EFFECT OF ACCLIMATIZATION RATE ON BIOGAS PRODUCTION FROM ANAEROBIC DIGESTION OF BIODIESEL WASTE PRODUCTS

by

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Dedicated to my parents and grandparents, who taught me to hope and work for a better world; and particularly to Amy Stevens, for her unfailing belief in me.

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LIST OF ABBREVIATIONS

AD: Anaerobic digestion

AW: Inoculum from a mixed agro-industrial waste digester

BDW: Biodiesel wastewater

CG: Crude glycerol

CGWW: Mixture of crude glycerol and biodiesel wastewater (15-85% v/v respectively)

COD: Chemical oxygen demand

GHG: Greenhouse gas

OLR: Organic loading rate

STP: Standard temperature and pressure

TS: Total solids

VS: Volatile solids

WT: Inoculum from a local wastewater treatment plant

ABSTRACT

Anaerobic digestion can be used to sustainably treat the organic byproducts of the biodiesel process (crude glycerol and biodiesel wastewater) while generating a renewable natural gas to be used for heating or electricity generation. The purpose of this thesis was to (1) investigate the possibility of co-digestion of biodiesel byproducts without use of external substrates or pretreatment and (2) assess the impact of various acclimatization rates on the stability and efficiency of such a system. Two inocula (effluent from a wastewater treatment plant digester and from an agro-industrial waste digester) and two acclimatization rates were studied. The results showed that co-digestion of crude glycerol and biodiesel wastewater at high organic loading rates (up to 6.8 g COD L^{-1} day-^{1}) is possible without addition of other substrates or pretreatment. The cumulative biogas production of the digesters using inoculum from the agro-industrial waste digester was statistically greater than the digesters using the wastewater treatment plant digester, indicating that similar inoculum could be useful for additional experiments. In addition, maximum efficiency due to a slower rate of acclimatization was higher for both inocula, up to a maximum average daily biogas yield of 621 mL biogas g^{-1} COD added. Finally, comparison of two methods for measuring gas production (mass difference and volumetrically using a syringe) revealed a reasonable correlation (R^2 = 0.97) between the methods. Additional validation could lead to use of the mass difference method as a validation method or an alternative gas production measurement method.

1. INTRODUCTION

1.1 Background and Significance

Global climate change necessitates renewable energy solutions that reduce greenhouse gas emissions. Increasing awareness of the need for environmental and economic stability in energy production is leading to increased growth of renewable fuel alternatives. Biodiesel can be produced from low value oil, including waste cooking oil, making it a desirable alternative to fossil fuel diesel. The popularity of biodiesel has grown immensely over the last two decades, with global production increasing more than 100-fold since 2000 (EIA, 2016). As a result, the amount of biodiesel byproducts has also increased to the point of exceeding demand. Most biodiesel production processes produce approximately 10 wt% crude glycerol, leading to a glut on the glycerol market and large volumes of byproducts that must be treated if they cannot be reused or sold for another use (Johnson and Taconi, 2007). Biodiesel production usually also generates large amounts of wastewater (20-120 vol% depending on the process) with a high chemical oxygen demand (Daud et al., 2015; Phukingngam et al., 2011). This crude glycerol (CG) and biodiesel wastewater (BDW) could be used as substrates in anaerobic digestion. Anaerobic digestion (AD) biologically degrades organic waste using a mixed microbial consortium to produce renewable natural gas. This process reduces greenhouse gas emissions from fossil fuels and provides a clean source of energy. Use of AD is one potential method to sustainably treat the organic byproducts of the biodiesel process while concurrently generating a value-added product in the form of methane that can be used for heating or electricity generation. This combination has the potential to improve environmental and economic feasibility of biodiesel production. However, anaerobic digestion processes can be disrupted by inhibitors, such as salts and long chain fatty acids (LCFAs) which can slow or even kill the process. Biodiesel byproducts are highly biodegradable but they can also contain high levels of these inhibitors that could make using AD to add value from byproducts and wastewater difficult.

Many researchers have investigated the possibility of digesting one of these byproducts alone or in tandem with other substrates, a process known as co-digestion (Astals et al., 2012; Baba et al., 2013; Phukingngam et al., 2011; Siles López et al., 2009; Viana et al., 2012). However, co-

digestion with external substrates may not be feasible for all biodiesel companies. Siles et al. (2010) demonstrated co-digestion of CG and BDW without additional substrates. However, this study used pretreatments, which adds to the capital and operating costs (Siles et al., 2010). As a result, research is needed for co-digestion of only crude glycerol and biodiesel wastewater without external substrates or pretreatment.

Acclimatization is one potential strategy for improving AD response to inhibitors, where the AD system is gradually exposed to increasing concentrations of inhibitors or higher organic loading rates. While some researchers have demonstrated the use of acclimatization with AD of biodiesel byproducts (Baba et al., 2013; Ma et al., 2008; Rinzema et al., 1993; Viana et al., 2012), to my knowledge no one has specifically quantified the difference in efficiency or stability between different acclimatization rates using these substrates. A better understanding of the acclimatization response of AD systems is needed to improve the feasibility of using AD to generate energy from biodiesel byproducts. I hypothesize that a slower rate of acclimatization improves the ability of an AD system to adapt to high concentrations of crude glycerol when mixed with biodiesel wastewater due to microbial adaptation, and that this phenomenon can be seen when using different inocula. My objectives with this research were to (1) investigate the possibility of co-digestion of biodiesel byproducts without use of external substrates or pretreatment and (2) assess the impact of various acclimatization rates on the stability and efficiency of such a system.

1.2 Thesis Objectives

The objectives of this thesis are as follows:

1. Demonstrate digestion of crude glycerol and biodiesel wastewater without pretreatment or addition of external substrates.
2. Assess the impact of gradual acclimatization rates on AD efficiency and stability for two different inocula.

1.3 Organization of Thesis

This thesis is organized in a traditional format. Chapter 2 summarizes and analyzes the relevant background literature and provides a justification for the need for this research. Chapter 3 describes

the general methodology used for the experiment described. Chapter 4 describes the results of the experiment and discusses the implications of this work. Finally, Chapter 5 summarizes the findings of the thesis and describes potential future work that could be pursued as a result of this study.

2. LITERATURE REVIEW

2.1 Need for sustainable treatments of biodiesel byproducts

2.1.1 Motivation for biodiesel use and production

Increased production and use of renewable fuels can decrease national and global dependence on fossil fuels, in turn reducing greenhouse gas (GHG) emissions. Biodiesel is a renewable, high-energy density liquid fuel that could contribute to the reduction of GHG emissions, particularly with continuing improvements in biodiesel production efficiency. For example, during the past three decades comprehensive life-cycle analyses for soybean biodiesel have shown that its fossil energy ratio has improved from 3.2 to 5.54, meaning that for each unit of fossil fuel used to produce it, biodiesel generates more than five units of energy (Pradhan et al., 2011, 2009). In addition, soybean-based biodiesel can displace up to 96% GHG emissions compared to petroleum-based diesel fuel (Huo et al., 2008).

Although biodiesel produced from vegetable oils was first demonstrated in the 1890s (Orchard et al., 2007), the low cost of petroleum in the 1920s drove the market towards petroleum-based diesel fuels (Kong et al., 2016). However, beginning in the 1970s, oil crises and environmental concerns renewed interest in renewable alternatives (Talebian-Kiakalaieh et al., 2013). Worldwide biodiesel production has also increased dramatically since the turn of the century, as shown in Figure 1 (EIA, 2016). Since that time, biodiesel production has continued to rise, reaching 622 thousand barrels/day worldwide in 2017 (*Global Bioenergy Statistics*, 2019). Between 2001 and 2019, biodiesel consumption in the United States has increased by approximately two orders of magnitude to nearly two billion gallons per year (EIA, 2020). As of 2017, biodiesel represented approximately 3.2% of the diesel market of the United States (Moriarty et al., 2020). However, between 2008 and 2017, U.S. capacity for biodiesel production has been underutilized, not exceeding a 70% utilization rate during that time (Moriarty et al., 2020). Economic conditions are generally the drivers for underutilization of capacity, as production ceases when cost of production exceeds price of biodiesel or when tax incentives are unavailable (Moriarty et al., 2020).

