Optimization of Dairy Manure Based Polyhydroxyalkanoate Production through Reduced Aeration on Enrichment Cultures

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

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May 2015

Authorization to Submit Thesis

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Abstract

Polyhydroxyalkanoates (PHA's) are a biologically produced thermoplastic polymer with similar mechanical and thermal properties to many common petrochemical plastics (i.e. HDPE, polystyrene). PHA's are also a unique renewable product that can replace many petrochemical currently on the market with a biodegradable alternative. Current commercial PHA production is cost prohibitive to using synthetic feed stocks (i.e. acetic acid, or glucose) and pure cultures. Conversely, PHA production on waste organics presents an opportunity to recover resources (carbon) from an otherwise undesirable substrate. The work herein focused on advancing a three stage Aerobic Dynamic Feeding (ADF) process that has successfully produced PHA rich biomass on fermented dairy manure. The primary focus of this work was the reduction of costs associated with PHA production, with a specific emphasis on reducing aeration (a cost intensive aspect of operations). This work assessed the effect of a reduction in aeration in an ADF based PHA production system.

Acknowledgements

I would like to express my sincere appreciation to my advisor Dr. Erik Coats, for providing words of wisdom and encouragement. I would also like to thank my committee member Dr. Nielson and Dr. Moberly for being flexible and taking the time to review this work. Your comments and suggestions are much appreciated. I would also like to show my gratitude to Simon Smith whose quick wit that never left a pun untold and to Cindi Brinkman for bring light hearted humor to the environmental engineering lab. I would also like to thank my peers in the lab group for their help and guidance, especially Nick Guho for always having a technical solution, and Andrea Hanson for her literary advice. My appreciation goes out to the students at the University of Idaho Statistical Assistance Center for their words of wisdom. Last but not least my appreciation goes out to my friends and family who have been with me since the beginning.

Funding to conduct the research presented and discussed in this thesis was provided by the National Science Foundation under Grant #1235885. Any opinions, findings, and conclusions expressed in this material are those of the author and do not necessarily reflect the views of the funding agency.

Dedication

This work is dedicated to my parents Rick and Chris, and everyone who has helped me achieve my goals.

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Introduction

The growing human impact upon the natural environment has given rise to many inventions and projects, all aimed at reducing the global human impact. Currently most projects and inventions rely on a volunteer consumer-based, wherein the consumer must expend their own time and/or money to implement the solution. This method is not capable of realizing comprehensive, global-scale benefits because it is inherently limited to those who volunteer implementation. However, non-volunteer based global solutions do exist and research is ongoing. To be clear, a "non-volunteer" solution is not necessarily regulation driven; rather, it can be an economically favorable approach which replaces a current product with an equivalent cheaper product. If the product was economically and physically equivalent, the merit (and marketing) associated with its environmentally benign nature should be sufficient to earn it a significant market share.

The research presented and discussed herein focuses on one example of a "non-volunteer" based global solution - the production of polyhydroxyalkanoates (PHA's) utilizing otherwise undesired organic waste as feed substrate. PHA has similar thermoplastic properties to conventional petroleum based plastics, with the advantages of biodegradability, biocompatibility, and utilization of waste resources in the production of a commodity (Rostkowski, Criddle, & Lepech, 2012). Widespread use of PHA's presents a massive environmental benefit. Currently several methods utilizing waste resources are being researched for the production of PHA including: municipal sludge, palm oil mill effluent, and tomato cannery waste (Reddy, Ghai, Rashmi, & Kalia, 2003). The research presented and discussed herein is focused on PHA production within the context of a 3 stage dairy manure stabilization process. Dairy manure lends itself well to the PHA production process because it is globally available in large quantities, high in organic content, and generally low in toxic compounds that often affect more industrialized wastes. The first stage is acidogenic fermentation of the manure, followed by liquid-solids separation. Fermenter supernatant (enriched in volatile fatty acids (VFAs), which are excellent precursors for PHA synthesis) is discharged into a PHA producing system, while the residual solids are processed via

methanogenic anaerobic digestion. This integrated suite of processes not only reduces the dairy environmental impact by reducing the carbon footprint and limiting nutrient runoff, but also recovers carbon from the manure in the form of PHA. PHA is capable of replacing many single use plastic products however cost remains a potential barrier to broader use. The current cost of industrially produced PHA is 4-9 times that of oil derived plastics such as high density polyethylene (HDPE) (Serafim, Lemos, Torres, Reis, & Ramos, 2008). For PHA to gain a market share the cost to the customer must be reduced. The work herein addresses a key cost component of PHA production, aeration costs, while concurrently seeking to improve PHA yield and assessing the nitrogen removal capacity of PHA bioreactors. Addressing these three integrated elements will help reduce the cost of PHA production ultimately reducing the cost to the customer.

Chapter 1: Background

1.1 Three Phase Dairy Manure Resource Recovery and Treatment

The human impact on the natural environment is becoming an ever greater concern; consequently ongoing efforts to minimize impacts are gaining traction. As an example of the anthropogenic impact on the natural environment, the dairy industry produces a considerable amount of nutrient waste, greenhouse gases, and solid wastes in the process of generating dairy products for human consumption. Therefore this industry is looking for technologies which can be used to reduce its environmental impact. Dairy waste has many valuable nutrients, and it is a prime raw material for the development and application of resource recovery systems.

The proposed three stage resource recovery and treatment system currently under investigation in Dr. Coats' lab at the University of Idaho will produce bio-gas, PHA saturated biomass, solids for compost, and nutrient rich water for algae farms (Erik R. Coats et al., 2013). Figure 1 is a schematic diagram of the proposed process.

On typical dairies, manure collected from loafing barns and milking parlors is distributed on fields. For our proposed process, once the manure has been collected, the solids would be diluted with tap water to a desired solids concentration. This slurry then enters a fermenter where solids will be hydrolyzed and fermented by bacteria to produce a VFA rich substrate. After solids/liquid separation, the residual solids will be processed via Anaerobic Digestion (for biogas production), while the VFA-rich centrate will be used in the process of PHA Enrichment and Production. The PHA Enrichment reactor will produce and sustain biomass capable of storing excess quantities of PHA. The Production reactor will provide conditions suitable for the biomass from Enrichment reactor to achieve maximum PHA concentration, hence forth referred to as "PHA saturation." Residual solids from the digestion process can be composted or reutilized as a bedding substitute. All effluent liquid streams can be land applied as irrigation water, partially returned to the head of the system as dilution water for raw manure, and/or further processed as nutrient supplement for algae farms.

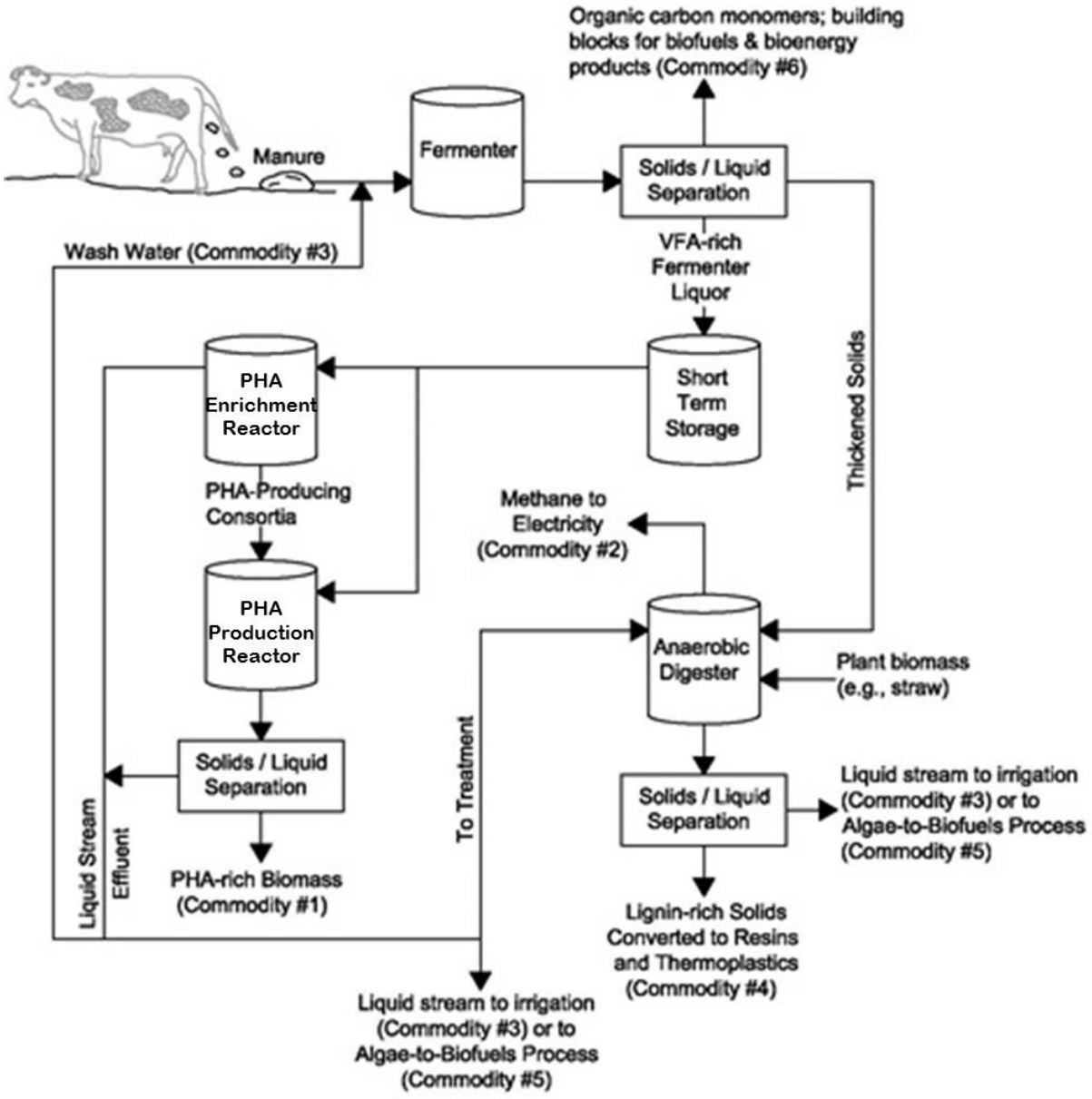


Figure 1: Schematic of Experimental Dairy Manure System.

1.2 Useful Biopolymers

Biologically produced products are of increasing interest due to their typically low environmental impact, ability to biologically degrade, and utilization of renewable resources. Regarding biopolymers, currently the commercial market is focused on three different types: poly(Lactic acid) (PLA), starch based polymers, and Polyhydroxyalkanoates (PHA's) (Yates & Barlow, 2013). These polymers all have similar material characteristics but

differ in their means of production. Both PLA and starch based polymers are plant based processes, which forces them into competition for arable land with food crops (Yates & Barlow, 2013). To this effect, PHA is of interest because it is biologically produced by bacteria utilizing organic carbon which can be harvested from existing waste streams. This process requires minimal land and still produces a commercially viable product capable of replacing many existing petrochemical plastics.

1.3 PHA Applications

PHA's are a class of biopolymers that exhibit physicochemical properties similar to thermoplastics (i.e. common packaging plastics). The largest current application is "dissolving" surgical sutures made from PHB, with a 2010 market value exceeding US$1.3 billion (Gumel, Annuar, & Chisti, 2013). Blends of PHA polymer have successfully been used in packaging materials, disposable commodities, and controlled-release agents for medicines or pesticides (Reddy et al., 2003). With continued research focused on reducing PHA cost, future applications of PHA products will provide a renewable, biodegradable, non-toxic replacement for many of the worst plastic pollutants currently present as waste in our natural environment.

1.4 Benefits of PHA

Plastic pollution is a global concern that can be partially remedied with the substitution of PHA for petrochemical plastics. Due to its biodegradable nature, PHA can help reduce pollution worldwide. In this regard, one of the worst affected ecosystems is the world's oceans, many marine mammals are being killed through entanglement with or ingestion of plastics (Eriksen et al., 2013). Swirling ocean currents create natural collection points called gyres. These gyres are showing increasing concentrations of waste, with most material being petrochemical-derived plastics. In these gyres, peak plastic concentrations range from 732 g per km^{-2} in the south Pacific, to 5,100 g per km^{-2} in the northern hemisphere

(Moore, Moore, Leecaster, & Weisberg, 2001). It is in these environments where the beneficial effects of a biodegradable plastic could have the greatest impact. In a marine environment PHA has been shown to degrade to 70% of dry mass in only 49 days (Thellen et al., 2008). If PHA's replace petrochemical plastics, garbage patches could be cleaned and eventually become a minimal threat.

Beyond reducing the volume of plastic in the ocean, PHA can also be used to reduce the world oil demand from plastic production and in so doing reduce CO_2 production (Gurieff & Lant, 2007); this is discussed in more detail in the "Costs and Life Cycle Comparisons of PHA Production" section. PHA is also useful when dealing with solid wastes. For example, PHA garbage bags would allow landfills to operate more efficiently by degrading faster and allowing bacteria open access to organic waste inside the bags.

1.5 Costs and Life Cycle Comparisons of PHA Production

Currently, commercial PHA commodity price is approximately 4-9 times that of petrochemical plastics (Serafim, Lemos, Albuquerque, & Reis, 2008), the high cost is driven by an expensive production process. At this time PHA is produced commercially using pure substrates, such as acetic acid or corn-derived sugar, and pure microbial cultures (wild or genetically modified). The feedstock is a primary expense, accounting for up to 40% (Dias et al., 2006) of the production cost, while maintaining axenic conditions coupled with downstream processing (e.g., PHA extraction from the microbial biomass) account for the remaining 60%. Opportunities exist in both areas to improve efficiencies and reduce the costs associated with PHA production. However even with improved operation efficiency the commodity price will still be very high and the pure substrate and mono-culture approach is not currently viewed as an economically viable petrochemical plastic replacement. PHA production on waste substrates has not yet been achieved commercially. However, waste substrates will reduce the substrate cost considerably (possibly completely) and could provide the key to an economically viable PHA product (Gurieff & Lant, 2007).

The potential environmental benefit of PHA use is often assessed through Life Cycle Assessments (LCA's). Many LCA's have been conducted comparing various petroleum-derived plastics to both pure culture PHA and waste feed PHA cultures (Akiyama, Tsuge, & Doi, 2003; Gerngross, 1999; Gurieff & Lant, 2007; Harding, Dennis, von Blottnitz, & Harrison, 2007). LCA's are a convenient tool for comparing products and processes, in that LCA attempts to calculate a product's life time costs; these costs can be energy consumption, monetary cost, and/or environmental costs. A typical LCA will attempt to assess the costs associated with a product from raw materials through to end of life processing in an attempt to create unbiased comparisons of various products. Environmental LCA's often attempt to calculate carbon dioxide release or consumption associated with a production, use, and disposal of a product.

As related to PHA, Gerngross (1999) completed an energy-focused LCA to compare PHA with polystyrene, a common petrochemical that is a candidate for replacement by PHA. His work demonstrated that a monoculture (pure culture) fed corn glucose will consume more fossil fuel per kg of PHA produced than would be consumed by a polystyrene production process (Gerngross, 1999). The pure culture fed corn glucose would approximately consume 2.39 kg fossil fuel/kg PHA, while polystyrene consumes 2.26 kg fossil fuel/kg polymer. The analysis demonstrated the high environmental cost (measured in fossil fuel equivalents) of both substrate and sterilization; the two accounted for 40% of the 2.39 kg of fossil fuel. Downstream processes accounted for 38%, while the biological process (aeration and mixing) consumed 22% of the fossil fuel.

In contrast to the study by Gerngross (1999), a LCA performed by Gurieff and Lant (2007) compared a mixed consortium PHA ($_{MC}$PHA) reactor fed an unidentified industrial waste that had been reduced to VFA's through acidogenic fermentation. In this study both financial costs and environmental impacts (measured in CO2 equivalents, CO2eq) were used as comparison standards. $_{MC}$PHA and High Density Polyethylene (HDPE) were directly compared, with a LCA performed by Akiyama (2003) on pure culture PHA was used as a reference (Akiyama et al., 2003). According to this analysis, the HDPE exhibited the largest

environmental cost at 25.2 kg CO_2eq/kg Polymer while pure culture PHA production was estimated at 15.3 kg-eq/kg PHA and $_{MC}$PHA was estimated at 20.4 kg-eq/kg PHA. The larger environmental cost of $_{MC}$PHA relative to pure culture production was due to lower cell densities requiring larger reactors, more energy required for mixing, and increased aeration demands (an issue relevant to this thesis). Energy production, principally for aeration, accounted for 40% of the non-renewable CO_2 equivalents released by the $_{MC}$PHA process. CO_2eq were released during aeration due to the energy for aeration being supplied by coal power plants, which produce a large quantity of CO_2 per watt produced. Use of green power (i.e., wind, solar, or combustion of anaerobic digester gas) will enable the $_{MC}$PHA process to reduce CO_2eq, ultimately producing fewer CO_2eq then pure culture PHA production. After energy use the next highest source of CO_2eq was substrate, $_{MC}$PHA released a negative CO_2eq because the substrate required little preparation and the production of PHA treated the waste reducing the environmental impact of the waste. Pure culture PHA requires agricultural products that must be highly purified releasing a large mass of CO_2eq in the process.

$_{MC}$PHA was financially more favorable when compared to pure culture PHA production, as demonstrated by Gurieff and Lant (2007). Financial cost was assessed using 2005 $USD and a price per kg of polymer. Gurieff and Lant (2007) assumed a 25% internal rate of return (IRR) was necessary to attract investors to this as yet untested technology. HDPE costs $1.85 per kg, while pure culture-derived PHA costs $4.33 per kg or $1.68 at a 10 year breakeven cost and $_{MC}$PHA $3.52 per kg ($0.87 breakeven). This analysis assumed a $_{MC}$PHA mean cell density of 6 g/L in the PHA production reactor and a mean PHA content of 60% on a dry wt. basis (g PHA/g dry matter). The pure culture method assumed 90 g/L final mean cell density and mean PHA content of 90% on a dry wt. basis. Although $_{MC}$PHA proved financially more favorable, the combined environmental and financial LCA identified pure culture PHA production would be the most competitive with HDPE, and also produce the fewest CO_2 equivalents. If an IRR lower than 25% was deemed acceptable, then both $_{MC}$PHA and pure culture could be very competitive with the HDPE market. Gurieff and Lant (2007)

demonstrated the necessity for $_{MC}$PHA processes to reduce the environmental footprint, in this instance aeration rate.

1.6 Viable Carbon Sources

Carbon molecules form the backbone to PHA and are necessary for both polymer production and bacterial growth. There are a wide range of sources which can supply acceptable carbon. These sources can be valuable products (i.e., sugars, food crops) to low/zero value wastes (i.e., manure, food processing waste). As noted, current commercially produced PHA uses a pure culture to produce PHA from a highly refined and valuable substrate (Harding et al., 2007). These substrates are typically agricultural based sugars or starches, which require purification and processing and add considerably to the cost and environmental footprint of PHA production. The additional costs associated with food-grade substrate can be as much as 50% of the total cost of PHA production (Table 1) (Reddy et al., 2003). Due to the high cost of the substrate, research into commercial PHA production has started to focus on biodegradable waste streams with a high organic content coupled with a $_{MC}$PHA system. In fact, many agricultural by-products and some industrial wastes have the necessary organic carbon content to be suitable for $_{MC}$PHA production. The ideal waste products would exhibit high VFA concentrations (either directly, or as a precursor such as simple carbohydrates), as this substrate is very quickly consumed by bacteria which is important to the PHA enrichment strategy. For carbohydrate-rich waste substrates, acidogenic fermentation is commonly used to maximize production of VFA's. Acidogenic fermentation allows many agricultural and industrial wastes to be utilized as substrate in biomass enrichment and production of PHA. Some of the waste carbon sources used in PHA processes include: dairy waste, municipal waste, bananas, damaged food grains, pea shells, apple pomace, palm oil mill effluent, and tomato cannery waste (Reddy et al., 2003). The work herein utilizes dairy waste; it is carbon rich and is universally available around the world.

Table 1: Substrate Cost, Yield, and Production.
(Reddy et al. 2003)

Substrate	Substrate price (US$ kg^{-1})	P(3HB) yield (g P(3HB) (g substrate)$^{-1}$)	Product cost (US$ (kg P(3HB))$^{-1}$)
Glucose	0.493	0.38	1.30
Sucrose	0.290	0.40	0.72
Methanol	0.180	0.43	0.42
Acetic acid	0.595	0.38	1.56
Ethanol	0.502	0.50	1.00
Cane molasses	0.220	0.42	0.52
Cheese whey	0.071	0.33	0.22
Hemicellulose hydrolysate	0.069	0.20	0.34

1.7 PHA Physical Properties

The physical properties of PHA are of critical interest to the commercial market. PHA shares many mechanical and thermal properties with polyethylene (PE) and polypropylene (PP) while still retaining its biodegradation properties (Thellen et al., 2008). The tensile strength (40MPa) and Young's Modulus (3.5 GPa) of typical PHB and PHBV (Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) another common PHA polymer) films are comparable to PP. One area of critical interest is elongation to break, a test that measures how much length a material will stretch (increase) before breaking. Typical pure PHB films have an elongation to break of 6% for which is very low compared to ~400% achieved by PP films. The elongation to break can be increased through increased HV content, thereby improving the application potential of the films. One key benefit PHB and PHBV films have over other polyolefin films is an improved oxygen barrier (Thellen et al., 2008). This is an important parameter in the food packaging industry, as the presence of oxygen can lead to food spoilage.

Physical properties of $_{MC}$PHA can vary according to hydroxyvalerate (HV) content. Literature does not widely report the physical properties of mixed culture PHA (Laycock, Halley, Pratt, Werker, & Lant, 2013). Reports that have published the physical properties show the waste derived polymer is suitable for industrial application (Dobroth, Hu, Coats, & McDonald, 2011). Contrary to waste derived polymers, commercial and pure culture PHA monomer and polymers have been reviewed (Braunegg, Lefebvre, & Genser, 1998; Laycock et al., 2013; Reis et al., 2003). The mechanical and thermal properties of a PHA polymer are dependent on both the synthesis of the polymer and processing of the polymer. Synthesis

of the polymer affects the molecular weight of the polymer, which affects mechanical properties such as stiffness, strength, and toughness (Kim & Zukoski, 2013). If the molecular weight of a polymer is too low the commercial applications are limited. Both PHB and PHBV have molecular weight ranging from $1x10^4$ to $3x10^6$ g/mol (Sudesh, Abe, & Doi, 2000). The degree of crystallinity in the polymer also affects physical properties such as elongation to break and Young's modulus. Crystallinity is most affected by the processing of PHA polymer, specifically the temperature of the extrusion processes (Thellen et al., 2008). Most PHB and PHBV polymers fall within a range of 55-80% crystallinity. A higher HV content polymer helps reduce crystallinity and improve physical properties such as elongation to break (5% for PHB and 50% for HV content of 20% (Sudesh et al., 2000)). Increased HV content also helps improve oxygen and water vapor barrier properties of the polymer (Thellen et al., 2008).

Table 2: Properties of PHA Polymers.
(Sudesh et al., 2000)

Sample	Melting temperature (°C)	Glass-transition temperature (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	180	4	3.5	40	5
P(3HB-*co*-20 mol% 3HV)	145	−1	0.8	20	50
P(3HB-*co*-6 mol% 3HA)[a]	133	−8	0.2	17	680
Polypropylene	176	−10	1.7	38	400
Low-density polyethylene	130	−30	0.2	10	620

[a] 3HA units: 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxyoctanoate (<1 mol%), 3-hydroxy-*cis*-5-dodecenoate (<1 mol%).

1.8 Biodegradation of PHA

As noted, one of the real values of using PHA as a substitute for conventional petro-plastics in commercial applications is the ability to biodegrade. PHA has been shown to be highly biodegradable in many natural environments; for example soil, lake water, sea water, compost, sewage sludge, and anaerobic digestion (Braunegg et al., 1998; Reddy et al., 2003; Sudesh et al., 2000; Thellen et al., 2008). The rate of degradation is a critical concern when considering pollution/environmental effects of PHA materials. While complete degradation of PHA is typical in all environments, the rate of degradation is strongly influenced by the

environment. Even in the biologically unfavorable conditions of Lake Lugano, Switzerland, where temperatures did not exceed 6^{o}C, 85% degradation by dry weight of PHA film was achieved in 254 days (Reddy et al., 2003). In more favorable conditions the rate of degradation is much higher. For example, Thellen (2008) reported 88-99% degradation by dry weight in only 49 days under ideal static laboratory condition listed in ASTM D6691. Thellen (2008) went on to examine degradation in sea water by simulating dynamic ocean conditions. The simulation continuously pumped sea water from Woods Hole, Massachusetts into the reactor to allow fluctuating sea condition to be replicated in the laboratory setting. Under these conditions, the 49 day degradation ranged from 33-50% on a dry weight basis. The fluctuation of temperature, pH, and bacterial load of the dynamic experiment were all considered possible limiting factors in PHA degradation. The degradation rate can also be affected by the physical characteristics of the PHA. The most significant characteristics are degree of crystallization and polymer composition (Thellen et al., 2008). When PHA is present outside of the cell the degradation process proceeds through enzymatic hydrolysis. Water insoluble PHA is broken down in two steps. First the enzyme must be adsorbed onto the film surface through the binding domain of the enzyme. Second the polymer must be hydrolyzed into water soluble monomers and oligomers which can be transported into the cell for utilization as a carbon source (Sudesh et al., 2000). The same steps apply if the PHA product is broken down through activated sludge systems. This would reduce solid waste and act as a means to “recycle” the carbon in PHA (Harding et al., 2007; Rostkowski et al., 2012).

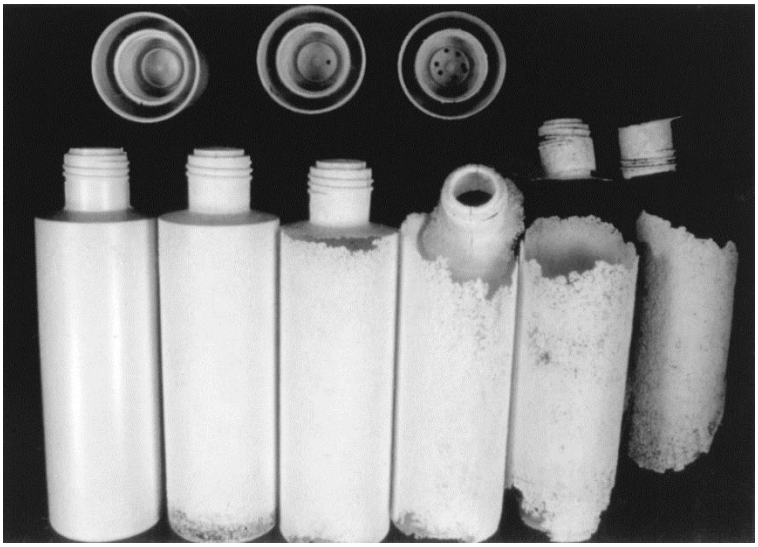


Figure 2: P(3HB-3HV) Degradation in Aerobic Sewage Sludge.
Left to Right: 0, 2, 4, 6, 8, and 10 Weeks of Treatment. (Madison & Huisman, 1999)

1.9 Summary

Biodegradable products are fast gaining popularity, however most products still rely upon natural resources (i.e. food crops, trees, or oil derived plastics). However, the bacterial product PHA is unique, because it can be produced with little to no use of nature resources. PHA can be biologically synthesized from waste streams, which not only recycle the carbon, but also reduces pollution from the utilized waste stream. In order for PHA to gain ground against more common oil derived plastics in the market it must be produced in both an economically and financially sustainable manner. Current market practices do not produce large scale quantities of PHA in a financially or economically sustainable manner. Due to those limitations research interest is turning toward mixed microbial PHA production utilizing waste based substrate. This technology is not yet optimized and is still in development. The work herein is focused on reducing the cost of PHA by reducing the aeration to only what is necessary for the system to operate. Aeration is a direct financial burden (due to power input) to the PHA process it is estimated to constitute 40% of the total biological phase cost (Akiyama et al., 2003). A secondary benefit of the reduction in aeration comes from enhanced nitrogen removal achieved through microaerophilic (low residual dissolved oxygen) conditions.

Chapter 2: Review of Relevant Bacterial/Biochemical Processes

Biological systems are extremely complex, with many processes, cycles, and reactions simultaneously occurring to achieve a desired result. Environmental engineers have the delicate task of combining the highly structured and controlled knowledge of microbiology to the loosely defined and often changing environment of waste treatment. This requires a background understanding of the microbial processes, and a solid grasp of the variability encountered in a real waste stream. This chapter is focused on the biochemical and bacterial processes that relate to the research presented and discussed herein. While actual measurements and analysis at this level of detail were not performed, the review herein is for background information.

