2015

Optimization of Glycerol and Biodiesel Waste Prefermentation to Improve EBPR

Marzieh Ghasemi
University of Central Florida

OPTIMIZATION OF GLYCEROL OR BIODIESEL WASTE PREFERMENTATION TO IMPROVE EBPR

By

MARZIEH GHASEMI
B.S. University of Tabriz, 2007
M.Sc. Isfahan University of Technology, 2011

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Civil, Environmental and Construction Engineering
in the College of Engineering and Computer Science
at the University of Central Florida
Orlando, Florida

Fall Term
2015

Major Professor: Andrew A. Randall

ABSTRACT

The enhanced biological phosphorus removal (EBPR) process efficiency relies on different operational and process conditions especially the type of carbon source available in the wastewater. Acetic acid and propionic acid are the two major volatile fatty acids (VFAs) found in domestic wastewater which can drive biological phosphorus (P) removal to the desired level. However, often domestic wastewater does not have a sufficient amount of VFAs. Due to high acetate and propionate production-cost, it is not economic to add acetate and propionate directly in full-scale wastewater treatment plants. This brought up the idea of using external carbon sources (e. g. molasses has been used successfully) in EBPR systems that can be converted to VFAs through a fermentation process. On the other hand, biodiesel fuels have been produced increasingly over the last decade. Crude glycerol is a biodiesel production major by-product that can be used as an external carbon source in wastewater treatment plant. Therefore, the main objective of this research is to optimize the glycerol/biodiesel waste fermentation process' operational conditions in pursuit of producing more favorable fermentation end-products (i. e. a mixture of acetic acid and propionic acid) by adding glycerol to a prefermenter versus direct addition to the anaerobic zone or fermentation with waste activated sludge. For this reason, different prefermenter parameters namely: mixing intensity, pH, temperature and solids retention time (SRT), were studied in a small scale fermentation media (serum bottles) and bench scale semi-continuous batch prefermenters. Experimental results revealed that glycerol/biodiesel waste fermentation resulted in a significant amount of VFAs production with propionic acid as the superior end-product followed by acetic acid and butyric acid. The VFA production was at its highest level when the initial pH was adjusted to 7 and 8.5. However, the optimum pH with respect to propionic acid production was 7. Increasing the temperature in serum bottles favored the total VFA production,

specifically in the form of propionic acid. Regarding the mixing energy inconsistent results were obtained in the serum bottles compared to the bench scale prefermenters. The VFA production in mixed serum bottles at 200 rpm was higher than that of un-mixed ones. On the other hand, the unmixed or slowly mixed bench scale prefermenters showed higher VFA production than the mixed reactors. However, the serum bottles did not operate long enough to account for biomass acclimation and other long-term effects that the prefermenter experiments could account for. As a consequence one of the most important and consistently results was that VFA production was significantly enhanced by reducing mixing intensity from 100 rpm to 7 rpm and even ceasing mixing all together. This was true both for primary solids and glycerol. In addition propionate content was high under both high and low intensity, and adding glycerol also increased the fraction of primary solids that formed propionic acid instead of acetic acid. Increasing the SRT from 2 to 4 days increased the VFA production about 12% on average. In order to investigate the effect of glycerol on EBPR process efficiency two identical A^2/O systems were monitored for 3 months. Experimental results suggested that glycerol addition could increase the P removal efficiency significantly. Adding glycerol to the prefermenter rather than the anaerobic zone resulted in a lower effluent soluble ortho phosphorus (SOP) (0.4 mg-P/L vs. 0.6 mg-P/L) but the difference was apparently statistically significant. Future experimentation should be done to determine if this effect is consistent, especially in carbon poor wastewaters. Also it would be desirable to conduct a longer pilot study or a full scale study to determine if this improvement in effluent SOP remains true over a range of temperature and changing influent conditions.

To My Family

ACKNOWLEDGMENTS

First and foremost, I would like to express my thanks and my deepest gratitude to Dr. Andrew A. Randall; for his guidance as well as his friendship throughout this study. He has made this whole experience possible and enjoyable. None of this would have been possible without his mentorship and help.

My sincere thanks also goes to the members of my dissertation committee, Dr. Steven J. Duranceau, Dr. Woo Hyoung Lee and Dr. Jose Jimenez for their time and feedback.

I also would like to thank Glendale Wastewater Treatment Plant (Lakeland, Florida), Iron Bridge Water Reclamation Facility (Oviedo, Florida) and SAKAL LLC-GROUPO LAKAS (Panama, Panama).

And last, but most certainly not the least, I would like to express my deepest love and gratitude to my family; I cannot thank them enough for their love and support.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 Enhanced Biological Phosphorus Removal

Phosphorus is among the required nutrients that are essential for growth and maintenance of living organisms. However, excess amounts of phosphorus causes eutrophication which results in oxygen depletion in water bodies. Therefore, it is necessary to be able to keep the concentration of phosphorus in treated wastewater discharges within an acceptable range. Biological nutrient removal (BNR) is a prevalent technology that facilitates nutrient removal from wastewater through biochemical reactions. Initially, BNR systems were designed with the intention of removing organic matter and nitrogen from wastewater. Earlier BNR systems consisted of anoxic and aerobic zones which provided nitrogen and organic matter removal. Later on, it was discovered that by adding an anaerobic zone prior to the anoxic reactor, enhanced biological phosphorus removal (EBPR) can occur in the same system. An EBPR process provides the means to remove phosphorus (P) through an anaerobic/aerobic sequence. In the anaerobic phase, short chain volatile fatty acids (VFAs) are taken up by bacteria known as poly-p accumulating organisms or PAOs. The PAOs consume and store the VFAs as polyhydroxyalkanoates (PHAs). The required energy and reducing agents are supplied by glycogen consumption and intracellular polyphosphate degradation which in turn increases the bulk water soluble P concentration. Under the subsequent aerobic condition intracellular PHAs are oxidized to produce required energy for maintenance and growth. In this stage, PHA consumption is accompanied with intracellular glycogen and polyphosphate replenishment. Therefore, during the aerobic metabolism the bulk P concentration decreases. Since P uptake in the aerobic zone is higher than P release during the anaerobic zone, there is a net P removal in EBPR systems (Chen et al., 2004). At the end, P removal is obtained by wastage of P enriched sludge. In activated sludge systems there is a fraction of PAOs, called

facultative PAOs, which are capable of removing phosphorus in anaerobic/anoxic cycle. In the anoxic zone, these bacteria can use nitrate as the external electron donor instead of oxygen (Ng et al., 2001). However, because of the competition between facultative PAOs and denitrifying bacteria for the limited substrate, in most cases there is a net P release in the anoxic zone (Barker and Dold, 1996). Figure 1-1 illustrates PAO metabolism in anaerobic and aerobic phases.

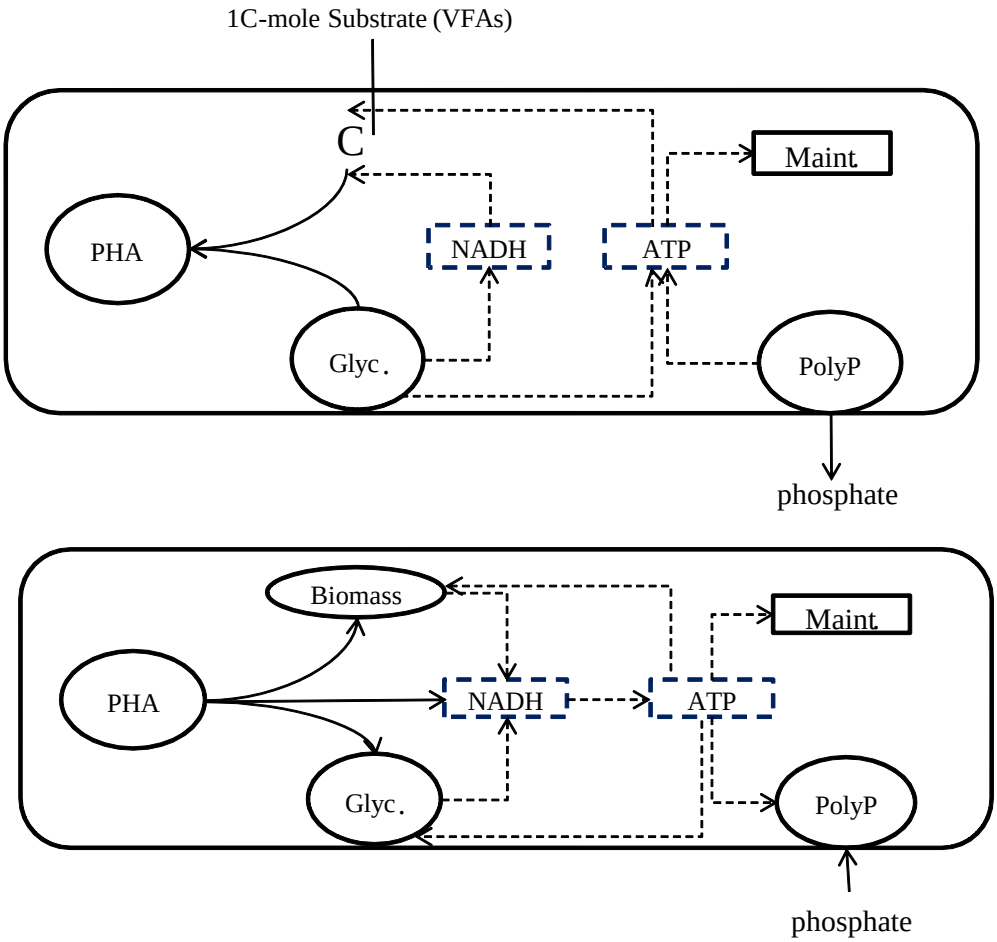

Figure 1-1: Anaerobic and Aerobic metabolisms of poly-P accumulating organisms (PAOs) (Smolders et al., 1995)

Among the different BNR system configurations an A^2/O system was employed in the current research to evaluate its capability in nitrogen and phosphorus removal under the imposed

process conditions. The A^2/O system consists of a sequence of anaerobic, anoxic and aerobic zones. One of the main concerns in operating the EBPR systems is to prevent nitrate from entering the anaerobic zone. The reason for that is facultative microorganisms will use nitrate and VFAs as their external electron acceptor and carbon source, respectively, reducing the substrates available for PAOs and consequently decreasing the P removal efficiency of the system. The use of the anoxic zone in an A^2/O system not only enables the N removal through the anoxic/aerobic cycle but also it decreases the amount of nitrate fed to the anaerobic reactor through the return activated sludge (RAS). The configuration of the A^2/O system is shown in Figure 1-2.

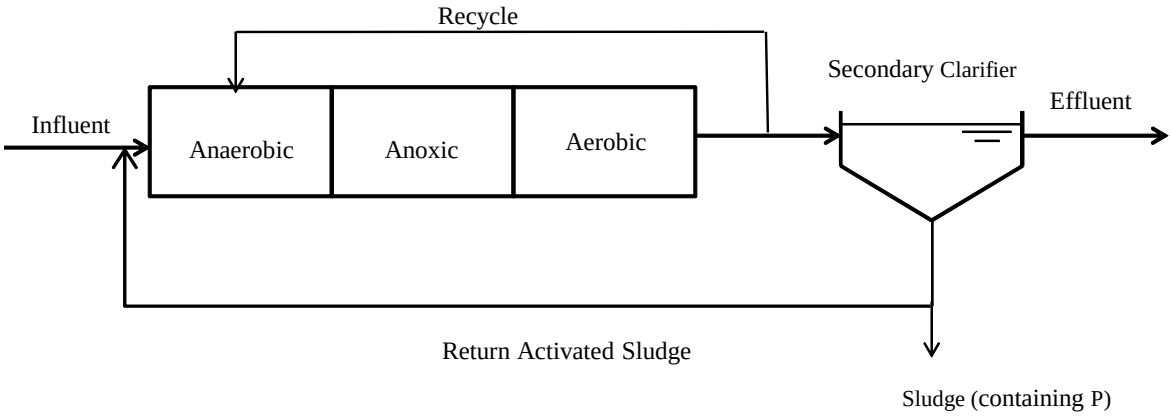

Figure 1-2: A^2/O configuration (Metcalf and Eddy, 2003).

The EBPR system efficiency relies on different operational and process conditions especially the type of carbon source available in the wastewater. As acetic and propionic acid are the two major VFAs found in domestic wastewater, several studies have been conducted to investigate their potential for phosphorus removal (Smolders et al., 1994a; Chen et al., 2004; Oehmen et al., 2005; Lopez-Vasquez, 2009). Experimental results revealed that both acetic acid and propionic acid were effective in P removal however a mixture of these two acids gave more

efficient P removal than either acid alone. However, often domestic wastewater does not have sufficient amount of VFAs to drive P removal to the desired level. On the other hand, due to high acetate and propionate production-cost, it is costly to add acetate and propionate directly in full-scale wastewater treatment plants. This brought up the idea of using external carbon sources in EBPR systems that can be converted to VFAs through a fermentation process.

1.2 Biodiesel

Biodiesel or bio-based fuels are commonly produced from vegetable oils or animal fats. Biodiesel is increasingly considered as a good replacement for diesel fuels. Structurally, biodiesel is composed of methyl and ethyl esters of fatty acids (FAME) which is produced via transesterification (alcoholysis) of triglycerides with an alcohol. Figure 1-3 presents the transesterification reaction of triglycerides. During the first stage of biodiesel production, a catalyst such as potassium hydroxide (KOH) or sodium hydroxide (NaOH) is dissolved in water by stirring intensely in a small reactor. Afterwards, both triglycerides and the catalyst mixture are pumped into the biodiesel reactor and stirred intensely for almost 2 hours. During the transesterification process which is carried out in this step the viscosity of a fat or oil triglyceride is lowered and a mixture of biodiesel and glycerol is produced. At the end of an effective reaction a two phased mixture, composed of biodiesel and glycerol, is produced. This mixture is allowed to sit for several hours to have complete separation of liquid phase. At the end of the quiescent period, the biodiesel layer at the top is collected (Demirbas, 2008). Biodiesel fuel is a renewable source of energy that has been attracting increasing attention because of its environmental benefits. It is a non-toxic, biodegradable fuel and is free of sulfur and aromatics. This means it produces lower exhaust emissions than petroleum fuels while it has the same efficiency. On the other hand, biodiesel prices

are higher than those of fossil fuels. The high biodiesel-production cost is strongly linked to the price of the feedstock which is almost 80% of the operating cost (Demirbas, 2008).

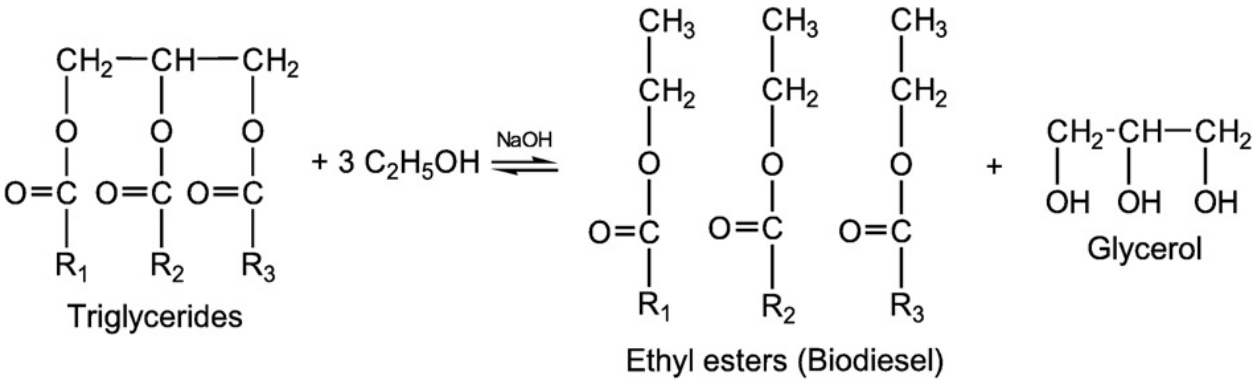

Figure 1-3: Transesterification reactions between triglycerides and ethanol, adapted from da Silva et al. (2009)

One promising way to offset the biodiesel-production cost is recovery of glycerol. Glycerol is a principle biodiesel production by-product. It is stated that a typical biodiesel waste consists of almost 56-60% glycerol, and 10-12% methanol (Bodik et al. 2009). The feasibility of using glycerol in EBPR has been investigated in a few studies. This is because VFAs, especially acetic and propionic acid, are the only known substrates which can directly drive EBPR (Hood and Randall, 2001; Chen at al. 2004). Fermentation of glycerol is a likely way to produce propionate. This fermentation can occur in the BNR anaerobic zone. Barbirato et al (1997) conducted a research on glycerol fermentation end-products in a reactor inoculated with *propionibacterium*. The experimental results showed that propionic acid with a yield of (0.844 mole-propionic acid/mole-glycerol) was the main glycerol fermentation end-product followed by succinic acid (0.055 mole-succinic acid/mole-glycerol), acetic acid (0.023 mole-acetic acid/mole-glycerol), and formic acid (0.02 mole-formic acid/mole-glycerol). Further experimental results reported by other scientists were consistent with Barbirato on propionic acid being the main glycerol fermentation

end-product by *propionibacterium* (Himmi et al., 2000; Zhang et al., 2009). The reason for that is related to the substrates' oxidation level. To explain, during the fermentation process glycerol is oxidized to get the same oxidation level of carbohydrates. As a result, propionic acid, the most reduced product, is produced in higher steps than other more oxidized products so that NAD^+ can be regenerated. Figure 1-4 depicts the glycerol metabolism by *propionibacterium*.

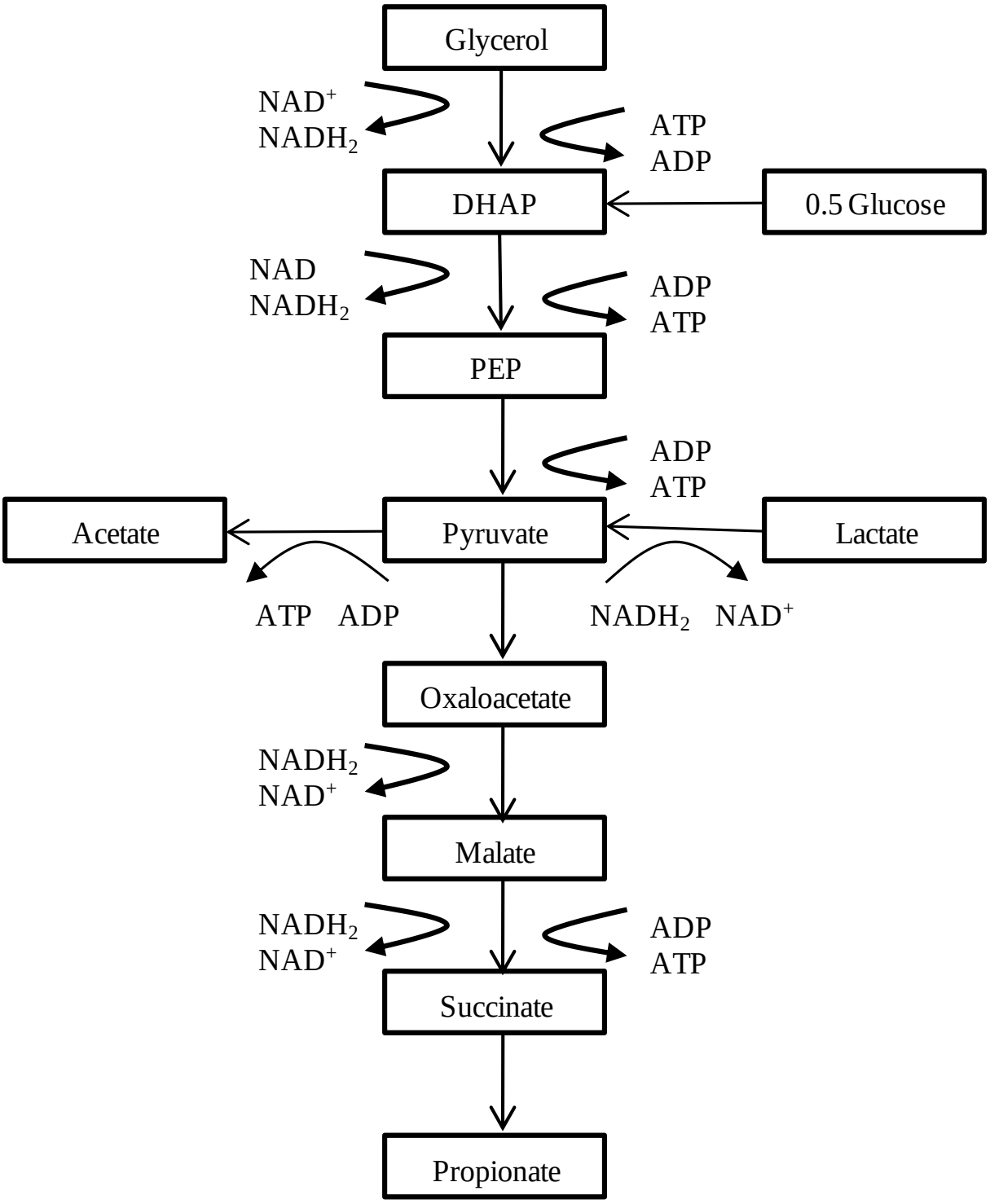

Figure 1-4: Propionic acid fermentation pathways from glycerol (Barbirato et al., 1997)

Distribution of glycerol fermentation end-products is affected by the dominant type of microorganisms and the operational conditions. The propionic acid bacteria, which produce propionic acid during the glycerol fermentation process, are aerotolerant microorganisms. They produce energy during the fermentation process. In order to increase the propionic acid production in glycerol fermentation, the propionic acid bacteria should be present in the sludge. In addition to the dominant type of microorganism, operational conditions such as temperature, pH, mixing, and SRT can also affect the glycerol fermentation. In order to have a desired state of fermentation it is important to understand the fermentation process pathways which are described briefly in the next section.

1.3 Fermentation

Fermentation of glycerol to short chain VFAs such as acetate and propionate is one promising strategy to make it more efficient in driving the EBPR process. A short explanation of fermentation processes is required to fully understand biological phosphorus removal in wastewater treatment plants. A two-stage fermentation process is shown in Figure 1-5. The first stage is called hydrolysis. In this stage, particulate materials and high molecular weight polymers are converted to simple monomers. The second step consists of two main reactions: acidogenesis and acetogenesis. During the acidogenesis reaction amino acids, sugars, and some fatty acids are converted to short chain VFAs with 2-5 carbon atoms. The 3 to 5 carbon VFAs can be then fermented to produce acetic acid, hydrogen gas, and carbon dioxide. These reactions are called acetogenesis and they are affected by the partial pressure of hydrogen gas in the reactor headspace. Fermentation occurs in the absence of oxygen and the organic substrate acts as the both electron donor and acceptor. In wastewater treatment plants fermentation processes can occur in the anaerobic zone and to varying extents in the sewer system. Under the anaerobic condition,

fermentative bacteria can convert particulate matter and complex organic compounds to VFAs which are known as the most favorable carbon sources for EBPR.

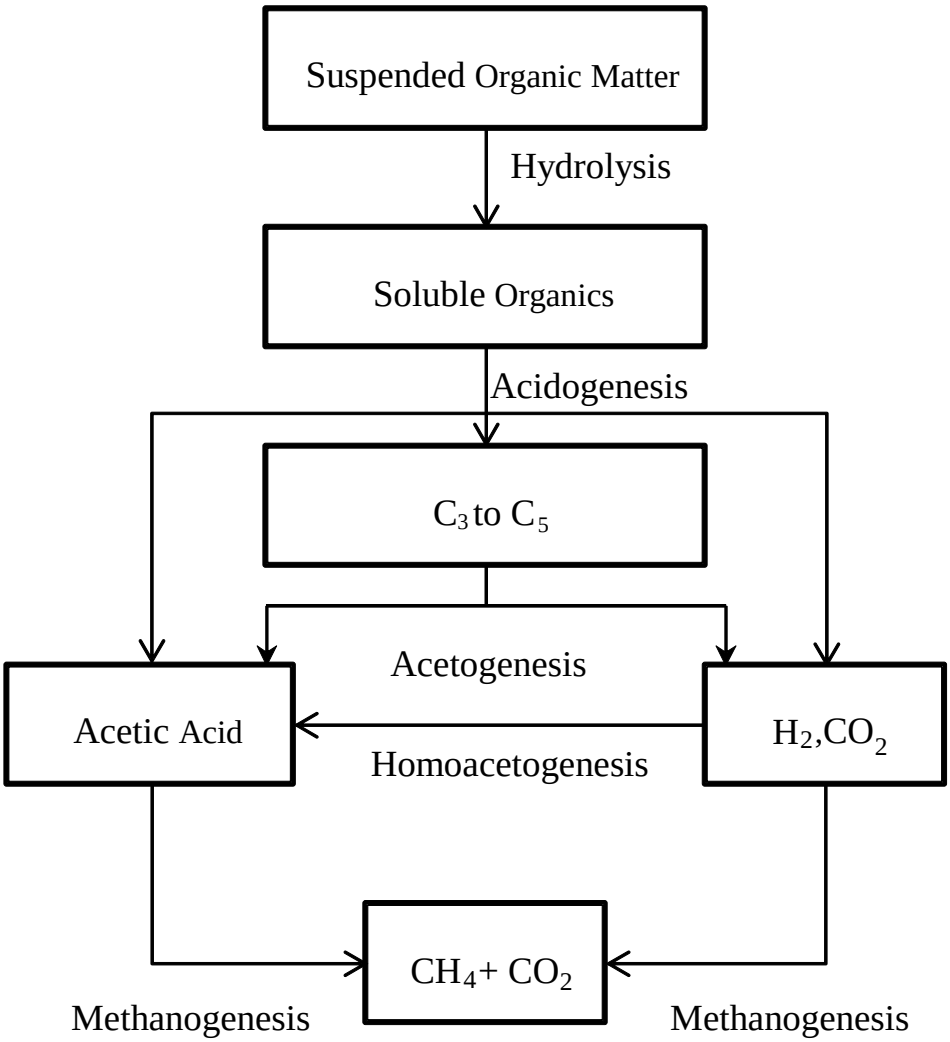

Figure 1-5: Fermentation/methanogenesis process (adapted from Appels et al. 2008)

In general one can classify the reactions that occur in the anaerobic reactor of EBPR systems into two main types: firstly, fermentation of complex molecules and particulate matter to simpler molecules (including VFAs) and then VFA uptake by poly-p organisms or their competitors the glycogen accumulating organisms or (GAOs) as the first step of phosphorus removal. However,

some plants are equipped with an independent unit process which precedes the anaerobic zone called a prefermenter. The main goal of having a prefermenter in such systems is to generate VFAs which then enter the anaerobic zone (and/or the subsequent anoxic zones if step feed is used). Most full-scale prefermenters are fed with the raw wastewater. These types of prefermenters are called in-line prefermenters. In contrast, side-stream prefermenters are fed with the sludge from primary clarifiers. According to the number of tanks that are being used, prefermenters are designed in 4 different configurations. An activated primary tank (APT) is an in-line prefermenter that consists of a primary clarifier that is fed with raw wastewater. VFAs are produced by the sludge blanket that has been formed at the bottom of the tank (the sludge blanket is allowed to accumulate more than would occur in a normal primary clarifier). The VFA enriched sludge, which also includes active fermenting microorganisms, is recycled back to the inlet of the tank. This recycling process results in effective contact between the fermenting microbes with the particulate matter of the influent as well as elutriation of the VFA produced in the sludge blanket. Single-stage prefermenters are side-stream prefermenters that consist of either a gravity thickener (static prefermenter) or a mixing tank (complete mix prefermenter). Both static and mixing tank prefermenters are fed with primary solids from an up-stream primary clarifier. In the case of a static prefermenter, the VFA-enriched overflow can be directly sent to the anaerobic reactor. However for the complete-mix prefermenter the VFA-enriched overflow of the mixing tank is then returned back to the primary clarifier where the mixing with the influent takes place. The sludge age in the APT or static prefermenters are controlled by the sludge height at the bottom of the prefermenter. In the complete-mix prefermenter sludge age is affected by the amount of biomass leaving in the tank effluent and a mass balance must be conducted to determine the sludge age. Additional amounts of biomass can be wasted from the complete-mix tank if a lower sludge age

is desired. A side-stream two-stage prefermenter (complete-mix tank followed by a gravity thickener) is fed with primary solids from an up-stream primary clarifier to the mixing tank. The overflow from the preceding mixing tank is conveyed to the thickener. The VFA-enriched overflow and the sludge of the thickener are then sent to the anaerobic reactor and the mixing tank respectively (Rössle et al. 2001). This allows direct control of the SRT by wasting the desired fraction of the complete-mix reactor. For prefermenters the SRT must be low enough to avoid methanogenesis. Methanogenesis results in the consumption of the VFAs. The SRT must then be high enough to allow fermenters to grow, but not high enough for methanogens to stay in the system. At high temperatures it may not be possible to wash out the methanogens and in such cases periodic sparging with air is used to suppress methanogens via oxygen toxicity (fermenters are much more aerotolerant than methanogens).

