHA; indeed, PHA production was less than anticipated. Peak values observed from these 2 day SRT assessments were 11.5 and 11.6 Cmmol/L (242.4 and 244.8 mg/L) for PR and PRS, respectively, on operational day 85. Although peak intracellular PHA content was similar for ER and ERS, the observed peaks occurred on different operational days: 19% (day 70) for ER and 21% (day 85) for ERS on a TSS (w/w) basis. Although the observed singular assessment maximum PHA production values were higher in ERS than ER, the reactors produced similar quantities of PHA on a regular basis (Table 4.2). Average peak PHA values over the four assessments were 7.4 and 6.6 Cmmol/L (154.6 and 140.0 mg/L) and 14.3% and 13.5% TSS (w/w) for ER and ERS,

respectively. The PHV content is also important since increased PHV fractions correlate with a more ductile polymer. Production was thus assessed based on the composition of PHV present with peak values of 38 and 41 %PHV (mol/mol) for ER and ERS (operational day 70), respectively; average PHV content was 25.5% and 28.8% (mol/mol) for ER and ERS, respectively. Ultimately, with different microbial stresses applied on ER and ERS, and resultant differences in %PHV produced, it would appear that the reactors enriched for MMC that utilize diverse metabolic pathways for carboxylate uptake and PHA synthesis. Overall, PHA values were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Table 4.2 summarizes data for each 2-day SRT sampling assessment to assess ER and ERS performance.

Table 4.2: 2-Day SRT Enrichment Reactor Summary

Operational Day	**21**		**70**		**79**		**85**	
Parameter	**ER**	**ERS**	**ER**	**ERS**	**ER**	**ERS**	**ER**	**ERS**
MLSS (mg/L) at t=0	367	165	350	123	465	98	650	380
MLVSS (mg/L) at t=0	290	140	350	118	365	77	575	337
MLSS (mg/L) at t=180	1310	1470	1040	780	960	950	1080	1520
MLVSS (mg/L) at t=180	1120	1370	810	610	840	810	1050	1330
F-F Ratio	0.12	0.19	0.15	0.20	0.12	0.29	0.16	0.18
q_{VFA} (Cmmol/gVSS*min)	0.57	0.80	0.34	0.88	0.34	1.13	0.23	0.33
q_{PHA} (Cmmol/gVSS*min)	0.07	0.13	0.10	0.33	0.12	0.28	0.13	0.19
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.15	0.17	0.29	0.39	0.36	0.24	0.48	0.59
Peak PHA (Cmmol/L)	3.7	3.3	6.2	7.4	8.0	4.2	11.5	11.6
Peak PHA (mg/L)	77.9	71.0	129.5	155.3	168.6	88.8	242.4	244.8
PHA, %TSS (w/w)	6.0	4.8	12.5	18.6	17.6	10.2	20.9	16.1
%PHV (mol/mol)	13.7	12.9	37.8	40.8	26.3	30.9	24.1	29.5

4.1.2. Production Phase Evaluations

The PHA production assessments were performed concurrently with enrichment reactor assessments on operational days 70, 79, and 85. The 2-day SRT production results are presented in Figure 4.3.

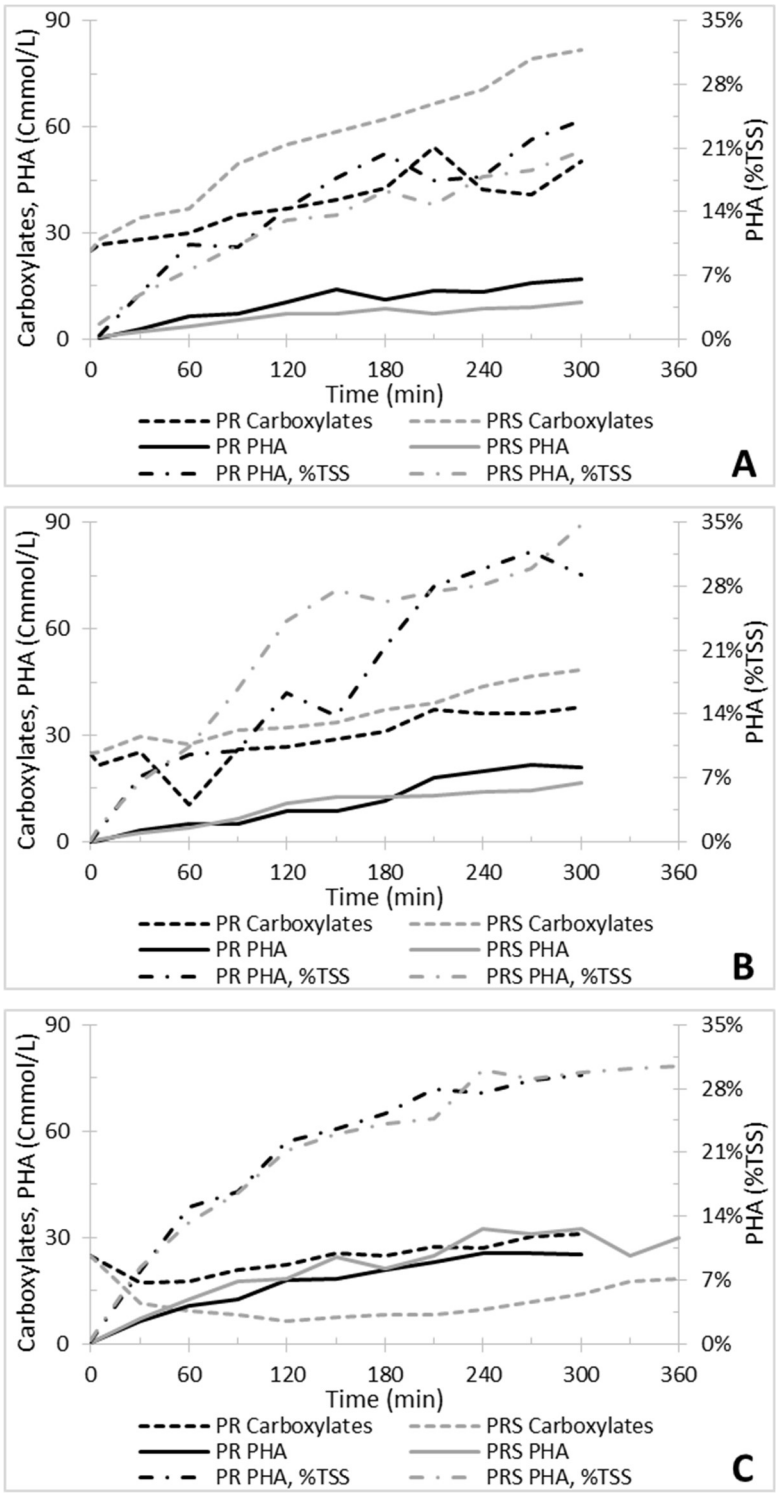

Figure 4.3: 2-Day SRT Production Sampling Assessments

(A, B, and C represent operational days 70, 79, and 85, respectively)

Each production assessment – specifically, the substrate feed rate – was designed based on specific carboxylate uptake rate data from the respective enrichment reactor MMC. A central operational goal was to manage bulk solution carboxylate concentrations in the production reactors to prevent inhibition of PHA synthesis; some research has suggested that in-reactor carboxylate concentrations exceeding 60 Cmmol/L can be detrimental to PHA production [18]. Operationally, and based on empirical guidance [18], substrate feed rates to the production reactors were established to maintain carboxylate concentrations consistently near the 25 Cmmol/L concentration (at t=0) applied to the enrichment reactors.

Predicting the MMC carboxylate uptake rate in the production phase *a posteriori* (i.e., based on enrichment carboxylate uptake) proved difficult. In particular, the respective MMC exhibited lower specific uptake rates in the production reactors, which were fed substrate continuously, in contrast to the enrichment reactors, which were fed a single pulse of substrate. As discussed in Section 2.8.4, substrate uptake rates can be highly variable from reactor to reactor; changing to a continuous feeding mechanism in the production stage ultimately caused carboxylate uptake rates to be significantly different than enrichment stage uptake rates. To a certain degree, this outcome was anticipated; continuous feeding operations in the production reactor would tend to induce intrinsic kinetics vs. the extant kinetics maintained in the enrichment reactors [108]. These kinetic differences, paired with uncertainties in operating a settling ADF enrichment reactor system, ultimately led to accumulation of carboxylates in the production reactors during each assessment; the effect was more profound for PRS vs. PR (i.e., Figure 4.3 A, B).

Despite the resultant kinetic differences, reduced uptake rates and associated accumulation of carboxylates realized in the reactors did not appear to impair PHA production; similar output values were obtained in each sampling assessment. Moreover, as production assessments proceeded, process adjustments were made – informed by previous and ongoing operations – to adjust substrate feed rates to the reactors and better align with the metabolic capabilities of the MMC. For example, to counteract the lower carboxylate uptake rates observed during production sampling, for production assessments performed on operational days 79 and 85, the continuous feed start was delayed by 30 minutes after the initial pulse of carboxylates was fed

to the reactors, thereby giving the MMC more time to consume part of the initial pulse and to synthesize the enzymes required for carboxylate uptake and PHA accumulation.

Solids concentrations were evaluated at the start of each cycle to determine the substrate feed rate based on the amount of biomass present. Reactor TSS values were equal to the concentrations obtained from the volume wasted from the enrichment phase. Average values for PR and PRS were 999 and 950 mg/L for MLSS and 889 and 875 mg/L for MLVSS, respectively; however, the mass of VSS in each production reactor – used to determine actual specific rates – was not similar due to differing initial reactor volumes, 1.5 L for PR and 0.5 L for PRS. Resultant average observed values for operational specific carboxylate uptake rates (q_{VFA}) in PR and PRS remained similar, at 0.20 and 0.21 Cmmol/gVSS*min, respectively. Due to a greater quantity of VSS present in PR, the MMC of the PR more efficiently consumed carboxylates than the MMC of PRS. Specific PHA synthesis rates (q_{PHA}) were not similar - 0.14 and 0.07 Cmmol/gVSS*min for PR and PRS, respectively - signifying the PR MMC more efficiently accumulated PHA than did reactor PRS. Specific rates were averaged to assess the continuously fed production systems; rates were calculated based on Equation 3.3 and Equation 3.4 and were evaluated on linearity assuming a zero-order kinetic relationship.

Average observed yields at the end of each assessment for PR and PRS were 0.60 and 0.44 $Cmmol_{PHA}/Cmmol_{VFA}$, respectively. These values fall within the range reported for other continuous-fed production evaluations utilizing fermented dairy manure (0.24–0.76 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]); however, the MMC in PRS consistently underperformed PR on a carboxylate-to-PHA conversion basis.

For 2-day SRT assessments, the maximum peak PHA production values obtained were 25.6 and 32.6 Cmmol/L (539.0 and 689.9 mg/L) for PR and PRS, respectively, on operational day 85; maximum intracellular PHA content was similar for both reactors with PR at 32% and PRS at 35% on a TSS (w/w) basis. While the maximum observed PHA production value was higher in PRS than PR, overall the MMC in reactor PR consistently outperformed reactor PRS (Table 4.3). Average peak PHA values were 21.4 and 19.9 Cmmol/L (451.5 and 419.8 mg/L) for PR and PRS, respectively, with an average of 28.6% PHA content (TSS basis, w/w) in both reactors. Regarding polymer quality, average values for peak %PHV were 30.3 and 24.7% (mol/mol) for PR and PRS, respectively, whereas ER consistently had a lower %PHV content

than ERS. Differing quantities of %PHV indicate that each MMC exhibited different metabolic pathways for PHA synthesis in both settling vs. non-settling and enrichment to production environments by producing more PHB or PHV, despite receiving the same carboxylate-rich substrate. Collectively, production results were consistent within the typical range reported in other ADF investigations [16, 23].

As a final comparison, in the production stage PHA was quantified based on the initial mass of inocul to determine the true increase in PHA in the reactor. Over all three assessment days, average values for maximum PHA based on inocula were 79.6% and 37.9% (w/w) for PR and PRS, respectively. Low values for PRS were due to diminishing reactor volume over the testing cycle, creating low volumes at the end of each assessment that significantly reduces calculated values. The large discrepancy in these values suggests that more of the solids added throughout the production cycle were attributed to PHA accumulation in PR than PRS. Table 4.3 summarizes the parameters evaluated to assess ER and ERS 2-day SRT performance in the production phase.

Table 4.3: 2-Day SRT Production Reactor Evaluation

Operational Day	**70**		**79**		**85**	
Parameter	**PR**	**PRS**	**PR**	**PRS**	**PR**	**PRS**
Initial MLSS (mg/L)	1300	1220	768	846	930	785
Initial MLVSS (mg/L)	1220	1140	717	786	730	700
Average q_{VFA} (Cmmol/gVSS*min)	0.15	0.12	0.19	0.24	0.25	0.27
Average q_{PHA} (Cmmol/gVSS*min)	0.08	0.05	0.18	0.05	0.17	0.10
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.55	0.46	0.50	0.39	0.74	0.47
Peak PHA (Cmmol/L)	17.1	10.5	21.6	16.5	25.6	32.6
Peak PHA (mg/L)	362.0	221.9	453.5	347.7	539.0	689.9
Max. PHA, %TSS (w/w)	24.0	20.7	31.7	34.8	29.5	30.4
PHA based on Inocula, % (w/w)	54.9	22.9	92.8	32.8	91.1	58.0
%PHV (mol/mol)	25.8	19.8	32.9	30.1	29.9	23.8

4.2. 3-Day SRT

4.2.1. Enrichment Reactor Evaluations

Upon completion of the 2-day SRT investigations, the enrichment reactors were transitioned to a 3-day SRT. Following 12 days to allow for process stabilization, comprehensive sampling for the 3-day SRT enrichment assessments was then performed on operational days 98, 106, 112,

and 119. Figure 4.4 displays carboxylate uptake and PHA accumulation for each comprehensive 3-day SRT sampling assessment.

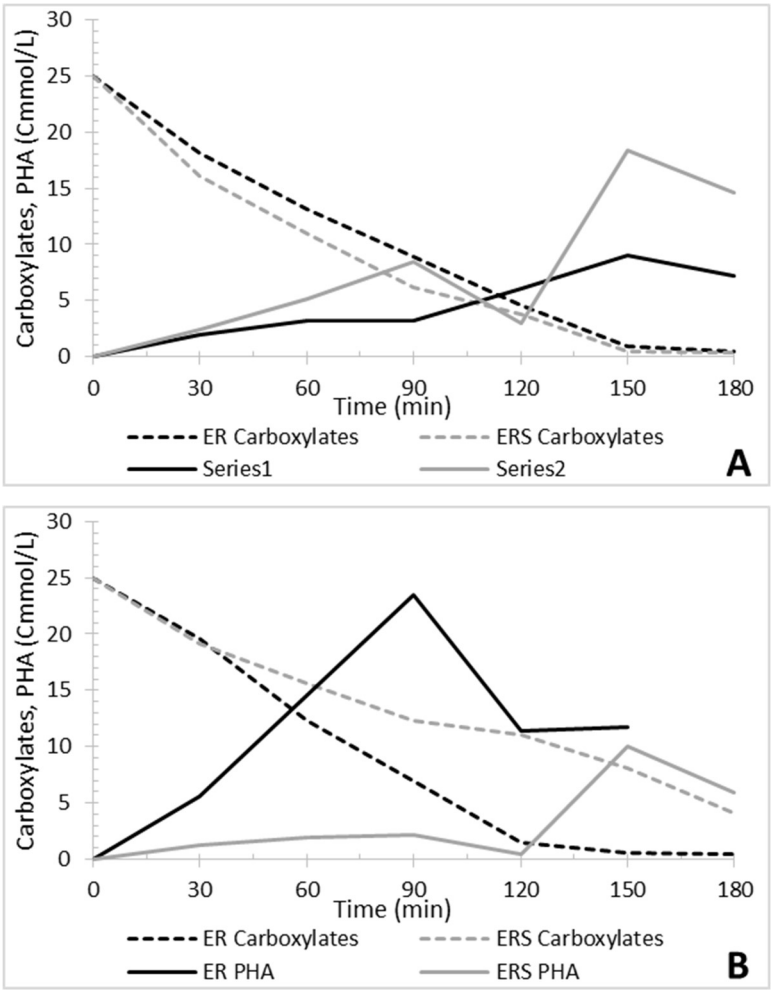

Figure 4.4: 3-Day SRT Enrichment Sampling Assessments
(A and B represent operational days 98 and 106, respectively)

Figure continued on the next page

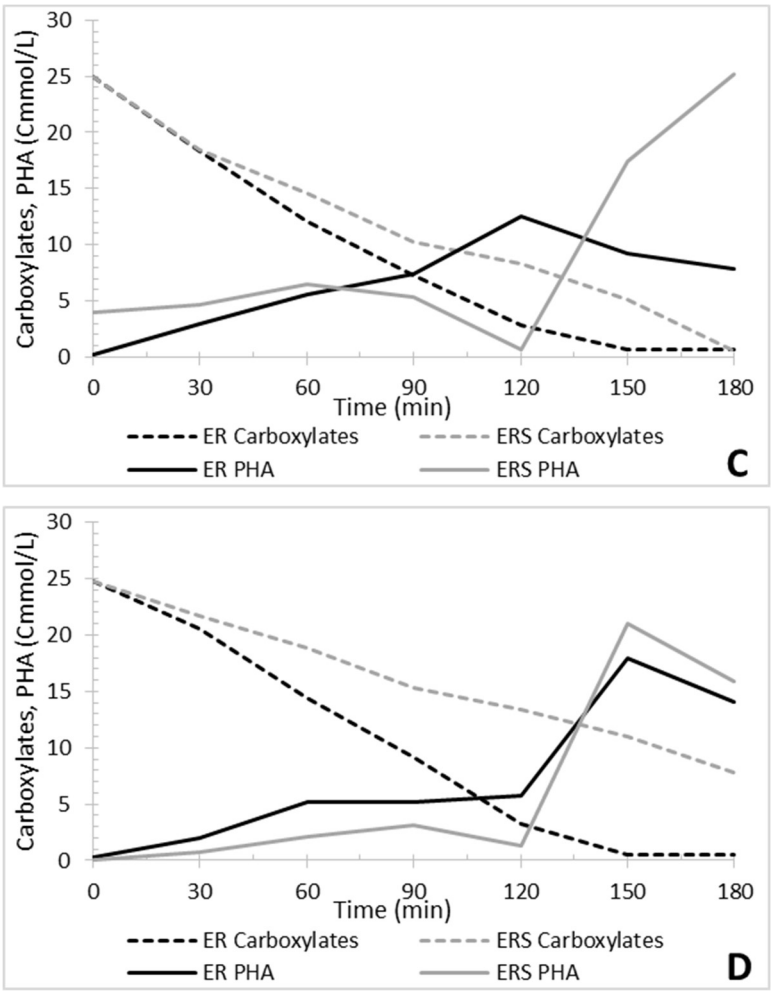

Figure 4.4: 3-Day SRT Enrichment Sampling Assessments
(C and D represent operational days 112 and 119, respectively)

Carboxylate uptake, as shown, appeared to occur more rapidly and became more consistent between assessments when the reactors were adjusted to the 3-day SRT. Additionally, the reactors produced higher amounts of PHA with the SRT change. The MMC of reactor ER again exhibited higher rates of carboxylate consumption vs. that in ERS, resulting in generally shorter comparative feast lengths; however, reduced carboxylate uptake rate did not necessarily result in lower PHA production. While reactor ER generally exhibited more rapid carboxylate depletion, the feast length of ERS was not consistently limited to 3 hours. To assess ERS decant, t=120 minute samples avoided collecting settled biomass; therefore, decreased solids and PHA values were expected.