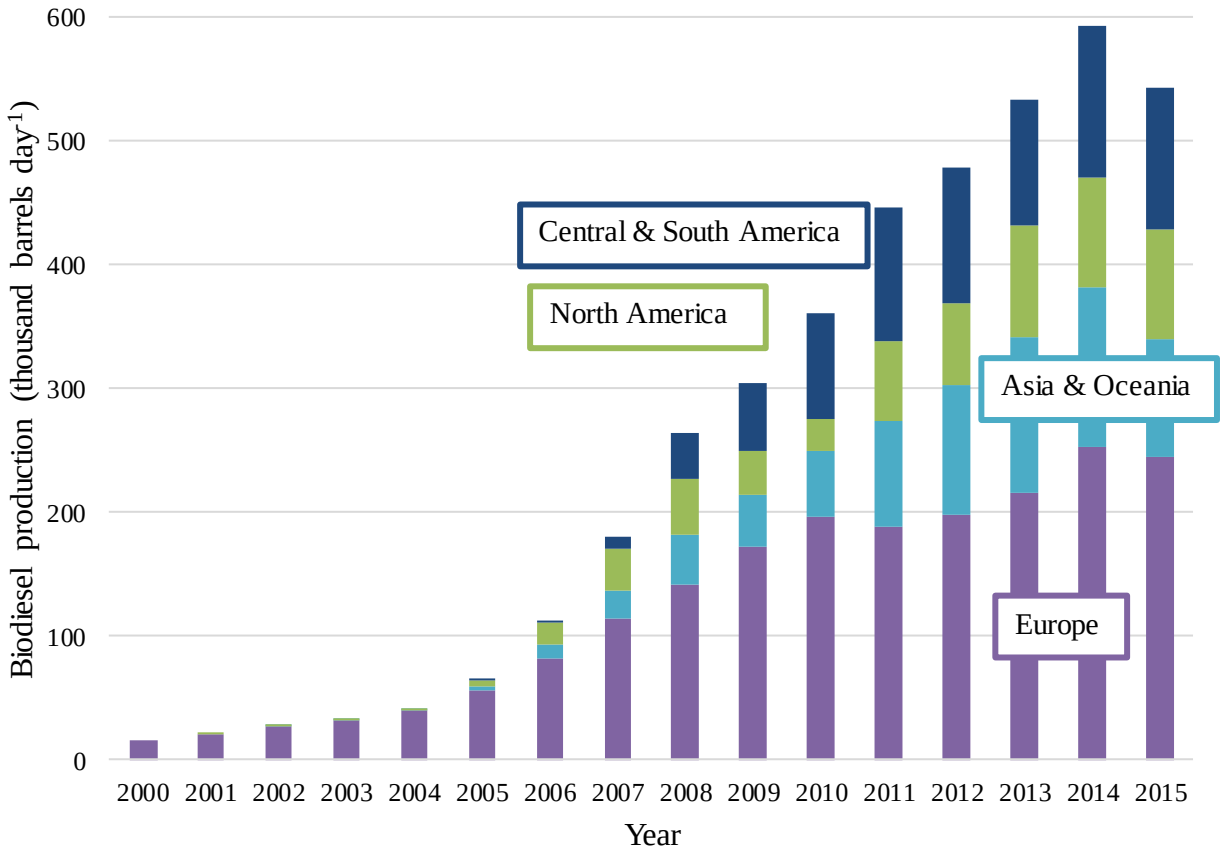

Figure 1: Biodiesel production by continent (Eurasia, Africa, and the Middle East are excluded since each represents less than 1% of the total world production) (EIA, 2016).

Since biodiesel is used for generators, manufacturing, and construction, as well as being a nearly ideal drop-in transportation fuel, it is a valuable renewable energy resource (Daud et al., 2015). In addition, the growth of the biodiesel industry has an important economic and labor impact. Between 2006 and 2015 in the United States, biodiesel production increased its economic impact and number of direct jobs 600% or more ($1.4 billion to $8.4 billion annually and 7,000 to 47,400 jobs respectively) (Moriarty et al., 2020).

2.1.2 Biodiesel byproducts

The most common method of producing biodiesel is transesterification with alcohol, which can optionally use a catalyst to break triglycerides found in oils into fatty esters (Talebian-Kiakalaieh et al., 2013). The fatty esters are then used as biodiesel. A substantial amount of glycerol (also

known as glycerin) is produced as a byproduct of this reaction at a rate of approximately 10 wt%. Due to the impurities in this byproduct, including water, catalysts, fatty acids, and salts, it is typically referred to as crude glycerol (CG) (Monteiro et al., 2018).

As a result, the dramatic increase in biodiesel production has also led to a glut on the market for crude glycerol (Johnson and Taconi, 2007; Viana et al., 2012). Specifically, the price of crude glycerol dropped to approximately \$0.05 lb^{-1} in the United States in 2007, a dramatic 80% decrease from historic prices (Johnson and Taconi, 2007; Yang et al., 2012). While crude glycerol can be used in limited applications, cost of purification and low glycerol prices due to excess supply often mean that disposal is necessary (Thompson and He, 2002). In addition to crude glycerol, in conventional biodiesel production, the biodiesel uses a water wash to remove contaminants that would otherwise decrease quality, including soaps, catalyst, free glycerol, alcohol, free fatty acids, and water (Daud et al., 2015). Like the crude glycerol, this wastewater must also be disposed of, treated, or used. Although the actual amount generated fluctuates depending on the processing procedure, the quantity ranges from 20-120 vol% of the biodiesel generated (Phukingngam et al., 2011; Siles et al., 2011). Treatment of these byproducts substantially increase operating and/or capital costs of biodiesel production depending on the method selected and can decrease the profitability of the final biodiesel product. To increase the economic viability of biodiesel, the treatment or use of biodiesel byproducts would ideally extract additional value from them to offset the required cost of their disposal.

2.2 Anaerobic digestion of biodiesel byproducts

Anaerobic digestion (AD) is one potential method for treatment of biodiesel waste products that also generates a value-added product in the form of renewable natural gas. This renewable natural gas could in turn be used to offset the heat or electrical requirements of the biodiesel plant or the digestion facility. While anaerobic digestion has been used worldwide for centuries for sustainable treatment of organic waste and generation of renewable energy, it has been most commonly used for treatment of manure or sewage sludge. Harnessing its full capabilities to generate renewable natural gas and provide sustainable waste treatment methods requires use of less common substrates including agro-industrial waste (dos Santos Ferreira et al., 2018; Jacob et al., 2020).

2.2.1 Demonstrations of AD of biodiesel byproducts

Researchers have repeatedly demonstrated the successful digestion of crude glycerol (Baba et al., 2013; Monteiro et al., 2018; Siles López et al., 2009; Viana et al., 2012) and biodiesel wastewater (Phukingngam et al., 2011; Siles et al., 2011) alone or in tandem with other substrates (a process also known as co-digestion). Key examples are summarized in Table 1. While co-digestion can be an attractive option for both the digestion facility and the biodiesel company, it may not always be feasible for the biodiesel company to outsource this treatment. However, few examples exist of the co-digestion of crude glycerol with biodiesel wastewater with no other co-substrates. While Siles et al. (2010) successfully demonstrated co-digestion using a pre-treated mixture of 85-15 vol% biodiesel wastewater with crude glycerol, any form of pretreatment adds to the capital and operating costs of treatment. To my knowledge, no researchers have investigated the possibility of co-digesting only crude glycerol and biodiesel wastewater without pretreatment.

Table 1: Examples of previously conducted studies on AD of biodiesel waste products, the organic loading rates studied (OLR), and the gas production as reported (*=converted to STP, **=assumed STP as temperature and pressure were not reported).

Biodiesel byproduct substrate	Co-digestion	Reactor type	Pretreatment	OLR (kg COD m^{-3} d^{-1})	Gas produced
Biodiesel wastewater (Phukingngam et al., 2011)	No	Anaerobic baffled reactor	Yes	0.5-3.0 (1.5 optimum)	0.4 L biogas g^{-1} COD removed**
Biodiesel wastewater (Siles et al., 2011)	No	Fed batch	Yes	0.4-3.0	0.297 L CH_4 g^{-1} COD removed
Crude glycerol (Siles López et al., 2009)	No	Fed batch	Yes	0.92-2.00	0.326 L CH_4 g^{-1} COD removed*
Crude glycerol + biodiesel wastewater (Siles et al., 2010)	No	Fed batch	Yes	1.33-1.60	0.284 L CH_4 g^{-1} COD removed*
Crude glycerol (Baba et al., 2013)	Yes	Continuous, pilot plant scale (30 m^3)	No	1.48	0.358 L CH_4 g^{-1} COD removed** (0.149 L CH_4 g^{-1} COD added)**
Crude glycerol (dos Santos Ferreira et al., 2018)	Yes	Batch	Yes	5.6 kg/m^3	0.08 L CH_4 g^{-1} COD added**
Crude glycerol (Ma et al., 2008)	Yes	Up-flow anaerobic sludge blanket	Yes	11.7	0.69 L biogas g^{-1} COD added (0.39 L CH_4 g^{-1} COD added)
Crude glycerol (Hutňan et al., 2009)	No	Up-flow anaerobic sludge blanket	No	6.5	0.53 L biogas g^{-1} COD added**
Crude glycerol (Kolesárová et al., 2011)	Both tested	Continuous mixed	No	1.3-2.2	0.52 L biogas g^{-1} COD added*

2.2.2 Obstacles to anaerobic digestion of biodiesel waste products

Anaerobic digestion uses a consortium of microorganisms to biologically degrade substrates. This mixed microbial consortium can be divided into four functional guilds corresponding to the steps of anaerobic degradation: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Angelidaki et al., 2011). The process is illustrated in Figure 2.

