Effect of Low Aeration and Heat Drying on Anaerobic Digester Performance and Microbial Community

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By

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Abstract

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This thesis describes three research projects on anaerobic digestion (AD) that investigated functional differences and relationships between microbial composition and digester function. Both archaeal and bacterial communities were characterized using high throughput (Illumina) sequencing technology with universal 16S rRNA gene primers.

In the first project, limited aeration, and limited aeration with an aerotolerant enrichment culture were investigated as possible methods to increase digester functional performance in comparison to a strictly anaerobic control digester. No functional differences were observed at quasi-steady state between the limited aerated digester (LAD), limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE), and strictly anaerobic control digester (ACD). After limited aeration, Total Kjeldahl Nitrogen (TKN) concentration was less for the LAD than the ACD, and percent difference between the LAD and ACD total TKN concentrations was 6.5%, ($p=0.044$, $n=5$). Specific methanogenic activity (SMA) tests for acetate, propionate, non-fat dried milk, and non-fat dried milk and air determined that the LAD did not show an increased methanogenic rate in comparison to the ACD. However, the LADE had a greater SMA with propionate than the ACD. At quasi-steady state operation, differently operated digesters were shown to contain similar archaeal communities during steady state operation. The LAD, LADE, and ACD had a high relative abundance of *Methanosaeta*, an acetoclastic methanogen, which ranged from 58 to 77% in each digester.

In the second project, a novel enrichment technique, that involved exposing methanogenic sludge to >100°C for 24 hours, was used to develop a culture dominated by hydrogenotrophic rather than acetoclastic methanogens and potentially establish the presence of syntrophic acetate oxidizing bacteria (SAOB). The enrichment technique was used on sludge samples obtained from the LAD, LADE, and ACD. LAD, LADE and ACD cultures exposed to high temperature drying were referred to LAD-D, LADE-D and ACD-D, respectively. These dried cultures were then rehydrated and used to seed new enrichment cultures. In SMA tests with non-fat dried milk and acetate, the LAD, LADE, and ACD (wet cultures) had statistically greater SMA than the LAD-D, LADE-D, and ACD-D (dried cultures) enrichments. In contrast, the dry culture enrichments had significantly greater SMA than wet cultures in the H_2/CO_2 SMA tests. The SMA decrease with non-fat dried milk was correlated to an increased relative abundance of *Rikenella.* Increased SMA with H_2/CO_2 was correlated to an increase in relative abundance of hydrogenotrophic methanogens such as *Methanobacterium* and *Methanothermobacter*, and a decreased *Methanosaeta* relative abundance.

The third project investigated the effect of ammonia-nitrogen (NH_3-N) concentration on the methanogenic rate of wet and dried cultures. Wet cultures had a higher SMA than dried cultures at 0, 3, 6, and 9 g/L NH_3-N. However, heat drying increased the subsequent SMA value with H_2/CO_2. Dried cultures also had a higher relative abundance of *Methanosarcina siciliae*. Further research is needed to investigate enrichment and survivability of *Methanosarcina siciliae* at higher temperatures. *Methanosarcina siciliae* has potential use as an augment to reduce and extract energy from methanol, trimethylamine, and dimethyl sulfide (DMS) present in AD systems.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

Anaerobic digestion (AD) is the process of converting organic matter, in the presence of microorganisms, to byproducts such as methane (CH_4) and carbon dioxide (CO_2). The AD process is both naturally occurring, such as in rivers, peat bogs, and sediments, and in engineered systems like water resource recovery facilities (WWRFs) (Botheju and Bakke, 2011). Phases of AD include hydrolysis, fermentation (acidogenesis), acetogenesis (acetate generation) and methanogenesis (CH_4 generation) (Rajeshwari et al., 2000). AD has applications in municipal and industrial applications in degradation of organic matter for production of CH_4. In comparison to aerobic systems, AD provides advantages including lower operational costs from lack of aeration requirements, production of CH_4, which can be used to operate plant equipment and heat the digesters, and less biomass production, which reduces sludge handling costs (Suryawanshi et al., 2010).

Environmental factors such as temperature and pH effect digester stability, as well as substrate characteristics and organic loading rate. More specifically, microbial inhibition can result from high concentration of ammonia or other potentially inhibitory chemicals. High ammonia concentration, for example, can result in a shift from ammonium (NH_4^+) to free ammonia (NH_3 or FA), which is inhibitory to the growth of methanogens. High free ammonia concentration can also cause microbial inhibition through the increase of intracellular pH (Wittmann et al., 1995; Chen, Ren, Wang, Liu, & Lee, 2010). Syntrophic acetate oxidizing bacteria (SAOB) have been shown to convert

acetate to H_2/CO_2 in NH_3 concentrations greater than 3 g/L, which is beneficial to AD systems that have influent NH_3 concentrations such as slaughterhouse waste. Therefore, this thesis describes one research project to develop a methanogenic culture potentially containing SAOB and hydrogenotrophic methanogens that were hypothesized to increase digester function when free ammonia concentrations were high.

Three projects are described in this thesis. For the first project, the effects of adding a limited oxygen loading based on 10% chemical oxygen demand (COD) influent to anaerobic digesters were investigated. Oxygen loading rate was based on previous work done by (Bocher et al., 2015). Three digesters were operated with biomass from an anaerobic control digester (ACD), limited aerated anaerobic digester (LAD), and limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE) to monitor for changes in functions. Digesters were each fed a synthetic industrial wastewater (non-fat dried milk) and operated at a 10-day HRT for LAD and ACD or 5-day HRT for LADE. Functional parameters measured periodically throughout quasi steady state period from days 270 to 290 included pH, influent COD, effluent COD and soluble COD (SCOD), volatile fatty acids (VFAs), and CH_4 concentrations. Based on work by (Tale et al., 2015), it was expected that the addition of an aerotolerant culture would improve anaerobic digester function with an increase in CH4 production, and reduction in COD.

For the second project, the effects of exposing methanogenic biomass to low oxygen concentrations and to drying at >100°C for 24 hours were initially investigated to develop a methanogenic culture containing significant populations of hydrogenotrophic methanogens. Three 15 L anaerobic digesters were fed non-fat dried milk in basal

nutrient solution (Speece, 2008), at a COD loading rate of 1 g/L-day and a 10-day HRT. The LAD, LADE, and ACD digesters were operated for over 6 months to attain quasi steady state. After which, 1L of the daily effluent biomass of the three digesters was centrifuged to 30 mL, and then dried at 104°C for 24 hours and stored in a desiccator. This process was performed for 2 months to obtain at least 40 grams of dried biomass. Dried biomass of each digester was then used to seed three sets of five 500 mL anaerobic digesters. These sets of digesters are the limited aerated digester-dried (LAD-D), limited aerated digester with augmented with an enrichment culture-dried (LADE-D), and anaerobic control digester-dried (ACD-D) based on the source of the dried seed biomass. Each digester received an initial 4 grams of biomass and 400 mL of a basal nutrient solution containing essential inorganic nutrients (Speece, 2008) for a solids concentration of 10 g/L (total solids) TS. R studio was used to perform nonmetric multidimensional scaling (NMDS) plots, using the Double Wisconsin Transformation, of effluent of anaerobic community sequence data gathered from Illumina sequencing.

The objective of the third project was to investigate NH_3-N inhibition between the 15L biomass and rehydrated dried biomass. An anaerobic toxicity assays (ATA) test was performed at varying NH_3-N concentrations from 0,3,6 and 9mg/L NH_3-N. ATA assays were monitored for methanogenic rate and compared to the wet and dry culture microbial community data gathered from Illumnia sequencing.

2. Overview of Anaerobic Digestion

2.1 Introduction

In the AD processes, with the absence of electron acceptors such as oxygen, nitrate, or sulfate, organic matter is degraded by differing trophic groups. Methane (CH_4) is also 50-70% of the biogas, while carbon dioxide (CO_2) and other gases in small sum are 30-40% (Batstone et al., 2002). Facultative and fermentative bacteria initiate digestion, through the process of hydrolysis of initial proteins and polysaccharides. Butyric acid is one possible intermediate product and is typically more rapidly degraded than propionate (Boone and Xun, 1987). Fermentation of organic acids results in end products such as acetate, formate, methanol, H_2, and CO_2 by the process of acidogenesis. The final step is methanogenesis. Methanogens are classified as anaerobic archaea, which then can be sub-divided into hydrogenotrophic species or acetoclastic methanogens. Hydrogenophilic or hydrogenotrophic species form CH_4 by reduction of H_2/CO_2, and acetoclastic or acetotrophic methanogens generate CH_4 by acetate decarboxylation (Rajeshwari et al., 2000; Veeken et al., 2000). Each step in the AD process is a component of a different microbial trophic group, which may rely on the microbes of different steps for substrate production.

Zitomer & Shrout (1998), who studied the effects if different air and oxygen headspace volumes in anaerobic batch reactors, determined that limited aeration application was a potential strategy for improving AD performance. Lim & Wang (2013) showed that limited aeration was successful in enhancing hydrolysis during anaerobic

digestion. As hydrolysis step degrades both insoluble organic material and molecular compounds such as lipids, polysaccharides, proteins, and nucleic acids into products that are further used in the acidogenesis and methanogenesis stages, hydrolysis is sometimes the rate limiting step in CH_4 production (Dewil et al., 2007). Enhancing the hydrolysis stage is important, as it may lead to a larger CH_4 yield from anaerobic digesters. Zitomer & Shrout (1998) showed that aerated complete-mixed digesters recovered from pH decrease more quickly than un-aerated reactors. In a study by Tang, Shigematsu, Ikbal, Morimura, & Kida (2004), researchers determined that limited aeration did not significantly alter phylogenetic diversity in the observed thermophilic (53°C) anaerobic digesters. Therefore, limited aeration may not result in dramatic differences in microbial community structure. Understanding of oxygen effects in anaerobic digestion is important to the research of this proposal as biosolids were dried in an atmosphere that contained oxygen.

2.1.2 Microbial Communities in Anaerobic Digestion

The AD process is carried out by organisms in different trophic groups. These groups are characterized as hydrolysis, acidogenesis, syntrophic acetogenesis, and methanogenesis groups. Hydrolysis is the process in which extracellular enzymes breakdown complex polymers and monomers into products such as glucose, amino acids, and particulate and soluble inert material (Batstone et al., 2002). Solubilization of these organic compounds are accomplished by hydrolytic bacteria. Due to volatile fatty acid (VFA) accumulation being limited by the slow rate of extracellular breakdown of these complex monomers, hydrolysis is often seen as the rate limiting step of the AD process. Hydrolysis rate is dependent on particle size, pH, and production, diffusion, and

adsorption of extracellular enzymes (Aldin, 2010). Previous techniques to increase VFA production and solubilization of organic compounds in the hydrolysis step have included limited aeration, thermal pretreatment, chemical and physical treatment, enzymatic treatment, and alkaline digestion (Lim and Wang, 2013; Yi et al., 2013)

Soluble organic matter is further converted to VFAs in the acidogenic (acidification) stage. VFA production is accomplished from two separate groups of aciodgens which convert the hydrolyzed organic matter into hydrogen (H_2), CO_2, and VFAs (Batstone et al., 2002). VFAs produced include acetate, propanoate, butyrate, iso-butyrate, iso-valeric, and valeric. The acidification stage is predominantly composed of facultative anaerobes which include the genera: *Pseudomonas*, *Bacillus*, *Clostridium, Micrococcus*, or *Flavobacterium* (Krzysztof Ziemiński, 2012).

Syntrophic acetogenesis involves the conversion of VFAs into acetate, H_2 gas, and CO_2 by two separate acidogenic groups. These groups degrade butyrate, valerate, and propionate (Batstone et al., 2002). Included in these acidogenic groups are the genera *Syntrophomonas* and *Syntrophobacter*. These bacteria convert VFAs into acetate and H_2. *Methanobacterium suboxydans* degrades pentanoic acid to propionic acid, and *Methanobacterium propionicum* degrades propionic acid to acetic acid (Liu et al., 2004).

Methanogens are classified as anaerobic archaea, which then are sub-divided into hydrogenotrophic species or acetoclastic methanogens. Hydrogenotrophic species form CH_4 by reduction of H_2/CO_2, and acetoclastic methanogens generate CH_4 by acetate decarboxylation (Rajeshwari et al., 2000; Veeken et al., 2000). Methanogenic archaea and sulfur reducing bacteria (SRB) are also responsible for consumption of H_2 gas (Tang et al., 2004). Inhibition of methanogens can result from pH change, hydrogen, and free

ammonia (FA) (Batstone et al., 2002). Hydrogen sulfide (H_2S), which is produced by SRBs is also inhibitory to methanogens at high concentrations (approximately 50 mg/L or more) (Speece, 1996).

2.2 Limited Aeration in Anaerobic Systems

2.2.1 Introduction

Limited aeration, which refers to the addition of small quantities of air, may enhance biochemical process and stabilize operating conditions in AD systems (Msontalvo et al., 2016). Limited aeration can also increase biomass production and methanogenic activity. Zitomer & Shrout (1998) determined that methanogenic activity can be similar or greater in micro-aerated systems than strictly anaerobic ones.

CH_4 generation did decrease with increasing oxygen loads for zero to 2.5%, 5%, and 10% (% of chemical oxygen demand (COD) influent load) (Botheju et al., 2010). However, no inhibition or damage to the biomass was seen from oxygenation. Increased CO_2 production has been observed at increasing oxygen levels. Oxidation of readily available organic substrate leads to increased aerobic respiration (Johansen and Bakke, 2006; Zhu et al., 2009). Díaz & Fdz-Polanco (2010) in a study with pilot scale AD systems showed that limited oxygen supply did not affect maximum CH_4 production. However, limited oxygen addition did not reduce average CH_4 production in comparison to the non-aerated AD control systems.

In previous studies limited aeration has been shown to have a positive effect on CH_4 production. Nguyen, Kuruparan, & Visvanathan (2007) noted increased reactor performance with an increase in biogas and CH_4 production, and an increase in volatile

solids (VS) and COD removal with limited aeration. Botheju, Lie, & Bakke (2010) and Botheju, Saramakoon, et al. (2010) showed a positive response in tested AD systems with limited aeration from an oxygenation load range of 0-16% of feed COD. Fu, Wang, Shi, & Guo (2016) saw an increase in CH_4 yield and VS removal efficiency by 16.5% and 10.3% in comparison to an anaerobic control.

According to Luostarinen, Luste, Valentin, & Rintala (2006), COD removal with intermittent aeration improved from 42 to 68%. COD removal also decreased after each sequential decrease of aeration, which shows that limited aeration did positively impact COD removal efficiency in the tested AD system. In a study of limited aeration and leachate replacement by Jagadabhi, Kaparaju, & Rintala (2010), a limited aeration flow rate of 1 L/min resulted in a significant increase of VFA production and no increase in effluent soluble COD (SCOD). Acetic acid was 80% of total VFAs in the leach-bed reactors. A 4 L/ min aeration rate resulted in a decrease in SCOD for tested one-stage leach-bed reactors. Results from aeration of the one-stage leach-bed reactor showed that limited aeration can be used to improve the conversion of SCOD into VFAs.

2.2.2 Increased Hydrolysis from Limited aeration of AD Systems

Hydrolysis can be improved through limited aeration in addition to increased CH_4 production, and increased COD removal. Limited aeration may increase the rate of degradation of biodegradable compounds (Luostarinen et al., 2006; Chen et al., 2010). In a study by Lim & Wang (2013), limited aeration pretreatment was used to enhance hydrolysis of a batch AD system using brown water, which is human fecal matter mixed with water, and food waste as the substrate. With an oxygenation loading rate of 37.5 mL O_2/ $L_{r\text{-}day}$, Chen et al. (2010) determined that limited aeration resulted in higher COD

solubilization, greater VFA, production, and greater conversion of short chain fatty acids to acetate. Enhancement of the hydrolysis stage due to limited aeration could be the result of increased activity of hydrolytic and acidogenic bacteria with the addition of oxygen. In a study by Fu et al. (2016), relative abundance of *Firmicutes,* class *Clostrida* and order *Clostridiales*, which are associated with the hydrolysis step in the AD process, increased under limited aeration conditions. *Methanosarcina*, which is slightly tolerant to oxygen addition, and *Methanobacterium* both doubled in relative abundance with microaerated compared to nonaerated conditions.

2.2.3 Increased Ammonia-Nitrogen (NH_3-N) Removal from Limited aeration of AD Systems

Limited aeration or a combination of anaerobic and aerobic process have been shown to improve nitrogen removal. Ammonia in anaerobic sludge can be oxidized to nitrite or nitrate with the addition of air. Oxidation of anaerobic sludge can occur with electron donors such as ammonia, sulfide, and biodegradable matter (Parravicini et al., 2008). Parravicini et al. (2008) showed that post aeration of anaerobic sludge resulted in an average of 45.6% total nitrogen reduction. Denaturing gradient gel electrophoresis (DGGE) analysis of the aerated sludge determined the presence of ammonia oxidizing bacteria (AOB) *Nitrosomonas* cluster. In a study by Zupančič & Roš (2008), two-stage anaerobic-aerobic semi-continuous digesters were analyzed for NH_3-N removal. Nitrogen was analyzed using Total Kjeldahl Nitrogen (TKN), ammonia, nitrate, and nitrite. In two-stage experiments, nitrogen removal was 38% at an HRT of 5 days. At an HRT of 6 to 7 days, NH_3-N removal was greater than 70%.

2.2.4 Removal of Hydrogen Sulfide with Limited aeration of AD Systems

Biogas produced by AD systems can be utilized in engine operation, and as an alternative to natural gas (Bacenetti et al., 2013). However, biogas composition can be a concern as the gas components such as H_2S and sulfur oxides can corrode engines and boilers or cause air pollution. Several studies have shown that limited aeration can be used to oxide sulfides present in AD systems (Chen et al., 2010; Jenicek et al., 2010; Krayzelova et al., 2015). Zitomer & Shrout (2000) determined that aerated methanogenic fluidized bed reactors (FBRs) had a greater COD removal efficiency than anaerobic FBRs with and oxygen transfer of 28%. Under inhibitory sulfate conditions, higher aeration resulted in lower H2S concentrations. COD removal also increased from 25 to 75% from the anaerobic to aerated FBR. Díaz, Pérez, Ferrero, & Fdz-Polanco (2011) showed removal of H_2S in biogas through sulfide oxidation in to pilot-scale digesters operated at a volume of 200 L. Sulfide was oxidized to elemental sulfur and was shown to accumulate in the headspace of the tested pilot-scale digesters. Díaz et al. (2011) determined the presence of sulfur oxidizing bacteria (SOB) and saw removal efficiencies of H_2S of 98.8% in operation days 41 to 80, and 99.8% in operation days 81 to 120. Nghiem, Manassa, Dawson, & Fitzgerald (2014) determined that oxidation of H_2S to elemental sulfur in the AD process is associated with SOB such as *Thiobacillus*, which are ubiquitous in AD systems. The study determined that limited aeration successfully reduced H_2S without inhibiting the AD process. H_2S concentration decreased by 58% after six days of limited aeration conditions.