Solids concentrations were evaluated at the start of each operational cycle to assess changes over time. Values for reactor WAS represent solids at the end of each operational cycle. Reactor solids at t=0 were determined based on WAS concentrations, assuming no solids added from the substrate. As expected, solids values were greater than for the 2-day SRT samples. Figure 4.5 represents the mixed liquor solids inventory for each 3-day SRT comprehensive sampling assessment.

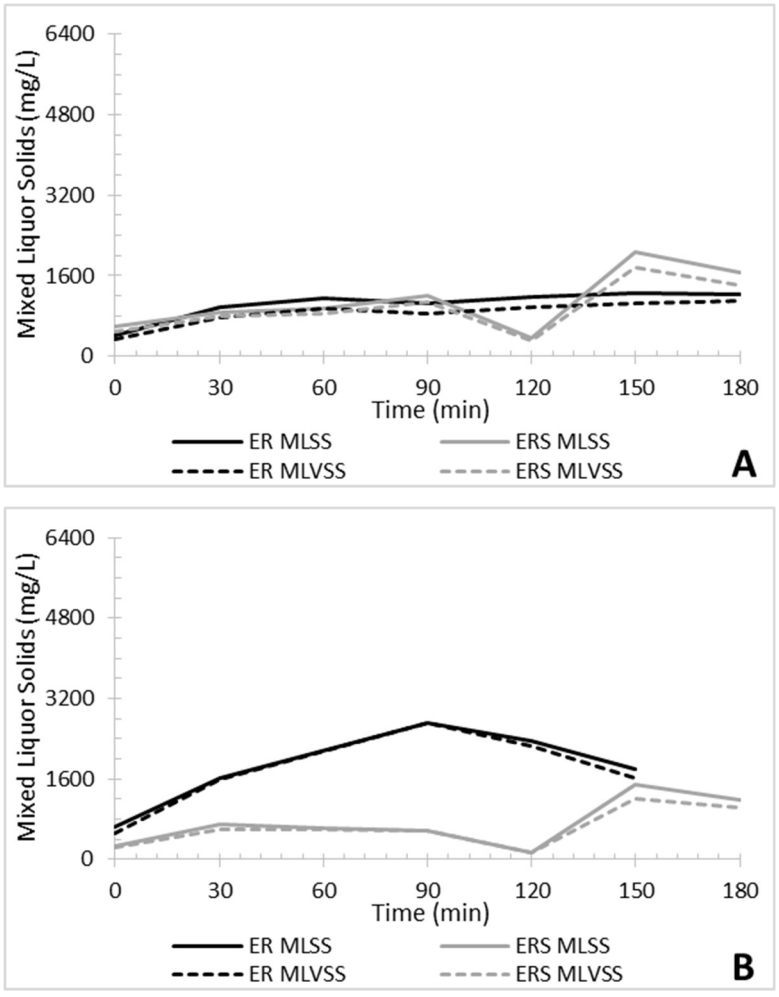

Figure 4.5: 3-Day SRT Enrichment Mixed Liquor Solids Inventory
(A and B represent operational days 98 and 106, respectively)

Figure continued on the next page.

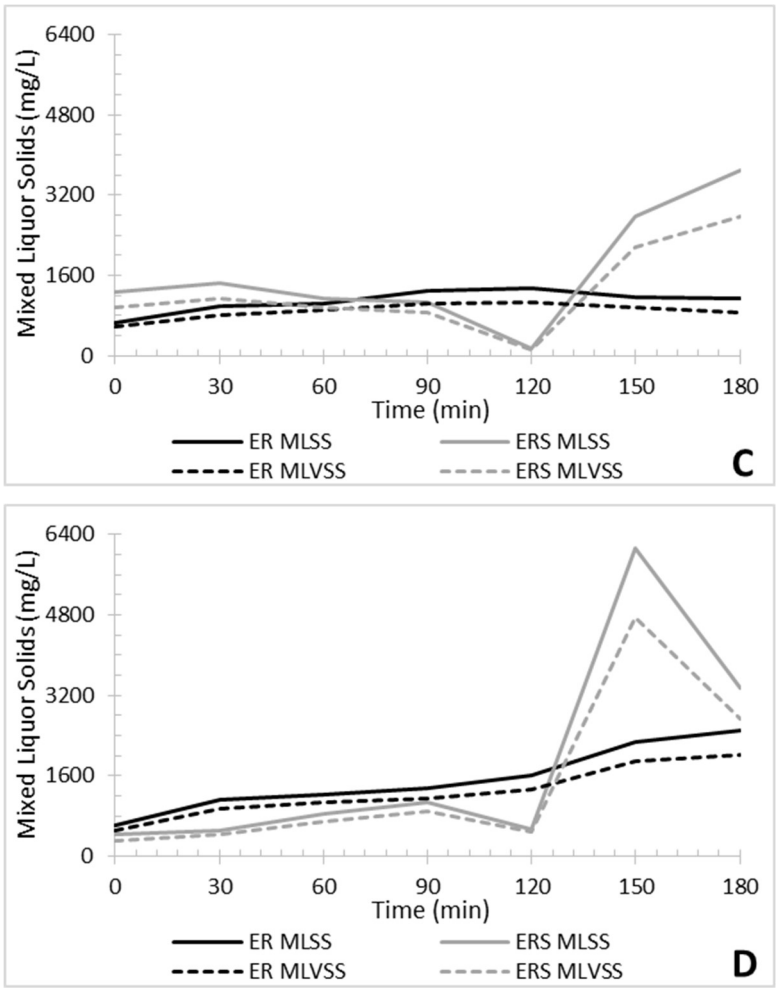

Figure 4.5: 3-Day SRT Enrichment Mixed Liquor Solids Inventory
(C and D represent operational days 112 and 119, respectively)

Average WAS values for ER and ERS were 875 and 1920 mg/L for TSS, and 725 and 1483 mg/L for VSS; average VSS ratios were 80.7% and 85.0 %. The higher concentration of solids in ERS WAS can be attributed to the settling/decant stage, as described under the 2-day SRT narrative. Average calculated values for ER and ERS at t=0 were 583 and 640 mg/L for MLSS and 483 and 494 for MLVSS, respectively. Differences in initial ER and ERS values are due to the larger volume decanted for each ERS each cycle. As shown, solids concentrations increased over the duration of the feast period for both ER and ERS. The initial solids increase was due to solids added with the DFL; average TSS values were 700 ± 257 mg/L (n= 8) for the DFL. Subsequent increases in solids concentrations were the result of growth of MMC biomass and accumulation of PHA. Average solids values at t=180 for ER and ERS were 1665 and 2470

mg/L for TSS, respectively, and 1398 and 1990 mg/L for VSS; average VSS ratios were 84.0% and 82.4%. With exception of ER WAS, solids values increased in both ER and ERS at each sampling point as anticipated with an increased SRT. While most of the solids concentrations increased compared to the 2-day SRT sampling, the mass of solids in the WAS decreased due to the decrease in reactor volume wasted for the 3-day testing while other solids mass values all increased. Table 4.4 summarizes solids data for each ER and ERS 3-day SRT sampling assessment.

Table 4.4: 3-Day SRT Enrichment Reactor Solids Inventory (Avg. ± SD)

Time	Parameter	ER	ERS
WAS (n=4)	***TSS (mg/L)***	875 ± 173	1920 ± 1309
	TSS (g)	0.87 ± 0.17	0.96 ± 0.65
	VSS (mg/L)	725 ± 149	1483 ± 969
	VSS (g)	0.72 ± 0.15	0.74 ± 0.48
t=0 (n=4)	***MLSS (mg/L)***	583 ± 115	640 ± 436
	TSS (g)	1.75 ± 0.35	1.92 ± 1.31
	MLVSS (mg/L)	483 ± 99	494 ± 323
	VSS (g)	1.45 ± 0.30	1.48 ± 0.97
t=180 (n=4)	***MLSS (mg/L)***	1665 ± 634	2470 ± 1236
	TSS (g)	5.00 ± 1.90	3.71 ± 1.85
	MLVSS (mg/L)	1398 ± 521	1990 ± 896
	VSS (g)	4.19 ± 1.56	2.99 ± 1.34

While average values for initial biomass were higher in ERS than ER, values for ERS dramatically fluctuated for each assessment as shown by considerably larger standard deviations. These deviations are thought to be caused by difficulties in accurately pipetting the large flocs in ERS; constantly changing values created operational challenges in the production stage. Although initial biomass concentrations in ERS were higher than ER, the feast length remained longer, resulting in a larger F-F ratio. Average F-F ratios were 0.12 for ER and 0.17 for ERS; these values are both less than the values obtained during the 2-day SRT operation, indicating increased microbial stresses (i.e., longer famine period) as the SRT increased.

Higher initial biomass concentrations also caused inconsistent but larger average values for specific carboxylate uptake rate (q_{VFA}) for ERS compared to ER. Average carboxylate uptake rates (r_{VFA}) were 0.14 and 0.12 Cmmol/min and average PHA synthesis rates (r_{PHA}) were 0.14 and 0.10 Cmmol/min for ER and ERS, respectively. Average carboxylate uptake rates remained similar to what was observed during the 2-day SRT sampling, while average PHA accumulation increased considerably for both reactors; this increase in carbon storage efficiency suggests a positive impact of an increased SRT on the ADF process. Average specific rates were 0.39 and 0.62 Cmmol/gVSS*min for q_{VFA}, and 0.27 and 0.24 Cmmol/gVSS*min for q_{PHA} for ER and ERS, respectively. Specific rates for VFA uptake and PHA synthesis increased for both reactors when switching from a 2 to 3-day SRT operation.

Average PHA yields for each reactor were 0.75 and 0.83 $Cmmol_{PHA}/Cmmol_{VFA}$ for ER and ERS, respectively. These values are consistent with values observed by others using a similar DFL feedstock (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$). Both yield values are nearly double the values reported for the 2-day SRT assessments, indicating increased efficiency for carboxylate-to-PHA conversion. Yield values were determined at the point of peak PHA (Cmmol/L) for each assessment. The MMC in ERS continued to outperform ER on a carboxylate-to-PHA conversion basis for 3-day SRT assessments.

Peak PHA values observed from the 3-day SRT assessments occurred on different operational days: 23.5 (day 106) and 25.2 (day 112) Cmmol/L (495.6 and 535.2 mg/L) for PR and PRS, respectively. Peak intracellular PHA content was similar for both ER and ERS with the observed peaks again occurring on different operational days: 20% (day 112) for ER, and 19% (day 98) for ERS, on a TSS (w/w) basis. In addition to similarities in maximum peak values,

the respective MMCs regularly produced similar quantities of PHA (Table 4.5). Average peak PHA values over the four assessments were 15.8 and 18.7 Cmmol/L (333.0 and 368.2 mg/L), and 17.5% and 14.3% TSS (w/w) for ER and ERS, respectively. Peak values of 28% (day 106) and 32% (day 119) (mol/mol) PHV content were observed for ER and ERS, respectively; average PHV content was 23.3% and 24.0% (mol/mol) for ER and ERS, respectively. Overall, PHA values were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Table 4.5 summarizes data for each 3-day SRT sampling assessment to assess ER and ERS performance.

Table 4.5: 3-Day SRT Enrichment Reactor Summary

Operational Day	**98**		**106**		**112**		**119**	
Parameter	**ER**	**ERS**	**ER**	**ERS**	**ER**	**ERS**	**ER**	**ERS**
MLSS (mg/L) at t=0	413	600	640	273	667	1263	613	423
MLVSS (mg/L) at t=0	340	490	527	230	567	950	500	307
MLSS (mg/L) at t=180	1230	1650	1790	1190	1130	3700	2510	3340
MLVSS (mg/L) at t=180	1110	1410	1610	1040	850	2780	2020	2730
F-F Ratio	0.13	0.12	0.12	0.18	0.12	0.15	0.13	0.22
q_{VFA} (Cmmol/gVSS*min)	0.54	0.56	0.34	1.01	0.32	0.28	0.36	0.62
q_{PHA} (Cmmol/gVSS*min)	0.06	0.12	0.26	0.07	0.10	0.14	0.12	0.09
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.38	0.75	1.31	0.60	0.57	1.04	0.74	0.93
Peak PHA (Cmmol/L)	9.0	18.4	23.5	10.1	12.5	25.2	18.0	21.0
Peak PHA (mg/L)	192.1	390.3	495.6	212.9	265.2	535.2	379.1	334.5
PHA, %TSS (w/w)	15.4	18.9	18.3	14.4	19.6	14.5	16.6	10.0
%PHV (mol/mol)	17.1	19.9	18.5	22.6	19.5	20.3	28.0	31.8

4.2.2. Production Phase Evaluations

Similar to the 2-day SRT evaluations, PHA production assessments using WAS from the respective enrichment reactors were performed concurrently with enrichment reactor assessments on operational days 98, 106, 112, and 119. Results are presented in Figure 4.6.

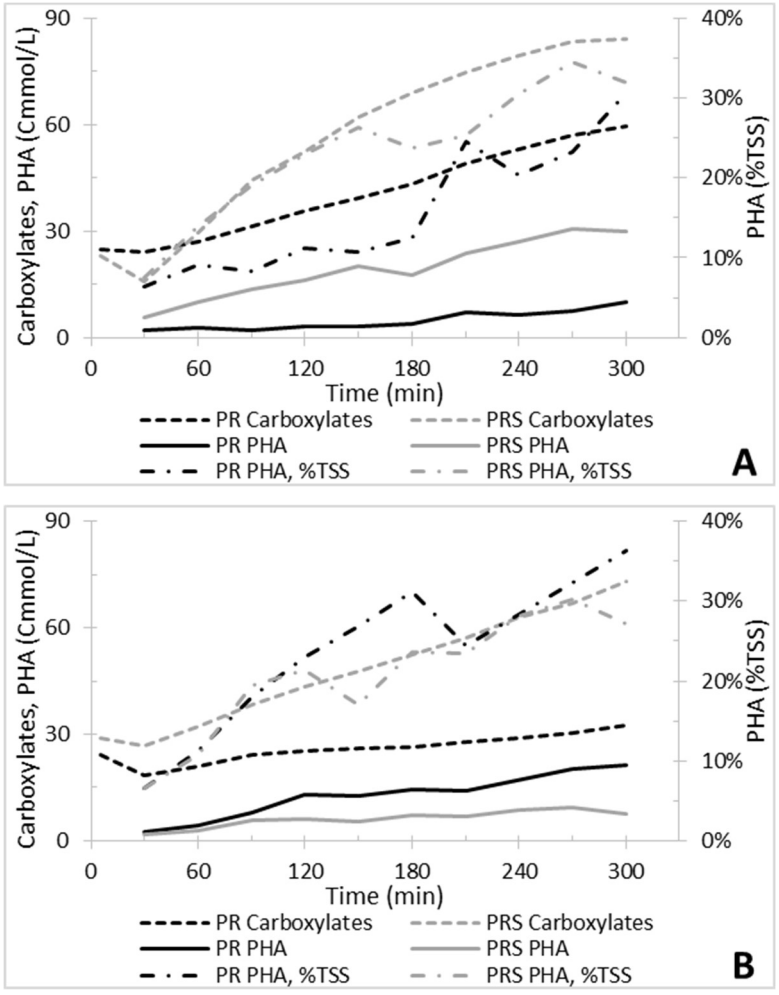

Figure 4.6: 3-Day SRT Production Sampling Assessments
(A and B represent operational days 98 and 106, respectively)

Figure continued on the next page.