2.1 Terminal Electron Acceptors

Energy is a basic requirement for all life; bacteria can either generate energy from the sun (phototrophs) or chemical oxidation reactions (redox reactions performed by chemotrophs). To produce energy, a chemical oxidation reaction requires an electron donor, often referred to as substrate, and an electron acceptor. Electron acceptors can take many forms; Table 3 lists several common electron acceptors and the general categories of bacteria that use them (Tchobanoglous, Burton, Stensel, Metcalf, & Eddy, 2003). The amount of energy derived from the redox reaction can be predicted thermodynamically by the Gibbs free energy change. A visual technique used to understand the energy release is to line up reductants and oxidants in a column with highest reducing power on top and strongest oxidants on the bottom; this is known as the “electron tower” (Figure 3). The difference in potential between the electron donor and electron acceptor indicates the potential energy contained in the reaction (Madigan & Martinko, 2006). However, Gibbs free energy is still required to calculate the energy released. Not all bacteria are capable of using all electron acceptors; for example obligate aerobes can only use oxygen while facultative aerobic bacteria are capable of using nitrate/nitrate when oxygen concentrations are low or not present.

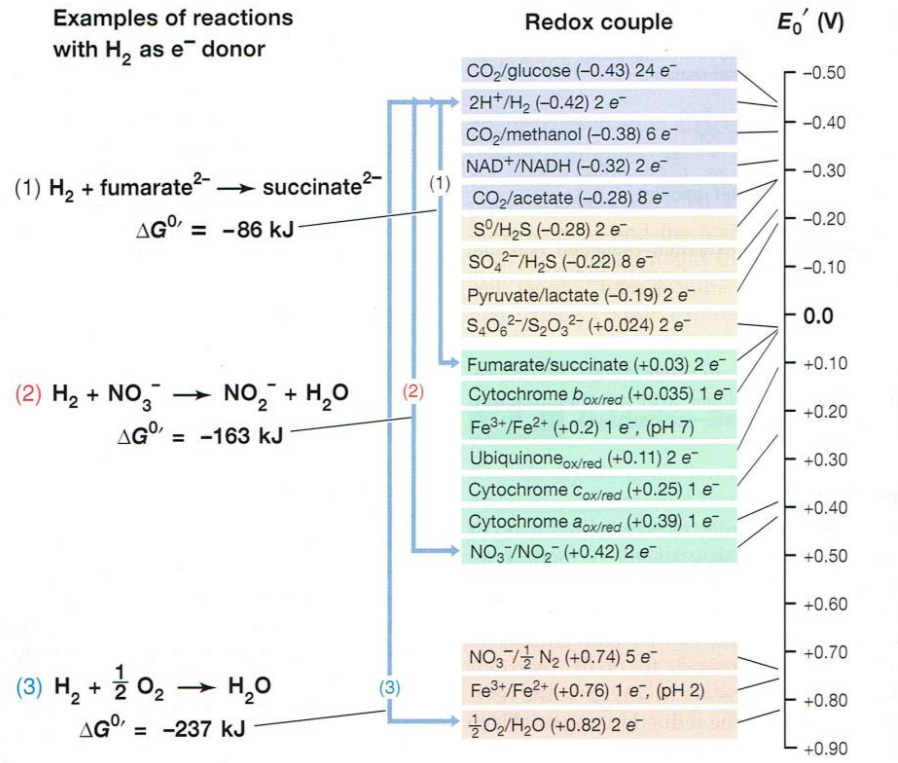


Figure 3: Electron tower
(Madigan & Martinko, 2006)

The resource recovery process employed herein to produce PHA enriches for two bacterial consortia. The fermenter and anaerobic digester illustrated in Figure 1 are dominated by anaerobic heterotrophs. These bacteria can utilize various organic and inorganic compounds (e.g., CO_2, Fumarate) as terminal electron acceptors; the by-product of these reaction are volatile fatty acids, or methane (Tchobanoglous et al., 2003). Conversely, the PHA enrichment and production reactors are aerated reactors; therefore, oxygen is the primary terminal electron acceptor. However, if residual oxygen concentrations are low, facultative heterotrophic bacteria may utilize nitrate and/or nitrite as a terminal electron acceptor.

Table 3: Bacteria Classification Based on Carbon Source and Electron Acceptor. (Tchobanoglous et al., 2003)

Type of bacteria	Common reaction name	Carbon source	Electron donor (substrate oxidized)	Electron acceptor	Products
Aerobic heterotrophic	Aerobic oxidation	Organic compounds	Organic compounds	O_2	CO_2, H_2O
Aerobic autotrophic	Nitrification	CO_2	NH_3^-, NO_2^-	O_2	NO_2^-, NO_3^-
	Iron oxidation	CO_2	Fe (II)	O_2	Ferric Iron Fe (III)
	Sulfur oxidation	CO_2	H_2S, S°, $S_2O_3^{2-}$	O_2	SO_4^{2-}
Facultative heterotrophic	Denitrification anoxic reaction	Organic compounds	Organic compounds	NO_2^-, NO_3^-	N_2, CO_2, H_2O
Anaerobic heterotrophic	Acid fermentation	Organic compounds	Organic compounds	Organic compounds	Volatile fatty acids (VFAs) (acetate, propionate, butyrate)
	Iron reduction	Organic compounds	Organic compounds	Fe (III)	Fe (II), CO_2, H_2O
	Sulfate reduction	Organic compounds	Organic compounds	SO_4	H_2S, CO_2, H_2O
	Methanogenesis	Organic compounds	Volatile fatty acids (VFAs)	CO_2	Methane

2.2 NADPH vs. NADH

NADH and NADPH are both intracellular electron carriers for microbial cells. NADH is used by bacterial cells for energy production (catabolic processes), and NADPH is used for biomacromolecule synthesis (anabolic processes). While the two electron carriers serve different purposes, uniformity does not exist in the literature on the metabolic model of the PHA production system. Some authors choose to show NADH (NAD+) (Nicotinamide Adenine Dinucleotide) when modeling PHA synthesis associated with the reduction of CoA (Third, Newland, & Cord-Ruwisch, 2003; vanAalastvanLeeuwen, Pot, vanLoosdrecht, & Heijnen, 1997), while others show NADPH (NADP+) (Nicotinamide Adenine dinucleotide Phosphate) (Dias et al., 2008),(Reis et al., 2003). There is no functional difference between the two enzymes; both are a means of storage and conveyance to supply reducing power within the cell. The difference lies in the processes and pathways that synthesize and utilize the two enzymes. Due to the lack of a functional difference, this over-simplification is often acceptable on models that assume a constant pool of NADPH/NADH is readily available in the cell (Third et al., 2003; vanAalastvanLeeuwen et al., 1997).

The main function of NADPH and NADH is to enable normally energetically unfavorable reactions to proceed in an efficient manner. Both molecules have a reduction potential of -0.32V on the electron tower, and therefore both are good electron donors. Functionally both molecules achieve the same result of transporting two electrons and two hydrogen atoms to intracellular reactions. One difference between the two is the synthesis pathways. NADH is typically produced in the catabolic TCA cycle during the synthesis of ATP. NADPH is produced in the pentose phosphate pathway, or enzymatically via a transhydrogenase. Although the pentose phosphate pathway is not the exclusive means of producing NADPH, most cells have mechanisms to convert NADH to NADPH (Madigan & Martinko, 2006). The second difference is where the enzymes are utilized. NAD+ is utilized in energy generation (catabolic) reactions, such as proton motive force and beta oxidation of fatty acids. NADPH is utilized in the production of biosynthetic (anabolic) reactions, such as PHA or Glycogen. The additional phosphorus in NADPH allows it to bind to a different set of coenzymes thus allowing a cell to "control" where the reducing potential is being used.

2.3 Beta-Oxidation

Bacteria rely on beta oxidation (Figure 4) to oxidize volatile fatty acids (VFA's) to shorter chain VFA's and acetyl-CoA. The beta oxidation pathway is important in this research, as short-chain VFA's (2-5 carbon) are a primary substrate for mixed culture PHA reactors. The research herein relies upon VFA's produced in a dairy fermenter to provide substrate for the PHA process. Beta oxidation is a repetitive process, and each cycle will break 2 carbons from longer chain VFA's (i.e., butyrate, valerate, and caproic acid), until acetate (2 carbon) or propionate (3 carbon) is reached. First the VFA is activated with coenzyme A, which results in the activation of acetyl-CoA. Next electrons are transferred to Flavin-adenine dinucleotide (FAD) in a dehydrogenation reaction. Third the electrons are transferred to NAD+ in the second dehydrogenation reaction (creating NADH). Finally the acetyl-CoA is cleaved off, to be consumed in the Tricarboxylic Acid Cycle (TCA) or (preferably) condensed to other intracellular intermediates such as PHA (Madigan & Martinko, 2006).

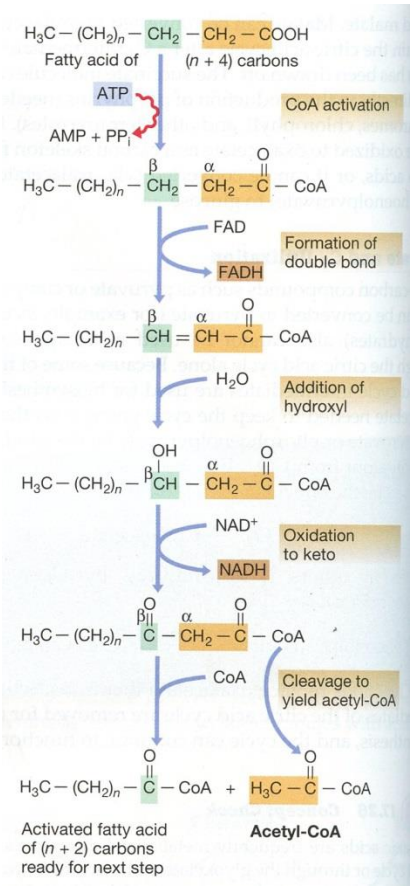


Figure 4: Beta Oxidation Diagram. (Madigan & Martinko, 2006)

2.4 Tricarboxylic Acid Cycle

The Tricarboxylic Acid Cycle (TCA) is the main mechanism bacteria utilize to produce reducing equivalents (NADH) for energetic needs. The cycle begins with the decarboxylation of a carbon compound (pyruvate), producing one molecule of NADH and Acetyl-CoA. The acetyl-CoA combines with the four carbon compound oxaloacetate yielding citric acid. The cyclical process produces cellular intermediates and reducing equivalents NADH and FADH. It also produces several important biosynthetic compounds such as α-ketoglutarate and oxaloacetate amino acid precursors (Madigan & Martinko, 2006). A lack of intermediates for the TCA cycle is one theory for the production of PHA in the non-nutrient limited conditions of the waste substrate $_{MC}$PHA reactors used in this work, this is discussed in more detail in Section 2.8.2 Aerobic Dynamic Feeding Metabolism3.1 ADF-Enrichment Reactor.

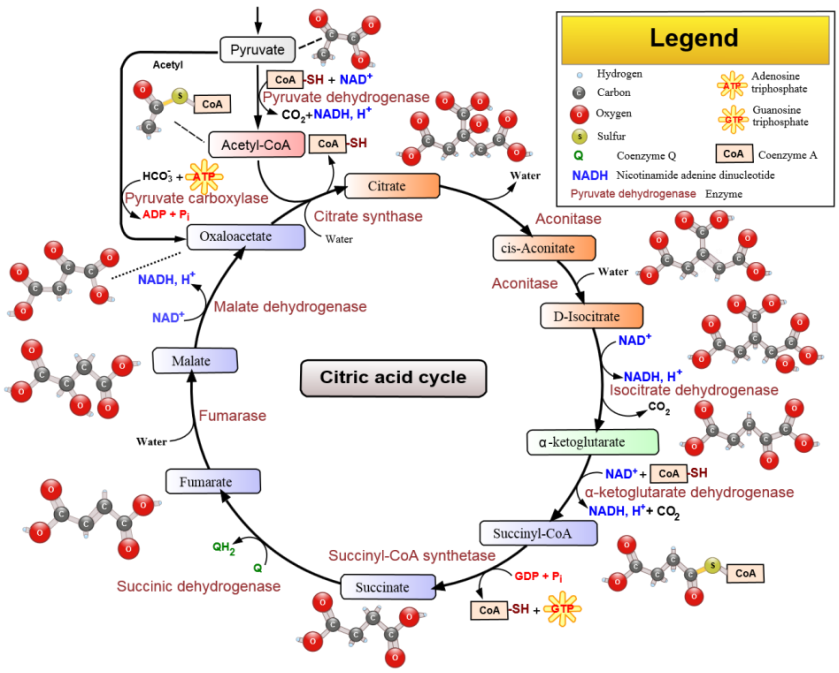


Figure 5: Citric Acid Cycle/TCA Cycle.
(Citric Acid Cycle by: Narayanese, CC BY-SA 3.0)

2.5 Pentose-Phosphate Pathway

The Pentose-Phosphate Pathway (PPP) is important to the PHA process, as it produces the reducing equivalent NADPH necessary for the synthesis of PHA as well as other intermediates necessary for growth. The PPP requires glycolysis to produce glucose 6-phosphate from glucose. The glucose 6-phosphate is then converted into pentose which generates NADPH and ribulose-5-phosphate. Ribulouse-5-phosphate is utilized in the formation of nucleic acids, precursors for deoxyribose nucleic acid (DNA). The PPP also generates sugar derivatives which are used in bacterial growth. If glucose is not present in solution it can typically be derived from polysaccharides. Starch and cellulose are common polysaccharides (Madigan & Martinko, 2006) found in dairy manure. Alternately, bacteria can also process pyruvate backward through the glycolysis pathway (referred to as gluconeogenesis) to provide substrate for the PPP.

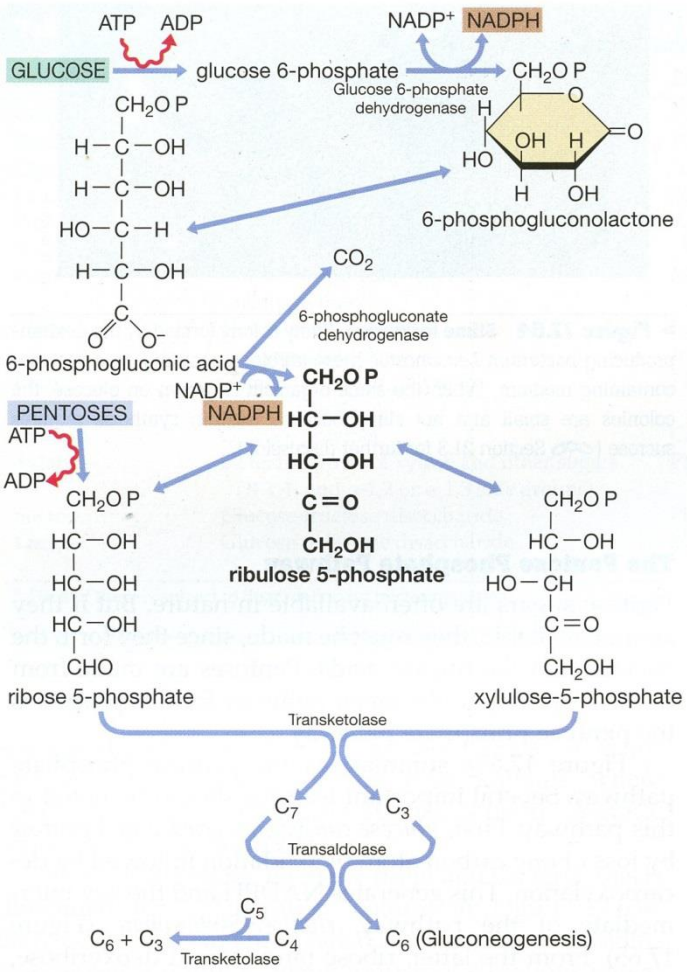


Figure 6: Pentose Phosphate Pathway.
(Madigan & Martinko, 2006)

2.6 Oxidative Phosphorylation

Under respirative conditions, oxidative phosphorylation is the mechanism which produces ATP (Adenosine Triphosphate). ATP is the principle energetic reserve for almost all biological activity. ATP is synthesized using energy captured and generated via the proton motive force (PMF). The PMF is generated via redox reactions inside the microbial cell, with captured electrons transported within the cell membrane and ultimately transferred to a terminal electron acceptor (TEA). When the TEA is exogenously provided (i.e., oxygen, nitrate), a PMF is generated when protons are concurrently pumped outside the cell wall. Protons re-enter the cell via the ATP Synthase mechanism, generating ATP from inorganic

phosphate plus ADP (Adenosine Diphosphate); ATP synthase is a membrane bound protein complex (Madigan & Martinko, 2006). In the absence of a suitable external electron acceptor, oxidative phosphorylation will not proceed. Electron donors can be either external substrate or stored interal substrates such as PHA.

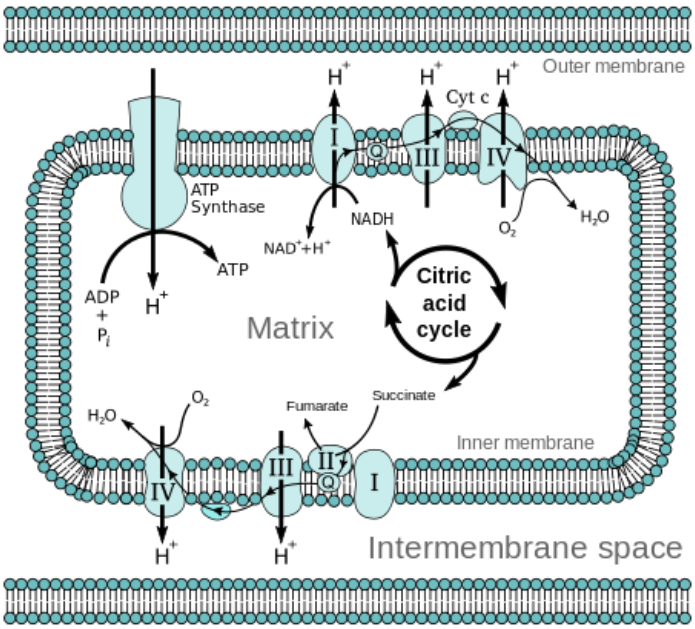


Figure 7: Oxidative Phosphorylation.
(Oxidative Phosphorylation by: Tim Vickers – CC)

2.7 Acidogenic Fermentation

Within the context of the PHA process being studied in this research, the primary objective of acidogenic fermentation is the conversion of biodegradable organic matter into volatile fatty acids (VFA's). The main difference between a fermentation system and anaerobic digestion is the solid retention time SRT. Specifically, SRT is used to control the bacterial population present in the reactor (see Figure 8). The bacteria in the reactor hydrolyze complex carbohydrates, proteins, and lipids, ultimately fermenting the substrate into VFA's (see Figure 9). In reactors with longer SRT's (greater than 4 days at 35^{o}C (Grady, Daigger, Love, & Filipe, 2011)) a different set of microorganisms (methanogenic archaea) convert the VFA's into methane and carbon dioxide. By reducing the SRT, the slower growing methanogenic organisms are not capable of growing to a critical mass in the system and are

washed out. This allows the process to significantly increase the VFA concentration in the reactor. Some methane is produced by the faster growing hydrogen oxidizing methanogens (hydrogenotrophs), which consumes exogenous VFA's. However the hydrogenotrophic activity is useful to the processes as it prevents the hydrogen partial pressure from exceeding inhibitory levels. Anaerobic oxidation of long chain fatty acids into smaller chain fatty acids (i.e., propionate, butyrate; VFAs important to PHA production) will be impaired as hydrogen partial pressures increase. Thus hydrogen oxidizing methanogens actually aid the acidogenic fermentation process by maintaining lower hydrogen partial pressures.

The second difference between fermentation and AD is the operational temperature. Bacterial kinetics increase or decrease with changes in temperature; typically kinetics increase with increased temperature. Fermentative bacteria exhibit considerably faster growth rates than methanogenic bacteria, and this difference in growth rate increases as temperatures decrease. Therefore a fermenting reactor that is not heated can allow for an increase in the SRT without suffering from methanogenic bacteria consuming VFA's. This allows more time for hydrolysis to occur; however a longer SRT also requires an increase in tank volume, therefore an optimization between SRT, heating cost, and capital cost would be necessary to optimize VFA production while minimizing cost. Figure 8 demonstrates the biochemical conversions occurring in a reactor as SRT is extended, the reactor modeled in the diagram was maintained at a constant temperature of 35^{o}C (Grady et al., 2011).

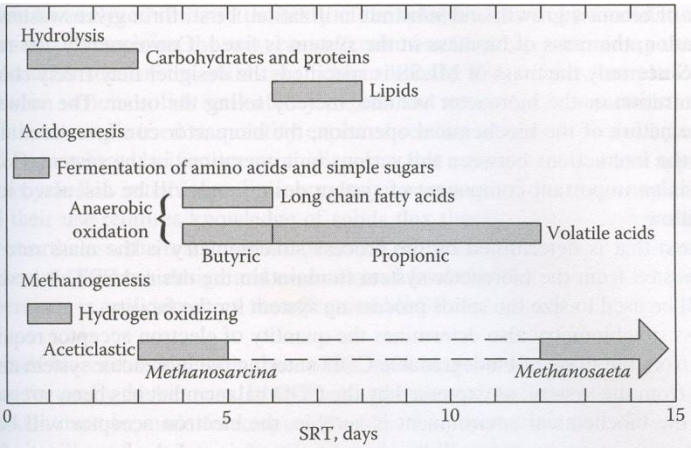


Figure 8: Biochemical Conversions Based on SRT.
(Grady et al., 2011)

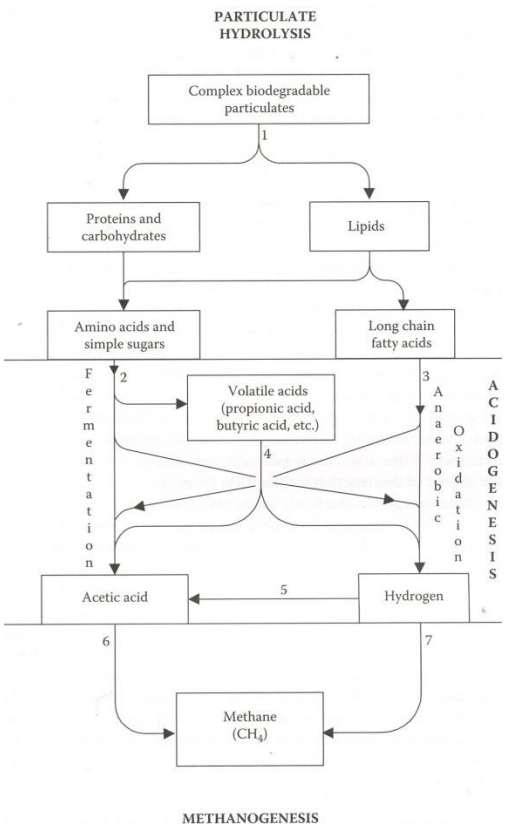


Figure 9: Multistep Methanogenic Processes.
(Grady et al., 2011)

2.8 Metabolic Pathways associated with PHA Production

Mixed microbial consortia are known to store PHA when cultured under "stressful" conditions. The two most common methods used with mixed microbial consortia to induce "stress" and associated PHA storage are (i) Anaerobic/Aerobic (An/Ae) cycling and (ii) Aerobic Dynamic Feeding (ADF). The Ae/An process induces PHA synthesis due to a lack of terminal electron acceptor (oxygen, nitrate, nitrite), with the most common application in the liquid-stream wastewater treatment process known as biological phosphorus removal (BPR) (Filipe, Daigger, & Grady, 2001; Schuler & Jenkins, 2003; vanAalastvanLeeuwen et al., 1997). Conversely, the ADF process applies a selective pressure to the microbial culture by inducing cycles of carbon feast (electron donor surplus) and carbon famine (lack of external electron donor; ultimate depletion of electron donor), all while ensuring sufficient terminal electron acceptor is available (oxygen, nitrate, nitrite). In both Ae/An and ADF, bacteria

capable of storing carbon as PHA sustain a longer period of growth, which gives them a competitive advantage. Thus, both process configurations can enrich for a PHA producing consortium. The exact metabolism for PHA production by bacteria in an ADF system is not well understood. Most theories have their origins in the better researched Ae/An systems, therefore this review covers both to provide a clearer understanding of the various PHA metabolic pathways.

Current commercial production of PHA utilizes pure cultures of wild or genetically modified organisms. In this process the culture is grown to a critical density, typically cell density can exceed 90 g/L (Gurieff & Lant, 2007). The culture is then subjected to growth limiting conditions to induce PHA synthesis. The most common growth limitation applied is a deficiency in the macro nutrients nitrogen and/or phosphorus. This growth limited approach is also popular for mixed microbial consortium being feed synthetic waste, or a waste substrate that is nutrient deficient.

2.8.1 Anaerobic/Aerobic PHA Metabolism

The An/Ae cyclical pattern enriches for a bacterial consortium capable of anaerobic carbon uptake and storage as PHA. Two general categories of bacteria can achieve anaerobic carbon uptake, Glycogen Accumulating Organisms (GAO's) and Polyphosphate Accumulating Organisms (PAO's). The PAO fraction relies on glycogen and polyphosphate stores to produce ATP and reducing equivalents, while polyphosphate cycling anaerobically and aerobically ultimately achieves excess phosphorus removal. GAO's rely only on glycogen for ATP and reducing equivalents, and do not achieve phosphorus removal.

To achieve BPR, substrate with a large quantity of readily biodegradable substrate is required. Under anaerobic conditions both PAOs and GAOs will transport acetate and other VFA's across the cell membrane, utilizing stored energy in the form of polyphosphate and glycogen. Once across the membrane the VFA's are condensed to Acetyl-CoA (acetate or other even carbon VFA's) or Propionyl-CoA (propionate or other odd carbon VFA's) through β-oxidation. ATP is generated through the hydrolysis of polyphosphate to orthophosphate and catabolization of glycogen. The acetyl-CoA is reduced to synthesize PHA, with the

reducing equivalents provided through glycogen metabolism and the Pentose Phosphate Pathway.

Glycogen can be catabolized through one of two pathways: Entner-Doudoroff (ED) or Embden-Meyerhof-Parnas (EMP) pathway. The differences in these two pathways provide a fundamental difference between PAO's and GAO's. PAO's utilize the ED pathway, which produces 6 ATP per 3 moles glycogen; however, this does not provide enough ATP to meet the energy needs of the cell. Therefore PAO's also rely on polyphosphate consumption to produce an additional 6 ATP for 6 mols of orthophosphate produced. The ED pathway is also capable of producing both NADPH, which is required for PHA synthesis, and NADH, which is required for cell maintenance and ATP production. PAO's use of polyphosphate provides the specific consortium with a unique metabolic advantage. Due to bacteria accumulating excess phosphorus from the influent, the mass of phosphorus in the treatment plant is coupled to the SRT instead of just the mass entering the system. The accumulation of phosphorus will allow the reactor to achieve higher (compared to influent) and more stable concentrations of phosphorus. This increased stability allows PAO's to survive short fluctuations in available carbon better than GAO's, since they have a stable additional source of ATP storage in the form of polyphosphate. The EMP pathway used by GAO's is capable of producing 9 ATP for every 3 mols of glycogen consumed (Maurer, Gujer, Hany, & Bachmann, 1997), making it more efficient than the ED pathway. The EMP pathway, however, only produces NADH, which means the bacteria must use other means to produce NADPH. The two available pathways are the Pentose Phosphate Pathway, which utilizes stored glycogen, or a transfer mechanism capable of switching NADH to NADPH.

Upon entering aerobic conditions the wastewater is low (or depleted) in exogenous organic carbon, but PAO's and GAO's contain a large reserve of stored PHA. The wastewater has high concentrations of inorganic phosphate, while the PAO's are low in stored polyphosphate. As oxygen is now available as an electron acceptor, PAO's utilize the PHA as an electron donor and perform normal aerobic metabolisms for growth. The bacteria also use the ATP generated via the TCA cycle to consume inorganic phosphate for

polyphosphate synthesis, thus replenishing their poly-P reserves. Glycogen is replenished through gluconeogenesis. Due to the stored carbon and energy present in aerobic conditions, the bacteria still have the capacity to grow, which increases the capacity for stored phosphorus consequently ensuring excess phosphorus is removed from the system. The continued cycling between An/Ae provides a competitive advantage to PAO's, because the poly-P stores allow them to consume organic carbon when other heterotrophs are incapacitated by the anaerobic conditions (Grady et al., 2011).