2.2.5 Microbial Communities Involved with Limited aeration of Anaerobic Digesters

Strict anaerobes have been thought to have no or very little tolerance to oxygen, as strict anaerobes cannot synthesize the enzyme superoxide dismutase. Because of this, oxygen has been commonly seen as inhibitory and toxic to the AD process. In contrast, however, other previous studies have shown that methanogenic organisms have a capacity to tolerate microaerated conditions for an extended period (Kiener & Leisinger, 1983; Kato, Field, & Lettinga, 1993; Chu, Zhang, Li, & Yang, 2005; Botheju, Lie, et al., 2010).

Oxygen tolerance of anaerobes can be associated with the oxygen uptake activity of facultative bacteria in an AD system. Facultative fermentative microbes can protect anaerobes by rapidly consuming dissolved oxygen in AD systems. Uptake by facultative bacteria creates an oxygen shielding effect for anaerobes (Kato et al., 1993; Song and Logan, 2004). In microaerated conditions, facultative microbes can grow more quickly which leads to more cellulose and hydrolytic enzymes, and a more rapid hydrolysis of complex polymers (Fu et al., 2016). If oxygenation levels are too high, however, facultative microbes may out compete strict anaerobes due to higher growth rates. Microbial competition for a given substrate may also act as a form of methanogenic inhibition (Conklin et al., 2007). Microbial granules, floc, or biofilm formation may also shield anaerobes by limiting oxygen diffusion, and through oxygen consumption by facultative or aerobic microorganisms (Kato et al., 1993; Zitomer & Shrout, 1998; Bakke, Kommedal, & Kalvenes, 2001; Botheju, Rathnasiri, & Bakke, 2008).

In an investigation of limited aeration condition in anaerobic digestion in

comparison to non-aerated anaerobic digestion by Tang et al. (2004), researchers determined that the domain *Bacteria* dominated both digesters in comparison to *Archaea* using fluorescence in situ hybridization (FISH). Phylogenetic analysis of 16s rRNA gene clones and real-time quantitative polymerase chain reaction (qPCR) by DGGE there was no phylogenic difference between *Bacteria* from aerated and non-aerated digesters, and *Firmicutes* dominated both systems. Using 16s rRNA gene clones and real time qPCR, there was an increase of *Methanosarcina* and decrease of *Methanoculleus* due to limited aeration.

2.3. Activity of Methanogenic Cultures After Heat Drying

Enrichments with dried anaerobic cultures have been shown to produce to produce CH_4 from H_2/CO_2 and acetate once reconstituted (Bhattad et al., 2017). In a study by Bhattad et al. (2017), effluent obtained from a 15 L laboratory digester was concentrated to 20 mL from 300 mL and heat dried at 104°C for 24 hours. Reconstituted biomass was able to consistently produce CH_4, however, dried cultures were shown to have a longer lag time till maximum CH_4, production. Microbial techniques showed survivability of methanogens, and methanogenic tolerance to oxygen. In dried cultures there was significant decrease in the acetoclast, *Mehtanosaeta,* while *Methanospirillium,* a hydrogenotroph, was more resilient to heat drying. Dried cultures had shorter lag times when fed H_2/CO_2 compared to acetate which suggests that hydrogenotrophic activity is better preserved in the heat drying process than acetoclastic.

Tale et al. (2015) applied an aerotolerant propionate consuming, methanogenic culture for bioaugmentation. In the study, the micro-aerated cultures performed better

than strictly anaerobic cultures when used for bioaugmentation. Digesters bioaugmented with the micro-aerated cultures had higher specific methanogenic activity (SMA) with propionate as the substrate, and shorter recovery time after organic overload (Tale et al., 2015). Application of an aerotolerant culture in bioaugmentation to improve digester performance may also be commercially applicable as the culture could be easily handled in ambient air (Zitomer, 2013).

2.4 Inhibition in Anaerobic Digestion from High Ammonia Concentrations

Inhibition is defined as an adverse decrease in microbial population or growth and can be the result of organic or inorganic substances (Yenigün and Demirel, 2013). Previous literature has shown that inhibition is a result of the FA portion of Total Ammonia-Nitrogen (TAN) concentration (Hansen Angelidaki & Ahring, 1998; Nakakubo, B. Møller, M. Nielsen, & Matsuda, 2008; Prochazka, Dolejs, Maca, & Dohanyos, 2012). TAN is a combination of FA and NH_4^+. With high concentrations of TAN, increase in pH can be inhibitory to the growth of methanogens as there is a shift to FA. Diffusion into the cell by FA potentially result in protein imbalance, and nutrient deficiencies (Chen, Cheng, & Creamer, 2008). Ammonia inhibition can result in VFA accumulation, and a decrease in pH, as VFAs become neutral. Decrease in pH is a higher hydrogen ion concentration, which can also be inhibitory to methanogens (Kim, 2003). Of the microbial communities involved in the AD process, methanogens are the least tolerant to ammonia addition and pH change (Chen et al., 2008). The TKN test was used in this study to measure TAN and Total Organic Nitrogen (TON) concentrations. Thygesen, Triolo, & Sommer (2012) determined that the average TAN fraction was 71%

and 75% of the TKN in their liquid fraction samples. The relationship between TAN and TKN was constant and linearly related with an r^2 value of 0.92.

Bacteria and archaea have shown partial resilience to high ammonia concentrations in anaerobic digesters. Several studies have shown that anaerobic reactors in high ammonia conditions treat waste via hydrogenotrophic methanogenesis, since acetoclastic methanogens are more sensitive to higher ammonia conditions (Mosbaek et al., 2016; Westerholm et al., 2011; Westerholm, Moestedt, & Schnürer, 2016). However, acetoclastic methanogenesis are also able to adapt to high ammonia conditions. For example, Westerholm et al. (2011) observed acetoclastic methanogens at 800 to 6900 mg/L. In previous work by Calli, Mertoglu, Inanc, & Yenigun (2005) there was an observed gradual COD removal with digesters operating at 700 mg/L FAN/L in the tested upflow anaerobic reactors. For 700 mg/L FAN/L *Methanosaeta* species decreased in activity. Hydrogenotrophs such as *Methanobacterium* and *Methanospirillium* were identified in DGGE analysis. *Methanosarcina*, an acetoclastic methanogen, had the highest abundance detected in all the reactors. Esquivel-Elizondo et al. (2016) saw increase of hydrogenotrophic and acetoclastic methanogens over time with reactors operating at approximately 2000 mg NH_3-N/L. Hydrogenotrophs identified included *Methanoculleus, Methanogenium,* and *Methanobrevibacter. Methanosaeta* was the sole acetoclastic methanogen identified. Both bacteria and archaea became less diverse with increased levels of NH_3-N/L. Enriched species included acetoclastic methanogens (*Methanosaeta);* Clostridia known to do forward acetogenesis (the conversion of VFAs in acetic acid, CO_2, and H_2) and reverse acetogenesis (where acetate is converted to H_2 and CO_2) such as *Clostridium* and *Clostridiaceae spp*); hydrogenotrophic methanogens

(Methanoculleus, Methanobrevibacter, and Methanogenium); fatty-acid producers; and syntrophic fatty acid fermenters (Esquivel-Elizondo et al., 2016). The study showed that hydrogenotrophic and acetoclastic methanogens could co-exist in high ammonia concentrations by establishing a syntrophic relationship with fermenters. This suggests that anaerobic digesters could potentially acclimate to high ammonia conditions over time.

2.5 Syntrophic Acetate-Oxidizing Microbes

In addition to the diverse groups of microorganisms that makeup the AD process and the conversion of acetate to CH_4 from acetoclastic methanogenesis, it is important to understand the microbial communities and metabolic steps involved with the oxidation of acetate to H_2 and CO_2. From the critical review paper on SAOB by Westerholm et al. (2016), the initial step of the syntrophic acetate oxidation (SAO) reaction is the oxidation of acetate to H_2, CO_2, and formate. Hydrogenotrophic methanogens then consume these products and produce CH_4. Known SAOB that are thermophiles or thermotolerant are *Thermacetogenium phaeum*, *Pseudothermotoga lettingae*, and *Tepidanaerobacter.* For the mesophilic group, known SAOB include *Clostridium ultunense*, and *Syntrophaceticus schinkii*. Genes associated with SAOB have been detected in a variation of digester operating conditions, such as varying ammonia concentrations, temperature, substrate characteristics, hydraulic residence time, and digester configuration. Abundance of SAOB is correlated with acetate degradation pathway, and production of the formyl tetrahydrofolate synthetase (FTHFS) enzyme, which is key in catalyzing formate (Westerholm et al., 2016).

3. Function of Low-Aerated versus Anaerobic Methanogenic Systems

A revised version of this chapter will be submitted to the journal *Bioresource Technology* as: Dylan, F., Venkiteshwaran, K., and Zitomer, D. Function of Low-Aerated versus Anaerobic Methanogenic Systems, B*ioresource Technology*.

3.1 Introduction

Anaerobic digestion (AD) is the process of converting organic matter, in the presence of microorganisms, to products such as methane (CH_4) and carbon dioxide (CO_2). Previously, anaerobes were thought to have no or little tolerance to oxygen, as strict anaerobes cannot synthesize the enzyme superoxide dismutase. Subsequent studies, however, have found that low aeration does not cause significant inhibition of CH_4 production. For example, in a study of anaerobic fluidized bed reactors, Zitomer and Shrout (1998) determined that methanogenic activity can be similar or greater in micro-aerated systems compared to strictly anaerobic systems. Additional studies have shown that methanogenic organisms have a capacity to tolerate microaerated conditions for an extended period (Kiener & Leisinger, 1983; Kato et al., 1993; Botheju, Lie, et al., 2010; Botheju, Saramakoon, et al., 2010). Limited aeration may enhance biochemical process and stabilize operating conditions in AD systems (Montalvo et al., 2016). In the study by Montalvo et al.(2016), limited aeration as an initial pretreatment was shown to increase CH_4 generation by 211%, increase hydrolysis of complex polymers to proteins, sugars, and soluble compounds, and a greater solids removal. Despite some success, limited aeration in AD systems is still at a preliminary stage. Further research is needed to show increased digester function with a continuous supply of oxygen (O_2) to AD systems and

comparison to a non-aerated system.

In this study, it was hypothesized that a continuous aeration of an AD system would result in increased CH_4 yield, chemical oxygen demand (COD) removal, and decreased volatile fatty acid (VFA) concentrations (Botheju et al., 2010; Botheju & Bakke, 2011). Three digesters were seeded with differing biomass to obtain differences in function and microbial communities using high throughput Illumnia sequencing.

3.2 Material and Methods

3.2.1 Lab-Scale Anaerobic Digesters

The effects of adding O_2 equivalent to 10% of the COD influent loading to an anaerobic digester was investigated. This oxygen loading rate was based on previous work done by others (Bocher et al., 2015; Tale et al., 2015) Three digesters were seeded with biomass from the anaerobic control digester (ACD), limited aerated anaerobic digester (LAD), and limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE) to monitor for changes in function.

Each 15L anaerobic digester was fed non-fat dried milk in basal nutrient solution (Speece, 2008) at a COD loading rate of 1 g/L_R-day at a 10 day hydraulic residence time (HRT). The ACD was seeded with biomass obtained from a 15L anaerobic bioreactor fed non-fat dried milk with nutrients and operated for more than 15 years. The LAD and LADE were seeded from 2L methanogenic bioreactors that had received oxygen equivalent to 10% of the COD loading and operated for 1 year and fed non-fat dried milk and operated under similar conditions as the 15 L digesters. Effluent biomass from each seed source was stored for 2 months to obtain a working volume of 15L. The LAD and

LADE received 5.625L of air daily. Air addition was performed via a digital peristaltic pump (Cole Parmer Masterflex US) to the digester headspace. For the LAD and ACD, 1.5L were removed daily via peristaltic pump (Cole Parmer Masterflex US) and replaced with 1.5L of nutrient feed with non-fat dried milk. Effluent biomass (3L) was removed from the LADE daily. Half of this effluent (1.5L) was concentrated through centrifugation at 4200 RPM (Centrifuge 5804 Eppendorf) for 14 minutes to achieve complete settling of biomass. Liquid was then removed, and solids were mixed with the remaining 1.5L of LADE effluent to concentrate the effluent to 500mL, separated in 5 150mL glass bottles. LADE effluent was then dried at 104°C in the VWR oven for 24 hours then stored in a desiccator for 24 hours. The dried biomass was then added back into the LADE the next day by reconstituting it into 1.5L of deionized water along with 1.5L of feed. The dried biomass, deionized water and feed mix was then fed to the LADE each day. Functional parameters measured periodically through quasi steady state from days 270 to 290 included biogas and CH_4 production of the LAD, LADE, and ACD systems, and pH, COD, soluble COD (SCOD) and VFA concentrations of the ACD, LAD, and LADE biomass. Influent COD was also measured.

3.2.2 Basal Nutrient Media

Basal nutrient media, as modified from Speece (2008), contained the following [mg/L]: NH_4Cl [400]; $MgSO_4.6H_2O$ [250]; KCl [400]; $CaCl_2.2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3.6H_2O$ [55]; $CoCl_2.6H_2O$ [10]; KI [10]; the salts $MnCl_2.4H_2O$, NH_4VO_3, $CuCl_2.2H_2O$, $Zn(C_2H_3O_2)_2.2H_2O$, $AlCl_3.6H_2O$, $Na_2MoO_4.2H_2O$, H_3BO_3, $NiCl_2.6H_2O$,

$NaWO_4.2H_2O$, and Na_2SeO_3 [each at 0.5]; $NaHCO_3$ [6000]; and resazurin [1].

3.2.3 Specific Methanogenic Activity Assay (SMA) Tests

Five SMA tests were performed using biomass from each 15L digester using 160mL serum bottles. The first SMA test employed acetate, the second SMA test employed propionate, the third SMA test employed hydrogen (H_2) and CO_2, fourth SMA test employed non-fat dried milk, and the fifth SMA test employed non-fat dried milk with an addition of 40mL of air, based on an O_2 equivalent to 10% of the COD loading the COD influent, into the serum bottle headspace.

For individual SMA tests, effluent (45mL) from each digester was added and 5 mL of substrate stock solution to yield 10 g/ L acetate, 2 g/L propionate and 2 g/L non-fat dried milk combined with nutrient solution. Assays bottles were prepared in triplicate, sealed and sparged with an O_2-free gas (N_2: CO_2, gas mix 70:30 v/v), and incubated at 35 °C and 150 rpm in an incubator-shaker. Each SMA was calculated by measuring the maximum CH_4 production rate divided by the total volatile suspended solids (VSS) in each assay bottle (Bhattad et al., 2017).

For the H_2/CO_2 SMA tests, 60 mL of H_2/CO_2 gas mix (4:1 v/v) was injected into each assay bottles containing 25mL of biomass. The H_2/CO_2 SMA biogas produced at a given time was calculated as the difference in the gas volumes (initial gas volume minus volume remaining). The H_2/CO_2 CH_4 production was estimated as the volume of H_2/CO_2 SMA biogas produced divided by the stoichiometric ratio 4 (i.e., 4 moles of H_2 and 1 mole of CO_2 consumed per 1 mole of CH_4 produced) (Bhattad, 2012). Maximum CH_4 production rate (mL CH_4/day) was calculated by linear regression using biogas slope of

each assay till rate of change was negligible. SMA was then determined by measuring the maximum CH_4 production rate divided by the VSS in each assay bottle (Bhattad, 2012).

3.2.4 Total Kjeldahl Nitrogen (TKN)

The distillation method for Total Ammonia Nitrogen (TAN), which is a combination of free ammonia (FA) and ammonium (NH_4^+), was performed using Standard Methods 4500-NH_3 (APHA, 2012). In this method, LAD and ACD biomass samples were diluted with deionized water. A borate buffer was then added, and each solution pH was adjusted to 9.5 with 6 N NaOH. Samples were boiled, and distillate was captured in 500 mL Erlenmeyer flasks containing a standard boric acid solution. At least 200 mL of each sample was then titrated with 0.02 N sulfuric acid until the methyl red indicator dye turned lavender color. Total Organic Nitrogen (TON), which is organically bound nitrogen but does not include nitrate-nitrogen (NO_3-N) or nitrite-nitrogen (NO_2-N) was also performed using standard methods (APHA, 2012). To determine TON, samples were digested after ammonia distillation using sulfuric acid digestion and boiling, which converts TON to TAN. Titration was then used to measure amount of TON and reported as TAN. The sum of TAN and TON was reported as TKN for the LAD and ACD biomass. Influent and effluent TKN concentrations were determined for LAD and ACD for a mass balance of ammonia, and a comparison of aerated versus non-aerated digestion regarding ammonia-nitrogen fate.

3.2.5 Analytical Methods

Biogas volume was measured by connecting latex tubing from each digester headspace to a gas sampling bag (Cole Parmer Kynar PVDF 20.3L). The biogas volume

collected in the gas sampling bag was measured daily using a wet test meter (Precision Scientific). COD was measured according to standard methods (APHA, 2012). Soluble COD (SCOD) was measured by filtering each sample through a 0.45 um pore-size membrane syringe filter and determining filtrate COD by standard methods (APHA, 2012) CH_4 concentration in biogas was measured by gas chromatography (GC System 7890B, Agilent Technologies, Irving, TX, USA) with a thermal conductivity detector. VFA concentrations were measured by gas chromatography using a flame ionization detector (GC-FID). Solids analyses total solids (TS), volatile solids (VS), total suspended solids (TSS), and VSS were performed by standard methods (APHA, 2012). Statistical analyses, such as the ANOVA single factor, and post-hoc two-sample Student t-test with equal variance, were performed using Microsoft Excel 2016 and built-in macro functions. The ANOVA single factor test and Bonferroni correction were used to adjust the significance to avoid statistical error when comparing groups greater than 2 samples.