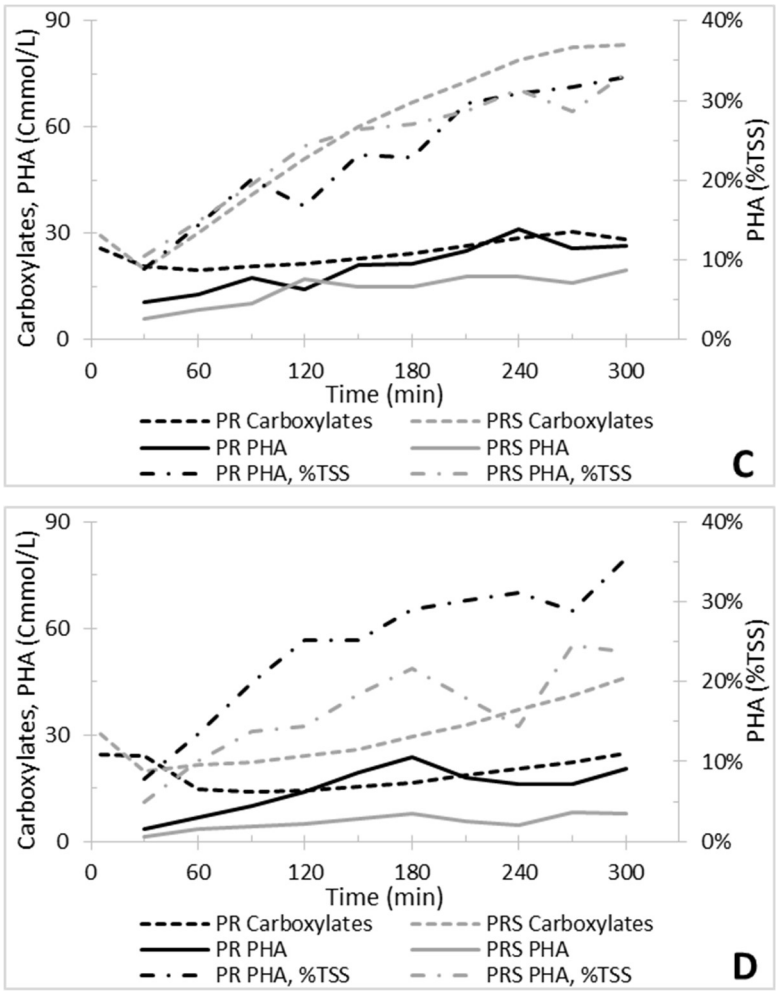

Figure 4.6: 3-Day SRT Production Sampling Assessments
(C and D represent operational days 112 and 119, respectively)

Utilizing the empirical operational information derived from the 2-day SRT assessments, the continuous feed was delayed 30 minutes after the initial pulse of carboxylates was added to the reactors; this was in an attempt to counteract lower carboxylate uptake rates observed during production sampling in all of the 3-day SRT production assessments. While carboxylate concentrations in PRS increased beyond the theoretical 60 Cmmol/L inhibition threshold, it did not seem to drastically inhibit PHA production. Higher levels of carboxylates occurred in PRS due to the substrate feed rate not adjusting proportionally to the decreasing reactor volume.

Solids concentrations were evaluated at the start of each cycle to determine the substrate feed rate based on the amount of biomass present. Reactor TSS values were equal to the concentrations obtained from the volume wasted from the enrichment phase. As expected for

an increased SRT, average values for PR and PRS solids increased from values reported for the 2-day SRT testing at 1045 and 2040 mg/L for MLSS, and 885 and 1620 mg/L for MLVSS, respectively. Regardless of largely differing initial concentrations, the mass of VSS added to each production reactor remained similar due to different initial reactor volumes: 1.0 L for PR and 0.5 L for PRS. Average values for specific carboxylate uptake rates (q_{VFA}) remained similar between the reactors and SRT assessments at 0.19 and 0.17 Cmmol/gVSS*min for PR and PRS, respectively. Conversely, specific PHA synthesis rates (q_{PHA}) were not similar between the reactors or SRT assessments - 0.09 and 0.04 Cmmol/gVSS*min for PR and PRS, respectively, indicating more efficient accumulation of PHA at a lower SRT. Specific rate values for VFA uptake and PHA synthesis were averaged to assess the continuously-fed production systems.

Average observed yields at the end of each assessment for PR and PRS were 0.61 and 0.19 $Cmmol_{PHA}/Cmmol_{VFA}$, respectively. Yield values for PR remained similar to the 2-day SRT values, whereas PRS values dramatically decreased. While yield values for PR are within the range reported for other continuously-fed production systems using fermented dairy manure (0.24 – 0.76 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]), PRS yield values are below the reported range. Significant differences in reactor volume resulted in lower calculated quantities of PHA (Cmmol) produced in PRS, creating the large discrepancy between PHA yield values. The MMC of PRS was able to constantly consume similar quantities of carboxylates to PR, indicating deficiencies in efficiently utilizing the substrate for PHA production; PRS consistently underperformed PR on a carboxylate-to-PHA conversion basis.

For the 3-day SRT assessments, the maximum observed peak PHA production values observed were 31.2 (day 112) and 30.7 (day 98) Cmmol/L (660.0 and 653.7 mg/L) for PR and PRS, respectively; maximum intracellular PHA content achieved was also similar for both reactors, with PR at 36% (day 106) and PRS at 35% (day 98) on a TSS (w/w) basis. Average peak PHA values were notably lower than observed single day maximums at 21.6 and 16.9 Cmmol/L (455.1 and 360.0 mg/L), with average PHA on a %TSS (w/w) of 33.8% and 30.7% for PR and PRS, respectively. Average peak PHA values on a %TSS basis were similar to the maximum peak values observed, whereas average values on a Cmmol/L basis were not similar to the maximum peak values. Differences in these averages and the respective maximums was due to a larger fluctuation in initial solids during these assessments than observed for the other

operational configurations; the reactors regularly produced similar values for peak PHA on a %TSS basis, while values for peak PHA on a Cmmol/L varied greatly between the assessments. Production of PHA on a %TSS basis was approximately double the values observed within the parent enrichment reactors for each sampling assessment. Regarding polymer quality, average values for peak %PHV present were 28.7 and 16.7% (mol/mol) for PR and PRS, respectively; whereas ER consistently had a higher %PHV content than ERS. Collectively, production results were generally within the typical range reported for other ADF investigations [16, 23].

Over all four assessment days, average values for maximum PHA based on inocula were 45.4% and 20.5% (w/w) for PR and PRS, respectively. Low values for PRS were due to diminishing reactor volume over the testing cycle, as described under the 2-day SRT narrative. Instances where maximum PHA based on inocula values were less than peak PHA %TSS were attributed to decreasing reactor volumes and variable amounts of initial solids. The large discrepancies between values indicate that more of the solids added throughout the production cycle were attributed to PHA accumulation in PR than PRS. Table 4.6 summarizes the parameters evaluated to assess ER and ERS performance during the production phase.

Table 4.6: 3-Day SRT Production Reactor Evaluation

Operational Day	**98**		**106**		**112**		**119**	
Parameter	**PR**	**PRS**	**PR**	**PRS**	**PR**	**PRS**	**PR**	**PRS**
Initial MLSS (mg/L)	1300	2280	960	820	1000	3790	920	1270
Initial MLVSS (mg/L)	1150	2020	790	690	850	2850	750	920
Average q_{VFA} (Cmmol/gVSS*min)	0.04	0.20	0.23	0.20	0.22	0.12	0.25	0.15
Average q_{PHA} (Cmmol/gVSS*min)	0.03	0.09	0.10	0.02	0.12	0.03	0.10	0.02
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.64	0.31	0.43	0.12	0.79	0.24	0.58	0.09
Peak PHA (Cmmol/L)	10.0	30.7	21.3	9.3	31.2	19.5	23.8	8.7
Peak PHA (mg/L)	211.2	653.7	449.5	199.8	660.0	414.5	499.7	172.2
Max. PHA, %TSS (w/w)	30.6	34.6	36.3	30.3	32.9	33.4	35.4	24.6
PHA based on Inocula, % (w/w)	17.2	49.9	50.5	11.6	61.8	14.1	52.1	14.1
%PHV (mol/mol)	22.3	16.0	27.4	7.5	25.1	17.2	40.0	26.1

4.3. 4-Day SRT

4.3.1. Enrichment Reactor Evaluations

Once 3-day SRT investigations were concluded, the enrichment reactors were adjusted to a 4-day SRT. Following 14 days to allow for process stabilization, comprehensive sampling was performed on operational days 134, 140, and 148. Figure 4.7 displays carboxylate uptake and PHA accumulation for each comprehensive 4-day SRT sampling assessment.

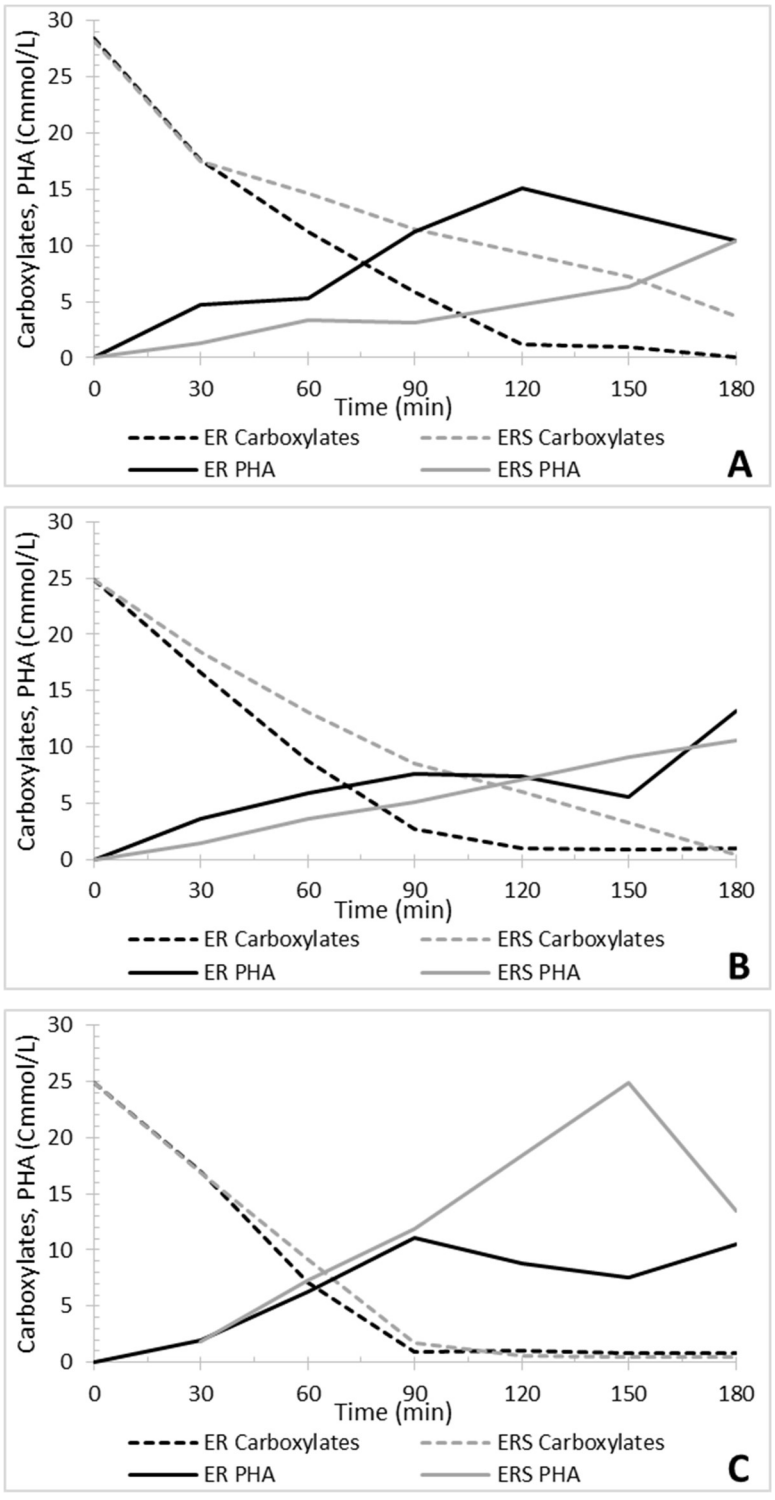

Figure 4.7: 4-Day SRT Enrichment Sampling Assessments

(A, B, and C represent operational days 134, 140, and 148, respectively)

As shown, carboxylate uptake further improved with an increased SRT; however, PHA production decreased slightly with the adjustment from the 3-day SRT. With a 4-day SRT, the reactors experienced similar rates of carboxylate consumption, which resulted in similar feast lengths and PHA production. To assess ERS decant, t=120 minute samples avoided collecting settled biomass; therefore, decreased solids and PHA values were anticipated.

Solids concentrations were evaluated at the start of each operational cycle to assess changes over time. Values for reactor WAS represent solids at the end of each operational cycle. Reactor solids at t=0 were determined based on WAS concentrations, assuming no solids added from the substrate. As expected with an increased SRT, solids values with a 4-day SRT increased over the 3-day SRT sampling. Figure 4.8 represents the mixed liquor solids inventory during the 4-day SRT comprehensive sampling assessments.

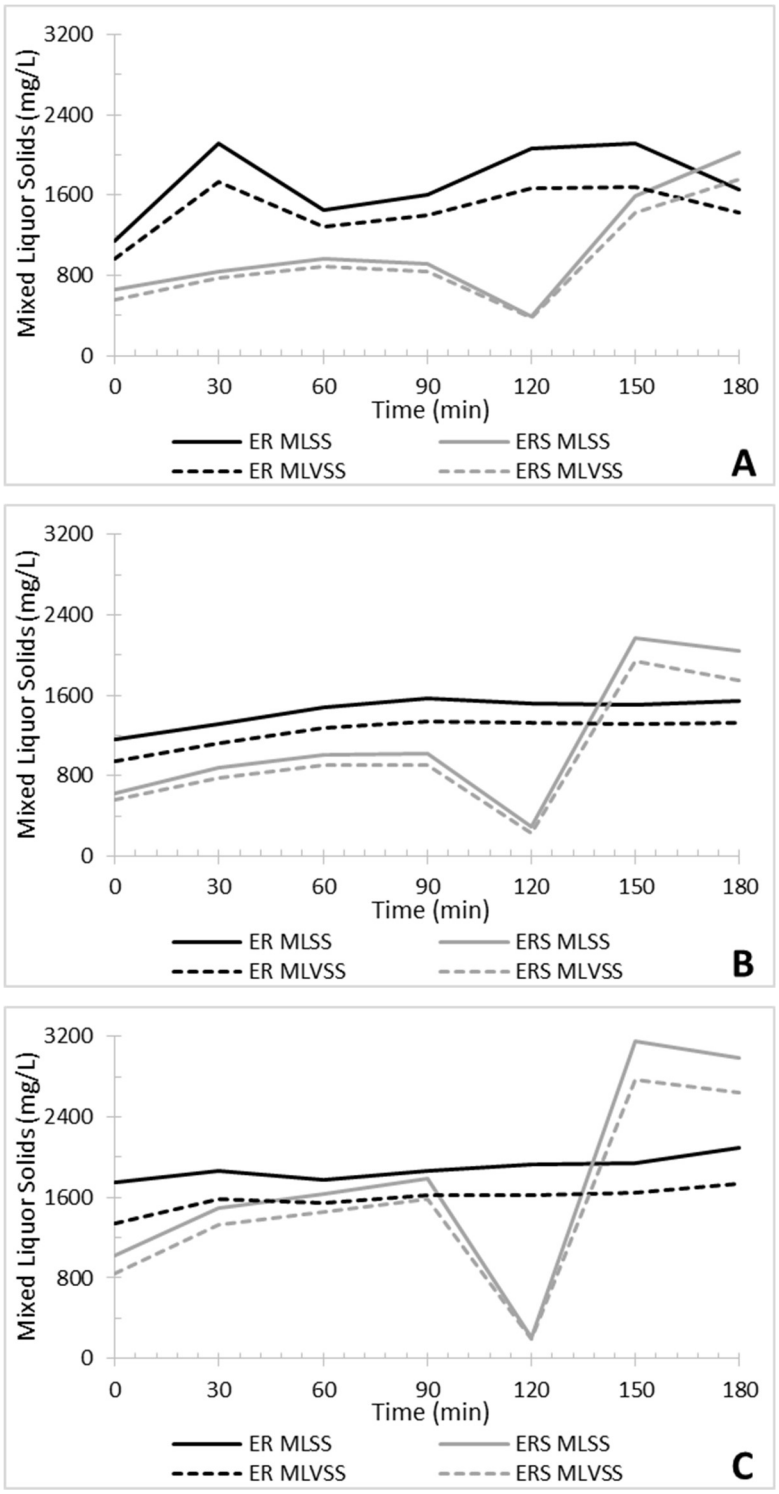

Figure 4.8: 4-Day SRT Enrichment Mixed Liquor Solids Inventory
(A, B, and C represent operational days 134, 140, and 148, respectively)

Average WAS values for ER and ERS were 1797 and 2050 mg/L for TSS and 1440 and 1733 mg/L for VSS; average VSS ratios were 80.7% and 84.1 %. Average calculated values for ER and ERS at t=0 were 1347 and 769 mg/L for MLSS, and 1080 and 650 for MLVSS, respectively, with identical average VSS ratios as the WAS. The higher concentration of solids in ERS WAS can be attributed to the settling/decant stage, as described previously. As shown, solids concentrations increased over the duration of the feast period for both ER and ERS. The initial solids increase was due to solids added with the DFL; average TSS values were 700 ± 257 mg/L (n= 8) for the DFL. Subsequent increases in solids concentrations were the result of growth of the MMC biomass and PHA accumulation. Average solids values at t=180 for ER and ERS were 1837 and 2350 mg/L for TSS and 1527 and 2050 mg/L for VSS; average VSS ratios were 83.2% and 87.5%. Larger values in ERS vs. ER after t=120 were attributed to decanting the low solids supernatant after settling, thus increasing concentrations in the remaining volume. With exception to ERS TSS at t=180, solids concentrations increased in both ER and ERS at each sampling point as was expected with a longer SRT. Solids mass in the WAS increased for ER due to the much larger concentration compared to the 3-day SRT sampling, but decreased in ERS due to the smaller volume for a 4-day SRT. Mass values increased for both reactors at t=0 and for ER for t=180, but decreased for ERS TSS at t=180 compared to 3-day SRT assessments. Table 4.7 summarizes solids data for each ER and ERS 4-day SRT sampling assessment.