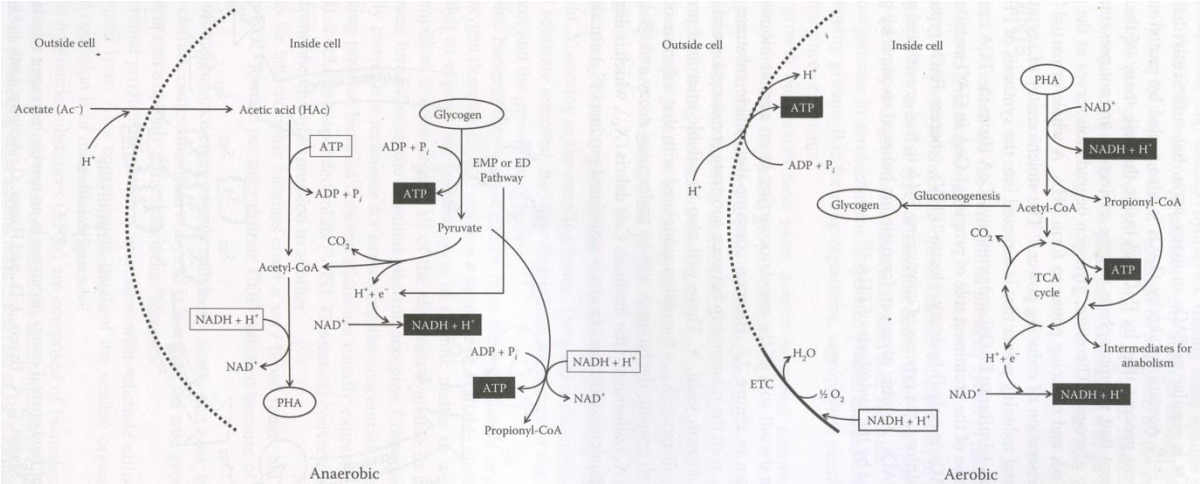


Figure 10: GAO Metabolic Diagram.
(Grady et al., 2011)

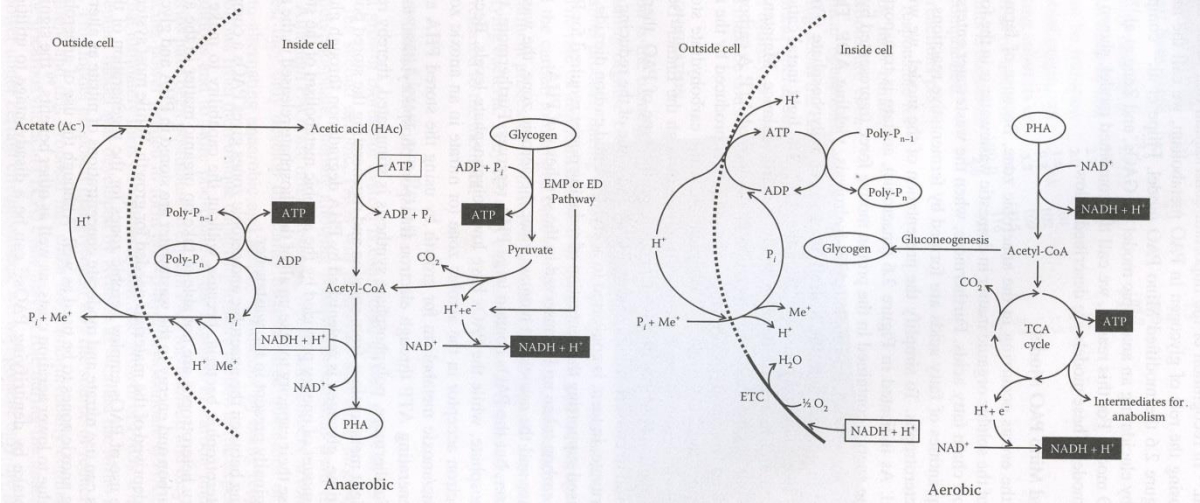


Figure 11: PAO Metabolic Diagram.
(Grady et al., 2011)

2.8.2 Aerobic Dynamic Feeding Metabolism

The ADF metabolism, which is the driving metabolism for $_{MC}$PHA synthesis and thus the core metabolism of interest for this research, is induced by cyclically exposing mixed microbial consortium to cycles of carbon surplus and deficiency, with the end result being an enrichment of bacteria capable of hyper PHA synthesis. Most highly biodegradable carbon substrates are viable for waste fed reactors, although processing may be required depending on the form of carbon. For example, a carbohydrate-rich waste stream would need to be processed through acidogenic fermentation to produce the desired VFA's. Once VFA's are available, the substrate is fed in a single, short-duration pulse to the Enrichment reactor, inducing carbon surplus at the beginning of an operational cycle (batch reactors) or at the inlet of a continuous flow system (i.e., series of continuously stirred tank reactors CSTR, or a plug flow reactor PFR). The period of carbon availability is referred to as the "feast" stage. Once the bacteria have consumed all available external carbon (VFA's), the feast stage has ended and the "famine" stage begins. The length of the feast phase is controlled by the biomass concentration, the specific uptake rate of the carbon substrate, and the mass of carbon substrate. The length of famine cycle is the total cycle length minus the length of feast. The ratio between feast and total cycle length has been identified as a critical design factor for PHA enrichment reactors (Majone, Massanisso, Carucci, Lindrea, & Tandoi, 1996; vanLoosdrecht, Pot, & Heijnen, 1997), and is referred to as Feast to Cycle ratio (Feast/Cycle) (See section 3.1 for more information on Feast/Cycle ratio).

The selection factor applied by ADF is based on carbon available for growth. Bacteria that consume the most carbon will be capable of achieving the most replication and will dominate the culture. However, growth is a relatively slow process, thus bacteria that can utilize storage to increase the carbon uptake rate can realize a competitive advantage over other heterotrophic bacteria. An additional selection factor is the necessity to survive the long famine period. Bacteria will consume available PHA and ultimately degrade much of their bio-machinery to ensure their survival. At the beginning of the subsequent feast phase, bacteria are low in intracellular growth intermediates for processes like the TCA cycle (mainly enzymes and proteins) (Albuquerque, Torres, & Reis, 2010). The lack of

intracellular intermediates delays the start of the growth phase due to time required to generate growth intermediates. This delay further enhances the advantages of PHA synthesis bacteria. The storage of carbon allows the replication process to be delayed until the growth bio-machinery is restored. Once growth starts, PHA storage is often observed to continue simultaneously with the growth processes. It is important to note that due to the mixed consortium and waste feedstock, a small fraction of non-PHA bacteria may survive on carbon from endogenous decay, slowly biodegradable carbon sources, or through bacteria predation. This lack of complete control of the microbial consortium is part of the limitation imposed by open cultures and waste feedstocks on the $_{MC}$PHA technology. However, life cycle analysis proves the limitations are negligible when comparing the benefits of $_{MC}$PHA to pure culture PHA; the cost savings and reduced carbon footprint make $_{MC}$PHA the most viable PHA production means currently available (Gurieff & Lant, 2007).

While exact metabolic pathways in the ADF-enriched consortia remain undefined, it is assumed they are similar to the known pure culture pathways. PHA is produced under growth limiting conditions; in the feast phase of an ADF system it is assumed that a limitation of growth intermediates (i.e., enzymes and RNA), induced by the famine period, initiates the PHA synthesis response (Serafim, 2008). For bacteria to sustain high substrate uptake rates requires excess ATP. The ATP is produced through the TCA cycle and oxidative phosphorylation, which creates a surge of reduced by-products from these processes. Under static conditions, these by-products are consumed through growth and cellular respiration. Due to the limited ability to grow, this overly reduced state can be balanced by PHA synthesis, which is a simple three enzymatic step process (Figure 12). Acetate and propionate substrate are transported into the cell and activated into acetyl-CoA (acetate) or propionyl-CoA (propionate); longer chain VFA's must be oxidized through beta oxidation. This process produces large amount of NADH, creating a potential redox imbalance. As the concentration of acetyl/propionyl-CoA increases in the cell, NADPH sythesis is activated and condenses the acetyl-CoA to hydroxybutyrate. This has the advantage of both consuming excess acetyl-CoA and helping maintain the redox balance in the cell (vanAalastvanLeeuwen et al., 1997)

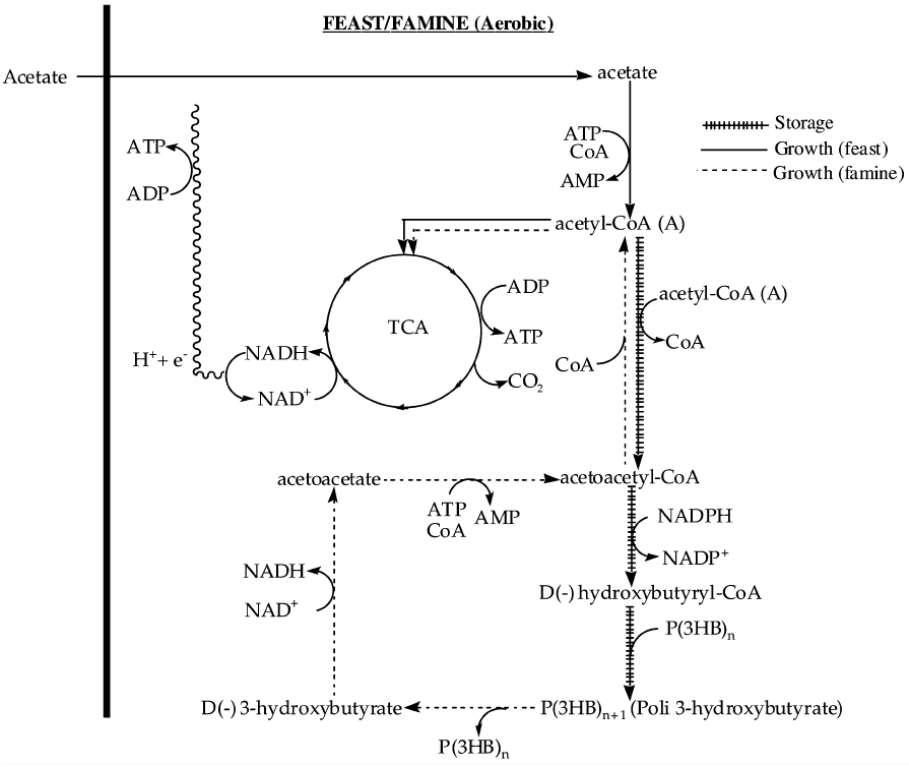


Figure 12: Possible Feast/Famine Metabolic Pathway.
(Reis et al., 2003)

2.9 Nitrification and Denitrification

The processes of nitrification and denitrification are well understood biological processes that are commonly utilized by wastewater treatment plants (also known as Waste Resource Recovery Facilities, or WRRFs) to remove wastewater nitrogen. The process is commonly described as utilizing two loosely defined groups of bacteria under two different environments. The first step is nitrification in which autotrophic bacteria sequester carbon from CO_2 and oxidize ammonia to nitrite (NO_2) then nitrate (NO_3). The organisms responsible for nitrification grow very slowly, requiring extended SRT's, typically in excess of four days to achieve reliable nitrification. Denitrification is a heterotrophic process where bacteria reduce NO_3 to NO_2 to NO to N_2O and finally N_2. Many bacteria are capable of achieving denitrification if a better electron acceptor (i.e. O_2) is not present. Nitrification and denitrification typically requires two separate phases or reactors. The first phase is aerated to allow nitrification to proceed. The second phase is not aerated and residual DO

concentrations should be less than 0.5 mg/L (Tchobanoglous et al., 2003). Because nitrate and nitrite are present, conditions are considered “anoxic” instead of “anaerobic.” The second phase consumes carbon as an electron donor and the nitrate or nitrite as an electron acceptor (Tchobanoglous et al., 2003).

2.10 Aeration Based on Two-Film Theory

This thesis is focused on reducing the aeration cost of the ADF enrichment system; it is thus important to understand the fundamentals behind the mass transfer of oxygen that is occurring. For this research the Two-Film theory was used to explain the adsorption of oxygen into the reactor volume during aeration. While many liquid oxygen transfer models have been proposed, the Two-Film theory has remained popular due to its simplicity and wide range of applications, and its ability to represent phenomena experienced in biological wastewater treatment reactors (Tchobanoglous et al., 2003). As the name implies, the Two-Film theory describes oxygen mass transfer through two films, one gas the other liquid. This model is used for both absorption (the transfer of dissolved gas from a gas phase into the liquid phase) and desorption (the transfer of gas out of solution and into the gas phase). The model is based on the assumption that the liquid and gas volumes are completely mixed, therefore partial pressures in both liquid and gas solutions can be assumed constant. Based on that assumption a mass balance is built on the theory of Fick’s First Law under steady state conditions (Equation 1).

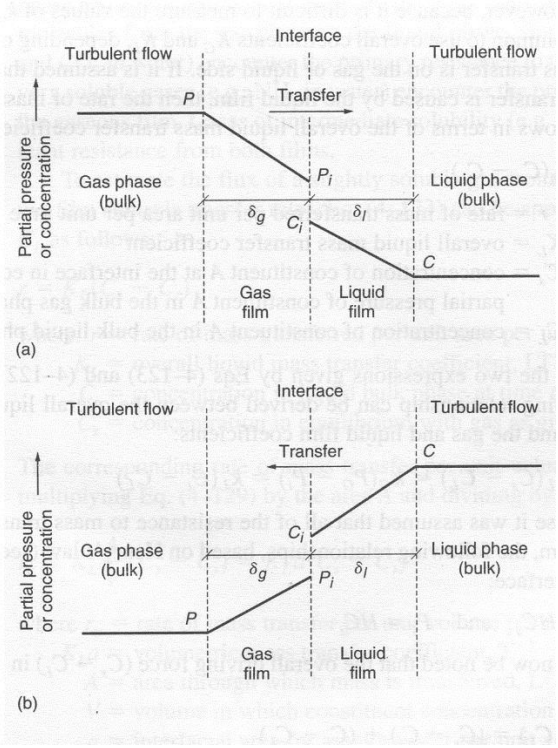


Figure 13: Gas Transfer Models.
(a) Adsorption (b) Desorption (Tchobanoglous, Burton, & Stensel, 2011)

$$\mathbf{r = k_g * (P_g - P_i) = k_L * (C_i - C_L)}$$

Equation 1: Fick's First Law

Where:
r= Rate of Mass Transfer
k_g= Gas Film Mass Transfer Coefficient
P_g= Concentration of constituent in bulk gas phase
P_i= Concentration of constituent in gas film
k_L= Liquid Film Mass Transfer Coefficient
C_i= Concentration in Liquid Film
C_L= Bulk liquid Concentration

Due to the difficulty in measuring the concentration at the liquid and gas film interfaces, it is necessary to assume the resistance to mass transfer is from one film (i.e. liquid or gas film), thus no concentration gradient exist in the other film (Figure 14)(Garcia-Ochoa & Gomez, 2009). In the case of adsorption, the mass balance is written assuming the all resistance is from the gas film. If the application is desorption (stripping) the mass balance is written on

the liquid side. The adsorption assumption has been heavily tested with slightly soluble gases (i.e., N_2, O_2, CO_2) and it has been demonstrated that the majority of resistance is controlled by the gas phase, validating the assumption (Crittenden, Montgomery Watson, & Wiley). In this research the adsorption of oxygen is being modeled, thus, it is assumed all resistance occurs in the gas film which simplifies the above mass balance into Equation 2.

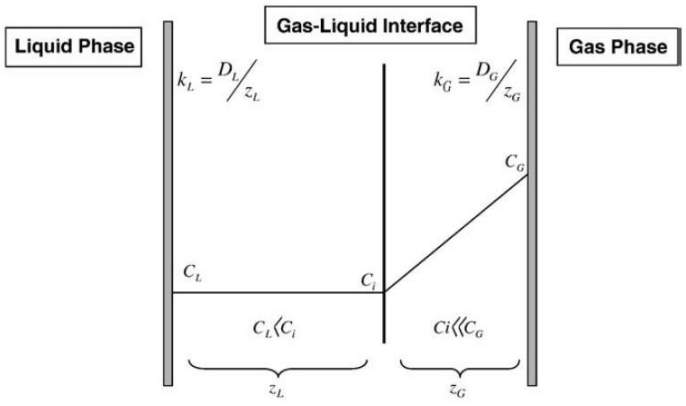


Figure 14: Adsorption model diagram. (Garcia-Ochoa & Gomez, 2009)

$$\mathbf{r = k_L * (C_s - C_L)}$$

Equation 2: Mass balance following gas transfer into solution.

Where:
K_L=Overall Liquid Mass Transfer Coefficient
C_L=Constituent concentration in bulk phase
C_s= Saturation concentration constituent
r= Rate of mass transfer

C_s can be calculated using the gas concentration in equilibrium as given by Henry's Law. C_t is the concentration of the gas at time t. It is often convenient to modify and present the equation in terms of a unit volume and per unit time. This is accomplished by multiplying the equation by A/V (Equation 3).

$$\mathbf{r_v = k_L * \frac{A}{V} * (C_s - C_t) = k_L a * (C_s - C_t)}$$

Equation 3: Mass Balance Corrected for unit Volume and unit Time.

Where:
A= Area through which mass is transferred
V= Volume in which the constituent concentration is increasing
k_La= Volumetric mass transfer coefficient
r_v= Rate of mass transfer per unit volume per unit time

The term k_La is known as the volumetric mass transfer coefficient, and is dependent on water quality, tank geometry, mixing characteristics, and aeration equipment used (Garcia-Ochoa & Gomez, 2009). Numerical values for k_La are determined experimentally, because aeration equipment installation, tank geometries, and mixing characteristics are often unique and difficult to measure on separately for modeling purposes. The transfer of oxygen is a key component of many waste water treatment facilities and as such many methods exist to quantify the k_La parameter at both lab scale and full scale applications (Garcia-Ochoa & Gomez, 2009; Stenstrom, Leu, & Jiang, 2006). See section 4.1 for more information on how k_La was assessed.

Chapter 3: Waste Fed ADF Review

The reviewer is directed to Figure 1, as related to the narrative that follows.

3.1 ADF-Enrichment Reactor

The production of PHA by bacteria has been well documented under growth limiting conditions, with the first work being published in the 1980's (Daigger & Grady, 1982). In the mid 1990's work was published on the use of the Feast/Famine or ADF feeding regime for the enrichment of PHA producing microbial consortiums (Majone et al., 1996; vanLoosdrecht et al., 1997). The purpose of the Enrichment reactor is to select a stable PHA producing consortium, with which to inoculate the Production reactor. The ideal reactor will reliably produce a culture that is highly enriched in PHA producing microbes and achieve very high concentration of PHA in the Production reactor.

Research has not yet focused on a single set of operating conditions for PHA reactors (i.e. SRT, cycle length, or OLR); Enrichment reactors have been successfully operated over a wide range of operating parameters (Table 4) (Dias et al., 2006). However, what has become a common indicative parameter of successful Enrichment reactors is the Feast/Cycle ratio (also referred to as the feast to famine ratio). A Feast/Cycle ratio less than 0.2 has been deemed a potential cutoff between reliable and variable selection of PHA producing consortium in an ADF Enrichment reactor (Dionisi, Majone, Vallini, Di Gregorio, & Beccari, 2007); note that there are no explanations as of yet that describe the relevance of this ratio at a molecular level, so it remains phenomenological. The Feast/Cycle ratio cannot be controlled directly, as it is a function of substrate uptake rate, cycle length, and mass of substrate fed. The ratio can be manipulated through modifications of SRT (solid retention time), cycle length, and organic loading rate (OLR). Many different combinations of operating conditions have been applied, while the Feast/Cycle ratio has remained a useful indication of conditions suitable for selecting a PHA producing culture.

Table 4: Review of reactor operating conditions

Source	SRT (Day)	Cycle Length (Hour)	HRT (day)	OLR Cmmol/L *gCOD/L	Reported Feast/Cycle	Substrate Source	Carbon Source
Albuquerque et al., 2010	10	12	1	30-60	0.21-0.22	Fermented Molasses	VFA's
Johnson et al., 2009	1	12	1	13.5	Not Reported	Synthetic Feed	VFA's
Morgan-Sagastume et al., 2013	1.5	1.5	2.1	3.1*	Not Reported	Synthetic Feed	VFA's
Dionisi et al., 2007	1	1 - 8	1	20*	0.1 - 0.5	Synthetic Feed	VFA's

3.2 Production Reactor

The goal of a PHA Production reactor is to maximize the PHA content in a reactor, and is intended to generate commercial level quantities of PHA. Proper operation of the Production reactor not only increases the mass of PHA recovered, but drastically reduces the chemicals required to extract a unit mass of PHA. To reach PHA saturation in a reactor, substrate should be continuously available to the culture.

Two feeding strategies are commonly applied to Production reactors: continuous substrate addition, or substrate addition on demand. The substrate addition on demand process requires an indicator parameter that must be constantly monitored to indicate the need for substrate. The two most common parameters that would be monitored to indicate the need for substrate addition are DO or pH; a rapid DO increase indicates that substrate has been depleted, and an increase in pH indicates depletion of organic acids. To utilize a pH approach requires a low alkalinity water to remain sensitive enough to detect consumption of VFA's, therefore the DO based approach is the most commonly used method. Johnson et al (2009) achieved the highest reported 89% peak PHA on a dry weight basis (pkPHA%) for $_{MC}$PHA. This was accomplished using an ADF enrichment reactor and Production reactor

with pH-based feed on demand. However, the work utilized a VFA based synthetic substrate with nitrogen deficiencies, causing growth limited conditions.

To increase the PHA storage response, the substrate is often altered to induce growth limiting conditions; this is typically accomplished through limiting nitrogen and/or phosphorus concentrations (Albuquerque et al., 2010; Johnson, Jiang, Kleerebezem, Muyzer, & van Loosdrecht, 2009). This nutrient limitation reduces or prevents the growth of the bacteria, which ensure a higher percentage of carbon substrate goes to intracellular PHA. While very effective, this technique is difficult to reproduce with waste-derived feedstocks which are typically high in nitrogen and phosphorus; moreover, this approach is most commonly applied to pure cultures. While the growth limited production processes are more efficient and achieve higher yields, non-growth limiting conditions nonetheless does not inhibit a high storage response (Johnson, Kleerebezem, & van Loosdrecht, 2010). Johnson et al. (2010) demonstrated that a non-nutrient limited reactor was capable of achieving high concentrations of PHA. The non-nutrient limited reactor achieved a pkPHA% concentration of 69% PHA (Dry Wt. Basis) with ammonia excess, while the ammonia starved reactor achieved 88% PHA (Dry Wt. Basis). These results were achieved with an $_{MC}$PHA culture and a synthetic VFA-based substrate for both enrichment and production. Ultimately, producing a waste substrate deficient in N or P is not common or easy, as most organic waste streams contain ample nitrogen and phosphorus concentrations. Some industrial waste streams may be sufficiently deficient in macro nutrients to be applicable. The dairy manure based substrate used for this investigation was not macro nutrient deficient.

3.3 Reduced Aeration

To produce an economically viable product using ADF enriched MMC and PHA production on waste substrate, operational costs must be reduced. An easy target for optimization is the removal of excess aeration. One of the largest costs in a mixed microbial PHA Enrichment system is aeration (Akiyama et al., 2003). A common practice in literature and previous work in Dr. Coats' research group was to exposed bioreactors to excess aeration ensuring that dissolved oxygen was never a limiting factor in the enrichment of a PHA producing culture (Guho, 2010; Johnson et al., 2009; Johnson et al., 2010). Conversely, research conducted as part of this thesis focused on reducing the aeration rate and assessing the associated process effects. As of this publication, only one other research group had specifically assessed the effects of reduced aeration, and that work focused specifically on PHB production and used synthetic substrate (Third et al., 2003; Third, Sepramaniam, Tonkovic, Newland, & Cord-Ruwisch, 2004). In contrast, this work focuses on real wastewater and the synthesis of a more value form of PHA (i.e., PHBV).

As of this writing Third et al. (2003, 2004) has completed the most work on the topic. However, in direct contrast to this thesis, the focus of their work was on reducing the carbon utilization in the aerobic periods and conserving it for post anoxic denitrification; the end goal of their work was to reduce the supplemental carbon required for post-anoxic denitrification. The important conclusion Third et al. (2003) demonstrated was an increase PHA yield (PHA/VFA Consumed) by reducing the mass transfer coefficient k_La. The end application of their work is different than the focus herein. Due to this difference, no PHA Production reactors were used in Third et al. (2003, 2004**).** The results from their work were based on a synthetic feed with balanced nutrients, and all carbon being supplied by acetate. This allowed detailed mass balances and a model fitting to be attempted. To prevent nitrification from consuming oxygen, Third et al. (2003) added Thiourea to inhibit ammonia oxidation, a common tactic often utilized in PHA research (Albuquerque et al., 2010).

3.4 NOx as a Terminal Electron Acceptor

Theoretically, by allowing nitrification to proceed within the Enrichment reactors, the microbial culture gains additional terminal electron acceptors (NO_2 and NO_3). This supplemental electron acceptor will be most applicable in the Feast phase when residual DO is low. Any consumption of NO_2 or NO_3 reduces the oxygen required to oxidize the substrate (VFA's). This has an additional benefit of resulting in a cleaner effluent due to denitrification removing nitrogen from the system. The possibility of coupling PHA enrichment and waste treatment could drastically reduce the opportunity cost associated with production of PHA from waste materials.

Chapter 4: Materials and Methods

4.1 Quantification of k_La

Typical aeration kinetics reported in the literature are as volume of air provided per volume of reactor, or as a range of DO concentrations (Guho, 2010; Morgan-Sagastume et al., 2014), while some only report the equipment used and nothing else (Albuquerque et al., 2010; Dionisi, Majone, Vallini, Di Gregorio, & Beccari, 2006; Villano et al., 2014). Such an approach is acceptable when reactors are excessively aerated to ensure oxygen is not a limiting nutrient, which is the typical approach for PHA investigations. However, properly quantifying aeration characteristics is of the utmost importance if the research is to be applied at a larger scale. Moreover, for research focused on controlled aeration (such as this thesis), it is most applicable to quantify the aeration kinetics and the mass transfer coefficient for aeration.

As discussed above in the two-film theory (Section 2.10), a mass transfer coefficient is necessary to properly calculate the oxygen transfer to a reactor. For this work and that of Third et al. (2003, 2004) the mass transfer coefficient was quantified and reported; this allowed aeration kinetics and experiment results to be compare both within literature and for future scale up. Utilizing k_La instead of other gross parameters (i.e. Vol Air/Vol reactor, or mass flow of air) is necessary due to k_La becoming a design parameter when applied to a scale up design. Unlike other parameters, k_La is independent from reactor geometry and aeration equipment.

The oxygen transfer parameter k_La was the design variable in this experiment. The oxygen uptake rate (OUR) and k_La were measured following the Dynamic Degassing method as first proposed by (Bandyopa.B & Humphrey, 1967) and further refined by Garcia-Ochoa and Gomez (Garcia-Ochoa & Gomez, 2009). The k_La was measured during the famine state of the Enrichment reactor to prevent fluctuation in OUR associated with VFA catabolism in the feast phase from affecting the analysis. Any change to OUR will directly affect the residual DO concentration (C_t) because all other variables in the model are constant (Equation 4), thus causing errors in the calculation of k_La.

The residual DO in the reactor was measured and logged using a Hach HQ40 fitted with an LDO101 probe (Hach Company, Loveland CO, USA). As part of the k_La quantification, air flow to the reactor was first stopped and DO was allowed to decrease to approximately 2.0 mg/L. This allowed quantification of the OUR using linear regression. Once the DO reached 2 mg/L, air flow was returned to the reactor and the DO concentrations recorded. The residual DO saturation curve was plotted in Excel (Figure 15 presents an example data plot). Equation 4 (Garcia-Ochoa & Gomez, 2009) was fit to the data by modifying the k_La coefficient to achieve the best fit as measured by R^2. It should be noted that $qo_2 * C_x$ =OUR .

$$C_t = \left(C_s - \frac{qo_2 * C_x}{K_L a}\right) - \left(C_s - C_0 - \frac{qo_2 * C_x}{K_L a}\right) * e^{(-K_L a * \Delta t)}$$

Equation 4: Residual Oxygen Model.