1.4 Objective and Scope

As fermentation processes are highly affected by operational conditions, it is important to be able to control the operational factors to enhance the prefermenters' efficiency. The type of carbon source, temperature, pH, SRT and the mixing intensity are among the factors which impact the prefermenter's function. The main objective of this research is to study the effect of SRT, pH and mixing intensity on glycerol and biodiesel waste fermentation to produce more favorable fermentation end-products (i. e. a mixture of acetic acid and propionic acid) for the microorganisms which can drive phosphorus removal in EBPR systems. The second chapter of the current study is dedicated to a brief analysis of the effect of mixing intensity, glycerol/biodiesel waste optimum initial dosage, temperature and pH on pure glycerol/biodiesel waste fermentation in serum bottles. The study will then be focused on bench scale prefermenters which were running as semi-continuous batch reactors. The corresponding results are shown and described thoroughly in three

separate chapters (Chapter 3, Chapter 4 and Chapter 5). In chapter 3 the effect of SRT and the type of substrate (pure glycerol vs. biodiesel waste) on VFA production was evaluated. Chapter 4 includes the effect of pH on glycerol and primary solids fermentation. Chapter 5 describes the effect of mixing energy on primary solids and pure glycerol fermentation in two phases. In Chapter 6 two BNR systems are observed to quantify the effects of glycerol fermentation on EBPR.

1.5 References

Appels, Lise, et al. "Principles and potential of the anaerobic digestion of waste-activated sludge." *Progress in Energy and Combustion Science* 34.6 (2008): 755-781

Barker, P. S., and P. L. Dold. "Denitrification behavior in biological excess phosphorus removal activated sludge systems." *Water Research* 30.4 (1996): 769-780.

Bodík, I., et al. "Biodiesel waste as source of organic carbon for municipal WWTP denitrification." *Bioresource technology* 100.8 (2009): 2452-2456.

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36

Demirbas, Ayhan. Biodiesel. Springer London, 2008

Himmi, E. H., et al. "Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii." *Applied microbiology and biotechnology* 53.4 (2000): 435-440

Hood, Cathy R., and Andrew Amis Randall. "A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates." *Water research* 35.11 (2001): 2758-2766

Lopez-Vazquez, Carlos M., et al. "Modeling the PAO–GAO competition: effects of carbon source, pH and temperature." *Water Research* 43.2 (2009): 450-462

Metcalf, Eddy. Inc.(2003), "Wastewater engineering treatment and reuse."

Ng, W., S. Ong, and J. Hu. "Denitrifying phosphorus removal by anaerobic/anoxic sequencing batch reactor." *Water Science & Technology* 43.3 (2001): 139-146.

Oehmen, Adrian, et al. "Anaerobic metabolism of propionate by polyphosphate- accumulating organisms in enhanced biological phosphorus removal systems." *Biotechnology and bioengineering* 91.1 (2005): 43-53.

Rössle, W. H., and W. A. Pretorius. "A review of characterisation requirements for in-line prefermenters Paper 2: Process characterisation." *Water S. A.* 27.3 (2001): 413-421.

Smolders, G. J. F., et al. "Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence." *Biotechnology and Bioengineering* 43.6 (1994): 461-470.

Smolders, G. J. F., et al. "A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process." *Biotechnology and bioengineering* 47.3 (1995a): 277-287.

Zhang, An, and Shang-Tian Yang. "Propionic acid production from glycerol by metabolically engineered< i> Propionibacterium acidipropionici</i>." *Process Biochemistry* 44.12 (2009): 1346-1351.

CHAPTER 2: OPTIMIZATION OF PURE GLYCEROL/BIODIESEL WASTE FERMENTATION IN SERUM BOTTLES

2.1 Introduction

EBPR is an efficient technology for removing phosphorus from wastewater. The efficiency of EBPR systems relies on the availability of short chain VFAs which are the most suitable carbon sources in driving phosphorus removal. However, typical domestic wastewater in temperature climates has insufficient amounts of VFAs. Therefore, in order to meet the effluent phosphorus limits it is necessary to increase the VFA content of the wastewater. Prefermentation is an efficient process in converting biodegradable organic compounds to VFAs. This unit process is employed preceding the anaerobic zone and has its own biomass. In this chapter we evaluate the effect of mixing, external substrate initial dosage, pH and temperature on fermentation process. It should be noticed that the serum bottle tests were a screening technique. They helped us to develop the analytical procedure and generate some preliminary information about glycerol and biodiesel waste fermentation. Therefore, not too much observations were conducted in this phase of study.

2.1.1 Mixing energy

Mixing is one of the parameters affecting fermentation processes. Mixing keeps the content of a reactor in suspension and provides a suitable contact between the microorganisms and the particles. Danesh and Oleszkiewicz (1997) stated that reduction of mixing period from 6 hr/cycle to 0.25 hr/cycle in a bench-scale prefermenter operating as a sequencing batch reactor (SBR) increased the primary solids fermentation. Banister and Pretorius (1998) reported that unmixed reactors showed a higher primary solids fermentation and net VFA production yield than that of mixed reactors. In research carried out by Yuan et al. (2011) on WAS fermentation, it was

showed that mixing decreases the propionic acid to acetic ratio by 16%. It is thought that lower mixing and stratification facilitates inter-species hydrogen transfer in methanogenic reactors. An analogous phenomena may be responsible for the observations of Danesh and Oleszkiewicz (1997) and the other researches mentioned. Both homoacetogenesis and production of propionic acid are hydrogen (H_2) consuming reactions just as hydrogen utilizing/CO_2 respiring methanogenesis.

2.1.2 External substrate

The two most common VFAs which are naturally present in septic domestic wastewater are acetic acid and propionic acid. Chen et al. (2004) monitored two SBR systems: one was cultivated with real wastewater and 2.5mM-C acetic acid and the other one was fed with the same amount of wastewater and 2.5mM-C propionic acid. Both SBRs were run under the same anaerobic and aerobic process conditions operating under the identical temperature, pH, and mean cell residence time. The experimental results revealed higher propionic acid to acetic acid ratio led to higher P removal in long-term cultivation. Oehmen et al. (2006) conducted research on synthetic wastewater samples spiked with either propionate or acetate as the sole carbon source in two sequencing batch reactors. During the 180 operating days, the propionate-fed reactor exhibited a significantly more stable performance compared to that in the acetate-fed reactor. It was shown that in the propionate-fed batch reactor P removal happened rapidly and around 98% of initial P content was removed during nearly all of operating days. However, in the acetate-fed batch reactor the effluent P concentration was changing over the operation time and near complete P removal was seldom observed.

2.1.3 Temperature

Temperature is one of the main factors which affect biochemical reactions in different ways including reaction rate and pathway, microorganism growth rates, and population dynamics. Previous studies showed that both hydrolysis and fermentation rate constants of primary solids increased as temperature goes up (Mahmoud et al., 2004). Gonzalez-Barcelo and Gonzalez-Martinez (2007) observed that the acidification of COD was almost doubled when the temperature increased from 22°C to 31°C. Yuan et al. (2011) observed that waste activated sludge (WAS) fermentation at 24.6°C and 14°C was almost complete in 5 and 14 days respectively. Temperature also affects the distribution of WAS fermentation end products. Referring to Yuan et al. (2011) propionic acid to acetic acid ratio increased from 36% to 68% when the temperature increased from 4°C to 24.6°C. Carol et al (2008) investigated the effect of temperature on the propionic acid production through the fermentation of glycerol. It was reported that the propionic acid production decreased with increasing the temperature from 30 to 37°C, the opposite of the effect observed for fermentation of WAS by Yuan et al. (2011).

2.1.4 pH

The pH value can change the distribution of fermentation products. Investigation showed that at low pH acetic acid and butyric acid were the major products of waste activated sludge fermentation whereas under alkaline condition acetic acid and propionic acid were the main products (Apples at al., 2008). Gonzalez-Barcelo and Gonzalez-Martinez (2007) studied the effect of pH on solubilization and acidification of primary solids in a sequencing batch reactor. Experimental results revealed that decreasing the pH from 7.7 to 5.5 increased the acidification of COD from 50% to 63%. Zeng et al. (2006) reported that the acidification of primary solids reached

the maximum value in a neutral pH range (6 to 7). Chen et al. (2007) reported that at a constant temperature both hydrolysis and VFA production of WAS were much higher under alkaline conditions than any other pH environment. To explain the effect of pH on hydrolysis, Chen et al. (2007) hypothesized that the alkaline pH leads to dissociation of acidic groups in proteins and carbohydrates which in turn increase the repulsions between the negatively charged extracellular polymeric substances. As a result, the solubility of proteins and carbohydrates are increased. The more soluble proteins and carbohydrates are, the higher the concentration of SCOD will be, which is an indication of hydrolysis. The pH of the fermenter also affects VFA production during the fermentation process. Chen et al. (2007) stated two possible reasons for this phenomenon, as follows: First, there is a higher hydrolysis rate in an alkaline environment which provides more SCOD available for acidification. Moreover, methanogenic activity is ceased under alkaline conditions. In the case of pure glycerol fermentation by *propionibacteria*, low pH values might lead to the system shutdown (Vorobjeva, 1999). The proposed reason for that was stated as the probable pH effect on the concentration of un-dissociated organic molecules which are toxic for bacterial cells. Since un-dissociated propionic acid molecules are more toxic to *propionibacteria* than un-dissociated acetic acid in a glycerol-spiked reactor with high propionate concentration, it is highly recommended to keep the pH high enough (pH value of 8) to prevent bacterial toxicity.

2.2 Materials and Methods

To study the effect of operational conditions (mixing energy, external substrate dosage, pH and temperature) on pure glycerol/biodiesel waste fermentation, initial tests were conducted in serum bottles. For this reason, 50 ml primary solids were diluted with 50 ml primary effluent. The mixture was then added to a 120 ml serum bottle and sealed using aluminum crimped caps equipped with butyl rubber septa to reduce the effect of oxygen exposure during the fermentation

time. Both the primary solids and the primary effluent used in this phase were collected from the Glendale Wastewater Treatment Plant (Lakeland, Florida). The collection of primary solids and the primary effluent was performed once a week and stored in a walk-in cooler at 4°C. During the serum bottles experiments mixing energy was provided by a rotary shaking table at 200 rpm. The glycerol concentration of the biodiesel waste was determined by using the modified colorimetric method (Bondioli et al., 2005). Glycerol constituted only 20% of the biodiesel waste received during the study which was on the low end of the range that might be expected from typical biodiesel waste. External substrate dosage was defined in a unit of mg/L. for this reason specific amount of substrate (in the unit of mass) was added to the serum bottles. Initial pH adjustment was carried out by addition of 1 M NaOH or 1 M HCl to the samples. Temperature experiments were conducted by locating the samples in a temperature controlled room which was set to the target temperature.

2.2.1 VFA analysis

VFA analysis was conducted by using a Shimadzu gas chromatography GC-14A (Shimadzu, Columbia, Maryland). For this reason a Nukul capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm) from Supelco was used. The analysis started at the oven temperature of 110°C and increased to 190°C at the rate of 5°C/min. The column was maintained at 190°C for an additional 10 minutes. The injector and detector port were kept at 220°C. The carrier gas was helium and it was provided at 20 cm/min linear velocity. The injection was conducted with an auto injector AOC-20i (Shimadzu, Columbia, Maryland). Pretreatment of the samples included centrifuging and then filtering the sample supernatant through 0.45 µm membrane filters. 1 ml of filtered samples were then transferred to 1.5 ml GC vials, sealed with aluminum crimp caps, and stored

frozen until the analysis. Prior to injection samples were acidified with 0.5 ml formic acid (5%) to have a pH of 3 or less. Calibration curves were developed by injecting the Shimadzu volatile free acid mix (46975-U; Shimadzu, Columbia, Maryland) which contained 10 mM of short chain volatile fatty acids with 2 to 7 carbon molecular chain length in deionized water.

2.2.2 Solids

Total suspended solid (TSS) and volatile suspended solid (VSS) were measured in accordance with Standard Method sections 2450 D and E (1995). Both tests were conducted using Whatman glass fiber filters 934/AH (Whatman, Pittsburgh, Pennsylvania). Before testing, filters and cleaned evaporating dishes (aluminum trays and ceramic Gooch crucibles) were rinsed with DI and dried overnight at 105°C to measure TSS. In order to measure VSS Gooch crucibles with the filters in were put in a furnace at 550°C for an hour prior to analysis to remove all volatile particles that might be attached to the crucibles and filters. The sample volume depended on the concentration range of the sample and the surface area of the filters. A typical raw wastewater TSS analysis might be carried out by filtering 40 ml of sample using dried, pre-weighed, aluminum trays and 4.25 cm diameter glass filters. As the primary solids were much thicker than wastewater 10 to 15 ml of 50:50 (V/V) diluted primary solids (diluted with wastewater influent) were sufficient to do TSS analysis in the case of using 2 cm diameter glass filters located in Gooch crucibles. Subsequently, crucibles were dried for an hour at 105° C. The VSS test was carried out by placing the same crucibles in a furnace at 550° C for an hour after being weighed for TSS. Then the crucibles with filters were placed in a desiccator for 1 hour and weighed after cooling to room temperature. The difference between the initial and final weight was equal to the mass of solids in

the samples. Then the mass was divided by the original sample volume to obtain the sample concentration.

2.2.3 Chemical oxygen demand

Chemical oxygen demand (COD) is a test to measure the organic carbon of the samples. In the current study high range (0-1500 mg COD/L) colorimeter COD vials (Lovibond, Sarasota, Florida) were used to measure COD according to the dichromate method which is described in Standard Methods (1995) section 5220 C. In this method, samples are heated for 2 hours and undergo a digestion process with acid, in the presence of potassium dichromate which is a strong oxidizer. Silver and, often, mercury is present in the digestion. Silver is a catalyst and mercury is used to eliminate the chloride interferences. 2 ml of sample is added to the vial, heated for two hours and oxidizable organic compounds reduce the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). After cooling down, the absorbance of samples were read with a spectrophotometer model Hach DR5000 at 620µm (Hach, Loveland, Colorado). As COD of primary solids were higher than the range (0-1500 mg/L) to measure both total and soluble COD (the fraction of COD that passed through Whatman glass fiber 934/AH filters) samples were diluted properly to be within the range.

2.2.4 Glycerol

Glycerol analysis was conducted using a modified colorimetric method for aqueous solutions (Bondioli ae al. 2005). Two required reagents in this method were 10 mM sodium periodate solution and 0.2 M acetylacetone solution. The 10 mM sodium periodate solution was prepared by dissolving approximately 21 mg sodium meta periodate in 5 ml of 1.6 M acetic acid

solution and then adding 5 ml of 4 M ammonium acetate solution. The 0.2 M acetylacetone solution was prepared by dissolving approximately 200 µL acetylacetone in 5 ml of 1.6 M acetic acid solution and 5 ml of 4 M ammonium acetate solution. To identify the glycerol concentration samples were first centrifuged. The supernatant of the centrifuged samples were then filtered through a 0.45 µL membrane filters. As in this method glycerol can be measured in a narrow range (0 to 30 mg/L), they were properly diluted with DI water. 2 ml of diluted samples were then transferred to 10 ml vial. Next, 1.6 ml of 10 mM sodium periodate solution was added to each vial and shook vigorously for 30 seconds. This reagent was needed to oxidize the glycerol to formaldehyte. In the next step, 1.6 ml of 0.2 M acetylacetone solution was added to vials and samples were then transferred to a water bath and kept there at 70°C for 1 minute. The latter reagent reacted with formaldehyte and gave a quantifiable color to the solution. After completion the reaction time samples were immersed in a beaker containing tap water at room temperature at least for 10 minutes to cool down and color development. The intensity of color was measured with a spectrophotometer model Hach DR5000 at 410µm (Hach, Loveland, Colorado). The standard solutions were made by dissolving specific amount of pure glycerol in the working solvent which was DI water (in the original method a 50:50 (V/V) mixture of distilled water and 95% ethanol was used as the working solvent). The accuracy and the precision tests conducted to evaluate the modified colorimetric method is shown in appendix A.

2.3 Results and discussion

2.3.1 Mixing energy

The effect of mixing energy on glycerol fermentation was studied through running 6 identical serum bottles (Control sample was run in triplicate and the mixed samples were run

in duplicate). The operational conditions are described in Table 2-1. The average VFA production in mixed samples was 863 mg COD/l whereas in the un-mixed sample it was 631 mg COD/l. These preliminary results proved that mixing increases the VFA production considerably. This could be related to the sufficient contact and hence better mass transfer between the corresponding microorganisms and the substrate. Mixing did not change the VFA composition significantly. In both mixed and un-mixed samples propionic acid was the predominant product. The propionic acid/acetic acid ratio in the mixed and un-mixed samples were 1.10 and 1.29 respectively. Therefore, due to about 37% improvement in total VFA production in mixed samples it was decided to provide mixing in future tests.

Table 2-1: VFA production and composition in serum bottles; VFAs and glycerol are in the unit of mg COD/L and HAc:HPr ratio is in the unit of C-mmole/C-mmole.

Sample	Substrate	Substrate Dosage	Final pH	Temp. (C)	Mixing (rpm)	HAc	HPr	VFA	HAc/HPr
Control	-	-	6.2	22	-	255	139	395	2.03
Unmixed	glycerol	608.5	5.9	22	-	275	356	631	0.86
Mixed	glycerol	608.5	6.1	22	200[1]	410	452	863	1.01

1: Shaker table rpm

2.3.2 Substrate Dosage

This phase of study was conducted in two steps. At first, 120 ml serum bottles containing 100 ml fresh primary solids were dosed with pure glycerol at different initial dosages: 0 mg/L, 100 mg/l, 500 mg/l, 1000 mg/l and 2000 mg/l and mixed for 24 hr on a shaker table at 200 rpm. In the second step samples were run under the same conditions but biodiesel waste was used to dose the identical serum bottles at the same concentration (mass basis). As it is indicated in Table 2-1 and Table 2-2 both glycerol and biodiesel waste addition affected the VFA production and

composition significantly. The VFA production was consistently increased by increasing the substrate initial dosage and in all glycerol/biodiesel waste dosed bottles propionic acid was the dominant fermentation end-product followed by acetic acid and butyric acid. The maximum VFA specific production rate and VFA production rate were observed in the bottles dosed at 2000 mg/l glycerol (2434 mg glycerol-COD/L). These values are in a good agreement with the rates observed for primary solids in the literature (Zeng et al. 2006). The presented results in both tables are the average of duplicates.

Table 2-2: VFA production and composition (mg COD/L), acetic acid :propionic acid ratio (C-mmole/C-mmole), total VFA specific production rate (mg COD/gVSS/hr) and total VFA production rate (mg COD/L/hr) with respect to the glycerol (mg COD/L) initial concentration at room temperature (22°C) and 200 rpm.

Sample	Substrate Dosage[1]	HAc	HPr	HBu	VFA	HAc/HPr	Specific rate	Production rate
Ctrl	-	57	16	-82[2]	-9	4.18		-
S1	122	84	107	-65	126	0.93	0.33	5.24
S2	609	124	294	-27	391	0.50	1.22	16.30
S3	1217	113	416	17	546	0.32	2.03	22.76
S4	2434	152	475	46	672	0.37	2.59	28.01

1: As theoretical oxygen demand of glycerol is 1.217 mg COD/mg glycerol the second column was calculated by multiplying the substrate dosage (0 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L and 2000 mg/L) by 1.217.
2: The negative number shows that the VFA concentration of the sample after 24 hr fermentation time was less than that at time 0.

Table 2-3: VFA production and composition (mg COD/L), acetic acid:propionic acid ratio (C-mmole/C-mmole) and total VFA production rate (mg COD/L/hr) with respect to the biodiesel waste initial concentration (mg COD/L) at room temperature (22°C) and 200 rpm.

Sample	Substrate Dosage[1]	HAc	HPr	HBu	VFA	HAc/HPr	Specific rate [2]	Production rate
Ctrl	-	107	-46[1]	0	62	-		2.56
S1	195	115	61	0	176	2.20	N/A	7.35
S2	975	159	226	0	385	0.82	N/A	16.04
S3	1950	144	235	0	379	0.72	N/A	15.81
S4	3900	250	417	0	667	0.70	N/A	27.79

1: The negative number shows that the VFA concentration of the sample after 24 hr fermentation time was less than that at time 0.

2: Solids data for these experiments was inadvertently lost.

3: The total COD value of biodiesel waste was measured as 1.95 mg COD/mg biodiesel waste. Hence the second column was calculated by multiplying substrate dosage (0 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L and 2000 mg/L) by 1.95.

2.3.3 Temperature and pH

This phase of study was carried out by dosing the serum bottles either with pure glycerol or biodiesel waste at the optimum initial dosage (2000 mg/L) achieved in the previous phase. All samples were run under the same process conditions. Figure 2-1 and Figure 2-2 show the VFA production in samples dosed with pure glycerol and biodiesel waste respectively. It should be mentioned that tests were done in different weeks with different wastewater characteristics such as initial VFA concentration, pH, TCOD and SCOD, solids and this explains the difference in VFAs between the a and b graphs at 22°C. For this reason, results from different weeks are shown on separate graphs. In addition comparisons of Figure 2-1 (a) and (b) and Figure 2-2 (a) and (b) are affected by this variation. Experimental results revealed that in spite of the temperature and the source of substrate the optimum initial pH with respect to the total VFA production was somewhere between 7 and 8.5 (for the pH ranged studied). It was observed that the initial pH also affected the VFA composition. Acetic acid, propionic acid and butyric acid production in pure glycerol-fed and biodiesel-fed bottles are shown in Figure 2-3 to Figure 2-8. Regardless of the

temperature propionic acid to total VFA production ratio in pure glycerol-fed bottles was at the maximum level when the initial pH was adjusted to 7. However, HAc to total VFA ratio did not follow a consistent trend in the aforementioned bottles. In the biodiesel waste-fed bottles, on the other hand, a pH range between 5.5 and 7 was the most favorable pH to increase the HPr to total VFA ratio whereas the basic environment resulted in HAc to total VFA ratio enhancement in the above-mentioned bottles. Butyric acid formed a small fraction of the total VFA production in the samples. As it can be seen in the graphs increasing the temperature has a favorable effect on VFA production. For example, at pH 8.5 increasing the temperature from 22°C to 36°C increases the VFA production from 1233 mg COD/L to 1988 mg COD/L for pure glycerol and from 997 mg COD/L to 2097 mg COD/L for biodiesel waste. Temperature also affected the VFAs composition. Regardless of the pH, increasing the temperature increased the fraction of propionic acid in pure glycerol-fed bottles. However, in the biodiesel waste-fed bottles there was not a consistent trend. In neutral and basic environments as the temperature increased the HPr to VFA ratio increased whereas in the acidic environment an opposite trend was observed. In other words, increasing the temperature in the acidic environment resulted in a smaller propionic acid fraction in total VFA produced during the fermentation.

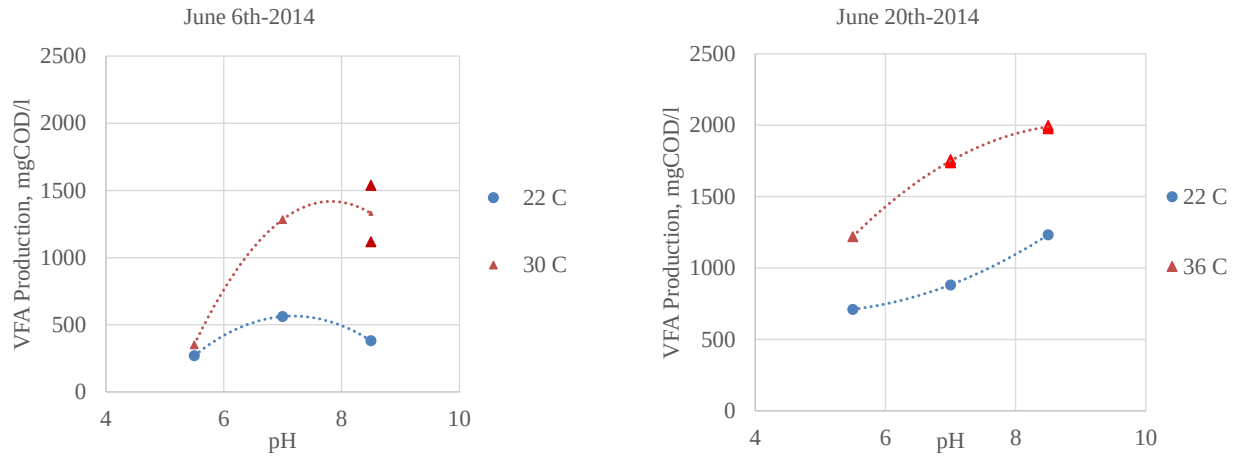

Figure 2-1: Total VFA production vs temperature and pH dosed with pure glycerol at 2000 mg/L and mixed at 200 rpm.

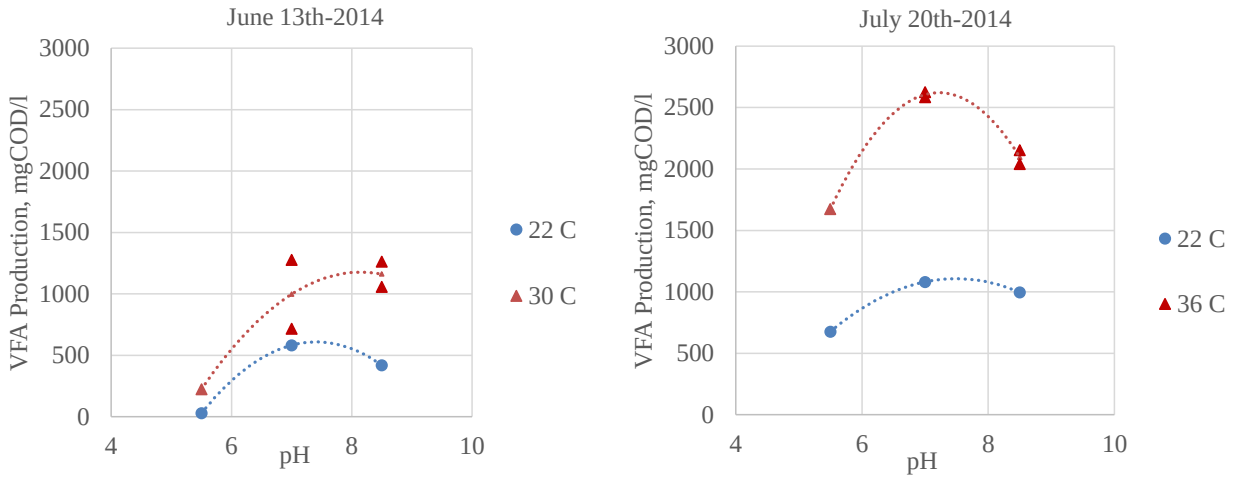

Figure 2-2: Total VFA production vs. temperature and pH dosed with biodiesel waste at 2000 mg/L and mixed at 200 rpm.