Table 4.7: 4-Day SRT Enrichment Reactor Solids Inventory (Avg. ± SD)

Time	Parameter	ER	ERS
WAS (n=3)	***TSS (mg/L)***	1797 ± 462	2050 ± 582
	TSS (g)	1.35 ± 0.35	0.77 ± 0.22
	VSS (mg/L)	1440 ± 303	1733 ± 439
	VSS (g)	1.08 ± 0.23	0.65 ± 0.16
t=0 (n=3)	***MLSS (mg/L)***	1347 ± 346	769 ± 218
	TSS (g)	4.04 ± 1.04	2.31 ± 0.65
	MLVSS (mg/L)	1080 ± 228	650 ± 165
	VSS (g)	3.24 ± 0.68	1.95 ± 0.49
t=180 (n=3)	***MLSS (mg/L)***	1837 ± 227	2350 ± 554
	TSS (g)	5.51 ± 0.68	3.52 ± 0.83
	MLVSS (mg/L)	1527 ± 185	2050 ± 511
	VSS (g)	4.58 ± 0.56	3.08 ± 0.77

Lower initial biomass concentrations in ERS resulted in a longer average feast length compared to ER and an equally larger F-F ratio; however, on operational day 148, the feast length was identical between the reactors. Average F-F ratios were 0.09 for ER and 0.12 for ERS; these values were less than the values obtained during the 3-day SRT operation, further indicating increased microbial stresses upon lengthening the SRT.

Lower initial biomass concentrations also resulted in a larger specific carboxylate uptake rate (q_{VFA}) for ERS than ER. Average rates were 0.15 and 0.14 Cmmol/min for carboxylate uptake

(r_{VFA}) and 0.11 and 0.09 Cmmol/min for PHA synthesis (r_{PHA}) for ER and ERS, respectively. Average carboxylate uptake rates remained similar to those observed during 2-day and 3-day SRT samplings; average PHA accumulation remained similar to 3-day SRT values for both reactors. Average specific rates were 0.14 and 0.22 Cmmol/gVSS*min for q_{VFA}, and 0.10 and 0.14 Cmmol/gVSS*min for q_{PHA} for ER and ERS, respectively. Specific rates decreased for both reactors compared to values obtained for both 2-day and 3-day SRT sampling.

Average observed PHA yields for each reactor were 0.52 and 0.63 $Cmmol_{PHA}/Cmmol_{VFA}$ for ER and ERS, respectively. These values are consistent with the values observed by others using a similar DFL feedstock (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$); however, both yield values are lower than the values reported for the 3-day SRT assessments, but higher than values obtained with the 2-day SRT sampling; These results suggest that the 4-day SRT results in a reduced efficiency in converting carboxylate consumption to PHA synthesis when compared to reactors with a 3-day SRT. Yield values were determined at the point of peak PHA (Cmmol/L) for each assessment. The MMC in ERS continued to outperform ER on a carboxylate-to-PHA conversion basis with the 4-day SRT assessments.

Peak PHA values observed with the 4-day SRT assessments occurred on different operational days: 15.1 (day 134) and 24.9 (day 148) Cmmol/L (317.8 and 529.6 mg/L) for PR and PRS, respectively. Peak intracellular PHA content was similar for both ER and ERS, although again the observed peaks occurred on different operational days: 18.0% (day 140) for ER, and 16.8% (day 148) for ERS on a TSS (w/w) basis. In addition to similarities in maximum peak values, the respective MMCs regularly produced similar quantities of PHA (Table 4.8). Average peak PHA values over the four assessments were 13.1 and 15.3 Cmmol/L (276.7 and 982.5 mg/L), and 15.3% and 12.9% TSS (w/w) for ER and ERS, respectively. Peak values of 36% and 34.5% PHV (mol/mol) content were observed on operational day 134 for ER and ERS, respectively; average PHV content was 28.6% and 28.2% (mol/mol) for ER and ERS, respectively. Overall, PHA values were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Table 4.8 summarizes data from the 4-day SRT sampling assessment to compare PHA performance between ER and ERS.

Table 4.8: 4-Day SRT Enrichment Reactor Summary

Operational Day	**134**		**140**		**148**	
Parameter	**ER**	**ERS**	**ER**	**ERS**	**ER**	**ERS**
MLSS (mg/L) at t=0	1140	660	1155	626	1747	1020
MLVSS (mg/L) at t=0	960	555	937	555	1342	840
MLSS (mg/L) at t=180	1650	2020	1770	1640	2090	2217
MLVSS (mg/L) at t=180	1430	1760	1410	1430	1740	2640
F-F Ratio	0.10	0.16	0.09	0.13	0.07	0.07
q_{VFA} (Cmmol/gVSS*min)	0.16	0.25	0.14	0.24	0.12	0.16
q_{PHA} (Cmmol/gVSS*min)	0.13	0.10	0.08	0.11	0.09	0.20
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.36	0.43	0.55	0.44	0.44	0.55
Peak PHA (Cmmol/L)	15.1	10.5	13.2	10.6	11.1	24.9
Peak PHA (mg/L)	317.8	219.5	277.1	233.4	235.2	529.6
PHA, %TSS (w/w)	15.4	10.9	18.0	11.0	12.6	16.8
%PHV (mol/mol)	36.0	34.5	33.0	33.1	16.7	17.1

4.3.2. Production Phase Evaluations

PHA production assessments were performed concurrently with enrichment reactor assessments on operational days 134, 140, and 148. Results are presented in Figure 4.9.

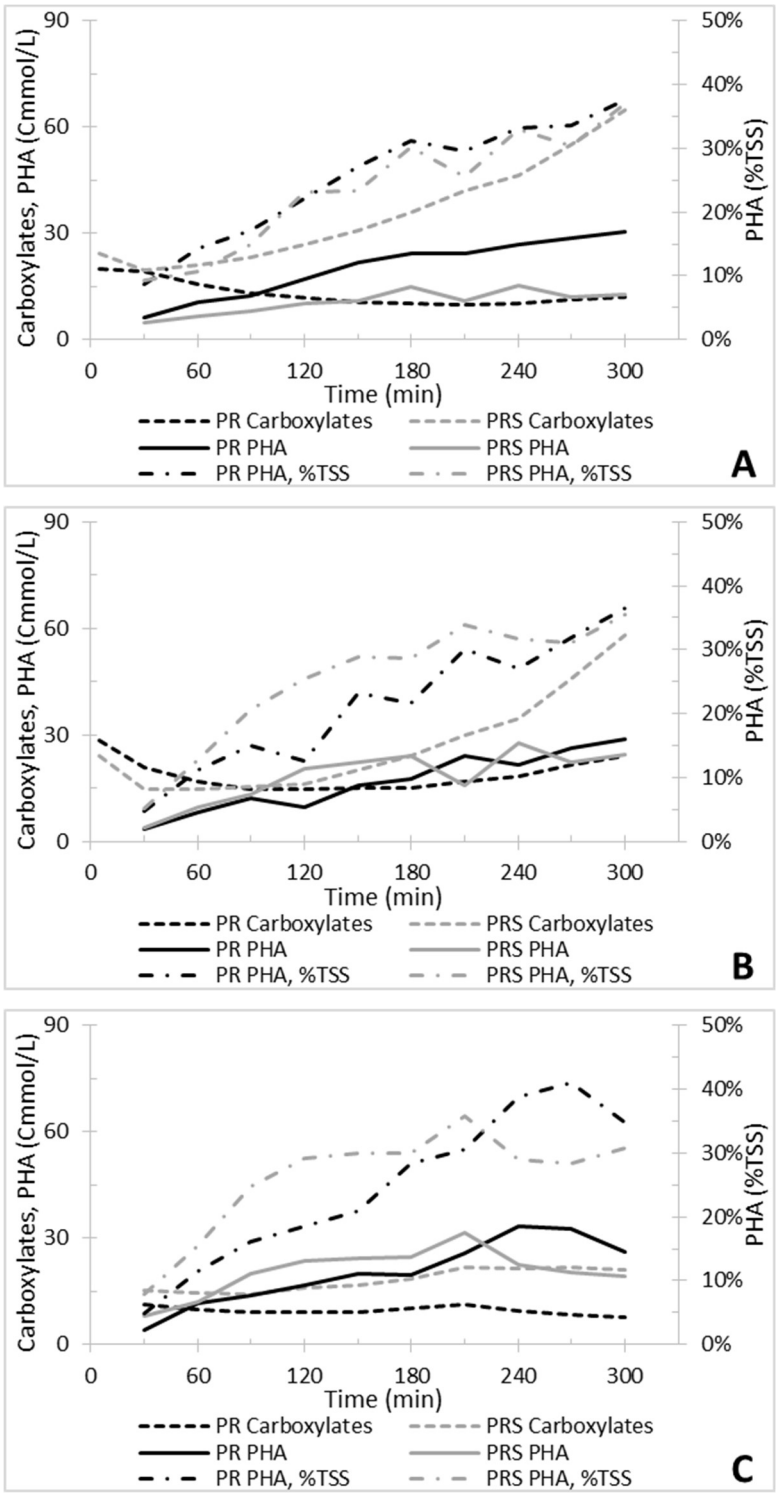

Figure 4.9: 4-Day SRT Production Sampling Assessments

(A, B, and C represent operational days 134, 140, and 148, respectively)

To counteract the lower carboxylate uptake rates observed during production sampling in all 4-day SRT production assessments, and consistent with operational modifications adopted for the other SRT evaluations, commencement of continuous feed was delayed 30 minutes after the initial pulse of carboxylates was added to the reactors.

Solids concentrations were evaluated at the start of each cycle to determine the substrate feed rate based on the amount of biomass present. Reactor TSS values were equal to the concentrations obtained from the volume wasted from the enrichment phase. As expected with a larger SRT, average values for PR and PRS solids increased from values reported for the 2-day and 3-day SRT testing: 1045 and 2040 mg/L for MLSS, and 885 and 1620 mg/L for MLVSS, respectively. Average values for PR and PRS were 1797 and 2050 mg/L for MLSS and 1440 and 1733 mg/L for MLVSS, respectively; however, the mass of VSS in each production reactor was not similar due to differing initial reactor volumes (0.75 L for PR and 0.375 L for PRS). Average observed values for carboxylate uptake rates (q_{VFA}) in PR and PRS remained similar between SRT assessments but not between reactors, at 0.20 and 0.14 Cmmol/gVSS*min respectively. Likewise, specific PHA synthesis rates (q_{PHA}) were similar to 2-day SRT values, but not similar between reactors at 0.12 and 0.07 Cmmol/gVSS*min for PR and PRS, respectively. Specific rates were averaged to assess the continuously fed production systems.

Average observed yields at the end of each assessment for PR and PRS were 0.37 and 0.12 $Cmmol_{PHA}/Cmmol_{VFA}$, respectively. While yield values for PR are within the range reported for other continuously-fed production evaluations using fermented dairy manure (0.24 – 0.76 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]), PRS yield values were lower than the reported range. Differences in yield values are amplified due to differing reactor volumes, as described previously; however, both yield values are lower than the values reported for the 2-day and 3-day SRT assessments, indicating a loss in production reactor efficiency in converting carboxylate consumption to PHA synthesis with a 4-day enrichment stage SRT. The MMC in PRS continued to underperform PR on a carboxylate-to-PHA conversion basis for the 4-day SRT assessments.

For 4-day SRT assessments, the maximum observed peak PHA production values were 33.4 and 31.6 Cmmol/L (707.7 and 670.6 mg/L) on operational day 148 for PR and PRS,

respectively; maximum intracellular PHA content achieved for PR was 41.0% (day 148) and for PRS 37.1% (day 134) on a TSS (w/w) basis. In addition to larger maximum peak PHA values observed in PR vs. PRS, the MMC of PR regularly outperformed PRS (Table 4.9). Average peak PHA values were 30.9 and 24.8 Cmmol/L (649.9 and 521.0 mg/L), with average PHA on a %TSS (w/w) of 38.4% and 36.2% for PR and PRS, respectively. In contrast to results obtained from the 3-day SRT assessments, average peak PHA values for 4-day SRT testing remained similar to maximum peak values observed due to smaller fluctuations in monitored parameters between sampling assessments. Regarding polymer quality, average values for peak %PHV present were 32.4% and 34.6% (mol/mol) for PR and PRS, respectively. Collectively, production results were consistent within the typical range reported for other ADF investigations [16, 23].

Over all three assessment days, average values for maximum PHA based on inocula were 37.4% and 16.1% (w/w) for PR and PRS, respectively. Low values for PRS were due to diminishing reactor volume over the testing cycle, creating a low volume at the end of each assessment that significantly reduced calculated values. Occurrences of maximum PHA based on the inocula being less than peak PHA %TSS were caused by decreasing reactor volumes and solids, as stated previously. The large discrepancy in these values indicates that more of the solids added throughout the production cycle may be attributed to PHA accumulation in PR compared to PRS. Table 4.9 summarizes the parameters evaluated to assess the 4-day SRT ER and ERS performance in the production phase.

Table 4.9: 4-Day SRT Production Reactor Evaluation

Operational Day	**134**		**140**		**148**	
Parameter	**PR**	**PRS**	**PR**	**PRS**	**PR**	**PRS**
Initial MLSS (mg/L)	1520	1760	1540	1670	2330	2720
Initial MLVSS (mg/L)	1280	1480	1250	1480	1790	2240
Average q_{VFA} (Cmmol/gVSS*min)	0.18	0.13	0.21	0.16	0.21	0.13
Average q_{PHA} (Cmmol/gVSS*min)	0.12	0.04	0.12	0.09	0.12	0.08
$Y_{PHA/VFA}$ (Cmmol/Cmmol)	0.43	0.09	0.33	0.11	0.34	0.18
Peak PHA (Cmmol/L)	30.5	15.1	28.7	27.6	33.4	31.6
Peak PHA (mg/L)	640.9	315.8	601.0	576.5	707.7	670.6
Max. PHA, %TSS (w/w)	37.7	37.1	36.4	35.6	41.0	35.9
PHA based on Inocula, % (w/w)	33.5	10.1	34.3	18.3	44.3	19.8
%PHV (mol/mol)	35.7	38.4	40.4	39.0	21.2	26.4

4.4. Integrating Results and Assessing Thesis Research Questions

Data collected from the SRT evaluations were collectively compiled and evaluated with regard to the posed Research Questions.

4.4.1. Effect of Settling

Statistical analysis was performed on data collected from all comprehensive sampling assessments from both the enrichment and production reactors to determine the potential effect of settling on PHA accumulation (i.e., RQ 1).

4.4.1.1. Statistical Analysis of Enrichment Reactor Settling

Effects of settling on enrichment reactor performance parameters were compared using two sample t-test evaluations to determine if the differences in mean reactor output were statistically significant. Statistical calculations incorporated data from all SRT assessments for ER and ERS. Parameters used to evaluate enrichment reactor PHA production performance included F-F ratio, PHA yield, and peak PHA production on both a Cmmol/L and %TSS (w/w) basis. Before conducting evaluations, the data were visualized using boxplots to illustrate the mean value and distribution of observed values for each parameter by reactor. Figure 4.10 illustrates the plots generated for each PHA enrichment reactor assessment parameter.