Where:
C_s= Oxygen Saturation (for local conditions)
qo_2= Specific Oxygen Uptake rate
C_x= Biomass concentration
C_t= Dissolved Oxygen Concentration at time t
C_0= Dissolved Oxygen Concentration at T_0

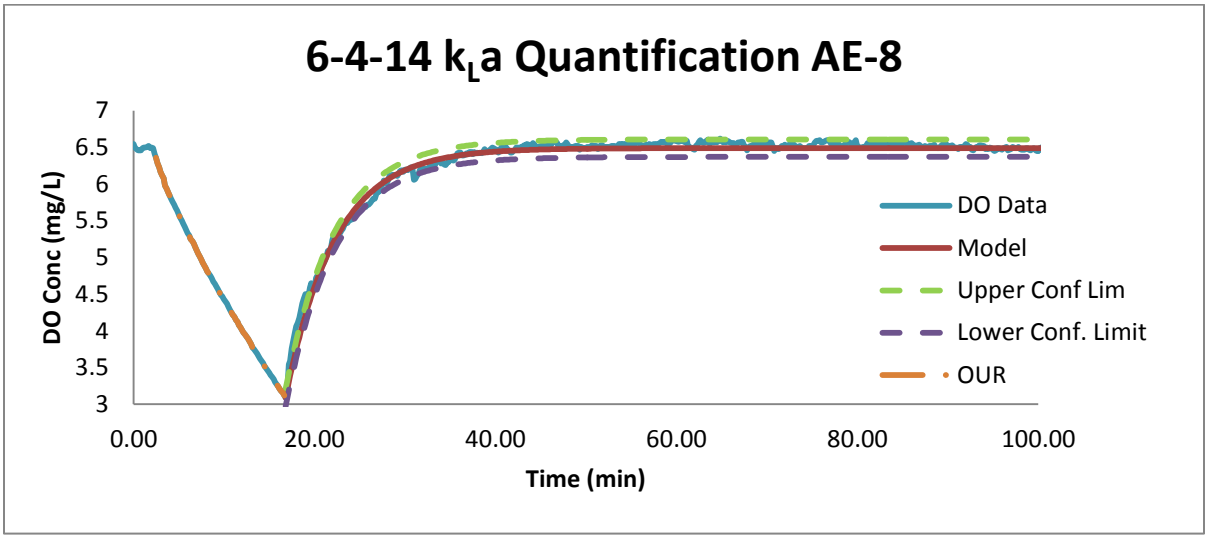


Figure 15: Example k_La Fitting Model.

The k_La coefficient with the best fit was selected using the residual squared sum of errors. All modeled saturation curves utilizing the selected k_La fit the data collected with an R^2

greater than 0.999. The best fit k_La was corrected for temperature based on the exponential function approximating the van't Hoff-Arrhenius relationship (Equation 5). A theta value of 1.024 was used in the correction, as recommended by (Tchobanoglous et al., 2003). Final experimental design k_La values are shown in Table 5 (see Ch. 5).

$$K_L a_{20c} = \frac{K_L a_T}{\theta^{T-20}}$$

Equation 5: van't Hoff-Arrhenius Temperature Correction.

T=Temperature in Degrees Celsius
Θ= Empirical Correction Coefficient

4.2 Effects of Wastewater Characteristics on Aeration

The oxygen transfer efficiency of each reactor was quantified based on the parameter α (Tchobanoglous et al., 2003), which is a unitless coefficient relating the k_La of a wastewater system to the k_La of the identical system filled with tap water.

$$\alpha = \frac{k_L a_{20c}\ wastewater}{k_L a_{20c}\ k_L a\ tapwater}$$

Equation 6: Calculation of Alpha Aeration Characteristics.

The value of α is often used in the design of aeration systems for full-scale systems. To quantify α, the k_La was first measured in each reactor following the process described above, and then corrected to 20°C and used in the numerator as the "wastewater" k_La. Next the reactor biomass content was transferred to a temporary holding vessel where it was aerated and mixed. The now empty reactor was filled with deionized water and the k_La was analyzed again. With the clean water there was no biological oxygen consumption; therefore, sodium sulfite and cobalt chloride were used to remove oxygen from solution. The parameter fitting system as described above was used to determine a clean water k_La value. This k_La was corrected to 20°C and used as the denominator or "tapwater" k_La

4.3 Reactor Stoichiometry and Kinetic Calculations

Reactor stoichiometry and kinetics are useful parameters when comparing results, understanding effects of changes, and troubleshooting processes. The sections below discuss calculation of several kinetic expressions common to PHA producing systems.

4.3.1 Yield PHA from VFA's

As noted, the goal of this research was to help advance the PHA process (Figure 1) toward commercialization. In this regard, the PHA/VFA yield is a useful parameter for assessing the potential performance differences between the biomass cultured in the four Enrichment reactors. The yield is a measure of the efficiency of PHA production from VFA's achieved by a biomass cultured under specific Enrichment reactor operating conditions. The yield parameter can provide some insight to the successful selection of PHA producing biomass achieved by the Enrichment reactor. Higher yields indicate more of the carbon uptake was diverted to PHA instead of growth, while lower yields indicate more carbon to growth; however, one cannot conclude from the yield parameter alone that the PHA-bacteria enrichment is lower, as PHA producing bacteria may be growing rather than producing PHA. One of the only means to accurately identifying the bacteria present in the mixed culture is through molecular biology techniques such as qPCR. While biomass has been preserved for future qPCR analysis, the method is not fully developed in the Coats' lab for use in this thesis.

The most commonly applied calculation of PHA/VFA yield is measured at the end of a production run by calculating the PHA concentration at time t divided by total VFA's consumed over time t (see Equation 7).

$$\text{Yield PHA}_t = \frac{\text{PHA}_t}{-\text{VFA}_t + \sum \text{VFA}_{\text{fed}}}$$

Equation 7: Yield PHA/VFA

Due to this experiment involving repeat measures, a linear regression model was applied to the data to establish the most representative yield for the entire data set, as this avoids errors associated with taking multiple averages to assess a single representative yield for

the entire data set. The data set comprised all seven Production evaluations or all five Enrichment evaluations separated into groups based on reactor k_La. The regression model predicts PHA concentration based on VFA's consumed (the intercept parameter was forced to zero, as no PHA was present at the start of any Enrichment or Production evaluations). Applying this method, the linear β_1 parameter (Equation 8) represents the best fit yield coefficient for the data set as described in Equation 7. Results are presented and discussed in Ch. 5.

$$\mathbf{PHA\ Conc} = \mathbf{0} + \boldsymbol{\beta_1} * \mathbf{VFA\ Consumed}$$

Equation 8: VFA Yield Model.

4.3.2 VFA Uptake Rate

The VFA uptake rate was calculated using a linear regression model of the entire Production or Enrichment evaluation data set separated into groups based on reactor k_La. Two uptake rates were calculated: first the overall VFA uptake rate (rVFA), and second, the specific VFA uptake rate (qVFA). The overall rate is simply VFA consumed per time with units mass/time (Cmmol/L min). The specific rate normalizes the overall rate to the mass of bacteria in the mixed liquor volatile solids (MLVSS , Biomass). The overall rate is divided by MLVSS (mass/volume) this yields units of mass/mass time (Cmmol/g min). The linear regression model intercept is forced through zero because no VFA's are consumed prior to the evaluation starting. The model then becomes a single variable: time (rVFA), or MLVSS*time (qVFA). The resulting coefficients from the regression are the uptake rates (rVFA or qVFA).

$$VFA_{Consumed} = \beta_1 * Time \quad Specific\ VFA_{Consumed} = \beta_1 * Time * MLVSS$$

Equation 9: VFA Uptake Rate Model

4.3.3 PHA Synthesis Rate

The PHA synthesis rate was calculated using a linear regression model of the entire Production or Enrichment evaluation data set separated into groups based on reactor k_La. Two rates were calculated, the total rate of synthesis for each reactor (rPHA), and the

specific synthesis rate (qPHA). Similar to the VFA uptake rate, the specific PHA production rate normalizes the rate based on biomass concentration (measured as MLVSS g/L). The units are mass PHA/time (rPHA, Cmmol/L min) and mass PHA/ biomass time (qPHA, Cmmol/g min). Both rate parameters values are useful for scaling up process and predicting how future systems may operate. Linear regression was used to model PHA concentration given time or MLVSS*time (Equation 10).

$$PHA_{Conc} = \beta_1 * Time \;\; Specific\, PHA_{Conc} = \beta_1 * (MLVSS * Time)$$

Equation 10: PHA Synthesis Model.

4.4 Sample Analysis

4.4.1 PHA Content

PHA content was analyzed as a methyl ester derivative through GC/MS (Gas Chromatography and Mass Spectroscopy). The method was originally proposed by (Braunegg, Sonnleitner, & Lafferty, 1978) and it has been modified due to the equipment used.

Samples were collected from the bioreactors and treated with 1mL of commercial sodium hypochlorite to lyse open the cells the solution was then centrifuged at 10,000 rpm for 4 minutes. The supernatant was poured off and the remaining solids rinsed with deionized water twice. The biomass was then dried at 100-105˚C for a minimum of 24 hours. Once dry a 10-20 mg sample of the biomass was weighed and placed into a test tube for digestion. Two mL of glacial methanol acidified with H_2SO_4 (3% V/V basis) and one mL of chloroform with 0.25 mg/L benzoic acid was added. The sample was then subjected to a 4 hour digestion at 100˚C in a HACH DRB 200 automated digestion block (Hach Company, Loveland CO, USA). Samples were allowed to cool before adding 2mL of deionized water and vortexed for 30 seconds. The samples sat for a minimum of 5 minutes to allow the organic and aqueous phases to separate. The organic phase was then pipetted and filtered through a Pasteur pipette column of anhydrous sodium sulfate into a 2mL screw top glass vial with Teflon®-sealed cap (Thermo Fisher Scientific Inc., Waltham, MA, USA). The

samples were injected into a ThermoQuest Trace™ GC with a Finnigan PolarisQ iontrap using a ThermoQuest AS2000 autosampler (Thermo Fisher Scientific Inc., Waltham, MA, USA). The inlet was operated in split mode, with a temperature of 210°C. Separation was achieved using a ZB1 (30m x 0.25mm ID) capillary column (Phenomenex, Torrance, CA, USA) which was ramped from an initial 40°C to 200°C at 5°C per minute. 3-hydroxybutyrate and 3-hydroxyvalerate monomers were verified by mass spectra at 103 m/z and retention time matching based on commercial standards (Sigma-Aldrich Co., St. Louis, MO, USA). The standard curves for quantification had correlation coefficients of r^2>0.95 (3-Hydroxyvalerate) and r^2>0.98 (3-Hydroxybutyrate).

4.4.2 Nutrients

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^{-1} then to 150°C at 10°C min^{-1} and hold for 3 min; finally, ramp to 200°C at 25°C min^{-1} and hold for 12 min) with helium as the carrier gas (1.2 mL min^{-1}). The split/splitless injector and detector were operated isothermally at 210 and 300°C, respectively. Prior to analysis, samples were acidified to a pH of 2 using 2.0M HNO_3. 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).

Nitrate was measured using Orbeco-Hellige test kits number RS5580 (Tintometer Inc., FL, USA). Results were measured on a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sample concentrations were determined from a five point standard curve with r^2>0.99.

Nitrite was measured using Diazotization method or HACH method 10019 (Hach Company, Loveland CO, USA), the equivalent Standard Method is 4500-NO_2-B (Standard Methods 1998). Results were measured on a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sample concentrations were determined from a five point standard curve with r^2>0.99.

Ammonia was measured using Orbeco-Hellige test kits number RS5650 (Tintometer Inc., FL, USA). Results were measured on a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sample concentrations were determined from a five point standard curve with r^2>0.99.

Dissolved oxygen (DO) was measured using a HACH IntelliCAL™ LDO101 probe connected to a HACH HQ40d Multi-Parameter Meter. Data was logged using the USB and A/C power adapter.

pH was measured electrochemically using an Accumet® pH probe and Accumet® AP85 portable multi-parameter meter. The probes were calibrated using standard NIST traceable pH solutions 4.01, 7, and 10.01 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Total and volatile suspended solids were measured utilizing Standard Methods 2540 D and 2540E respectively (Standard Methods 1998). Weight tins with dry Glass filters 0.45 micrometer (Milipore, Billerica, MA, USA) were weighed. The filter was then used to filtrater a known volume of sample, filters and residual solids were then dried at 103-105^{o}C for 24 hours before being weighted. The change in mass was divided by the sample volume to achieve a mass of solids/volume of sample. Volatile solid samples were then incinerated at 550^{o}C and reweighed.

Total organic carbon (TOC), total nitrogen (TN), and total carbon (TC) were completed by the University of Idaho Analytical Sciences Laboratory (Moscow, ID).

4.5 Statistical Analysis

Statistical analysis was carried out by Microsoft® Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and by SAS 9.3 (SAS Institute Inc., NC, USA).

Proc GLM is a powerful analysis of variance tool utilized by SAS. Proc GLM utilizes the method of least squares to fit general linear models (GLM) to data sets. The model is especially useful when comparing continuous dependent, independent variables and unbalanced data sets. The process fits several general linear models to the data in the MODEL statement. These general linear models are used to compare variables entered into the proc GLM MODEL statement. The method compares each variables impact on the general linear model and compares any variable interactions effects entered into the MODEL statement. For the majority of analyses presented herein, proc GLM was used to fit the data from all reactors; an interaction between k_La and another variable was included in the model statement. If the interaction effect had a statistically significant effect on the model, then the effect of k_La on the enrichment reactor was statistically significant to the comparison variable. If the interaction was not statistically significant, then the k_La had no effect or the effect was overshadowed by a large variance in the modeled data. The proof of statistical significance for this procedure relies on an F-test and acceptance or rejection of the null hypothesis that model changes with the inclusion of the interaction. The F-test is most easily summarized by a probability or Pr>F in this case statistical significant is based on a Pr>F of less than 0.05 (this term is often referred to as α). Proc GLM can also be used for the following statistical analyses; regression, multiple regressions, analysis of variance (ANOVA), response surface models, and many other tests (Inc., 2014).

Proc Reg is a general regression modeling system which utilizes nine methods to select the best fit, and displays model fitting plots (i.e. residual fit, data normality, Cook's D, and quantile-quantile (Q-Q) residual plots). The command can be used for multiple model statements, interactive changes in model and data, test of linear hypotheses, collinearity diagnostics, and many other useful regression related tests (Inc., 2014). In this work it was used to fit linear models to the data, and to provide relevant model fitting information.

Chapter 5: Reduced Aeration Investigation

The purpose of the research presented and discussed in this thesis was to evaluate the effect of aeration on the enrichment of a PHA-producing mixed microbial culture grown on a real waste stream (VFA-rich fermenter liquor from fermented dairy manure). The investigation is based on work performed by Third et al. (2003) with key differences: use of real waste stream, end goal is PHA Production, and nitrification/denitrification was not inhibited. Due to uninhibited nitrification of ammonia, both Enrichment and Production reactors will have elevated concentrations of nitrate and nitrite. This may lead to a dilution effect from the autotrophic bacteria responsible for nitrification. As described, the PHA Enrichment reactor maintains a biomass capable of excess PHA synthesis, with waste from said reactor used to generate commercial quantities of PHA in the Production reactor (Figure 1). Metrics to assess the process focused on the PHA/VFA yield sustained in the enrichment reactors as well as the peak PHA (pkPHA%) on a dry mass basis achieved in a Production reactor. The goal was to establish a range of acceptable aeration rates (k_La) that incurs limited or no negative impacts on the enrichment process or downstream production process. During the progression of this work, nitrogen removal was observed in the Enrichment reactor. Nitrogen is an environmental pollutant of real concern for dairies, and technologies are needed to reduce the nitrogen concentration in dairy wastewater effluent. This is of interest to full scale systems, and therefore it was quantified.

5.1 Experimental Design

The investigation was set up with a single design factor - k_La; all other operating conditions were held constant (see Table 5). The experimental k_La's were selected after a preliminary two-reactor evaluation experiment was conducted; one reactor was aerated at a high rate (k_La=23 hrs^{-1}) while a second reactor was aerated at a much lower rate (k_La=6 hrs^{-1}). The high rate reactor was aerated at the same vol air/vol reactor as used in earlier PHA work (Guho, 2010), and was measured and used as a "control" reactor. The low aeration reactor was intended to provide preliminary data to ensure the low end of the aeration range

tested would still be capable of supporting biomass that exhibited a Feast Famine response (i.e. a residual DO spike was observed during the cycle). The goal of this preliminary experiment was to guide the selection of k_La for the research constituted by this thesis. Since the preliminary experiment demonstrated a k_La of $6hr^{-1}$ was acceptable, aeration was reduced further (in the full experiment) to find the lowest acceptable aeration rate. It was expected that the reactor with the lowest aeration rate would fail. The minimum setting of the Aera Mass Flow controllers (MFC) is 1% full scale (10mL/min) therefore the lowest k_La achievable was $4hr^{-1}$. The rest of the range was split equally with one reactor at $8hr^{-1}$ and another at $12hr^{-1}$. While not as applicable for comparisons, it is convenient to note the reduction in air flow provided by the reduction to k_La (Table 5). In other systems an identical reduction in k_La will not always yield the same % reduction in air flow as calculated here, but it should remain similar.

5.2 Experimental PHA Enrichment Reactors

The experiment consisted of four bench-scale PHA Enrichment reactors (Table 5). The reactors were operated as sequencing batch reactors (SBRs) and were continually stirred with no settling phase (i.e., chemostats). The reactors were operated with an SRT/HRT of 4 days and cycle length of 24 hours. Operating parameters were selected based on previous research in the Coats lab (Guho, 2010). Aeration was accomplished with a 2.0 micron gas sparger (Williams Brewing, San Leandro CA, USA) with air flow rates controlled by Aera PI-98 MFC's rated to 0-1000 ±1 standard cubic cm per min (sccm) (Hitachi Metals America, San Jose, California, USA). Each reactor was equilibrated to a target k_La by adjusting the air flow rate until the desired k_La was achieved. The k_La was checked on a biweekly basis for the duration of the experiment. Mixing was achieved with Thermo Scientific Cimarec magnetic stir plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 1.5-in long Teflon coated magnetic stir bars. Mixing intensity was maintained at a constant level 5 on the magnetic stir plates for all Enrichment reactors. The reactors were scrubbed daily and the aerations stones were soaked in 1N HCL for a minimum of five minutes before rinsing with deionized

water and cleaning with Kimwipe© then re-submerging in the reactor. The 1N HCL soak and cleaning was used to prevent biofilms from blocking the aeration stone pores and affecting the bubble properties. After reactor cleaning, 300 mL of mixed liquor was removed and either disposed of, or used in a second phase PHA Production reactor. The reactor was then re-filled with 300 mL of substrate comprised of 40% dairy fermenter liquor and 60% tap water by volume. For this study batches of substrate mixed every three days and stored at 4^{o}C until used. Evaporation affected reactor volumes therefore; reactors were topped off as needed with tap water prior to feeding and cleaning.

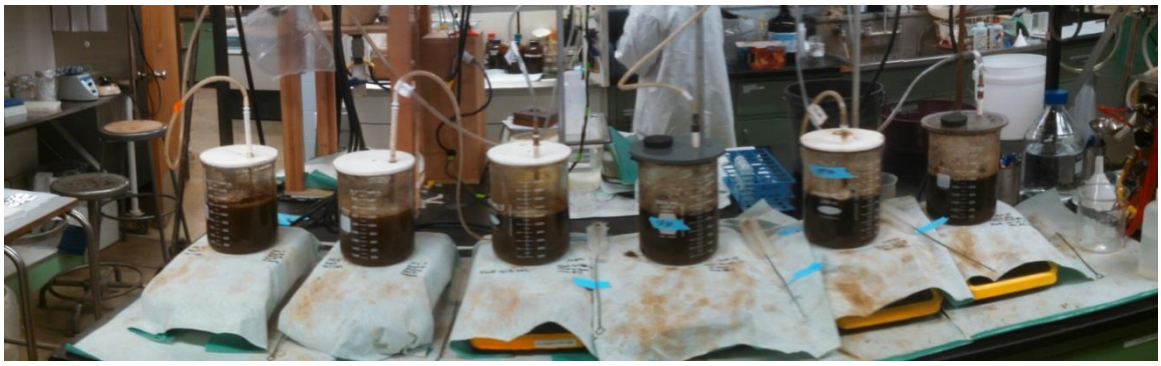


Figure 16: Experimental Enrichment reactors

Table 5: Experimental Reactor Operating Conditions.

Reactor	Operating Volume (L)	k_La (hr^{-1})	Avg. Air Flow (mL/min)	[1]Avg. Flow Reduction from Control	Mixing Level	SRT/HRT (day)
AE-4	1.2	4	15	97.5%	5	4
AE-8	1.2	8	80	87%	5	4
AE-12	1.2	12	245	59%	5	4
AE-20 (control)	1.2	20	600	NA	5	4

1: Avg. flow reduction from control is calculated by; dividing the difference between reactor air flow and control air flow by control air flow.

5.3 Experimental PHA Production Reactors

In total, seven sets of independent PHA production reactor evaluations were conducted (Table 6) on biomass wasted from each Enrichment reactor. All PHA Production reactors had an initial operational volume of 300 mL (i.e., the daily waste/inocula for the respective Enrichment reactors), and air flow was controlled with an Applikon Flow Console (Applikon Bio-Technology, Delft, Netherlands). The same aeration stones and mixing plates were used in the production phase as in the enrichment phase (Figure 17). In contrast with Enrichment reactor operations, the PHA Production rector substrate was undiluted dairy fermenter liquor. Each production test consisted of periodically pulse feeding undiluted fermenter liquor, with the aim to induce and sustain PHA synthesis (i.e. “feast” like conditions). The PHA Production reactor evaluation process evolved through three distinct stages as experience was gained and the process refined (Table 6). Stage 1 operations relied exclusively on reactor DO to indicate the need for pulse addition of substrate, as well as to signal the end of the production run. Substrate addition was triggered by a spike in residual DO, while the production evaluation was ended when substrate addition to the reactor did not cause a residual DO drop. Samples for VFA and PHA analysis were collected 10 and 30 minutes after each pulse, unless another pulse was added before the 30 minutes mark. In Stage 1, the volume of substrate pulsed to the reactor achieved the same volumetric loading as the Enrichment reactor regardless of production reactor volume changes due to sampling. The Enrichment reactors received 120mL of undiluted fermenter liquor daily (10% of reactor volume) therefore the Production reactors received 30mL (10% of reactor volume). However, periodic sampling removed volume from the reactor (approx. 20 mL for every sample) therefore the production reactor volume was constantly changing but the feed remained constant (30mL). After experiencing operational challenges (Discussed in Section 5.6.1) with Stage 1 operations, the Production strategy was modified and referred to as Stage 2.

For Stage 2 operations, the PHA Production reactors were pulsed substrate at a set time interval (45-min for run 3, 30-min for run 4), or upon a spike in residual DO (similar to Stage 1). The selection of time interval was based on the previous day Enrichment reactor VFA

uptake rate and VFA concentration of the Production reactor substrate. By assessing the inocula "feast" characteristics, it was envisioned that performance of the PHA production could be maximized. This time-based substrate addition strategy had the theoretical benefit of ensuring ample VFA's were continuously present to the biomass, instead of the biomass experiencing a short duration of carbon limited condition (i.e., the cause of a DO spike) between substrate depletion and the next pulse of substrate. This also theoretically helped reduce the chances biomass would consume stored PHA, due to deficient exogenous VFA's. Each pulse volume was held volumetrically constant to the volume of the production reactor (10% of reactor volume at time of pulse). This method accounted for the net loss of reactor volume due to sampling as the evaluation progressed. The sampling rate was also increased to 3 samples per substrate addition cycle. The purpose of the enhanced sampling was to form a more robust sample set with a focus on VFA consumption and PHA synthesis between substrate additions.

The final phase of evaluations (Stage 3) employed a consistent 30 minute feeding rate regardless of DO; the sampling rate for VFA and PHA analysis was also reduced. Specifically, sampling did not start in stage 3 until the end of the 2nd substrate addition cycle. Once sampling began, one sample was collected immediately prior to substrate addition, for a total of 7 samples. In Stage 3 operations, the volume of substrate supplied to the reactor was maintained at 30 mL regardless of the reactor volume. However, in this Stage 3 analysis, the reactor volume slowly increased due to additional substrate volume exceeding sampling volume. This strategy sustained a minimum concentration of VFA's for PHA synthesis (theoretically sustaining a "feast" condition). The first pulse addition of substrate for all Production reactors occurred at time zero.

Table 6: Modifications to Production Run Process.

Production Stage	Feeding Trigger	End of Production	Volume of Feed Pulse	Production Runs
1	DO	DO	10% of Initial Volume	1, 2
2	DO or Time	7 Feedings	10% of Reactor Volume	3, 4
3	30 min	7 Feedings	10% of Initial Volume	5, 6, 7

During the operation of the PHA Production reactors, dissolved oxygen (DO) was monitored continuously with a Hach LDO101 probe and HQ40 meter (Figure 17) (Hach Company, Loveland CO, USA). The rate of aeration was not controlled in the production evaluations. The k_La was not measurable in these reactors, as changes in reactor volume, water chemistry, and OUR happened too quickly to accurately assess the k_La. Instead, air flow rate was set so that DO concentration were close to 1.0 (mg/L). At this DO concentration, small changes in the OUR cause large changes in the DO concentration and were thus easier to identify.

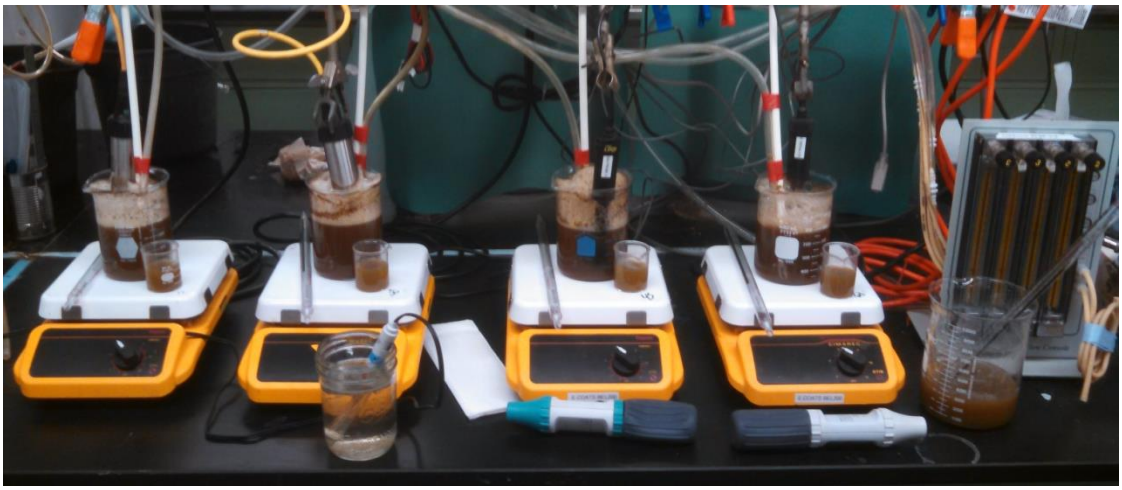


Figure 17: Production evaluation, aeration control on right.

5.4 Fermenter Operations

The PHA Enrichment and Production reactor substrate was produced in a 20L lab-scale dairy manure fermenter. The fermenter was loaded at 8.75-g VS/L Day and operated at an SRT/HRT of 4 days. The fermenter reactor was continuously stirred using a 3.75" diameter helical impeller driven by an Oriental Motor (San Jose, CA, USA) USM315-401W 15 W AC speed control motor connected to 3GN35SA reduction gearbox. The operating motor speed ensured uniform mixing within the reactor. Reactor temperature was ambient room temperature 22-25^{o}C. To minimize gas transfer from the reactor headspace, two draft tubes were installed. The tubes were constructed of Schedule 40 ¾" PVC pipe and extended from lid to just below the low water level. One tube housed the mixing drive

shaft while the other tube was used to supply feed. Effluent was removed through the bottom of the reactor through a large 2" ball lock valve. Head space gas was vented to an airlock using Tygon tubing inserted through a grommet in the reactor lid. The airlock was disconnected during feeding to prevent negative pressure from forming, atmospheric exposure to the reactor was limited to approximately 5-L per day. Daily effluent was centrifuged for solid separation. Residual manure solids and 1-2L of centrate were fed to anaerobic digesters, while the residual fermenter liquor centrate was used for this PHA research.

Manure was collected every 1-2 weeks and stored in a lab refrigerator at 4^{o}C. The manure was collected in 5 gallon buckets from the University of Idaho dairy loafing barns, typically in the eating area which reduced bedding material being collected with the manure. Samples of each manure batch were tested for TS and VS to facilitate a constant VS loading on the fermenter and digestion system.

5.5 Results and Discussion

The performance of biomass in each PHA Enrichment reactor was evaluated in both the enrichment and production stages, with each assessment day separated by a minimum of three SRT's (12 days). A total of five enrichment evaluations and seven production evaluations were conducted over a 230 day period. The data was summarized and analyzed using SAS 9.3 (SAS Institute Inc., NC, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Reactor performance was compared using the k_La as an identification class variable, and data was separately analyzed with k_La as a group variable. Points of interest were statistical significance in VFA/PHA yield, statistical significance in Max PHA Production, models for PHA produce, and models for VFA uptake rates. These parameters have value for future scale up or further understanding the kinetics of the reactors.