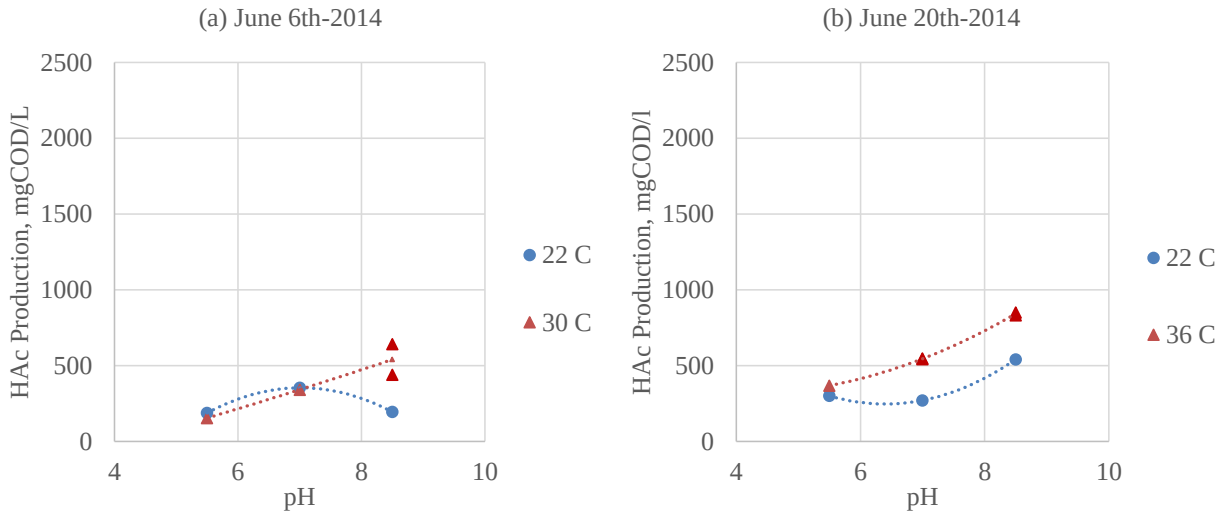

Figure 2-3: Acetic acid production vs temperature and pH dosed with pure glycerol at 2000 mg/L and mixed at 200 rpm

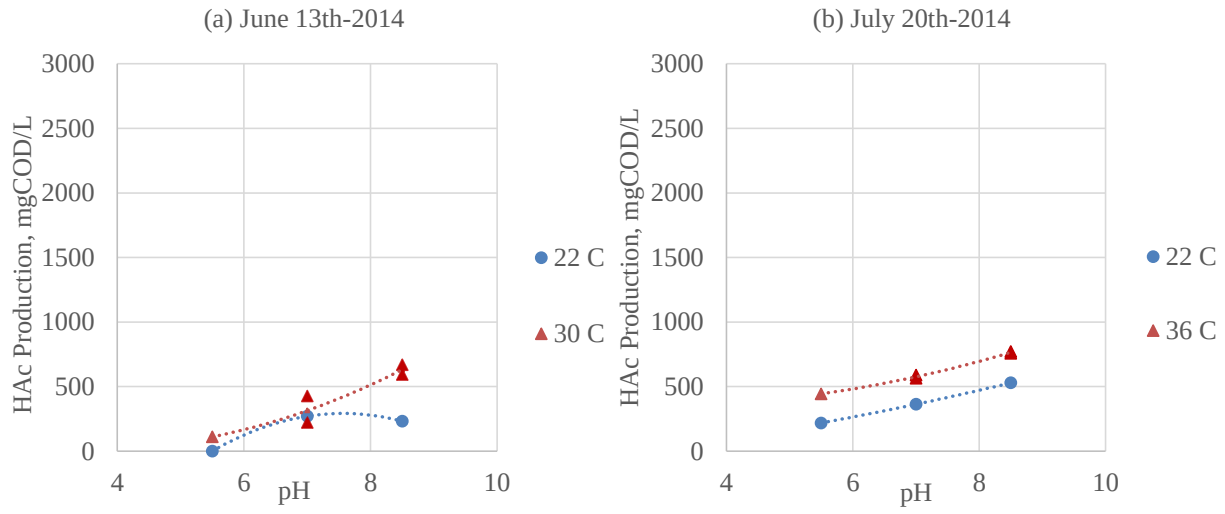

Figure 2-4: Acetic acid production vs. temperature and pH dosed with biodiesel waste at 2000 mg/L and mixed at 200 rpm

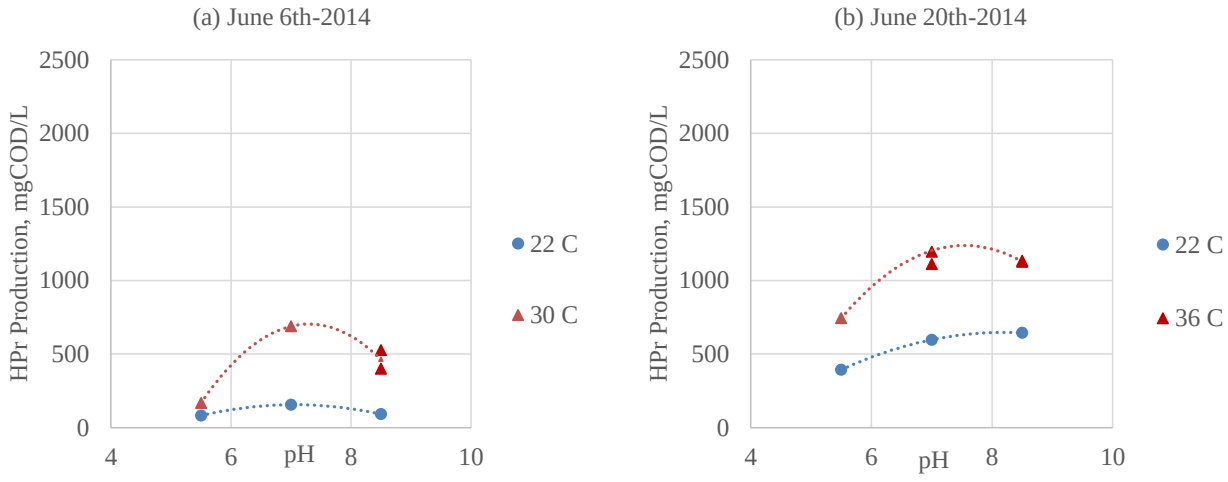

Figure 2-5: Propionic acid production vs temperature and pH dosed with pure glycerol at 2000 mg/L and mixed at 200 rpm

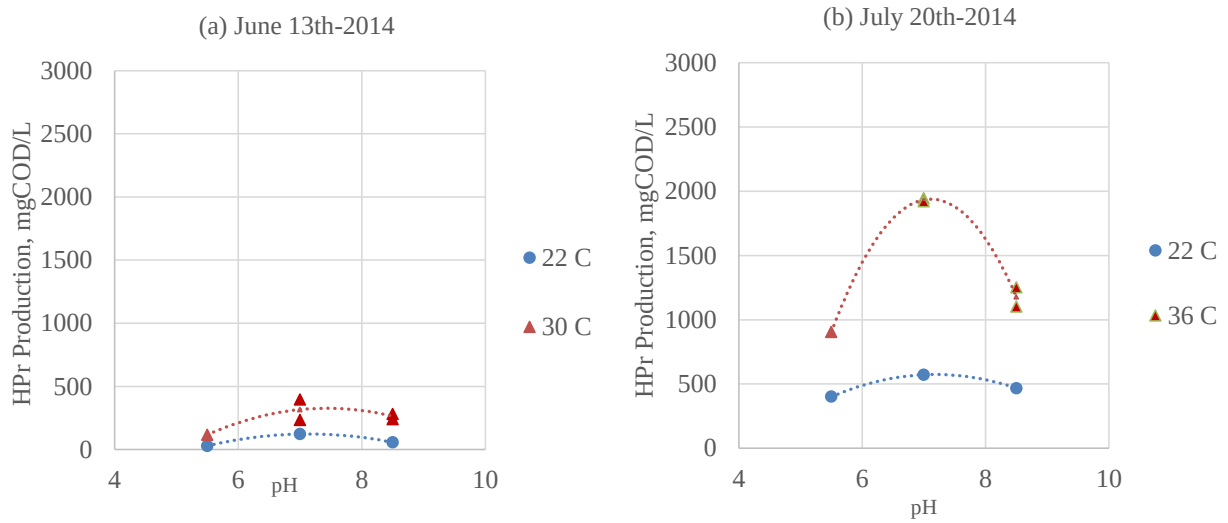

Figure 2-6: Propionic acid production vs. temperature and pH dosed with biodiesel waste at 2000 mg/L and mixed at 200 rpm

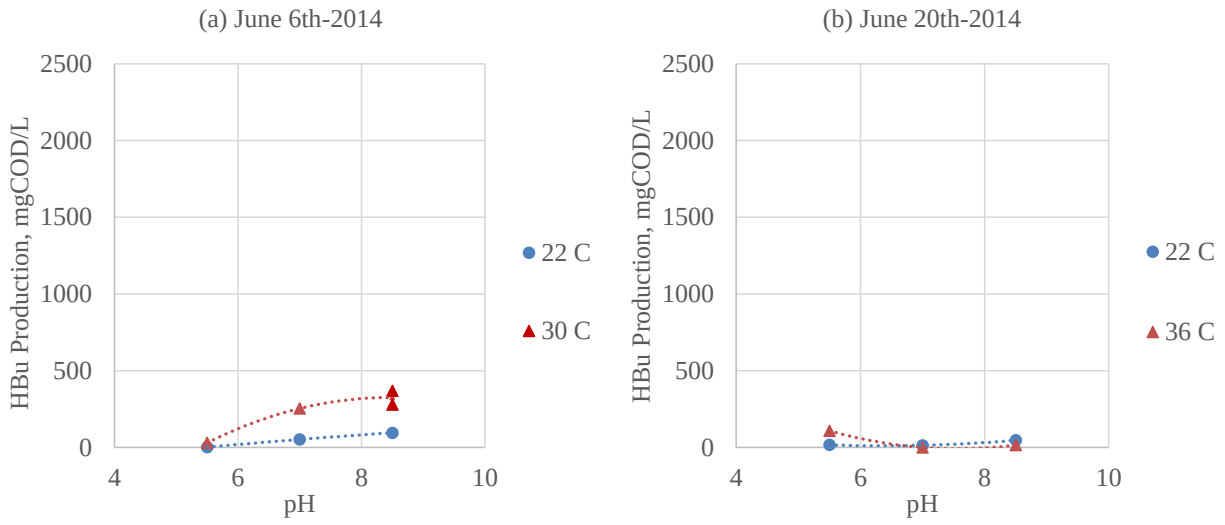

Figure 2-7: Butyric acid production vs temperature and pH dosed with pure glycerol at 2000 mg/L and mixed at 200 rpm

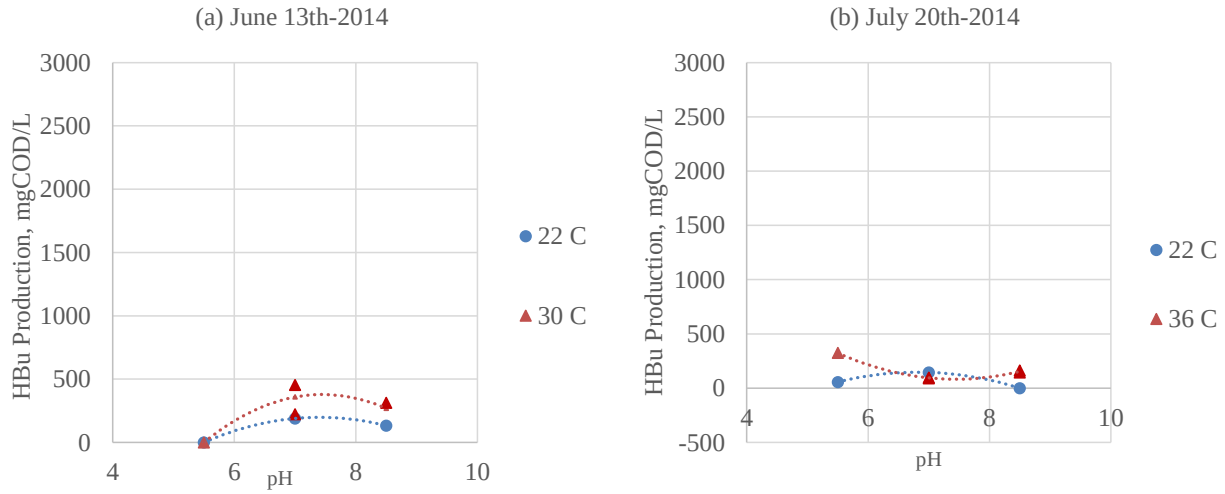

Figure 2-8: Butyric acid production vs. temperature and pH dosed with biodiesel waste at 2000 mg/L and mixed at 200 rpm

2.4 Conclusion

Experimental results revealed that mixing energy increased the glycerol fermentation to VFAs about 37%. Loading the serum bottles with glycerol and biodiesel waste as the external carbon source not only increased the VFA production but also affect the VFA composition. The propionic acid production was continuously increased as the initial substrate dosage was increased from 100 mg/L to 2000 mg/L. Propionic acid to acetic acid ratio in bottles dosed with 2000 mg/L pure glycerol and 2000 mg/L biodiesel waste were 3.13 and 1.67 respectively. It was observed that regardless of pH value increasing the temperature led to greater VFA production. Temperature also affected the VFA composition. In both pure glycerol-fed and biodiesel waste-fed bottles raising the temperature from 22°C to 36°C increased the HPr/VFA ratio at all pH levels (except in the biodiesel waste-fed bottle at pH of 5.5). The optimum pH value regarding the total VFA production was varied between 7 and 8.5. However, the propionic acid production was at its maximum when the pH was adjusted to 7.

2.5 References

Appels, Lise, et al. "Principles and potential of the anaerobic digestion of waste-activated sludge." *Progress in Energy and Combustion Science* 34.6 (2008): 755-781.

Banister, S. S., and W. A. Pretorius. "Optimisation of primary sludge acidogenic fermentation for biological nutrient removal." *WATER S. A.* 24.1 (1998): 35-42

Bondioli, Paolo, and Laura Della Bella. "An alternative spectrophotometric method for the determination of free glycerol in biodiesel." *European journal of lipid science and technology* 107.3 (2005): 153-157.

Coral, Jefferson, et al. "Batch fermentation model of propionic acid production by Propionibacterium acidipropionici in different carbon sources." *Applied biochemistry and biotechnology* 151.2-3 (2008): 333-341.

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36

Chen, Yinguang, et al. "Hydrolysis and acidification of waste activated sludge at different pHs." *Water Research* 41.3 (2007): 683-689.

Danesh, Shahnaz, and Jan A. Oleszkiewicz. "Volatile fatty acid production and uptake in biological nutrient removal systems with process separation." *Water environment research* 69.6 (1997): 1106-1111.

González-Barceló, O. G., and Simón González-Martínez. "Anaerobic prefermentation and primary sedimentation of wastewater in a sequencing batch reactor." *Water SA* 32.4 (2007): 577-583

Mahmoud, Nidal, et al. "Anaerobic stabilisation and conversion of biopolymers in primary sludge—effect of temperature and sludge retention time." *Water Research* 38.4 (2004): 983991

Oehmen, Adrian, et al. "Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources." *Journal of Biotechnology*123.1 (2006): 22-32.

Vorobjeva, Lena I. Propionibacteria. *Springer*, 1999

Yuan, Q., R. Sparling, and J. A. Oleszkiewicz. "VFA generation from waste activated sludge: Effect of temperature and mixing." *Chemosphere* 82.4 (2011): 603-607.

Zeng, R., Z. Yuan, and J. Keller. "Effects of solids concentration, pH and carbon addition on the production rate and composition of volatile fatty acids in prefermenters using primary sewage sludge." *Water Science & Technology* 53.8 (2006): 263-269.

CHAPTER 3: THE EFFECT OF EXTERNAL SUBSTRATE (PURE GLYCEROL VS BIODIESEL WASTE) AND SRT ON VFA PRODUCTION IN BENCH-SCALE PREFERMENTERS

3.1 Introduction

Biological Nutrient Removal (BNR) is an established technology that enables the removal of nutrients, namely nitrogen and phosphorus, from waste water through biochemical reactions. EBPR is a specific BNR process which is known as a powerful mechanism to remove phosphorus. Volatile Fatty Acids (VFAs) are the most suitable carbon sources that can drive phosphorus (P) removal. It is reported that for 1 mg/L P to be removed about 7-9 mg/L VFA as COD is needed (Barnard, 1993). However, wastewater often doesn't have enough VFAs to develop phosphorus removal to the desired level, especially in temperature climates. One way to increase the VFA concentration in the wastewater is prefermentation of the primary sludge which provides hydrolysis and acidification of the biodegradable COD of the influent primary solids. EBPR removal efficiency varies depending on different parameters such as the type of carbon source (e. g. type of VFA, fermentable rbCOD) and the operational conditions (e. g. solid retention time, temperature, etc.). Numerous studies have reported that both acetic acid and propionic acid were effective for phosphorus removal. In short term cultivation, acetic acid showed a better Soluble Ortho Phosphorus (SOP) removal than propionic acid (Abu-ghararah and Randall, 1991). In sequencing batch reactor (SBR) systems with long-term cultivation, the SOP removal efficiency in reactors with a higher influent propionic acid to acetic acid ratio removed more P than low acetic acid to propionic acid ratio influent reactors (Chen et al. 2004). Due to high propionic acid production-cost (propionate is produced industrially by using petroleum hydrocarbons), it is expensive to purchase supplemental propionate in full-scale wastewater treatment plants. On the other hand glycerol is the main constituent of biodiesel waste and can be used as an external carbon

source in BNR systems. Barbirato et al. (1997) reported that regardless of the propionic acid bacteria strain (*Propionibacterium acidipropionici, Propionibacterium acnes* and *Clostridium propionicum*) propionic acid was the dominant glycerol fermentation end-product followed by succinic acid, acetic acid, and propanol. Himmi et al. (2000) showed that propionic acid formation by propionic acid bacteria (*Propionibacterium acidipropionici* and *Propionibacterium freudenreichii ssp. shermanii*.) was about 2 times greater on glycerol than glucose. This leads to the idea of using glycerol as the external substrate in prefermenters in order to produce propionic acid which can be subsequently used as carbon source to drive phosphorus removal in EBPR systems. As EBPR efficiency is highly affected by propionic acid levels and acetic acid to propionic acid ratios it is important to optimize the operational conditions of prefermenters. In the current study prefermenter SRT value and mixing energy were studied for primary solids with supplemental glycerol added.

SRT (solids retention time also known as mean cell residence time or MCRT), is a term referring to the time that solids or microorganisms stay in a reactor system. Increasing the SRT up to a specific value increases the prefermenter VFA production because it increases the quantity of microorganisms in the reactor. On the other hand methanogens have lower specific growth rates than that of fermentative microorganisms. Therefore longer SRT values leads to higher concentration of methanogens in prefermenters, which consume the VFA produced from fermentation. Danesh et al. (1997) reported that increasing the SRT in the range of 4 to 13 days increased the VFA volumetric production rate as mgVFA/L-d in bench scale prefermenters. Elefsiniotis and Oldham (1991) found that increasing the SRT value from 5 to 15 days (5 d, 10 d and 15 d) enhanced the VFA specific production rate. Bouzas et al. (2007) investigated the simultaneous effect of SRT and the recirculation sludge flow-rate on both side-stream and in-line

prefermenters. It was reported that although in the lab scale experiments increasing the SRT increased the VFA production, in the real full scale plant the results were not consistent. However, the highest VFA production was observed in the side-stream prefermenter when the SRT was adjusted to 6 days and the recirculation sludge flow-rate was 4.5 L/hr.

3.2 Materials and methods

In order to study the effect of external substrate and the SRT on fermentation process 4 prefermentation reactors were operated. The liquid volume inside the reactors was 1500 mL. Mixing energy was applied to all reactors at 50 rpm. The pH was not adjusted or changed in any of the reactors and the temperature was 22°C. The SRT in Reactor# 1, Reactor # 2, Reactor # 3 and Reactor # 4 was 4 days, 2 days, 2 days and 4 days respectively. Depending on the SRT value the proper amount of solids inside the reactor was wasted, and the reactor was reloaded with the same volume of fresh primary solids on a daily basis. Fresh primary solids were obtained from the Glendale Wastewater Treatment Facility (Lakeland, Florida) weekly and kept in a cooler at 4°C. Reactor # 1 and Reactor # 2 were dosed with 1500 mg pure glycerol/cycle. Therefore the initial increase in glycerol concentration in Reactor # 1 and Reactor # 2 was 1000 mg/L. Reactor # 3 was run as a control without glycerol addition. Biodiesel waste was received from SAKAL LLC (Panama, Panama) and kept at 4°C for the entire study period. According to the HPLC method conducted at Mid-west Laboratories (Omaha, Nebraska) and the colorimetric method conducted in the environmental lab at the University of Central Florida the glycerol concentration in the biodiesel waste batches was approximately 20%. Reactor # 4 was dosed with 1500 mg (by weight) biodiesel waste per cycle which resulted in an approximate glycerol concentration of 200 mg/L. Table 3-1 shows the experimental conditions of the four reactors. As shown in Table 3-1 the SRT was the isolated parameter between Reactor # 1 and Reactor # 2, whereas the effect of

pure glycerol addition was the isolated parameter between Reactor # 2 and Reactor # 3. Reactor# 1 and Reactor # 4 were run the same but with a different substrate type (pure glycerol vs. biodiesel waste). Sampling was conducted over 7 weeks. Since after 4 weeks the VFA production suddenly increased significantly the presented values are the average of 6 sampling events conducted in the last 3 weeks when the system seemed to achieve steady-state. The feed characteristics is stated in appendix B.

Table 3-1: Experimental conditions (MLVSS and MLSS data are the average values from December 2nd-2015 to December 18th-2015)

	Temp. (°C)	pH	MLSS (mg/L)	MLVSS (mg/L)	Mixing (rpm)	Substrate	Substrate dosage (mg COD/L)	SRT (days)
R1	22	4.68	16153	14620	50	Pure glycerol	1217	4
R2	22	4.79	13947	12613	50	Pure glycerol	1217	2
R3	22	5.05	17200	14880	50	-	-	2
R4	22	4.85	13511	14427	50	Biodiesel Waste	1950	4

1: pH, MLSS and MLVSS data show the average values inside the reactors.
2: Theoretical oxygen demand of glycerol was calculated as 1.217 mg COD/mg glycerol. Therefore, the substrate dosage in Reactor # 1 and Reactor # 2 was 1000 mg/L × 1.217 mg COD/mg glycerol = 1217 mg COD/L. The total COD value of biodiesel waste was measured as 1.95 mg COD/mg biodiesel waste. Hence the substrate dosage in Reactor # 4 was 1000 mg/L × 1.95 mg COD/mg biodiesel waste = 1950 mg COD/L.

3.2.1 Analytical methods

VFAs were measured by gas chromatography using a Shimadzu GC14-A which was equipped with a Supelco Nukul Column (30m × 0.25mm I.D. × 0.25µm; Supelco, St. Louis). The column initial temperature was increased from 110°C to 190°C at the rate of 5°C/min and stayed at the highest temperature for additional 10 min to remove all the residuals from the column. The injection port and detector port both were kept at 220°C. The carrier gas helium was provided at 20 cm/min linear velocity. Samples were centrifuged and then filtered with 0.45µm membrane

filters. Samples were acidified before injection with 5% formic acid in 1.5 ml GC vials (the pH must be 3 or less). 1 µl acidified sample was injected by an auto-injector AOC-20i (Shimadzu, Columbia, Maryland). Total suspended solids and volatile suspended solids were measured according to Standard Methods section 2450 D and E (1995). Chemical oxygen demand was determined by using the high range (0-1500 mg COD/L) colorimeter COD vials (Lovibond, Sarasota, Florida). The absorbance of the samples were then measured using a Hach DR5000 spectrophotometer at 620µm (Hach, Loveland, Colorado). Glycerol concentration was determined by modified colorimetric method for aqueous solutions (Bondioli and Bella, 2005). In the modified method DI water was used as the working solvent to make the glycerol standard solutions whereas in the original method a 50:50 (V/V) mixture of distilled water and 95% ethanol was used as the working solvent (APPENDIX A). (2 ml of filtered (via 0.45µm membrane filters) samples were transferred to 10 ml amber vials and 1.2 ml of a 10 mM sodium periodate solution was then added into each vial. Next, the vials were shaken vigorously for 30s. Afterwards, 1.2 ml of a 0.2 M acetylacetone solution was added to each sampling amber vial and the vials were then put in a water bach at 70 °C for 1 min. The vials were transferred to a beaker containing tap water at room temperature and kept there for 10 min to cool down and allow color development. The absorbance was read by a Hach DR5000 spectrophotometer at 410µm.

3.3 Results

3.3.1 Solids retention time

Experimental results showed that changing the SRT from 2 to 4 days did not affect the fermentation process significantly. The average VFA production (the average of all dates shown in Figure 3-1) in Reactor # 1 (SRT= 4 days) and Reactor # 2 (SRT= 2 days) was 10307 mg COD/L

and 9205 mg COD/L respectively. It should be noted that the VFA production of each cycle was calculated by subtracting the VFA concentration of the inflow at time zero from the VFA concentration of the corresponding reactor at the end of the cycle (24 hr. fermentation time). Despite significant changes in VFA production from Nov. 6th to Dec. 18th the values were always similar. With respect to propionic acid Reactor # 1 had a slightly higher production but both produced the desired mix of propionic and acetic acid (Table 3-2). The average propionic acid production in Reactor # 1 was 6221 mg COD/L whereas in Reactor # 2 the average propionic acid production was 4886 mg COD/L. The average acetic acid production in Reactor # 1 and Reactor # 2 were 3658 mg COD/L and 3809 mg COD/L respectively. The average propionic acid to acetic acid ratio was increased from 2.30 to 3.30 as mg COD/mg COD when the SRT increased from 2 days to 4. The average specific VFA production rate did not change with respect to SRT and in both reactors were equal at 37.9 mg COD/(mgVSS/hr). It is possible that SRT has a significant impact on VFA and propionic acid production outside the SRT range analyzed (i.e. 2 to 4 days), however, due to time constraints the effect of SRTs outside this range were not studied. Table 3-2 summarizes the fermentation composition and specific VFA production rate in Reactor # 1 and Reactor # 2. The numbers presented in Table 3-2 are the average of the last 6 data points (from December 2nd-2015 to December 18th-2015).

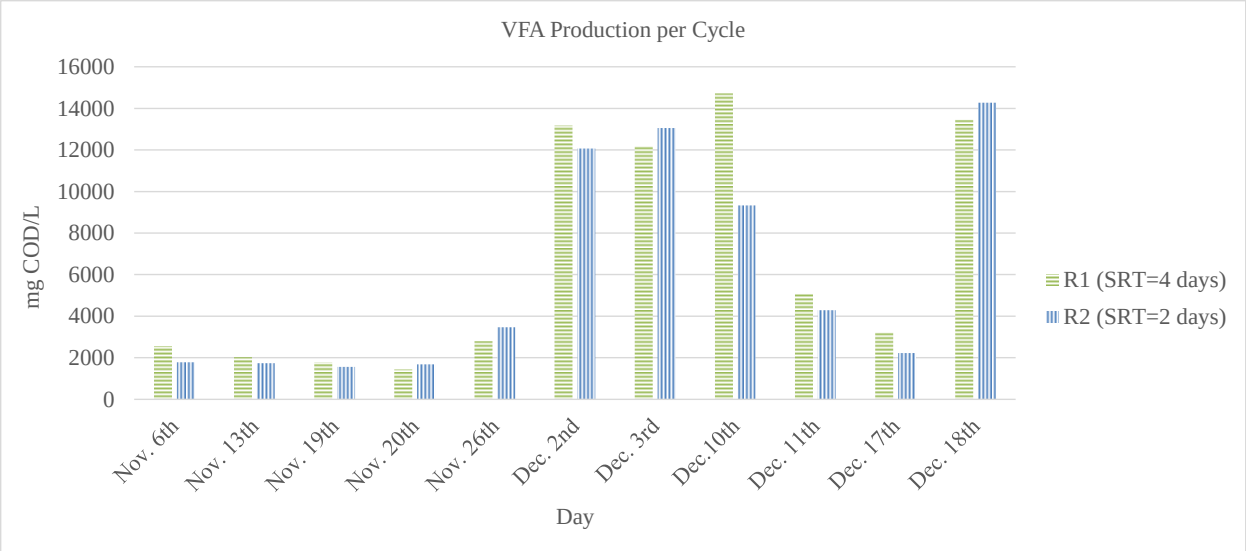

Figure 3-1: VFA production in Reactor # 1 (4 days SRT) and Reactor # 2 (2 days SRT)

Table 3-2: Fermentation composition and the average VFA production per cycle (from December 2nd-2015 to December 18th-2015) in Reactor # 1 vs. Reactor # 2 (SRT as the isolated experimental variable).

	SRT (days)	HAc[1] (mg COD/L)	HPr[2] (mg COD/L)	HBu[3] (mg COD/L)	VFA (mg COD/L)	Specific rate (mg COD/mgVSS/hr)
R1	4	3658 ± 2415	6221 ± 2149	428 ± 854	10307 ± 4875	37.9 ± 22
R2	2	3809 ± 2811	4886 ± 2119	510 ± 916	9205 ± 4933	37.9 ± 20.5

1. HAc: Acetic acid
2. HPr: Propionic acid
3. HBu: Butyric acid
4. The COD of acetic acid, propionic acid and butyric acid was calculated by multiplying the column 4, 5 and 6 by 1.06, 1.51 and 1.82 (their theoretical CODs per unit mass) respectively.

3.3.2 Glycerol addition

Comparison of Reactor # 2 versus Reactor # 3 isolates pure glycerol as the sole experimental variable. From this it can be seen that most of the glycerol was fermented to propionic acid. In fact the molar-C yield is greater than 1.0, which implies that some of the carbon from the primary solids was fermented to propionic acid in Reactor # 2 that was not fermented to propionic acid in Reactor # 3. There is more than one possible explanation for this but one theory

would be that if glycerol is present and favors bacteria that produce propionic acid as their fermentation end product then their population will be larger in Reactor # 2 than in Reactor # 3. As a result some of the primary solids are also fermented by this larger population and end up as propionic acid in Reactor # 3, explaining why the propionic acid yield is greater than the amount of that can be attributed to the glycerol that was added.

Table 3-3: Fermentation composition and VFA production increases and molar yields as carbon for Reactor # 2 vs. Reactor # 3 (purified glycerol versus Control Reactor). Substrate dosage and VFAs are in the unit of mg COD/L. The specific production rate is in the unit of mg COD/gVSS/hr.

	Substrate	Substrate dosage	HAc	HPr	HBu	VFA	Specific rate
R2	glycerol	1217	3809 ± 2811	4886 ± 2119	510 ± 916	9205 ± 4933	37.9 ± 20.5
R3 (Control)	-	-	3486 ± 2411	1915 ± 789	301 ± 714	5702 ± 2694	13.4 ± 9.5
Increase Relative to Control			323	2971	209	3503	-

3.3.3 Pure glycerol vs. biodiesel waste

Reactor 1 and 4 were run under the same experimental conditions but with different external substrate. Table 3-4 shows that the VFA production in the biodiesel waste-fed reactor (Reactor 4) was slightly lower than that in the pure glycerol-feed reactor (Reactor # 1). The possible reason for that is the biodiesel waste had a lower glycerol concentration and it consisted of only 20% glycerol. Therefore, the initial increase in glycerol concentration in Reactor # 4 was 243 mg COD/L. The VFA specific production rate (mgVFA/gVSS/hr) in the pure glycerol loaded reactor was higher than that in the biodiesel waste loaded reactor. In addition, the pure glycerol loaded reactor was more effective in propionic acid production. However, even with the biodiesel waste,

approximately 45% of the VFAs present were propionic acid indicating it can be used to obtain the desired propionic:acetic acid mixture.

Table 3-4: Fermentation composition and VFA production in Reactor # 1 vs. Reactor # 4 (pure glycerol versus biodiesel waste. Substrate dosage and VFAs are in the unit of mg COD/L. The specific production rate is in the unit of mg COD/gVSS/hr.