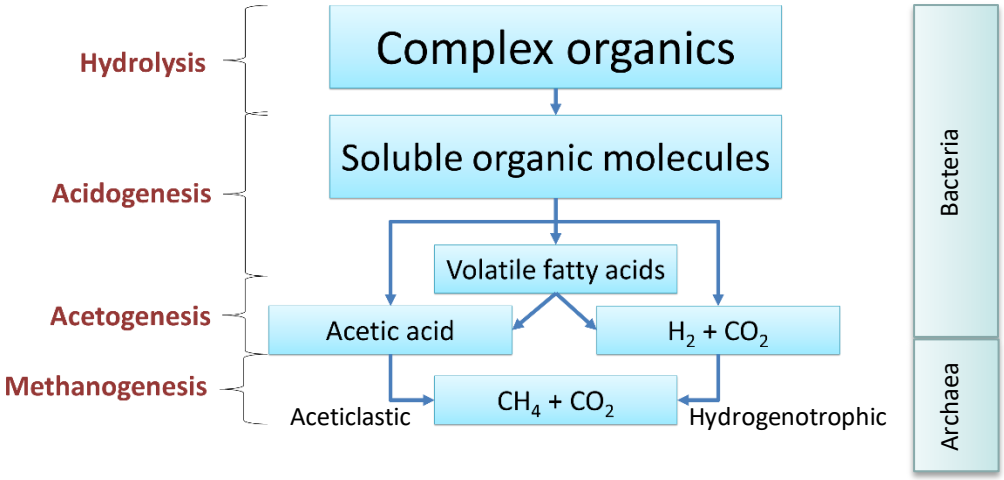

Figure 2: Flow diagram of the anaerobic digestion process.
Adapted from Angelidaki et al., 2011.

This microbial consortium is highly interdependent and thus susceptible to inhibition by many compounds, including byproducts from the AD process. Although glycerol is readily biodegradable, if the acidogenesis step proceeds too rapidly, the methanogens can be overwhelmed by the accumulation of acidic intermediates, which lowers the pH (dos Santos Ferreira et al., 2018).

Other inhibitors common in anaerobic digestion of biodiesel waste products include inorganic salts, such as chlorides or sulfates (dos Santos Ferreira et al., 2018; Viana et al., 2012), and long-chain fatty acids (LCFAs) (Viana et al., 2012). High salinity, or high ionic strength, increases osmotic pressure on cells and can lead to dehydration or inhibition of critical enzymes that play important roles in cell metabolism (dos Santos Ferreira et al., 2018). Solutions to this problem include dilution (dos Santos Ferreira et al., 2018; Suehara et al., 2005; Viana et al., 2012), co-digestion (Kolesárová et al., 2011; Siles et al., 2010), and acclimatization (Ma et al., 2008). Additionally, sulfur compounds increase competition among anaerobic organisms as sulfate reducing bacteria use the COD for their metabolism, producing hydrogen sulfide as an unwanted

gaseous byproduct (Viana et al., 2012). Finally, while LCFAs are biodegradable, at high concentrations they can adhere to cell walls, which either inhibiting cell function or causing flotation and thus washout (Viana et al., 2012). Acclimatization of digesters to high concentrations of LCFAs has been demonstrated (Rinzema et al., 1993). Other solutions often involve pretreatment of the biodiesel byproducts prior to use as substrates, which could add capital and operational costs to the overall treatment (Viana et al., 2012). Thus, the combination of gradual acclimatization to potentially inhibitory substrates and use of co-digestion is worth further investigation for AD of biodiesel byproducts.

Anaerobic digesters also require appropriate macro- and micronutrients in order to function properly. Carbon, nitrogen, and phosphorus are important macro-nutrients, and other researchers have outlined some of the critical micronutrients (Wheatley, 1990). As biodiesel byproducts often lack the needed nitrogen and/or phosphorus for AD, addition of these nutrients, or co-digestion with substrates that include these nutrients may be necessary (Baba et al., 2013; Hutňan et al., 2009; Kolesárová et al., 2011; Viana et al., 2012). In addition, researchers have discovered several other micronutrients that are critical for AD functions that may need to be added manually or by use of co-digestion substrates (Viana et al., 2012; Wheatley, 1990).

2.3 Acclimatization of anaerobic digesters

2.3.1 Gradual acclimatization of anaerobic digestion

Since anaerobic digestion is a biologically mediated process consisting of an interdependent and diverse microbial consortium, it has been hypothesized that some microbial consortium can be adapted, or acclimatized, to compounds or concentrations of compounds that would otherwise be inhibitory. Acclimatization to ammonia inhibition in particular has been researched in detail with some success (Yenigün and Demirel, 2013). In addition, it has been suggested that slowly increasing the organic loading rate could improve the digestion of LCFAs (Rinzema et al., 1993) and crude glycerol (Baba et al., 2013; Ma et al., 2008). This finding is of great importance in AD of biodiesel waste products since LCFAs are often present in crude glycerol. Others have shown similar mechanisms may improve tolerance towards other inhibitory compounds potentially present in biodiesel byproducts including salts (Viana et al., 2012). However, to my knowledge,

this is the first study specifically examining the impact of different rates of gradual acclimatization of inocula on a co-digestion system utilizing only CG and BDW as substrates. If acclimatization has a demonstrated impact on efficiency and stability, a clearer understanding of that impact could improve future research in the area of AD of biodiesel byproducts by opening additional possibilities for higher organic loading rates. It may also assist in optimization of start-up procedures for industrial AD of biodiesel byproducts.

3. MATERIALS AND METHODS

3.1 Inoculum and substrate characterization

Two inocula were selected based on the likely availability to a biodiesel manufacturer. The first inoculum was the effluent from a mixed waste mesophilic digester that co-digests a variety of agro-industrial waste, including some biodiesel waste products (AW). The second inoculum was the effluent from a mesophilic anaerobic digester from a local wastewater treatment plant (WT). The AW inoculum was collected on October 4, 2019 and the WT inoculum was collected on October 3, 2019. In both cases, the inoculum was used to start the digesters the same day they were collected. Feeding of the substrate began on October 5, 2019. The physical and chemical characteristics of each inoculum is shown in Table 2.

The substrates (biodiesel wastewater and crude glycerol) were collected fresh on October 4, 2019 from a local biodiesel company that generates biodiesel from cooking oil waste. The biodiesel wastewater (85 v/v%) was mixed with the crude glycerol (15 v/v%) at approximately the ratio that these substrates are produced at this biodiesel facility. This mixture is subsequently referred to as CGWW, and was stored at 4°C. No other substrates were added to the treatments. Pure glycerol (PG) was used for a positive control for each inoculum. No other substrates were added to the positive controls. The characteristics of the substrates are shown in Table 2.

Table 2: Physical and chemical characteristics of inocula and substrates.

	Total Solids (g solids kg^{-1} substrate)	**Volatile Solids** (g solids kg^{-1} substrate)	**Chemical Oxygen Demand** (g COD L^{-1})
WT	19	12	18
AW	100	73	71
CGWW	296	291	340
PG	990	991	1286

3.2 Digester design

As shown in Figure 3, each digester was composed of a 1-Liter Büchner flask (Bomex) capped with a #10 rubber stopper. The rubber stopper contained a single port constructed out of a 10 mL

Falcon pipet, which extended below the liquid surface. A piece of tubing was attached to the top of the port in order to allow the tubing to be closed between sampling and facilitated the removal of effluent and addition of substrate using plastic syringes. Gas was collected in either 1- or 3-Liter Tedlar bags with a septum and a valve. The gas bags were connected to the digester by tubing to the flask's side arm. Each digester also contained a magnetic stir bar.

Figure 3: Experimental apparatus schematic.

3.3 Experimental design and set up

The digesters were set up on the day that the inoculum was collected. For each digester, the mass of the digester was measured using an Adventurer ARA520 balance (OHAUS, Parsippany, NJ), then 750 mL of inoculum was measured using a graduated cylinder. The mass of the inoculum was measured after being added to the digester. The rubber stoppers were inserted and pushed tightly closed, with the tubing clamp sealing the digester. The gas bags were added to the tubing and the valves and tubing clamps were opened. The digesters were then placed in water baths with the temperature set point that matched the temperature of the digester of origin.

For this experiment, two inocula were used as described above. One blank (inoculum only) digester, one positive control (pure glycerol) digester, four slow acclimatization rate digesters (Treatment 1), and four fast acclimatization rate digesters (Treatment 2) were used for each

inoculum. The number of digesters used in each treatment (20 digesters total) are shown in Table 3 and the chosen rates are discussed further in Section 3.4.

Table 3: Experimental design: number of digesters and digester IDs, with water bath temperature per inoculum indicated.