5.6 Enrichment Reactors Performance Assessment

As described, the PHA Enrichment reactors were operated identically, with the exception of the one experimental variable (k_La). The data analysis discussion and summary that follows includes all five evaluations, and all analyses were performed with SAS 9.3. Before presenting the comprehensive analysis of all the enrichment data, two evaluations have been selected to illustrate the trends observed in the Enrichment reactor evaluations (Figure 19 through Figure 21).

5.6.1 Enrichment Evaluation 1

The first enrichment evaluation example (Figure 19 and Figure 20) was performed on 12-12-13 and was the second of the five enrichment evaluations. Only three DO probes were available, thus only two reactors (AE-4 and AE-8) have continuous residual DO data. The other two reactors only have periodic DO checks performed just before a sample was collected. Liquid and biomass samples were collected and analyzed. The liquid was analyzed for three forms of nitrogen (NH_3-N, NO_2-N, and NO_3-N) and VFA's. The biomass was tested for PHA.

First examining the terminal electron acceptor, reactors AE-12 and AE-20 exhibited a typical ADF DO response Figure 18, with residual DO increasing substantially upon depletion of VFA's (Figure 19 –C, D). However, AE-4 and AE-8 did not exhibit the typical large spike in residual DO trend (Figure 19-A, B); rather, residual DO remained relatively high (≈4 mg/L) throughout the feast phase. However VFA's were still rapidly consumed (within 120 minutes; Feast/Cycle ratio = 2hrs/24hrs = 0.083). It was expected that AE-4 and AE-8 would experience the longest low residual DO concentrations (<1.0 mg/L) due to oxygen becoming a limiting nutrient while the biomass consumed the VFA substrate (feast phase), however that was not the case. One possibility that explains the relatively high residual DO during the feast phase is the availability of an alternative terminal electron acceptor (i.e., NO_3 or NO_2). Supporting this theory, in part, is the observed fluctuation in NO_2 concentrations for reactor AE-8 (Figure 20-B), suggesting that some of the terminal electron acceptor requirements were being fulfilled by NO_2. It would appear that the DO was trending down

when NO_2 was available. NH_3 concentrations remained relatively stable during this phase, which was unexpected, as concentrations should decrease given the spike of NO_2 (an intermediate of nitrification). This lack of NH_3 fluctuations could be explained by NH_3 replenishment from degrading organic matter present in the feed. It is not evident what caused the high residual DO in AE-4 (Figure 20-A). Partial nitrification was also observed in reactors AE-12 and AE-20 (Figure 20-C, D), although much less so in the most highly aerated reactor (AE-20).

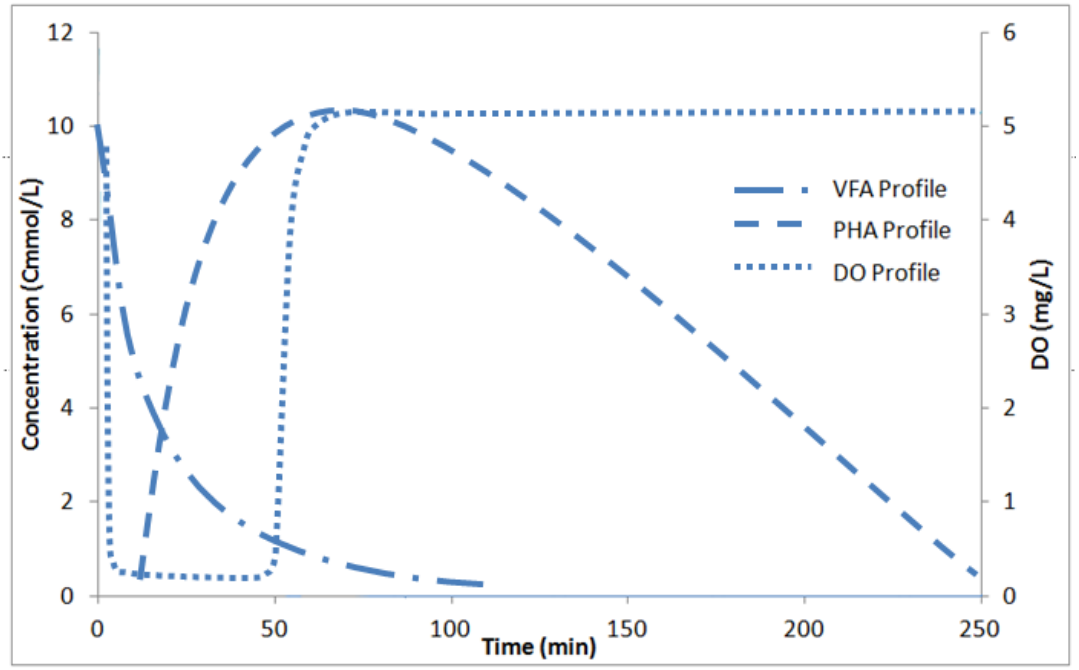


Figure 18: Idealized "Typical" enrichment reactor data.

Considering carbon cycling, the only reactor that showed a noticeable difference in PHA production and VFA consumption is AE-20 (Figure 19-D) with peak PHA and VFA exhaustion occurring at approximately 60 minutes , as compared with approximately 120 minutes in reactors AE-4, -8, and -12. The higher synthesis and uptake rate is likely caused by the constant abundance of excess DO available to this biomass. The other three reactors exhibited very similar performance, with peak PHA and VFA exhaustion being reached around 120 minutes. All four reactors achieved similar peak PHA concentrations in the Production evaluation performed on 12/17/13 (see section 5.7.1 PHA Production Assessment, Stage 1 for details).

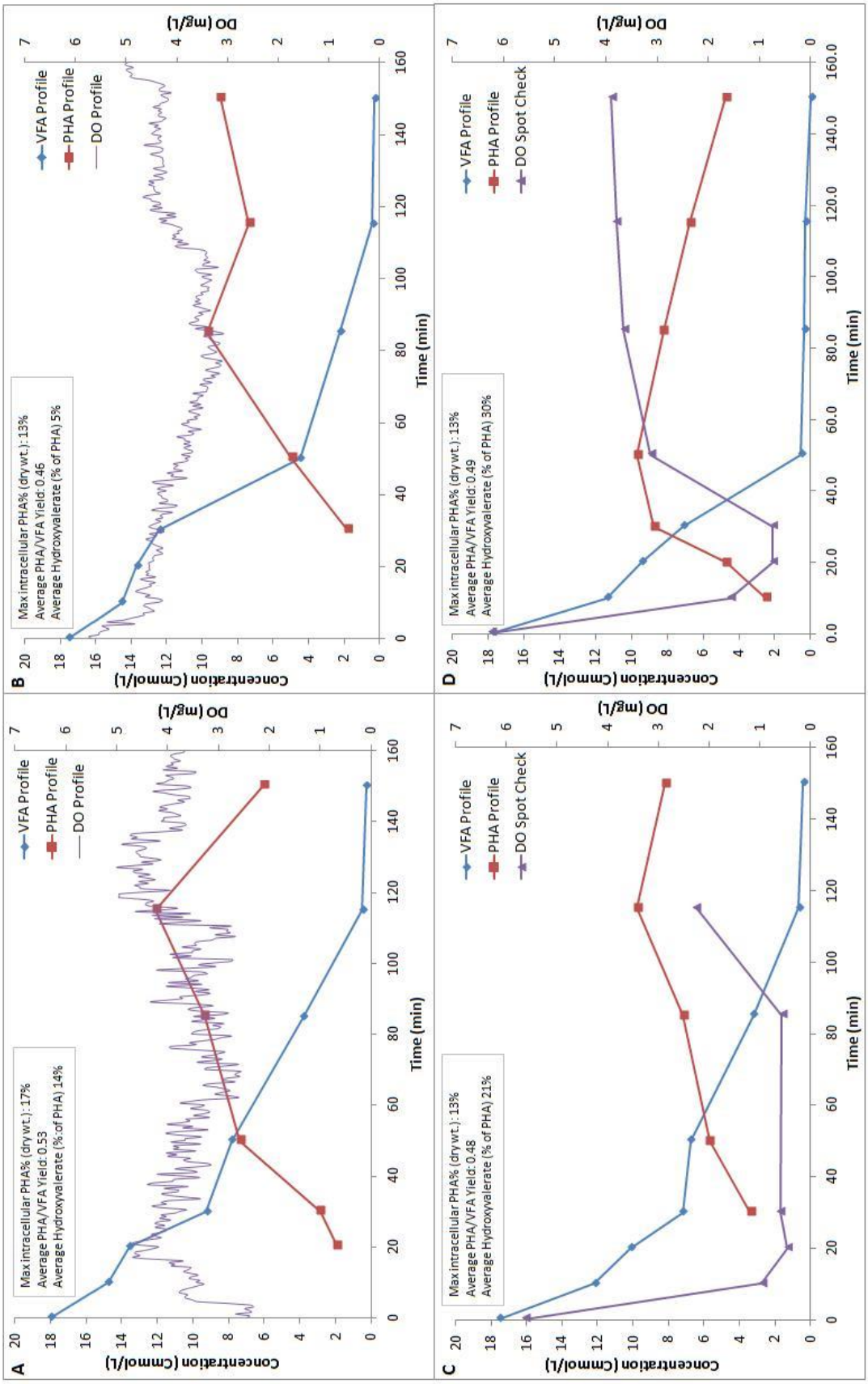


Figure 19: Carbon data, Enrichment evaluation 12-12-13
AE-4 (A), AE-8 (B), AE-12 (C), AE-20 (D)

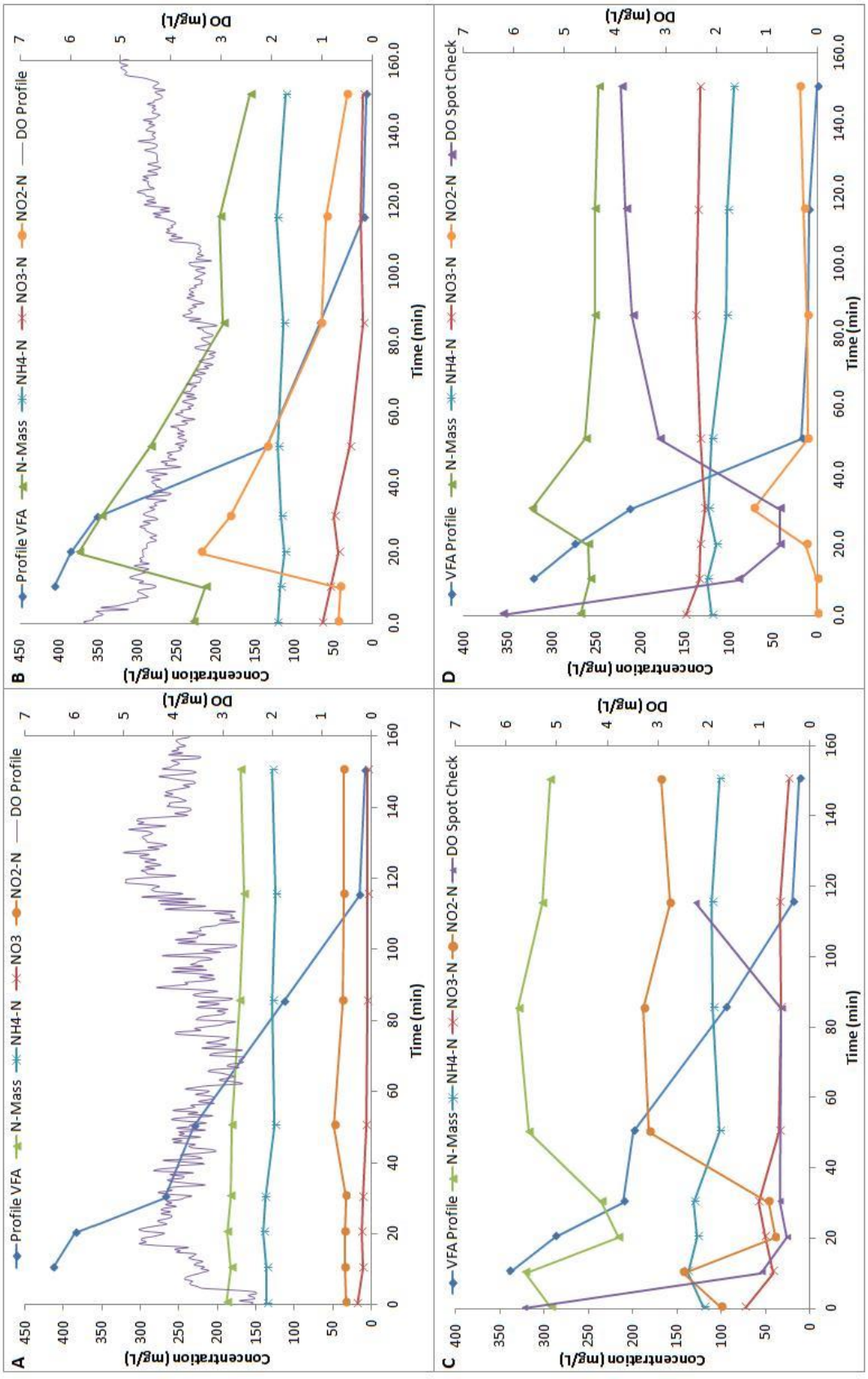


Figure 20: Nitrogen data Enrichment evaluation 12-12-13.
AE-4 (A), AE-8 (B), AE-12 (C), AE-20 (D)

5.6.2 Enrichment Evaluation 2

The second enrichment evaluation example was performed on 4-29-14 and was the fourth of the five enrichment evaluations. Four DO probes were available for this performance assessment, allowing all reactors to have continuous residual DO data. Liquid and biomass samples were collected and analyzed. The liquid was analyzed for three forms of nitrogen (NH_3-N, NO_2-N, and NO_3-N) and VFA's. The biomass was tested for PHA. To better understand nitrogen cycling, sampling was continued long after carbon cycling had finished (≈250 min). This evaluation suffered a computer glitch that shut off the mass flow controllers for reactors AE-8 and AE-12 (Figure 22). However, the aeration shutdown occurred late in the operational cycle, after carbon (VFA and PHA) depletion at ≈250 min; at this time the nitrate concentration was elevated in the reactors and thus the reactors did not experience a lack of terminal electron acceptor. This aeration failure could have affected the nitrogen cycles observed, although no sudden changes in nitrogen concentration were observed in any of the reactors regardless of aeration shutdown.

For this fourth sampling analysis, all PHA Enrichment reactors demonstrated a net removal of nitrogen over the sampling period (Figure 22). Reactor AE-4 achieved the greatest nitrogen removal (32% or 56.1 mg/L) and AE-20 achieved the lowest nitrogen removal (5% or 11.9 mg/L). These results could be predicted, given that they represent the lowest and highest aeration rates, respectively. In other words, low aeration would be expected to remove more nitrogen through its use as an alternative terminal electron acceptor while higher aeration rates would cause little to no nitrogen removal as dissolved oxygen provides all the terminal electron acceptors necessary and nitrogen is only required for growth.

In contrast to the observations for the 12-12-13 performance assessment, all four reactors exhibited a noticeable increase in residual DO upon VFA depletion. Reactor AE-4 and AE-8 (Figure 21) exhibited very small spikes in residual DO, increasing from 0.02 to 0.25 (mg/L) and 0.5 to 0.68 (mg/L), respectively; this relatively small increase is likely due to the OUR (associated with catabolism of PHA, NH_3, and NO_2) still being considerably larger than the oxygen transfer rate. Reactors AE-12 and AE-20 (Figure 21) realized much larger spikes in

residual DO(2.0 to 3.75 mg/L and 0.5 to 5.0 mg/L, respectively); these larger increases can be explained by a drastic reduction of the OUR but also a much higher oxygen transfer rate present in these reactors.

The carbon cycling for all reactors was not entirely captured due to a loss of the last PHA samples collected in the enrichment evaluations. However, all reactors had consumed the majority of PHA produced by the 175 minute sample. All reactors also achieved very similar concentrations of PHA at their peak (≈10 Cmmol/L, Figure 21); as was observed for the 12-12-13 analysis, only AE-20 achieved an early peak (≈50 minutes), while all other reactors peaked at approximately 100 minutes (Figure 21). AE-20 and AE-8 achieved VFA depletion at approximately 50 minutes, while AE-4 and AE-12 achieved VFA depletion at approximately 75 minutes. The delayed depletion experience by AE-12 was unexpected, as AE-12 had a higher availability of oxygen due to a higher OTR than AE-8. Therefore, theoretically the mixed microbial consortium should be capable of consuming VFA's faster.

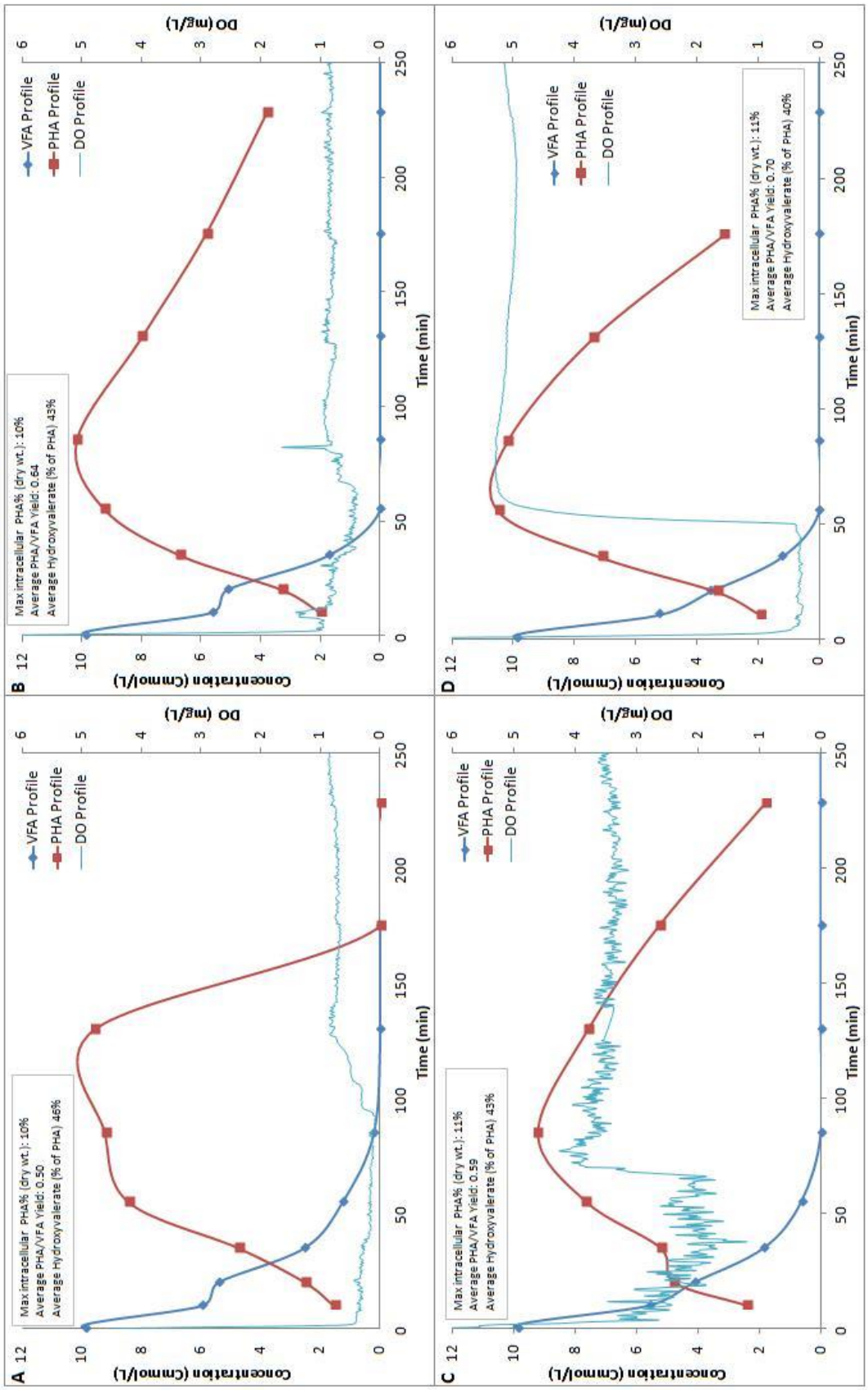


Figure 21: Carbon data, Enrichment Evaluation 4-29-14. AE-4 (A), AE-8 (B), AE-12 (C), AE-20 (D)

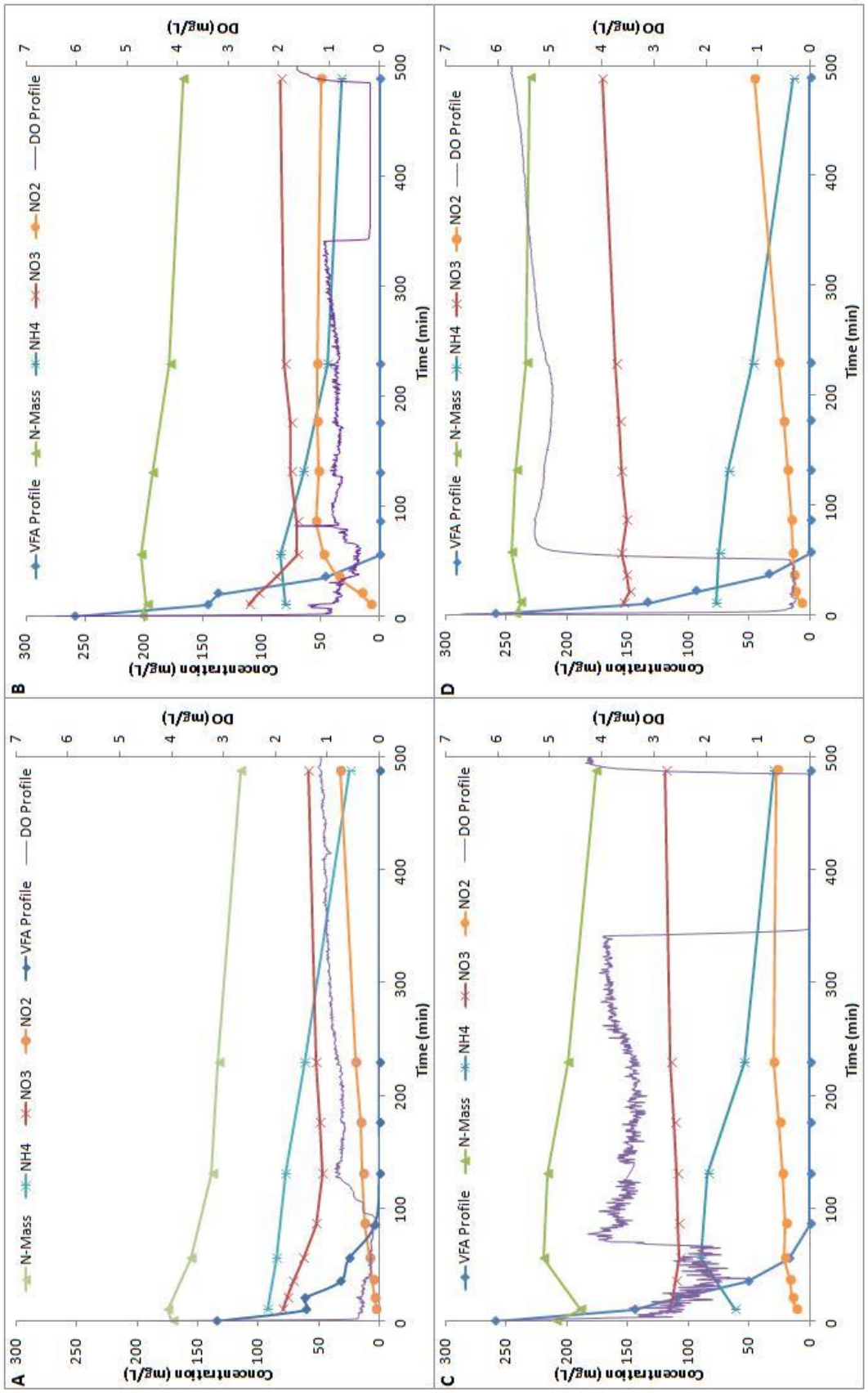


Figure 22: Nitrogen Data, Enrichment evaluation 4/29/14. AE-4 (A), AE-8 (B), AE-12 (C), AE-20 (D)

5.6.3 Alpha Aeration Parameters

The Alpha aeration parameter is useful for understanding the effects of aeration equipment and water chemistry on the oxygen transfer. It is presented herein as reference to industrial processes; however, it is of little significance to the investigation. An Alpha of 1.0 indicates identical aeration characteristics to tap water, while values less than one represent an inhibition in oxygen transfer. Typical values for diffusion and mechanical aeration systems range from 0.4 to 1.2 (Tchobanoglous et al., 2003). Reactor alpha's are as follows; AE-20 α=0.98, AE-12 α=1.01, AE-8 α=.088, and AE-4 α=1.1. These values were measured only once and may not be representative of the entire investigation period. The values being close to 1.0 is not surprising given that 60% of the substrate was tap water, and the dairy manure added to the fermenter was also diluted with tap water.

5.6.4 Feast to Cycle Ratio

The Feast/Cycle ratio as described in section 3.1 is a potentially important parameter when assessing the ability of a PHA Enrichment reactor to select for PHA producing cultures (Majone et al., 1996; vanLoosdrecht et al., 1997). As such the Feast/Cycle ratio was calculated with every enrichment evaluation, as well as several other periodic observations during the course of the investigation.

The Feast/Cycle ratio was calculated from 24-hr DO plots recorded on Hach HQ40d meters and LDO101 probes for PHA Enrichment reactors. The end of the carbon "feast" period was established when the residual DO concentration rapidly increased. While some carbon remains after the end of this defined "feast" period (e.g., PHA; some unmeasured COD potentially remaining in solution), denoting the depletion of VFAs is a reliable and consistent method for establishing this useful process metric. However, a potentially confounding variable particular to this research relates to nitrification; the residual DO plots for low aeration rates (i.e., k_La 4 and 8 hr^{-1}) reflect nitrifying bacteria activity that can also exhibit a significant oxygen demand. Thus, the residual DO can remain depressed for periods well after VFA exhaustion. Best judgment was used in analyzing the more difficult

residual DO plots. Reactors AE-4 and AE-8 were monitored more extensively than reactors AE-12 and AE-20 due to the more variable nature of residual DO in those reactors.

A Feast/Cycle ratio of less than 0.2 is recommended in the literature (Johnson et al., 2009), based on the empirical data showing successful selection of PHA producing consortiums that have a Feast/Cycle ratio less than 0.2. The ratio calculated for reactor AE-4 is above the desired Feast/Cycle ratio for the first 150 days (Figure 23). However, after day 150 the biomass in the reactor AE-4 achieved a ratio very similar to the other reactors, and remained low and stable thereafter. One potential explanation for the high Feast/Cycle ratio is excess OUR caused by nitrifying bacteria "masking" the residual DO rise caused by VFA exhaustion. This masking would prevent an accurate estimation of the Feast/Cycle ratio. The other potential explanation is a change in bacterial population present in the reactor that drastically increased the VFA uptake rate. The other reactors, with few exceptions, consistently exhibited a Feast/Cycle ratio of 0.2 or less.

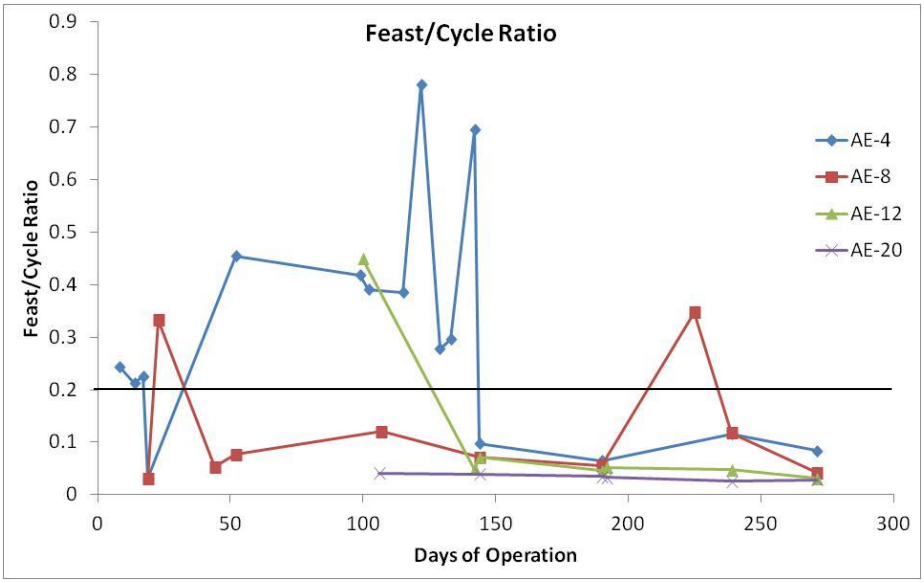


Figure 23: Feast/Cycle Ratios.