	SRT (day)	Substrate	Substrate dosage	HAc	HPr	HBu	VFA	Specific rate
R1	4	G[1]	1217	3658 ± 2415	6221 ± 2149	428 ± 854	10307 ± 4875	37.9 ± 22
R4	4	BDW[2]	1950	5365 ± 2377	3512 ± 1308	244 ± 654	9120 ± 2812	26 ± 13

1: Pure glycerol
2: Biodiesel waste

3.4 Conclusion

In order to enhance the prefermenters' performance regarding VFA production, the effect of external substrate and SRT were studied in this research. It was observed that adding the external substrate increased the VFA production. Adding the pure glycerol at the initial dosage of 1000 mg/l increased the VFA production about 67% on average (Reactor # 2 vs. Reactor # 3). Regardless of the SRT propionic acid was the dominant fermentation end-product followed by acetic acid and butyric acid. It was revealed that the SRT value did not have a major effect on glycerol/biodiesel waste fermentation. Under the same process and environmental conditions increasing the SRT from 2 to 4 days improved the VFA production about 12%. However, the pure glycerol-fed reactor showed a better performance than a comparable mass of biodiesel waste, but glycerol concentrations were also much lower in the biodiesel waste. The VFA production was increased about 81%, 60% and 61% versus the control in Reactor # 1, Reactor # 2 and Reactor# 3 respectively. The VFA specific production rate varied widely over a range of 26 mgVFA-COD/gVSS/hr to 38 mgVFA-COD/gVSS/hr. Adding the pure glycerol increased the specific VFA production rate from 13.4 to 37.9 mgVFA-COD/gVSS/hr at an SRT of 2days. Biodiesel waste

increased the specific rate to 26 mgVFA-COD/gVSS/hr which was much higher than the control but significantly less than that of pure glycerol fed reactors at 37.9 mgVFA-COD/gVSS/hr (Reactor # 1 and Reactor # 2).

3.5 References

Abu-Ghararah, Z. H., and C. W. Randall. "The effect of organic compounds on biological phosphorus removal." *Water Science & Technology* 23.4-6 (1991): 585-594

Barbirato, F., D. Chedaille, and A. Bories. "Propionic acid fermentation from glycerol: comparison with conventional substrates." *Applied Microbiology and Biotechnology* 47.4 (1997): 441-446.

Barnard JL. Prefermentation in biological nutrient removal plants. Proceedings of the Joint CSCE-ASCE National Conference on Environmental Engineering, Montreal, Que., Canada, 12 – 14 July; 1993. p. 1767 – 74

Banister, S. S., and W. A. Pretorius. "Optimisation of primary sludge acidogenic fermentation for biological nutrient removal." *WATER S. A.* 24.1 (1998): 35-42

Bondioli, Paolo, and Laura Della Bella. "An alternative spectrophotometric method for the determination of free glycerol in biodiesel." *European journal of lipid science and technology* 107.3 (2005): 153-157.

Bouzas, A., et al. "Fermentation and elutriation of primary sludge: Effect of SRT on process performance." *Water research* 41.4 (2007): 747-756

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36.

Danesh, Shahnaz, and Jan A. Oleszkiewicz. "Volatile fatty acid production and uptake in biological nutrient removal systems with process separation." *Water environment research* 69.6 (1997): 1106-1111.

Elefsiniotis, P., and Oldham, W.K. (1991) The Effect of Operational Parameters on the Acid-Phase Anaerobic Fermentation in the Bio logical Phosphorus Removal Process. Proc Am. Soc Civ. Eng. Nati. Conf. Environ. Eng., Reno, Nev., 325

Himmi, E. H., et al. "Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii." *Applied Microbiology and Biotechnology* 53.4 (2000): 435-440.

CHAPTER 4: THE EFFECT OF pH ON GLYCEROL/BIODIESEL WASTE FERMENTATION IN SEMI-CONTINIOUS BATCH REACTORS

4.1 Introduction

Biological nutrient removal (BNR) is a successful technology for removing excess amounts of nutrient from wastewater via biochemical reactions. Enhanced biological phosphorus removal (EBPR) is achieved with a specific biological nutrient removal configuration in which P removal is accomplished in an anaerobic/aerobic sequence. In order to have an EBPR system running successfully it is important to have enough volatile fatty acids (VFAs) in the system. It is reported that for 1 mg biological P removal 7 to 9 mg VFA is needed (Abu-Ghararah and Randall, 1991). However, domestic wastewaters often have a limited amount of VFA. One way to increase the VFA concentration in the wastewater is employing a separate unit process named a primary prefermenter which receives primary solids from primary clarifier underflow. Fermentation in the prefermenter produces considerable amounts of VFAs from the biodegradable solids. The performance of a prefermentor is affected by different parameters such as temperature, solids retention time (SRT), pH and so on. Gonzalez-Barcelo and Gonzalez-Martinez (2007) reported that decreasing the pH from 7.7 to 5.5 increased the acidification of primary solids in a sequencing batch reactor. In contrast, Zeng et al. (2006) investigated the effect of pH on acidification of primary solids in a batch system. It was observed that the acidification of primary solids decreased significantly when the pH decreased from 7 to 5.5. The optimum pH was reported between 6.5 and 7. Danesh and Oleszkiewicz (1997) observed that regardless of the SRT, decreasing the pH from 7-7.6 to 6.1-6.4 resulted in a lower VFA volumetric production rate. We et al. (2009) indicated that, regardless of SRT, increasing the pH from 3 to 11 increased the VFA production significantly. It was shown that at a SRT of 5 days the majority of VFA production change occurred when the pH was raised from 3 to 7. Bengtsson et al. (2009) examined the effect of pH on fermenting 4

different types of wastewater in a batch system designed based on activated primary tank prefermenters (i. e. a primary clarifier with a recycle). They observed that the optimum pH with respect to VFA production depends on the type of wastewater but generally ranged between 5.25 and 6. A dramatic VFA production drop was seen in a lower pH environment. In addition, VFA composition was also affected by the pH and regardless of the type of wastewater increasing the pH led to more propionic acid and less acetic acid formation. Given the importance of pH on the fermentation process, the objective of this research was to identify the optimum pH to increase the VFA production and to control the VFA composition in prefermenters which were dosed with either pure glycerol or biodiesel waste to favor propionic acid.

4.1.1 Materials and methods

The effect of pH on external substrate (i. e. pure glycerol or biodiesel waste) fermentation was investigated in 2 phases. In the first phase, a mixture of 50 ml primary solids and 50 ml primary effluent was transferred to serum bottles and dosed with either 2000 mg/l pure glycerol or biodiesel waste. Biodiesel waste was dosed using the weight of the waste, i. e. 200 mg of waste was put into a serum bottle containing 100 ml of diluted primary solids). Bottles were then crimped with butyl rubber septa and aluminum crimp caps and run for 24 hours on the shaking table which provided mixing at 200 rpm. The experiment was conducted at room temperature (22°C) and three different initial pHs: 5.5, 7, and 8.5 using 1M HCl or 1M NaOH. The biodiesel waste was received from Grupo-Lakas and stored at 4°C. The glycerol concentration of the biodiesel waste was measured with a colorimetric method (Bondioli and Bella, 2005) at the environmental engineering laboratory at the University of Central Florida, and by an HPLC method at Mid-west Laboratories (Omaha, Nebraska). Referring to the results the glycerol constituted 20% of the biodiesel waste which is a

significantly lower glycerol content than most biodiesel waste (Boodik et al. 2009) which can have as much as 60% glycerol content. However, the COD analysis revealed that the COD level of the biodiesel waste (1.95 mg COD/mg biodiesel waste) was much higher than that of pure glycerol (1.217 mg COD/mg pure glycerol) probably due to methanol and other organics. In the second phase 4 identical reactors with 2 L volume were run under anaerobic conditions. The liquid volume of each reactor was 1.5 L. The solid retention time (SRT) in all reactors was set at 4 days and they operated as semi-continuous fed batch reactors. Therefore, 375 ml of mixed liquor was removed and the reactors were loaded with the same amount of fresh primary solids approximately every 24 hours. The primary solids were obtained from Glendale Wastewater Treatment Facility (Lakeland, Florida) once a week and stored at 4°C. The reactors were mixed continuously with mechanical mixers at 50 rpm. Reactor # 1, 2, and 3 were dosed with pure glycerol at 1000 mg/L and Reactor # 4 were dosed with the same mass of biodiesel waste (giving an approximately glycerol concentration of 200 mg/L). The initial pH of the reactors were adjusted to 5.5, 7, and 8.5 using the 1M HCl or 1 M NaOH solutions. The experiment was conducted at room temperature (22°C). Table 4-1shows the experimental conditions of the 4 reactors.

Table 4-1: Experimental conditions of 4 prefermenter reactors performing at 22°C (the MLSS and MLVSS data of R1 and R2- R4 are the average of 5 and 8 data points respectively at the end of each cycle)

Reactor #	Initial pH	SRT	External Substrate	Substrate dose (mg/L)	MLSS (mg/L)	MLVSS (mg/L)	Mixing (rpm)
R1	5.5	4	Pure glycerol	1000 [1]	12467	10693	50
R2	7	4	Pure glycerol	1000	13217	10358	50
R3	8.5	4	Pure glycerol	1000	12725	10333	50
R 4	7	4	Biodiesel waste	1000	14925	12533	50

1: Approximately 200 mg/L glycerol

4.1.2 Analytical methods

VFA analysis was conducted using gas chromatography. A Shimadzu GC-14A equipped with a Supelco Nukul Column (30m × 0.25mm I.D. × 0.25µm; Supelco, St. Louis) was used to measure the VFA concentration. The analysis started at the column initial temperature of 110 °C and increased to 190 °C at 5°C/min. Both the injection and detection ports were kept at 220 °C. The carrier gas was helium and it was provided at 20 cm/min. Samples were centrifuged and filtered through 0.45 µm membrane filters. Prior to the injection samples were acidified with 5% formic acid to lower the pH below 3. 1 µl acidified sample (5% formic acid) was then injected by an auto-injector AOC-20i (Shimadzu, Columbia, Maryland). pH was measured with a handheld Oakton pH meter (Vernon Hills, Illinois). Total suspended solids and volatile suspended solids were measured according to the Standard Method Sections 2450 D and E (1995). Chemical oxygen demand was determined by using the high range (0-1500 mg/L) colorimeter COD vials (Lovibond, Sarasota, Florida). The absorbance of the digested samples were measured by Hach spectrophotometer DR5000 at 620 µm (Hach, Loveland, Colorado).

4.2 Results

4.2.1 Serum bottles

As it is shown in Figure 4-1 (a) and (b), regardless of the external substrate the maximum VFA production occurred in neutral and basic environments. Except for one data set from June 20th (pure glycerol) the neutral environment was more effective than the basic environment. It should be noted that the tests were done in different weeks with different wastewater characteristics such as initial VFA concentration, pH, COD and solids. This could explain the difference in VFA production of identical samples conducted at different times.

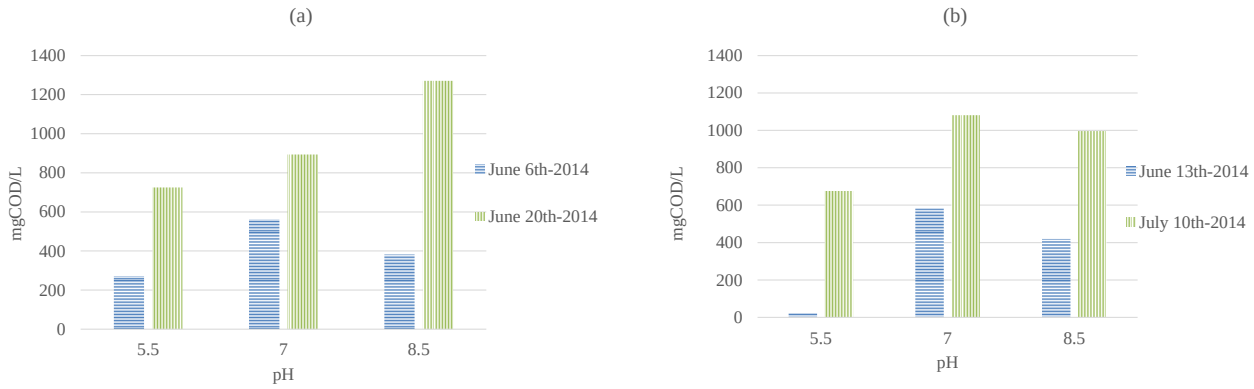

Figure 4-1: VFA production after 24 hour fermentation time in serum bottles (a) dosed with pure glycerol (b) dosed with biodiesel waste.

Table 4-2 and Table 4-3 summarize the average VFA production, VFA composition and propionic acid to acetic acid ratio in pure glycerol fed-bottles and biodiesel waste fed-bottles respectively. Detailed study of VFA compositions revealed that the neutral environment was the optimum environment with respect to propionic acid production especially in biodiesel waste-fed bottles. The average acetic acid: propionic acid ratio in pure glycerol-fed bottles and biodiesel waste-fed bottles at pH 7 were 0.97 and 1.06 C-mmole/C-mmole respectively which implied that the pure glycerol-fed bottles were provided with a better external substrate for propionic acid production which, might be a result of higher glycerol concentration in pure glycerol-fed bottles.

Table 4-2: Average VFA production, composition and propinic acid:acetic acid in serum bottles dosed with pure glycerol (VFAs are in the unit of mg COD/L and HAc/HPr is in the unit of C-mmole/C-mmole).

pH	HAc	HPr	HBu	VFA	HAc/HPr	% of VFAs (HAc:HPr:HBu)
5.5	245	237	16	498	1.2	49:48:3
7	313	376	39	728	0.97	43:52:5
8.5	368	368	91	827	1.16	45:45:10

5. HAc: Acetic acid
6. HPr: Propionic acid
7. HBu: Butyric acid
8. The COD of acetic acid, propionic acid and butyric acid was calculated by multiplying the column 4, 5 and 6 by 1.06, 1.51 and 1.82 (their theoretical CODs per unit mass) respectively.

Table 4-3: Average VFA production, composition and propinic acid:acetic acid in serum bottles dosed with biodiesel waste (VFAs are in the unit of mg COD/L and HAc/HPr is in the unit of C-mmole/C-mmole).

pH	HAc	HPr	HBu	VFA	HAc/HPr	% of VFAs (HAc:HPr:HBu)
5.5	108	215	26	349	0.58	31:62:7
7	317	347	168	832	1.06	38:42:20
8.5	380	262	66	708	1.68	54:37:9

4.2.2 Semi-continuous batch reactors

Figure 4-2 illustrates the VFA production in 8 sampling events. The optimum pH in terms of VFA production, was between 7 and 8.5. In some observations the VFA production was dominant at neutral pH and in some observations a basic environment (pH=8.5) was the most effective environmental. Zeng et al. (2006) reported the same trend between VFA production vs. the pH value. They identified the neutral pH as the optimum pH for producing VFAs. Regarding the VFA composition, propionic acid and butyric acid production were at their maximum levels when the initial pH was adjusted to 7 (Table 4-4) which is consistent with the results from serum bottles. The propionic acid production in Reactor# 1 (pH=5.5) and Reactor # 3 (pH=8.5) vs. Reactor # 2 (pH=7) were 42% and 85% respectively. On the other hand, the maximum acetic acid

production occurred in Reactor # 3 where the pH was adjusted to 8.5. In other reactors (Reactor # 1, Reactor # 2 and Reactor # 3) acetic acid was consumed in some cycles which suggests that there might have been minor acetocalstic methanogenesis active in them, but the amounts consumed were very small and may indicate that there was no significant change in the amount of acetic acid.

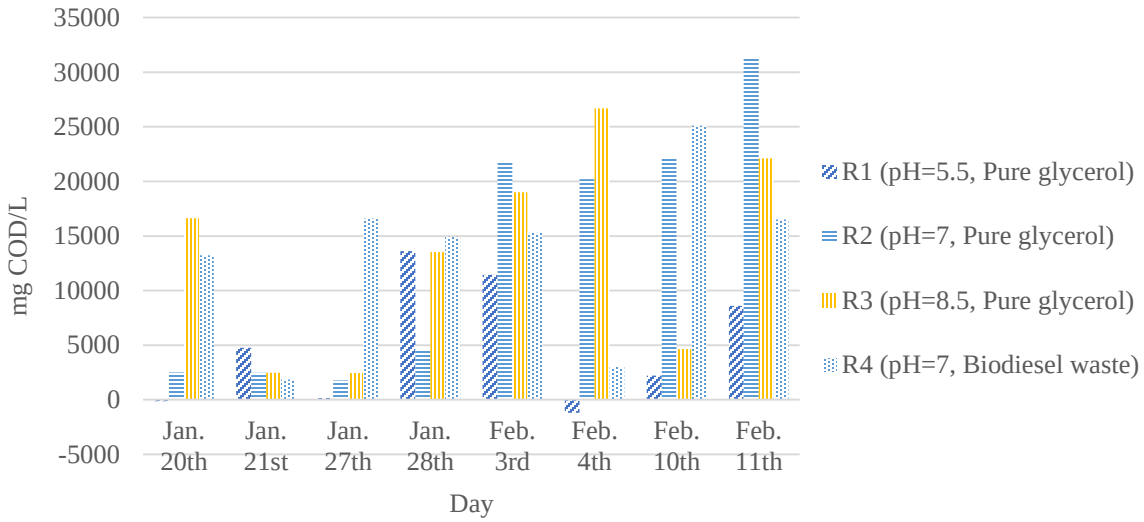

Figure 4-2: VFA production in 4 reactors vs. the pH and the external substrate

Comparing the overall performance of Reactor # 2 (dosed with pure glycerol) and Reactor # 4 (dosed with biodiesel waste) revealed that although both reactors had almost the same amount of VFA production on average, propionic acid production in Reactor # 2 was higher than that in Reactor # 4 whereas acetic and butyric acid production in Reactor # 4 surpassed Reactor # 2. The lower propionic acid production was probably due to the low glycerol content (20%) of the biodiesel waste. However, the total COD of the biodiesel waste was 1950 mg/L compared to 1217 mg/L as total COD in Reactor # 2, and apparently a significant portion of this was fermentable COD which could be converted to acetic and butyric acid. Reactor # 2 and Reactor # 3 showed

almost the same amounts of total VFA production indicating that both neutral and basic environments were favorable regarding total VFA production. However, VFA production in a neutral environment mostly occurred in the form of propionic acid. Regarding the VFAs specific production rate, Reactor # 3 and Reactor # 4 were behaved almost similarly with 60.95 mg COD/gVSS/hr and 60.72 mg COD/gVSS/hr respectively followed by Reactor # 2 and Reactor # 1 with 53.85 mg COD/gVSS/hr and 27.04 mg COD/gVSS/hr. Although the VFAs specific production rate in Reactor # 2 was less than that of Reactor # 3 and Reactor # 4 all three reactors had a close VFAs volumetric production rate (Table 4-5). Solid analysis results' revealed that although all reactors were loaded with the same inflow and were at the same SRT value, the average MLSS and MLVSS in Reactor # 4 was higher than that in other reactors.

Table 4-4: Average VFA production, composition in 4 reactors (VFAs are in the unit of mg COD/L and the HAc/HPr are in the unit of C-mmole/C-mmole).

Reactor	Initial pH	VFAs	HAc	HPr	HBu	HAc/HPr	Percent of VFA HAc:HPr:HBu
R1	5.5	4926 ± 4740	934 ± 2400	2866 ± 1756	1126 ± 882	0.38	19:58:23
R2	7	13350 ± 9824	4912 ± 4975	6834 ± 3580	1604 ± 1555	0.84	37:51:12
R3	8.5	13442 ± 7812	6830 ± 4562	5785 ± 2416	826 ± 1245	1.37	51:43:6
R4	7	13319 ± 6531	6153 ± 4338	4789 ± 1809	2377 ± 1264	1.49	46:36:18

Table 4-5: Average VFAs specific production rate and volumetric rate in 4 reactors.

Reactor	Initial pH	MLSS (mg/L)	MLVSS (mg/L)	VFAs (mg COD/L)	Specific rate (mg COD/gVSS/hr)	Volumetric rate (mg COD/hr)
R1	5.5	12467	10693	4926 ± 4740	27.04 ± 24.9	70.8 ± 49.4
R2	7	13217	10358	13350 ± 9824	53.85 ± 35.9	139.1 ± 102
R3	8.5	12725	10333	13442 ± 7812	60.95 ± 45.6	140.0 ± 81.4
R4	7	14925	12533	13319 ± 6531	60.72 ± 38.17	138.7 ± 68

4.3 Conclusion

The most consistent result for both the serum bottle experiments and the reactor experiments was that an initial acid pH of 5.5 resulted in very significant inhibition of VFA production. In the

reactor experiments the degree of inhibition was greatest for acetic acid, then propionic acid and least for butyric acid which had higher production for the pH 5.5 reactor than the pH 8.5 reactor. However, total VFA production was virtually the same in all reactors except the pH 5.5 reactor (R1) which produced less than 37% of the VFAs produced by the next lowest reactor (i. e. R4, the biodiesel waste reactor). All three of the other reactors (R2, R3, and R4) produced almost identical VFA and quantity mixtures of propionic acid and acetic acid consistent with optimizing EBPR beyond what may be obtained with only acetic acid. Lopez-Vazquez et al. (2009) found EBPR was optimal with mixtures of 1:1 and 75:25 carbon molar ratio acetic acid: propionic acid. Chen et al. (2004) found a 1:1 molar ratio of acetic:propionic acid to be of the greatest benefit. In this study the ratio for R2, R3 and R4 were 0.84, 1.37 and 1.49 C-mmol/C-mmole respectively. Considering the prefermenter effluent will mix with the un-perfermented primary clarifier effluent which tends to be dominated by acetic acid the most beneficial stream would be R2 (pure glycerol, initial pH of 7) effluent which had the highest quantity of propionic acid and a total VFA production equal to R3 and R4. Note, that much of the VFAs and propionic acid originated with the primary solids since 1000 mg/L glycerol can't explain the large quantities of VFA produced. The one major of the results is the impact of butyric acid. Butyric acid has been studied in some brief batch experiments with un-acclimated biomass (Hood and Randall 2001) but to our knowledge had never been studied in long term cultivation. The biodiesel waste produced the most butyric acid, while alkaline pH produced the least butyric acid. Future research should look at the impact of butyric acid on the overall EBPR since this effect could impact the decision of which prefermentation condition is optimal. In the absence of this information R2 (pure glycerol, pH of 7) is the probable optimal condition. Another important aspect of estimating the impact of prefermentation is how much these concentrations increase the VFA: TP ratio when blended with the primary effluent

stream. In this case approximately 100 mg/L VFA will be added to the influent stream which is enough to drive 14 mg/L P removal (in excess of what is needed for a typical domestic wastewater) especially a septic wastewater with atypical influent TP of 6 to 8 mg/L).

4.4 References

Abu-Ghararah, Z. H., and C. W. Randall. "The effect of organic compounds on biological phosphorus removal." *Water Science & Technology* 23.4-6 (1991): 585-594

Bengtsson, Simon, et al. "Acidogenic fermentation of industrial wastewaters: effects of chemostat retention time and pH on volatile fatty acids production." *Biochemical Engineering Journal* 40.3 (2008): 492-499.

Bouzas, A., et al. "Fermentation and elutriation of primary sludge: Effect of SRT on process performance." *Water research* 41.4 (2007): 747-756.

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36

Danesh, Shahnaz, and Jan A. Oleszkiewicz. "Volatile fatty acid production and uptake in biological nutrient removal systems with process separation." *Water environment research* 69.6 (1997): 1106-1111

González-Barceló, O. G., and Simón González-Martínez. "Anaerobic prefermentation and primary sedimentation of wastewater in a sequencing batch reactor." *Water SA* 32.4 (2007): 577-583.

Hood, Cathy R., and Andrew Amis Randall. "A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates." *Water research* 35.11 (2001): 2758-2766.

Lopez-Vazquez, Carlos M., et al. "Modeling the PAO–GAO competition: effects of carbon source, pH and temperature." *Water Research* 43.2 (2009): 450-462.

Wu, Haiyan, et al. "The effect of pH on anaerobic fermentation of primary sludge at room temperature." *Journal of hazardous materials* 172.1 (2009): 196-201.

CHAPTER 5: THE EFFECT OF MIXING ON GLYCEROL/BIODIESEL WASTE FERMENTATION

5.1 Introduction

The enhanced biological phosphorus removal (EBPR) process has been widely used in wastewater treatment over the last few decades. The success of this process depends on the wastewater volatile fatty acids (VFAs) content. Abu-ghararah and Randall (1991) reported that approximately 7 mg VFAs is required to biologically remove 1 mg phosphorus (P) from the wastewater. As wastewater often has an insufficient amount of carbon sources, specifically in the form of VFAs it is crucial to provide the influent with enough VFAs. Prefermentation is a beneficial process able to increase the VFA concentration of the influent. Numerous studies have been done to assess the prefermentation potential of wastewater in terms of VFA production and composition. Gonzalez-Barcelo and Gonzalez-Martinez (2007) evaluated the fermentation efficiency of a sequencing batch system (SBR) treating the entire wastewater stream with an 8 hr cycle and 340 min anaerobic time. They reported that in the organic loading range of 0.27 to 1.30 gCOD/gMLVSS/d more than 70% of the dissolved COD of the prefermenter effluent was in the form of VFAs and the acidification of the dissolved COD of the influent was always higher than 50%. Barajas et al. (2002) observed 27% acidification of the influent dissolved COD in an activated primary tank. Rössle and Pretorius (2001) indicated that 1 to 70 mgVFA/L/hr could be generated as a result of primary solids fermentation in side-stream prefermenters. In order to increase the EBPR efficiency not only the quantity of the VFAs is important but also the VFAs composition must be taken into account. Lopez-Vazquez et al. (2008) reported that EBPR efficiency for a mixture of acetic acid and propionic acid was higher than that of a wastewater containing either acetic acid or propionic acid as the sole carbon source. Chen at al. (2004) observed that the acetic acid:propionic acid ratio (carbon molar ratio) of 1:1 resulted in the

optimum EBPR performance. However, due to high production cost it is not economic to add acetic acid and propionic acid directly to the EBPR process. One way to provide these components is to control the fermentation operational conditions (such as temperature, mixing and pH) to favor greater VFA production and the desired composition. Mixing is one of the parameters which affects the fermentation process. The purpose of mixing is to suspend the particulate matter in the mixture to increase the contact between the particles and the microorganisms. Danesh and Oleszkiewicz (1997) investigated the effect of mixing intensity on primary solids fermentation in bench-scale prefermenters. Experimental results revealed that decreasing the mixing period from 6 hr/cycle to 0.25 hr/cycle enhanced both the volumetric VFA production rate and the specific VFA production rate. Banister and Pretorius (1998) observed that the net VFA yield in fermentation of primary solids in unmixed reactors was higher than that of mixed reactors. On the other hand, fermentation of crude glycerol as the main component of the biodiesel waste showed promising results in VFA production specifically in the form of propionic acid. Zhang and Yang (2009) reported that fermentation of crude glycerol in a fibrous-bed bioreactor at 32°C and pH of 7 resulted in 0.54 to 0.71 gram propionic acid production per gram of crude glycerol in the presence of *P.acidipropionici* bacteria. They observed that propionic acid was the dominant crude glycerol fermentation end-product followed by succinic and acetic acid. Zhu et al. (2010) studied glycerol fermentation in a 7 liter batch reactor cultivated with a pure culture and reported that up to 44.62 g/L propionic acid was produced after 220 hr fermentation time when glycerol (as the sole carbon source) was added at an initial dosage of 30 g/L and fed continuously at a rate of 0.01 L/hr after 72 hr for a duration of 48 hr. With all these in mind, the objective of this research was to investigate the pure glycerol/biodiesel waste potential in VFA production via fermentation process in a mixed

culture side stream reactor and to optimize the mixing intensity to favor the VFA production and composition (e. g. high propionic/acetic acid mix) to the desired level.

5.2 Materials and methods

This research was conducted in two separate phases. In the first phase 5 reactors with liquid volumes of 1500 ml were operated under the same environmental and process conditions with mixing energy as the experimental variable ranging from 0 to 100 rpm. Mixing was provided by U shape plastic blades with 0.31 inch shaft diameter and 13.8 inch shaft length (Cole-Parmer, Vernon Hills, Illinois) connected to Grainger gear motors (Orlando, Florida) at different rpms. The reactor conditions are described in Table 5-1. After running the reactors for 3 weeks to reach the steady state condition sampling was started and conducted twice a week (on two successive days) for 5 weeks. Fresh primary solids was obtained from Glendale Wastewater Treatment Facility (Lakeland, Florida) once a week and stored in a walk-in cooler at 4°C. The solids retention time (SRT) value was set at 4 days. For this reason 375 ml of primary solids from the reactors was replaced with the same amount of fresh primary solids on a daily basis. Reactors were dosed with 1000 mg/L pure glycerol daily.