3.2.6 Microbial Analysis

DNA was extracted from the 15L digesters on day 446 using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer instructions. The biomass samples were subjected to bead beating on a vortexer (Model 58816-121, VWR International, Radnor, PA, USA) for 10 minutes. Sequencing was performed by a commercial laboratory (MR DNA, Shallowater, TX, USA) using an Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA, USA) following manufacturer guidelines. Barcodes and primers were removed from Q25 filtered sequences and processed as previously described (Dowd et al., 2008). Briefly, data were refined by removing sequences <200 bp, sequences with

ambiguous base calls, and sequences with homopolymers >6 bp. Denoised sequences were clustered into operational taxonomic units (OTUs) having 97% similarity. Singleton sequences and chimeras were removed. BLASTn was used to taxonomically classify OTUs against a curated data- base derived from GreenGenes, RDPII, and NCBI (CME, 2015; DeSantis et al., 2006; NCBI, 2015). A sample-based rarefaction was performed on the combination of archaea and bacteria operational taxonomic units (OTUs). Relative abundance was calculated for each OTU at the genus level by dividing the number of OTU sequence count by the total number of sequence counts in the sample. Heat maps were generated using relative abundance data.

3.3 Results and Discussion

3.3.1 Comparison of Digesters Based on Function

Digesters reached quasi steady-state operation at day 269 defined by the coefficient of variation (cv) being less than 10% for digester biogas production in a two-week period. For quasi steady state analysis during days 269 to 286, daily gas removed from the LAD, LADE, and ACD are shown in Table 1. Gas removed from LAD and LADE included some N_2 and possible residual O_2 in digester headspace resulting from air addition during feeding each day. During quasi steady state, the average digester pH and average effluent TS, VS, TSS, VSS, TCOD, and SCOD concentrations were similar among the three digester types ($p<0.05$, $n=5$) (Table 1). TCOD concentration for the influent is shown in Table 1. Based on the average influent COD concentration of 10±0.31 g/L, average TCOD removal values for the LAD, LADE, and ACD systems were 79±3%, 81±2%, and 79±4%. VFA concentrations at quasi steady state operation

for the LAD and ACD were below the detection limit of 50 mg/L. For the LADE, effluent average total VFA concentration was 250±80 mg/L. Acetic acid was the dominant acid and made up 60% of the total VFA concentration.

Table 1. Influent and digester effluent performance parameters.

	Influent	LAD	LADE	ACD
Daily gas removed (L/d)		13.1±0.16	12.8±0.44	8.84±0.27
pH		7.69±0.04	7.45±0.17	7.52±0.07
TS (g/L)		9.8±0.2	6.5±0.15	10.1±0.12
VS (g/L)		2.2±0.09	1.80±0.07	2.7±0.08
TSS (g/L)		3.34±0.2	2.02±0.09	3.1±0.11
VSS (g/L)		3.1±0.1	1.75±0.1	2.4±0.13
TCOD (g/L)	10±0.31	2.05±0.23	1.90±0.16	2.06±0.18
SCOD (g/L)		0.15±0.02	0.19±0.06	0.15±0.014

The LAD, LADE, and ACD systems produced an average of 0.36±0.022 L-CH_4 L_R-d, 0.39±0.003 L-CH_4/ L_R-d, and 0.36±0.21 L-CH_4/ L_R-d (Figure 1). Theoretical CH_4 production was calculated to be 0.395 L-CH_4/ L_R-d for the LAD, LADE, and ACD systems based on an influent COD of 1 g/L_R-d at a HRT of 10 days. The ANOVA test, post-hoc student t-test for two-sample assuming equal variance, and a 95% confidence interval ($p<0.05$) were used to determine if digesters were statistically different in CH_4

production. Based on the ANOVA and post-hoc student t-test using the Bonferroni correction for multiple samples, there were no significant differences among CH_4 production rates for the LAD and LADE ($p=0.07$, $n=5$), LAD and ACD ($p=0.67$, $n=5$), and LADE and ACD ($p=0.15$, $n=5$). Based on an extensive review of limited aeration in AD systems by Botheju and Bakke (2011) it was expected that aeration would increase CH_4 yield in comparison to the non-aerated system. Potential reasons for similar CH_4 production would be that one, 15L digesters were operated over 500 days, allowing for acclimation to the substrate (non-fat dried milk) by anaerobic microbial communities. Secondly, non-fat dried milk was potentially not complex enough, in that it contained mostly carbohydrates that can be easily solubilized, to show beneficial effects of increased hydrolysis of complex polymers and monomers from limited aeration.

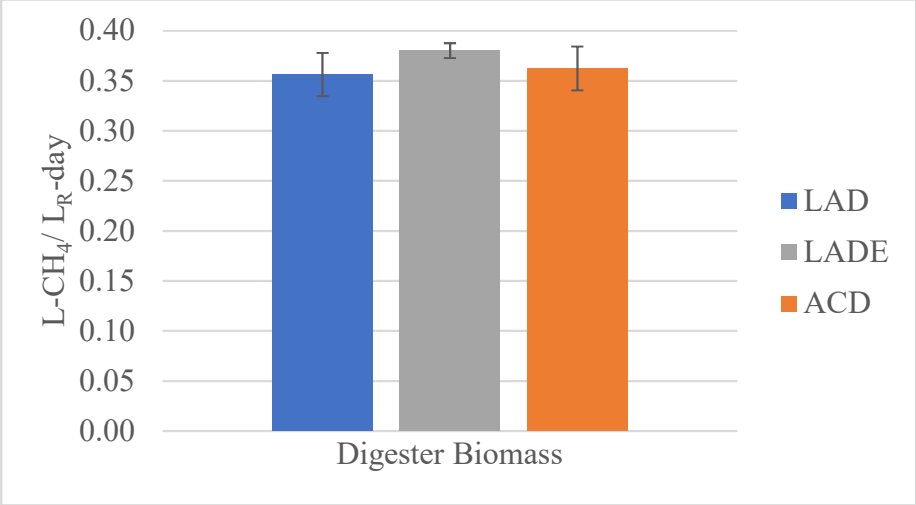

Figure 1. Average CH_4 volume produced per liter reactor per day for the LAD, LADE, and ACD. The graph shows the average CH_4 volume observed during quasi steady state operation. The error bars represent standard deviation from five measurements of CH_4.

3.3.2 TKN Analysis

Aeration resulted in reduced average effluent total and soluble TKN concentrations compared to non-aeration AD systems. The LADE biomass was not included in TKN analysis as the objective was to determine TKN difference between aerated and non-aerated digesters, excluding augmentation with an aerotolerant culture. It was expected that LAD would have a lower effluent TKN concentration due to oxidation of ammonia with aeration to nitrite ($N0_2^-$) and nitrate (NO_3^-) (Parravicini et al., 2008).

Table 2 shows total TKN results for the influent, LAD and ACD effluent. Figure 2 shows fraction of TAN and TON for Total TKN. The average total TKN effluent concentrations for the LAD and ACD were significantly different ($p=0.0007$, $n=5$). The average effluent LAD TKN concentration was 6.5% less than that of the ACD. Table 3 shows soluble TKN results and Figure 3 shows fraction of TAN and TON for soluble TKN. Average effluent soluble TKN concentration values for LAD and ACD were not significantly different ($p=0.09$, $n=5$). This indicates the ACD biomass retained a higher TKN concentration than the LAD biomass.

Table 2. Digesters Total TKN concentrations (mg/L).

Digester Sets	Influent	LAD	ACD
Average TAN	160.5 ±22,6	392±24.34	400 mg/L±16.92
Average TON	509.9±17.7	197.5±22.4	230.5±16.11
Average TKN	670.4±27.1	589.6±9.8	630.5±13.4

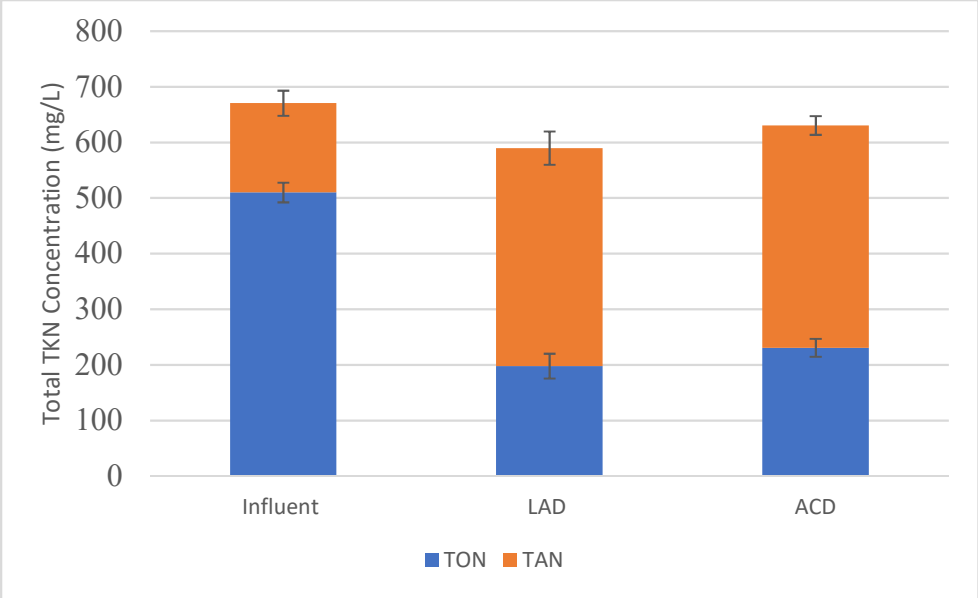

Figure 2. Total TAN and TON concentrations for the LAD and ACD. The error bars represent standard deviation from five measurements of TKN.

Table 3. Data table for soluble TKN (mg/L).

Digester Sets	Influent	LAD	ACD
Average TAN	107.5 ±7.0	364.6±6.6	360.1±7.3
Average TON	457.6±26.9	70.0±20.8	51.2±12.2
Average TKN	565.1±7.0	434.3±6.6	411.8±7.3

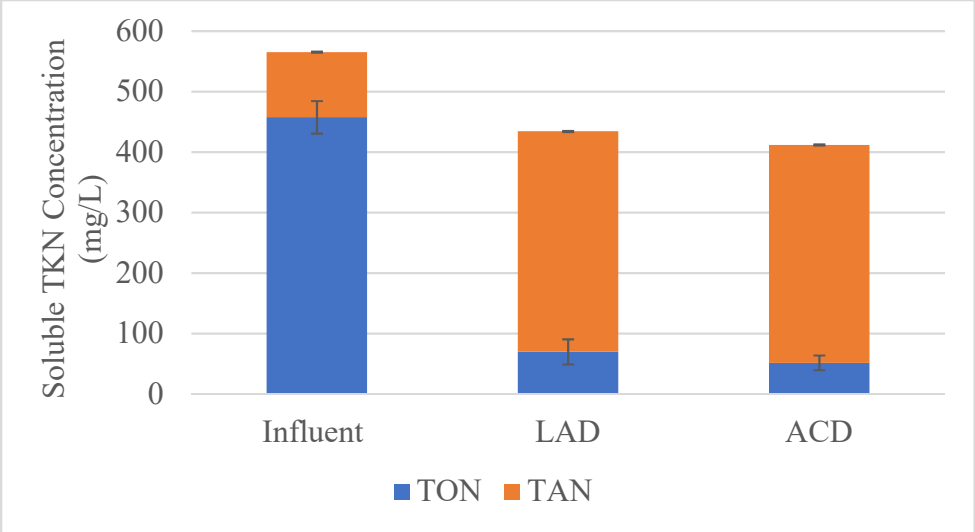

Figure 3. Soluble TAN and TON in the LAD and ACD systems. The error bars represent standard deviation from five measurements of TKN.

3.3.3 Archaeal Community Analysis in 15L Digesters

A total of 14, 15, and 13 archaeal OTUs based on 96 to 99% similarity were identified among each LAD, LADE and ACD digester sample analyzed. Seven Archaeal OTUs represented 95 to 99% of the archaeal relative abundance in all three digester types. These 7 OTUs were most similar to the genera *Methanosaeta, Methanolinea, Methanobrevibacter, Methanoculleus, Thermogymnomonas, Methanospirillum,* and *Methanococcus* (Table 4). The LAD, LADE, and ACD biomass samples were distinguished by a high relative abundance of *Methanosaeta*, an acetoclastic methanogen, which ranged from 58 to 77% in each digester. Relative abundance of archaeal communities was similar between the LAD, LADE, and ACD effluent. However, the LADE biomass had a higher relative abundance of the hydrogenotrophic methanogens, *Methanospirillum* (23.5%) and *Methanococcus (*1.55%) (Table 4). A nonmetric

multidimensional scaling plot (nMDS) and principal component analysis (PCA) of the archaeal data are shown in Chapter 4. Figure 4 gives a visual representation of the differences between the three wet cultures in relative abundance of the top 7 OTUs on the genus level for archaea.

Table 4. Relative abundance values for 7 archaeal OTUs representing 96 to 99% of total abundance.

Genus	LAD %Abundance	LADE %Abundance	ACD % Abundance
Methanosaeta	77.0	63.6	58.1
Methanolinea	13.2	1.9	28.5
Methanobrevibacter	2.1	6.1	9.5
Thermogymnomonas	2.5	No Detected	1.2
Methanospirillum	Not Detected	23.5	Not Detected
Methanococcus	3.3	1.3	1.9
Methanoculleus	Not Detected	3.3	Not Detected

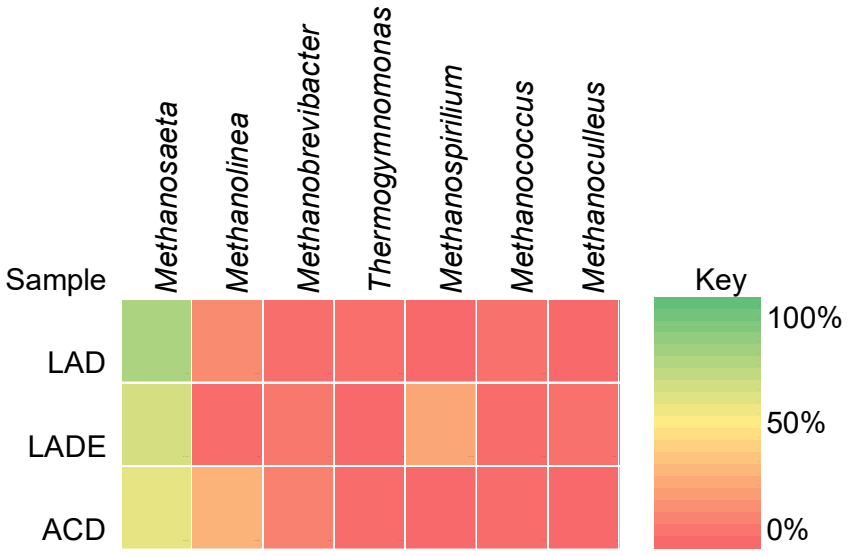

Figure 4. Heatmap showing percent abundance of archaea for each biomass.

3.3.4 Bacterial Community Analysis in 15L Digesters

For the LAD, LADE, and ACD 214, 222, and 197 OTUs were identified on the genus level for each biomass samples analyzed. Bacterial communities in all three digester sets were dominated by OTUs most similar to fermenters belonging to the phyla *Bacteroidetes, Firmicutes, Proteobacteria,* and *Actinobacteria, Synergistetes, and Chloroflexi*, which made up 98% or greater of total abundance for phylum OTUs (Table 5).

Table 5. Percent abundance of most common bacteria phylum OTUs.

phylum	LAD %Abundance	phylum	LADE %Abundance	phylum	ACD %Abundance
Bacteroidetes	37.4	Bacteroidetes	40.75	Bacteroidetes	12.41
Firmicutes	33.2	Firmicutes	30.27	Firmicutes	27.02
Proteobacteria	20.0	Proteobacteria	14.81	Proteobacteria	36.79
Actinobacteria	3.8	Actinobacteria	9.71	Actinobacteria	9.45
Synergistetes	2.1	Synergistetes	1.85	Synergistetes	10.01
Chloroflexi	2.3	Chloroflexi	0.91	Chloroflexi	1.92

The most abundant bacterial OTUs observed in the digesters were most similar to the genera *Clostridium, Petrimonas, Pseudoalteromona, and Parvimonas* (Table 6). Table 6 shows the 30 OTUs that contributed to approximately 90% of the total bacteria sequence relative abundance in the LAD, LADE, and ACD. LAD and LADE biomass samples were distinguished from ACD by a higher relative abundance of *Petrimonas* and *Parvimonas*. ACD biomass was dominated by *Pseudoalteromonas and Clostridium*

(Table 6). A nMDS and PCA of the bacterial data are shown in Chapter 4. Figure 5 gives a visual representation of the differences between the three wet cultures in relative abundance of top 20 OTUs on the genus level for bacteria.

Table 6. Percent abundance of most common bacteria genus OTUs for the LAD, LADE, and ACD.