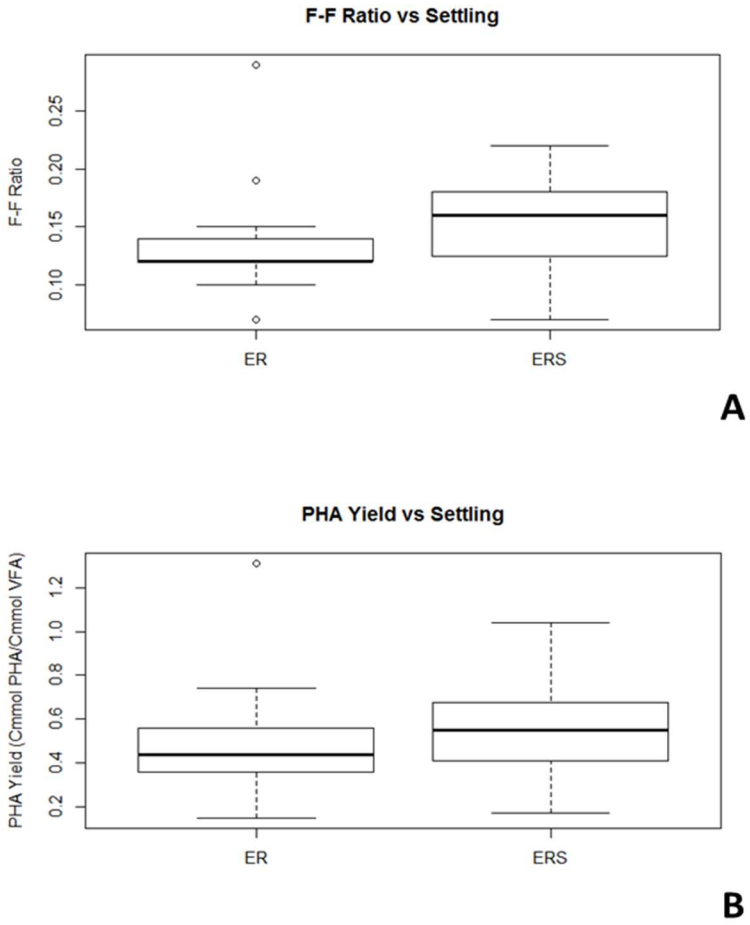

Figure 4.10: Enrichment Reactor PHA Production Parameters vs. Settling
(A and B represent the F-F Ratio and PHA Yield response parameters, respectively)

Figure continued on the next page.

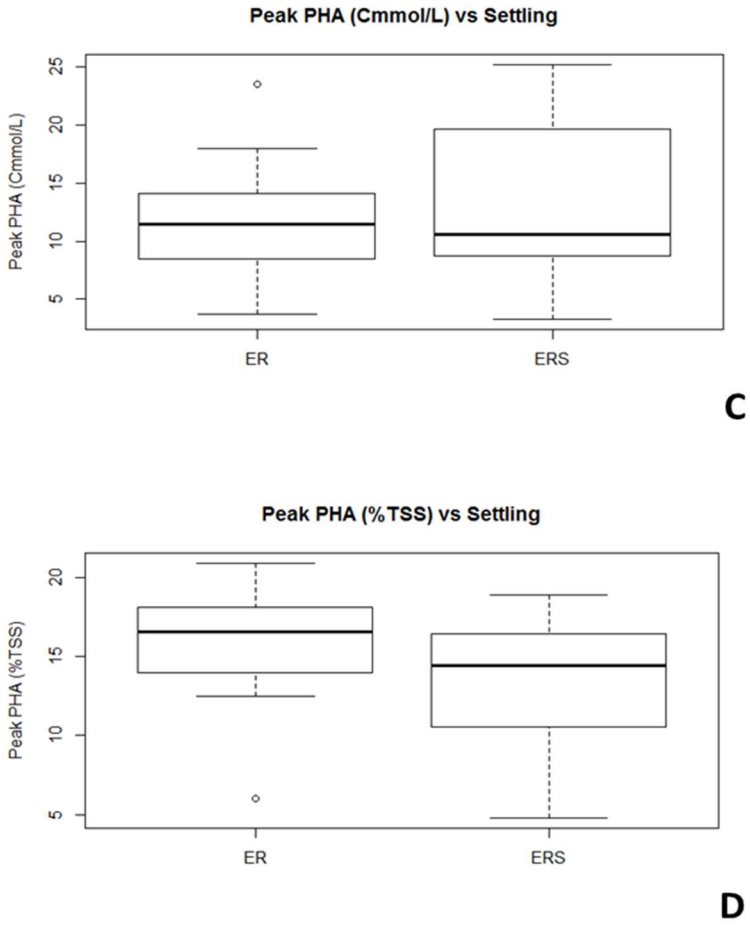

Figure 4.10: Enrichment Reactor PHA Production Parameters vs. Settling
(C and D represent the Peak PHA Cmmol/L and Peak PHA %TSS response parameters, respectively)

Feast length was consistently shorter for reactor ER than ERS; operational days 98 and 148 (3- and 4-day SRTs, respectively) were the only instances ERS had a feast length shorter than or equal to ER. A shorter feast length resulted in a smaller F-F ratio. Average F-F ratio values were 0.14 and 0.15 for ER and ERS, respectively. These values are both less than the recommended maximum ratio of 0.20 [86], indicating an adequate famine length to induce requisite stress to induce the feast PHA accumulation response for both the settling and non-settling operational regime. Statistical tests showed that there is not a statistically significant difference between the average ER and ERS F-F ratios. These results suggest that implementation of a settling phase does not induce more metabolic stress on a MMC.

Observed PHA yield was consistently higher in reactor ERS than reactor ER, although observed yield was higher in ER on operational days 79, 106, and 140 (2-, 3-, and 4-day SRTs, respectively). Average observed PHA yield values were 0.51 and 0.56 $Cmmol_{PHA}/Cmmol_{VFA}$ for ER and ERS, respectively. These values are within the range of values observed by others using a similar DFL feedstock (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]). Statistical testing showed that there was no statistical significance between the average values for observed PHA yield between ER and ERS, indicating that implementation of a settling phase does not affect the yield of PHA in the enrichment reactor.

Peak PHA values were similar between ER and ERS throughout all SRT assessments. Average peak PHA values were 12.0 and 13.4 Cmmol/L, and 15.7 and 13.3 %TSS (w/w) for ER and ERS, respectively. Overall, enrichment reactor PHA results were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Statistical testing showed no statistical significance between the average values for peak PHA produced between ER and ERS on both a Cmmol/L and %TSS basis, signifying that implementation of a settling phase does not impact peak PHA production in the enrichment reactor.

The overall enrichment stage results and statistical analyses do not support the original hypothesis and demonstrate that implementation of a sedimentation phase at the end of the feast period does not enhance enrichment stage operational performance.

4.4.1.2. Statistical Analysis of Production Reactor Settling

Effects of settling on production reactor performance parameters were compared using two sample t-test evaluations to determine statistical significance in mean reactor output differences. Statistical calculations incorporated data from all SRT assessments. Parameters used to evaluate production reactor PHA production performance were PHA yield and peak PHA production on both a Cmmol/L and %TSS basis. Figure 4.11 illustrates the boxplots generated for each PHA production reactor assessment parameter.

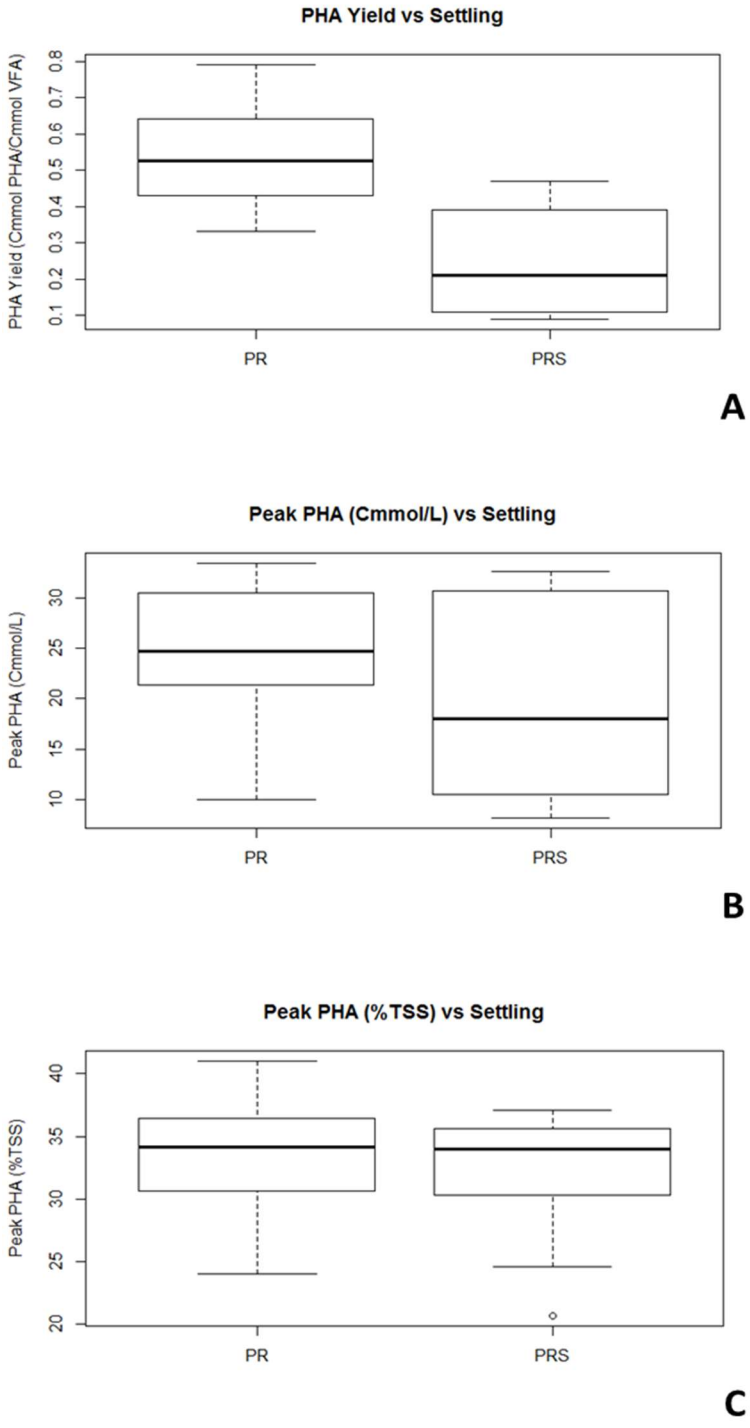

Figure 4.11: Production Reactor PHA Production Parameters vs. Settling
(A, B, and C represent response parameters PHA Yield, Peak PHA Cmmol/L and Peak PHA %TSS, respectively)

Observed PHA yield was greater in PR vs. PRS for each sampling assessment. Average PHA yield values were 0.53 and 0.23 $Cmmol_{PHA}/Cmmol_{VFA}$ for ER and ERS, respectively. These values are within or near the range of values observed by others using a similar DFL feedstock (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]). Consistently higher PHA yield values in PR demonstrates a more efficient carboxylate-PHA conversion and potentially indicates a larger population of PHA-accumulating microbes. Statistical tests determined that there is a statistically significant difference in mean PHA yields between PR and PRS; demonstrating that implementation of a settling phase negatively impacts PHA production.

Peak PHA values were similar between PR and PRS throughout all SRT sampling assessments. Average peak PHA values were 24.3 and 20.2 Cmmol/L, and 33.6 and 31.7 %TSS (w/w) for PR and PRS, respectively. Production of PHA on a %TSS basis was approximately double the values observed in the parent enrichment reactors for each sampling assessment. Overall, production reactor PHA results were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Statistical testing showed no statistical significance between the average values for peak PHA produced between PR and PRS on both a Cmmol/L and %TSS basis, suggesting that implementation of a settling phase does not impact overall peak PHA synthesis in the production reactor.

Total results and statistical analysis indicate that implementation of a sedimentation phase at the end of the feast period of the enrichment stage does not increase overall PHA production performance as hypothesized.

4.4.1.3. Nutrient Removal

In addition to PHA production, effluent nutrient concentrations are of particular interest, as a concurrent goal of the dairy manure-based ADF operations would be to achieve wastewater treatment. To assess full-scale impacts of implementing the settling phase, potential losses in nutrient removal associated with the removal of the supernatant after 2 hours from ERS were evaluated. Nutrient removal was determined on the basis of chemical oxygen demand (COD), ammonia (NH_3), nitrate (NO_3), and phosphorus (P). Nutrient concentrations were determined as averages recorded for each reactor at t=0, 60, 120, and 180 over all SRT assessments. End of cycle conditions were assumed to be equal to t=0 values as concentrations at t=0 were

determined using WAS and reflect the state of each reactor before substrate was added. Figure 4.12 illustrates the differences between ER and ERS average nutrient concentration conditions.

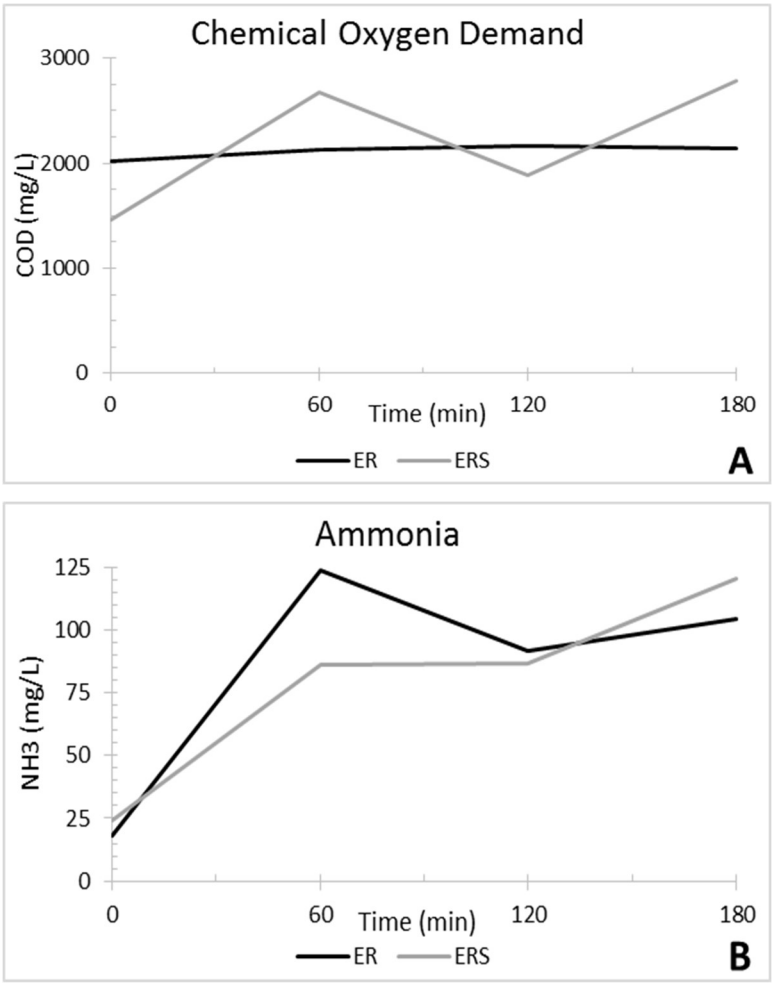

Figure 4.12: Enrichment Reactor Nutrients

(A and B represent concentrations for COD and Ammonia, respectively)

Figure continued on the next page.

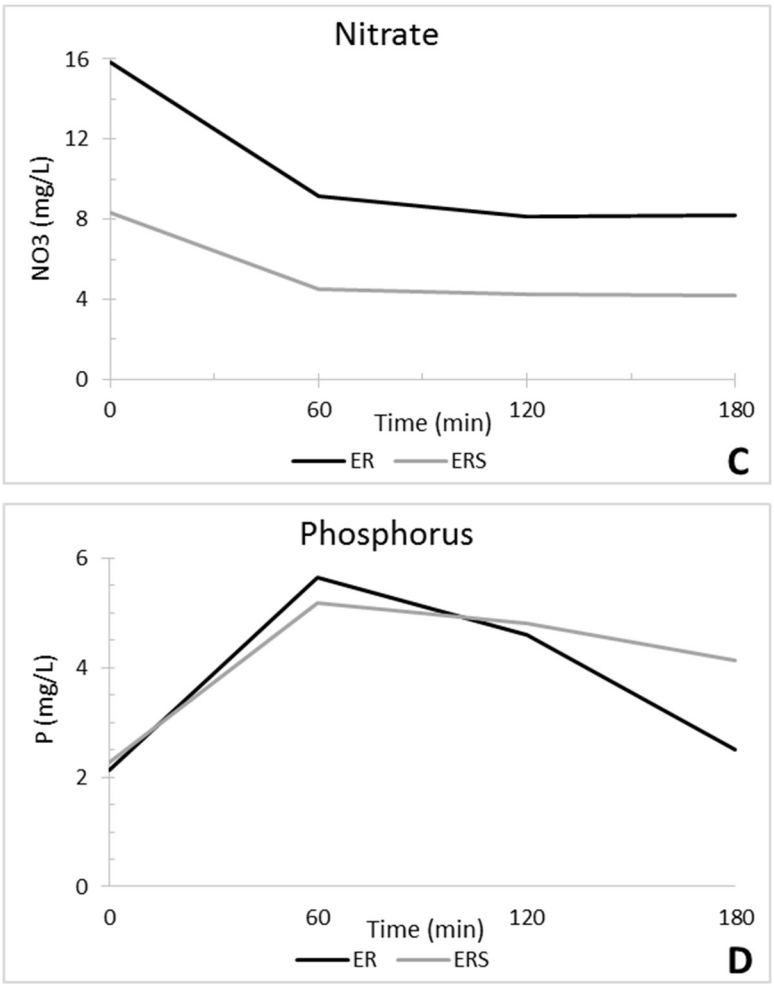

Figure 4.12: Enrichment Reactor Nutrients

(C and D represent concentrations for Nitrate and Phosphorus, respectively)

As shown, effluent nutrient values remained similar to or lower in ERS compared to ER. Increases in COD, ammonia, and phosphorus concentrations from t=0 to t=60 were caused by the addition of the DFL substrate to the reactors. Return of ammonia and phosphorus concentrations to the lower t=0 values indicates the ability of the enrichment reactor MMC to induce similar levels of nitrification and biologically remove phosphorus. Nitrate concentrations decrease to approximately half of the t=0 values after 60 minutes, indicating simultaneous nitrification-denitrification occurring in both ER and ERS. While overall nutrient values were typically lower in ERS than ER, samples taken at t=120 minutes for reactor ERS indicate the nutrient concentrations of the volume decanted from the reactor. Therefore, as shown for the ERS operation, a large quantity of nutrients would require additional treatment under full-scale operations. Due to the loss of treatment seen with implementation of a settling

stage, reactor ER experienced better overall nutrient removal than ERS. The COD concentrations in the decanted supernatant of ERS were much lower than expected values; indicating that settling removed inadequate levels of SDS to enhance the enrichment of PHA-producing bacteria in the MMC or metabolic famine metabolism (RQ 1).