5.6.5 OLR Stability and F:M Ratio

Since all PHA Enrichment reactors received substrate from the same source, all mixed microbial cultures realized the same Organic Loading Rate (OLR; g COD/L day). The OLR was a measure of VFA concentration in the feed. Figure 24 shows the variation of substrate OLR

over the course of this investigation. Variation of the OLR is cause by changing concentrations of VFA's in the fermenter liquor. Due to the variable nature of manure fermentation the process has inherent variability which is highlighted in the OLR plot. It is important to note that no trend linking maximum PHA production and OLR is exhibited (Figure 25); The PHA production data shown is from the PHA Production reactors. The relatively low OLR from day 100 to 200 were caused by an unknown condition, while fermenting organic waste streams is a reliable method to enrich for VFA's the process can always be affected by variability in the influent. The inherent influent variability imposed on a waste fed system is one of the challenges faced by $_{MC}$PHA Production as unknown upstream factors can cause upsets in downstream reactors.

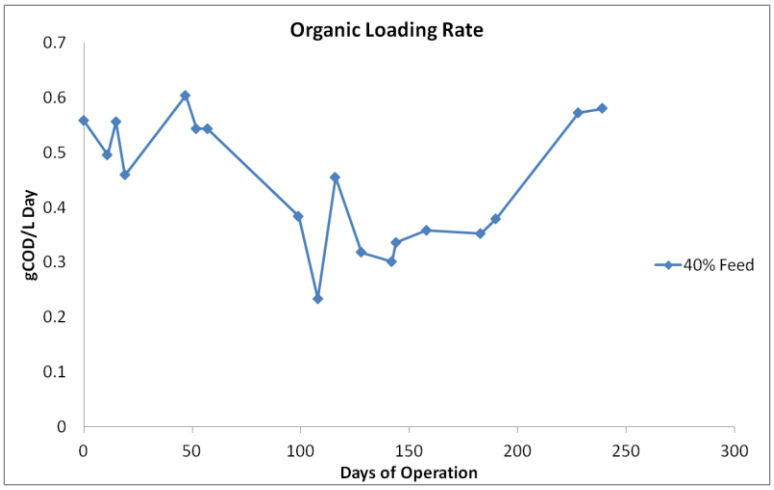


Figure 24: Organic Loading Rate (OLR) gCOD/ L Day.

The food to microorganism (F:M) ratio was popularized by wastewater treatment operations. In effect it compares growth conditions of bacteria by normalizing the substrate present to the mass of bacteria present. In this application the daily mass of VFA's (gCOD) is divided by g MLVSS present in the reactor. The F:M could prove a useful parameter to PHA Enrichment reactors by maintaining a low F:M the famine period is extended thus increasing the competitive advantage of PHA producing microorganism; however, if the F:M is too low the biomass will not be stable. Figure 25 shows the F:M ratio during the course of the experiment, also plotted are the pkPHA% achieved in the Production reactor. If a correlation was present it would be easily identifiable. Due to the variable nature of the

fermented dairy manure substrate the OLR, and subsequently the F:M is variable throughout the investigation. Future work may use F:M as a control parameter in optimizing PHA enrichment reactors; however, the variance of the parameter in this study prevent further conclusions.

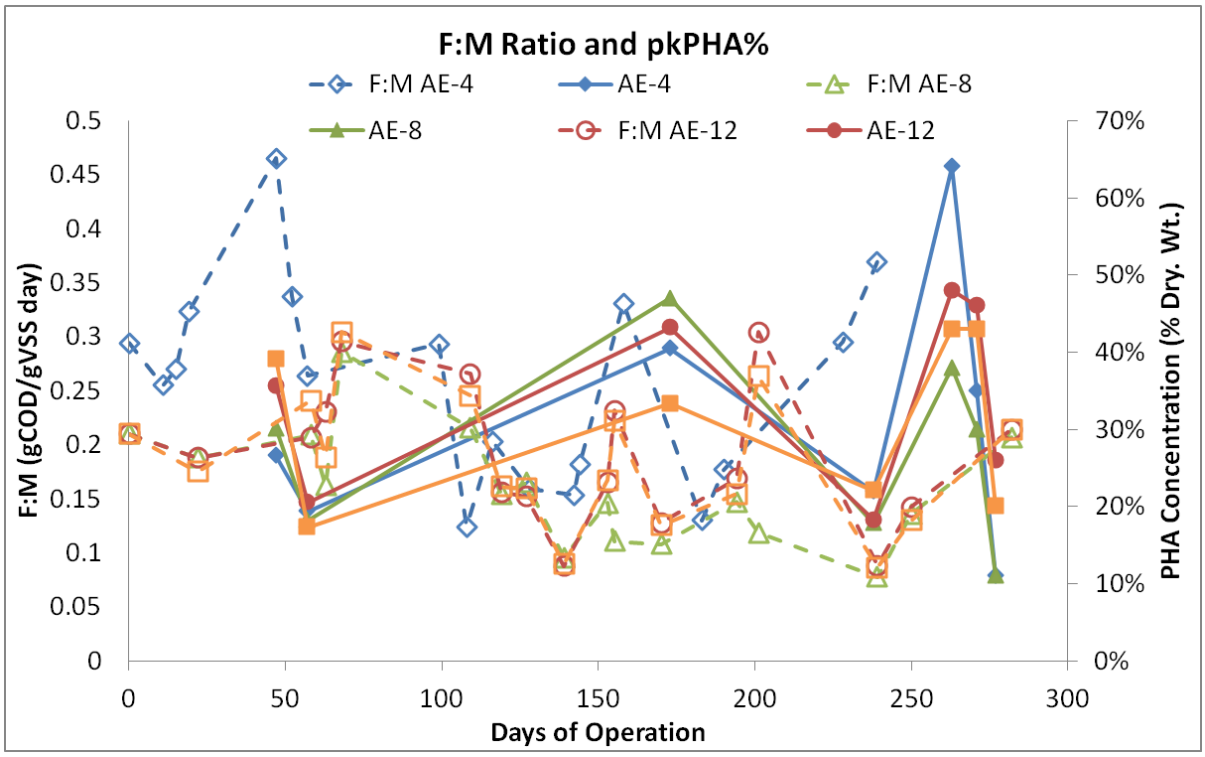


Figure 25: F:M Ratio and pkPHA%;
F:M- Food to Microorganism ratio

5.6.6 Reactor Solids Content

Performance of the PHA Enrichment systems was inherently variable due to the changing characteristics of manure and associated changes in OLR. The reactor solids concentrations (reported as MLSS, mixed liquor suspended solids) were regularly checked in all Enrichment reactors. Figure 26 shows the variations in reactor MLSS over the course of the experiment. The average MLSS concentration followed 90% confidence interval in mg/L are as follows; 2309±572 (AE-20), 2248±655 (AE-12), 2454±674 (AE-8), and 2238±321 (AE-4). It was anticipated that the reduced oxygen in the lower aeration reactors would decrease the respective biomass concentrations. However, MLSS was relatively consistent across all

reactors until 150 days of operation when, AE-20 and AE-12 MLSS content decreased substantially while AE-4 and AE-8 remained relatively constant. All through the investigation reactors AE-20 and AE-12 had very similar MLSS concentration, while AE-8 and AE-4 seemed to have a more independent response. This could likely be caused by the effect of k_La on the growth of bacteria. The sudden variability in MLSS later (days 150 and 200) in the experiment could be caused by sampling error. The sample collected for analysis was 10-mL compared to the 1200-mL of reactor volume, thus if a non representative sample was collected (i.e., due to clumping of sludge or sludge adhering to reactor walls) it is possible to cause a significant error. AE-12 and AE-20 reactors both had a significant drop in MLSS before peaking to higher than normal concentrations and ultimately returning to normal on days 200, 245, and 250. No causes are apparent during this period the reactors OLR was stable around 0.4 (gCOD/L day) and gradually rose to 0.6 (gCOD/L day) this is the likely reason for the large increase in MLSS.

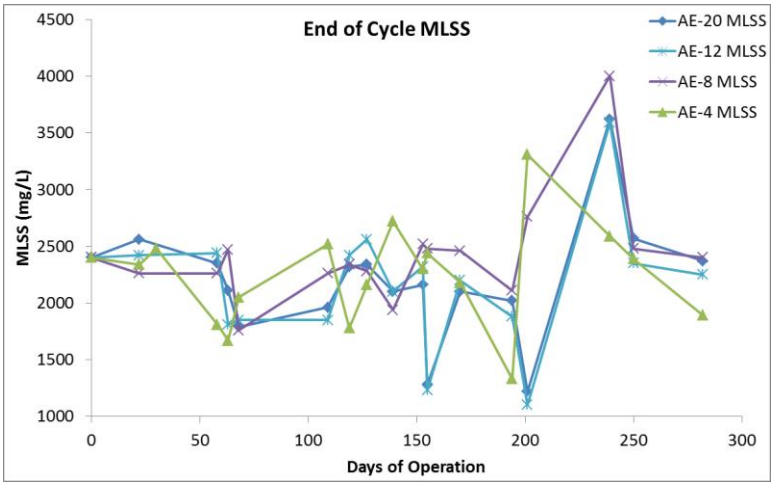


Figure 26: End of Enrichment cycle MLSS concentrations.

5.6.7 PHA/VFA Yield

The PHA/VFA yield values reported in Table 7 were calculated using procedures described in Chapter 4, sections 4.3.1 and 4.5. Proc GLM was used to analyze the effects of VFA Consumed, k_La, and the interaction between k_La*VFA Consumed. This analysis

demonstrated a correlation between PHA concentration and VFA consumed, as would be expected. The analysis also demonstrated the significant impact of k_La*VFA Consumed, with a Pr>F value of 0.0282. This analysis demonstrates that k_La had a statistically significant effect on the Enrichment reactors' PHA/VFA yield. The linear regression coefficients (Table 7) were calculated using proc REG in SAS. The model was forced to intercept at zero due to no PHA being present when no VFA's had been consumed; the data and regressions are presented in Figure 27. Reactor AE-20 achieved the highest yield coefficient at 0.796 (Cmmol PHA/Cmmol VFA); reactors AE-4 and AE-8 achieved similar values 0.624 and 0.650 (Cmmol/Cmmol), respectively. AE-12 had the lowest coefficient with 0.509 (Cmmol/Cmmol). Reactors AE-4 and AE-8 models achieved a high degree of fit R^2 (i.e. low variance) values of 0.94 and 0.91 respectively. The low variance suggests a more reliable and consistent biomass is present in those reactors compared to the AE-12 and AE-20, which exhibited R^2 values of 0.77 and 0.82 respectively. This is contrary to what was expected based on the work of Third et al. (2003), in which a reduction in k_La caused an increase in PHB/VFA yield. This is discussed further in section 5.6.11.

Table 7: PHA/VFA Yield (Cmmol/Cmmol); based on linear regression of five Enrichment evaluations

k_La	n	Model Pr>F	Adj R^2	Yield (Cmmol/Cmmol)
4	31	<0.0001	0.94	0.624
8	28	<0.0001	0.91	0.650
12	30	<0.0001	0.77	0.509
20	28	<0.0001	0.82	0.796

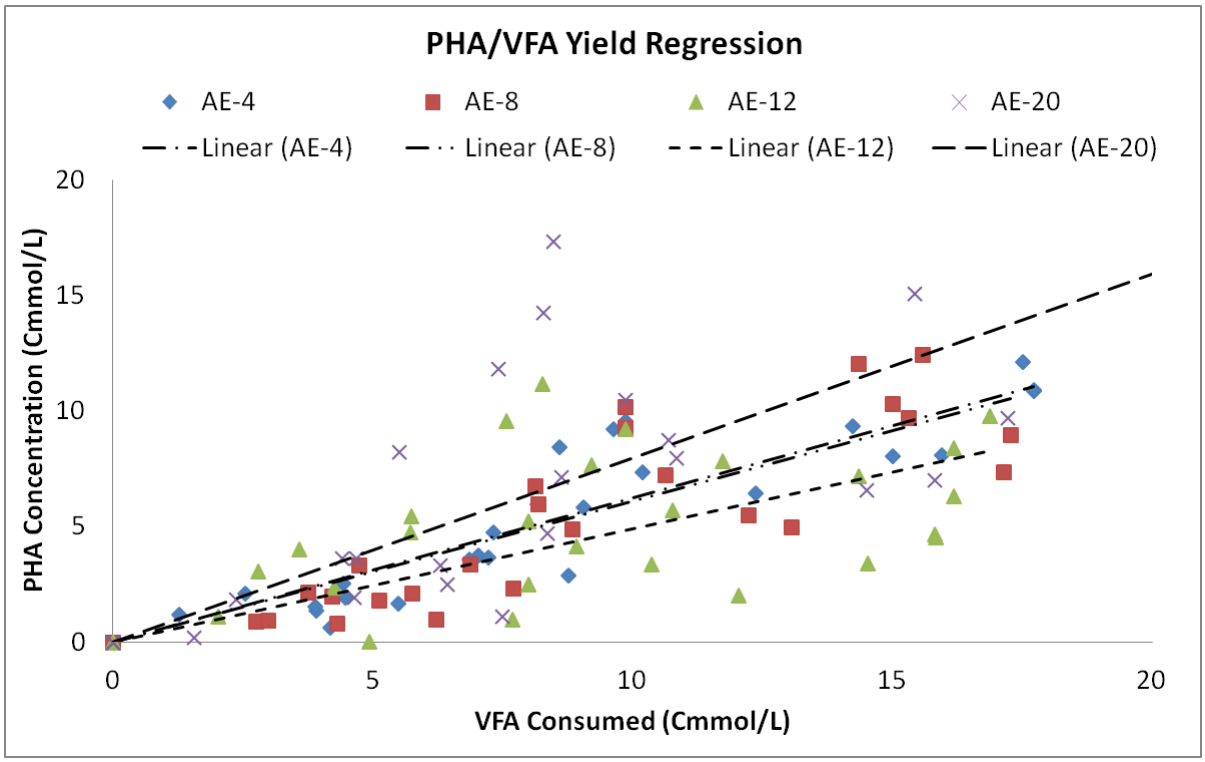


Figure 27: Regression results and data, used to model reactor Enrichment yield (PHA/VFA).

5.6.8 Maximum PHA Content

The maximum PHA concentration (Cmmol/L) was selected for each of the five enrichment runs and then analyzed with proc GLM for statistical differences. The peak intracellular PHA content for each of the five enrichment runs is shown in table (

Table 8). The model compared regressions using the variables date, k_La, and k_La*Date interaction. Date was included to allow comparison within each Enrichment evaluation and prevented the whole experiment variance from having an effect. The results of this analysis showed no statistically significant differences, with Pr>F value of 0.193 for the interaction effects of k_La*Date. The plot of maximum concentrations (Figure 28), demonstrates some interesting patterns. The most notable trend is on both the 12-12-13 and 6-16-14 evaluations the reactors achieved very similar maximum PHA concentrations. A large spread between Enrichment reactors max PHA content was observed on 11-9-13, 3-14-14, and 7-19-14. The range of max PHA content on a g PHA per g Dry biomass shows typical values ranging from 9-15% PHA, while some reactor went as low as 4.4% (AE-12 11/9/13) and others achieved a peak of 22.3% (AE-20 7/19/14).

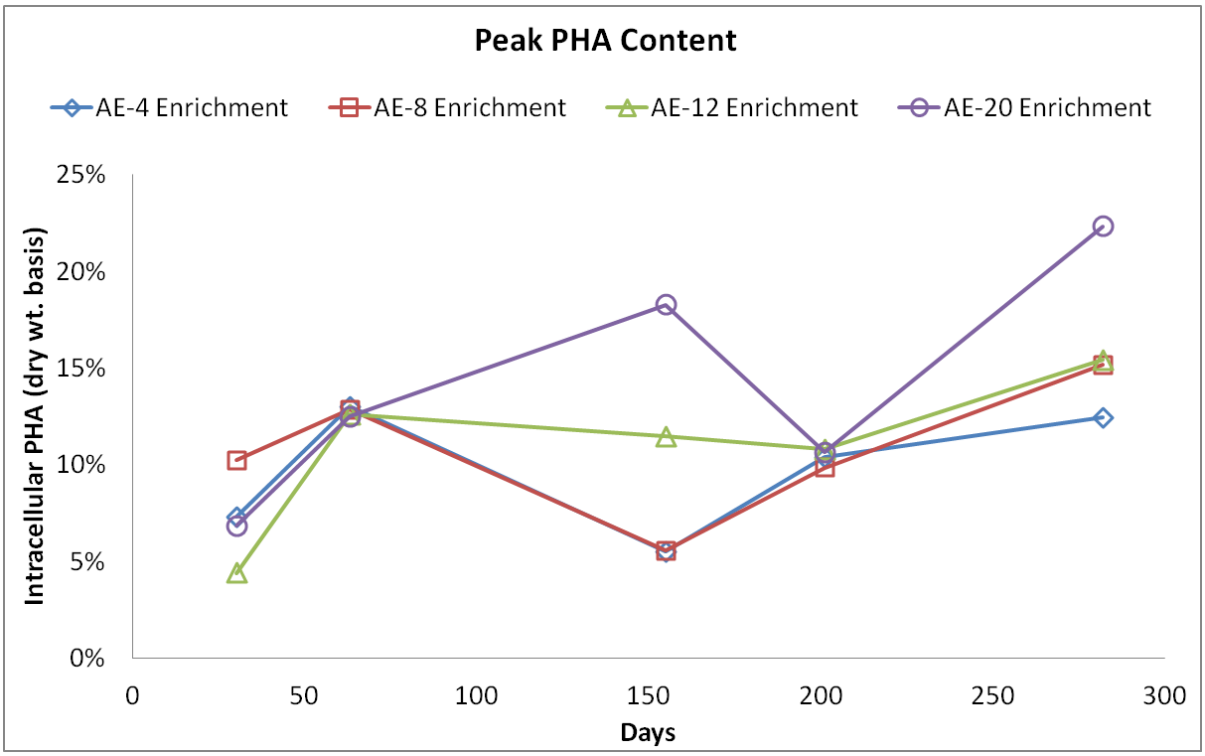


Figure 28: Plot of max PHA concentrations for each Enrichment evaluation.

Table 8: Enrichment evaluation max PHA concentration and averages.

K_LA	Date	PHA Cmmol/L	PHA% PHA g/g Biomass	HV% HV g/g PHA	*Average PHA %
4	11/9/2013	8.09	7.3%	36%	9.7 ± 2.2
	12/12/2013	12.12	13.0%	6%	
	3/14/2014	5.96	5.5%	29%	
	4/29/2014	9.58	10.4%	0.44	
	7/19/2014	10.90	12.5%	49%	
8	11/9/2013	10.33	10.3%	36%	10.7 ± 2.9
	12/12/2013	9.71	12.8%	14%	
	3/14/2014	5.98	5.6%	0.198	
	4/29/2014	10.18	9.9%	0.393	
	7/19/2014	12.44	15.2%	0.464	
12	11/9/2013	4.68	4.4%	20%	10.9 ± 2.9
	12/12/2013	9.80	12.6%	2%	
	3/14/2014	11.18	11.5%	0.26	
	4/29/2014	9.22	10.8%	0.38	
	7/19/2014	8.39	15.4%	0.55	
20	11/9/2013	7.00	6.9%	21%	14.1 ± 4.4
	12/12/2013	9.71	12.5%	22%	
	3/14/2014	17.35	18.3%	37%	
	4/29/2014	10.47	10.6%	0.37	
	7/19/2014	17.82	22.3%	59%	

***Average ± 90% Confidence Level.**

5.6.9 VFA Uptake Rate Analysis

The rate of VFA Uptake was calculated using the process described in section 4.3.2, the data set used all samples from all 5 enrichment evaluations. Proc GLM was used to calculate the interaction between Time, or Time*MLSS, and k_La thus comparing the reactors to each other. This analysis determines statistical significance caused by a change in reactor k_La. No statistical significant differences were present between the Enrichment reactors for either qVFA or rVFA; The Pr>F values were 0.261 and 0.839 for qVFA and rVFA respectively. This is surprising as the reduction in k_La was expected to reduce the rate at which bacteria were capable of taking up the VFA's (Tchobanoglous et al., 2003; Third et al., 2003), however this does not appear to be the case. This result could be caused by two conditions; a parameter common to all reactors could be controlling substrate uptake, or the variance within the data set was too large to statistically identify differences.

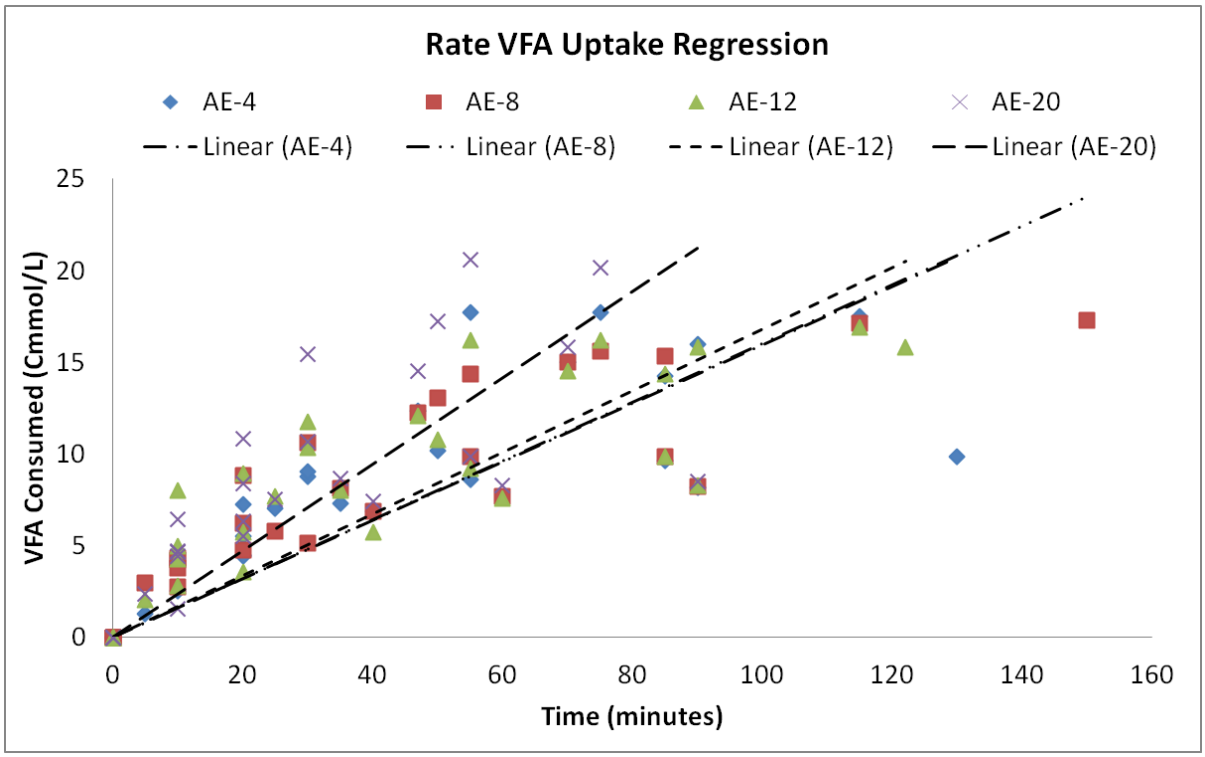
Proc Reg was used to build linear models; these models were designed so that the coefficients would relate to commonly used design yields. Specifically, two models were developed to calculate rVFA and qVFA; a plot of the models used for analysis of covariance is in Figure 29.

While none of these rates demonstrated a statistically significant difference, likely due to large variance in the data, the general trends are still worth noting. The mean highest substrate uptake rate occurred in the reactor with the greatest aeration rate where oxygen was never a limiting factor. The reactors AE-8 and AE-12 appear very similar suggesting condition were very similar. Reactor AE-4 had the lowest uptake rates likely due to a lower concentration of terminal electron acceptors available to the bacteria. The high level of variance and associated poor fit of the models is likely caused by gradual changes in bacterial population changing the VFA uptake rates. The variance and poor fit is also present in the qVFA which demonstrates the variance in uptake rate was not caused by the observed changes in MLSS concentration.

Table 9: Rate of VFA uptake; based on linear regression of five Enrichment evaluations.

k_La	n	Adj R-Sq	qVFA Cmmol/gVSS min	*±95% CL	Adj R-Sq	rVFA Cmmol/L min	*±95% CL
4	32	0.64	0.047	0.010	0.77	0.109	0.021
8	31	0.76	0.067	0.011	0.81	0.126	0.023
12	30	0.70	0.067	0.015	0.76	0.122	0.025
20	29	0.70	0.086	0.021	0.73	0.142	0.033

***Bounds for a 95% Confidence Level.**



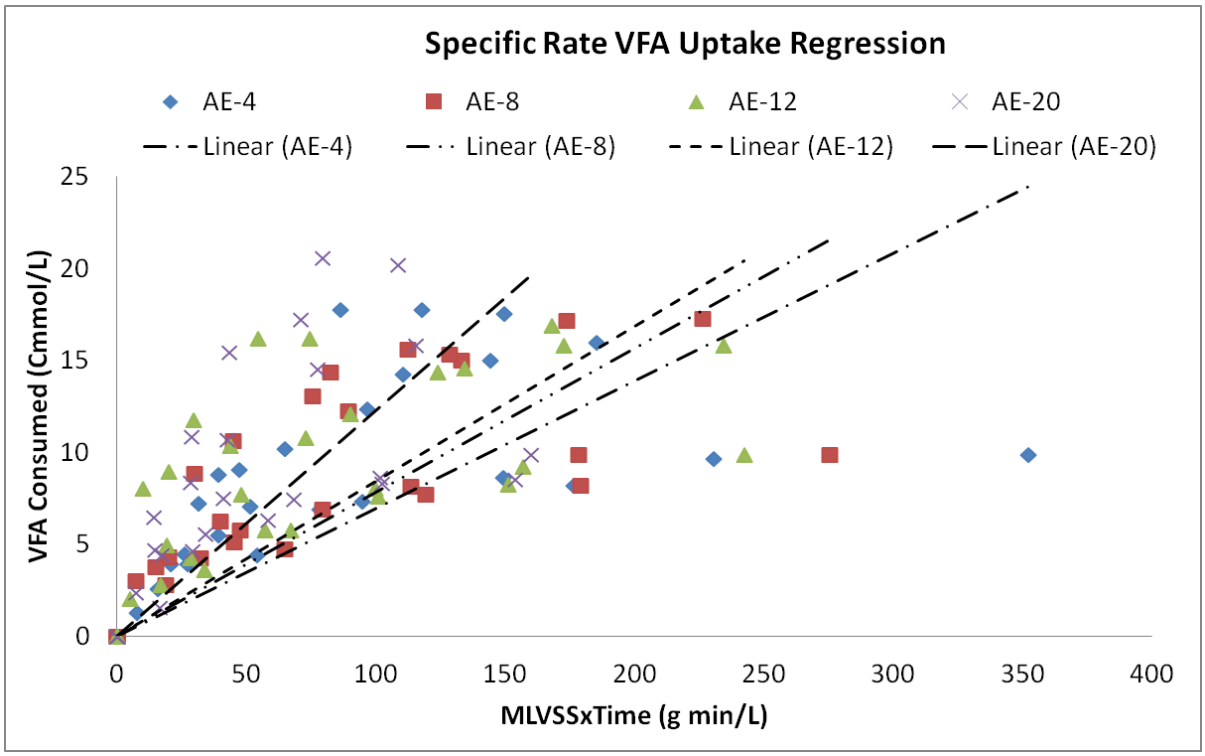


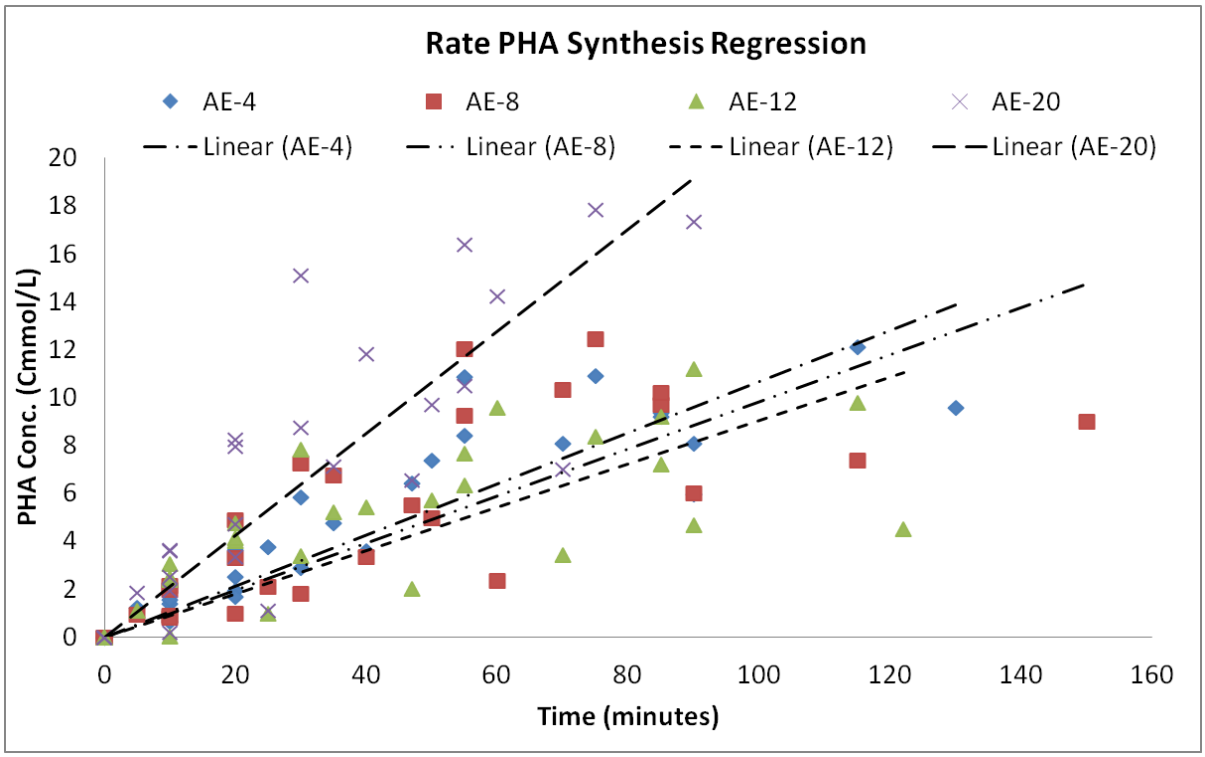
Figure 29: Enrichment VFA Uptake Models.