OTU	genus	LAD %Abundance	LADE %Abundance	ACD %Abundance
1	clostridium	17.5	11.3	18.1
2	petrimonas	16.7	16.5	2.7
3	pseudoalteromonas	11.8	8.1	29.3
4	parvimonas	10.5	11.5	4.0
5	sphingobacterium	6.2	Not Detected	Not Detected
6	rikenella	5.9	6.1	4.2
7	syntrophobacter	3.2	2.0	Not Detected
8	rubrobacter	3.1	Not Detected	7.2
9	parabacteroides	2.9	0.4	Not Detected
10	desulfomicrobium	2.1	Not Detected	Not Detected
11	synergistes	2.0	1.7	9.7
12	bellilinea	2.0	0.5	1.6

13	solitalea	1.7	6.2	0.9
14	cytophaga	1.5	1.6	2.3
15	bacteroides	1.4	2.5	0.8
16	bacillus	0.7	2.3	Not Detected
17	syntrophorhabdus	0.7	0.6	1.7
18	anaerophaga	0.6	6.3	0.9
19	lachnoclostridium	0.5	Not Detected	Not Detected
20	thermoanaerobacter	0.5	Not Detected	Not Detected
21	desulfonispora	Not Detected	Not Detected	0.6
22	desulfobulbus	Not Detected	1.1	0.6
23	desulfofaba	Not Detected	Not Detected	0.8
24	atopobium	Not Detected	Not Detected	1.7
25	saccharibacter	Not Detected	Not Detected	0.7
26	spirochaeta	Not Detected	Not Detected	0.8
27	smithella	Not Detected	Not Detected	1.8
28	sulfuricurvum	Not Detected	1.3	Not Detected
29	olsenella	Not Detected	9.2	Not Detected

30	calderihabitans	Not Detected	0.5	Not Detected

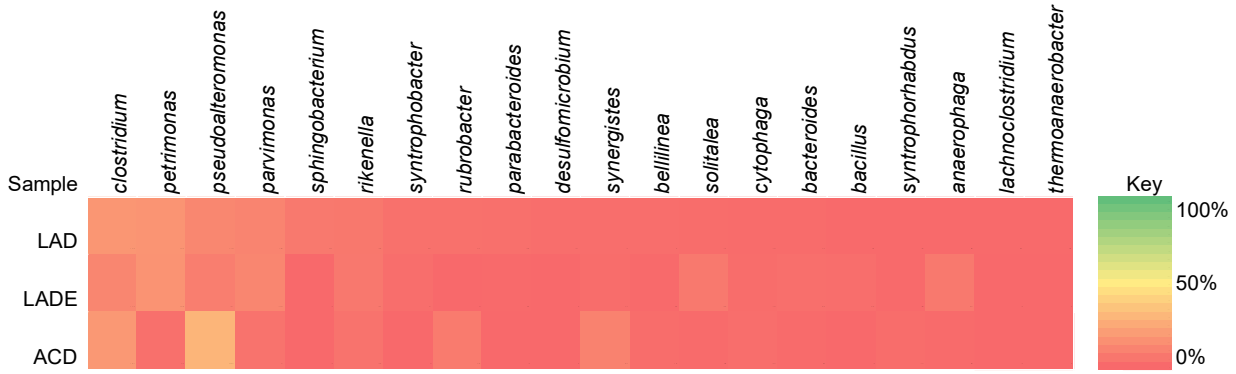

Figure 5. Heatmap showing percent abundance of bacteria for each biomass.

3.3.5 SMA Analysis

Based on the ANOVA single factor test, biomass samples were statistically significant from each other with acetate ($p<0.05$). The observed SMA from the ACD was statistically higher ($p<0.05$, $n=6$) than both LAD and LADE digesters, respectively. The average ACD SMA value (191.7± 1.2 mL CH_4/g VSS-day) was 33% greater than that of the LAD (129.3±10.1 mL CH_4/g VSS-day) and 22% greater than the LADE (150.4±4.4 mL CH_4/g VSS-day) with acetate as the substrate (see Figure 6). The LAD and LADE were not determined to be statistically different ($p<0.05$, $n=6$). Results showed that non-aerated biomass had a higher SMA with acetate versus aerated and aerated with an enrichment culture. Results confirmed previous studies that aeration and drying reduced biomass SMA with acetate in comparison to a non-aerated system (Bhattad, 2012).

However, based on relative abundance of acetoclastic methanogens in each biomass, specifically *Methanosaeta,* it was expected that the LAD (77% relative abundance) and LADE (63.6% relative abundance) would have greater SMA values than the ACD (58.1%). A potential reason for higher SMA with acetate as the substrate for the ACD biomass was that each digester had higher or similar relative abundance of formative bacteria and known acidogenic bacteria such as *Clostridium* (see Table 6).

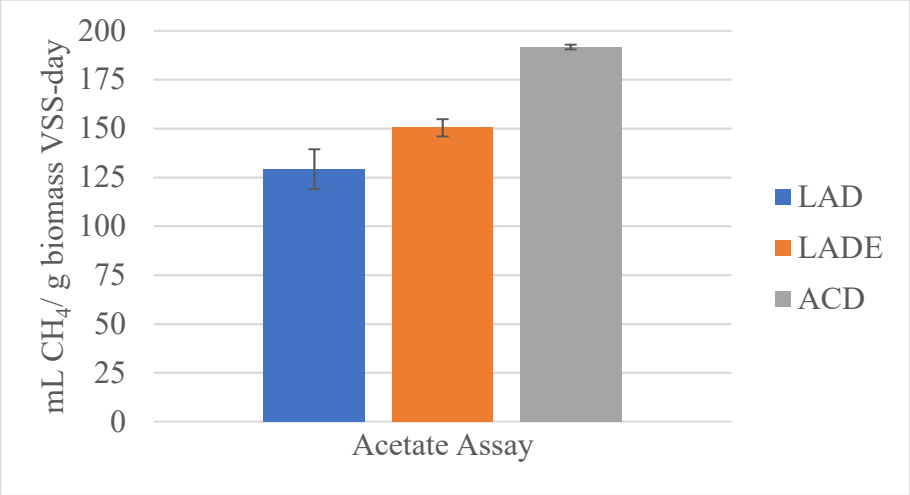

Figure 6. Acetate SMA Values for LAD, LADE, and ACD Systems. The error bars represent standard deviation from triplicate observations.

Figure 7 shows mL CH_4/g VSS-day for the LAD, LADE, and ACD SMA test with propionate as the substrate. The greater SMA with propionate may indicate a higher abundance of syntrophic propionate-oxidizing bacteria (McCarty & Smith, 1986; Speece, Boonyakitsombut, Kim, Azbar, & Ursillo, 2006). LADE SMA value (79.6± 2.3 mL CH_4/g VSS-day) was 38% and 27% greater than that of LAD (49.7±1.43 mL CH_4/g VSS-day) and ACD (57.9±0.92 mL CH_4/g VSS-day. The observed SMA from the LADE was statistically higher ($p<0.05$, $n=6$) than both LAD and ACD digesters, respectively.

Results confirm previous studies that heat drying biomass results in a greater SMA with propionate (Schauer-Gimenez et al. 2010; Bhattad et al. 2017). Unlike the LAD and ACD biomass, the LADE biomass had detection of the genus *Methanolinea* and *Methanospirillum,* which both play important roles in propionate utilization (Schauer-Gimenez et al. 2010; Tale et al. 2015). The LADE biomass also had greater relative abundance of the propionate oxidizing bacteria *Syntrophobacter* (see Table 6).

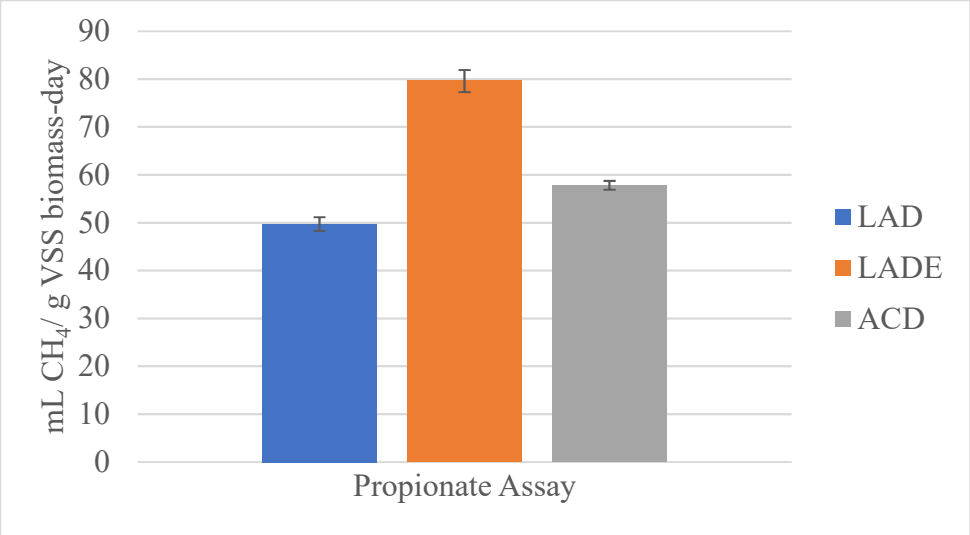

Figure 7. SMA values for the LAD, LADE, and ACD systems with propionate. The error bars represent standard deviation from triplicate observations.

Figure 8 shows SMA values for the LAD, LADE, and ACD SMA tests with H_2/CO_2 as the substrate. The observed SMA from the LADE was statistically the same ($p<0.05$, $n=10$) as both LAD and ACD digesters. The average LADE SMA value was 5% greater than that of LAD (0.59± 0.006 mL CH_4/g VSS-day) and was 5% greater than that of the ACD (0.59±0.06 mL CH_4/g VSS-day). Results disprove the hypothesis that limited aeration and limited aeration and drying would result in a higher SMA with H_2/CO_2.

However, microbially, the LAD, LADE, and ACD biomass had similar relative abundance of hydrogenotrophic methanogens contributing 20-40% of the total archaeal taxa, which may be a potential reason for similar SMA with H_2/CO_2.

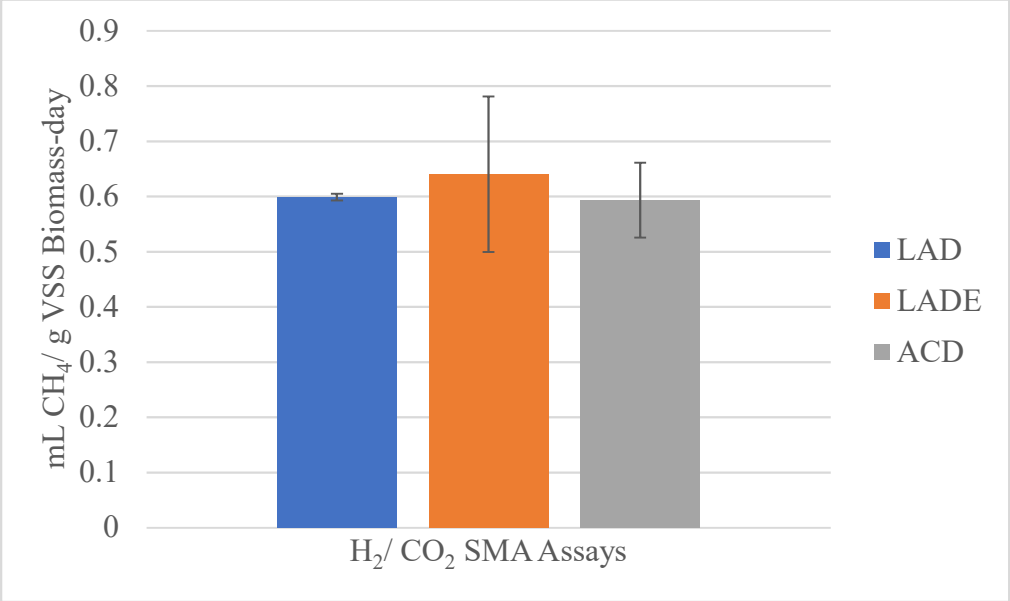

Figure 8. SMA values for LAD, LADE, and ACD with H_2/CO_2. The error bars represent standard deviation from triplicate observations.

Figure 9 shows LAD and ACD SMA values with non-fat dried milk as the substrate. The average ACD SMA value with non-fat dried milk (54.0± 2.31 mL CH_4/g VSS-day) was 31% greater than that of the LAD systems (37.0±1.95 mL CH_4/g VSS-day). The observed SMA from the ACD biomass was statistically higher ($p<0.05$, $n=4$) than the LAD digester. This result disproves the hypothesis that limited aeration would improve SMA with a complex substrate such as non-fat dried milk by increasing hydrolysis of complex monomers. A potential reason for this is that non-fat dried contains more carbohydrates which can be solubilized without the addition of O_2, and are

more easily solubilized than proteins and fats (Vidal et al., 2000).

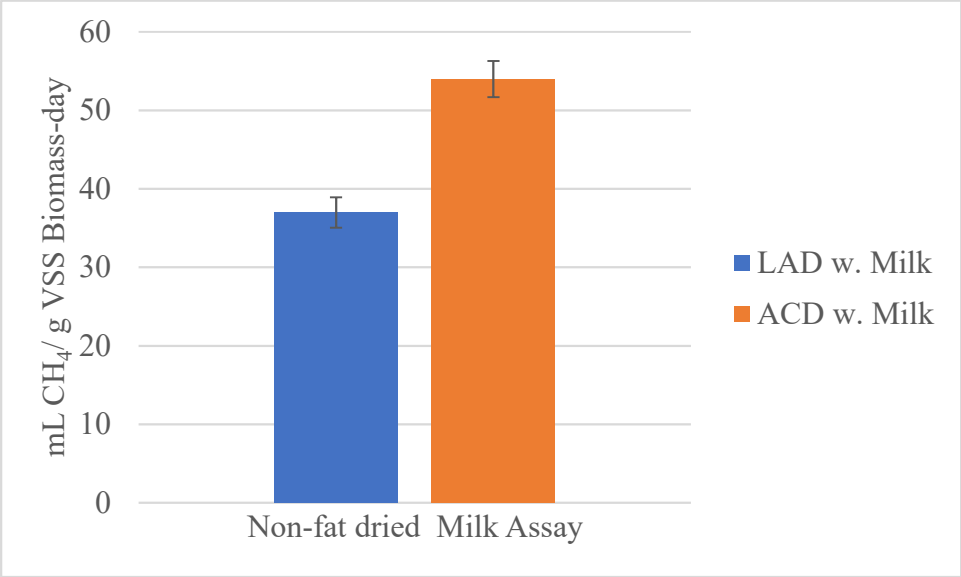

Figure 9. SMA values for LAD and ACD with non-fat dried milk. The error bars represent standard deviation from triplicate observations.

Figure 10 shows SMA values for LAD and ACD biomass (0.045L) with milk and O_2 (i.e., air). For the milk and air SMA tests, the average LAD biomass SMA value (41.5± 2.4 mL CH_4/g VSS-day) was 5% greater than that of the ACD biomass (39.4±7.6 mL CH_4/g VSS-day). The observed SMA from the LAD biomass was statistically the same ($p<0.05$, $n=4$) as the ACD digester. Due to the lower SMA with milk and air than non-fat dried milk alone, limited aeration may have been inhibitory to the ACD biomass. No change was seen between the SMA values for the LAD with non-fat dried milk and milk+air, which indicates that methanogenic communities may have been more resilient after air addition due to enrichment of aerotolerant methanogenic communities.

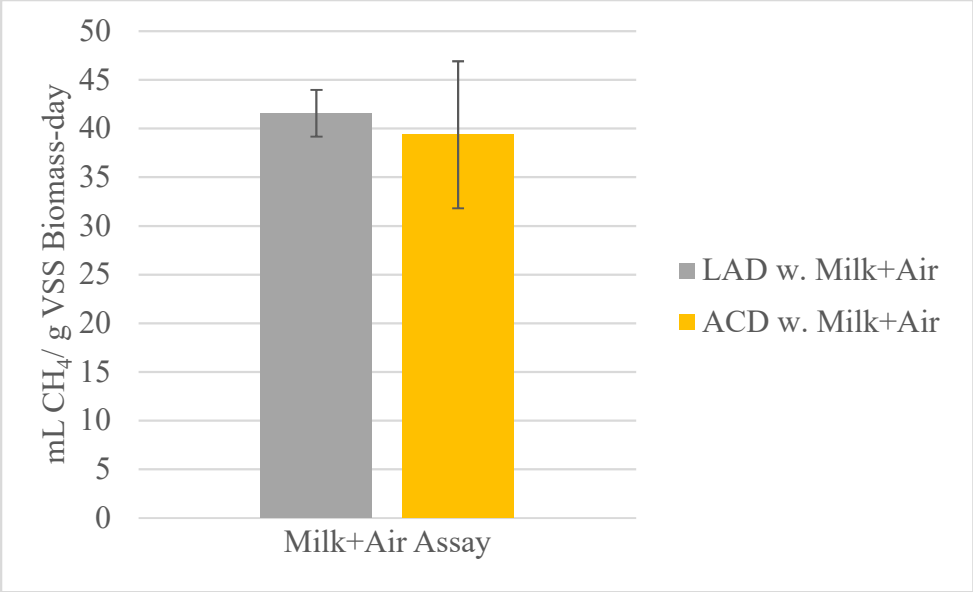

Figure 10. SMA values for LAD and ACD for the non-fat dried milk and limited oxygen test conditions . The error bars represent standard deviation from triplicate observations.

3.4 Conclusion

The ACD as well as LAD and LADE systems demonstrated similar quasi steady state functional parameters, including CH_4 production rate, COD and SCOD removal, pH, and effluent VFA concentrations. Aeration and aeration and augmentation with an aerotolerant culture resulted in no additional benefits in comparison to a non-aerated digester. However, the study showed that aeration and aeration with a dried culture where not inhibitory to digester function. The observed TKN concentration from the LAD biomass was statistically lower ($p<0.05$, $n=5$) than the ACD biomass. Therefore, aeration may have further oxidized TKN to NO_2-N and NO_3-N, and further denitrification to N_2 gas, which would have accumulated in the headspace.

The LAD, LADE, and ACD biomass samples were distinguished by a high

relative abundance of *Methanosaeta*, an acetoclastic methanogen, for which relative abundance values ranged from 58 to 77% in each digester. The most common bacterial taxa detected in the digesters were the genera *Clostridium, Petrimonas, Pseudoalteromona, and Parvimonas*. The combination of aeration and augmentation with a dried aerotolerant enrichment culture did result in a higher SMA with propionate then aeration and the anaerobic control. Microbially, this was represented by an increase in *Methanospirillium* and increase in propionate oxidizing bacteria in the LADE. Aeration did not result in an increase in hydrogenotrophic activity as each biomass had a similar SMA with H_2/CO_2. Microbial community data correlate with H_2/CO_2 SMA results as each biomass had similar relative abundance of hydrogenotrophic methanogens. Aeration with non-fat dried milk did not increase SMA of the LAD and ACD biomass in comparison to SMA with non-fat dried milk. It may be possible to observe hydrolysis enhance with aeration with a more complex substrate such as organic municipal solid waste.

4. Activity of Methanogenic Cultures After Heat Drying

A revised version of this chapter will be submitted to the journal *Bioresource Technology* as: Dylan, F., Venkiteshwaran, K., and Zitomer, D. Activity of Methanogenic Cultures After Heat Drying, B*ioresource Technology*.

4.1 Introduction

The development of aerotolerant methanogenic cultures has benefits in reducing required storage of biomass and potential use in bioaugmentation of AD systems. Air drying concentrates the biomass and reduces volume of storage. Dried aerotolerant methanogenic cultures also have practical use in biochemical methane potential (BMP) tests and as seed for laboratory digesters. Bhattad et al. (2017) determined that anaerobic biomass could be dried and stored in an air atmosphere and still produce methane (CH_4) from hydrogen (H_2)/ carbon dioxide (CO_2), or acetate once reconstituted (dried biomass in an aqueous solution). Reconstituted dry cultures also showed survivability of methanogens, and methanogenic tolerance to oxygen. In dried cultures there was a significant decrease in 16S rRNA transcript/gene copy numbers of the acetoclast, *Methanosaeta,* while *Methanospirillum* a hydrogenotroph, was more resilient to heat drying. Dried cultures had shorter lag times when fed H_2:/CO_2 compared to acetate which suggested that hydrogenotrophic activity was better preserved in the heat drying process than acetoclastic activity.