Inoculum	**AW (T = 38.2°C)**		**WT (T = 38.9°C)**	
	Digester, n	**Digester ID**	**Digester, n**	**Digester ID**
Blank	1	A1	1	W1
Positive control	1	A2	1	W2
Treatment 1 (Slow)	4	A3-A6	4	W3-W6
Treatment 2 (Fast)	4	A7-A10	4	W7-W10

3.4 Digester operation

Feeding of the digesters began on October 5, 2019 and proceeded every other day. This approximately 48-hour spacing was likely sufficient for near-complete degradation of the substrates (Siles et al., 2011). Before the feeding process began, the digester was dried with a cloth and the mass was measured without the gas bag, with a tubing clamp closing off the tubing that was attached to the gas bag. During feeding, the digester was stirred using a magnetic stir bar. A sample of the effluent was removed using a plastic syringe through the feeding port, approximately equal to the mass that would be added. Although a minor vacuum was drawn using this method, the digester was kept sealed using tubing clamps during the entire operation. The digester was weighed again while the effluent sample's pH was measured. Another syringe was used to add the CGWW mixture (or pure glycerol for the positive control) of a predetermined amount equivalent to the amount removed and the digester was weighed again. The gas volume was measured using a 140-mL syringe.

Periodically, gas composition was determined using a 5000 Biogas Analyzer (LANDTEC North America, Inc., Colton, CA). If the gas volume was insufficient (<500 mL) to get a stable reading using the Biogas Analyzer, the biogas was diluted with air in a separate gas bag and the dilution ratio was used in the calculations to determine the final composition. Due to technical challenges with the composition measurements during the experiment, including some procedural changes in the sample collection and equipment used for measurement, the biogas composition was not measured daily. However, it was measured periodically, giving approximate estimates of its value over time. The biogas was vented from the gas bags following every volume measurement, thus

the biogas composition measured would have reflected the composition of the headspace during the last day or two since measurement.

Following feeding and measuring, tubing clamps were used to seal the feeding port. On days when the digesters were not fed, if the digesters produced excessive gas (i.e., the biogas volume exceeded gas bag capacity), the digesters were weighed and the gas measured, without feeding or removal of material.

Figure 4 illustrates the feeding schedule for each treatment as it proceeded during the experiment, as minor adjustments were made to the initial schedule based on failure of the digesters. Feeding proceeded until all the digesters in a treatment failed completely, meaning they ceased to produce any biogas. In a few cases, specifically AW Fast and AW Slow, a little substrate was reintroduced after a brief break to see if the digesters would recover. The digesters were usually monitored for several days after failure.

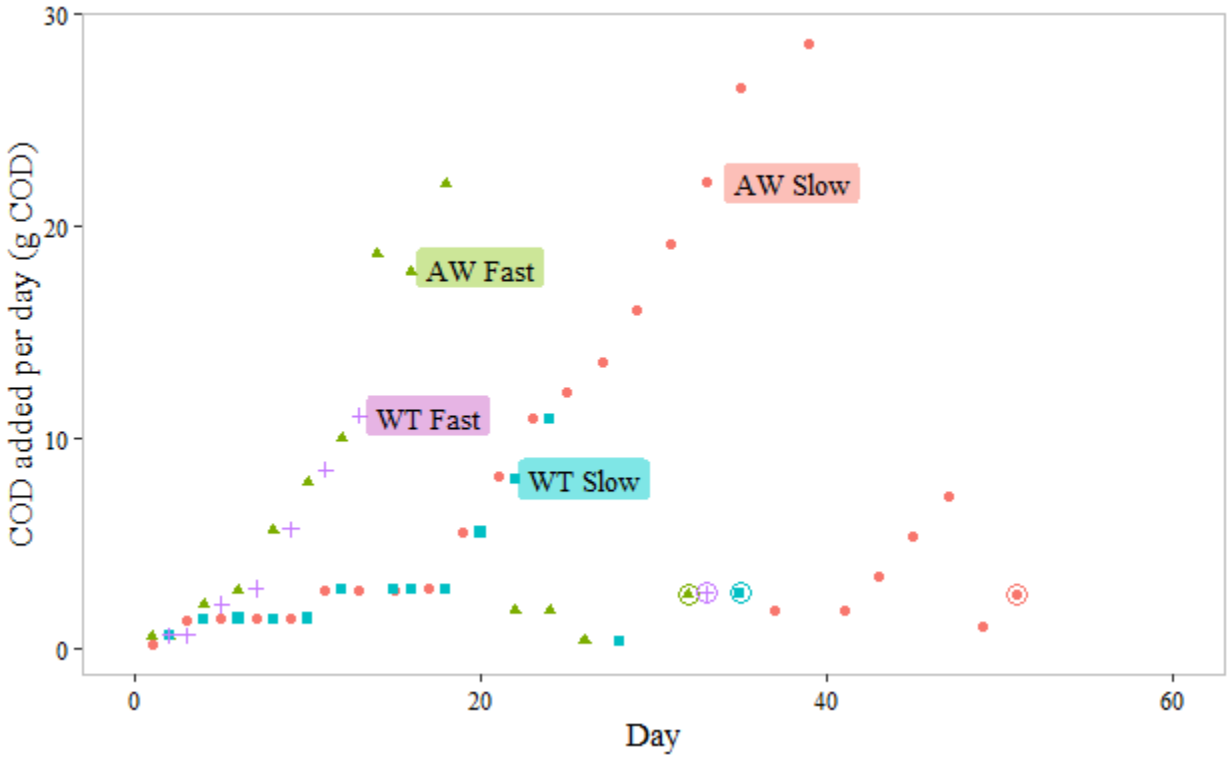

Figure 4: Feeding schedule for each treatment expressed as g COD added per day; addition of supplemental nutrients shown (○).

3.5 Supplemental nutrients

The digesters were spiked with a supplemental nutrient mixture after their efficiency had decreased severely in order to ensure that failure was not due to lack of micro or macro-nutrients. In this case, the failure of the digesters was defined as the point at which the efficiency dropped to close to zero; in other words, the cumulative biogas production plateaued despite the addition of substrate. The composition of this nutrient mixture, based on work done by previous researchers (Baba et al., 2013; Bougrier et al., 2018; Speece, 1983), is shown in Table 4 and the day of addition is indicated in

Figure 4.

Table 4: Supplemental nutrients added to digesters.

Chemical	**Source**	**Concentration in digesters** (g/L)
NH_4Cl	Baker	0.4170
KH_2-PO_4	Sigma	0.0033
$CaCl_2 \cdot 2H_2O$	Sigma	0.0052
$MgCl_2 \cdot 6H_2O$	Sigma	0.0052
$FeCl_2$	Mallinckrodt	0.0063
$CoCl_2 \cdot 6H_2O$	Mallinckrodt	0.0011
$NiCl_2 \cdot 6H_2O$	Acros	0.0011
$MnCl_2 \cdot 4H_2O$	Fisher	0.0012
Yeast extract	Fisher	0.0104
$CuSO_4$ (anhydrous)	Fisher	0.0032

3.6 Chemical and physical analyses

The total and volatile solids (TS and VS) analyses were conducted according to the Standard Methods of the APHA (1992). Chemical oxygen demand (COD) was measured using the Hach TNTplus Ultra-High Range COD Vial Test (TNT823) and a Hach DR3900 Benchtop Spectrophotometer (Hach Company, Loveland, CO). The ammonia nitrogen (NH_3-N) was measured using the Nessler Method and Hach Spectrophotometer (Hach Company, Loveland, CO). Dilutions were done on a mass basis to get samples to the needed detectable range for the chemical analyses. pH was measured using a Jenco Digital pH meter (Model 60), which was calibrated periodically.

3.7 Data analysis

3.7.1 Subtraction of inoculum contribution

For all digesters, the contribution of the inoculum to the volume of gas produced was subtracted prior to analysis. A fitted regression was used to estimate the contribution of the inoculum in the digesters based on the gas production of the blanks.

3.7.2 Statistical analysis

As biological systems, anaerobic digesters often experience a great deal of variability, even between biological replicates. As a result, this experiment used four replicates per treatment group. Most of the statistical analysis was done in R (https://www.r-project.org/). An ANOVA analysis of the cumulative biogas produced by each digester at the end of the experiment was conducted between all treatments. Tukey's test of the same metric was used to identify pair-wise differences between individual treatments. A significance level of $p < 0.01$ was used. Levene's test for homogeneity of variance was used to verify the appropriate use of ANOVA for this analysis. A Kologorov-Smirnov test was used to verify a normal distribution for each treatment. The cumulative biogas production at the end of the conclusion of the experiment was used as a metric to smooth out sample-to-sample variability that may occur on a day-to-day basis with biological systems.

One digester out of every treatment had a cumulative gas volume production at the end of the experiment outside of three standard deviations of the average, indicating significant variation from the other digesters in the treatment. Since biological variability is expected in AD systems, to examine further if these unusual digesters were outliers, Dixon's test for a single outlier was used (https://contchart.com/outliers.aspx). This approach was recommended in the literature for determining outliers for AD biomethane potential tests (Holliger et al., 2016). A p value of $p < 0.05$ was used to test for significance. Additional discussion of potential outliers is found in Section 4.1.