5.6.10 PHA Synthesis Rate Analysis

The calculation of PHA synthesis rates (rPHA) and specific PHA synthesis rates (qPHA) was accomplished with SAS 9.3 and utilized data from all five Enrichment evaluations. Proc GLM was used to assess for statistically significant differences between reactors. Both rPHA and qPHA models were deemed to have a high level of statistical difference for the interaction effects of k_La and Time (or TimexMLVSS), with Pr>F values of <0.0001 and 0.0006 respectively. Reactors AE-4, AE-8, and AE-12 all had similar synthesis rates, ranging from 0.10 to 0.12 rPHA (0.04 to 0.05 qPHA); however, biomass in reactor AE-20 realized a doubling of the synthesis rate to 0.2 rPHA (0.1 qPHA) (Table 10). This increase in synthesis rate is likely due to the abundance of residual DO. The linear regression models utilized data from all five enrichment evaluation (Figure 30); both models (rPHA and qPHA) have a high R^2, indicating that through the five evaluations the correlation was repeatable, predictable, and stable. Comparing the reported specific synthesis rates to values reported in literature shows that data in this study are slightly less than average, though still well within reported ranges. Typical values for $_{MC}$PHA, including waste substrates, ranged from 0.013 to 0.402 (Cmmol/g min) with an average of 0.117 (Cmmol/g min) (Reis et al., 2003; Serafim, Lemos, Albuquerque, et al., 2008)

Table 10: Rate of PHA synthesis; based on linear regression of five Enrichment evaluations.

k_La	n	Adj R^2	qPHA (Cmmol/gVSS min)	*95% CL	Adj R^2	rPHA (Cmmol/L min)	*95% CL
4	31	0.911	0.048	0.009	0.79	0.107	0.012
8	28	0.851	0.054	0.011	0.772	0.122	0.020
12	30	0.856	0.052	0.010	0.777	0.101	0.015
20	28	0.872	0.112	0.023	0.786	0.213	0.032



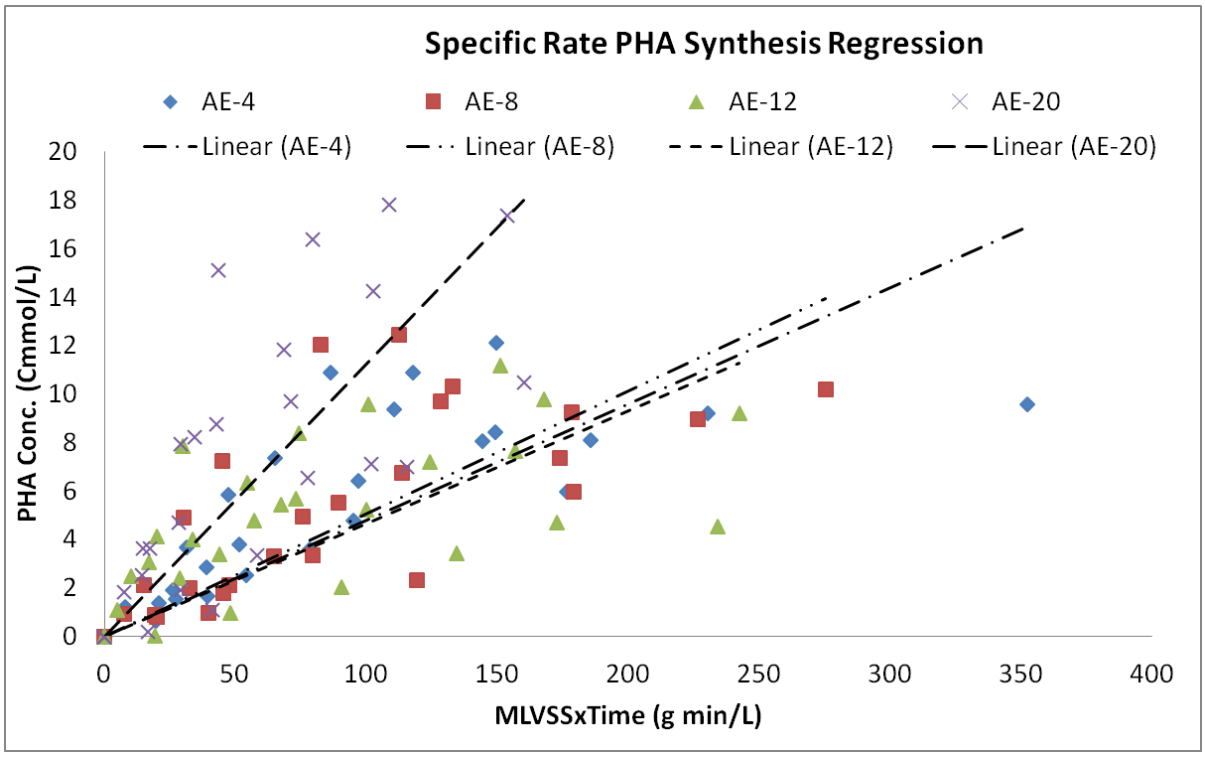


Figure 30: Enrichment evaluation PHA synthesis models.

5.6.11 Nitrogen Removal

As discussed, some nitrogen removal was observed in the Enrichment reactors. Nitrogen removal is of interest due to the reduction of oxygen demand provided when nitrates are utilized in place of oxygen as a terminal electron acceptor. In addition, the effluent from the reactor will be cleaner with a reduced nitrogen load to downstream systems. Proc GLM did not demonstrate a statistically significant difference between Enrichment reactors for the mass of nitrogen removed. The interaction between mass of nitrogen removed and k_La had Pr>F value of 0.3903; this is likely due to large variation in nitrogen removal from the system. The system also appeared to have an unidentified source of nitrogen, with total nitrogen present in the system increasing at times, indicating negative nitrogen removal. This unidentified source is likely break down of particulate organic matter present in the reactor substrate. The un-quantified nitrogen increases the large variation observed in the models (Figure 31).

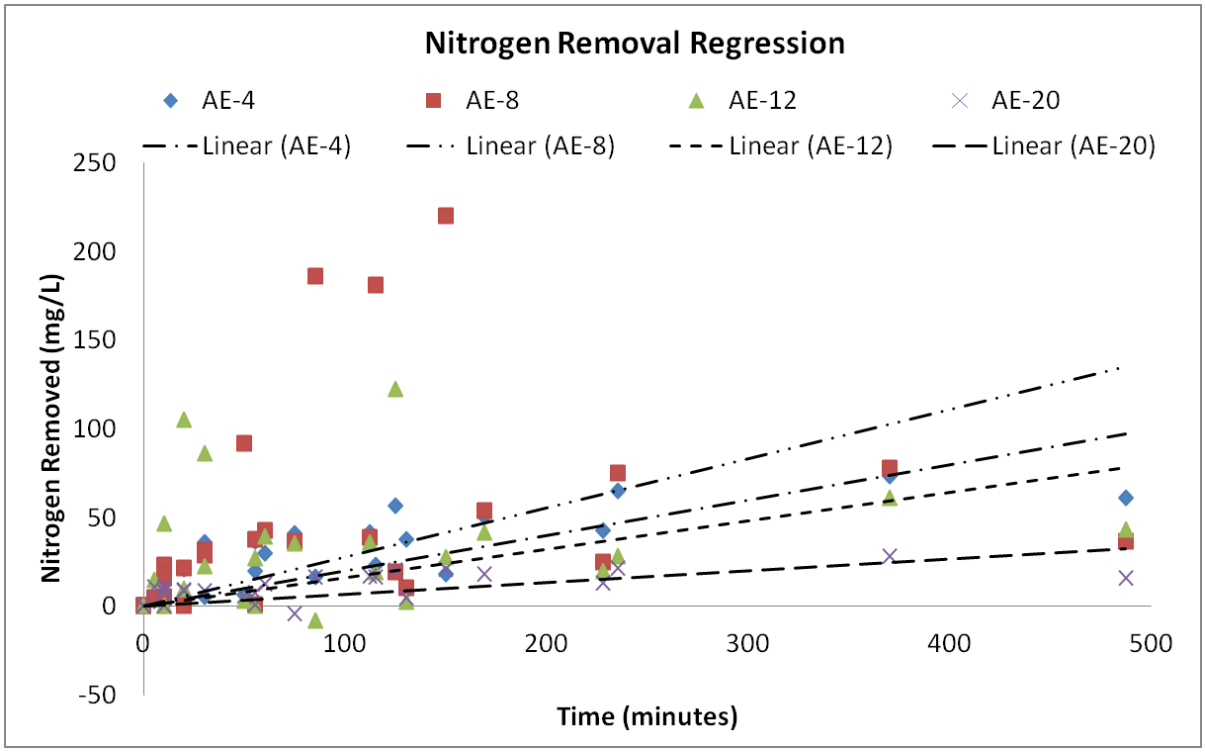


Figure 31: Nitrogen removal models.

5.6.12 Summary and Conclusions of Enrichment Data

The PHA Enrichment reactors achieved stable operational conditions quickly in the investigations, with consistent mixed liquor solids observed across all reactors; however a brief, unstable operational period was realized, before the process re-stabilized toward the end of the evaluation period. The organic loading rate (Figure 25) also remained relatively consistent until 150 days when a gradual decrease occurred before it increased to original levels later in the investigation. The reduction in reactor MLSS could be caused by the fluctuations in reactor OLR. Ultimately these fluctuations did not affect either the reactor PHA/VFA yield or Feast/Cycle ratio observed during the evaluation period. The yield was highly repeatable across all five Enrichment evaluations, realizing very high R^2 values. The hypothesis of an increase in PHA/VFA yield associated with higher aeration rate did not occur; instead the yields were AE-12 > AE-20 > AE-8 > AE-4 (Table 7). The hypothesis was based on work performed by Third et al. (2003) in which a correlation was established that demonstrated an increase in the PHA/VFA yield as the aeration decreased (k_La range tested 6 to 51 hr^{-1}). This work did not observe a similar correlation between k_La and PHA yield, likely due to the differences between substrates. Third et al. (2003) utilized a synthetic substrate with acetate as the only carbon source and nitrification inhibiting concentrations of Thiourea. The difference between synthetic substrate and a real waste stream with multiple species of VFA present would play a significant role in the reactor response to low aeration. As the substrate in this work was a real waste based substrate (and not sterilized), complete control was not achievable; thus non-VFA carbon sources could enter the reactor with substrate changing the reactor response. Further, this work did not inhibit nitrification, allowing an alternative terminal electron acceptor to be present in solution. This extra terminal electron acceptor would have confounded the rigorously controlled experiment performed by Third et al. (2003); however, one purpose of this experiment was to mimic the real world application as closely as possible thus it was necessary to allow nitrification to occur.

The Feast/Cycle ratios did exceed the recommended 0.2 (Figure 23) in the AE-4 reactor, however, the Enrichment evaluation demonstrated that VFA depletion occurred before a

residual DO spike therefore the Feast/Cycle ratios greater than 0.2 may be misleading. Operationally all reactors appeared to be very stable regardless of the aeration rate applied, which suggests a cost savings can be successfully realized in the enrichment phase through the reduction of aeration to the reactors. However the main industrial interest is in the quantity of PHA produced during the production process, which may be affected by reduction in Enrichment aeration. Thus, analysis of the PHA production reactors follows.

5.7 PHA Production Reactors Analysis

As noted, biomass recovered from each Enrichment reactor was used as inocula to evaluate and assess PHA production potential. To maintain process stability, biomass must be wasted from the enrichment reactor on a daily basis. In a future commercialization model, commodity PHA production would be realized using daily biomass wasted from Enrichment reactors. In total, seven independent PHA production evaluations were conducted on biomass from each Enrichment reactor; discussed below are results from three of the production evaluations, which were selected to be representative of the results of the complete investigation. Data from all seven production evaluations were compiled and analyzed using SAS 9.3. The analyses calculated relevant design parameters and statistically compares the reactors for significant differences.

5.7.1 PHA Production Assessment, Stage 1

Results from the second PHA production evaluation are illustrated in Figure 32 and are representative of both the first and the second production evaluation. Times for substrate addition are indicated by solid vertical lines on the charts; only three DO probes were available, therefore continuous residual DO data was only available for Production reactors AE-4 and AE-8, while residual DO in reactors AE-12 and AE-20 (Figure 32) were periodically checked with the third probe. As shown, an initial increase in PHA accumulation for all inocula was observed between 0-30 minutes, followed by a slower rate of PHA accumulation from 30 minutes until the end of evaluation. The VFA consumed plot shows a very inconsistent uptake rate in all reactors, suggesting a highly variable metabolic state.

Moreover, as shown, the biomass in all Production reactors realized periods of VFA depletion. This outcome is likely due to the use of residual DO to trigger substrate addition. Specifically, the concentration of VFA's had to reach levels low enough to change the bacteria's metabolism and induce a change in the OUR, ultimately resulting in a measurable increase in residual DO. This cyclical carbon surplus to carbon limitation and constant changing of metabolisms was thought to be one reason for the reduced pkPHA% concentrations. Total intracellular pkPHA% accumulated in these reactors ranged from 17-20% on a dry wt. basis, which is considerably lower than has been achieved with other waste streams (i.e., tomato cannery waste, primary sludge, or palm oil mill effluent) reporting 25% to 48% PHA on a dry weight basis (Serafim, Lemos, Albuquerque, et al., 2008). The hydroxyvalerate (HV) content (important to the material properties of PHA) was very close to values reported in literature. This production evaluation realized 20% to 35% gHV/gPHA and Serafim et al. (2008) a review article reporting 11% to 65% gHV/gPHA for a wide array of substrates. DO profiles in (Figure 32) for AE-4 and AE-8 demonstrate the lack of clearly defined peak events which caused confusion on the timing of substrate addition. Ultimately, these results were used to design the Stage 2 procedure.

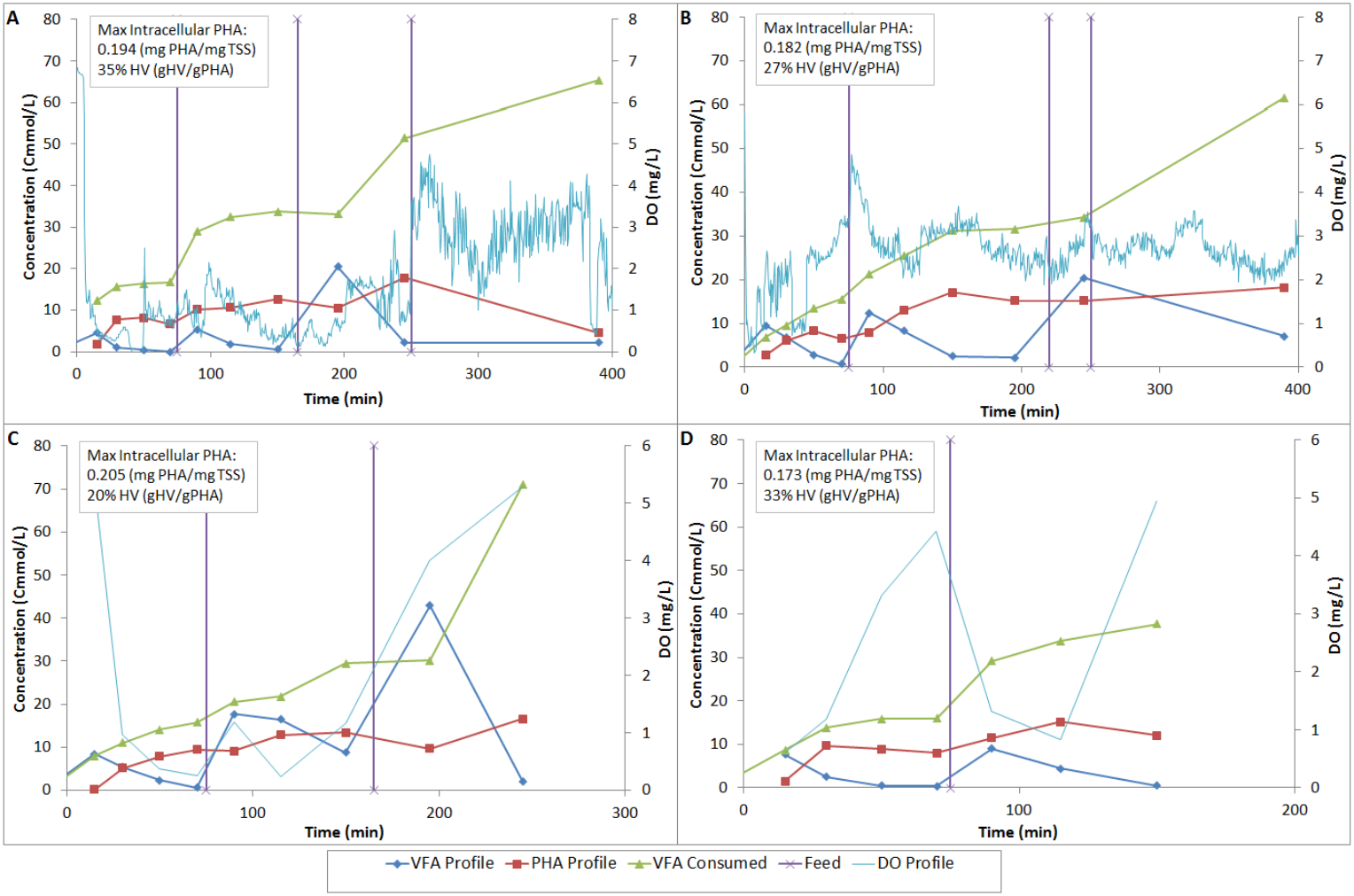


Figure 32: Production Data from 12/17/13, Stage 1.
A) AE-4 B) AE-8 C) AE-12 D) AE-20

5.7.2 PHA Production Assessment, Stage 2

The second stage PHA production evaluation utilized a time-based and residual DO-based method to determine when to add more substrate to sustain PHA production. Contrary to stage one the volume of substrate per pulse was adjust for each pulse event. This strategy prevented organic overloading late in the production run, when the Production reactor volume is low, the volumetric loading rate was maintained with substrate volume being equal to 10% of reactor volume at the time of feeding. This prevented excessive substrate concentrations which can potentially shock bacteria and impair PHA accumulation (E. R. Coats, VandeVoort, Darby, & Loge, 2011). As shown (Figure 33), inocula from two of the reactors (AE-4 and AE-20) achieved higher pkPHA% concentration at 27.7% and 27.5% respectively, as compared with the other reactors (AE-8 and AE-12) which peaked at 17.8% and 23.3% respectively. The increase in pkPHA% suggests an improvement in the production reactor process; however, it is still less than values reported for other waste substrates (Serafim, Lemos, Albuquerque, et al., 2008). The hydroxyvalerate content of the PHA realizes a large increase compared to Stage 1 evaluations. The reactors realized hydroxyvalerate content between 50% and 56% of total PHA. This high hydroxyvalerate content is still with ranges reported in literature however it is on the high end of the reported range (11-65% gHV/gPHA (Serafim, Lemos, Albuquerque, et al., 2008))

The sampling rate for these production runs was drastically increased in an effort to better characterize VFA consumption and PHA production. The increased sampling revealed some moderate decrease in PHA concentration between substrate pulses, though the general trend shows increasing PHA accumulation. As shown, the residual DO profile exhibited many well defined lows, however, the duration of the low was extremely short, which was not indicative of biological changes in OUR. Instead the low residual DO measurements were likely caused by a dilution effect from the addition of anaerobic (no residual DO) fermenter liquor, rather than changes in bacterial OUR. Another trend in the data is a very sudden reduction in VFA's consumed after the 7^{th} feeding in all reactors but AE-4. This observation would suggest that pkPHA% has been reached or exceeded in all reactors except AE-4. It is also important to note that residual VFA concentrations in the reactors

were gradually increasing as the production run progressed. In contrast to the Stage 1 operations, the bacteria appear to have been less affected by patterns of carbon excess followed by carbon limitation, although some carbon limitation may have been realized. This is evident in the relatively stable VFA consumed plots, with the VFA consumed maintaining a linear correlation with time until near the end of the production runs.

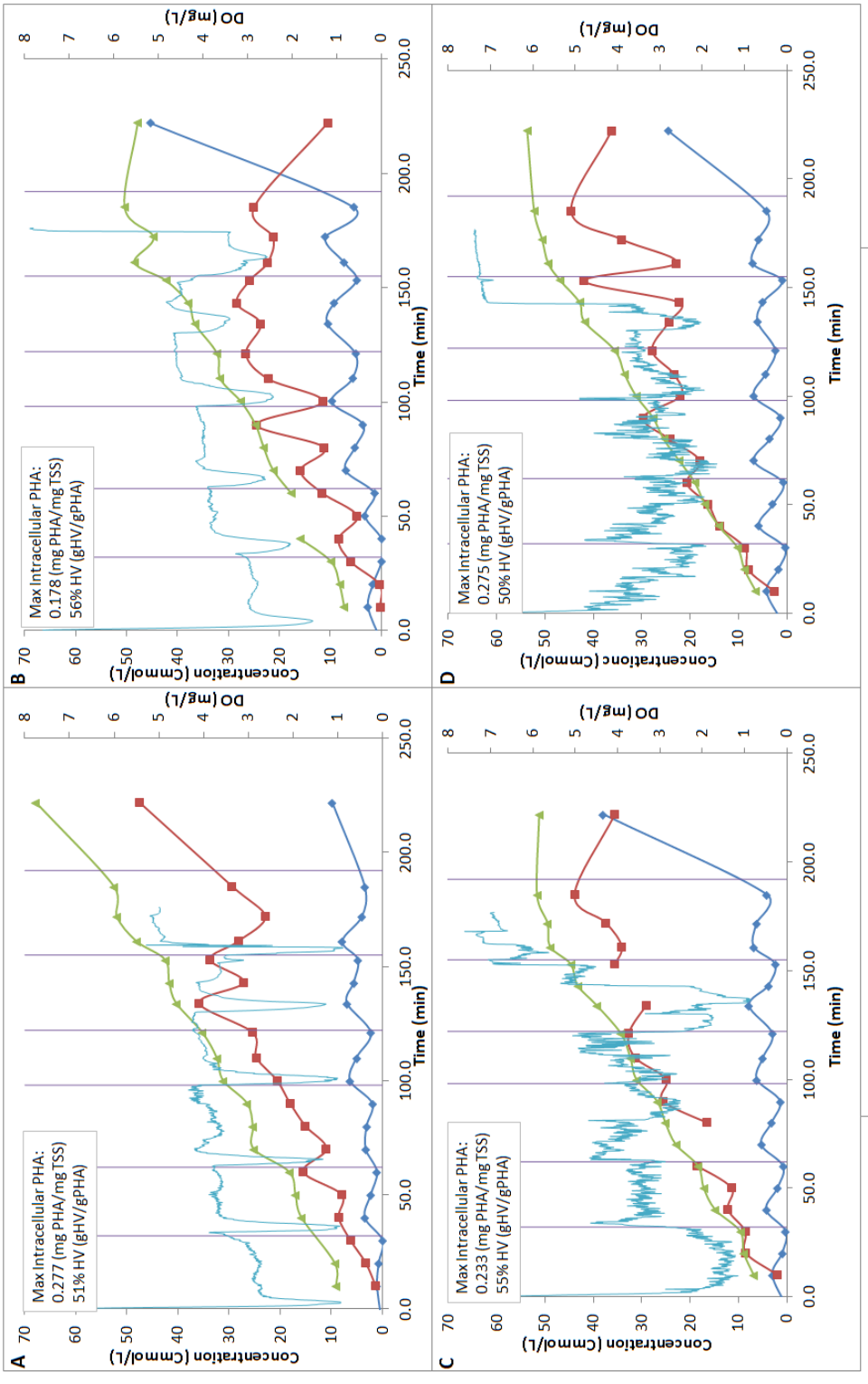


Figure 33: Production Run Data from 6/16/14, Stage 2.
A) AE-4 B) AE-8 C) AE-12 D) AE-20

5.7.3 PHA Production Assessment, Stage 3

The third and final stage in the PHA production evaluation and assessment utilized only time-based feeding events (Figure 34). As compared with Stage 2, the sampling rate was reduced to one sample every 30 minutes, starting at 55 minutes after the first substrate addition. The volume of substrate pulse-fed was kept constant, as in this Production reactor stage volume did not change much due to feeding (volume gained) and sampling (volume lost) being approximately equal. In this final procedure, pkPHA% concentrations approached values reported in literature (25-60%) (Serafim, Lemos, Albuquerque, et al., 2008). The best result was achieved by Production reactor AE-12 (Figure 34-C), which realized an intracellular peak of 46.1% PHA on a dry wt. basis. As shown, residual DO exhibited very short duration decreases, though they were longer than the results reported in section 5.7.2, likely caused by the addition of substrate. Similar to Stage 2, VFA concentrations continued to increase throughout the Production run, thereby ensuring that the bacteria were never carbon limited (i.e. sustained “feast” conditions). It is important to note that PHA Production reactors AE-4 and AE-8 (Figure 34) reached fairly high concentrations of VFA’s by the end of the production run; this was caused by a feeding rate that exceeded the VFA uptake rate. In this production stage, intracellular PHA concentrations in reactors AE-4 and AE-12 (Figure 34) are both continuing to rise at the end of the run, suggesting the reactor was capable of producing more PHA. The results also emphasize the need for more easily quantifiable parameter to indicate the end of a production run, rather than the procedure used.