4.4.2. Effect of SRT (Statistics)

Statistical analysis was performed on the data collected from comprehensive sampling assessments for both enrichment and production reactors to determine the potential effects of increasing enrichment stage SRT on overall PHA accumulation (i.e., RQ 2).

4.4.2.1. Enrichment Reactor SRT Statistical Analysis

The effect of SRT on enrichment reactor performance parameters were evaluated using an analysis of variance (ANOVA) testing to determine statistical significance between mean values. Statistical calculations incorporated data from ER and ERS for each SRT. Response variables used to evaluate enrichment reactor PHA production performance were F-F ratio, PHA yield, and peak PHA production on both a Cmmol/L and %TSS basis. Before conducting evaluations, the data was visualized using boxplots to illustrate the mean and distribution of observed values for each parameter by SRT. Figure 4.13 demonstrates the boxplots generated for each PHA enrichment assessment parameter.

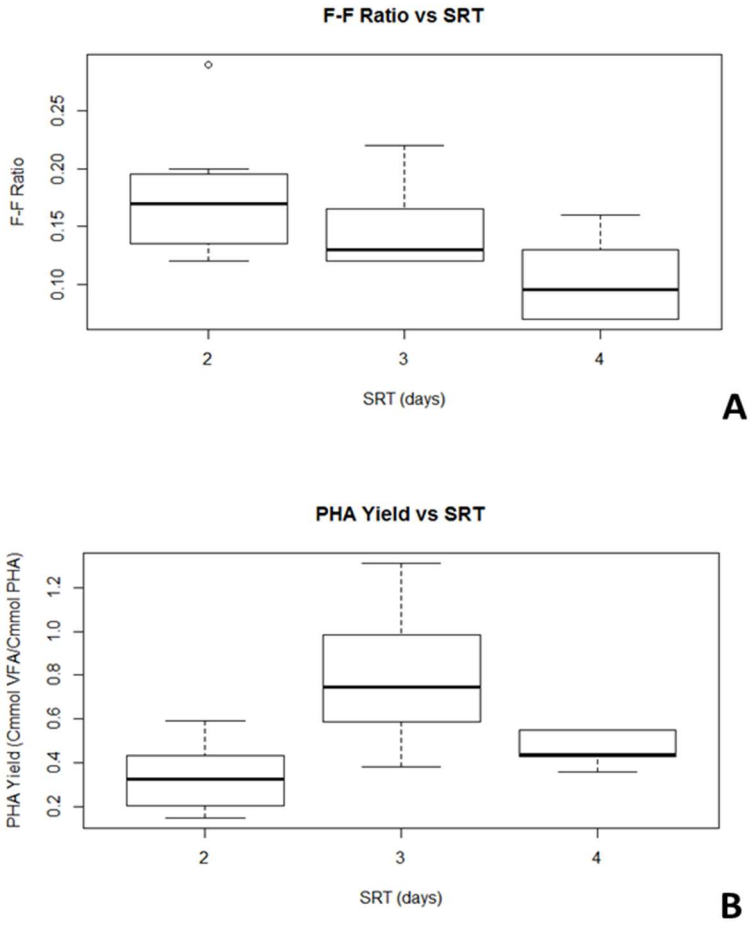

Figure 4.13: Enrichment Reactor PHA Production Parameters vs. SRT

(A and B represent the F-F Ratio and PHA Yield response parameters, respectively)

Figure continued on the next page

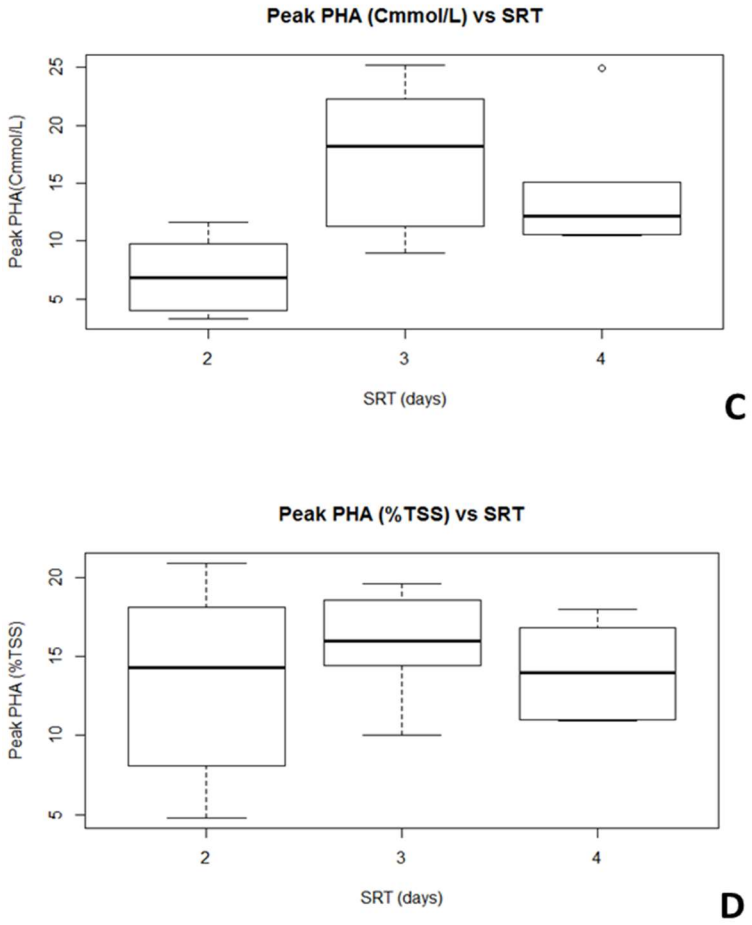

Figure 4.13: Enrichment Reactor PHA Production Parameters vs. SRT
(C and D represent the Peak PHA Cmmol/L and Peak PHA %TSS, respectively)

Feast length seemed to decrease as the SRT, and microbial populations, increased in the reactors. Shorter feast lengths resulted in improved F-F ratios as SRT increased. Average F-F ratio values were 0.18, 0.15, and 0.10 for SRTs of 2-, 3-, and 4- days, respectively. These values are all smaller than the recommended maximum ratio of 0.20 [86], indicating an adequate famine length to induce stress for PHA accumulation for an operational regime with SRT between 2 and 4 days. Statistical tests verified that there is a statistically significant difference between SRT F-F ratio means; therefore, increasing SRT potentially induces an increased metabolic stress on a MMC.

Observed PHA yield varied between each SRT assessment. Average observed PHA yield values were 0.38, 0.79, and 0.46 $Cmmol_{PHA}/Cmmol_{VFA}$ for an SRT of 2, 3, and 4-days,

respectively. While results observed for similar DFL feedstock studies used SRTs greater than 2-days, results in this study for 2-, 3-, and 4-day SRTs were all within the reported range for PHA yield (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]). While the results appear to differ greatly for each SRT (Figure 4.13), statistical testing showed that there was no statistical significance between the average values for observed PHA yield and the respective SRTs. These results suggest that SRT does not have a significant effect on PHA yield in the enrichment reactor.

Average peak PHA fluctuated between each assessment on a Cmmol/L basis; however, average peak intracellular PHA content, measured on a %TSS (w/w) basis, remained similar for each SRT tested. Average peak PHA values were 6.8, 17.2, and 14.2 Cmmol/L, and 14.0, 16.0, and 14.1 %TSS (w/w) for 2-, 3-, and 4-day SRTs, respectively. Overall, enrichment reactor PHA results were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Statistical analysis confirmed that there is a significant difference between average peak PHA produced (Cmmol/L), indicating that an SRT of 2-days does not produce a similar concentration of PHA as a 3- or 4-day SRT; however, testing also showed that there was no statistical significance between average values for peak intracellular PHA (%TSS), between the three SRTs. Differences in concentration and %TSS values are attributed to variations in reactor solids concentrations between PR and PRS.

Contrary to the initial hypothesis, operational results and accompanying statistical analyses indicate that increasing enrichment reactor SRT resulted in increased MMC output in the enrichment stage.

4.4.2.2. Statistical Analysis of Production Reactor SRT

Effects of SRT on production reactor performance parameters were evaluated using ANOVA testing to determine statistical significance between mean values. Statistical calculations incorporated data from ER and ERS for each SRT. Response variables used were PHA yield and peak PHA production on both a Cmmol/L and %TSS basis. Figure 4.14 presents the boxplots generated for each PHA production assessment parameter.

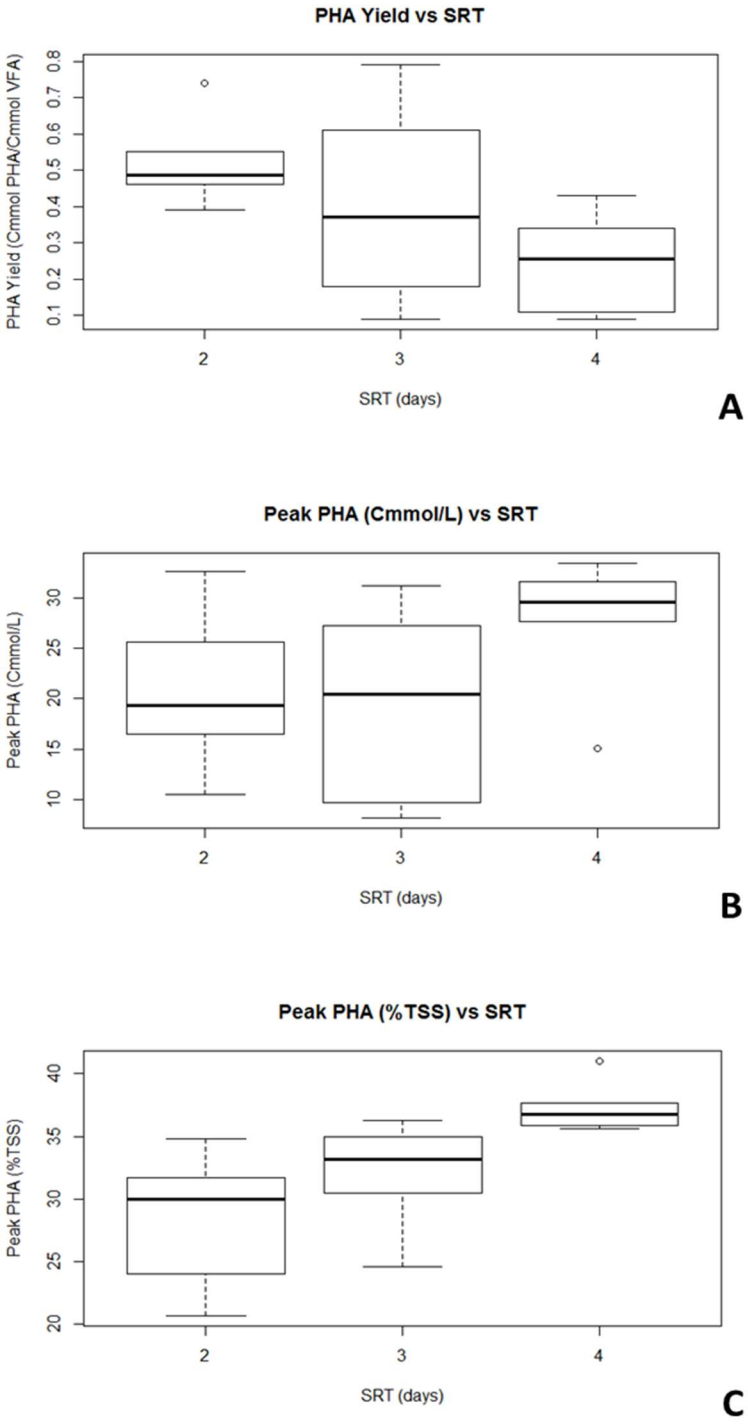

Figure 4.14: Production Reactor PHA Production Parameters vs. SRT
(A, B, and C represent response parameters PHA Yield, Peak PHA Cmmol/L and Peak PHA %TSS, respectively)

Average observed PHA yield values were 0.49, 0.40, and 0.25 $Cmmol_{PHA}/Cmmol_{VFA}$ for SRTs of 2-, 3-, and 4-days, respectively. While results observed for similar DFL feedstock studies used SRTs greater than 2-days, results for all SRTs evaluated in this study were within the reported range for PHA yield (0.24 – 1.13 $Cmmol_{PHA}/Cmmol_{VFA}$ [16, 17, 23]). Statistical testing showed that there was a statistical significance between the average values for observed PHA yield between SRTs, suggesting that enrichment stage SRT has a significant effect on production stage PHA yield.

Average peak PHA produced remained similar between each assessment on a Cmmol/L basis; however, average peak intracellular PHA content, measured on a %TSS (w/w) basis, fluctuated for each SRT tested. For the 3-day SRT assessments, average peak PHA values were notably lower than observed single day maximums, whereas average peak PHA values for the 4-day SRT testing remained similar to maximum peak values, likely due to smaller fluctuations in monitored parameters between sampling assessments. Average peak PHA values were 20.6, 19.3, and 27.8 Cmmol/L, and 29.0, 32.3, and 37.3 %TSS (w/w) for 2-, 3-, and 4-day SRTs, respectively. Overall, production reactor PHA results were within the reported range presented in the literature when utilizing a dairy manure feedstock [16, 17, 23]. Statistical analysis confirmed that there was no statistical significance between average values for peak PHA produced (Cmmol/L), but testing did illustrate that there is a statistically significant difference between average peak intracellular PHA (%TSS) for each SRT. These results suggest that intracellular PHA (%TSS) increases as SRT increases.

Overall results and statistical analysis indicate that increasing enrichment reactor SRT improved overall PHA output of the dairy manure-fed ADF system (RQ 2).

5. CONCLUSIONS

Research was conducted to assess impacts of operational parameters on a three-stage ADF PHA production process utilizing a diary manure substrate (Figure 2.7) with a specific focus on wastewater carbon effects coupled with SRT in the enrichment cycle. Two enrichment stage reactors were operated at SRTs of 2-, 3-, and 4-days, along with operation of corresponding production stage reactors. Evaluations were conducted to determine the potential effects of applied enrichment stage sedimentation and SRT on overall ADF PHA output. Sedimentation, with subsequent supernatant removal, was introduced following the enrichment feast period to remove potential SDS once the RDS (i.e., carboxylates) needed for PHA synthesis had been consumed. It was hypothesized that implementation of a sedimentation and subsequent decant phase would remove residual SDS, increasing the enrichment of a PHA-producing MMC while also enhancing the requisite famine metabolism, which would result in increased PHA yield in the production stage. Additionally, it was hypothesized that a shorter SRT in the enrichment stage would better enrich for a MMC capable of storing greater quantities of carboxylates as PHA. Conclusions based on this study include the following:

i. Implementation of a sedimentation and subsequent decant phase at the end of the feast period in the enrichment stage did not achieve necessary levels of substrate removal to induce a greater metabolic impact on the MMC. Resultant supernatant removal and effluent from the ERS operation would require additional treatment than the ER operational regime under full-scale ADF operations. Addition of a sedimentation phase increased operational complexity and did not improve PHA production for a three-stage ADF system utilizing a dairy manure substrate.

ii. Increasing enrichment stage SRT led to an enhanced enrichment stage feast-famine response and improved PHA synthesis in the production stage. Overall, increasing the SRT led to a more operationally stable and predictable ADF system.

5.1. Future Research

This research evaluated the impacts of controllable ADF enrichment stage operational parameters; however, additional effects and optimization of other controllable parameters within the enrichment stage have yet to be confirmed, including: OLR, HRT, CL, and volume.

Future investigations into optimizing the efficiency and output of the ADF process should focus on the relationships between and the effects of these parameters on the feast-famine process. Additionally, research needs to be conducted to develop the optimal controllable operational parameters of the production stage and potential correlations between ADF system stages.

While results presented in this research indicate the implementation of a sedimentation and decant phase does not improve ADF performance when using a fermented dairy manure substrate, further analyses need be performed to evaluate potential improvement for differing operational configurations.

Future investigations to advance this research include:

i. Determination of the quantity and type of microbial populations enriched for within the settling and non-settling enrichment reactors.

ii. Sedimentation process control to begin the settling phase at the end of the feast length as potentially measured by carboxylate depletion, or a large and sudden increase in dissolved oxygen.

iii. Experimentation with decant and total reactor volumes to potentially negate detrimental impacts associated with low volumes.

iv. Development of OLR range where PHA production is not inhibited for production and enrichment stage operations. Determination of optimal OLR for enrichment stage operation.