Based on previous work, dried methanogenic cultures have shown the ability to produce CH_4 once reconstituted. Previous studies have also used an initial augment culture but have not continuously augmented with an enrichment culture. Additional

understanding of methanogenic microbial composition is also still unknown. Further research is needed to observe continuously augmentation on digester performance and quantify archaeal and bacterial communities to provide a better understanding of community dynamics and comparison to wet culture counterparts.

In this study, a continuous novel enrichment technique that involve exposing methanogenic biomass to low oxygen concentrations and to drying at >100°C for 24 hours were used to develop a methanogenic culture resilient to heat and air drying. Biomass was used from the anaerobic control digester (ACD), limited aerated anaerobic digester (LAD), and limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE). Dried biomass was then reconstituted in a nutrient solution and monitored for performance, changes in function, and changes in microbial communities using high throughput Illumnia sequencing.

4.2 Material and Methods

4.2.1 Development of Dried Cultures

Three 15L anaerobic enrichment digesters were fed non-fat dried milk in basal nutrient solution (Speece, 2008), with a chemical oxygen demand (COD) loading rate of 1 g/L_R-day at a 10-day hydraulic retention time (HRT). Enrichment digesters included the LAD, LADE, and ACD. The LAD and LADE received 5.625L of air daily, with an oxygen loading equal to 10% of the influent COD load at 35°C and 1 atm. For ACD and LAD, 1.5L were removed daily via pump (Cole Parmer Masterflex US) and replaced with 1.5L of nutrient feed with non-fat dried milk (Kroger). For LADE, 3L of the digester contents were removed daily. The 1.5L of the LADE effluent biomass was

concentrated through centrifugation at 4200 RPM in the Centrifuge 5804 (Eppendorf) at 14 minutes to achieve complete settling of biomass. Liquid was then removed, and solids were mixed with the remaining 1.5L of LADE effluent. LADE effluent was then dried at 104°C in the VWR oven for 24 hours then stored in a desiccator for 24 hours. The dried biomass was then added into the LADE the succeeding day by reconstituting it into 1.5L of deionized water along with 1.5L of feed.

Enrichment digesters were operated for over 6 months to attain quasi steady state based on less than 10% variation in daily biogas production. After which, 1L of the daily effluent biomass of the three digesters was centrifuged to produce 30 mL of concentrated biomass that was then dried at 104°C in the VWR oven for 24 hours and stored in a desiccator. This process was performed for 2 months to obtain at least 40 g of dried biomass. Dried biomass from each digester was then used to seed five anaerobic batch systems with a total volume of 500mL. These batch systems were designated as the limited aerated digester-dried (LAD-D), limited aerated digester with augmented with an enrichment culture-dried (LADE-D), and anaerobic control digester-dried (ACD-D) batch systems. Each batch system initially received 4 g of biomass and 400mL of a basal nutrient solution containing essential inorganic nutrients (Speece, 2008), yielding a solids concentration of 10 g/ L_R total solids (TS). Digesters were also sparged with an O_2-free gas (N_2: CO_2, gas mix 70:30 v/v). Biogas production volume was measured using a glass syringe by inserting the needle through the rubber septa of each bottle. Biogas CH_4 content, volatile fatty acid (VFA) concentration and soluble COD (SCOD) concentration were measured using standard methods (APHA, 2012) every 10 days to monitor the batch systems.

4.2.2 Basal Nutrient Media

Basal nutrient media, as described by Speece (2008), contained the following [mg/L]: NH_4Cl [400]; $MgSO_4.6H_2O$ [250]; KCl [400]; $CaCl_2.2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3.6H_2O$ [55]; $CoCl_2.6H_2O$ [10]; KI [10]; the salts $MnCl_2.4H_2O$, NH_4VO_3, $CuCl_2.2H_2O$, $Zn(C_2H_3O_2)_2.2H_2O$, $AlCl_3.6H_2O$, $Na_2MoO_4.2H_2O$, H_3BO_3, $NiCl_2.6H_2O$, $NaWO_4.2H_2O$, and Na_2SeO_3 [each at 0.5]; and $NaHCO_3$ [6000].

4.2.4 Specific Methanogenic Activity (SMA) Tests

Three differing SMA tests were performed for wet and dry cultures. SMA tests used non-fat dried milk and nutrient solution, acetate, and a H_2/CO_2 gas mixture to compare CH_4 production rate of each biomass. SMA tests were performed at day 205 for reconstituted 500 mL batch systems and day 500 for 15L digesters. The first SMA test employed non-fat dried milk, the second SMA test employed acetate, and the third SMA test employed H_2 and CO_2 as substrates. Assays bottles were prepared in triplicate, sealed and sparged with an O_2-free gas (N_2: CO_2, gas mix 70:30 v/v), and incubated at 35 °C and 150 rpm in an incubator-shaker. For individual SMA tests, effluent (45mL) from each digester was added and 5 mL of substrate stock solution to yield 10 g/ L acetate, and 2g/L non-fat dried milk combined with nutrient solution. Each SMA was calculated by measuring the maximum CH_4 production rate divided by the total volatile suspended solids (VSS) in each assay bottle (Bhattad et al., 2017).

For the H_2/CO_2 SMA tests, 60 mL of H_2/CO_2 gas mix (4:1 v/v) was injected into each assay bottles (160mL) containing 25mL of biomass. The H_2/CO_2 SMA biogas

produced at a given time was calculated as the difference in the gas volumes (initial gas volume minus volume remaining). The H_2/CO_2 CH_4 production was estimated as the volume of H_2/CO_2 SMA biogas produced divided by the stoichiometric ratio 4 (i.e., 4 moles of H_2 and 1 mole of CO_2 consumed per 1 mole of CH4 produced) (Bhattad, 2012). Maximum CH_4 production rate (mL CH_4/day) was calculated by linear regression using biogas slope of each assay till rate of change was negligible. SMA was then determined by dividing maximum CH_4 production by the VSS in each assay bottle (Bhattad, 2012).

4.2.3 Analytical Methods

Biogas volume measured by inserting a needle with a wetted glass syringe through the septa of each serum bottle. SCOD was measured by filtering each sample through a 0.45um pore size membrane syringe filter and determining COD of filtrate by standard methods (APHA, 2012). The CH_4 concentration was measured by gas chromatography (GC System 7890B, Agilent Technologies, Irving, TX, USA) with a thermal conductivity detector. VFA concentrations were measured by gas chromatography using a flame ionization detector (GC-FID). TS, volatile solids (VS), total suspended solids (TSS), and VSS were measured by standard methods (APHA, 2012). Statistical analyses, such as the ANOVA single factor, and post-hoc two-sample Student t-test with equal variance, were performed using Microsoft Excel 2016 and built-in macro functions. The ANOVA single factor test and Bonferroni correction were used to adjust the significance to avoid statistical error when comparing groups greater than 2 samples.

4.2.5 Microbial Analysis

DNA was extracted from the 15L digesters on day 446 using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer instructions. The biomass samples were subjected to bead beating on a vortexer (Model 58816-121, VWR International, Radnor, PA, USA) for 10 minutes. Sequencing was performed by a commercial laboratory (MR DNA, Shallowater, TX, USA) using an Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA, USA) following manufacturer guidelines. Barcodes and primers were removed from Q25 filtered sequences and processed as previously described (Dowd et al., 2008). Briefly, data were refined by removing sequences <200 bp, sequences with ambiguous base calls, and sequences with homopolymers >6 bp. Denoised sequences were clustered into operational taxonomic units (OTUs) having 97% similarity. Singleton sequences and chimeras were removed. BLASTn was used to taxonomically classify OTUs against a curated data- base derived from GreenGenes, RDPII, and NCBI (CME, 2015; DeSantis et al., 2006; NCBI, 2015). A sample-based rarefaction was performed on the combination of archaea and bacteria operational taxonomic units (OTUs). Relative abundance was calculated for each OTU at the genus level by dividing the number of OTU sequence count by the total number of sequence counts in the sample. Custom R scripts using the phyloseq program for R studio were used to perform non-metric multidimensional scaling (nMDS) using the Double Wisconsin Transformation for community sequence data gathered from Illumina sequencing. Data was transformed to even sampling depth, and nMDS plots were created using the Double Wisconsin Transformation with the function, ordinate (“NMDS”,

"bray"). Principal component analysis (PCA) was also performed using R, and custom R scripts using the packages phyloseq, FactoMineR, and factoextra.

4.3 Results and Discussion

4.3.1 Reconstituted Dried Biomass Digester Function

Biogas production as well as increased SCOD and acetic acid concentrations were seen within 24 hours of initial startup of each triplicate 500 mL batch system, whereas CH_4 production was first observed after 20 days. This indicates that the methanogenic microbes were inhibited more with air drying compared to hydrolytic and fermentative bacteria, which were active immediately after incubation. Initial TSS and VSS concentrations of the 500 mL batch systems were observed to be 0.98±0.003 g/g and 0.67±0.01 g/g for LAD-D, 0.98±0.003 g/g and 0.66±0.014 g/g for LADE-D, and 0.98±0.004 g/g and 0.64±0.008 g/g for ADC-D.

Two days after startup, biogas production was observed in each batch system. The overall ACD-D set biogas production rate (6.0±0.91mL biogas/day) was 20% greater than the LAD-D (4.4±0.2 mL biogas/day) and LADE-D (4.4± 0.39mL biogas/day) (Figure 11). SCOD data is shown in Figure 12. The highest average SCOD concentration for the ACD-D, LAD-D, and LADE-D sets were 5.59 ±0.3 g/L, 3.13±0.15 g/L, and 3.16 ±0.51 g/L, respectively. These concentrations were observed on day 60 for LAD-D and ACD-D, and day 40 for LADE-D. SCOD concentrations gradually decreased to 0.02±0.02 g/L, 0.03±0.03 g/L, and 0.81±1.03 g/L on day 170 for LAD-D, LADE-D, and ACD-D, respectively. Results showed that biomass that has been dried and stored for 2 months can produce biogas from SCOD and VFAs utilization through endogenous

decay. The production of biogas, SCOD, and VFAs from dried biomass activity once rehydrated correlates to previous studies that showed methane production after heat drying (Tale et al. 2015; Bhattad et al. 2017).

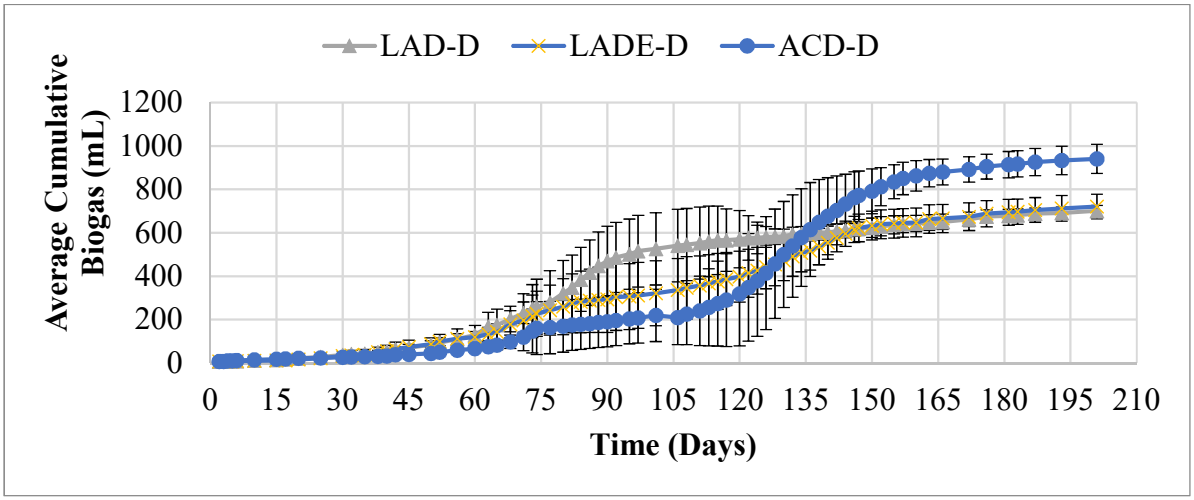

Figure 11. Average biogas production for the 500 mL batch systems. The plot shows the average biogas production observed in batch systems incubated with dried aero-tolerant biomass. The error bars represent standard deviation from triplicate digesters.

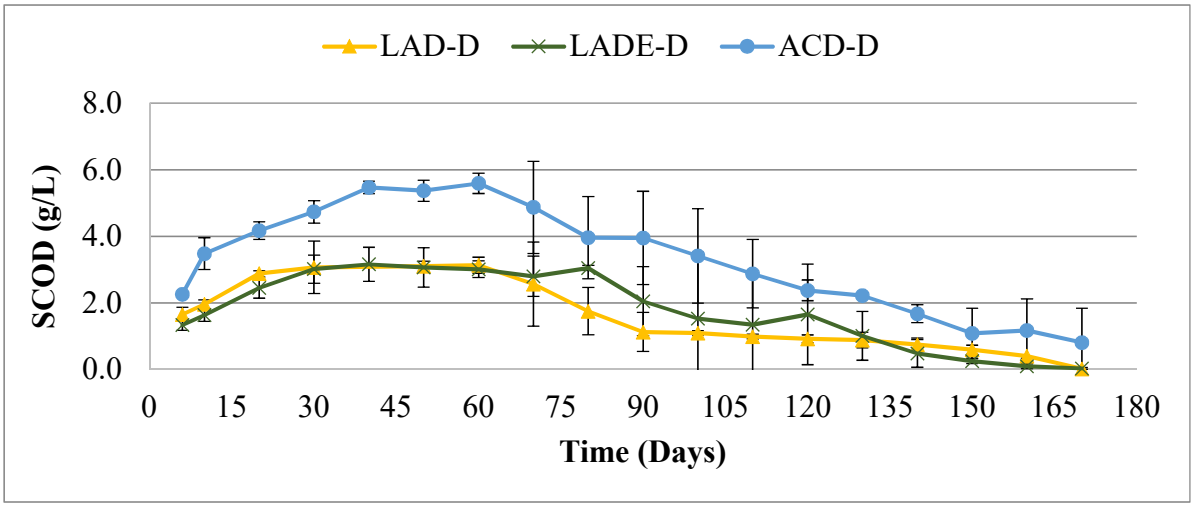

Figure 12. Average SCOD concentration for the 500 mL batch systems. The plot shows the average SCOD concentration profile observed in batch systems incubated with dried aero-tolerant biomass. The error bars represent standard deviation from triplicate digesters.

VFA data are shown for the LAD-D (Figure 13-A), LADE-D (Figure 13-B), and ACD biomass (Figure 13-C). The highest average total VFA concentration for the LAD-D, LADE-D, and ACD-D sets were 3.3±0.6 g/L, 3.1±0.5 g/L, and 5.4±0.4 g/L, respectively. These concentrations were observed on day 40 for LAD-D and LADE-D, and day 60 for ACD-D. VFA concentrations gradually decreased to 0.11±0.1 g/L, 0.06 ±0.03 g/L, and 0.72±1.1g/L on day 170 for LAD-D, LADE-D, and ACD-D, respectively. Acetic acid was the dominant VFA in the batch systems, accounting for 60-80% of total VFA concentration (Figure 13A-C).

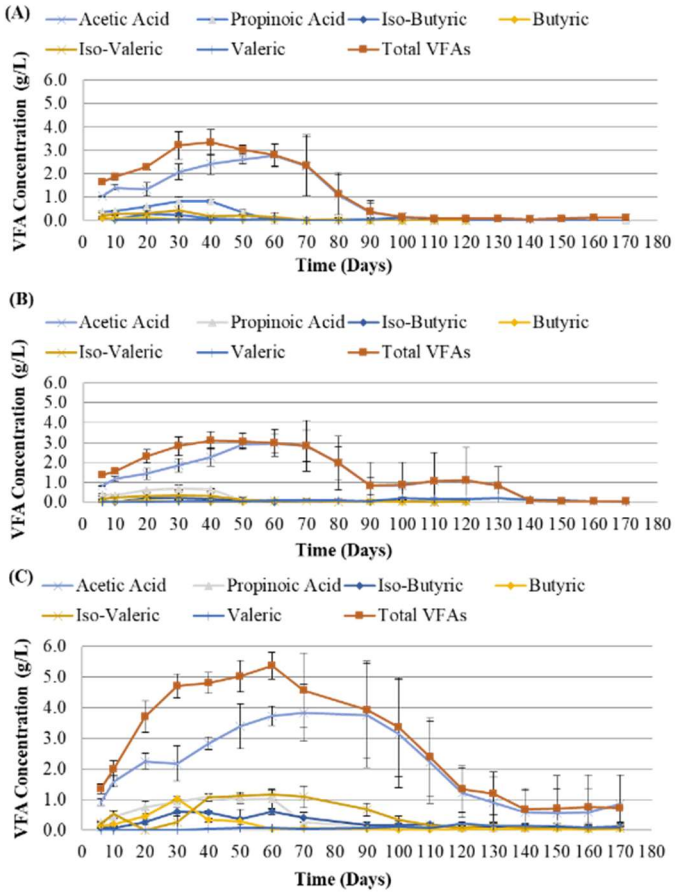

Figure 13. Average VFA concentrations from the effluent of the batch systems (A) LAD-D, (B) LADE-D, and (C) ACD-D during 170 days of operation. The error bars represent standard deviation from triplicate digesters.

CH_4 production was first detected in LAD-D and LADE-D batch systems after 20±0 days of incubation, and 26±6 days for the ACD-D batch systems. Figure 14 and Figure 15 show cumulative CH_4 production over time. The ACD-D CH_4 production rate from day 15 to day 170 was 6.3±0.84 mL CH_4/day. The LAD-D and LADE-D set CH_4 production rates were 4.7±0.48 and 4.6±0.21 mL CH_4/day (Figure 14). The ACD-D set CH_4 production rate was 25% and 27% greater than the LAD-D and LADE-D. However, CH_4 production rate was higher for both the LAD-D and LADE-D than the ACD-D during days 15 to 100. Figure 15 shows CH_4 production from days 20 to 100. For days 20 to 100, the ACD-D set CH_4 production rate (2.4±0.43 mL CH_4/day) was 62% and 54% lower than the LAD-D (6.3 mL±1.24 CH_4/day) and the LADE-D (5.2±2.09 mL CH_4/day). These data showed that the aerated dried biomass had a significantly shorter lag time (the amount of days till maximum CH_4 production or CH_4 max) compared to the anaerobic dried biomass regarding CH_4 production. For the LAD-D and LADE-D the CH4 max was approximately 90 days and approximately 140 days. Therefore, the combination of aeration and drying was shown to greatly reduce lag time to CH_4 max compared to the rehydrated anaerobic control biomass. Aeration and drying also reduced time to initial CH_4 detection. Research by Zitomer et al. (2016) noted CH4 detection at 3 months for 2L digesters bioaugmented with an aerotolerant culture. This may indicate that the microbial community from the aerated digesters had a higher tolerance to the heat drying process.