3.7.3 Analysis of gas volume measurements

Accurate measurement of gas production is a long-standing challenge for AD experiments. In previous experiments, the syringe method of volumetric measurement was shown to be adequate. However, other researchers have successfully used the difference in mass to determine the production of biogas (Hafner et al., 2015). Due to the time-consuming nature of the syringe method, it was of interest for this experiment to compare the mass difference and syringe methods to determine if the mass difference method could be employed for an experiment of this type (i.e., semi-continuous). To compare the two methods, the measured biogas volume using the syringe

method was converted to standard temperature and pressure (STP). The mass difference between feeding days (i.e. following the substrate addition one day and prior to the digestate extraction the next day) was converted to volume using the ideal gas law and STP. Initially, the gas composition was approximated as 60% methane and balance carbon dioxide due to the limited gas composition measurements. The justification for this estimate is found in Section 4.2.3. Later, actual composition measurements were also used to convert mass difference to volume.

4. RESULTS AND DISCUSSION

4.1 Outliers

Using the final cumulative gas volume of each digester as a metric, one digester from each treatment was identified as exceptionally different from the others in the treatment, as defined by a digester's gas production being outside three standard deviations of the treatment mean. Table 5 shows the digesters that were shown to be unusual by this definition. Using Dixon's test for one outlier and at a significance of $p < 0.05$, A5 and A10 were the only digesters that were shown to be true outliers.

Table 5: Digesters determined to be exceptional (based on cumulative biogas volume, end of experiment).

Treatment	Digester	True outlier?	High/Low biogas production	Treatment coefficient of variation excluding digester	Treatment coefficient of variation including outlier
WT Slow	W3	No	H	3.8%	9.3%
WT Fast	W7	No	H	11.6%	18.1%
AW Slow	A5	Yes	L	0.8%	11.1%
AW Fast	A10	Yes	H	5.8%	29.7%

The treatments between the two inocula statistically distinct; in other words, both WT treatments were different from both AW treatments. When all the digesters were included, the two AW treatments were not significantly different from each other at a p-value of 0.051, and the WT treatments were also not significant ($p = 0.47$). When the true outliers (see Table 5) were discarded, each of the treatments were statistically distinct from each other ($p < 0.001$). When only the most extreme outlier (A10), was discarded, the two AW treatments were significantly different, but the WT treatments were not significantly different ($p=0.057$).

To determine the source of the variability from the apparent outliers, data from total and volatile solids (mid- and end-point) and COD measurements were examined. In addition, the actual mass of substrate added throughout the experiment and the initial mass of the inoculum was reviewed. However, the source of the observed variations could not be determined definitively. A possible

explanation is digester-to-digester variability due to biological variation, but this could not be proven. As one of the two outliers had higher gas production (A10) than the other replicates, leaky digesters or gas bags are an unlikely explanation for that outlier. In addition, the other two digesters that exhibited exceptional behavior (W3 and W7) also had higher gas production. Since no clear qualitative reason for the outliers could be determined, the following analysis included all digesters. However, Figure 6 also indicates what the average gas volume production for the treatment would have been if A10 was not included.

4.2 Biogas production

4.2.1 Contribution of inoculum

The cumulative biogas volume produced by the inoculum only (or blank) digesters for both inocula appeared to follow a first-order rate law. Therefore, the Excel trendline was calculated based on this data. As demonstrated in Figure 5, the actual gas production for each blank (inoculum only) compared well to the equations used to fit the trendlines shown. These equations, weighted by initial mass of inoculum in each reactor, were used to calculate the contribution of the inoculum at any time point. This value was then subtracted from the measured gas volume.

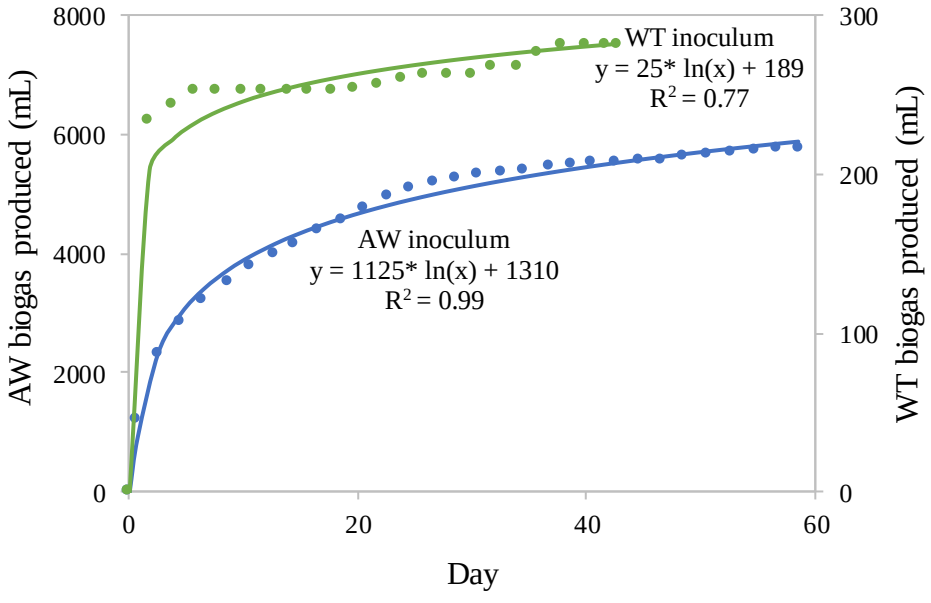

Figure 5: Comparison of measured blank (●) to fit equation used (line) to calculate contribution of the inoculum on each digester.

4.2.2 Cumulative biogas quantity

As daily biogas production varied per digester, the cumulative biogas production volume was used to indicate overall digester performance. Each digester's cumulative biogas volume was calculated, then an average for all the digesters in a treatment was calculated from the individual digesters (n=4). As shown in Figure 6, the WT treatments failed at a lower loading rate and with less cumulative biogas than the AW treatments. In this case, failure is defined as the complete cessation of biogas production while substrate is still being added, shown in Figure 6 as a plateau of cumulative biogas production. As discussed in Section 4.1, the WT treatments were statistically lower than the AW treatments. The difference between the WT and AW treatments is likely due to the AW inoculum containing a more active and robust microbial community as evidenced by a higher volatile solids concentration (see Table 2). In addition, it is possible that the additional residual COD leftover in the inoculum (see Table 2) stabilized the AW digesters while a rapidly biodegradable substrate was added. While this difference makes it difficult to compare the two inocula, it does indicate that the AW inoculum may be more robust and stable for this type of substrate. As a result, AW inoculum or similar could be preferable to WT inoculum or similar in other experiments of this type.

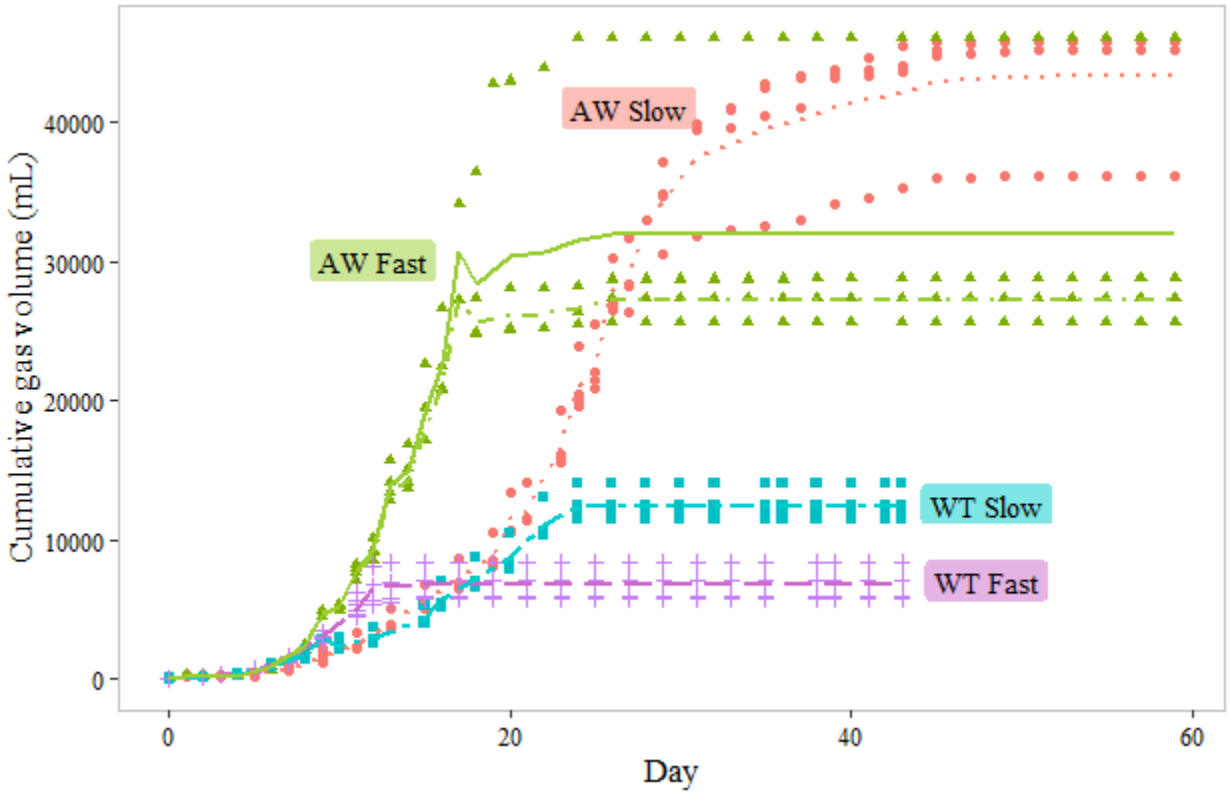

Figure 6: Cumulative volume of biogas produced (STP) for each treatment (n = 4 replicates), shown as individual measurements for each replicate (point) and the daily average (line); (-•- indicates average of AW Fast without the outlier A10).