6. REFERENCES

1. Chen, G. and M. Patel, *Plastics Derived from Biological Sources: Present and Future: A Technical and Environmental Review*, in *Chem. Rev.* 2012. p. 2082-2099.
2. Yates, M.R. and C.Y. Barlow, *Life cycle assessments of biodegradable, commercial biopolymers—A critical review.* Resources, Conservation & Recycling, 2013. **78**: p. 54-66.
3. Kadouri, D., et al., *Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates.* Critical Reviews in Microbiology, 2005. **31**(2): p. 55-67.
4. Holmes, P.A., *Applications of phb - a microbially produced biodegradable thermoplastic.* Physics in Technology, 1985. **16**(1): p. 32-36.
5. Laycock, B., et al., *The chemomechanical properties of microbial polyhydroxyalkanoates.* Progress in Polymer Science, 2012. **38**(3-4): p. 536-583.
6. Digregorio, B.E., *Biobased performance bioplastic: Mirel.* Chemistry & biology, 2009. **16**(1): p. 1.
7. Gerngross, T.U., *Can biotechnology move us toward a sustainable society?* Nat Biotechnol, 1999. **17**(6): p. 541-4.
8. Gerngross, T.U. and S.C. Slater, *How green are green plastics?* Sci Am, 2000. **283**(2): p. 37-41.
9. Serafim, L.S., et al., *Strategies for PHA production by mixed cultures and renewable waste materials.* Applied Microbiology and Biotechnology, 2008. **81**(4): p. 615-628.
10. Liebrand, C., *Cooperative approaches for implementation of dairy manure digesters*, ed. K.C. Ling and D. United States. Rural. 2009, Washington, D.C.: Washington, D.C. : USDA, Rural Development.
11. McCalib, R.J., *Opening the Gates of Cow Palace: Regulating Runoff Manure as a Hazardous Waste Under RCRA.(Resource Conservation and Recovery Act of 1976).* Michigan Law Review, 2017. **116**(3): p. 501.

12. Coats, E.R., et al., *An integrated 2-stage anaerobic digestion and biofuel production process to reduce life cycle GHG emissions from U.S. dairies.* BioFPR, 2013. **7**(4): p. 459-473.

13. Dionisi, D., et al., *Biodegradable polymers from organic acids by using activated sludge enriched by aerobic periodic feeding.* Biotechnology and Bioengineering, 2004. **85**(6): p. 569-579.

14. Majone, M., K. Dircks, and J. J. Beun, , *Aerobic storage under dynamic conditions in activated sludge processes. The state of the art:* Water Science and Technology, 1999. **39**(1): p. 61-73.

15. Hanson, A.J., et al., *Community proteomics provides functional insight into polyhydroxyalkanoate production by a mixed microbial culture cultivated on fermented dairy manure.* Appl. Biochem. Biotechnol., 2016. **100**(18): p. 7957-7976.

16. Carleton, B.S., *Toward maximal polyhydroxyalkanoate production from dairy manure : controlling and optimizing biosynthesis with implications for design*, ed. E.R. Coats. 2016: Thesis (M.S., Chemical Engineering)--University of Idaho,May 2016.

17. Guho, N.M., *Polyhydroxyalkanoate production coupled with waste treatment using a three-stage sequencing batch reactor system fed dairy manure*, E.R. Coats, Editor. 2010, Thesis (M.S., Civil Engineering)--University of Idaho, July 2010.

18. Serafim, L.S., et al., *Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions.* Biotechnology and Bioengineering, 2004. **87**(2): p. 145-160.

19. van Aalst-van Leeuwen, M.A., et al., *Kinetic modeling of poly(beta-hydroxybutyrate) production and consumption by Paracoccus pantotrophus under dynamic substrate supply.* Biotechnology and bioengineering, 1997. **55**(5): p. 773.

20. Huang, L., et al., *Insights into Feast-Famine polyhydroxyalkanoate (PHA)-producer selection: Microbial community succession, relationships with system function and underlying driving forces.* Water Research, 2018. **131**: p. 167-176.

21. Oliveira, C., et al., *Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities.* New Biotech., 2017. **37**(Pt A): p. 69-79.

22. Marang, L., M.C.M. Van Loosdrecht, and R. Kleerebezem, *Modeling the competition between PHA-producing and non-PHA-producing bacteria in feast-famine SBR and staged CSTR systems.* Biotechnology and Bioengineering, 2015. **112**(12): p. 2475-2484.

23. Watson, B., *Optimization of Dairy Manure Based Polyhydroxyalkanoate Production through Reduced Aeration on Enrichment Cultures*, E. Coats, R. Nielson, and J. Moberly, Editors. 2015, ProQuest Dissertations Publishing.

24. Coats, E.R., B. Watson, and C.K. Brinkman, *Polyhydroxyalkanoate Synthesis by Mixed Microbial Consortia cultured on Fermented Dairy Manure: Effect of Oxygen Mass Transfer on Process Rates/Yields and Microbial Ecology.* Water Res., 2016. **106**: p. 26-40.

25. Dionisi, D., et al., *Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate.* Journal of Chemical Technology & Biotechnology, 2005. **80**(11): p. 1306-1318.

26. Johnson, K., et al., *Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity.* Biomacromolecules, 2009. **10**(4): p. 670.

27. Albuquerque, M.G.E., et al., *Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses.* Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses, 2007. **130**: p. 411-421.

28. Sudesh, K., H. Abe, and Y. Doi, *Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters.* Progress in Polymer Science, 2000. **25**(10): p. 1503-1555.

29. Wei, L., et al., *Characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) biosynthesized by mixed microbial consortia fed fermented dairy manure.* Journal of Applied Polymer Science, 2014. **131**(11): p. 5516-5528.

30. Reis Maria, A., et al., *Metabolic modelling of polyhydroxyalkanoate copolymers production by mixed microbial cultures.* BMC Systems Biology, 2008. **2**(1): p. 59.

31. Doi, Y., *Microbial polyesters*. 1990, New York, N.Y.: New York, N.Y. : VCH.

32. Madison, L.L. and G.W. Huisman, *Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic.* Microbiology and Molecular Biology Reviews, 1999. **63**(1): p. 21.

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

34. Oehmen, A., et al., *Advances in enhanced biological phosphorus removal: From micro to macro scale.* Water Research, 2007. **41**(11): p. 2271-2300.

35. Probst, D.L., *Polyhydroxyalkanoate production within a novel WRRF configuration*, ed. E.R. Coats. 2016: Thesis (M.S., Civil Engineering)--University of Idaho,May 2016.

36. Bengtsson, S., et al., *Production of polyhydroxyalkanoates from fermented sugar cane molasses by a mixed culture enriched in glycogen accumulating organisms.* Journal of Biotechnology, 2010. **145**(3): p. 253-263.

37. Coats, E., et al., *Functional stability of a mixed microbial consortium producing PHA from waste carbon sources.* Appl. Biochem. Biotechnol., 2007. **137**: p. 909-925.

38. Appel, F., *Advancing a Post-Anoxic Biological Nutrient Removal Process Selecting for Nitritation*, E.R. Coats, K. Chang, and J. Moberly, Editors. 2015, ProQuest Dissertations Publishing.

39. Coats, E., et al., *Toward Polyhydroxyalkanoate Production Concurrent with Municipal Wastewater Treatment in a Sequencing Batch Reactor System.* J. Environ. Eng.-ASCE, 2011. **137**(1): p. 46-54.

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

41. Choi, J.-i. and S.Y. Lee, *Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation.* Bioprocess Engineering, 1997. **17**(6): p. 335-342.

42. Salehizadeh, H. and M.C.M. Van Loosdrecht, *Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance.* Biotechnology Advances, 2004. **22**(3): p. 261-279.

43. Majone, M., et al., *Influence of Storage of Kinetic Selection to Control Aerobic Filamentous Bulking.* Water Science and Technology, 1996. **34**: p. 223-232.

44. Beun, J.J., et al., *Stoichiometry and kinetics of poly-β-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures.* Biotechnology and Bioengineering, 2000. **67**(4): p. 379-389.

45. Guho, N., *Advancing bioplastic production from waste feedstocks using mixed microbial consortia by reconsidering the underlying biochemical basis: One step back, two steps forward*, in *Civil and Environmental Engineering*. 2014, University of Idaho. p. 27.

46. Russell, J.B., *The Energy Spilling Reactions of Bacteria and Other Organisms.* Journal of Molecular Microbiology and Biotechnology, 2007. **13**(1-3): p. 1-11.

47. Russell, J.B. and G.M. Cook, *Energetics of bacterial growth: balance of anabolic and catabolic reactions.* Microbiological Reviews, 1995. **59**(1): p. 48-62.

48. Dias, J.M.L., et al., *Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product.* Macromolecular Bioscience, 2006. **6**(11): p. 885-906.

49. Reis, M., et al., *Production of polyhydroxyalkanoates by mixed microbial cultures.* Bioprocess and Biosystems Engineering, 2003. **25**(6): p. 377-385.

50. Morgan-Sagastume, F., et al., *Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus.* Water Research, 2010. **44**(18): p. 5196-5211.

51. Choi, J. and S.Y. Lee, *Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation.* Applied Microbiology and Biotechnology, 1999. **51**(1): p. 13-21.

52. Metcalf, amp, and a. Eddy, *Wastewater engineering : treatment and resource recovery*. Fifth edition / revised by George Tchobanoglous, H. David Stensel, Ryujiro Tsuchihashi, Franklin Burton ; contributing authors, Mohammad Abu-Orf, Gregory Bowden, William Pfrang. ed, ed. G. Tchobanoglous, et al. 2014, New York, NY: New York, NY : McGraw-Hill Education.

53. Grady, C.P.L., *Biological wastewater treatment*. 2nd ed. rev. and expanded. ed, ed. G.T. Daigger and H.C. Lim. 1999, New York: New York : Marcel Dekker.

54. Reinecke, F. and A. Steinbüchel, *Ralstonia eutropha strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers.* Journal of molecular microbiology and biotechnology, 2009. **16**(1-2): p. 91.

55. Cherrington, C.A., et al., *Organic Acids: Chemistry, Antibacterial Activity and Practical Applications.* Advances in Microbial Physiology, 1991. **32**(C): p. 87-108.

56. Stephanopoulos, G., *Metabolic engineering : principles and methodologies*, ed. A.A. Aristidou and J.H. Nielsen. 1998, San Diego: San Diego : Academic Press.

57. Russell, J.B. and F. Diez-Gonzalez, *The Effects of Fermentation Acids on Bacterial Growth.* Advances in Microbial Physiology, 1997. **39**: p. 205-234.

58. Gottschalk, G., *Bacterial metabolism*. 2nd ed. ed. 1986, New York: New York : Springer-Verlag.

59. Madigan, M.T., *Brock biology of microorganisms*. 11th ed. ed. Biology of microorganisms, ed. J.M. Martinko and T.D. Brock. 2006, Upper Saddle River, NJ: Upper Saddle River, NJ : Pearson Prentice Hall.

60. Lemos, P.C., et al., *Metabolic Pathway for Propionate Utilization by Phosphorus-Accumulating Organisms in Activated Sludge: 13C Labeling and In Vivo Nuclear Magnetic Resonance.* Applied and Environmental Microbiology, 2003. **69**(1): p. 241.

61. Steinbüchel, A., *Biodegradable plastics.* Current Opinion in Biotechnology, 1992. **3**(3): p. 291-297.

62. Haywood, G.W., et al., *Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism Alcaligenes eutrophus.* FEMS Microbiology Letters, 1988. **52**(1): p. 91-96.

63. Haywood, G.W., et al., *The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism Alcaligenes eutrophus.* FEMS Microbiology Letters, 1988. **52**(3): p. 259-264.

64. Haywood, G.W., A.J. Anderson, and E.A. Dawes, *The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by Alcaligenes eutrophus.* FEMS Microbiology Letters, 1989. **57**(1): p. 1-6.

65. Oeding, V. and H.G. Schlegel, *Beta-ketothiolase from Hydrogenomonas eutropha H16 and its significance in the regulation of poly-beta-hydroxybutyrate metabolism.* The Biochemical journal, 1973. **134**(1): p. 239.

66. Doi, Y., et al., *Biosynthesis of Copolyesters in Alcaligenes Eutrophus H16 From exp 13 C-Labeled Acetate and Propionate.* Macromolecules, 1987. **20**(12): p. 2988-2991.

67. Doi, Y., et al., *Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate in Alcaligenes eutrophus from 5-chloropentanoic and pentanoic acids.* Macromol. Rapid Commun., 2005. **26**(13): p. 1027-1031.

68. Braunegg, G., G. Lefebvre, and K.F. Genser, *Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects.* Journal of Biotechnology, 1998. **65**(2): p. 127-161.

69. Doi, Y., et al., *Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus.* FEMS Microbiology Letters, 1992. **103**(2-4): p. 103-108.

70. Henderson, R.A. and C.W. Jones, *Poly-3-hydroxybutyrate production by washed cells of Alcaligenes eutrophus; purification, characterisation and potential regulatory role of citrate synthase.* Archives of Microbiology, 1997. **168**(6): p. 486-492.

71. Senior, P.J. and E.A. Dawes, *The regulation of poly-beta-hydroxybutyrate metabolism in Azotobacter beijerinckii.* The Biochemical journal, 1973. **134**(1): p. 225.

72. Jendrossek, D., *Polyhydroxyalkanoate Granules Are Complex Subcellular Organelles (Carbonosomes).* The Journal of Bacteriology, 2009. **191**(10): p. 3195.

73. Kawaguchi, Y. and Y. Doi, *KINETICS AND MECHANISM OF SYNTHESIS AND DEGRADATION OF POLY(3-HYDROXYBUTYRATE) IN ALCALIGENES-EUTROPHUS.* Macromolecules, 1992. **25**(9): p. 2324-2329.

74. Kobayashi, T., et al., *Purification and Properties of an Intracellular 3-Hydroxybutyrate-Oligomer Hydrolase (PhaZ2) in Ralstonia eutropha H16 and Its Identification as a Novel Intracellular Poly(3-Hydroxybutyrate) Depolymerase.* The Journal of Bacteriology, 2003. **185**(12): p. 3485.

75. Kobayashi, T., et al., *Novel Intracellular 3-Hydroxybutyrate-Oligomer Hydrolase in Wautersia eutropha H16.* The Journal of Bacteriology, 2005. **187**(15): p. 5129.

76. Uchino, K. and T. Saito, *Thiolysis of Poly(3-hydroxybutyrate) with Polyhydroxyalkanoate Synthase from Ralstonia eutropha.* The Journal of Biochemistry, 2006. **139**(3): p. 615-621.

77. Uchino, K. and T. Saito, *Thiolysis of poly(3-hydroxybutyrate) A novel biochemical reaction.* Canadian Journal of Chemistry, 2008. **86**(6): p. 516-519.

78. Kleerebezem, R. and M.C.M. van Loosdrecht, *Mixed culture biotechnology for bioenergy production.* Current Opinion in Biotechnology, 2007. **18**(3): p. 207-212.

79. Dionisi, D., et al., *Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor.* Biotechnology and Bioengineering, 2006. **93**(1): p. 76-88.

80. Frigon, D., et al., *Oligonucleotide probe hybridization and modeling results suggest that populations consuming readily degradable substrate have high cellular RNA levels.* Water Science and Technology, 2002. **45**(6): p. 115-126.

81. Marang, L., et al., *Impact of non-storing biomass on PHA production: An enrichment culture on acetate and methanol.* International Journal of Biological Macromolecules, 2014. **71**: p. 74-80.

82. Korkakaki, E., M.C.M. van Loosdrecht, and R. Kleerebezem, *Survival of the fastest: Selective removal of the side population for enhanced PHA production in a mixed substrate enrichment.* Bioresource Technology, 2016. **216**: p. 1022-1029.

83. Mas, J., C. Pedros-Alio, and R. Guerrero, *Mathematical model for determining the effects of intracytoplasmic inclusions on volume and density of microorganisms.* The Journal of Bacteriology, 1985. **164**(2): p. 749.

84. Lee, S., *Bacterial polyhydroxyalkanoates*, in *Biotechnol. Bioeng.* 1996. p. 1-14.

85. Albuquerque, M.G.E., C.A.V. Torres, and M.A.M. Reis, *Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection.* Water Research, 2010. **44**(11): p. 3419-3433.

86. Yang, J., et al., *Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor.* The ISME Journal, 2010. **5**(5): p. 896.

87. Valentino, F., et al., *Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels.* Water Research, 2015. **77**: p. 49-63.

88. Fernández-Dacosta, C., et al., *Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment.* Bioresource Technology, 2015. **185**: p. 368-377.

89. Coats, E.R., et al., *Production of natural fiber reinforced thermoplastic composites through the use of PHB-rich biomass.* Bioresour. Technol., 2008. **99**(7): p. 2680-2686.

90. van Loosdrecht, M.C.M. and J.J. Heijnen, *Modelling of activated sludge processes with structured biomass.* Water Science and Technology, 2002. **45**(6): p. 13-23.

91. Carta, F., et al., *Simultaneous storage and degradation of phb and glycogen in activated sludge cultures.* Water Research, 2001. **35**(11): p. 2693-2701.

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

93. Beun, J., et al., *Poly- beta -hydroxybutyrate metabolism in dynamically fed mixed microbial cultures.* Water Research, 2002. **36**(5): p. 1167-1180.

94. Villano, M., et al., *Effect of pH on the production of bacterial polyhydroxyalkanoates by mixed cultures enriched under periodic feeding.* Process Biochemistry, 2010. **45**(5): p. 714-723.

95. Garcia-Ochoa, F. and E. Gomez, *Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview.* Biotechnology Advances, 2009. **27**(2): p. 153-176.

96. Third, K.A., M. Newland, and R. Cord-Ruwisch, *The effect of dissolved oxygen on PHB accumulation in activated sludge cultures.* Biotechnology and Bioengineering, 2003. **82**(2): p. 238-250.