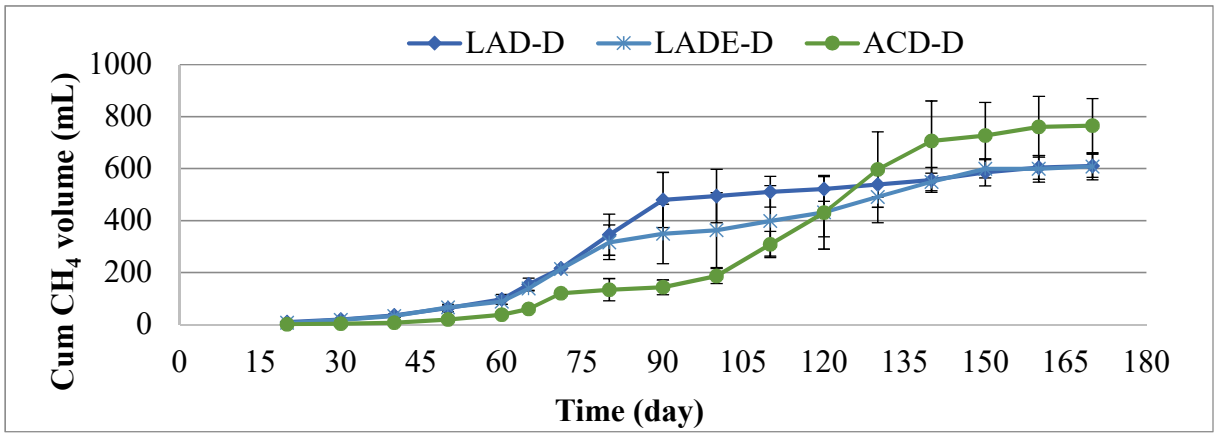

Figure 14. Average cumulative CH_4 volume for the 500mLbatch systems. The plot shows the cumulative CH_4 volume produced from the digesters incubated with dried aero-tolerant biomass for 0 to 170 days. The error bars represent standard deviation for triplicate digesters.

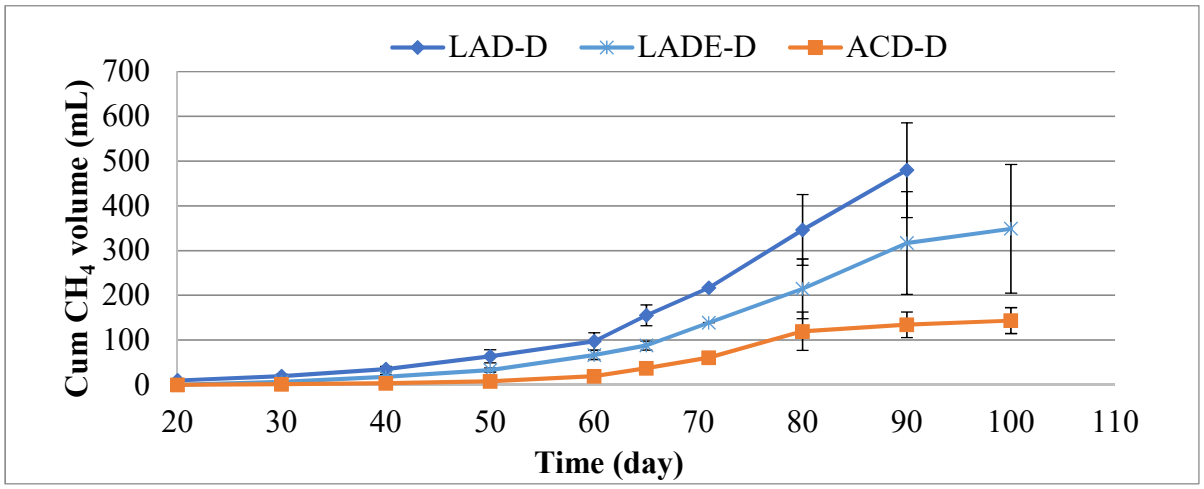

Figure 15. Average cumulative CH_4 volume for the 500mLbatch systems. The plot shows the cumulative CH_4 volume produced from the batch systems incubated with dried aero-tolerant biomass for 20-100 days. The error bars represent standard deviation for triplicate digesters.

4.3.2 SMA Analysis

SMA data showed survivability of methanogenic cultures after heat drying in an air atmosphere and storage for two months. VSS concentrations for LAD, LADE, and ACD were 2.27±0.27, 2.28±0.13, and 2.11±0.15 g/L, respectively. SMA tests for LAD, LADE, and ACD biomass were performed on day 501. VSS concentrations for LAD-D, LADE-D, ACD-D were 1.57±0.12 g/L, 1.29±0.067 g/L, and 1.79 ±0.17 g/L. SMA tests for LAD-D, LADE-D, and ACD-D were performed on day 205 of operation.

Figure 16 shows mL CH_4/ g VSS-d for the LAD, LADE, ACD (wet cultures), and the LAD-D, LADE-D, and ACD-D (dry cultures) for the SMA test with non-fat dried milk as the substrate. In comparison of solely dry cultures, the LAD-D SMA value (27.6± 5.6 mL CH_4/g VSS-day) was 18% greater than the SMA of LADE-D (22.6±8.7 mL CH_4/g VSS-day) and 60% greater than the SMA of ACD-D (11±0.62 mL CH_4/g VSS-day). The LAD-D and ACD-D ($p=0.03$, $n=8$) were significantly different for SMA rate with non-fat dried milk as the substrate ($p<0.05$). The LADE-D and ACD-D ($p=0.46$, $n=8$) and the LADE-D and LAD-D ($p=0.15$, $n=8$) were not significantly different. Therefore, aeration and aeration and augmentation were shown to better preserve SMA with non-fat dried milk than a non-aerated system after drying and storage of biomass then rehydration. Microbially, this was represented by an increase relative abundance in *Rikenella* in the LAD and LADE systems, and an increase in *Bacteroides* (see Table 12-14 in *Digesters Grouped Based on Bacterial Communities*).

In comparison of wet versus dry cultures, the average LAD SMA value (63.9±7.3 mL CH_4/g VSS-day) was 56% greater than the SMA of LAD-D (27.6± 5.6 mL CH_4/g

VSS-day); average LADE CH_4 production rate (52.04± 3.4 mL CH_4/g VSS-day) was 56% greater than the SMA of LADE-D (22.6±8.7 mL CH_4/g VSS-day); average ACD CH_4 production rate (58.6± 5.4 mL CH_4/g VSS-day) was 81% greater than the SMA of ACD-D (11±0.62 mL CH_4/g VSS-day). The wet cultures LAD and ACD were statistically higher than their dry culture counterparts for SMA rate with non-fat dried milk as the substrate ($p<0.05$, $n=6$). However, after the Bonferroni correction factor, the LADE-D and LADE biomass were not statistically different with non-fat dried milk as the substrate. This indicates that while CH_4 production is possible after air drying and rehydration, anaerobic digester microbial communities may be only partially tolerant to the combination of heat and air drying.

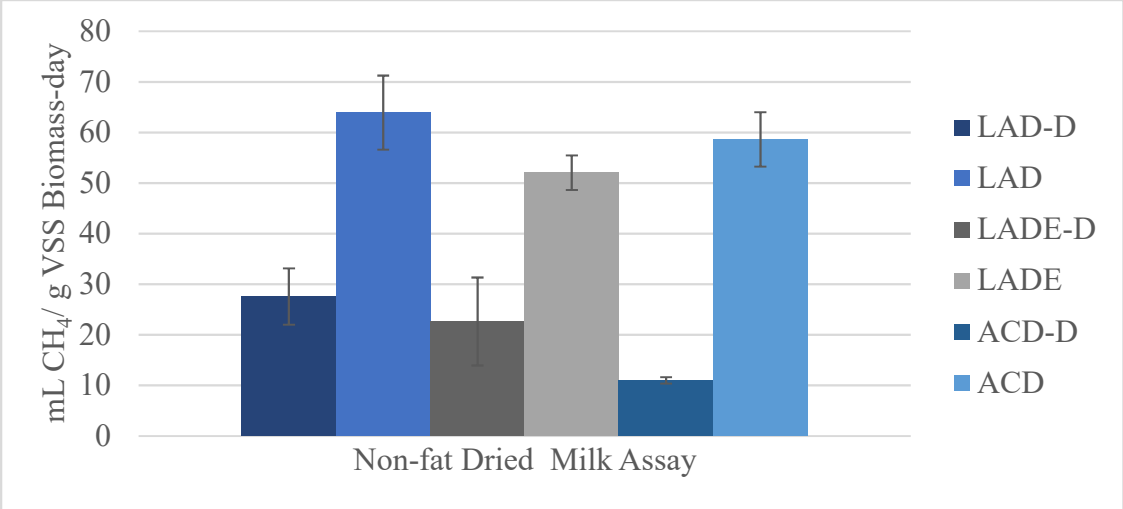

Figure 16. CH_4 production rates per gram of VSS-d for the LAD, LADE, and ACD, and the LAD-D, LADE-D, and ACD-D systems with non-fat dried milk. The error bars represent standard deviation from triplicate observations.

Figure 17 shows mL CH_4/g VSS-day for the wet and dry cultures for the SMA test with acetate as the substrate. For dry cultures, the average LADE-D SMA (250.4±

12.9mL CH_4/g VSS-day) was 24% greater than the SMA rate of LAD-D (190.1±1.9mL CH_4/g VSS-day) and 32% greater than the SMA rate of ACD-D (170.3±0.93mL CH_4/g VSS-day). The observed SMA from the LADE-D was statistically higher ($p<0.05$, $n=5$) than both LAD-D and ACD-D digesters, respectively. Therefore, the combination of aeration and augmentation resulted in the highest SMA with acetate in comparison to aeration and an anaerobic control after heat drying biomass and rehydration.

For wet versus dry cultures, the average LAD SMA (363.9±12.1mL CH_4/g VSS-day) was 48% greater than the SMA rate of LAD-D (190.1± 1.9mL CH_4/g VSS-day); average LADE-D CH_4 production rate (250.4± 12.9mL CH_4/g VSS-day) was 1% greater than the SMA rate of LADE (248±4.6mL CH_4/g VSS-day); average ACD CH_4 production rate (351.4±8.8mL CH_4/g VSS-day) was 52% greater than the SMA rate of ACD-D (170.3±0.93mL CH_4/g VSS-day). The LAD-D and LAD ($p=0.0014$, $n=4$) and ACD-D and ACD ($p=0.0007$, $n=4$) were significantly different for SMA rate with acetate as the substrate using the two-tailed student t-test with unequal variance and a 95% confidence interval ($p<0.05$). LADE-D and LADE ($p=0.79$, $n=4$) were not significantly different ($p<0.05$). Based on the acetate SMA test the LAD and ACD had statistically greater SMA than their dry culture counterparts. Only the LADE-D and LADE had statistically similar SMA with acetate. Higher SMA with acetate for wet cultures versus dry cultures correlate to previous research by Bhattad et al. (2017). A potential reason for a lower SMA with dried biomass is that hydrogenotrophic methanogens are better preserved in the heat drying process than acetoclastic methanogens (Tale et al., 2015; Bhattad et al. 2017). Microbially, this was represented by in an increase in hydrogenotrophic methanogens and a decrease in acetoclastic (see Table 7 for wet

cultures and Tables 8-10 for dry cultures).

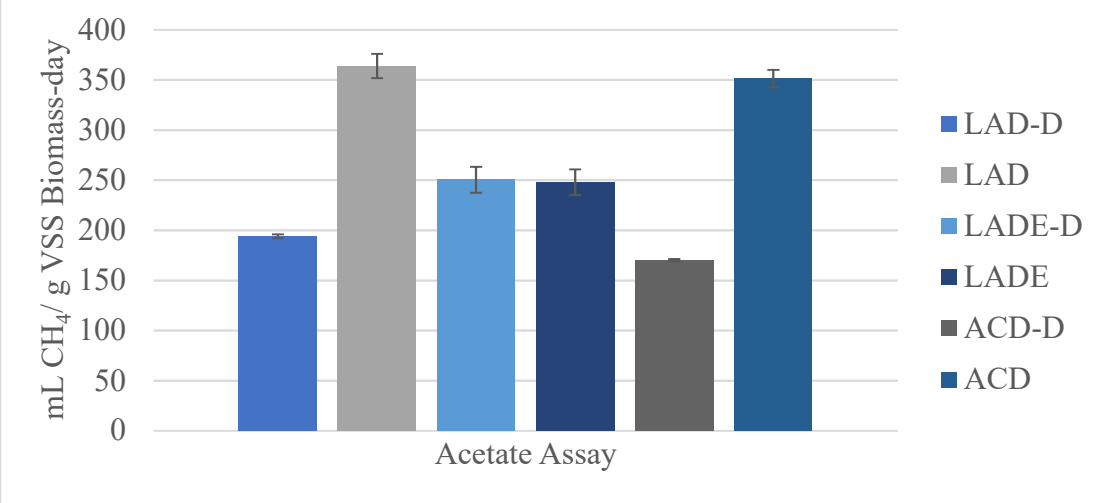

Figure 17. CH_4 production rates per gram of VSS-d for the LAD, LADE, and ACD, and the LAD-D, LADE-D, and ACD-D systems with acetate. The error bars represent standard deviation from triplicate observations.

Figure 18 shows mL CH_4/ g VSS-d for the wet and dry cultures for the SMA test with H_2/CO_2 as the substrate. The average LADE-D SMA (1.17± 0.075 mL CH_4/g VSS-day) was 13% greater than the SMA rate of LAD-D (1.08±0.1 mL CH_4/g VSS-day) and 36% greater than the SMA rate of ACD-D (0.71±0.12 mL CH_4/g VSS-day). The observed SMA from the LADE-D was statistically higher ($p<0.05$, $n=6$) than the ACD-D digesters. SMA for the LAD-D and LADE-D SMA values with H_2/CO_2 were not significantly different ($p=0.088$, $n=10$). Therefore, aeration and aeration with augmentation with an aerotolerant culture was shown to better preserve hydrogenotrophic activity than an anaerobic system after heat drying and storage.

For wet versus dry cultures, the average LAD-D SMA (1.08± 0.1 mL CH_4/g VSS-day) was 45% greater than the SMA of LAD (0.59± 0.006 mL CH_4/g VSS-day); average

LADE-D SMA (1.17± 0.075 mL CH_4/g VSS-day) was 45% greater than the SMA of LADE (0.64±0.14 mL CH_4/g VSS-day); average ACD-D CH_4 production rate (0.71±0.12 mL CH_4/g VSS-day) was 17% greater than the SMA of ACD (0.59±0.06 mL CH4/g VSS-day). The LAD-D and LADE-D dry cultures had significantly higher SMA than wet cultures ($p<0.05$, $n=10$). The ACD-D and ACD biomass was statistically the same with H_2/CO_2 as the substrate. Results showed an increased SMA activity with dry cultures in comparison to wet cultures with H_2/CO_2, which indicates an enrichment of hydrogenotrophic methanogens. These results confirm previous studies that drying enriches for hydrogenotrophic methanogens over acetoclastic (Tale et al., 2015 Bhattad et al. 2017). In methanogenic microbial communities, this was seen in the LAD-D, LADE-D, and ACD-D systems with a shift in the archaeal communities to a higher relative abundance of hydrogenotrophic methanogens. Specifically, *Methanobacterium* in the LAD-D biomass (Table 8), *Methanobacterium* and *Methanoculleus* in the LADE-D biomass (Table 9), and *Methanothermobacter* and *Methanobacterium* in the ACD-D biomass (Table 10).

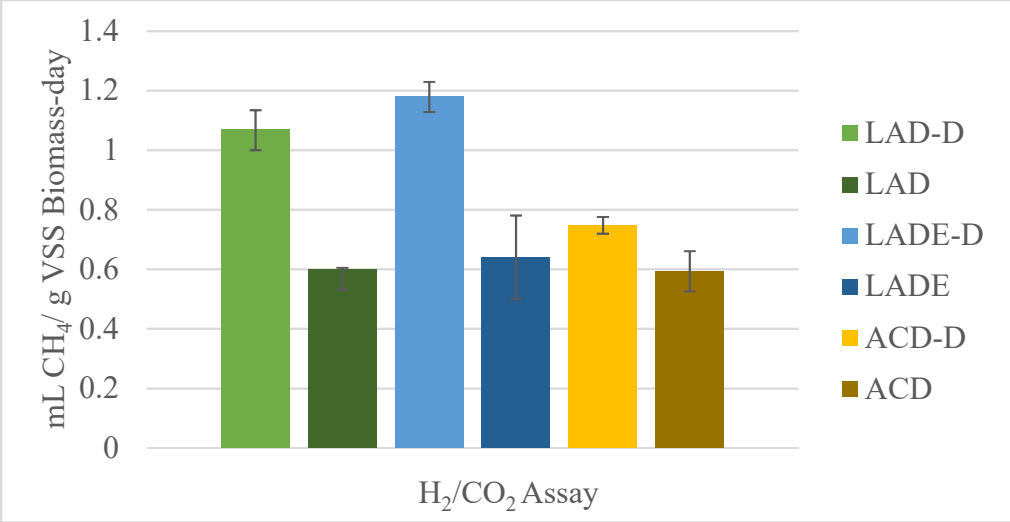

Figure 18 . CH_4 production rates per gram of VSS-d for the LAD, LADE, and ACD, and the LAD-D, LADE-D, and ACD-D systems with H_2/CO_2. The error bars represent standard deviation from triplicate observations.