Table 5-1: Phase I operational conditions of 5 reactors (pH, MLSS and MLVSS are the average of available data points (5 to 8 data points) at the end of each cycle)

	Temp. (°C)	Final pH	MLSS (mg/L)	MLVSS (mg/L)	Mixing (rpm)	External Substrate	Substrate dosage (mg COD/L)	SRT (days)
R1	22	4.29	19774	15700	0	Pure glycerol	1217	4
R2	22	4.38	21850	17350	7	Pure glycerol	1217	4
R3	22	4.42	19892	16133	30	Pure glycerol	1217	4
R4	22	4.42	19281	15283	50	Pure glycerol	1217	4
R5	22	4.41	18975	15943	100	Pure glycerol	1217	4

In the second phase of the current research the effect of mixing on fermentation process was studied in a narrower mixing range (0 and 7 rpm) by running 5 identical reactors, 2 of them were dosed with pure glycerol, 2 of them dosed with biodiesel waste, and 1 reactor working as a control with no glycerol addition. The test was run at room temperature (22°C). The pH was not changed during the test and the SRT was adjusted to 4 days. The experimental and operational conditions of phase II are stated in Table 5-2. In order to decrease the primary solids fermentation contribution in VFAs production and evaluate the external substrate (glycerol or biodiesel waste) fermentation end products the substrate's initial concentration was increased to 4333 mg/L (6500 mg per cycle) and the primary solids density was lowered by diluting it with raw influent received from the Iron Bridge Water Reclamation Facility (Oviedo, Florida) at a ratio of 1:1 (V/V). The glycerol concentration of the biodiesel waste was determined by the colorimetric method in the environmental laboratory at the University of Central Florida and the HPLC method at Mid-West Laboratories (Omaha, Nebraska). The glycerol concentration in the biodiesel waste received from SAKAL LLC (Panama, Panama) was lower than what was expected in a typical biodiesel waste which is about 56-60% (Bodik et al. 2009) and was approximately 20% on average. However, the COD analysis showed that the total chemical oxygen demand (TCOD) of biodiesel waste in the current research was 1.95 mg COD/mg biodiesel waste which is greater than that of pure glycerol (1.217 mg COD/mg glycerol).

Table 5-2: Phase II operational conditions of 5 reactors of phase II (pH, MLSS and MLVSS are the average of 8 data points at the end of each cycle).

	Temp. (°C)	Final pH	MLSS (mg/L)	MLVSS (mg/L)	Mixing (rpm)	Substrate	Substrate dosage[1] (mg COD/L)	SRT (days)
R1	22	5.8	9028	7709	0	-	-	4
R2	22	4.4	8145	6579	0	Pure glycerol	5274	4
R3	22	4.7	8650	7125	0	Biodiesel waste	8450	4
R4	22	3.9	7383	5952	7	Pure glycerol	5274	4
R5	22	4.7	9693	8146	7	Biodiesel waste	8450	4

1: 6500 mg external substrate was added to each reactor daily. As the COD of pure glycerol and biodiesel waste were 1.217 mg COD/mg-glycerol and 1.95 mg COD/mg-biodiesel waste and the reactors active volume was 1.5 L, the substrate dosage in the reactors were calculated as:

(6500 mg glycerol) × 1.217 mg COD/mg-glycerol)/1.5 L = 5274 mg COD/L

(6500 biodiesel waste) × (1.95 mg COD/mg-biodiesel waste)/1.5 L = 8450 mg COD/L

5.2.1 Analytical methods

VFA analysis was conducted by Nukul capillary column (30m × 0.25mm I.D. × 0.25µm; Supelco, St. Louis) which was installed on a Shimadzu GC-14A gas chromatograph (Shimadzu, Columbia, Maryland) with a FID detector. The initial column temperature was 110°C and it increased to 190°C by a ramp of 5°C/min and maintained at 190°C for an additional 10 min. Both injector and detector temperature were set at 220°C. The carrier gas was helium and it was provided at linear velocity of 20 cm/min. Samples were first centrifuged and the supernatants were then filtered with a 0.45 µm membrane filters. 1 ml filtered samples were transferred to 1.5 ml GC vials, sealed with aluminum caps and stored frozen until the injection time. Just before the injection time, after samples reached room temperature (22°C) acidification was conducted with 5% formic acid to lower the pH below 3. 1 µL acidified sample was injected automatically by an auto injector AOC-20i (Shimadzu, Columbia, Maryland). Solids analysis (total suspended solids and volatile suspended solids) was conducted in accordance to Standard Method sections 2450 D and E (1995).

COD analysis (total COD and soluble COD) was performed using the high range (0-1500 mg COD/L) colorimeteric COD vials (Lovibond, Sarasota, Florida). Glycerol measurement was done according to a colorimetric method (Bondioli et al. 2005).

5.3 Results and discussion

5.3.1 Phase I

Experimental results revealed that there was an inverse correlation between the mixing energy and VFA production (Figure 5-1). In other words, the lower the mixing energy the higher the VFA production. To calculate the VFA production it was assumed that the reactors achieved steady state conditions. Therefore the VFA production was calculated by subtracting the VFA concentration in fresh primary solids going inside the reactor from the VFA concentration in the reactors at the end of each cycle. Figure 5-1 depicts the VFA production with respect to the mixing intensity. Although outliers were seen in some sampling events, overall the VFA production in Reactor # 1 with 0 rpm and Reactor # 2 with 7 rpm were much higher than that of high intensity mixed reactors. Reactor # 5 with 100 rpm showed the least VFA production consistently. The average VFA production in Reactor # 1 to Reactor # 5 were 8156 mg COD/L, 5692 mg COD/L, 3035 mg COD/L, 2087 mg COD/L and 2232 mg COD/L in order. Since only 1217 mg COD/L pure glycerol was added in Phase I most of the VFAs had to originate from the primary solids.

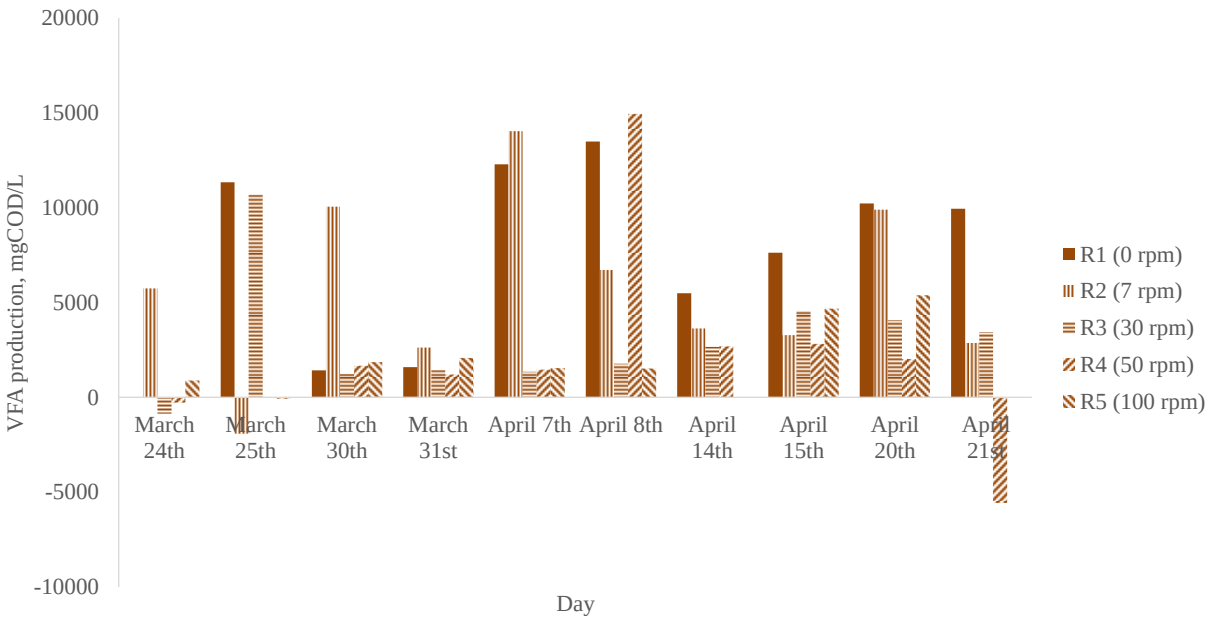

Figure 5-1: VFA production vs mixing energy in R1 (0 rpm), R2 (7 rpm), R3 (30 rpm), R4 (50 rpm) and R5 (100 rpm) in Phase I

Figure 5-2 and Figure 5-3 show the acetic acid and propionic acid production in Phase I. Lowering the mixing energy from 100 rpm to 0 rpm increased the propionic acid production almost 3 times on average. On the other hand, acetic acid production was inconsistent, and in some cases acetic acid consumption was observed. However, the amount was small, implying that acetoclastic methanogenesis was not significant. TCOD data showed little decrease which was consistent with low methanogenesis. Propionic acid was consistently produced and production was much higher at 0 to 7 rpm. VFA composition and the average production of each component are described in Table 5-3.

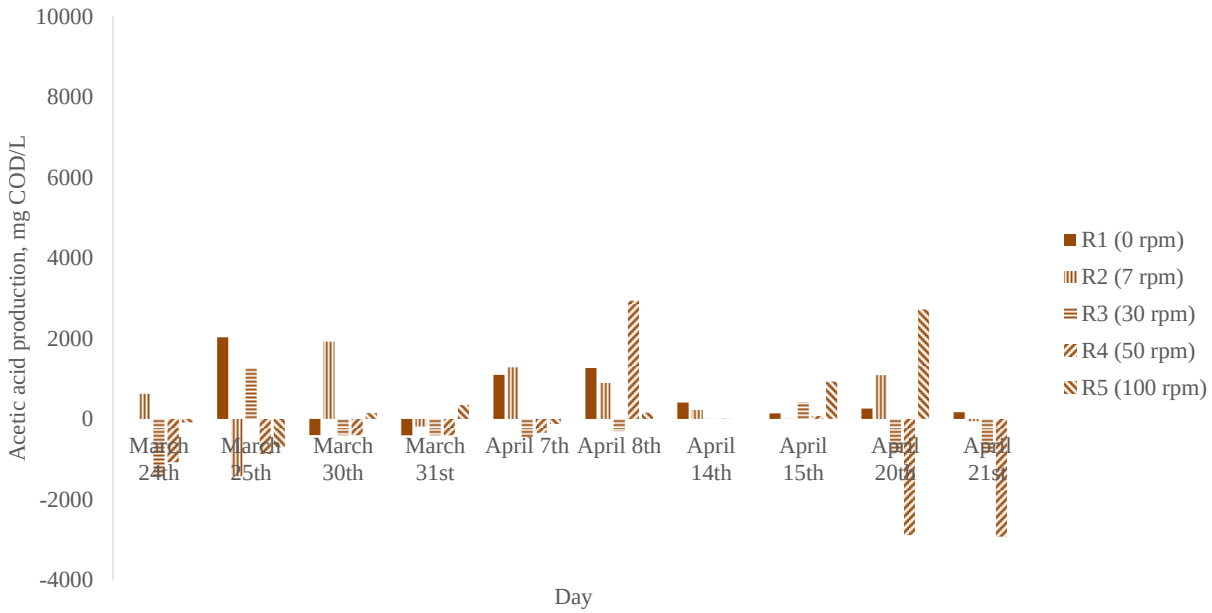

Figure 5-2: Acetic acid production in 5 batch prefermenters dosed with 1000 mg/L pure glycerol and mixed within the range of 0 to 100 rpm with R1 (0rpm), R2 (7rpm), R3 (30 rpm), R4 (50 rpm) and R5 (100 rpm).

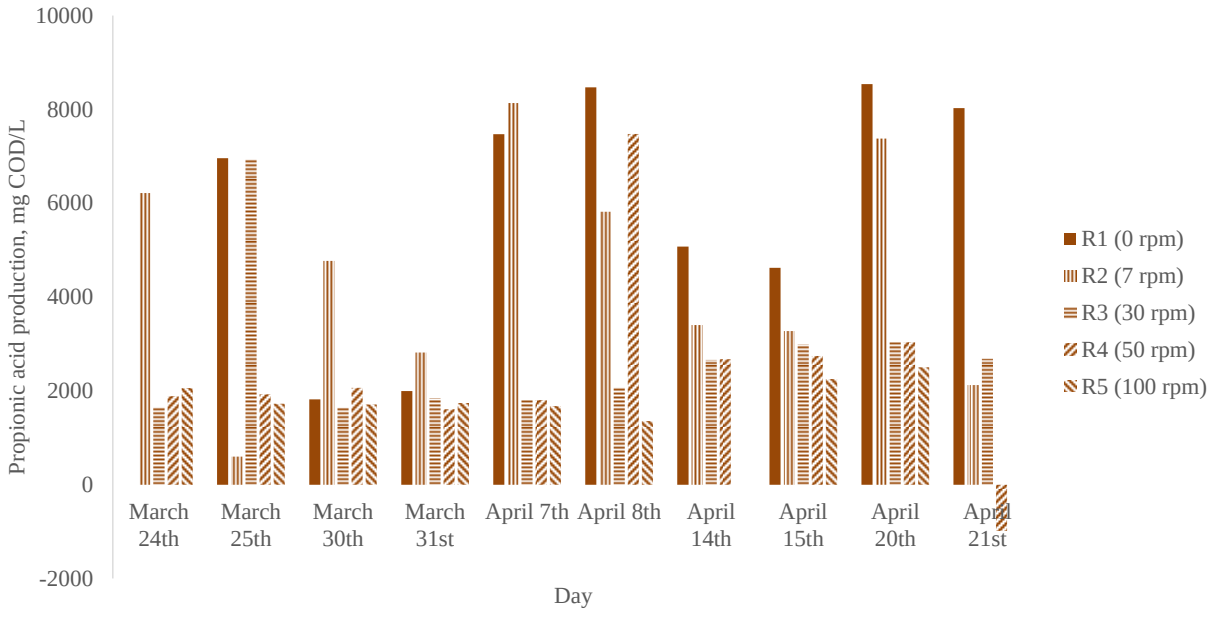

Figure 5-3: Propionic acid production in 5 batch prefermenters dosed with 1000 mg/L pure glycerol and mixed within the range of 0 to 100 rpm with R1 (0rpm), R2 (7rpm), R3 (30 rpm), R4 (50 rpm) and R5 (100 rpm).

Table 5-3: Average acetic acid, propionic acid and butyric acid and VFA production in 5 reactors of Phase I

Reactor	Mixing (rpm)	HAc (mg COD/L)	HPr (mg COD/L)	HBu (mg COD/L)	VFA (mg COD/L)	HAc/HPr (C-mmole/C-mmole)
R1	0	505 ± 621	5888 ± 2032	1762 ± 1178	8156 ± 3427	0.10
R2	7	435 ± 671	4456 ± 1703	802 ± 1332	5692 ± 3310	0.11
R3	30	-300 ± 527	2734 ± 1126	600 ± 795	3035 ± 2233	-
R4	50	-592 ± 1179	2424 ± 1497	255 ± 127	2087 ± 3666	-
R5	100	420 ± 867	1879 ± 308	-68 ± 677	2232± 1553	0.26

Table 5-4 shows the specific production rate of the 5 reactors. Reactor # 1 (0 rpm) had the highest VFAs specific production rate (20 mg COD/gVSS/hr) followed by Reactor # 2 (14 mg COD/gVSS/hr). Although Reactor # 2 had higher MLVSS concentration (17350 gVSS/L) than Reactor # 3 (16133 gVSS/L) and Reactor # 4 (15283 gVSS/L) due to a greater VFA production it showed a higher specific production rate. The volumetric production rate also followed the same trend. Reactor # 4 with the highest mixing energy had the lowest specific and volumetric production rates.

Table 5-4: VFAs specific and volumetric production rate in 5 reactors of Phase I. All reactors were dosed with 1217 mg COD/L pure glycerol

Reactor #	Mixing (rpm)	MLVSS (mg COD/L)[1]	Total VFA (mg COD/L)	Specific rate [2] (mg COD/gVSS/hr)	Volumetric rate (mg COD/L/hr)
R1	0	23236	8156 ± 3427	20 ± 14.7	85 ± 35.7
R2	7	25678	5692 ± 3310	14 ± 7.2	59.3 ± 34.5
R3	30	23877	3035 ± 2233	9 ± 8.8	31.6 ± 23.3
R4	50	22619	2087 ± 3666	5 ± 1.87	21.7 ± 37
R5	100	23596	2232 ± 1553	8 ± 5	23.2 ± 16.2

1: This column was calculated by multiplying the average MLVSS of each reactor (Table 5-1) by the biomass TCOD which was assumed to be 1.48 mg COD/mgVSS.

2: The presented value for R1, R2, R3, R4 and R5 was the average of 4, 7, 5, 3, and 4 for which solids data were available.

COD analysis was also conducted on the samples. As the material inside the reactor was so thick there is a high variability for COD measurements. However, the results showed that there

was a good agreement between the initial and final COD values meaning that methanogenesis was not significant in the reactors. Also solubilization occurred in the reactors. The SCOD/TCOD of the reactors' influent varied between 0.07 and 0.09 and it was increased up to 0.23 at the end of some cycles.

5.3.2 Phase II

Experimental results revealed that adding pure glycerol/biodiesel waste increased the VFA production in the reactors especially in Reactor # 2, Reactor # 3 and Reactor # 5 (Figure 5-4). However, the biodiesel waste-fed reactors had a better performance. Although the glycerol concentration in the biodiesel waste was about 20%, the high VFA production might be related to the other carbon sources such as methanol present in the biodiesel waste (the COD analysis showed that the COD of biodiesel waste was 1.95 mg COD/mg-biodiesel waste which is 60% higher than that of pure glycerol as 1.217 mg COD/mg-glycerol). Figure 5-4 shows the VFA production in the reactors. Reactor # 4 (7 rpm, pure glycerol) had the lowest efficiency with no VFA production in half of sampling events and a slight production in the other half with 681 mg COD/L as the maximum VFA production achieved on June 7th. It should be noted that for the first 4 sampling events (June 29th to July 8th) VFA consumption was observed in Reactor # 1 (no glycerol, 0 rpm, i. e. the control), however, for the last 4 sampling events (July 14th to July 22nd) VFAs were produced slightly in the same reactor. Overall, to be consistent with other reactors the average was taken from 8 data points resulted in an average VFA consumption of -21 mg COD/L which is effectively equal to zero. It was also observed that there was an inverse correlation between mixing energy and the VFA production in the reactors. Regardless of the external substrate, the VFA production in un-mixed reactors were higher than that of mixed reactors. This might be a result of

having more solids and interfaces in unmixed reactors which provided better hydrolysis and solubilization in the system. It also may be that interspecies H_2 transfer (a necessary substrate for propionic acid production but an end-product for acetic acid fermentation) was more efficient in a stratified reactor as well as the fact that glycerol has a 3 carbon chain length like propionic acid. The solid analysis on the samples showed that the average MLSS and MLVSS solid in biodiesel-fed reactors were higher than that of glycerol –fed reactors.

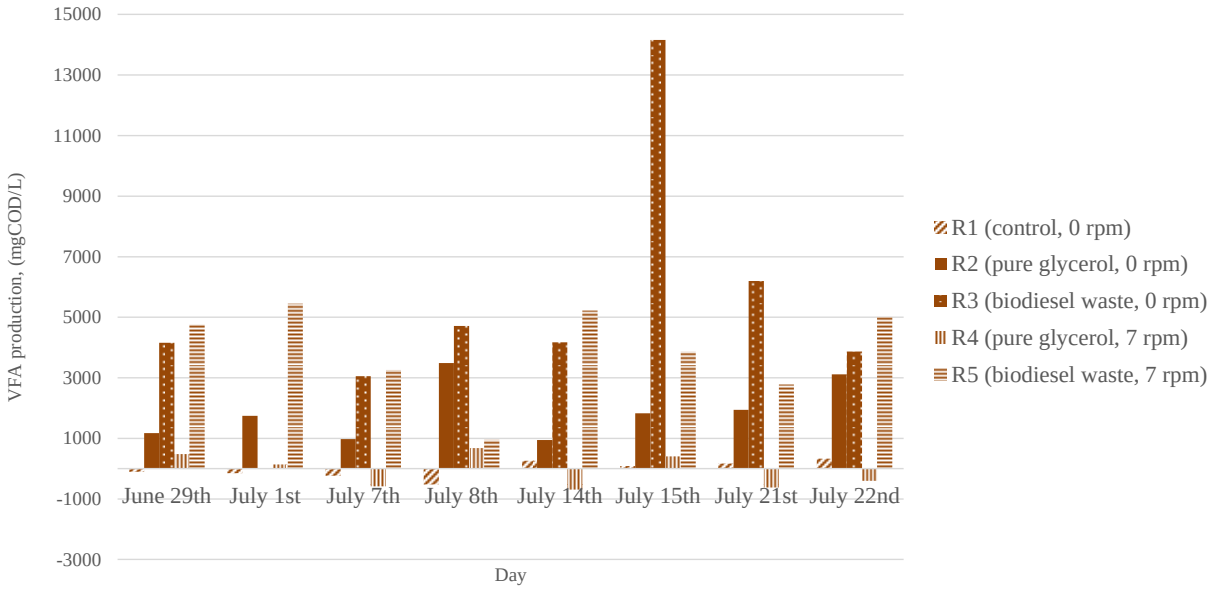

Figure 5-4: VFA production in Reactor # 1 to Reactor # 5 of Phase II

As it is shown in Figure 5-5 and Figure 5-6 VFAs were produced mostly in the form of propionic acid whereas acetic acid production in the reactors was negligible. A slight COD depression at the end of some sampling cycles (24 hr. fermentation) in addition to the acetic acid consumption in some observations suggested that there might be a minor amount of acetoclastic methanogenesis active in the reactors. The average VFA production and composition in the reactors are stated in Table 5-5. Comparing the VFAs specific and volumetric production rate revealed that the biodiesel waste fed-reactors had a considerable higher VFA production rate than

the pure glycerol fed reactors. Regardless of the external substrate increasing the mixing intensity decreased the VFAs production rate (both specific and volumetric production). The maximum VFAs specific and volumetric production rates were observed in Reactor # 3 (0 rpm, dosed with biodiesel waste) which were 31 mg COD/gVSS/hr and 52.5 mg COD/hr respectively.

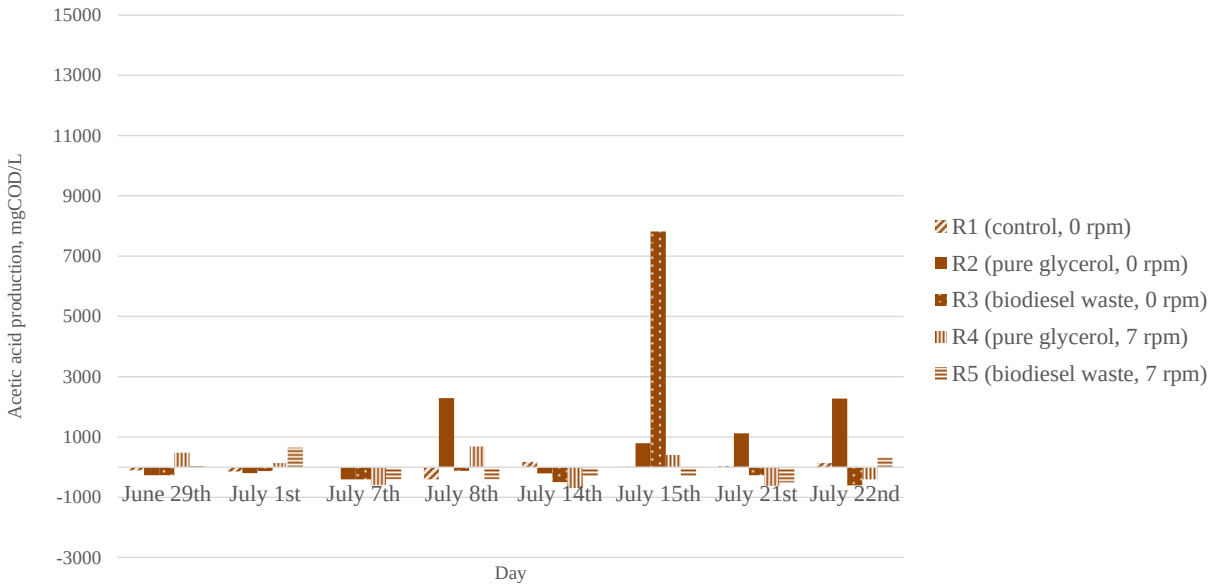

Figure 5-5: Acetic acid production in 5 reactors of Phase II

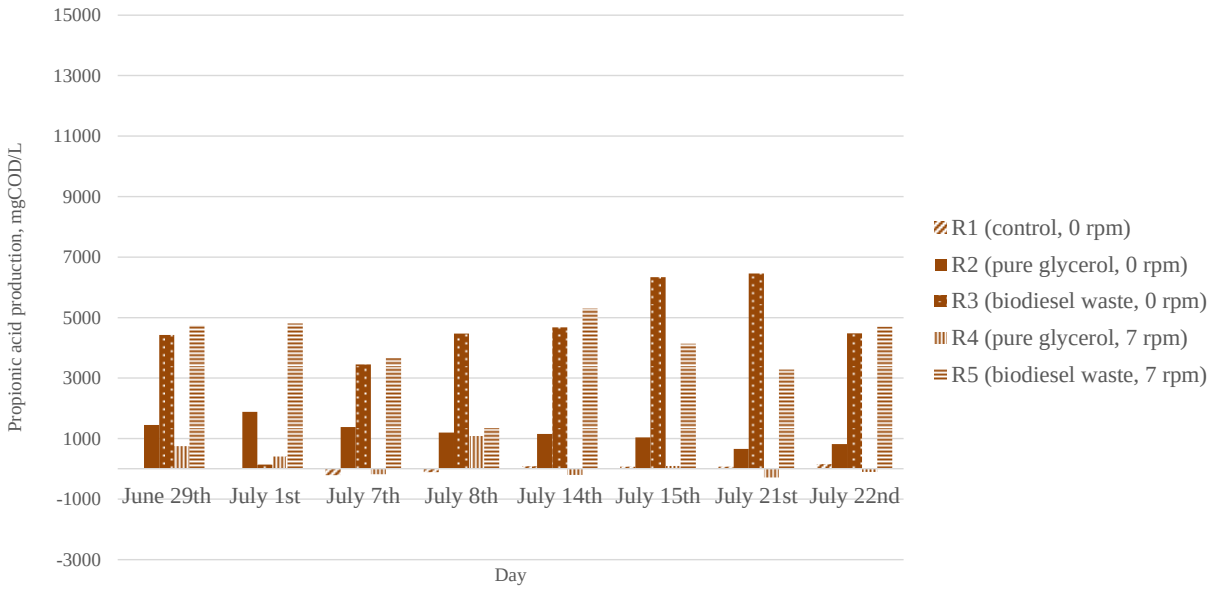

Figure 5-6: Propionic acid production in 5 reactors of Phase II

Table 5-5: Average VFA production and composition in 5 reactors at room temperature and SRT of 4 days (The presented data are the average of 8 data points from June 29th to July 22nd).

	Mixing (rpm)	Substrate	Dosage (mg COD/L)	HAc (mg COD/L)	HPr (mg COD/L)	HBu (mg COD/L)	VFA (mg COD/L)
R1	0	-	-	-42 ± 152	8 ± 99	13 ± 22	-21 ± 238
R2	0	G [1]	5274	675 ± 948	1196 ± 321	31 ± 47	1902 ± 795
R3	0	BDW [2]	8450	690 ± 2413	4304 ± 1644	45 ± 108	5040 ± 3414
R4	7	G	5274	-300 ± 223	197 ± 421	27 ± 63	-76 ± 469
R5	7	BDW	8450	-114 ± 341	4000 ± 1049	28 ± 62	3914 ± 1284

1: G = glycerol
2: BDW = biodiesel waste

Table 5-6: VFAs specific and volumetric production rate

	Mixing (rpm)	Substrate	Dosage (mg COD/L)	MLVSS (mg COD/L)	VFA (mg COD/L)	Specific rate [1] (mg COD/gVSS/hr)	Volumetric rate (mg COD/hr/L)
R1	0	-	-	11409	-	-	-
R2	0	G	5274	9737	1902 ± 795	16 ± 13	19.8 ± 8.3
R3	0	BDW	8450	10545	5040 ± 3414	31 ± 28	52.5 ± 35.6
R4	7	G	5274	8809	-	-	-
R5	7	BDW	8450	12056	3914 ± 1284	24 ± 8	40.8 ± 13.4

1: This column was calculated by multiplying the average MLVSS of each reactor (Table 5-2) by the biomass TCOD which was assumed to be 1.48 mg COD/mgVSS.
2: The presented data for R2, R3 and R5 are the average of 5, 6 and 6 data points.

In the second phase time series analysis was conducted on Reactor # 1 and Reactor # 2. For this reason 3 more sampling events were carried out. The VFA production in Reactor # 1 and Reactor # 2 vs. time is shown in Figure 5-7. As it can be seen in the VFA production in both reactors is consistently increasing (except one on July 8th) over time which might be a result of biomass acclimation.

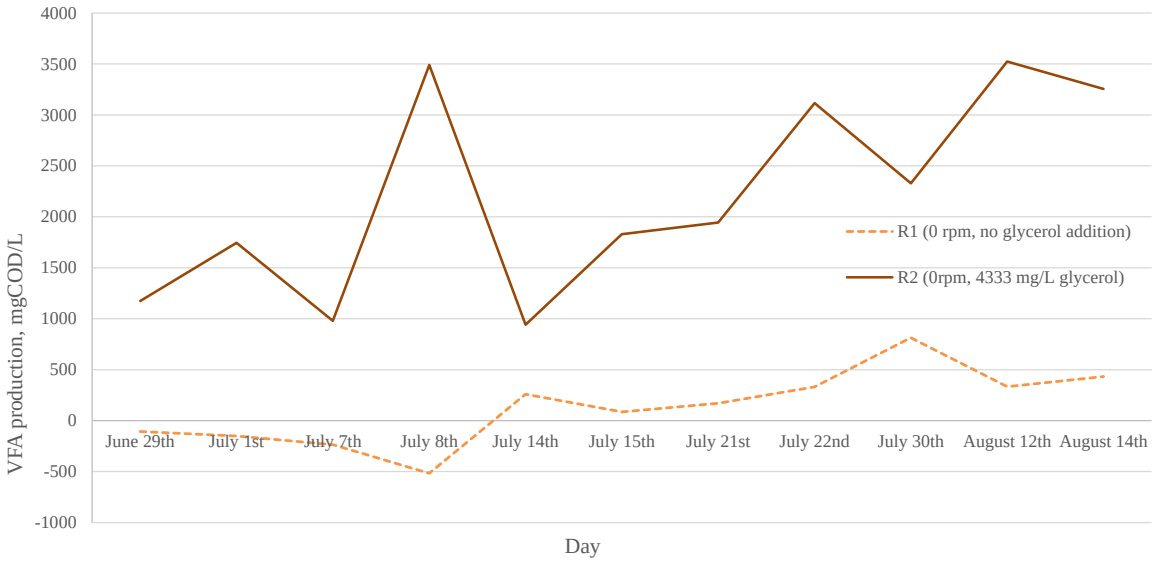

Figure 5-7: VFA production in Reactor # 1 (0 rpm, no glycerol addition) and Reactor # 2 (0rpm, 4333 mg/L glycerol) vs. time

Time series analysis was conducted on Reactor # 1 and Reactor # 2 from 3 sampling cycles (July 30th, August 12th and August 14th). However, as the data had a high variability none of the kinetic models (zero order, first order, and second order reaction models) could fit the data better than the other.