However, as discussed in Section 4.1, the AW Fast and Slow treatments were only statistically different from each other when A10 was excluded (mean for AW Fast with outlier excluded shown with a dashed line on Figure 6). The WT treatments were not statistically significant from each other unless both true outliers (A5 and A10) were excluded.

4.2.3 Biogas quality

Figure 7 shows the gas composition measurements by treatment type after day 15, when composition measurements started. Since the measurement accuracy below a dilution of <50 mL of biogas was not verified, only the measurements using >50 mL of biogas are shown. Although distinct trends are difficult to distinguish, most digesters were producing biogas at a composition of around 60% during the first several days of measurements. However, over time the fast digesters declined rapidly in methane composition. This decline in methane production appears to correlate

approximately with the timing of conversion efficiency (see Section 4.3). Although there was insufficient data to make conclusive statements about the biogas composition or the overall methane production, 60% methane is used as a benchmark for other comparisons when a composition is needed. Prior to the drops in biogas composition for each treatment due to the decrease in efficiency, it appears that the biogas composition was generally close to 60% methane ±10%. However, since there is a lack of composition data, this can only be considered a rough approximation.

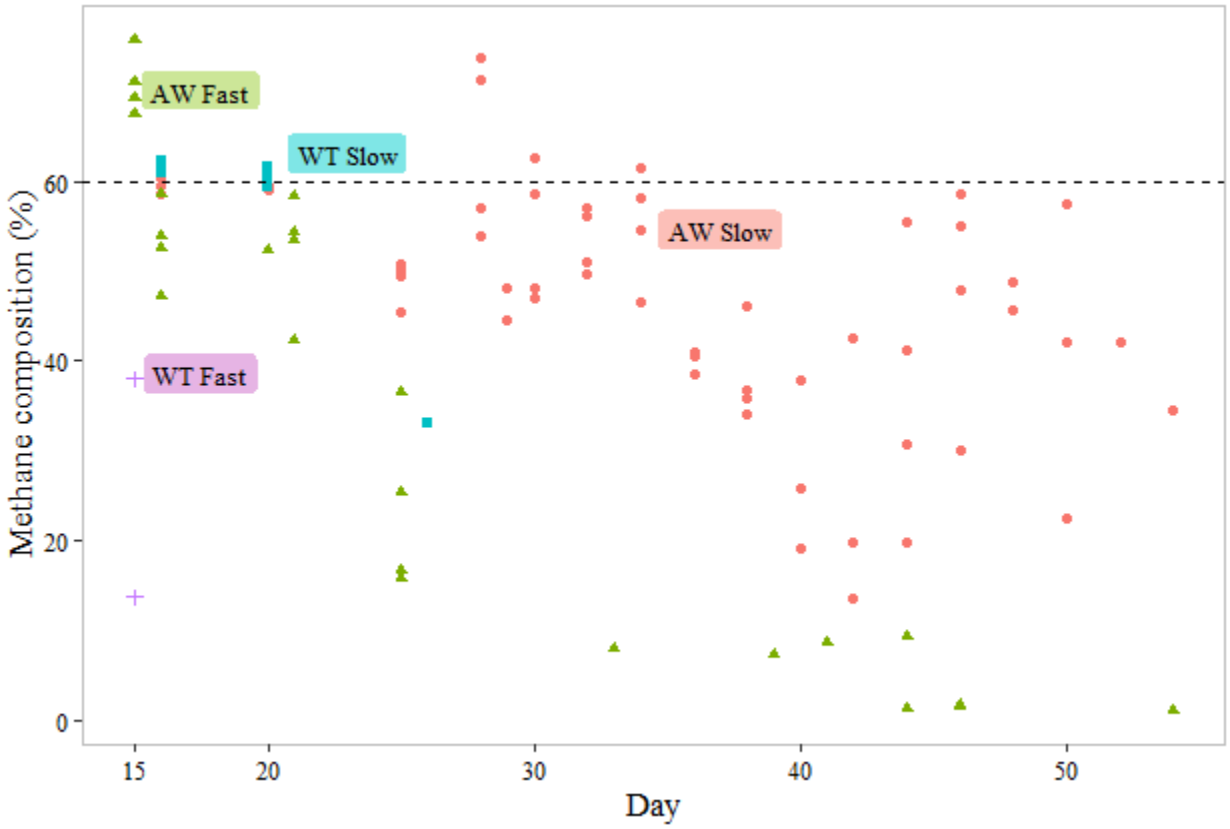

Figure 7: Gas composition over time by treatment type.

4.3 Conversion efficiency

For this experiment, conversion efficiency was defined as the volume of biogas produced per mass of COD added during the previous feeding occurrence. Although a daily conversion efficiency results in some noise due to residual COD, the trends are more visible with a daily conversion efficiency than a cumulative overall efficiency. The daily conversion efficiency was calculated for each digester, then the averages were calculated for each treatment. The conversion efficiency (X)

calculation is shown in Equation 1, where V = volume of biogas produced (mL, STP), m = mass of COD added (g COD), and the subscripts indicate the day on which the measurement was taken (i.e., 2 = day 2, 1 = day 1).

$$X_2 = \frac{V_2}{m_{COD,1}} \quad \text{Eq. 1}$$

The conversion efficiency of the slower acclimatization rate treatments was higher for a longer period of time, as demonstrated in Figure 8. In fact, for a maximum theoretical yield of 350 mL methane per gram of chemical oxygen demand (COD), (Heidrich et al., 2011) and assuming biogas composition of around 60%, which is a reasonable assumption as explained in Section 4.2, the AW Slow treatment was close to the theoretical maximum conversion (583.3 mL biogas g^{-1} COD) for several days. The others achieved maximum average efficiencies close to this (see Table 6), but for a shorter time frame. In addition, both slow treatments had a longer "plateau" period close to their average maximum efficiency than the Fast treatments. It is also clear from this figure that there was a tipping point long before complete failure at which the efficiency dropped dramatically. This phenomenon is marked on Figure 8 using colored, dashed lines and will be discussed in more depth in Section 4.4. No significant improvements to efficiency occurred following the addition of the nutrient supplemental solution, although it is possible that it was added too late to aid in recovery.