4.3.3 Digesters Grouped Based on Archaeal Communities

Wet and dry cultures were compared microbially to determine potential shifts in bacterial and archaeal communities. Based on work by Seib, Berg, and Zitomer (2016) and Bhattad et al. (2017), it was expected that dry cultures would show a significant shift in microbial communities in comparison to wet cultures. For wet cultures, a total of 14, 15, and 13 archaeal OTUs based on 96 to 99% similarity were identified among each LAD, LADE and ACD digester sample analyzed. Seven Archaeal OTUs represented 95 to 99% of the archaeal relative abundance in all three digester types. These 7 OTUs were most similar to the genera *Methanosaeta, Methanolinea, Methanobrevibacter, Methanoculleus, Thermogymnomonas, Methanospirillum,* and *Methanococcus* (Table 7). The LAD, LADE, and ACD were distinguished by a high relative abundance of *Methanosaeta*, an acetoclastic methanogen, which ranged from 58 to 77% in each

digester. In comparison to the LAD and ACD, the LADE digester had a higher relative abundance of the hydrogenotrophic methanogens, *Methanospirillium* (23.5%) and *Methanococcus (*1.55%) (Table 7).

A total of 11±1, 10±1, and 10. ±0.5 archaeal OTUs at the genus level were identified among the reconstituted dried biomass batch system sets LAD-D, LADE-D, and ACD-D samples analyzed. Tables 8-10 show archaeal communities that accounted for 96 to 99% of the total abundance of archaea for the triplicate sets of LAD-D, LADE-D, and ACD-D. The LAD-D was distinguished by the genera *Methanobacterium, Methanosarcina, and Methanoculleus*; the LADE-D was distinguished by the genera *Methanobacterium*, *Methanoculleus,* and *Methanosarcina*; the ACD-D archaea OTUs were most similar to the genera *Methanothermobacter, Methanobacterium, Methanoculleus,* and *Methanosarcina* (Tables 8-10).

The rarefaction curve performed using custom R scripts with phyloseq and is shown in Figure 19. The 12 sample points from the 6 biomass sets were clustered into 3 sample sets and 3 specific groups: the LAD, LADE, and ACD samples (wet cultures), and the triple reconstituted dried digesters operated in batch conditions, LAD-D1, LAD-D2, and LAD-D3 (Group 1), LADE-D1, LADE-D2, and LADE-D3 (Group 2), ACD-D1, ACD-D2, and ACD-D3 (Group 3). Group 1, 2, and 3 formed distinct clusters. In the nMDS plot there was a significant shift from the wet cultures from Groups 1-3 (Figure 20). In reconstituted dried biomass digesters, Groups 1-3, there was a significant increase in hydrogenotrophic methanogen relative abundance. Diversity in archaea also decreased. Dried biomass had fewer detected archaea on the genus level than wet biomass and were dominated by fewer archaea for relative abundance; in general, by hydrogenotrophic

methanogens (see Table 7 and Tables 8-10). Microbial data confirms previous work by Bhattad et al. (2017), who showed drying and storage reduced diversity of anaerobic microbial communities in comparison to wet cultures, and promoted hydrogenotrophic growth.

In comparison to wet cultures, which were dominated by *Methanosaeta* relative abundance, Group 1 was distinguished by *Methanobacterium* and *Methanosarcina* (Table 8), and Group 2 by *Methanobacterium, Methanosarcina,* and *Methanoculleus* (Table 9), and Group 3 by *Methanothermobacter, Methanobacterium,* and *Methanosarcina* (Table 10). In all 15L digester effluent that was dried and stored then reconstituted, there was an increase in hydrogenotrophic methanogen relative abundance, and a decrease of acetoclastic methanogen relative abundance, specifically *Methanosaeta.*

The PCA, which was subsequently performed with total OTUs analyzed, helps visualize the dominant archaea present in the wet, dried biomass, and rehydrated dried biomass (Figure 21). In the graph, the 15L digesters LAD, LADE, and ACD are represented by SMA.A, SMA.T, and SMA. C (Wet Samples). The 15L digester biomass that was dried at 104°C the LAD-DS, LADE-DS, and ACD-DS are represented by DS. A, DS. T, and DS.C, respectively (Dry Samples). The triplicate samples of LAD-D, LADE-D, and ACD-D are represented by A.1-3, T.1-3, C.1-3 (Reconstituted LAD, LADE, and ACD) (Figure 21). For archaea, vectors aligned with the OTUs that had the highest relative abundance in each sample, indicating the archaeal community difference across each sample was due to specific dominant OTUs in each sample. The wet and dry samples were driven by *Methanosaeta*, *Methanospirillium* and *Methanolinea;* the A.1-3 and T.1-2 samples were driven by *Methanobacterium and Methanosarcina*; the T.3 and

the C.3 samples were driven by *Thermogymnomonas*, and the C.1-2 samples were driven by *Methanoculleus* and *Methanothermobacter*.

Table 7. Archaea abundance on the genus level for the LAD, LADE, and ACD Systems.

Genus	LAD %Abundance	LADE %Abundance	ACD % Abundance
Methanosaeta	77.0	63.6	58.1
Methanolinea	13.2	1.9	28.5
Methanobrevibacter	2.1	6.1	9.5
Thermogymnomonas	2.5	Not Detected	1.2
Methanospirillum	Not Detected	23.5	Not Detected
Methanococcus	3.3	1.3	1.9
Methanoculleus	Not Detected	3.3	Not Detected

Table 8. Archaea abundance on the genus level for the triplicate samples of LAD-D.

Genus	LAD-D1 %Abundance	LAD-D2 %Abundance	LAD-D3 %Abundance
Methanobacterium	43.5	54.8	55.6
Methanosarcina	34.0	29.1	1.9
Methanoculleus	10.6	8.3	26.0
Thermogymnomonas	7.7	5.3	6.9
Methanobrevibacter	3.3	2.0	8.9

Table 9. Archaea abundance on the genus level for the triplicate samples of LADE-D.

Genus	LADE-D1 %Abundance	LADE-D2 %Abundance	LADE-D3 %Abundance
Methanobacterium	46.3	24.3	20.1

Methanoculleus	41.7	8.8	4.4
Thermogymnomonas	7.3	5.2	12.4
Methanobrevibacter	4.2	3.0	3.5
Methanosarcina	0.18	58.4	51.6
Methanothermobacter	0.18	0.1	0.15

Table 10. Archaea abundance on the genus level for triplicate samples of ACD-D systems.

Genus	ACD-D1 %Abundance	ACD-D2 %Abundance	ACD-D3 %Abundance
Methanothermobacter	55.1	59.8	15.6
Methanobacterium	21.9	1.2	41.5
Methanoculleus	15.8	15	4.4
Thermogymnomonas	6.6	9.8	4.4
Methanosarcina	0.4	13.8	33.7

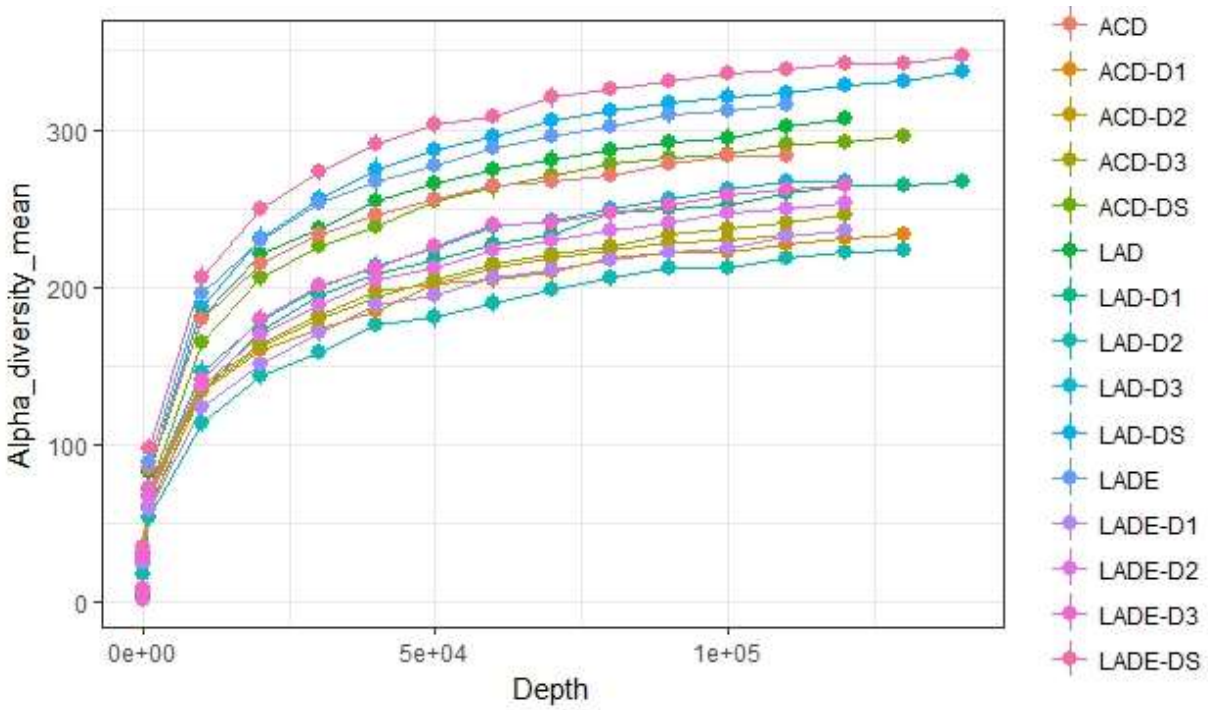

Figure 19. Rarefaction curve of total OTUs of archaea and bacteria.

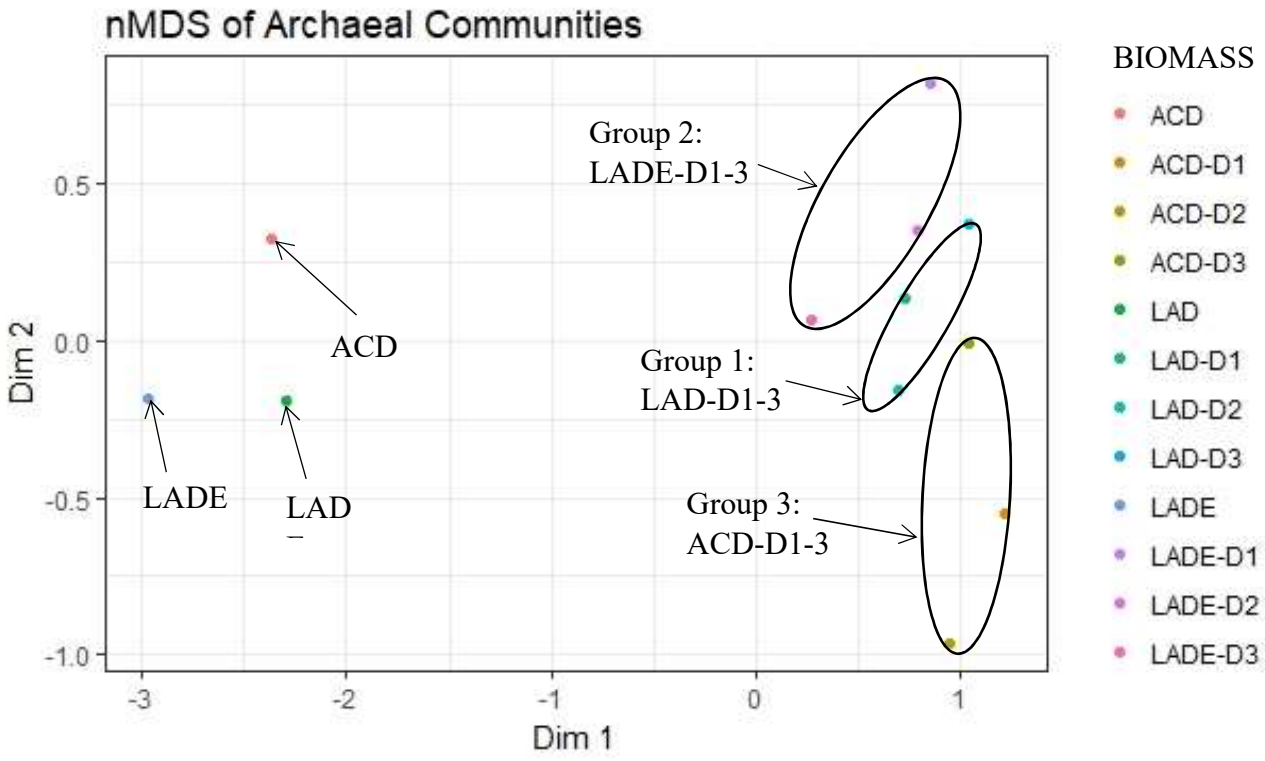

Figure 20. Archaeal sequence nMDS scaling plot. The LAD, LADE, and ACD effluent biomass are represented by separate sample points. Groups 1-3 represent triplicate reconstituted biomass digester sets (LAD-D1-3, LADE-D1-3, ACD-D1-ACD-D3). Groups 1-3 showed a significant shift from 15L samples.

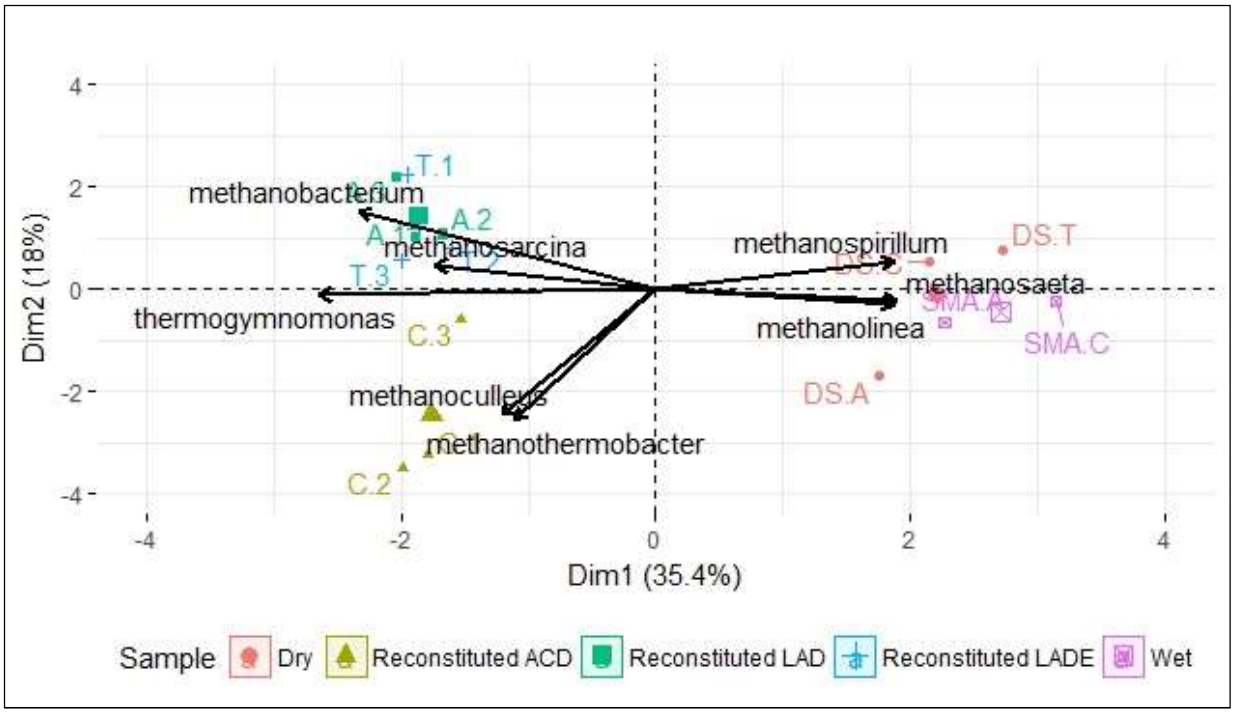

Figure 21. PCA analysis of Archaea 16S rRNA sequencing profiles for each AD biomass sample.

4.3.4 Digesters Grouped Based on Bacterial Communities

A total of 214, 222, and 197 bacterial OTUS were identified on the genus level for each of the LAD, LADE, and ACD biomass samples analyzed. The most common bacterial communities observed in the digesters were the genera *Clostridium, Petrimonas, Pseudoalteromona, and Parvimonas* (Table 11). Table 11 shows the 30 OTUs that contributed to approximately 92 to 96% of the total bacteria sequences in the LAD, LADE, and ACD. LAD and LADE were distinguished from ACD with a higher relative abundance of *Petrimonas* an *Parvimonas*. ACD was dominated mostly by *Pseudoalteromonas and Clostridium* (Table 11).

A total of 170 ±10, 166±9, and 162±2 bacterial OTUs on the genus level were identified among the reconstituted dried biomass digester sets LAD-D, LADE-D, and ACD-D samples analyzed. Tables 12-14 show bacterial communities that accounted for 96 to 99% of the total abundance of bacteria for the triplicate sets of LAD-D, LADE-D, and ACD-D. Bacterial relative abundance varied slightly between triplicate sets. The LAD-D was distinguished by the genera *Rikenella* and *Clostridiisalibacter*; the LADE-D was distinguished by the genera *Rikenella* and *Anaerophaga;* the ACD-D bacteria OTUs were most similar to the genera *Bacteroides, Clostridium, and Rikenella* (Tables 12-14).

The rarefaction curve performed using custom R scripts with phyloseq and is shown in Figure 19 in *Digesters Grouped Based on Archaeal Communities*. The 12 sample points from the 6 biomass sets were clustered into 3 sample sets and 3 specific groups: the LAD, LADE, and ACD samples (wet cultures), and the triple reconstituted dried digesters operated in batch conditions, LAD-D1, LAD-D2, and LAD-D3 (Group 1), LADE-D1, LADE-D2, and LADE-D3 (Group 2), ACD-D1, ACD-D2, and ACD-D3 (Group 3). Group 3 formed a distinct cluster, while 1 and 2 groups overlapped (Figure 22). In the nMDS plot there was a significant shift from the wet cultures to Group 1-3 (Figure 22). In Groups 1-3, there was a significant increase in *Rikenella* from the wet cultures. *Bacteroide* relative abundance was higher in Group 3 than in Groups 1 and 2 (Tables 12-14). *Clostridium* relative abundance also did not decrease significantly from the wet cultures to Group 3 (Table 11 and Table 14).