97. Dias, J.M.L., et al., *Mathematical modelling of a mixed culture cultivation process for the production of polyhydroxybutyrate.* Biotechnology and Bioengineering, 2005. **92**(2): p. 209-222.

98. Wei, Y., et al., *Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate Cupriavidus taiwanensis Strains.* Int. J. Mol. Sci., 2011. **12**(1): p. 252-265.

99. Venkata Mohan, S. and M. Venkateswar Reddy, *Optimization of critical factors to enhance polyhydroxyalkanoates (PHA) synthesis by mixed culture using Taguchi design of experimental methodology.* Bioresource Technology, 2013. **128**: p. 409-416.

100. Liu, Z., et al., *Optimization of polyhydroxybutyrate (PHB) production by excess activated sludge and microbial community analysis.* Journal of Hazardous Materials, 2011. **185**(1): p. 8-16.

101. Montiel-Jarillo, G., J. Carrera, and M.E. Suárez-Ojeda, *Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: Effect of pH and N and P concentrations.* Science of the Total Environment, 2017. **583**: p. 300-307.

102. Bren, A., et al., *The last generation of bacterial growth in limiting nutrient.* BMC systems biology, 2013. **7**: p. 27.

103. Venkateswar Reddy, M. and S. Venkata Mohan, *Effect of substrate load and nutrients concentration on the polyhydroxyalkanoates (PHA) production using mixed consortia through wastewater treatment.* Bioresource Technology, 2012. **114**: p. 573-582.

104. Fang, F., et al., *Identifying the influential priority of the factors governing PHB production by activated sludge with integration of uniform design and grey relational analysis.* Separation and Purification Technology, 2014. **136**: p. 111-114.

105. Clescerl, L.S., A.E. Greenberg, and A.D. Eaton, *Standard Methods for Examination of Water and Wastewater*. 1998: American Public Health Association.

106. Braunegg, G., B. Sonnleitner, and R.M. Lafferty, *A Rapid Gas-Chromatographic Method for the Determination of Poly-Beta-Hydroxybutyric Acid in Microbial Biomass.* European Journal of Applied Microbiology and Biotechnology, 1978. **6**(1): p. 29-37.

107. Henze, M., et al., *Wastewater and biomass characterization for the activated sludge model no. 2: Biological phosphorus removal*, M. Henze and W. Gujer, Editors. 1994. p. 13-23.

108. Grady, C.P.L., B.F. Smets, and D.S. Barbeau, *Variability in kinetic parameter estimates: A review of possible causes and a proposed terminology.* Water Res., 1996. **30**(3): p. 742-748.

109. Johnson, K., R. Kleerebezem, and M.C.M. van Loosdrecht, *Model-based data evaluation of polyhydroxybutyrate producing mixed microbial cultures in aerobic sequencing batch and fed-batch reactors.* Biotechnology and Bioengineering, 2009. **104**(1): p. 50-67.

APPENDIX A: ANALYSIS OF REACTOR PROCESS KINETICS

Process kinetics were evaluated in the enrichment stage PHBV reactors to determine potential differences in MMC capabilities. Specifically, data was analyzed to assess the specific rates of carboxylate uptake and PHA synthesis (i.e., qVFA and qPHA, respectively) of the respective MMC. The specific rates, qVFA and qPHA, were normalized using the initial amount of biomass present for each assessment (i.e., at t=0, representing the WAS). Normalization of the specific rates was required to assess microbe kinetics due to initial carboxylate feed concentrations (i.e., OLR) remaining constant throughout testing; normalization also allowed for comparisons to be performed on a similar basis between reactor configurations. Evaluating process kinetics then became an analysis of the specific rates with respect to the food to microorganism ratio (F:M), with the F:M being calculated solely on the concentration of carboxylates. This analysis was conducted to provide insight into how to properly calculate or model process kinetic rates in future assessments.

Analyses of process kinetics assumed zero-order rate relationships for qVFA. Comparisons between ER and ERS specific rates were evaluated using data for all sampling assessments, but were also separately conducted for each SRT configuration tested. Figure A.1 displays the kinetic relationships for qVFA vs. F:M

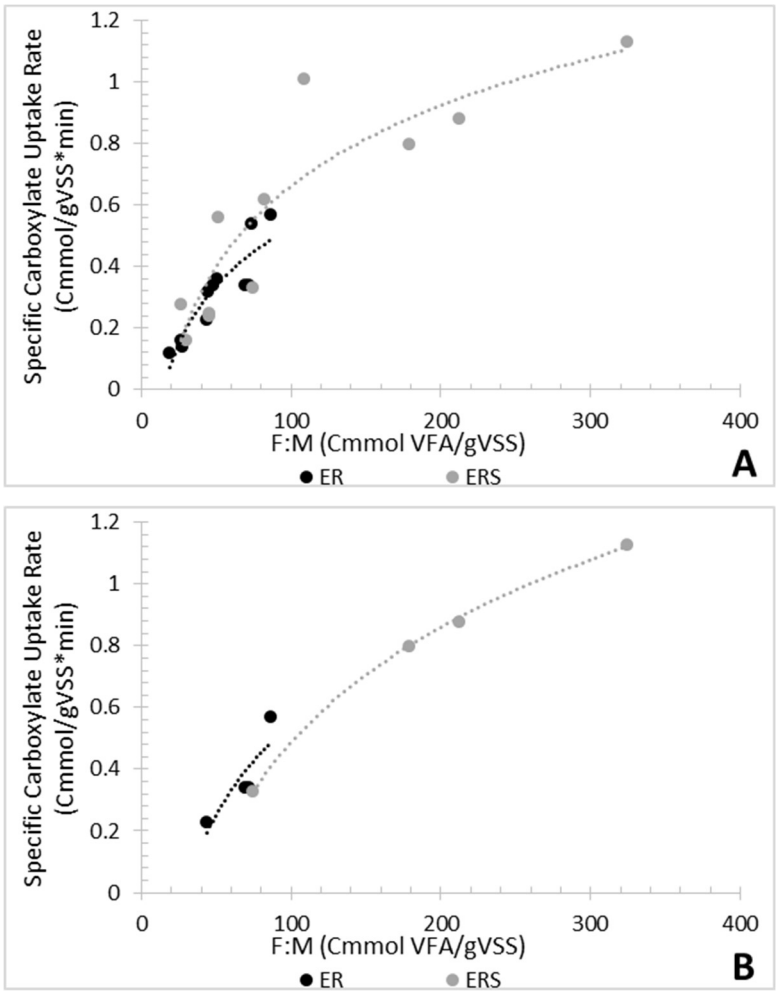

Figure A.1: Enrichment Stage qVFA Kinetic Analysis

(A and B represent data from all SRTs and the 2-day SRT, respectively)

Figure continued on the next page.

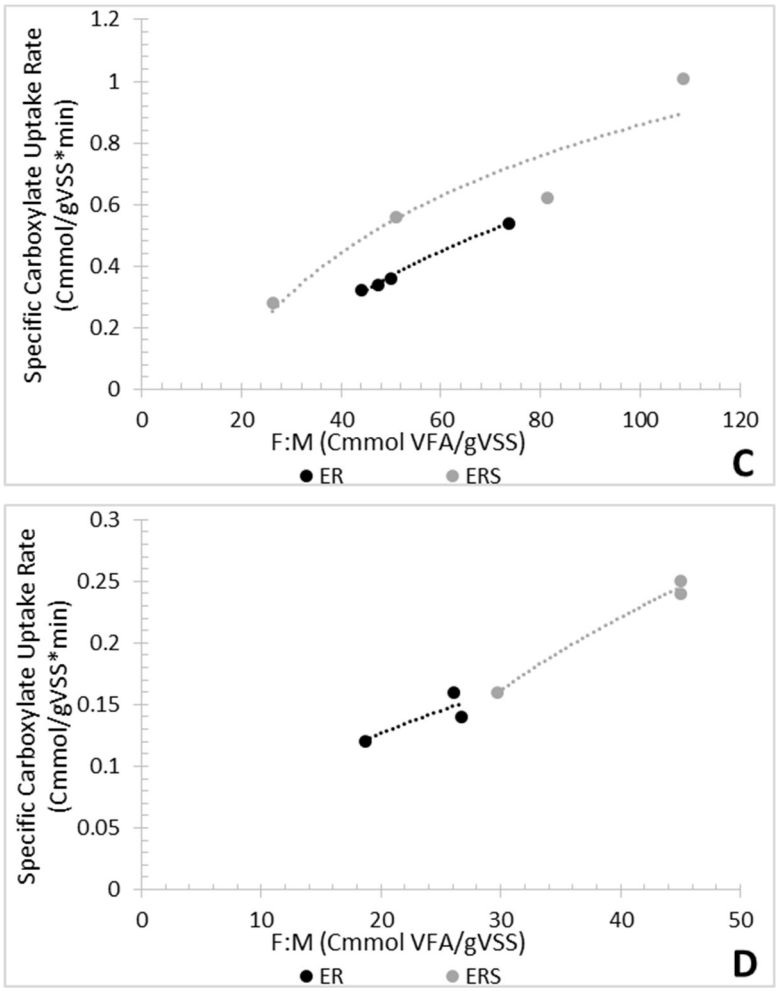

Figure A.1: Enrichment Stage qVFA Kinetic Analysis
(C and D represent data from the 3-day SRT and 4-day SRT, respectively)

Process kinetics for MMC-based systems are often modeled using a Monod relationship as shown in Equation 3.7. In this regard, as illustrated the qVFA vs. F:M association tends to follow a fairly strong logarithmic distribution for both the complete dataset and for each SRT. The observed correlation indicates that a zero-order rate relationship can accurately describe qVFA rates and that a Monod calculation may correctly model substrate uptake in ADF systems. Reactor ERS kinetics tended to occur at a faster rate for qVFA than reactor ER, indicating that the MMC may have a larger maximum specific carboxylate uptake rate. Distribution equations and coefficients of determination (R^2) of linear regression are summarized in Table A.1.

Table A.1: Enrichment Stage qVFA Analysis Summary

Parameter	Reactor	R^2	Equation
Complete Results	ER	0.81	y = 0.2713 * ln(x) – 0.7223
	ERS	0.81	y = 0.3763 * ln(x) – 1.0704
2-Day SRT	ER	0.76	y = 0.4285 * ln(x) – 1.4217
	ERS	0.99	y = 0.5386 * ln(x) – 1.9926
3-Day SRT	ER	0.99	y = 0.4425 * ln(x) – 1.364
	ERS	0.87	y = 0.4534 * ln(x) – 1.2292
4-Day SRT	ER	0.70	y = 0.0831 * ln(x) – 0.1223
	ERS	0.99	y = 0.2051 * ln(x) – 0.5359

Comparisons for qPHA between ER and ERS were similarly evaluated using data for all sampling assessments, and included separate assessments for each SRT configuration tested. Figure A.2 illustrates the evaluations for qPHA vs. F:M.

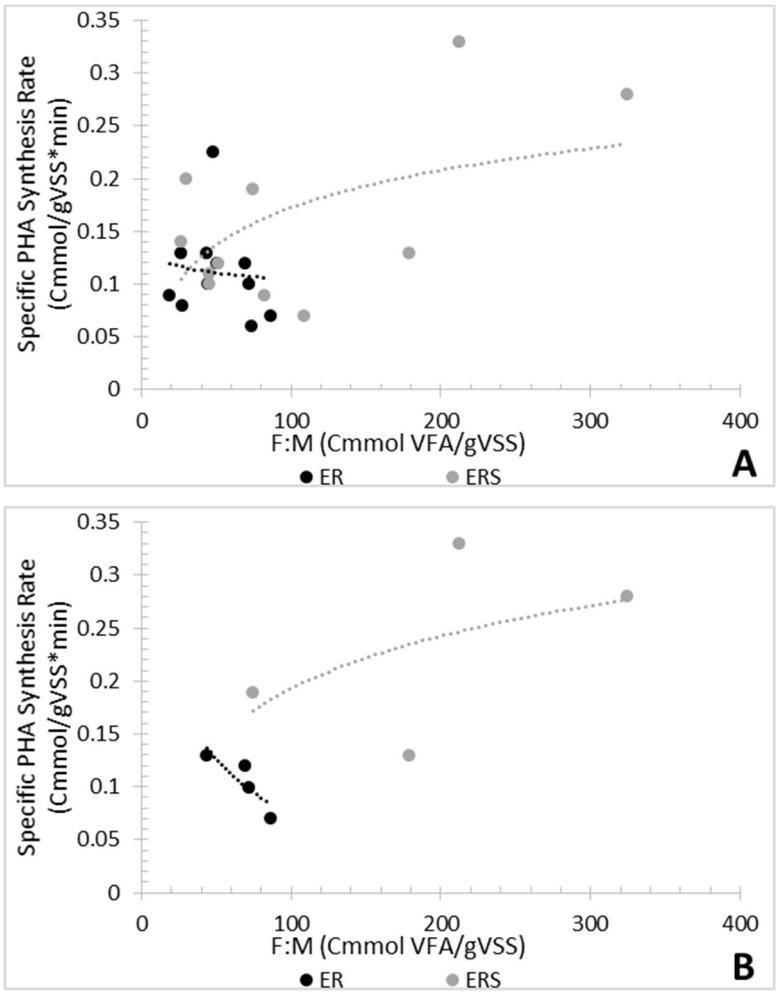

Figure A.2: Enrichment Stage qPHA Kinetic Analysis

(A and B represent data from all SRTs and the 2-day SRT, respectively)

Figure continued on the next page.

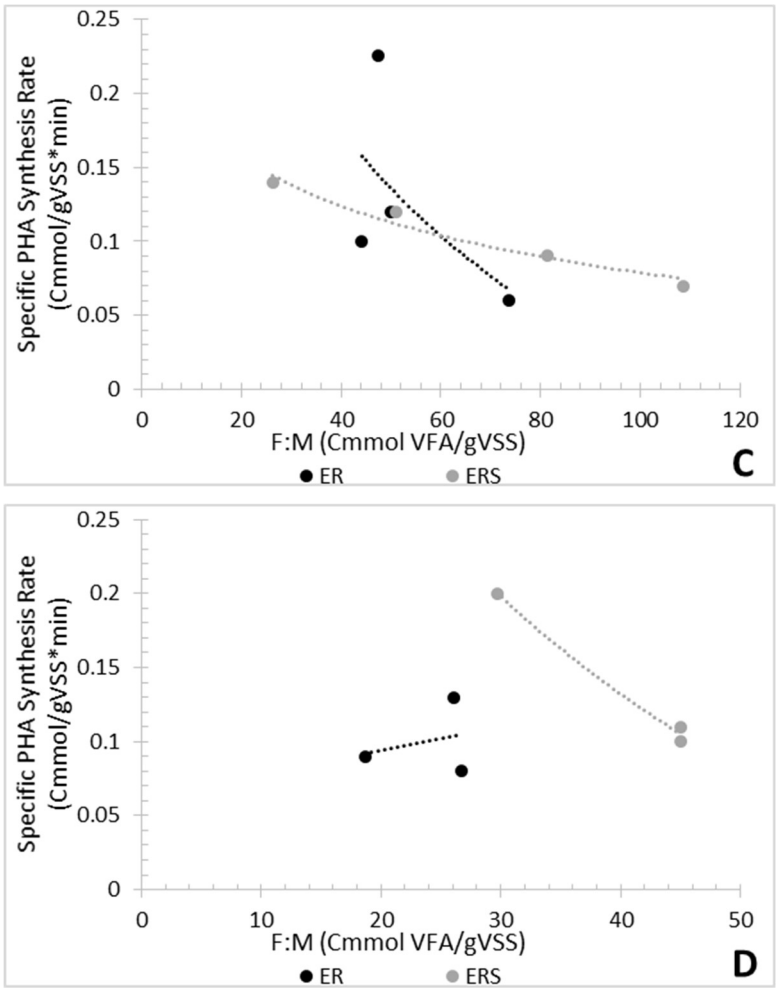

Figure A.2: Enrichment Stage qPHA Kinetic Analysis
(C and D represent data from the 3-day SRT and 4-day SRT, respectively)

In contrast to qVFA analyses, as shown the qPHA vs. F:M relationship did not tend to follow a discernable Monod distribution for any of the assessments. The observed, and calculated, lack of correlation is due in part to PHA synthesis not occurring under a zero-order rate relationship. These results indicate that qPHA does not follow a simple zero-order based Monod calculation and further analyses need to be performed to more accurately model specific PHA synthesis rates; indeed, others have suggested that MMC 'feast' PHA kinetics must include qVFA, growth, and microbial maintenance [19, 109], and thus additional interrogation of the data coupled with collection of additional data is needed. Distribution equations and coefficients of determination (R^2) of linear regression are summarized in Table A.2.

Table A.2: Enrichment Stage qPHA Kinetic Analysis Summary

Parameter	Reactor	R^2	Equation
Complete Results	ER	0.01	y = -0.009 * ln(x) + 0.1465
	ERS	0.26	y = 0.0509 * ln(x) – 0.0618
2-Day SRT	ER	0.73	y = -0.078 * ln(x) + 0.4294
	ERS	0.24	y = 0.0713 * ln(x) – 0.1354
3-Day SRT	ER	0.32	y = -0.176 * ln(x) + 0.8239
	ERS	0.96	y = -0.049 * ln(x) + 0.3056
4-Day SRT	ER	0.07	y = 0.0355 * ln(x) – 0.0122
	ERS	0.99	y = -0.229 * ln(x) + 0.9778