By measuring the glycerol concentration at the end of 6 cycles (July 15th to August 14th) the average VFA production yield with respect to the control reactor (i. e. the effect of glycerol) was calculated as 0.46 mg COD-VFA/mg COD-glycerol with the standard deviation of 0.13. Table 5-7 shows the VFA production, VFA production yield, VFA specific and volumetric production rate from July 14th to August 14th (7 sampling events).

Table 5-7: VFA production, VFA production rate and VFA production in Reactor # 1 and Reactor # 2 (the presented data are the average of 5 to 6 data points from July 14th to August 14th when a positive net VFA production was observed in Reactor # 1).

	Mixing (rpm)	Substrate	VFA (mg COD/L)	specific rate (mg COD/mgVSS-hr)	Volumetric rate (mg COD/L/hr)	yield (mg COD/mg COD)
R1	0	-	347 ± 247	2.1 ± 2.1	3.6 ± 2.6	-
R2	0	G	2419 ± 974	13 ± 6.31	25.2 ± 10.15	0.57 ± 0.13
R2 with respect to control			2073 ± 934	11 ± 5.8	21.6 ± 9.7	0.46 ± 0.14

1: G = pure glycerol

The glycerol analysis showed that almost all the glycerol was consumed after 24 hr fermentation time. Figure 5-8 depicted the glycerol concertation at different times in 3 sampling events.

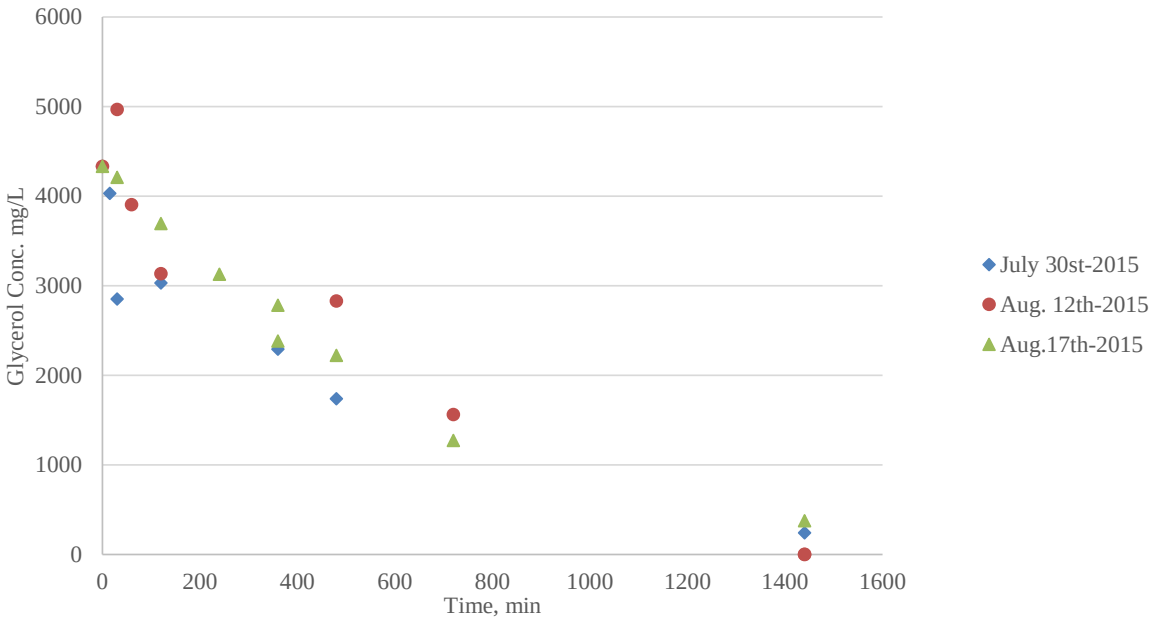

Figure 5-8: glycerol concentration in Reactor # 2 over 24 hr fermentation time

Experimental results revealed that the glycerol consumption rate in Reactor# 2 followed first order reaction kinetics. Equation (1) expresses a first order reaction (i. e. when the rate of reaction is proportional to the concentration of the reactant).

$$[A]_t = [A]_0 e^{-kt} \qquad (5.\ 1)$$

Where:

$[A]_0$ = the glycerol concentration at time 0.

$[A]_t$ = the glycerol concentration at time t.

k = rate constant

The K value changed between 0.0017 min^{-1} and 0.0026 min^{-1} with an average of 0.002 min^{-1}. A sample calculation is shown in Appendix E.

5.4 Conclusions

- Mixing energy had an unfavorable effect on VFA production. In both phases, regardless of the external substrate, higher mixing energy resulted in a lower VFA production.
- In Phase II it was observed that the biodiesel waste fed reactors had a better performance than the pure glycerol fed reactors in terms of VFA production, and VFA production rate and that most of the VFA was still propionic acid. Although the glycerol concentration in biodiesel waste fed reactors is less than that of pure glycerol fed reactors (glycerol constituted 20% of the biodiesel waste) other carbon sources present in biodiesel waste (e. g., methanol, ethanol, etc…) as unseparated biodiesel organics might be involved in VFA production.
- Glycerol analysis at the end of each cycle showed that more than 90% of initial glycerol was consumed over the fermentation cycle (except in Reactor # 2 from July 15th during which only 66% of the glycerol was consumed). The glycerol consumption was considerably higher than the total VFA production in phase II in terms of mg COD/L. This may mean some COD went into other organic end-products and maybe a small amount of reduced gases such as H_2.
- The VFA production yield in Reactor # 2 of phase 2 was measured as 0.46 mg COD-VFA/mg COD-glycerol.
- Comparing the results from Reactor # 1 of phase 1 (0rpm, 1217 mg COD/L pure glycerol, 15700 mg/L MLVSS) with Reactor # 2 of phase II (0 rpm, 5273 mg COD/L pure glycerol, 6579 mg/L MLVSS) revealed that although Reactor # 2 of Phase II was dosed with a significantly higher amount of glycerol it produced less

VFAs than Reactor 1 of phase I (the average VFA in Reactor # 1 of phase 1 and Reactor # 2 of Phase II were 8156 mg COD/L and 1902 mg COD/L respectively). This huge difference was most likely related to the lower influent VSS concentration of Reactor 2 of phase II (9737 mgVSS-COD/L) than that of R1 (23236 mgVSS-COD/L) since VSS solubilization and fermentation was a major part of VFA production along with glycerol.

- The glycerol consumption rate obeyed first order kinetics. The K constant (average of 3 values) was determined as 0.002 min^{-1}.

5.5 References:

Abu-Ghararah, Z. H., and C. W. Randall. "The effect of organic compounds on biological phosphorus removal." *Water Science & Technology* 23.4-6 (1991): 585-594.

Banister, S. S., and W. A. Pretorius. "Optimisation of primary sludge acidogenic fermentation for biological nutrient removal." *WATER S. A.* 24.1 (1998): 35-42

Barajas, María Guadalupe, Antoni Escalas, and Rafael Mujeriego. "Fermentation of a low VFA wastewater in an activated primary tank." *Water SA* 28.1 (2002): 89-98.

Bodík, I., et al. "Biodiesel waste as source of organic carbon for municipal WWTP denitrification." *Bioresource technology* 100.8 (2009): 2452-2456.

Bondioli, Paolo, and Laura Della Bella. "An alternative spectrophotometric method for the determination of free glycerol in biodiesel." *European journal of lipid science and technology* 107.3 (2005): 153-157.

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36.

Danesh, Shahnaz, and Jan A. Oleszkiewicz. "Volatile fatty acid production and uptake in biological nutrient removal systems with process separation." *Water environment research* 69.6 (1997): 1106-1111.

González-Barceló, O. G., and Simón González-Martínez. "Anaerobic prefermentation and primary sedimentation of wastewater in a sequencing batch reactor." *Water SA* 32.4 (2007): 577-583.

Lopez-Vazquez, Carlos M., et al. "Modeling the PAO–GAO competition: effects of carbon source, pH and temperature." *Water Research* 43.2 (2009): 450-462.

Rossle, W. H., and W. A. Pretorius. "A review of characterisation requirements for in-line prefermenters: Paper 1: Wastewater characterisation." *Water SA* 27.3 (2001): 405-412.

Zhang, An, and Shang-Tian Yang. "Propionic acid production from glycerol by metabolically engineered Propionibacterium acidipropionici." *Process Biochemistry* 44.12 (2009): 1346-1351.

Zhu, Yunfeng, et al. "Optimization and scale-up of propionic acid production by propionic acid-tolerant Propionibacterium acidipropionici with glycerol as the carbon source." *Bioresource technology* 101.22 (2010): 8902-8906.

CHAPTER 6: THE EFFECT OF GLYCEROL ADDITION ON EBPR

6.1 Introduction

Enhanced biological phosphorus removal (EBPR) is an efficient engineered biological process to remove excess phosphorus (P) from wastewater. The conventional EBPR process consists of a sequence of anaerobic and aerobic process. In the anaerobic zone poly-P accumulating organisms (PAOs), which are the most effective microorganisms in P removal, consume the readily biodegradable organic carbons (namely volatile fatty acids or VFAs) as the carbon substrate and store them as polyhydroxyalkanoates (PHAs). VFA uptake by PAOs in the anaerobic zone is accompanied with glycogen consumption and intracellular poly-P degradation as the source of energy and reducing agents. This results in a bulk P concentration increase. In the successive aerobic zone, PAOs in the presence of oxygen oxidize the PHAs to provide the required energy for growth and maintenance. PHA degradation in the aerobic condition is accompanied with glycogen and intracellular poly-P replenishment which results in bulk P concentration depletion. Since the aerobic P uptake is higher than anaerobic P release, a net P removal occurs in the system and P is removed by P enriched sludge. In activated sludge systems there is a fraction of PAOs, called denitrifying PAOs, which can use nitrate as the electron acceptor and are able to remove P through the anaerobic/anoxic sequence (Ng et al., 2001). However, due to the competition between the denitrifying PAOs and other denitrifying bacteria for the limited substrate in many cases a net P release is observed in the anoxic zone (Barker and Dold, 1996). The success of EBPR depends on both quantity and composition of VFAs. As acetic acid and propionic acid are the most common VFAs available in domestic wastewater numerous studies have been conducted to evaluate the potential of these carbon sources for EBPR (Smolders et al., 1994a; Chen et al., 2004; Oehmen et al., 2005; Lopez-Vasquez, 2009). Experimental results revealed that

although both acetic acid and propionic acid were appropriate carbon sources for P removal, the mixture of these two carbon sources at a specific ratio improved the EBPR efficiency considerably (Chen et al., 2004, Lopez-Vasquez, 2009). However, it is not economic to directly add these components to the wastewater. This brought up the idea of using other carbon sources which can be fermented to acetic acid and propionic acid.

Crude glycerol is the main by-product of biodiesel production. Typical biodiesel waste mixtures contains 56% to 60% crude glycerol (Bodik et al., 2009). As biodiesel has been produced increasingly over the last decade it is crucial to be able to manage the waste disposal environmentally or find an innovative eco-friendly application for the waste. Using the crude glycerol in activated sludge systems as an external substrate is a promising application that not only can solve the waste disposal complications but also can offset the biodiesel production cost to some extent (Da Silva et al. 2009). Several studies have been conducted on glycerol fermentation composition and introduced propionic acid as a major fermentation end product (Barbirato et al 1997, Himmi et al. 2000; Zhang et al. 2009, Yuan et al. 2010). Yuan et al (2010) investigated the potential of using the pure glycerol in an EBPR process through a sequencing batch reactor (SBR) which was seeded with the acetate-utilizing PAOs (mixed culture) treating a synthetic wastewater and observed only 30% of P removal from the influent. However, they reported that adding a prefermenter to the system increased the P removal up to 100% since the glycerol were first converted to the VFAs and then were available to be consumed by PAOs. Guerrero et al. (2012) reported that in an SBR system which was working based on EBPR process conditions (e. g. anaerobic/aerobic sequences) adding the glycerol could resulted in an efficient EBPR if the system had a sufficient anaerobic time (4.5 hr). They hypothesized that glycerol could not directly be consumed by PAOs and it first needs to be converted to consumable carbon sources

(essentially acetic and/or propionic acid). Coats et al (2015) observed that in a SBR worked based on the EBPR process, adding crude glycerol as an external substrate directly to the anaerobic zone resulted in a less anaerobic P release than that of VFA-dosed SBR. The P release per unit substrate in the glycerol-dosed SBR and the VFA-dosed SBR were reported as 0.17 P-mole/C-mole and 0.24 P-mole/C-mole respectively. Taya et al. (2015) stated that crude glycerol could be an effective external substrate to drive P removal if it was dosed at a proper concentration as at high concentration long chain fatty acids or LCFA (i. e. myristic acid and palmitic acid) content of crude glycerol might accumulate on the biomass layer and prevent the nutrients transfer to the biomass.

With all these in mind, the main objective of the current research is to study the potential of using pure glycerol in the EBPR process by determining the best location for adding glycerol in a continuous flow activated sludge system treating real wastewater. The study will determine if glycerol should be added to the prefermenter or to the anaerobic zone. For this reason two identical A^2/O systems were under investigation; one dosed with pure glycerol in the anaerobic zone and the other was dosed with the same amount of glycerol but to the prefermenter.

6.2 Materials and methods

Two identical A^2/O system were build using PVC pipes at Iron Bridge Water Reclamation Facility (Oviedo, Florida). The 400 L influent bucket was filled up with raw wastewater on a daily basis. The characteristics of the pilot influent (the preliminary phase and Phase I) are shown in Table 6-1 and Table 6-2.

Table 6-1: Average influent (pilot reservoir wastewater) characteristics of the preliminary phase (the presented values are the average of 10 data points from May 21st to July 29th).

Parameter	pH	TSS (mg/L)	VSS/TSS	SOP [1] (mg-P/L)	TKN (mg-N/L)	TCOD (mg/L)	SCOD (mg/L)
Average	7.43	92	0.85	5	38.5	317	194
STD deviation	0.1	56	-	0.7	5.31	117	28

1: For SOP the presented value is the average of 5 data points

Table 6-2: Average influent (pilot reservoir wastewater) characteristics of the phase I (the presented values are the average of 9 data points from August 5th to September 10th)

	pH	VFA (mg COD/L)	TSS (mg/L)	VSS/TSS	TKN [1] (mg-N/L)	TP (mg-P/L)	SOP (mg-P/L)	TCOD (mg/L)	SCOD (mg/L)
Ave.	7.5	23	72	0.85	42.3	5.2	3.7	252	155
STD	0.1	24	23	-	4.7	1.4	1.2	58	36

1: For TKN the presented value is the average of 8 data points.

The influent was conveyed to the anaerobic zones using peristaltic pumps (CO 7553-70; Cole-Parmer, Vernon Hills) with variable speed controllers coupled to a programmable timer. For the first 2 months (preliminary phase) no prefermenter was employed for both systems which included anaerobic, anoxic, and aerobic zones followed by a secondary clarifier. After the systems reached steady state conditions side stream primary prefermenters were linked to the anaerobic zone. The VFA enriched outflow from the prefermenter was discharged to the anaerobic zone using peristaltic pumps at the flowrate of 83.3 ml/min for a duration of one minute each hour which resulted in a flowrate of 2 L/d. The prefermenters had a liquid volume of 10 liters. 2 L of primary solids was put into the prefermenter manually each day. Effluent flow to the anaerobic zone was by gravity and equaled the prefermenter influent flow. Mixing in the prefermenters was at 50 rpm. When glycerol flow was added it had a 7000 mg/L concentration which flowed into the prefermenter in Pilot A and into the anaerobic zone of Pilot B. The glycerol pump operated at a

flow rate of 20.83 ml/min for a duration of 1 min each hour resulting in a total influent flow of 0.5 L/d (i. e. a glycerol mass of 3500 mg per day). The effective increase in the influent glycerol concentration was 13.5 mg/L for an influent flow of 52 L/d. Prefermenters were fed with fresh primary solids received once a week from the Glendale Wastewater Treatment Plant (Lakeland, Florida). The solids were removed to maintain an SRT of 5 days. Return activated sludge (RAS) was returned from the secondary clarifier to the anaerobic zone and nitrate recycle returned nitrate from the aerobic zone to the preceding anoxic zone. The returns were made of 3/8 inch MasterFlex tubes through peristaltic pumps (CO 7553-70; Cole-Parmer, Vernon Hills) with variable speed controllers. The aerobic zone was equipped with air diffusers to provide aeration and keep the mixed liquor suspended. A wooden topless box was built around the whole system using wood painted with water based paint to provide protection and water resistant (in case of spills) . Figure 6-1 depicts the schematic diagram of the A^2/O system in the current study. The average influent, effluent and recycles, and SRT are shown before (Table 6-3) and after (Table 6-4) linking the prefermenters. The active volume of all reactors are stated in Table 6-5.

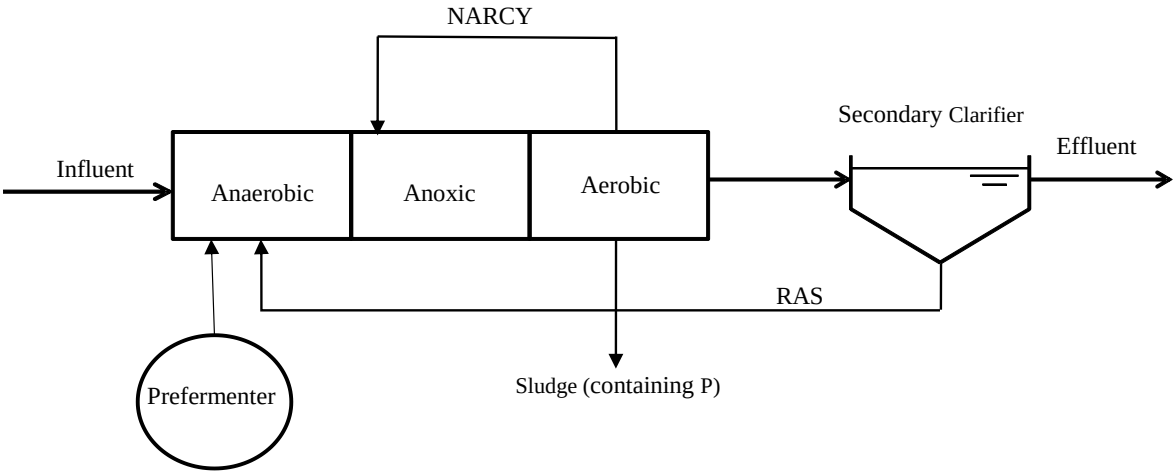

Figure 6-1: Schematic diagram of the A^2/O system employed in the current study

Table 6-3: Average flow rate of recycles, influent and effluent and SRT of Train (A) and Train (B) before linking the prefermenters. Presented values are the average of 10 data points from May 21th to June 29th.

	Influent (L/d)	NARCY (L/d)	RAS (L/d)	Effluent (L/d)	WAS (L/d)	SRT (1/d)
Flowrate	50	98.6	37.4	47.3	2.7	9.5

Table 6-4: Average flow rate of recycles, influent and effluent and SRT of Train (A) and Train (B) after linking the prefermenters. Presented values are the average of 10 data points from August 5th to September 10th.

	Influent (L/d)	Prefermenter (L/d)	NARCY (L/d)	RAS (L/d)	Effluent (L/d)	WAS (L/d)
Train (A)	59.8	2	129	37	59.2	2.7
Train (B)	59.8	2	134	38	58.4	2.7

Table 6-5: Volume of each reactor in both trains

Unit process	Anaerobic	Anoxic	Aerobic	2nd clarifier
Volume (L)	3.59	5.90	17.95	3.14

Sampling was done once a week during the first month of operation (June 17th to July 22nd) and increased to two times per week for the rest of the operational period (August 5th to September 10th). On-site filtration was conducted on samples to prevent the possible effects of solids hydrolysis on soluble ortho-phosphorus, ammonia, SCOD and VFA content of samples. VFAs analysis was conducted by using the GC method described in Chapter 2. TCOD and SCOD were measured using the high range (0-1500 mg/L) colorimeter COD vials (Lovibond, Sarasota, Florida). The absorbance of the digested samples were measured by Hach spectrophotometer DR5000 at 620 ηm (Hach, Loveland, Colorado). TSS and VSS of the samples were measured in accordance to Standard Method sections 2450 D and E (1995). pH was measured with a handheld

Oakton pH meter (Vernon Hills, Illinois). Phosphorus analysis was conducted as is described in the next sub section.

6.2.1 Phosphorus

Phosphorus in aqueous solutions is present in 3 different common forms: orthophosphate, poly-phosphate, and organic phosphate. Orthophosphates are phosphoric acid salts including PO_4^{3-}, HPO_4^{2-}, H_2PO^{4-}, and H_3PO^4. Polyphosphates are complex compounds composed of two or more phosphorus atoms. Organic phosphate has a low concentration level in domestic wastewater but the concentration of it is considerable in many industrial wastes. In the current research phosphorus analysis was conducted in accordance to the Molybdovanadate method (Standard Method section 4500-P C, 1995) using the total and reactive high range (0 to 100 mg-P/L) Hach reagent sets (Hach, Loveland, Colorado). In this method samples were first filtered with 0.45µm membrane filters. Phosphorus content of each sample was then quantified using molybdate under an acidic environment in which phosphate ions react with ammonium molybdate and form bright yellow complexes. Since for low P level samples this color is not discernible; vanadium is added to form vanadomolybdophosphoric acid to produce a more intense color. The intensity of color is then measured by a Hach spectrophotometer DR 5000 at the wavelength of 420 ηm. To measure the total phosphorus all the available phosphorus forms in the sample should be converted to ortho phosphorus. For this reason, the unfiltered samples were digested to convert all organic and polyphosphate compounds to orthophosphate. The persulfate oxidation method as described in Standard Method (section 4500-P B5, 1995), was used to convert the total P concentration to the orthophosphate form. After completion of persulfate oxidation, the samples were treated using the vanadomolybdophosphoric acid colorimetric method (just like filtered samples) to determine the

total P concentration. This works since following digestion all phosphorus is now present in the form of orthophosphate.

6.3 Results and discussion

6.3.1 VFA concentration

VFA analysis was conducted on the pilot reservoir wastewater from August 5th, 2015 to September 10th, 2015. The average VFA concentration of the pilot reservoir wastewater was 20 mg COD/L (The average was calculated from 8 data points as one of the measurements was an outlier). VFA analysis showed that acetic acid was the only VFA present in the pilot reservoir wastewater. Prefermenters were linked to the system on August 2nd 2015. The VFA analysis on the prefermenters' out-flow showed that there was a considerable amount of VFAs produced in both prefermenters, however, the prefermenter of Train (A), which was loaded with pure glycerol at the dosage of 3500 mg/d (i. e. 4260 mg COD/d) produced more VFAs than the prefermenter of Train (B). The average VFA concentrations in prefermenter (A) and prefermenter (B) were 1476 mg COD/L and 508 mg COD/L respectively. Figure 6-2 depicted the VFA concentration in the influent, prefermenter (A) and prefermenter (B). It should be mentioned that 2 data points from each train was ignored as an outlier. Experimental results revealed that acetic acid was the dominant VFA followed propionic acid. No butyric acid production was observed except 1 data point from August 10th in Train (A). Figure 6-3 and Figure 6-4 depicted the VFA production and composition in both trains.

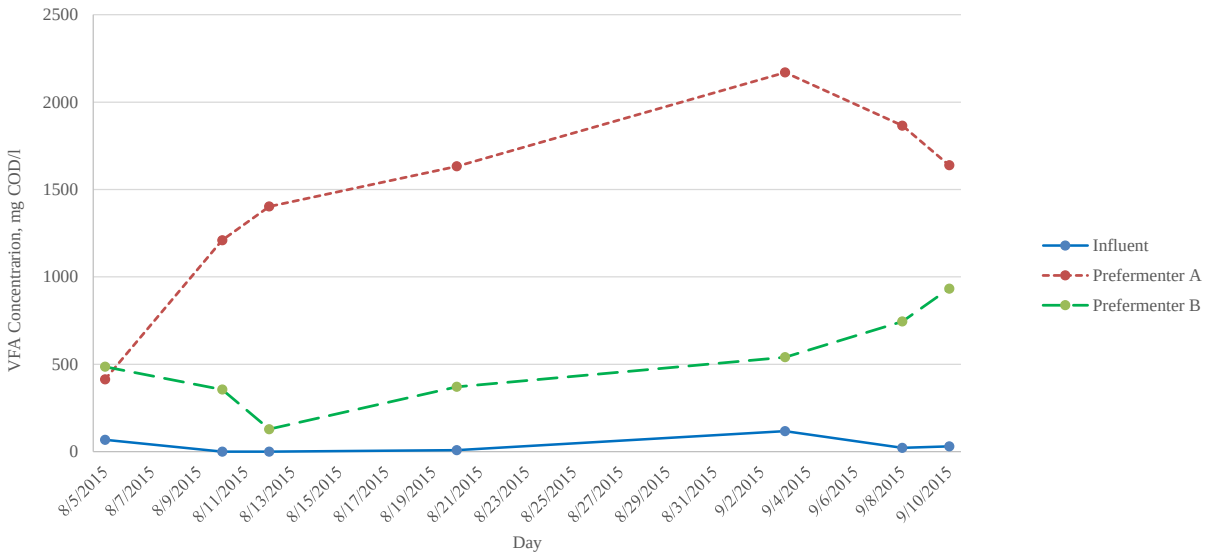

Figure 6-2: VFA concentration of the pilot reservoir wastewater, prefermenter (A) and prefermenter (B).

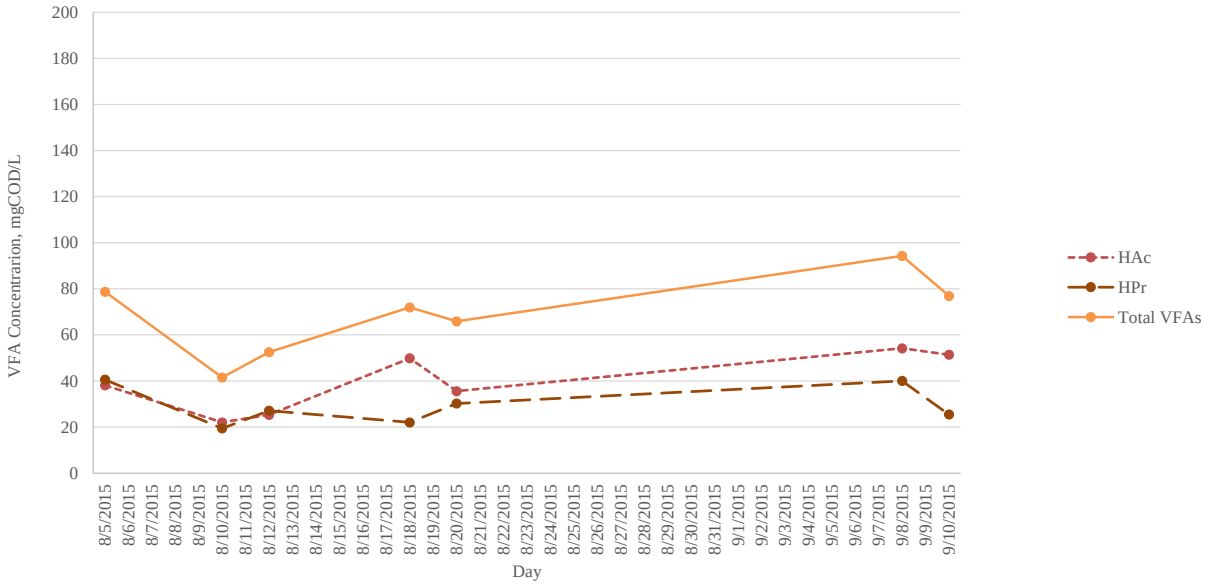

Figure 6-3: VFAs concentration and composition of the combined influent in Train (A)

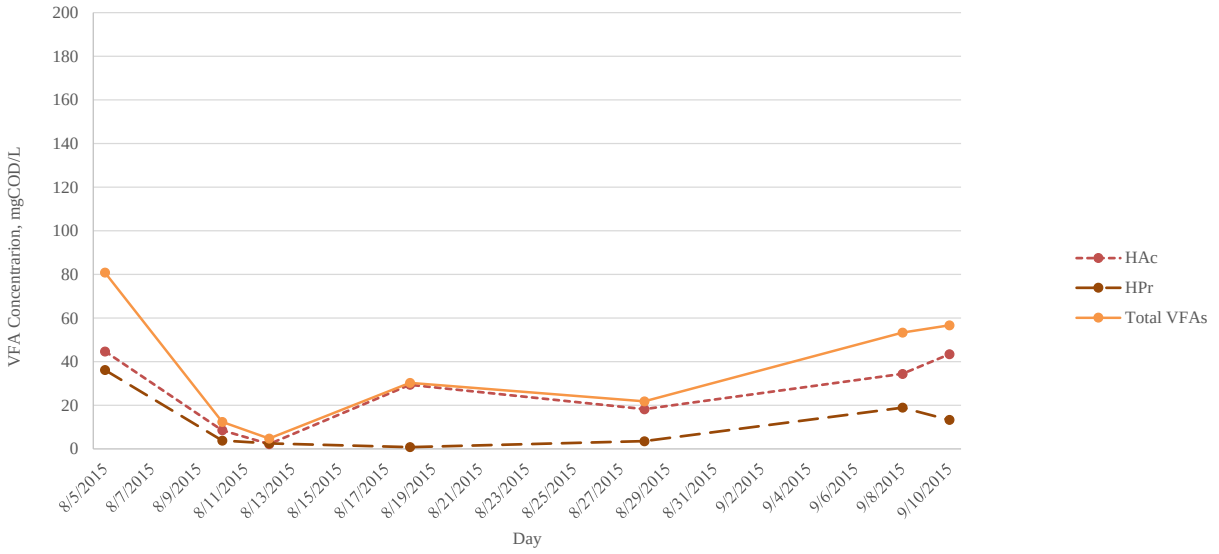

Figure 6-4: VFAs concentration and composition of the combined influent in Train (B)

The combined influent (pilot reservoir wastewater combined with the prefermenters' outflow) characteristics of Train (A) and Train (B) are summarized in Table 6-6. As is shown in Table 6-6 the average VFA concentration in Train (A) is 85% higher than that of Train (B). It should be noted that as the 3500 mg/d glycerol was added to the anaerobic zone of Train (B) the TCOD and SCOD concentration in the anaerobic reactor of Train (B) was about 82 mg COD/L higher than the number presented in Table 6-6 (assuming the total influent flowrate of 52 L/d). As it can be seen in Table 6-6 combined influent characteristics in both trains were almost the same except the VFA concentration which proves that they were working identically and the VFA concentration due to glycerol addition in Train (A) prefermenter is the only variable between the two trains.