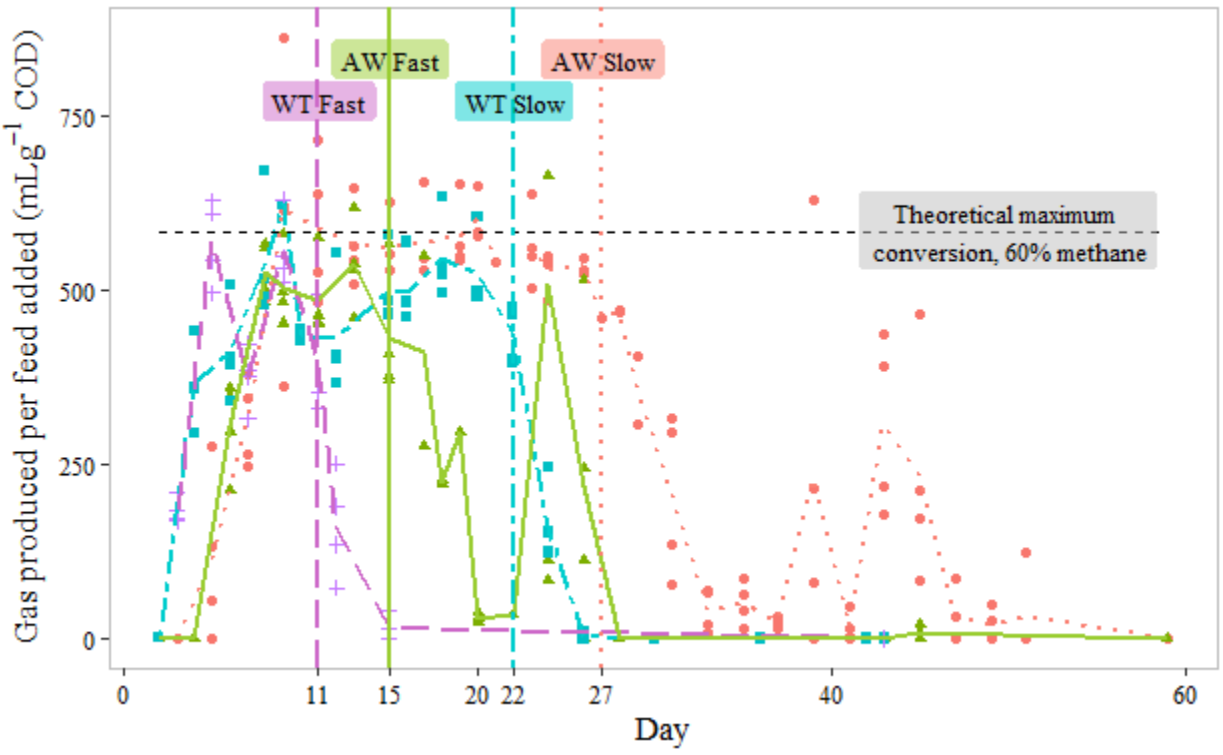

Figure 8: Efficiency of treatments as expressed by biogas volume produced per amount of COD added the previous feeding day (n = 4 replicates), shown as individual measurements for each replicate (point) and the daily average (line).

4.4 Maximum total load and failure

4.4.1 Cumulative total load prior to failure

Figure 9 shows the cumulative total COD in the form of CGWW added to each treatment prior to complete failure. Clearly, for both inocula, a higher cumulative total COD added was achieved; however, this difference was more dramatic for the AW inoculum than the WT inoculum.

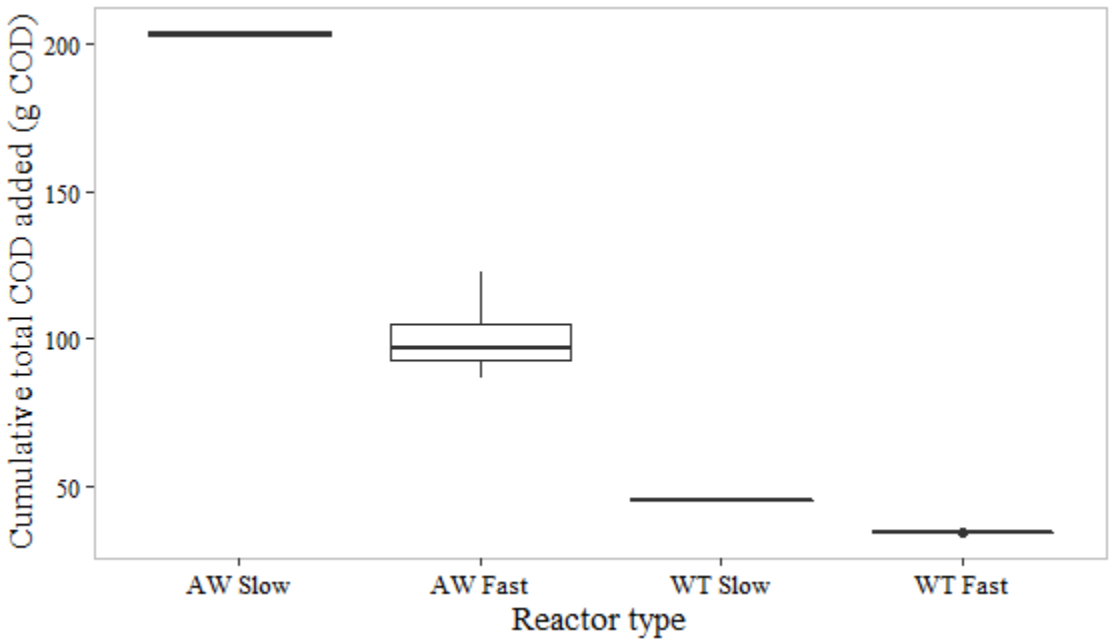

Figure 9: Total Cumulative COD load for each treatment.

4.4.2 Load prior to decreased efficiency

As Figure 8 demonstrates, after a plateau of maximum efficiency, each treatment rapidly dropped in efficiency, later followed by complete failure. The digesters continued generating a significant amount of gas after this point of decreased efficiency prior to total failure, however. As shown in loading rates are sustainable in full-scale digesters.

Table 6, both the cumulative mass of substrate added to the digesters prior to the drop in efficiency and the organic loading rate (OLR) prior to the efficiency drop was higher for the slow acclimatization rates for the WT treatments. However, despite the higher cumulative COD loading for the slower AW treatment, the OLR prior to the efficiency drop was lower on average for those treatments. This indicates that although the slower AW treatment could handle a greater cumulative amount of the substrate, the gradual acclimatization rate did not necessarily increase the organic loading rate. Nevertheless, despite this similarity in tipping points, the WT Slow digesters achieved a much higher cumulative load prior to failure due to the slower acclimatization speed. The OLR prior to the dramatic drop in efficiency for all treatments was in the range of OLRs that other researchers have studied for biodiesel byproducts, but on the higher end in comparison (see Table 1) (Baba et al., 2013; dos Santos Ferreira et al., 2018; Hutňan et al., 2009;

Kolesárová et al., 2011; Ma et al., 2008; Phukingngam et al., 2011; Siles et al., 2011, 2010; Siles López et al., 2009). Longer term experiments at a larger scale would be needed to determine if these loading rates are sustainable in full-scale digesters.

Table 6: Maximum efficiency and loading rate prior to drop in efficiency.

	AW Slow	AW Fast	WT Slow	WT Fast
Maximum average efficiency (mL biogas g^{-1} COD added)	615	535	621	568
Day average efficiency dropped >10%	27	15	22	11
OLR prior to drop (g COD L^{-1} day^{-1})	5.1	6.8	3.0	1.6
Cumulative substrate added prior to drop (g COD)	68	49	31	16
Maximum OLR prior to complete failure (g COD L^{-1} day^{-1})	10.7	7.0	4.1	4.1
Cumulative total COD added (g COD)	203	101	45	34

4.4.3 Ammonia overloading

Although this experiment did not specifically investigate the cause of failure in the digesters, samples from one digester from each treatment group revealed relatively minor concentrations of ammonia nitrogen. As shown in Table 7, none of the treatments reached ammonia concentrations above 3 g NH_3-N L^{-1}, which has been shown to be inhibitory in similar AD systems (Yenigün and Demirel, 2013). Additional studies could include further research into the mechanism for failure for these digesters.

Table 7: Ammonia content in digestate at end of experiment.

Digester ID	Treatment	Ammonia measurement (g NH_3-N L^{-1})
A5	AW Slow	1.92
A9	AW Fast	2.66
W4	WT Slow	0.53
W10	WT Fast	0.54

4.5 Gas volume measurement methods comparison

Finally, part of this experiment included determining whether the method of using mass difference to measure biogas production could be applied for an experiment of this type, as it was previously used with success by other researchers (Hafner et al., 2015). The syringe method (a volumetric method for measuring biogas production) used for this experiment was compared to mass difference data collected during the experiment.

Figure 10 illustrates the discrepancy between the gas volume measurements and the mass difference converted to volume.