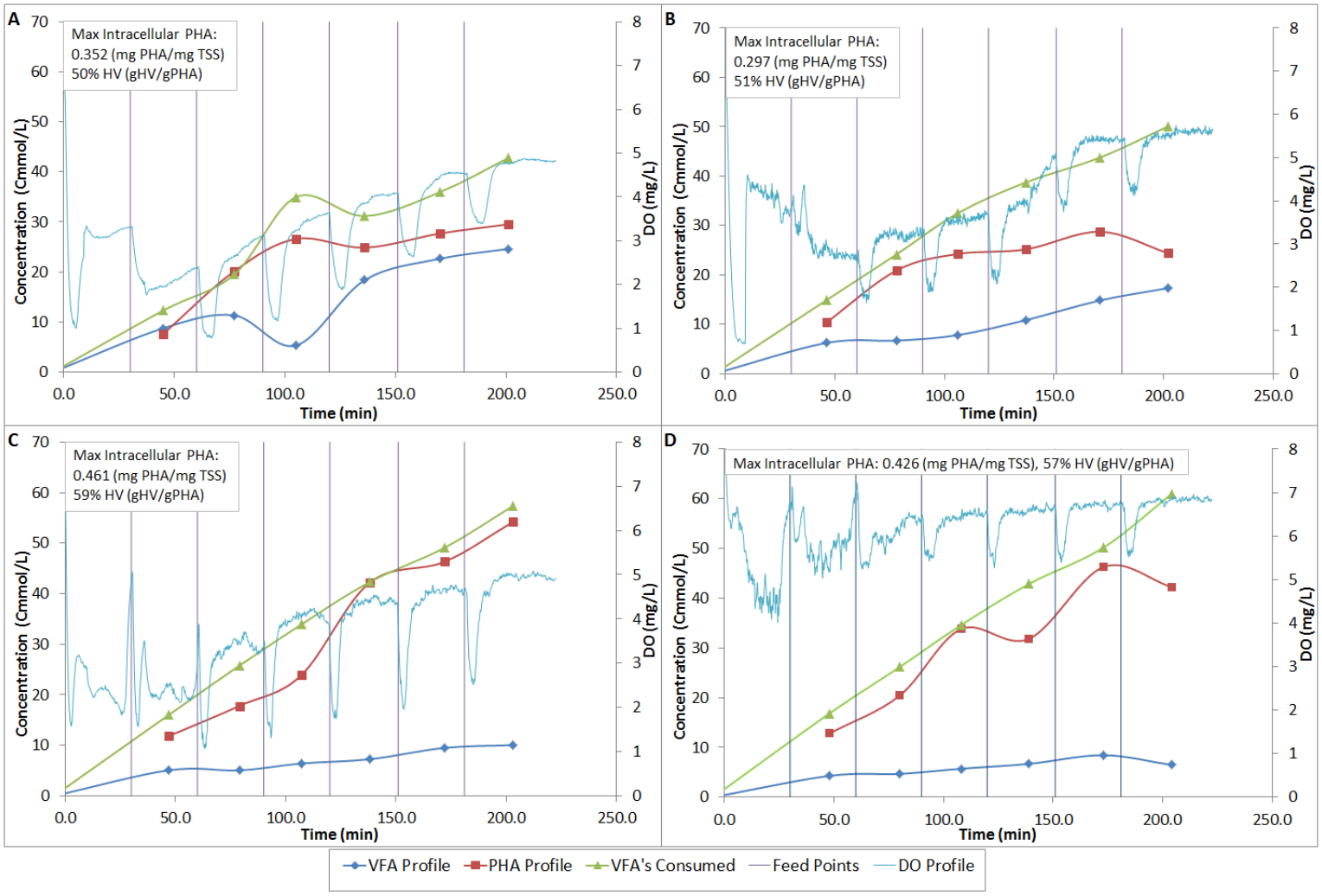


Figure 34: Production Run Data from 7/19/14, Stage 3.
A) AE-4 B) AE-8 C) AE-12 D) AE-20

5.7.4 PHA/VFA Yield

PHA yield is of particular interest to an industrial application, as it represents an efficiency of carbon invested to product (PHA) generated. While in the case of waste-derived substrates, the cost of carbon substrate is often low therefore lessening the industrial interest in yield; however, it is still a useful for estimating PHA production capabilities.

Proc GLM was used to identify any potential interactions between k_La and VFA Consumed (see section 4.5 for relevance of interactions effects). The k_La was entered as a class variable and the dependent variable was PHA concentration. The model variables were VFA's consumed, k_La, and the interaction between k_La and VFA's consumed (k_La*VFA_Cons). The interaction effect of k_La*VFA_Cons resulted in a Pr>F value of 0.1012, therefore the effect of k_La does not have a statistically significant effect on the VFA consumed based model. The yield coefficient calculated for each reactor does not bear statistically significant differences (Figure 35).

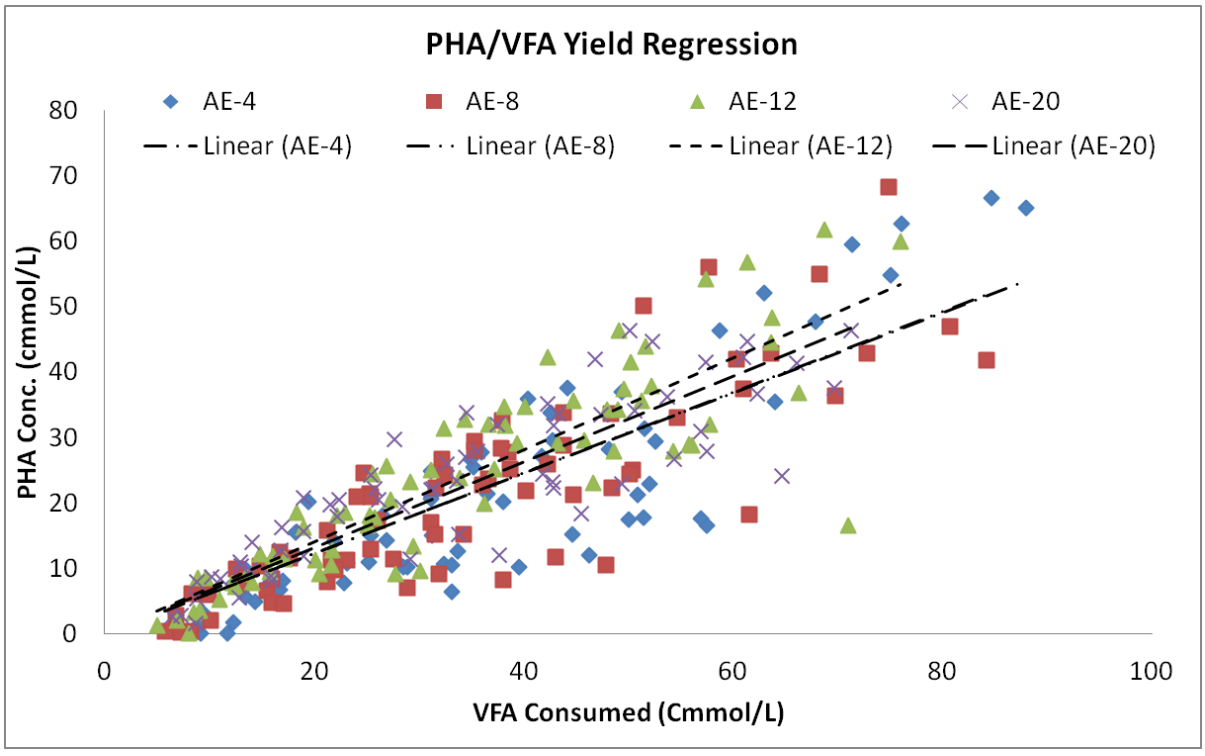


Figure 35: SAS Analysis for Statistically Significant Differences in Yield.

A linear regression analysis was performed utilizing data from all seven production evaluations to determine the yield coefficient associated with each reactor; the results are summarized in Table 11. The data has a high degree of fit (i.e. R^2>0.90), which indicates that the process was very stable and suffered little variance during all seven evaluations. The yields reported in Table 11 are well within typical values for mixed cultures which received a variety of different substrates (i.e., palm mill oil effluent, tomato cannery waste, or paper mill waste). Typically yield reported in literature ranged from 0.3 to 0.9 with an average reported yield of 0.54 (Dias et al., 2006; Reis et al., 2003; Serafim, Lemos, Albuquerque, et al., 2008).

Table 11: PHA/VFA Yield Coefficients; based on linear regression of seven production evaluations.

k_La	n	Model Pr>F	Adj R^2	Yield (Cmmol/Cmmol)	*±95% CL
4	70	<0.0001	0.904	0.614	0.048
8	71	<0.0001	0.911	0.615	0.046
12	71	<0.0001	0.934	0.703	0.044
20	63	<0.0001	0.941	0.654	0.041

***Bounds for 95% Confidence Interval**

5.7.5 Maximum PHA Production

From an operational standpoint, one of the most important parameters to be assessed in the process of evaluating the data (as related to future potential commercialization prospects) is peak intracellular PHA content, measured on a dry wt. basis (pkPHA%). The pkPHA% parameter is critical for downstream processing. Each batch of biomass rich in PHA requires the same mass of chemicals for downstream PHA purification, regardless of the intracellular PHA concentration. If the concentration of PHA in the biomass is higher, then the cost of the PHA per kg produced is reduced because the mass of chemical per kg of PHA is reduced.

The highest pkPHA% was selected from each of the seven productions evaluations for each of the four PHA Enrichment reactors (Table 12). The variation in Cmmol fed present in Table 12 is caused by variable concentration of VFA's in the fermenter effluent, as well was variations in the number of substrate additions. It is also important to note the presence of

PHA/VFA yields greater than 1(i.e., more PHA present than carbon consumed), while this should be impossible in this instance it is due to non-VFA carbon being utilized by bacteria to produce PHA.

The pkPHA% data was compared using the proc GLM procedure in SAS 9.3 (see Figure 37). From this analysis it was determined the reactors' pkPHA% were not statistically different (Pr>F 0.84). Therefore the variation observed in Table 12, is consistent across all reactors and the change to the Enrichment reactor k_La showed no statistical effect. This suggests the variation was caused by an un-quantified parameter. Figure 36 shows peak intracellular PHA for both Enrichment and Production reactors for reference purposes, no correlation exists between Enrichment pkPHA% and Production pkPHA%. Identifying and quantifying the unknown parameter(s) was beyond the scope this investigation; however, this could be a focus of continuing research.

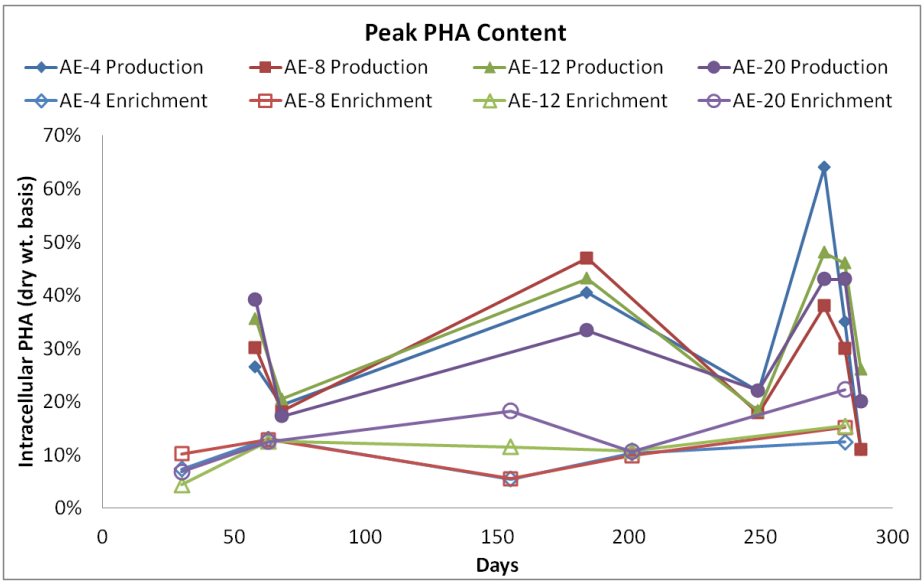


Figure 36: Peak intracellular PHA of all Enrichment and Production reactors.

Table 12: Peak Production PHA. (% Dry wt. Basis)
MLSS - Mixed liquor suspended solids; Pk Yield – Yield of at point of peak PHA concentration (Cmmol/Cmmol)
pkPHA – On a % dry wt. basis.; Avg. Yield – Average yield for evaluation (Cmmol/Cmmol)

k_LA (1/hr)	DATE	Cmmol Fed (Cmmol)	MLSS (mg/L)	Pk Yield (PHA/VFA)	pkPHA %	Avg. Yield (PHA/VFA)
4	12/7/2013	16.96	1820	53%	27%	29%
4	12/17/2013	19.20	2050	50%	19%	36%
4	4/12/2014	24.73	2590	85%	40%	70%
4	6/16/2014	16.37	2520	85%	22%	46%
4	7/11/2014	32.78	1750	64%	64%	35%
4	7/19/2014	26.56	1890	103%	35%	80%
4	7/25/2014	40.06	1560	47%	11%	26%
8	12/7/2013	12.72	2470	86%	30%	65%
8	12/17/2013	19.20	2260	64%	18%	47%
8	4/12/2014	26.06	2740	97%	47%	75%
8	6/16/2014	16.37	2490	99%	18%	44%
8	7/11/2014	32.78	1960	82%	38%	64%
8	7/19/2014	26.56	1780	87%	30%	72%
8	7/25/2014	40.06	1533	44%	11%	24%
12	12/7/2013	8.48	1810	71%	36%	53%
12	12/17/2013	7.86	1850	59%	21%	41%
12	4/12/2014	19.54	2540	95%	43%	78%
12	6/16/2014	16.37	2410	101%	18%	61%
12	7/11/2014	32.78	1790	85%	48%	67%
12	7/19/2014	26.56	1160	100%	46%	81%
12	7/25/2014	40.06	1687	79%	26%	54%
20	12/7/2013	8.48	2110	83%	39%	66%
20	12/17/2013	5.25	1960	70%	17%	44%
20	4/12/2014	19.25	1820	91%	33%	67%
20	6/16/2014	16.37	2460	109%	22%	66%
20	7/11/2014	32.78	1940	91%	43%	77%
20	7/19/2014	26.56	1687	98%	43%	84%
20	7/25/2014	40.06	1633	48%	20%	24%

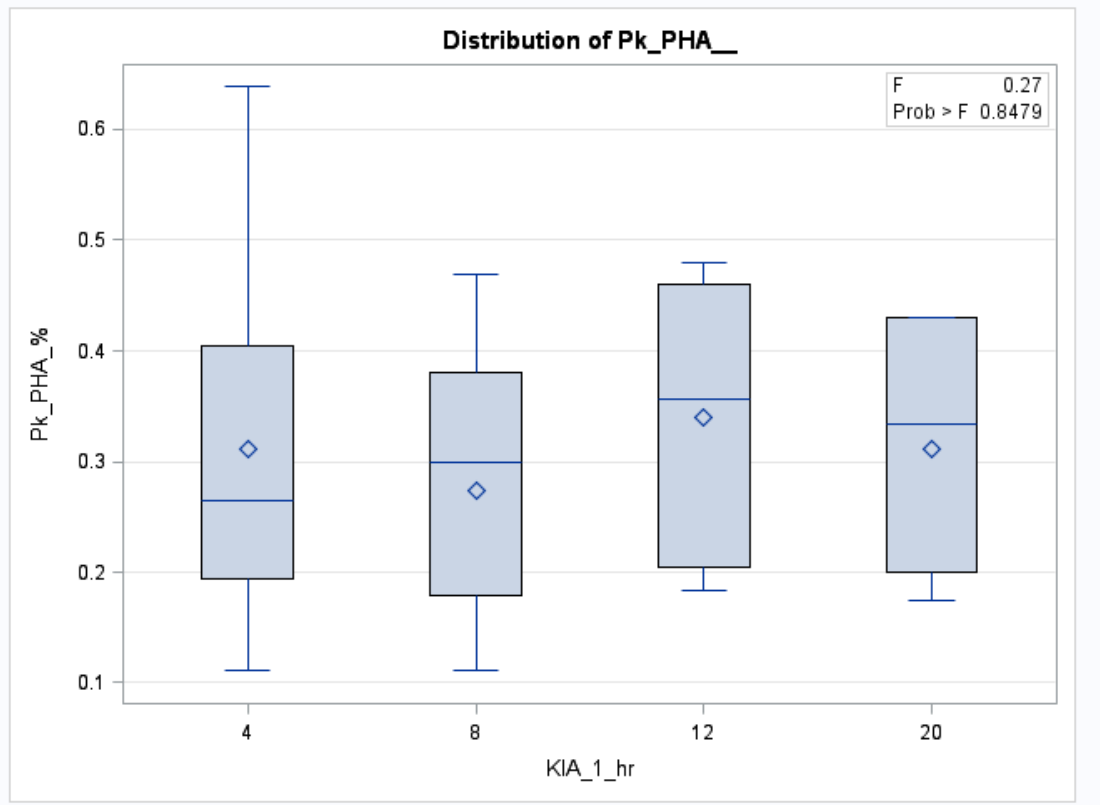


Figure 37: GLM Analysis of Mean pkPHA%

5.7.6 VFA Uptake Rate

The rate for VFA uptake is potential influential to future production runs, as it determines the time required to consume VFA's present in a pulse substrate addition, thus it can be used to calculate the time between substrate pulses. To determine if rVFA and qVFA rates were significantly different across the four aeration regimes, Proc GLM was used to compare the parameters time, and time*k_La (interaction). The qVFA rates realized by the four reactors were statistically different (Pr>F 0.0007), conversely the rVFA rates was not statistically different (Pr>F 0.238). All of the reactors were capable of similar goss VFA uptake rates regardless of MLVSS concentration, however when that rate is divide by MLVSS (qVFA) it becomes highly significant. Due to MLVSS being the only difference between the two statistical tests, it leads to the conclusion that the MLVSS is the cause of the change in significance. It can also be said that another parameter must be controlling

the rVFA rate causing similarity across the four reactors, while significance differences in Production reactor biomass leads to a statistical difference in qVFA.

The linear regression models utilizing samples from all seven Production reactor evaluations realized a very high fit (R^2 >0.90) (Table 13) indicating a consistent and stable process throughout the experiment duration. These rates can be affected by the rate of substrate addition, however, that was only believed to be the case during Stage 1 of the production procedure. The rates reported in Table 13 are similar to rates reported in earlier work completed in Dr. Coats' lab (Guho, 2010). All VFA uptake rates were very similar, with a standard deviation of only 0.007 for the rVFA rates. The qVFA has a slightly larger spread, with AE-20 achieving the highest rate (0.151 Cmmol/ mg min) and AE-8 achieving the lowest (0.123 Cmmol/mg min)). AE-4 and AE-12 were in the middle and achieved very similar rates with 0.139 and 0.135 (Cmmol/ L min) respectively.

Table 13: Rate of VFA Uptake; based on linear regression of seven production evaluations.

k_La	n	Model Pr>F	Adj R^2	qVFA (Cmmol/gVSS min)	*±95% CL	Adj R^2	rVFA (Cmmol/L min)	*±95% CL
4	70	<0.0001	0.971	0.139	0.006	0.97	0.257	0.011
8	71	<0.0001	0.92	0.123	0.009	0.933	0.239	0.015
12	71	<0.0001	0.902	0.135	0.010	0.963	0.248	0.013
20	63	<0.0001	0.961	0.151	0.008	0.958	0.247	0.013

***Bounds for a 95% Confidence Level.**

5.7.7 PHA Synthesis Rate

The PHA synthesis rate was calculated by combining all samples from the seven production evaluations into one data set separated by the class variable k_La. The rates were analyzed for statistical significance using Proc GLM to calculate the effects of reaction time and reaction time*k_La (interaction). The model dependent variable was PHA concentration while independent variables were reaction time (rPHA) or MLVSS* reaction time (qPHA). For both the overall rate and the specific rate, the interaction between k_La and time or MLVSS*time was deemed significant (Pr>F 0.0076 (rPHA) and 0.0457 (qPHA)).

The linear regression used to model the PHA synthesis is summarized in Table 14 with the raw data and regression lines in Figure 38. The fit is quite remarkable for these models

more variability was expected due to PHA being substrate dependent and not time dependent. PHA synthesis is induced by carbon surplus, which is supplied by a substrate addition; therefore, the rate can increase or decrease depending on the availability of carbon. So long as substrate is continuously available in non-limiting quantities (i.e. as experienced in Stages 2 and 3), it would appear that PHA synthesis should be relatively unaffected.

Table 14: Rates of PHA Synthesis; based on linear regression of seven production evaluations.

k_La	n	Model Pr>F	Adj R^2	qPHA (Cmmol/gVSS min)	*±95% CL	Adj R^2	rPHA (Cmmol/ min)	*±95% CL
4	70	<0.0001	0.926	0.088	0.006	0.844	0.155	0.016
8	71	<0.0001	0.829	0.075	0.008	0.800	0.143	0.017
12	71	<0.0001	0.870	0.096	0.009	0.899	0.174	0.014
20	63	<0.0001	0.879	0.098	0.009	0.866	0.158	0.016

***Bounds for a 95% Confidence Level.**

It is interesting to note that AE-20 exhibited the highest qPHA while AE-12 exhibited the highest rPHA. These differences are likely caused by less biomass (MLVSS) being present in AE-20 (Figure 26). Values reported in literature for both waste fed and pure culture were slightly higher than what is reported herein; values ranged from 0.402 to 0.013 (Cmmol/g min) with an average of 0.117 (Cmmol/g min) (Reis et al., 2003; Serafim, Lemos, Albuquerque, et al., 2008). For a full scale application, the difference in PHA synthesis rates only induces a longer reaction time until pkPHA%. Therefore, in general it is not expected that the lower rates present a significant cost difference between reactors.

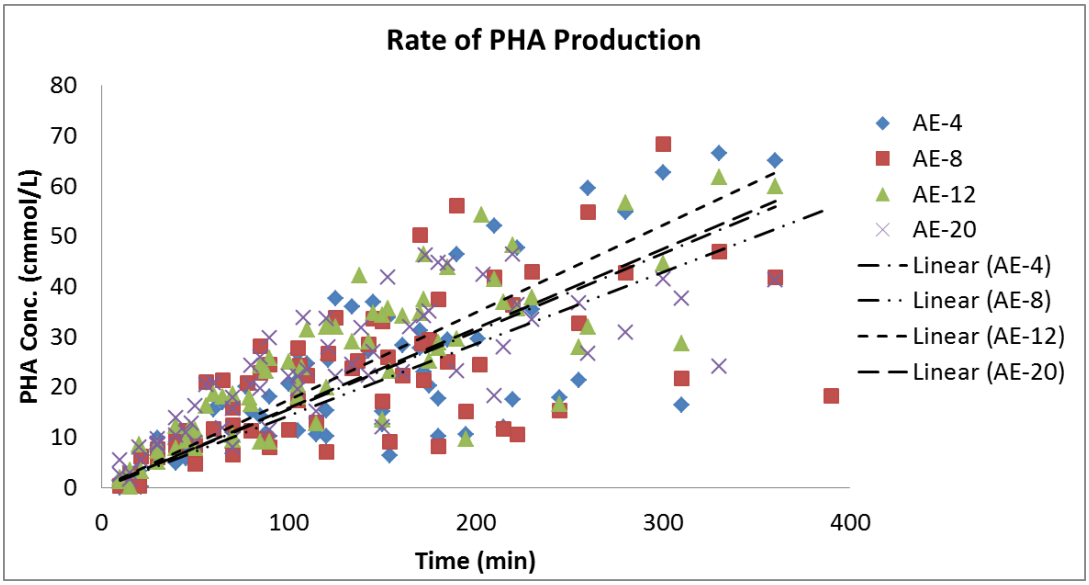


Figure 38: Rate of PHA Synthesis, linear regression and data.

5.7.8 Hydroxyvalerate Content

The hydroxyvalerate (HV) concentration in the polymer is important due to enhanced mechanical properties. Specifically, increased HV content will yield a larger elongation to break length, less brittle polymer, and better oxygen barrier properties. These properties are of keen interest to the packaging market, especially when packaged materials may spoil when exposed to oxygen.

Biomass mean HV content from all seven production evaluations, by reactor, was compared using proc GLM. The model included parameters for PHA concentration, k_La, and interaction between PHA concentration and k_La. PHA concentration is included in the model due to a statistically significant correlation between the %HV and PHA concentration (Pr>F of <0.0001). From the proc GLM, k_La and its interaction with PHA concentrations was found to be insignificant with respect to HV content (Pr>F 0.197) therefore no statistically significant differences were present. Further the model was shown to be statistically insignificant (Pr>F 0.705), therefore an average value was used to describe the data instead of a model.

Least square means was applied to calculate the mean HV content of the reactors. This average content of HV (Table 15) is promising for future applications of a dairy manure

produced PHA polymer. A high HV content is preferred for plastic films, packaging materials, and most PHA applications.

Table 15: Mean HV Concentration.

k_La	n	Mean HV Content gHV/gPHA	Standard Deviation
4	70	0.396	±0.18
8	71	0.401	±0.18
12	71	0.445	±0.16
20	63	0.424	±0.17

5.7.9 Summary and Conclusions of Production Data

From the difficulties experienced at the beginning of the production reactor assessment it became apparent that a major limiting aspect of the production process was the lack of real time VFA's monitoring. While currently limited technology exists that is capable of real time VFA's monitoring the production process is in need of a parameter capable of maintaining stable substrate concentrations. While the timed method was adequate it relies heavily on assessments completed the previous day and lacks the real time quantification. One possibility is the use of real time quantification of OUR (quantified through dissolved oxygen fluctuations); however, OUR relies on a microbial response to a lack of substrate which induces cyclical patterns of substrate depletion and availability. Thus, continued work is necessary to identify a parameter capable of real time quantification that can provide more insight to the system than is capable with dissolved oxygen based parameters.

From the results of these investigations, it can be concluded that the PHA/VFA yield as measured in the PHA production assessments did not exhibit a statistically significant difference between inocula from the four PHA Enrichment reactors. This result could be due to the aeration not being controlled in the Production reactor. The hypothesis of an increased selection of PHA producing bacteria as reactor k_La was reduced was not proven. Specifically none of the PHA Production reactors inoculated with biomass from the different PHA Enrichment reactors achieved a statistically significant difference in pkPHA%. A higher pkPHA% would be one indication of a better selection of PHA producing bacteria in a given

biomass. The production process demonstrated a remarkable robustness, with repeatable results being achieved even when influent characteristics had the same limited control that would exist in a full scale application of this technology. The effect of a large reduction in the Enrichment reactor aeration caused very few effects on the process. This is surprising given the biomass relying on aerobic oxidation for most of its required energy; in other words, one would expect aeration to be a very sensitive parameter. From this work it appears the reactors operating criteria are very robust in enriching for PHA producing biomass even at very low aeration rates. These observations, in turn suggest a significant cost savings can be achieved through the reduction of aeration, further enhancing the viability of future PHA producing systems.

5.7.10 Conclusion of Reduced Aeration Experiment

Aeration reduction in the PHA Enrichment reactors was selected as a means to reduce the cost for biologically produced $_{MC}$PHA from fermented dairy manure. Current literature indicated a reduction in aeration would cause an increase in the PHA yield (PHA/VFA). It was also hypothesized that the reduced aeration would cause an increase in the environmental selection process by allowing PHA producing bacteria another advantage over non-PHA producing bacteria.

Based on statistical significance; results from these investigations suggest no net negative effects were attributed to a reduction of the Enrichment reactor aeration. Parameters of interest associated with PHA production included; maximum intracellular PHA content, PHA/VFA yield, hydroxyvalerate content, and Enrichment reactor MLSS. None of these factors demonstrated a statistically significant difference between the four experimental aeration rates. This was partly due to high variation within the data sets; however the variation was expected due to natural variability associated with waste derived substrate; however, overall the reactors and associated biomass were remarkably robust throughout the investigation. The reactors also demonstrated the same robust response to reduction in aeration with the lowest aeration reactor (AE-4) receiving 97.5% less air than the highest

aeration reactor (AE-20) and still performing well in the production mode. This demonstrates a large reduction from current research aeration rates can be applied to future industrial PHA Production facilities.

5.8 Future Work

Due to the variability observed in the Production reactor peak PHA concentrations, an optimization of the Production reactor is recommended. The current method of production is strictly a means of analysis. The future work could focus on many parameters including OLR, feeding rates, residual dissolved oxygen concentration, and trigger parameter to optimize for peak performance from the Production reactor.

In this work bacteria were observed consuming nitrate in place of oxygen as a terminal electron acceptor; while this was anticipated, it was not part of the experiment. A future experiment could try to utilize an anoxic period in the Enrichment reactor to enhance the observed nitrogen removal and further reduce aeration. The highest oxygen demand of the current process occurs during the feast phase of the reactor cycle, thus this phase would be an ideal candidate for the anoxic conditions. Utilizing an anoxic feast phase would minimize oxygen requirements while maximizing the substrate available for nitrogen removal. This work demonstrated the ability of PHA enrichment reactors to perform at very low oxygen transfer rates therefore it is feasible that a completely anoxic-feast reactor may also perform well.

To date the Coats' lab has focused on hydraulic retention and substrate derived methods to control the ADF Enrichment reactor. Future work could utilize physical means to increase the ability for a PHA Enrichment reactor to select for PHA producing biomass. Three examples of physical means of separation include the use of nitrifying biofilms, modification to settling and decant phase, or the use of selective settling. All of these methods are of interest because they rely on physical systems which are more easily controlled and manipulated than the naturally variable and waste derived substrate.

Nitrifying biomass is often unavoidable in waste derived substrate due to high concentration of ammonia typically present in the waste substrate. The nitrifying biomass exhibits a dilution effect on the concentration of PHA producers in the mixed liquor therefore it is favorable to separate nitrifying biomass from the mixed liquor. This is possible with the use of submerged plastic media providing surface area for nitrifying biofilms to develop, thus the nitrifying biomass could be physically attached to plastic media and easily separated from the mixed liquor.

Another alternative is the reorientation of the settling and decant phase to a time immediately after the feast phase. Due to small quantities of slowly biodegradable carbon present in the waste derived substrate the ADF technique cannot achieve a true famine; by physically removing some of the slowly biodegradable carbon with the effluent, the effectiveness of the famine phase could be improved.

Last is the use of a selective settling method such as hydro-cyclones this technology could be used to improve both the Enrichment reactor and Production reactor. Biomass with high PHA content has a higher specific gravity therefore it is feasible that a hydro-cyclone system could be used to separate bacteria with high intracellular PHA content from the mixed liquor.

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