Table 6-6: Combined influent characteristics in Train (A) and Train (B)

	Train (A)		Train (B)	
	Average	SD	Average	SD
VFA (mg COD/L)	69	17.5	37	27.4
TCOD (mg/L)	458	54	453*	52
SCOD (mg/L)	211	22	179*	32
TSS (mg/L)	191	44	216	48
VSS/TSS	0.84	-	0.83	-
TP (mg-P/L)	6.4	1.2	6.4	1.1
SOP (mg-P/L)	4.2	1.1	4.3	1.1

*: Addition of glycerol increases these numbers by 82 mg COD/L.

6.3.2 EBPR

Phosphorus analysis showed that the SOP concentration in both trains were almost the same before linking the prefermenters but after August 2nd when the prefermenters were activated Train (B) showed a higher SOP concentration in the anaerobic zone (Figure 6-5). The average anaerobic SOP concentration before linking the prefermenters was 13 mg-P/L in both trains, however after linking the prefermenters it increased to 15 mg-P/L in Train (A) and 20.5 mg-P/L in Train (B). With respect to the aerobic SOP concentration, the bulk SOP concentration in the aerobic zones was slightly lower than the influent before employing the prefermenters. However, linking the prefermenters increased the EBPR function. As it is shown in Figure 6-6 after linking the prefermenters, SOP concentration in the aerobic zone was dropped to 0.4 mg-P/L (SD = 0.3) in Train (A) and 0.6 mg-P/L (SD = 0.2) in Train (B) in average (the averages were taken from August 10th to September 10th). This indicates that adding the glycerol to the prefermenter maybe more

favorable for EBPR than adding the glycerol directly to the anaerobic zone. The average P removal in Train (A) and Train (B) were 93.3% and 90.75% respectively.

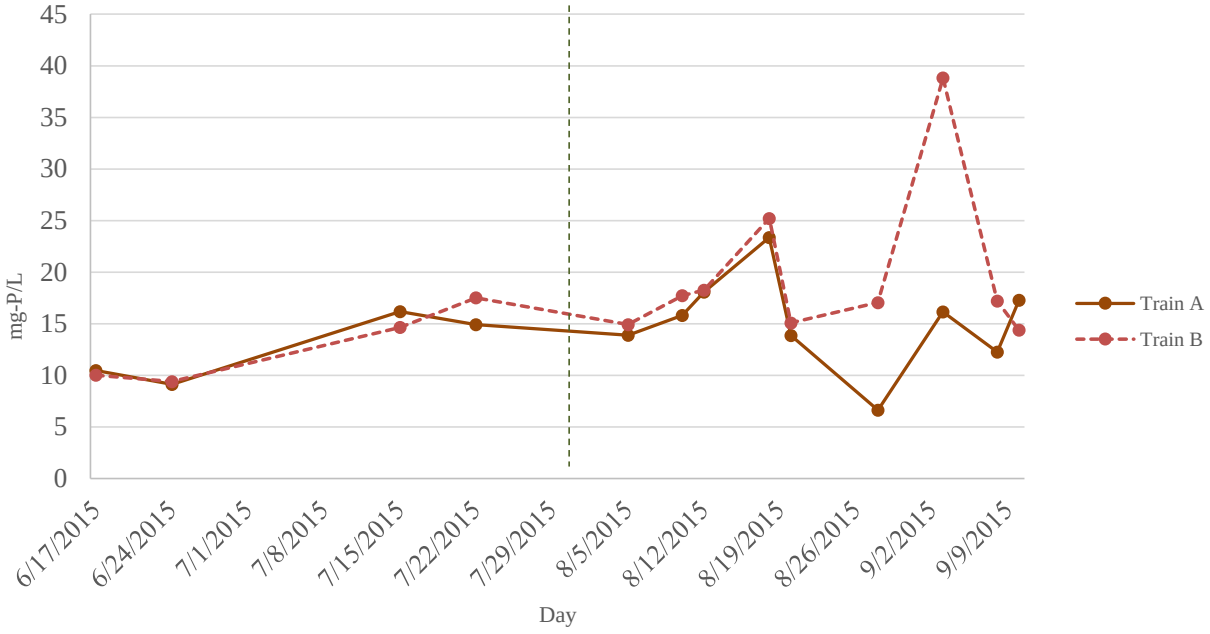

Figure 6-5: Anaerobic soluble ortho-P concentration in Train (A) and Train (B). Prefermenters were connected on August 2nd.

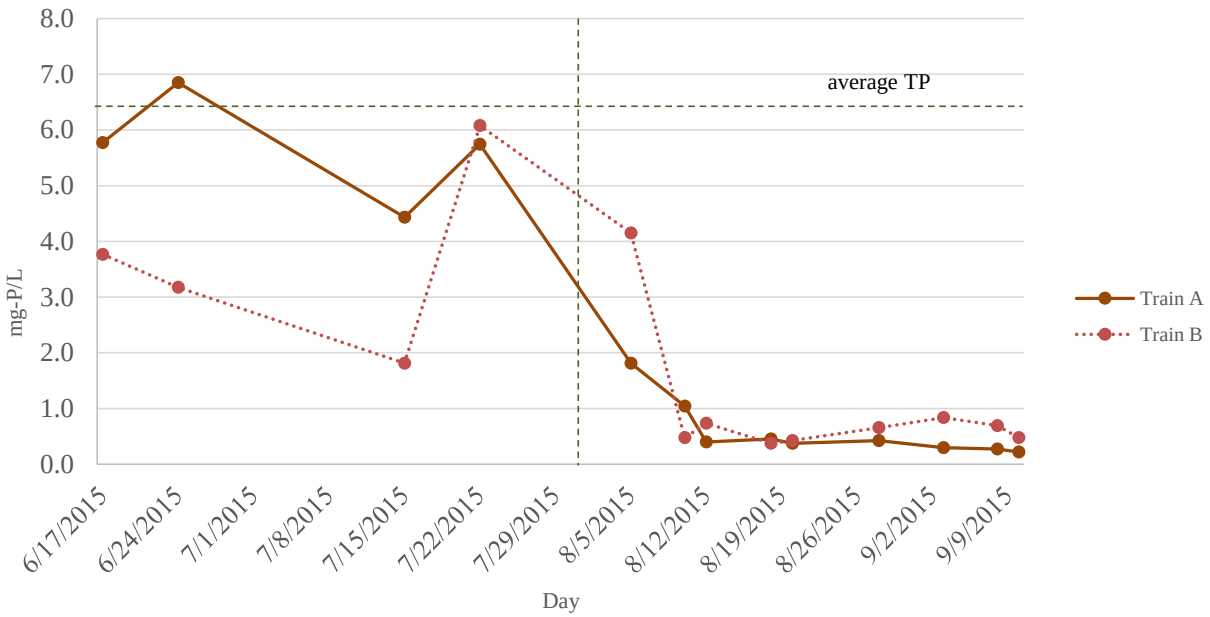

Figure 6-6: Aerobic soluble ortho-P concentration in Train (A) and Train (B). Prefermenters were connected on August 2nd

The average aerobic P uptake to anaerobic P release after linking the prefermenters in Train (A) and Train (B) were 0.84 and 1.02 respectively. However, by including the anoxic P uptake these ratios were increased to 1.17 in Train (A) and 1.15 in Train (B). As is shown in Table 6-7 before linking the prefermenters there was a net P uptake in the anoxic zone of Train (A). On the other hand, in Train (B) in half of the sampling events a net P uptake was observed in the anoxic zone whereas in the other half a net P release was seen in the same reactor. However, after linking the prefermenters there was a net P release in the anoxic zone of both trains. In order to investigate the VFAs availability for PAOs in the anaerobic zone further analysis was conducted on the nitrate concentration of RAS recycle. Experimental results showed that nitrate concentration of RAS recycles of both trains were almost similar and even at the highest values observed in the study the RAS nitrate concentration was not a significant variable.

Table 6-7: The average SOP change over the anaerobic, anoxic and aerobic reactors before and after linking the prefermenters. The presented numbers are mass balance data normalized to the influent flowrate.

	Train	ΔP An (mg-P/L)	ΔP AX (mg-P/L)	ΔP AE (mg-P/L)	ΔP AE /ΔP AN (mg-P/mg-P)	ΔP uptake/ΔP release
Before linking the prefermenters	(A)	+13.26	- 5.14 [1]	-7.50	0.51	0.89
	(B)	+13.79	1.74	-17.78	1.41	1.16
After linking the prefermenters	(A)	22.07	- 6.35	-18.84	0.84	1.17
	(B)	+27.74	-5.6	-25.90	1.02	1.15

1: Negative sign shows the P uptake and positive sign shows the P release

In EBPR systems phosphorus is mostly removed by the P enriched sludge. The poly-P organisms active mass has a P concentration of 0.38 mg-P/mg-VSS whereas the P concentration in other MLVSS groups (poly-P organism endogenous mass, usual organism active mass, usual organism endogenous mass, and non-biodegradable particulate COD or inert mass) is approximately 0.03 mg-P/mg-VSS. However, by calculating the P content of the WAS in the current study it was revealed that this fraction was much higher than 0.03 mg-P/mg-VSS (i. e. 3%). Figure 6-7 shows the P content of the WAS reached 10% in Train (B) and 8% in Train (A). Despite the lower aerobic SOP concentration (i.e. more phosphorus was removed from Train (A) by the biomass) it had a lower P content than Train (B). The reason for that was the higher mass flux (in terms of mgVSS/d) of Train (A) than that of Train (B). The average mass flux in Train (A) and Train (B) leaving the system were 7712 mgVSS/d (standard deviation of 1200 mgVSS/d) and 5963 mgVSS/d (standard deviation of 2089 mgVSS/L) respectively. Table 6-8 summarizes EBPR performance characteristics of both trains.

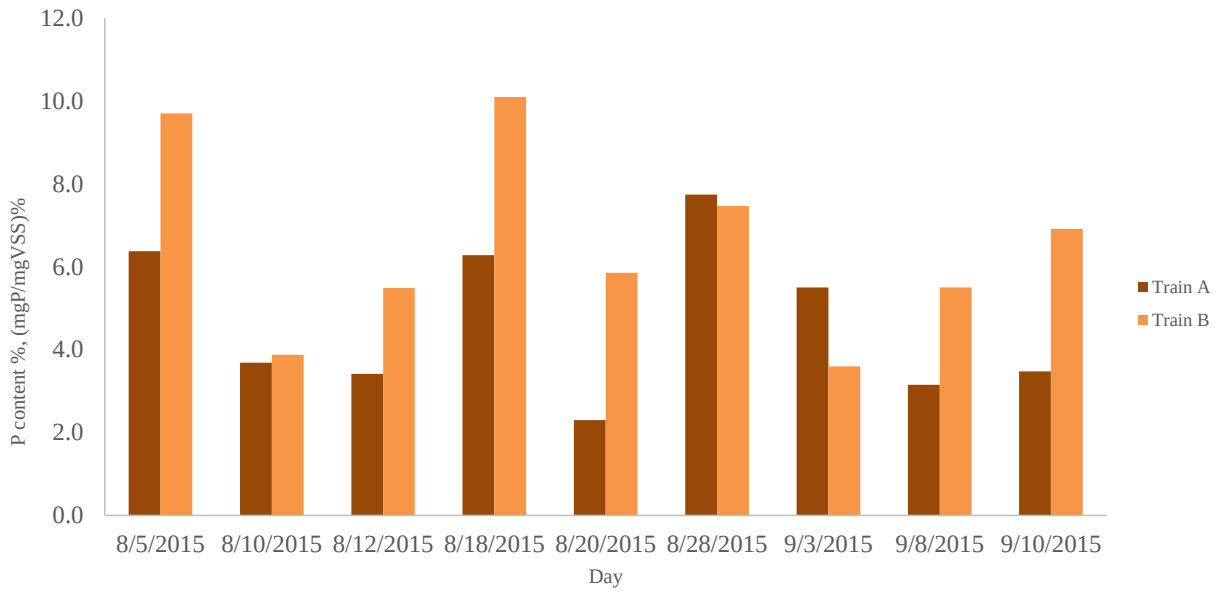

Figure 6-7: P content of the biomass leaving the system in Train (A) and Train (B)

The average effluent TCOD in both trains were equal at 33 mg COD/L. TSS concentration in the effluent of both systems were close at 6 mg/L in Train (A) and 4 mg/L in Train (B).

Table 6-8: EBPR performance characteristics in Train (A) and (B)

	Aerobic P release/ anaerobic P uptake	Overall Prelease/Puptake	SRT (1/d)	TSS_{Eff} (mg/L)	$TCOD_{Eff}$ (mg/L)
Train (A)	0.84	1.17	9.1	6	33
Train (B)	1.02	1.15	9.9	6	33

6.4 Conclusion

Before linking the prefermenters the total effluent P concentration was slightly less than that of the influent indicating that P was only removed by normal assimilation from the system. The probable reason for that might be the insufficient amount of VFA present in the system. After connecting the side stream prefermenters to the anaerobic zone the P removal efficiency increased to 93.3% in Train (A) and 90.75% in Train (B) in average. VFA analysis on prefermenter (A) and

prefermenter (B) showed that both propionic acid and acetic acid were produced although the VFA concentration in prefermenter (A) the one at which 3500 mg/d pure glycerol dosed was 85% higher than that of prefermenter (B). Propionic acid was the dominant VFA in prefermenter (A) whereas in prefermenter (B) acetic acid was the major primary solids fermentation end-product. With respect to anaerobic P release both systems were performing similarly in the first operational phase. However, employing the prefermenters resulted in higher bulk SOP concentration in the anaerobic zone, especially in Train (B). P uptake was performed successfully in the aerobic zone of both trains and anoxic P uptake was also an important part of EBPR in both systems. Overall, both trains showed excellent P removal after linking the prefermenters. Although the effluent SOP in Train (A) was lower than that of Train (B), it is unlikely that this difference was statistically significant. Therefore, it is possible that there may be an advantage to adding glycerol to the prefermenter rather than the anaerobic zone but our data was not conclusive. The definitive answer to this question requires further study, and in particular a full scale or large pilot scale investigation.

6.5 References

Barbirato, F., D. Chedaille, and A. Bories. "Propionic acid fermentation from glycerol: comparison with conventional substrates." *Applied Microbiology and Biotechnology* 47.4 (1997): 441-446

Barker, P. S., and P. L. Dold. "Denitrification behavior in biological excess phosphorus removal activated sludge systems." *Water Research* 30.4 (1996): 769-780.

Bodík, I., et al. "Biodiesel waste as source of organic carbon for municipal WWTP denitrification." *Bioresource technology* 100.8 (2009): 2452-2456.

Chen, Yinguang, Andrew A. Randall, and Terrence McCue. "The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid." *Water Research* 38.1 (2004): 27-36

Coats, Erik R., Zachary T. Dobroth, and Cynthia K. Brinkman. "EBPR Using Crude Glycerol: Assessing Process Resiliency and Exploring Metabolic Anomalies." *Water Environment Research* 87.1 (2015): 68-79.

Da Silva, Gervasio Paulo, Matthias Mack, and Jonas Contiero. "Glycerol: a promising and abundant carbon source for industrial microbiology." *Biotechnology advances* 27.1 (2009): 30-39.

Guerrero, Javier, et al. "Glycerol as a sole carbon source for enhanced biological phosphorus removal." *Water research* 46.9 (2012): 2983-2991.

Himmi, E. H., et al. "Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii." *Applied microbiology and biotechnology* 53.4 (2000): 435-440

Hood, Cathy R., and Andrew Amis Randall. "A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates." *Water research* 35.11 (2001): 2758-2766

Lopez-Vazquez, Carlos M., et al. "Modeling the PAO–GAO competition: effects of carbon source, pH and temperature." *Water Research* 43.2 (2009): 450-462

Ng, W., S. Ong, and J. Hu. "Denitrifying phosphorus removal by anaerobic/anoxic sequencing batch reactor." *Water Science & Technology* 43.3 (2001): 139-146.

Oehmen, Adrian, et al. "Anaerobic metabolism of propionate by polyphosphate- accumulating organisms in enhanced biological phosphorus removal systems." *Biotechnology and bioengineering* 91.1 (2005): 43-53.

Smolders, G. J. F., et al. "Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence." *Biotechnology and Bioengineering* 43.6 (1994): 461-470.

Tayà, Carlota, et al. "Assessment of crude glycerol for Enhanced Biological Phosphorus Removal: Stability and role of long chain fatty acids." *Chemosphere* 141 (2015): 50-56.

Yuan, Q., et al. "Enhancing biological phosphorus removal with glycerol." *Water Science and Technology* 61.7 (2010): 1837-1843.

Zhang, An, and Shang-Tian Yang. "Propionic acid production from glycerol by metabolically engineered< i> Propionibacterium acidipropionici</i>." *Process Biochemistry* 44.12 (2009): 1346-1351.

CHAPTER 7: CONCLUSION

VFA production enhancement in EBPR systems benefits P removal efficiency in rbCOD/VFA limited systems as more readily biodegradable COD is available for P removing microorganisms (i.e. poly-P accumulating organism or PAOs). One way to increase the VFAs concentration of the influent is to apply a prefermenter. As several parameters could affect the prefermenters' efficiency in terms of production and composition the current study was mostly dedicated to optimizing the glycerol/biodiesel waste fermentation operational conditions. Experimental results revealed that:

- Glycerol/biodiesel waste fermentation resulted in a significant VFA production, mostly in the form of propionic acid. The reason for that might be related to the fact that the carbon chain length is the same for glycerol and propionic acid (i. e. 3 carbons). Comparing pure glycerol and biodiesel waste, although glycerol constituted approximately 20% of the waste batch, it was still more efficient than pure glycerol. The higher VFA production in biodiesel waste fed reactors (and serum bottles) might be related to the other carbon sources such as methanol, ethanol or unidentified organics present in the biodiesel waste. The COD analysis revealed that the COD of biodiesel waste was 1.95 mg-COD/mg-biodiesel-waste which was higher than that of pure glycerol (1.217 mg-COD/mg-glycerol).
- The preliminary tests conducted in 120 ml serum bottles suggested that mixing benefits the VFA production and changed the glycerol consumption pathway as more propionic acid was produced comparing to un-mixed serum bottles. However, different results were achieved from semi-continuous bench scale prefermenters. The serum bottle tests were short-term and could not account for acclimation effects

or changing population due to selective competition. It was observed that un-mixed and slowly mixed prefermenters (up to 7 rpm) had a higher VFA production than that of more intensely mixed reactors (e. g. 50 rpm, 100 rpm). This might be a result of having more concentrated media in unmixed reactors which provided a better hydrolysis and solubilization induction in the system. It also may be that interspecies H_2 transfer (an important substrate for propionic acid production in many non-methanogenic fermentation reactors) was more efficient in a stratified reactor. Time series data on glycerol consumption in an unmixed reactor dosed with 6500 mg/L-cycle pure glycerol revealed that the glycerol consumption rate obeyed first order kinetics. The K constant (average of 3 values) was determined as 0.002 min^{-1}.

- The most consistent result for both the serum bottle experiments and the reactor experiments was that an initial acid pH of 5.5 resulted in very significant inhibition of VFA production. The optimum initial pH value regarding the VFA production (the experiments were conducted at 3 pH values: 5.5, 7 and 8.5) was determined somewhere between 7 and 8.5 both in serum bottles and bench scale prefermenters. However, with respect to propionic acid production the pH of 7 was more favorable than the other pH values. The acetic acid:propionic acid ratio at the pH of 7 was close to 1 (C-mmole/C-mmole) both in serum bottles and bench scale prefermenters. A 1:1 ratio been reported as the most beneficial ratio for successful EBPR (Chen et al, 2004; Lopez-Vazquez et al, 2009).
- The effect of temperature on the glycerol/biodiesel fermentation process was only evaluated in serum bottles. In serum bottles regardless of the carbon source and the

pH value increasing the temperature led to a higher VFA production mostly in the form of propionic acid since the experiments were too short to allow methanogens to build up due to their slow growth rates. As the temperature went up from 22°C to 36°C the VFAs specific production rate increased by a factor greater than 2.

- The effect of SRT on glycerol fermentation process was studied for two SRT values of 2 and 4 (d) in the bench scale prefermenters. It was observed that the SRT value was not a significant variable in VFA production and under the same process and environmental conditions increasing the SRT from 2 to 4 days improved the VFA production about 11% in average.
- Adding glycerol also increased propionic acid production from the primary solids suggesting it favored a larger biomass fraction or enzyme induction related to propionic acid as an end-product.

The effect of glycerol addition on VFA production and consequently on the EBPR process efficiency was studied in two identical A^2/O systems. The only difference between the systems was the reactor where glycerol was added. Train (A) was dosed with the 3500 mg/d pure glycerol in the prefermenter whereas the Train (B) was loaded with the same amount of glycerol but added to the anaerobic zone. Experimental results showed that

- Adding the glycerol to the prefermenter (Train A) resulted in 1476 mg-COD/L VFA production and increase the propionic acid fraction of total VFAs in the combined influent from 0 to 29 mg COD/L. Although linking the prefermenter itself increase the VFA concentration in the combined influent of Train (B) it was not adequate and comparing to Train (A) the average VFA concentration was lower than that of Train (A) (69 mg COD/L in Train (A) vs. 37 mg COD/L in Train (B)).

- Employing the prefermenters plus glycerol enhanced EBPR performance. The P removal efficiency after linking the prefermenters in Train (A) and Train (B) were 93.3% and 90.75% in average respectively.
- Pilot (A) which glycerol added to the prefermenter, had an average effluent SOP of 0.4 mg-P/L while Pilot (B) had an effluent SOP of 0.6 mg-P/L. However, there was enough effluent variability in both systems, and too few observations, to determine if the difference was statistically significant.

APPENDIX A: ACCURACY AND PRECISION DATA OF GLYCEROL COLORIMETRIC ANALYSIS

In the current study the glycerol concentration was measured by using the colorimetric method (Bondioli et al. 2005) with a minor modifications. In the modified method as is described in Chapter 2 DI water was used as the working solvent (instead of a 50:50 (V/V) mixture of distilled water and 95% ethanol which was used in the original method). The average percent recovery of the first 20 spiked samples were 93.35% (standard deviation was 19.3%). Figure A- 1 depicts the percent recover data vs. time which gives a quantitative method to assess accuracy.

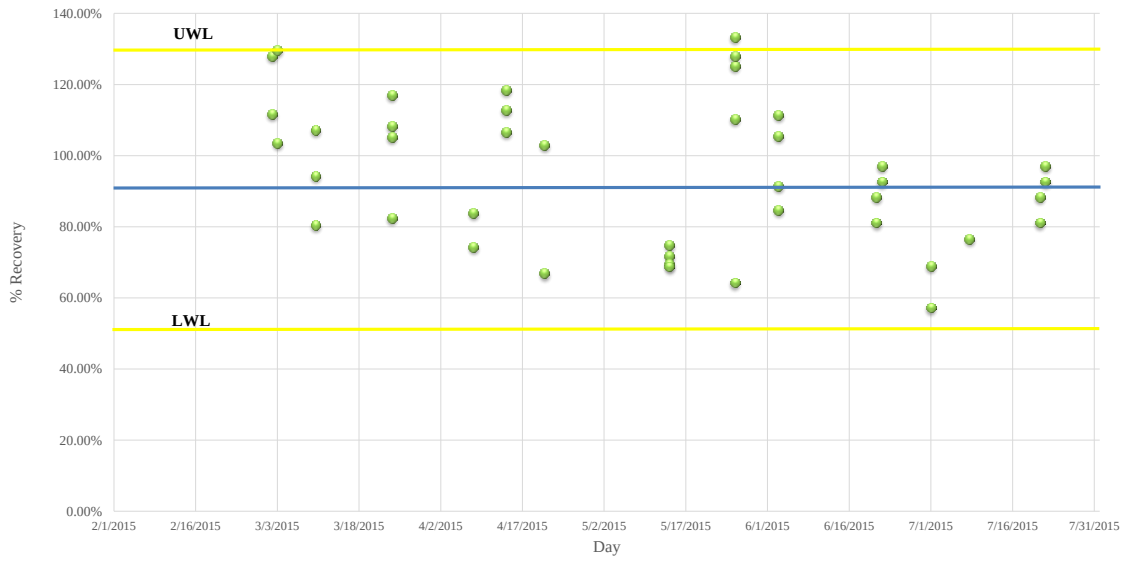

Figure A- 1: Percent recovery data of glycerol concentration using the modified colorimetric method.

The precision was also quantified for the modified glycerol colorimetric method. The average of relative percentage difference (RPD) for this method was calculated as 12.56% with a standard deviation of 10.54%. Table A- 1 shows the precision data.

Table A- 1: RPD data of glycerol concentration using the modified colorimetric method

Date	Sample	Conc. (mg/L)	Duplicate Conc. (mg/L)	RPD
1/27/2015	R3	31	30.63	1.2%
2/11/2015	R2	33	29	13.7%
4/8/2015	R4	49	48	1.3%
4/21/2015	R1	37	32	17.1%
4/21/2015	R3	31	30	3.4%
5/20/2015	R4	32	23	33.9%
5/26/2015	R4	15	12	23.1%
6/3/2015	R4	13	11	19.0%
6/21/2015	R3	463	453	2.1%
6/21/2015	R5	501	463	7.9%
8/12/2015	R2	77.0	0	-%
8/17/2015	R2	2782	2383	15.5%

Sample calculation for RPD is shown below using the data of R3 achieved on Jan. 27th, 2015:

$$RPD = \frac{X_1 - X_2}{\frac{X_1 + X_2}{2}} \quad \text{(A. 1)}$$

$X_1 – X_2$ = (31 mg/L) – (30.63 mg/L)

$X_1 – X_2$ = (0.37 mg/L)

$(X_1 + X_2)/2$ = [(31 mg/L) + (30.63 mg/L)]/2 = (30.8 mg/L)

RPD = 1.2%

APPENDIX B: CALCULATING THE ACETIC ACID: PROPIONIC ACID PRODUCTION RATIO

The acetic acid:propionic acid ratio in terms of C-mmole/C-mmole was calculated by multiplying the molar production of each component by the number of carbon atoms forming each component (acetic acid and propionic acid are two carbon and three carbon molecules). Sample calculation is conducted on the average VFA production and composition data of serum bottles dosed with pure glycerol at pH of 7 (Table B- 1).

Table B- 1: average VFA production and composition of serum bottles dosed with 2000 mg/L pure glycerol at pH of 7 and room temperature (22°C).

pH	HAc (mg COD/L)	HPr (mg COD/L)	HBu (mg COD/L)	VFA (mg COD/L)
7	313	376	39	728

Sample calculation:

HAc production = 313 mg COD/L

HAc theoretical Oxygen demand: 1.06 mg COD/mgHAc

(313 mg COD/L)/ (1.06 mg COD/mgHAc) = 295.28 mg/L as HAc

Molecular weight of acetic acid = 60.05 mg/ mmole

(295.28 mg/L)/ (60.05 mg/ mmole) = 4.9 mmole/L

(4.9 mmole/ L) × (2 mmole-C/mmole-HAc) = 9.83 mmole-C/L HAc was produced on average.