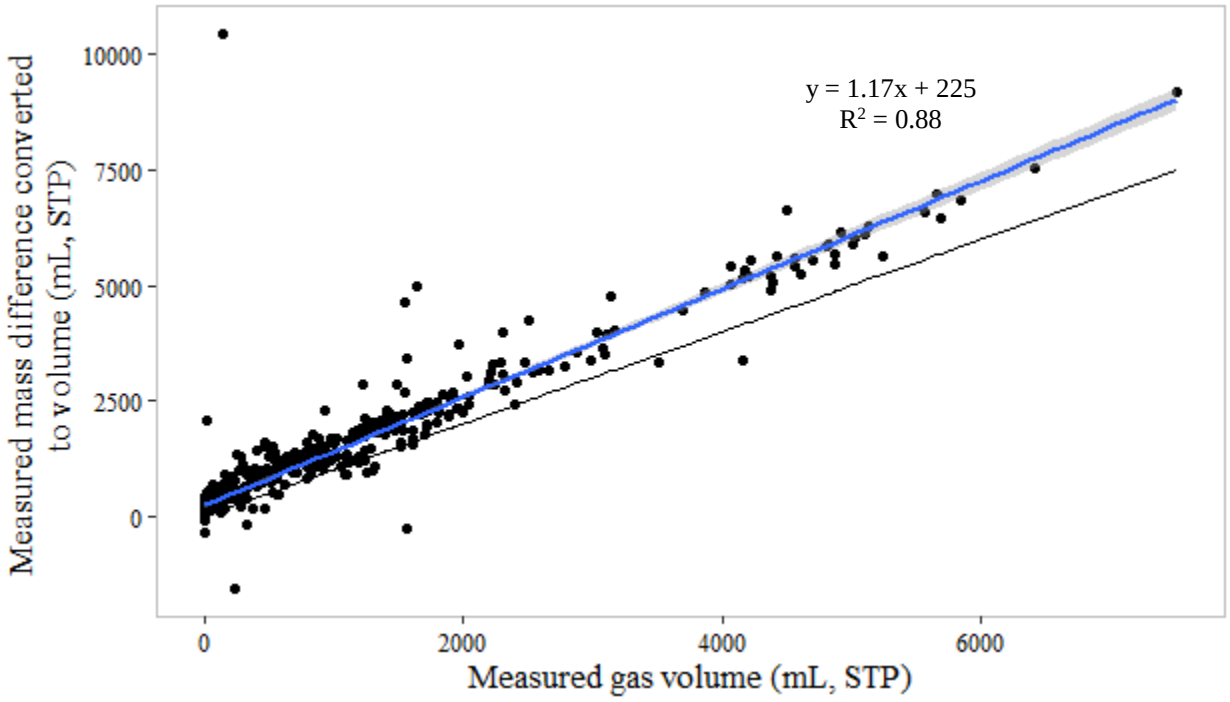

Figure 10: Comparison of daily biogas volume measurements directly using a syringe and calculated from mass difference (using 60% methane); black line represents the hypothetical ideal, or perfect agreement between the two values, and blue line is the linear regression.

As demonstrated in Figure 10, there are significant differences between the two methods of measurement. The slope is 17% higher than ideal, meaning that at higher measured masses and volumes the two calculated values diverge further. In addition, there is a clear offset to the values. Over time, these differences make a significant impact on the cumulative biogas production. One

cause of this discrepancy may have been the lack of composition data as accurate gas quality measurements are essential for correct conversion of the mass difference to volume. As discussed in Section 4.2, during this experiment, gas quality was not measured consistently, and gas composition measurements began late. As a result, some discrepancy can be anticipated between the two measurement types. In an additional analysis, shown in Figure 11, the limited composition data available was used to calculate the gas volume from the mass difference. In this analysis, a linear regression gives a slope of 1.04 and y-intercept of 309 with an R^2 of 0.97, which is significantly better.

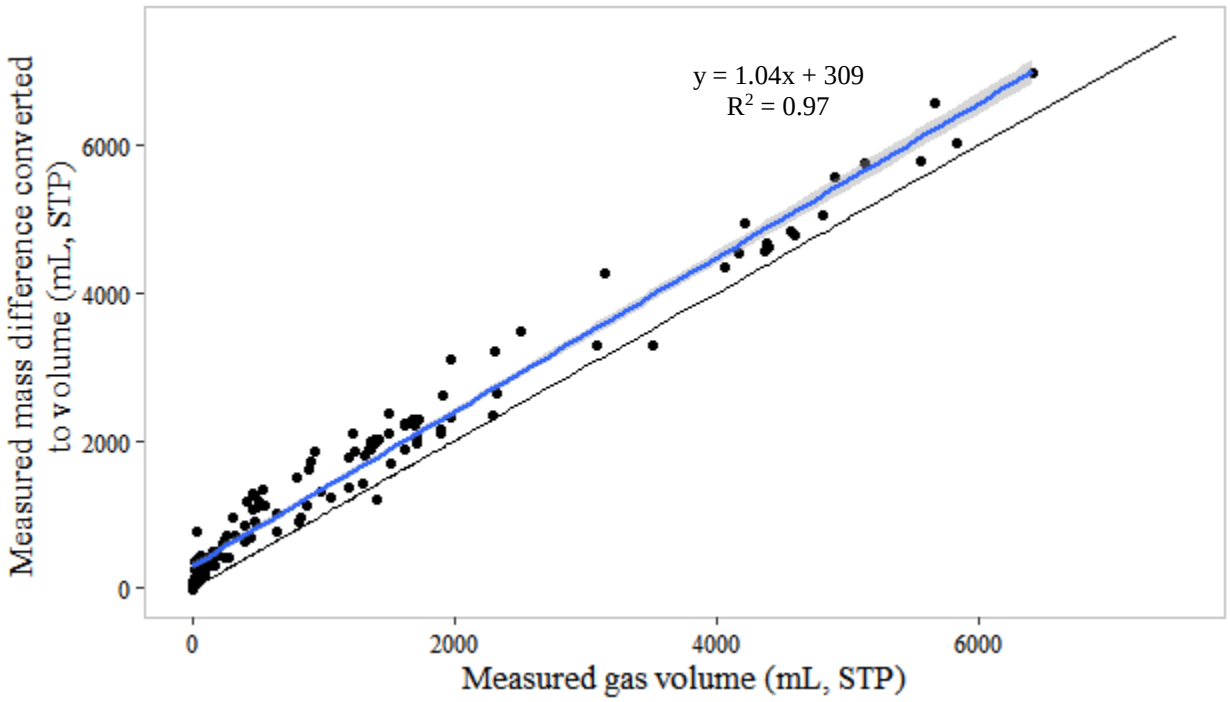

Figure 11: Comparison of daily biogas volume measurements directly using a syringe and calculated from mass difference (using composition data); black line represents the hypothetical ideal, or perfect agreement between the two values, and blue line is the linear regression.

Since this second regression gives a slope of close to one but with a y-intercept much higher than zero, I hypothesize that the steepness of the slope shown in Figure 10 is related to lack of composition data and that the offset is due to instrumental error. The data does indicate that digester mass loss can be linearly correlated with gas production. This is promising, as this method could be used in the future for verification of gas volume measurements or even as an alternative

measurement for gas production. However, a more complete data set including more gas composition measurements would be needed for additional validation,

Another cause of the discrepancy could be an inherent challenge in measuring the mass of the digesters at close to the scale's maximum capacity. Although calibration of the scale was verified prior to beginning the experiments, the filled digesters were approximately 1500 g, which was close to the scale's capacity to measure. The calibration masses were 50 g or less. As a result, if the calibration was off close to the scale's capacity, some error could have been introduced. Additional calibration and experiments would be needed to determine final causes. The data used for the data analysis for this thesis came from the direct volume measurement using the syringe method.

5. CONCLUSION

In conclusion, it is possible to co-digest crude glycerol and biodiesel wastewater at high OLRs without addition of other substrates or pretreatment. This study demonstrated that for the rates tested a slower acclimatization rate improved efficiency and stability at higher loading rates for the AW inoculum. The slower acclimatization rate also resulted in slight improvements in maximum cumulative load for the WT inoculum. The greatest efficiency (>600 mL biogas g^{-1} COD) and stability over time (efficiency drop at day 27) were observed with the AW Slow treatments, which may imply that a slow acclimatization rate with a robust inoculum would be preferable for future experiments.

In addition, for the inocula and rates tested a slower acclimatization rate results in improved maximum total load. Conversion efficiency from chemical oxygen demand to biogas remains higher for a longer period of time for the slower acclimatization rates. The AW inoculum (effluent from an agro-industrial waste digester) achieved higher loading rates than the WT inoculum (effluent from a domestic wastewater treatment plant). Therefore, additional research should be conducted on similarly robust inocula in the future.

Finally, it was shown that using the method of mass difference to monitor gas production was correlated closely to the volumetric measurements using the syringe method, which is promising for future use of this method as a validation method or alternative gas production measurement. However, this method does require some additional refining prior to use in future studies of this type. For example, accurate biogas composition is critical for accurate comparison. In addition, more thorough calibration checks on the balance at masses close to balance capacity would be needed.

5.1 Future work

In the future, researchers could build on the research done here by studying additional acclimatization rates and investigating the cause of process failure for a similar system. Methodically studying many acclimatization rates may enable optimization of start-up procedures

for AD systems of biodiesel byproducts and could lead to improvements in start-up procedures for AD of additional substrates. In this study, the addition of supplemental nutrients may have come too late to improve process performance. Further research on this could be beneficial. Similarly, it may be useful to definitively determine the cause of failure as this was not demonstrated in this study. Specifically, monitoring concentrations of ammonia, inorganic salts, and long-chain fatty acids may be instructive. In addition, a similar experiment employing microbial community analysis could reveal deeper insight into the changes the microbiome may experience due to the differences in acclimatization rate.

Additional research would be needed to determine the feasibility of this strategy for biodiesel companies. For example, a longer-term experiment at high loading rates at pilot-scale could help determine if the organic loading rates and acclimatization rates studied here would be feasible for a full-scale digester on-site at a biodiesel production facility. Finally, a similar experiment with different digester configurations, such as an up-flow anaerobic sludge blanket reactor, may give insight into design considerations for full-scale digesters.

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