HPr production = 376 mg COD/L

HPr theoretical Oxygen demand: 1.51 mg COD/mgHPr

(376 mg COD/L)/ (1.51 mg COD/mgHPr) = 249 mg/L as HPr

Molecular weight of propionic acid = 74.08 mg/ mmole

(249 mg/L)/ (74.08 mg/ mmole) = 3.36 mmole/L

(3.36 mmole/ L) × (3 mmole-C/mmole-HPr) = 10.08 mmole-C/L HPr was produced on average.

Therefore, the acetic acid: propionic acid production ratio in terms of C-mmole/C-mmole would be:

(9.83 mmole-C/L HAc)/ (10.08 mmole-C/L HPr) = (0.97 C-mmole HAc/C-mmole HPr)

APPENDIX C: FEED (PRIMARY SOLIDS) CHARACTERISTICS

Table C- 1: primary solids characteristics of Chapter 3

Parameter	Nov. 5-2014	Nov. 12-2014	Nov. 18-2014	Nov. 25-2014	Dec. 1-2014	Dec. 9-2014	Dec.16-2014
Total VFA (mg COD/L)	643	1887	1920	1787	2129	1805	2604
MLSS (mg/L)	2267	21600	15200	-	10000	18600	18933
MLVSS (mg/L)	2240	20400	12400	-	9800	16800	15600
pH	5.77	6.6	-	-	5.3	-	-
TCOD (mg/L)	3440	29940	23040	30080	21160	48680	29080
SCOD (mg/L)	2120	3220	2370	3790	2130	-	2420

Table C- 2: primary solids characteristics of Chapter 4

Parameter	Jan 19-2015	Jan. 26th-2015	Feb. 2-2015	Feb. 9-2015
Total VFA (mg COD/L)	3777	5898	8904	7258
MLSS (mg/L)	14133	17600	20909	16600
MLVSS (mg/L)	9733	16200	18000	13400
pH	5.5	5.3	5.5	5.4
TCOD (mg/L)	24160	43920	48040	29440
SCOD (mg/L)	2760	2570	3300	3970

Table C- 3: primary solids characteristics of Chapter 5 phase I

Parameter	March 23-2015	March 29-2015	April 6-2015	April 12-2015	April 19-2015
Total VFA (mg COD/L)	3778	1469	1728	0	7601
MLSS (mg/L)	-	-	-	12000	33000
MLVSS (mg/L)	-	-	-	10267	22000
pH	-	5.2	5.4	-	-
TCOD (mg/L)	50360	45040	36240	37200	51840
SCOD (mg/L)	2640	1810	1800	2230	2670

Table C- 4: primary solids characteristics of Chapter 5 phase II

Parameter	June 28th-2015	July 6th-2015	July 13th-2015	July 20th-2015	July 30th-2015	August 12th-2015	August 17th-2015
Total VFA (mg COD/L)	272	609	698	943	554	792	793
MLSS (mg/L)	6200	6667	5600	8833	4800	7600	8800
MLVSS(mg/L)	-	-	-	6500	3733	7600	-
pH	7						
TCOD (mg/L)	13680	15560	13320	15440	8680	16200	18400
SCOD (mg/L)	870	1145	1285	1225	1110	1030	1420

APPENDIX D: VFA PRODUCTION AND VFA SPECIFIC PRODUCTION RATE

VFA production per cycle was calculated when the system reached an approximate steady-state condition (3 SRTs). The production was measured by subtracting the VFA concentration of the inflow at time 0 from the VFA concentration of the sample at the end of the cycle (approximately 24hr) since the flow going in and out was the same at 375 ml/cycle.

Table D- 1: VFA concentration of the feed and Reactor # 1 measured on December 1st-2015 and December 2nd-2015 respectively.

Sample	Time	HAC (mg COD/L)	HPr (mg COD/L)	HBu (mg COD/L)	Total VFA (mg COD/L)	MLVSS (mgVSS/L)
Feed	0	724	793	612	2129	9800
Reactor 1	24hr	4565	9268	1472	15305	14000

$$\Delta A = CA_{end} - CA_0 \quad \text{(D. 1)}$$

Where:

CA_{end} = Concentration of component A at the end of the cycle

CA_0 = Concentration of the component A in the feed at time 0

By plugging in the data from Table the VFA production is calculated as follow:

ΔHAc = (4565 mg COD/L) - (724 mg COD/L)

ΔHAc = (3841 mg COD/L)

ΔHPr = (9268 mg COD/L) - (793 mg COD/L)

ΔHPr = (8475 mg COD/L)

ΔHBu = (1472 mg COD/L) - (612 mg COD/L)

ΔHBu = (860 mg COD/L)

ΔVFA = ΔHAc + ΔHPr + ΔHBu

ΔVFA = (3841 mg COD/L) + (8475 mg COD/L) + (860 mg COD/L)

ΔVFA = (13176 mg COD/L)

The VFA specific production rate is the rate of VFA production per unit of VSS of the influent as is shown in equation D-2.

$$Specific\ rate_{VFA} = \frac{\Delta VFA}{VSS*hr} \quad \text{(D. 2)}$$

VFA specific production rate = (13176 mg COD/L) /.(9.8 gVSS/L) / (24 hr)

VFA specific production rate = 56.02 mg COD/gVSS/hr

APPENDIX E: GLYCEROL CONSUMPTION RATE

Reaction rate is a term which shows the relationship between the rate of a reaction and the concentration of the reactants. For a simple chemical reaction of "$n_A A + n_B B \rightarrow$ product" the reaction rate is defined as Equation 1:

$$rate = k\,[A]^x[B]^y \qquad \text{(E. 1)}$$

Where:

[A]= Concentration of the reactant A

[B] = Concentration of the reactant B

K = Rate constant

X and Y= partial reaction order (the overall reaction order is equal to X + Y)

Reaction rates are often modeled by zero order, first order, and second order reaction equations. In zero order reactions, the reaction rate is independent from the concentration of reactants. However, in first order reactions, there is a linear relationship between the rate of the reaction and the concentration of one reactant. Second order reactions, are those in which the reaction rate is proportional to the product of the concentration of two reactants or the square of the concentration of one of the reactants (the overall order of second or reaction equals to 2). Equation 2 to 4 express the zero, first and second order reaction models:

$$\frac{dA}{dt} = k \qquad \text{(E. 2)}$$

$$\frac{dA}{dt} = k\,[A] \; or \; \frac{dB}{b} = k\,[B] \qquad \text{(E. 3)}$$

$$\frac{dA}{dt} = k\,[A]^2 \; or \; \frac{dA}{dt} = k\,[A][B] \qquad \text{(E. 4)}$$

The K (i. e. rate constant) of the mentioned reactions can be determined by plotting the concentration vs. time in zero order reactions, natural logarithm of the concentration of one reactant vs time in first order reactions, and the reciprocal of the concentration of one reactant vs. time in second order reactions. R-squared value of the linear regression line of the depicted plots is one index to help evaluate the validity of the reaction model. If the reaction model is valid the slope and the intercept will allow calculation of the rate constant.

Time series analysis on glycerol concentration (Chapter 5) revealed that the glycerol consumption follows the first order reactions. A sample calculation is stated below. The presented data are from August 17th.

Table E- 1: Glycerol time series data

Time (Sec)	Conc. (mole/L)	LN [(Conc.)]	1/[Conc.]
0	0.047	3.851	0.021
0.5	0.046	3.822	0.022
2	0.040	3.692	0.025
4	0.034	3.525	0.029
6	0.028	3.334	0.036
8	0.024	3.184	0.041
12	0.014	2.627	0.072
24	0.004	1.412	0.244

Assuming that the reaction rate follows the zero order reaction, the concentration of glycerol in terms of mole/L (column 2 of Table 1) was plotted vs. time (Figure 1). The R-squared value was calculated as 0.91 (R-squared is the coefficient of determination).

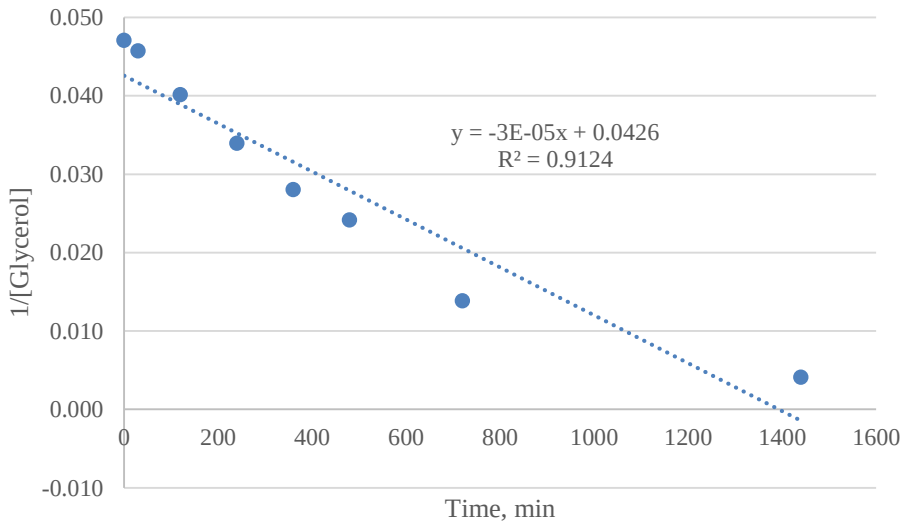

Figure E- 1: Zero order reaction evaluation for glycerol concentration over time

Assuming that the reaction rate follows the first order reaction, the concentration of glycerol in terms of mole/L (column 2 of Table 1) was plotted vs. time (Figure 2) with an R- squared of 0.99.

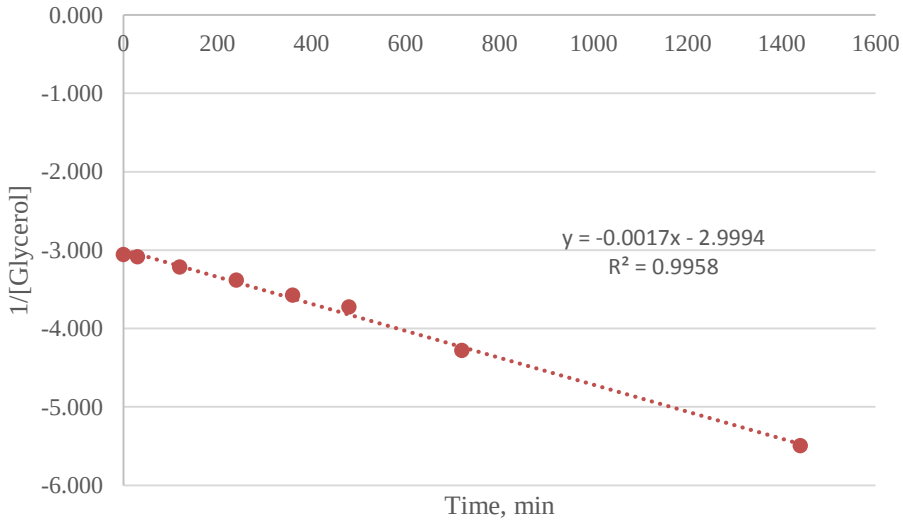

Figure E- 2: First order reaction evaluation for glycerol concentration over time

Assuming that glycerol consumption follows the second order reaction model the reciprocal concentration was plotted vs. time and the result is shown in Figure E- 3. The R-squared value was calculated as 0.89.

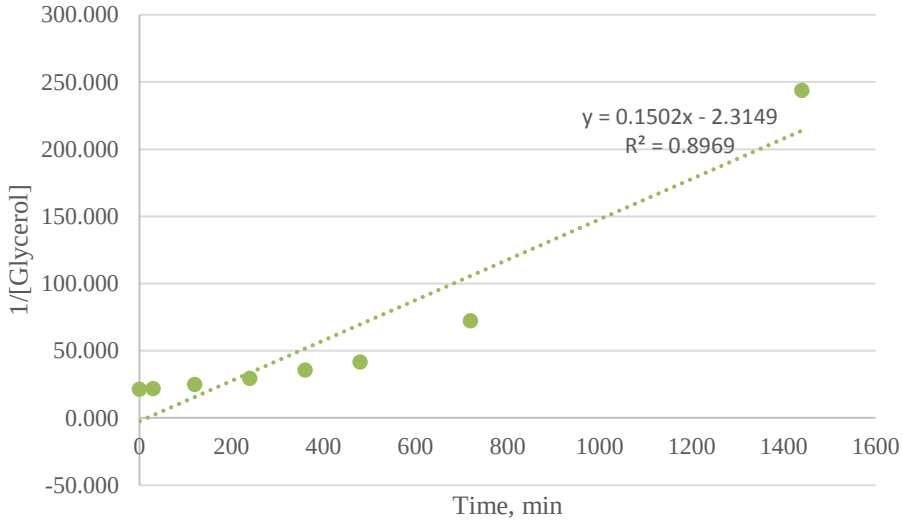

Figure E- 3: Second order reaction evaluation for concentration over time

Considering the R-squared values and clearly curvilinear nature of the other 2 plots, first order reaction model (R= 0.99) fit the data. The same calculations were conducted on two more data sets and the results were consistent in terms of reaction rate model.

APPENDIX F: C MASS BALANCE

Mass balance is a technique that enables us to identify the changes of a definable component occurring during biochemical reactions or bulk water transport (mass fluxes). The general mass balance formula is shown in Equation (F- 1):

$$Inflow + Generation = Accumulation + Consumption + Outflow \quad \text{(F. 1)}$$

The chemical oxygen demand (COD) mass balance in a BNR system is defined as Equation (F- 2):

$$MCOD_{IN} = MCOD_{OUT} + MCOD_{OXIDIZED} \quad \text{(F. 2)}$$

Where:

$MCOD_{IN}$ = mass of COD in the influent (mg COD/d)

$MCOD_{OUT}$ = mass of COD leaving the system through the effluent and waste sludge (mg COD/d)

$MCOD_{OXIDIZED}$ = mass of COD which is oxidized to CO_2 and water.

The mass of COD entering the system is calculated by multiplying the influent flowrate by the total COD concentration of the influent. The mass of oxygen consumed in the aerobic zone includes carbonaceous oxygen demand (mass of oxygen needed for complete oxidation of organic matters to CO_2 and H_2O) and the nitrogenous oxygen demand (the mass of oxygen required for nitrification reaction in the aerobic zone). The mass of COD leaving the system consists of COD mass leaving the system through the effluent and the COD mass leaving the system through the WAS recycle. As a sample calculation the COD mass balance is shown in the following. The data was obtained on August 28th -2015 from Train B (Table F- 1 and Table F- 2).

Table F- 1: Influent, effluent and recycles flowrate of Train (B) on August 28th-2015

	Influent	Prefermenter	NARCY	RAS	WAS	Effluent
Flowrate (L/d)	91.2	2	242	57.6	2.7	90.2

Table F- 2: Solids and COD data of Train (B) on August 28th-2015

	INF (mg/L)	PREF. EFF (mg/L)	AN (mg/L)	AX (mg/L)	AE (mg/L)	EFF (mg/L)	2nd Clarifier (mg/L)
TSS	50	4620	3310	3353	3240	3	13
VSS/TSS	0.82	0.82	0.83	0.82	0.82	0.82	0.82
TCOD	254	7007	-	-	-	36	-
sCOD	201	1390	75	39	49	-	31

The TCOD entering the system is calculated below. Note that for the prefermenter instead of $TCOD_{PREF}$ the $SCOD_{PREF}$ was used since the high $TCOD_{PREF}$ concentration made measurement difficult and biomass could not be differentiated either biodegradable or non-biodegradable organics. Also as the pure glycerol in Train (B) was added separately into the anaerobic zone it should be taken in to the account.

$$MCOD_{IN} = Q_{IN} \times TCOD_{IN} + Q_{PREF} \times SCOD_{PREF} + SCOD_{GLYCEROL} \quad \text{(F. 3)}$$

[(91.2 L/d) × (254 mg COD/L)] + [(2 L/d) + (695 mg COD/L)] + [(3500 mg/L) × (1.217 mg COD/mg glycerol)] = 30204 mg COD/d

The COD mass of the effluent was calculated by multiplying the effluent flowrate by its soluble COD concentration. The effluent VSS was treated the same way as the solid phase in the WAS flow. The COD mass of the solids phase in WAS cycle was measured by multiplying the biomass concentration (MLVSS) of the aerobic zone (i. e. the WAS cycle was originated from the

aerobic zone) by the WAS flowrate assuming the amount of oxygen removed per unit biomass unit is 1.48 mg COD/mgVSS. The soluble COD of the WAS cycle is calculated by multiplying the WAS flowrate by the SCOD of the aerobic reactor.

$$MCOD_{OUT} = Q_{EFF} \times TCOD_{EFF} + Q_{WAS} \times VSS_{AE} \times 1.48\ mg\ COD/mgMLVSS + Q_{WAS} \times SCOD_{AE} \quad \text{(F. 4)}$$

[(90.2 L/d) × (36 mg COD/L)] + [(2.7 L/d) × (2657 mgVSS/L) × (1.48 mg COD/mgVSS)] + [(2.7 L/d) × (49 mg COD/L)] = 14026 mg COD/d

Total oxygen demand in the aerobic zone is calculated by measuring the oxygen uptake rate (OUR) in the aerobic zone multiplied by the aerobic zone volume. On August 28th-2015 the OUR of the aerobic zone of Train (B) was equal to 1161.86 mgO_2/L/D. The nitrogenous oxygen demand is measured by multiplying the mass of nitrate formed in the aerobic zone by the mass of oxygen needed for producing a unit mass of nitrate which is assumed to be 4.57 mg O_2/mg nitrate. Carbonaceous oxygen demand is calculated by subtracting the nitrogenous oxygen demand from the total oxygen demand.

$$MCOD_{AE} = OUR \times V_{AE} - \Delta NO_3\text{-}Produced_{AE} \times 4.57\ mg\ O_2/mg\ NO_3\text{-}Produced \quad \text{(F. 5)}$$

[(1161.68 mg O_2/L/d) × (17.95 L)] – [(3859 mg NO_3-Produced/d) × 4.57 (mg O_2/mg NO_3-Produced)] = 3220 mg COD/d

The mass of COD needed for the denitrification process in the anoxic zone is calculated by multiplying the mass of nitrate reduced in the anoxic zone by the mass of oxygen required per mg nitrate removal in the anoxic zone which is assumed to be 2.86 mg O_2/mg NO_3-Denitrified .On August 28th the nitrate mass nitrate denitrified in the anoxic zone was 2826 mg NO_3-Denitrified.

$$MCOD_{DN} = \Delta NO_3\text{-}Denitrified/d \times 2.86\ mg\ O_2/mg\ NO_3\text{-}Denitrified \quad \text{(F. 6)}$$

(2826 mg NO_3-Denitrified/d) × 2.86 = 8082 mg COD/d

With all those calculations, the mass of COD leaving the system and oxidized during the biochemical reactions was:

$$MCOD_{TOTAL\ OUT} = MCOD_{OUT} + MCOD_{AE} + MCOD_{DN} \quad \text{(F. 7)}$$

(14026 mg COD/d) + (3220 mg COD/d) + (8082 mg COD/d) = 25328 mg COD/d

The COD recovery is then calculated as follow:

$$COD_{RECOVERY} = (MCOD_{IN}/MCOD_{TOTAL\ OUT}) \times 100 \quad \text{(F. 8)}$$

[(30204 mg COD/d)/ (25328 mg COD/d)] × 100 = 119%

APPENDIX G: P MASS BALANCE

In the current study the EBPR performance of an A^2/O system was studied in 2 parallel trains. Both trains were dosed with pure glycerol. In train (A) glycerol was added at the concentration of 3500 mg/d to the prefermenter whereas in train (B) glycerol was added at the same concentration and mass flux but to the anaerobic zone. Figure G- 1 depicts the configuration of both trains. As phosphorus cannot leave or enter a system in a gaseous form in order to calculate the P change in any BNR system, the P mass change in the liquid and solid phases are of concern.

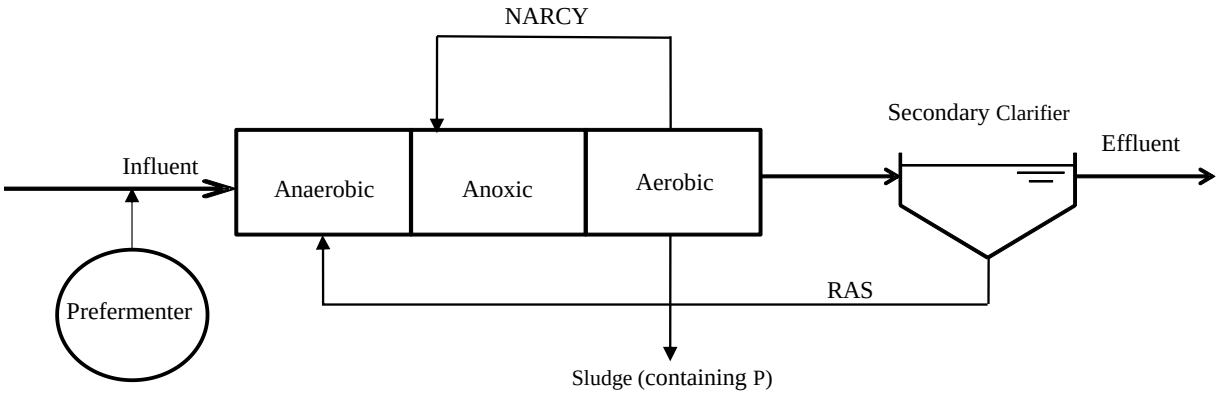

Figure G- 1: A^2/O configuration in the current study

Table G- 1 shows the phosphorus concentration in different reactors (liquid phase) of Train (B) achieved on August 28th-2015. The flowrate of influent, prefermenter, recycles (NARCY, RAS) and effluent are in Table G- 2.

Table G- 1: P concentration in different reactors of train (A) measured on August 28th-2015

	Influent	Prefermenter	Anaerobic	Anoxic	Aerobic	2nd Clarifier
TP (mg/L)	5.8	-	-	-	-	-
SOP (mg/L)	5.3	17.5	6.6	5	0.4	0.4

Table G- 2: Flowrate of the influent, prefermenter, effluent and recycles of Train (B) on August 28th-2015

	Influent	Prefermenter	NARCY	RAS	WAS	Effluent
Flowrate (L/d)	91.2	2	249	50.4	2.7	91.2

Change in P mass over the anaerobic zone is calculated by subtracting the total P mass leaving the anaerobic zone from the total P mass that enters the anaerobic reactor.

$$\Delta P_{AN} = (Q_{INF} + Q_{PREF} + Q_{RAS}) \times SOP_{AN} - (Q_{INF} \times TP_{INF}) - (Q_{PREF} - SOP_{PREF}) - (Q_{RAS} - SOP_{CLA}) \quad \text{(G. 1)}$$

By substituting the corresponding values stated in Table G- 1 and Table G- 2 the P change over the anaerobic reactor would be as follow:

[(91.2 L/d + 2 L/d + 50.4 L/d) × (6.6 mg-P/L)] – [(91.2 L/d) × (5.8 mg-P/L)] – [(2 L/d) × (17.5 mg-P/L)] – [(50.4 L/d) × (0.4 mg-P/L)] = 363.64 mg-P/d

It should be noted that in Equation the total P concentration was used as the influent P content. The reason for that is that the particulate P is rapidly converted to soluble ortho-phosphate upon contact with the biomass. As the $\Delta P_{Anaerobic} > 0$ there is a net P release in the anaerobic zone

which proves that poly-P accumulating organism were functioned as they were supposed in an EBPR process.

Change in P mass over the anoxic zone is calculated by conducting mass balance over the anoxic zone which is shown below:

$$\Delta P_{AX} = (Q_{INF} + Q_{PRF} + Q_{RAS} + Q_{NARCY}) \times SOP_{AX} - (Q_{INF} + Q_{PRF} + Q_{RAS}) \times SOP_{AN} - Q_{NARCY} \times SOP_{AE} \quad \text{(G. 2)}$$

Plugging in the values from Table G- 1 and Table G- 2 in the above-mentioned mass balance formula over the anoxic zone resulted in 415.64 mg-P/day P release in the anoxic zone which is calculated as follow:

[(91.2 L/d + 2 L/d + 50.4 L/d +249 L/d) × (5 mg-P/L)] – [(91.2 L/d + 2 L/d +50.4 L/d) × (6.6 mg-P/L)] – [(249 L/d) × (0.4 mg-P/L)] = 415.64 mg-P/d

By conducting the mass balance over the aerobic zone the change in P mass was calculated as 1805.96 mg-P/L. Note that $\Delta P_{AE} < 0$ means that there was a net P uptake in the aerobic zone. The calculations are stated below:

$$\Delta P_{AE} = (Q_{INF} + Q_{PRF} + Q_{RAS} + Q_{NARCY}) \times SOP_{AE} - (Q_{INF} + Q_{PRF} + Q_{RAS} + Q_{NARCY}) \times SOP_{AX} \quad \text{(G. 3)}$$

[(91.2 L/d + 2 L/d + 50.4 L/d +249 L/d) × (0.4 mg-P/L)] – [(91.2 L/d + 2 L/d + 50.4 L/d +249 L/d) × (5 mg-P/L)] = - 1805.96 mg-P/d

The P mass balance over the secondary clarifier is shown below. As is calculated there was a small net P uptake over the 2ndclarifier.

$$\Delta P_{CLA} = (Q_{EFF} + Q_{RAS}) \times SOP_{2nd\text{-}CLA} - (Q_{INF} + Q_{PRF} + Q_{RAS}) \times SOP_{AE} \quad \text{(G. 4)}$$

{(91.2 L/d + 50.4 L/d) × (0.4 mg-P/L)} - {(91.2 L/d + 2 L/d + 50.4 L/d) × (0.4 mg-P/L)} = (- 0.8 mg-P/d)

The net P removal is calculated by subtracting the total sum of SOP release from total some of SOP uptake which is equal to 1027.48 mg-P/d. Table G- 3 summarizes the net P release/uptake over the different reactors and the total P removal of the system.

Table G- 3: P change over the reactors of Train (B) on August 28th

	Anaerobic	Anoxic	Aerobic	2nd Clarifier	Entire Process
ΔP (mg/d)	+363.64	+415.64	-1805.96	-0.8	-1027.48

APPENDIX H: P CONTENT CALCULATION

Phosphorus (P) is one of the required elements for maintenance and growth of microorganisms. Poly-P accumulating organisms (PAOs) are specific facultative microorganisms that under an anaerobic condition consume their intracellular poly-P as a source of energy and release P into the bulk liquid. However, under an aerobic condition these microorganisms have the ability to uptake more P from the bulk liquid than they released in the preceding anaerobic reactor. This results in a net P removal. In the current study the approximate aggregate VSS P content (% mass) was calculated. A sample calculation is conducted below using the data observed on September 3rd from Train (A) (Table H- 1and Table H- 2).

Table H- 1: Charactristics of the inflent, effluent and invoved reactors in the A^2/O system of the current study

	INF	PREF	AN	AX	AE	EFF	2nd CLA
TSS (mg/L)	57	5560	2367	2890	3053	7	7
VSS/TSS	0.84	0.84	0.85	0.84	0.84	0.84	0.84
TCOD (mg/L)	183	9018	-	-	-	26	-
SCOD (mg/L)	119	2096	85	39	29	-	31
TP (mg-P/L)	5.3	50.9	-	-	-	0.6	-
SOP (mg-P/L)	2.8	20.7	16.2	8.0	0.3	-	0.2

Table H- 2: Flowrate of the influent, effluent and recycles in the A^2/O system of the current study

INF (L/d)	Pref. (L/d)	NARCY (L/d)	RAS (L/d)	WAS (L/d)	EFF (L/d)
58.5	2.0	97.9	47.5	2.7	53.3

The first step is to calculate the biomass flux leaving the system via the effluent and the WAS stream. It should be noted that the WAS stream was originated from the aerobic reactor.

$$Solids\ flux\ out = Q_{EFF} \times TSS_{EFF} \times (VSS/TSS)_{EFF} + Q_{WAS} \times TSS_{AE} \times (VSS/TSS)_{AE} \quad \text{(H. 1)}$$

Solids flux out = [(53.3 L/d) × (7 mg TSS/L) × (0.84 VSS/TSS)] + [(2.7 L/d) × (3053 mgTSS/L) × (0.84 VSS/TSS) = 7238 mgVSS/d

In the next step, the soluble P flux through the effluent and WAS cycle is to be calculated as follows:

$$Liquid\ phase\ P\ flux\ out = Q_{EFF} \times SOP_{2nd\ CLA} + Q_{WAS} \times SOP_{AE} \quad \text{(H. 2)}$$

Liquid phase P flux out = [(53.3 L/d) × (0.2 mg-P/L)] + [(2.7 L/d) × (0.3 mg-P/L)] = 11.47 mg-P/d

The total P removal via biomass is calculated by subtracting the effluent and the WAS soluble P content (calculated in the previous step) from the combined influent (i. e. including the prefermenter) total P content.

$$System\ net\ P\ removal = Q_{INF} \times TP_{INF} + Q_{PREF} \times TP_{PREF} - Liquid\ Phase\ P\ Flux \quad \text{(H. 3)}$$

System net P removal = [(58.5 L/d) × (5.3 mg-P/L)] + [(2 L/d) × (50.9 mg-P/L)] – (11.47 mg-P/d) = 400. 38 mg-P/d

By dividing the mass flux of P in the solids form (400.38 mg-P/L) by the biomass leaving the system (7238 mgVSS/d) the P content of the leaving VSS is calculated as:

$$P\ content\ \% = Solids\ Phase\ P\ flux / Solids\ flux \times 100 \quad \text{(H. 4)}$$

P content % = [(400.38 mg-P/L) / (7238 mgVSS/d] × 100 = 5.5%