The PCA in Figure 23 shows dominant bacteria present in the total digester biomass samples analyzed. In the graph, the 15L digesters LAD, LADE, and ACD are represented by SMA.A, SMA.T, and SMA. C (Wet Samples). The 15L digester biomass

that was dried at 104°C the LAD-DS, LADE-DS, and ACD-DS are represented by DS. A, DS. T, and DS.C, respectively (Dry Samples). The triplicate samples of LAD-D, LADE-D, and ACD-D are represented by A.1-3, T.1-3, C.1-3 (Reconstituted LAD, LADE, and ACD) (Figure 23). Like the results of the archaeal data, vectors representing bacterial OTUs aligned with different AD biomass samples. The wet and dry samples were driven by *Petrimonas, Pseudoalteromonas,* and *Bacteroides*; the A.1-3 and T.1-2 samples were driven by *Clostridiisalibacter* and *Rikenella*; The T.3 sample was driven by *Clostridiisalibacter, Rikenella,* and *Anaerophaga,* and the C.1-3 samples were driven by *Proteiniphilum*.

Table 11. Percent abundance of most common bacteria genus OTUs for LAD, LADE, and ACD.

OUT	genus	LAD %Abundance	LADE %Abundance	ACD %Abundance
1	clostridium	17.5	11.3	18.1
2	petrimonas	16.7	16.5	2.7
3	pseudoalteromonas	11.8	8.1	29.3
4	parvimonas	10.5	11.5	4.0
5	sphingobacterium	6.2	Not Detected	Not Detected
6	rikenella	5.9	6.1	4.2
7	syntrophobacter	3.2	2.0	Not Detected

8	rubrobacter	3.1	Not Detected	7.2
9	parabacteroides	2.9	0.4	Not Detected
10	desulfomicrobium	2.1	Not Detected	Not Detected
11	synergistes	2.0	1.7	9.7
12	bellilinea	2.0	0.5	1.6
13	solitalea	1.7	6.2	0.9
14	cytophaga	1.5	1.6	2.3
15	bacteroides	1.4	2.5	0.8
16	bacillus	0.7	2.3	Not Detected
17	syntrophorhabdus	0.7	0.6	1.7
18	anaerophaga	0.6	6.3	0.9
19	lachnoclostridium	0.5	Not Detected	Not Detected
20	thermoanaerobacter	0.5	Not Detected	Not Detected
21	desulfonispora	Not Detected	Not Detected	0.6
22	desulfobulbus	Not Detected	1.1	0.6
23	desulfofaba	Not Detected	Not Detected	0.8
24	atopobium	Not Detected	Not Detected	1.7

25	saccharibacter	Not Detected	Not Detected	0.7
26	spirochaeta	Not Detected	Not Detected	0.8
27	smithella	Not Detected	Not Detected	1.8
28	sulfuricurvum	Not Detected	1.3	Not Detected
29	olsenella	Not Detected	9.2	Not Detected
30	calderihabitans	Not Detected	0.5	Not Detected

Table 12. Percent abundance of most common bacteria genus OTUs for LAD-D1, LAD-D2, and LAD-D3.

OTUs	genus	LAD-D1 %Abundance	LAD-D2 %Abundance	LAD-D3 %Abundance
1	rikenella	44.50	49.46	39.26
2	clostridiisalibacter	13.19	17.59	10.41
3	pseudoalteromonas	8.31	9.62	5.58
4	clostridium	5.09	6.19	5.24
5	thermoanaerobacter	0.49	3.14	2.50
6	petrimonas	4.47	2.00	5.18
7	syntrophomonas	1.72	1.31	2.99
8	synergistes	1.76	0.35	1.20
9	proteiniphilum	0.90	1.08	2.09
10	alkaliphilus	1.00	0.69	1.54
11	moorella	1.35	0.51	0.63
12	parvimonas	1.22	0.45	1.19
13	bacteroides	1.11	2.09	9.22

14	kosmotoga	0.72	0.33	0.98
15	olsenella	0.67	Not Detected	0.93
16	bacillus	3.52	0.35	1.05
17	desulfotomaculum	0.72	0.49	0.76
18	desulfobulbus	Not Detected	Not Detected	0.52
19	desulfosporosinus	0.50	0.23	Not Detected
20	aminomonas	Not Detected	0.23	0.87
21	parabacteroides	2.09	0.46	Not Detected
22	proteiniborus	Not Detected	0.37	Not Detected
23	desulfonispora	Not Detected	Not Detected	0.49
24	exiguobacterium	1.80	Not Detected	Not Detected

Table 13. Percent abundance of most common bacteria genus OTUs for LADE-D1, LADE-D2, and LADE-D3.

OTUs	genus	LADE-D1 %Abundance	LADE-D2 %Abundance	LADE-D3 %Abundance
1	rikenella	47.97	20.9	6.9
2	pseudoalteromonas	9.77	10.8	Not Detected
3	clostridiisalibacter	4.46	Not Detected	4.4
4	clostridium	4.44	6.0	6.0
5	bacteroides	3.84	3.8	13.0
6	alkaliphilus	3.15	1.4	
7	petrimonas	2.68	4.8	4.6
8	proteiniphilum	2.30	4.2	2.5
9	pelotomaculum	2.29	Not Detected	Not Detected
10	olsenella	2.00	2.3	6.9
11	syntrophomonas	1.83	3.2	2.7
12	synergistes	1.32	2.7	2.6

13	kosmotoga	1.32	2.0	1.6
14	symbiobacterium	1.04	0.8	Not Detected
15	moorella	0.97	4.3	3.4
16	ralstonia	Not Detected	1.2	1.9
17	desulfotomaculum	0.93	1.2	1.1
18	desulfonispora	0.79	0.7	1.7
19	parvimonas	1.53	5.7	3.4
20	proteiniborus	0.72	Not Detected	Not Detected
21	bacillus	0.70	Not Detected	Not Detected
22	anaerophaga	Not Detected	11.7	16.5
23	anaerovirgula	Not Detected	Not Detected	1.2
24	thermoanaerobacter	Not Detected	5.4	1.2
25	desulfosporosinus	Not Detected	1.0	Not Detected
26	exiguobacterium	Not Detected	Not Detected	6.2
27	parabacteroides	Not Detected	Not Detected	2.8

Table 14. Percent abundance of most common bacteria genus OTUs for ACD-D1, ACD-D2, and ACD-D3.

OTUs	genus	ACD-D1 %Abundance	ACD-D2 %Abundance	ACD-D3 %Abundance
1	bacteroides	21.0	12.76	21.76
2	clostridium	15.2	12.76	18.49
3	rikenella	14.9	7.43	9.72
4	proteiniphilum	7.7	9.44	6.21
5	pseudoalteromonas	6.7	3.09	3.34
6	petrimonas	6.2	9.56	9.21
7	alkaliphilus	3.2	7.99	5.19

8	thermoanaerobacter	3.0	2.54	1.64
9	clostridiisalibacter	2.7	2.79	6.24
10	pelotomaculum	2.4	Not Detected	Not Detected
11	syntrophomonas	2.3	1.98	2.15
12	thermacetogenium	2.0	5.65	Not Detected
13	parvimonas	1.5	6.41	3.75
14	synergistes	1.3	0.71	Not Detected
15	symbiobacterium	1.1	1.02	0.59
16	cryptanaerobacter	1.0	0.86	1.21
17	desulfotomaculum	0.9	0.71	0.74
18	proteiniborus	0.7	1.34	1.21
19	moorella	0.7	2.32	Not Detected
20	bacillus	0.5	2.64	0.46
21	parabacteroides	Not Detected	Not Detected	0.55
22	stenotrophomonas	Not Detected	Not Detected	0.42
23	desulfosporosinus	Not Detected	Not Detected	0.93
24	tepidanaerobacter	Not Detected	Not Detected	0.74
25	tissierella	Not Detected	1.59	Not Detected

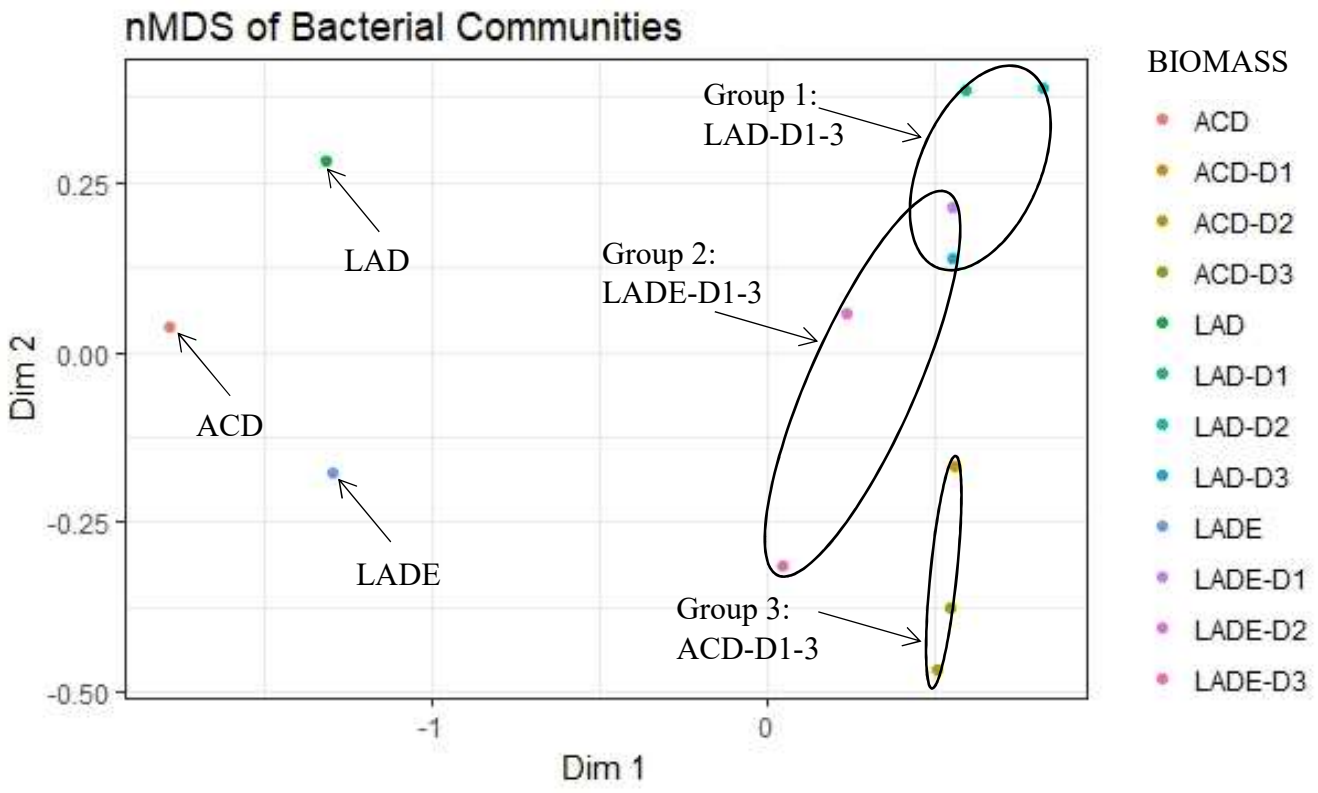

Figure 22. Bacterial sequence nMDS scaling plot. The LAD, LADE, and ACD effluent biomass are shown as separate sample points. The Groups 1, 2, and 3 represent triplicate reconstituted biomass digester sets (LAD-D1-D3, LADE-D1-D3, ACD-D1-D3). Groups 1-3 showed a significant shift from 15L samples.

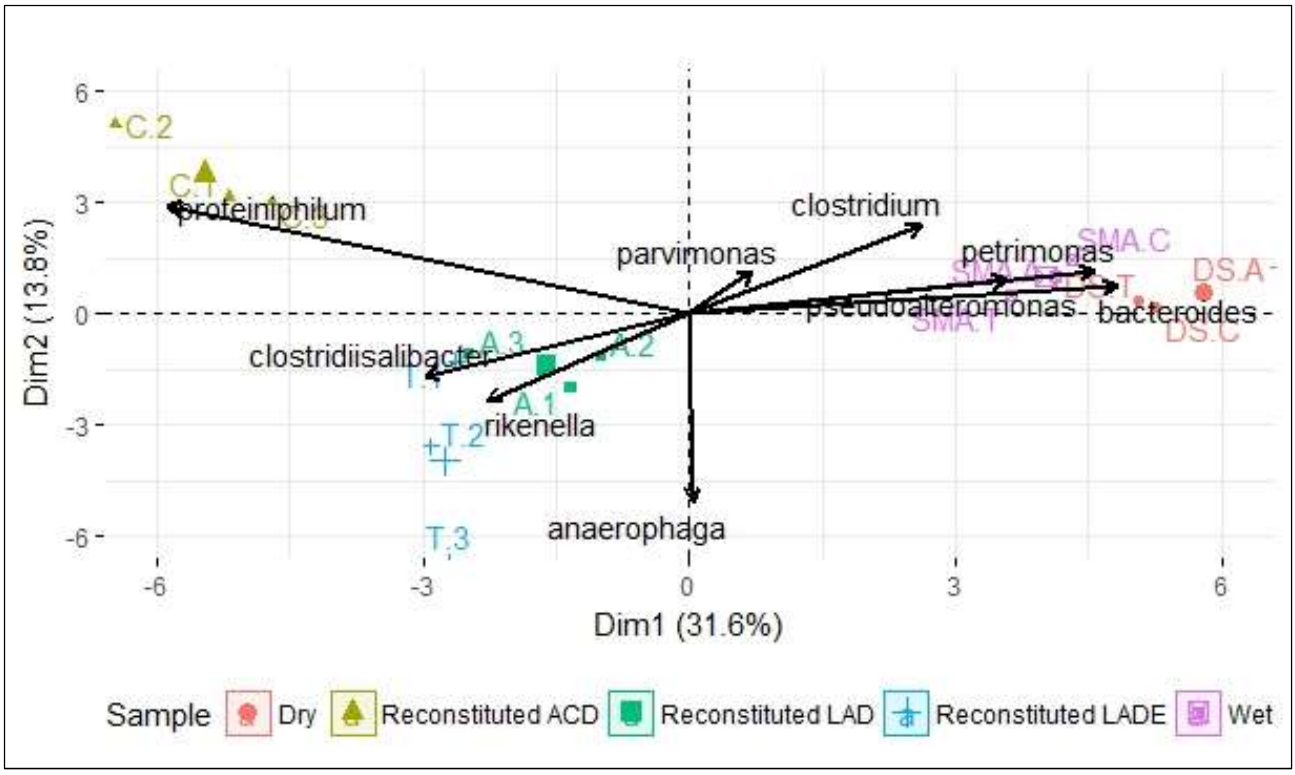

Figure 23. PCA analysis of Bacteria 16S rRNA sequencing profiles for each AD biomass samples.

4.4 Conclusion

The study investigated digester function of reconstituted biomass in a nutrient solution (Speece, 2008), SMA with non-fat dried milk, acetate, and H_2/CO_2, and whether microbial community composition data differed between 15L and reconstituted dried biomass digesters. All reconstituted digesters were operated identically in batch systems for 207 days of operation. Digester steady-state performance was similar for functional parameters, including CH_4 production rate, SCOD removal, pH and effluent VFA concentration. Dried biomass from a continuous aerated and aerated with augmented aerotolerant aerated culture resulted in a shorter lag time to maximum CH_4 production

than anaerobic biomass once reconstituted. In comparison to previous work, lag time was decreased from 3 months to 20 days to initial CH4 production.

From the non-fat dried milk test, the wet cultures had significantly greater SMA values than the dry cultures ($p<0.05$, $n=6$). This indicates that methanogenic communities may be only partially tolerant to heat drying and storage. Decrease in SMA in dry cultures were also represented by an increased relative abundance of *Rikenella*. The LAD and ACD had statistically greater SMA values with acetate than the LAD-D and ACD-D. The LADE and LADE-D had statistically similar SMA values with acetate ($p<0.05$, $n=4$). For the H_2/CO_2 SMA test, dry cultures had statistically greater SMA than the wet cultures (p,0.05, n=10). Increase in SMA correlated to an increase in relative abundance of hydrogenotrophic methanogens.

Heat drying of digester biomass did induce higher H_2/CO_2 activity and reduce diversity of bacteria and archaea. Microbially, this was represented in the nMDS and PCA plots as there was a significant shift for archaeal and bacterial communities from the wet to dry cultures. Hydrogenotrophic methanogens were dominated in dry cultures, whereas acetoclastic methanogens were dominate in wet cultures. For bacteria, dry cultures had higher relative abundance of *Rikenella*, *Clostridiisalibacter*, and *Proteiniphilum* than wet cultures.

5. Anaerobic Digester Performance with High Ammonia-Nitrogen Concentrations

A revised version of this chapter will be submitted to the journal *Bioresource Technology* as: Dylan, F., Venkiteshwaran, K., and Zitomer, D. Anaerobic Digester Performance with High Ammonia-Nitrogen Concentrations, B*ioresource Technology*.

5.1 Introduction

Formation of ammonia from degradation of protein rich materials requires strategies to prevent methanogenic inhibition (Westerholm et al., 2016). Free ammonia (NH_3) produced can passively diffuse into the cell, which can result in proton imbalance and potassium deficiency (Chen et al., 2008). Bacteria and archaea have demonstrated partial tolerance to NH_3 concentrations ranging from 4-5g NH_3/ L in anaerobic digesters (Chen et al., 2008). Anaerobic reactors with high NH_3 concentrations (>3 g/L NH_3-N) treat waste predominantly via hydrogenotrophic methanogenesis since acetoclastic methanogens are more sensitive to higher ammonia conditions. However, acetoclastic methanogens are also able to adapt to high NH_3 conditions (Westerholm, Roos, & Schnürer, 2010; Westerholm et al., 2016; Mosbaek et al., 2016).

In the anaerobic digester (AD) process, methane (CH_4) can be produced from acetate via a syntrophic acetate oxidizing (SAO) pathway. In this pathway, the acetate is oxidized to hydrogen (H_2) and carbon dioxide (CO_2) by syntrophic acetate oxidizing bacteria (SAOB), the H_2 and CO_2 are then converted to CH_4 by hydrogenotrophic methanogens (Westerholm et al., 2016). This pathway is alternate to the dominant pathway of CH_4 production from acetate, which involves direct conversion of acetate to

CH_4 by acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*). Currently known SAOB include strain advanced-oxidizing rod-shaped eubacterium (AOR) such as *Reversibacter*, *Clostridium ultunense*, *Thermacetogenium phaeum*, *Tepidanaerobacter acetatoxydans*, *Thermotoga lettingae*, and *Syntrophaceticus schinkii* (Fotidis et al., 2013; Hattori, 2008; Westerholm et al., 2016).

Previous studies have shown that under high NH_3 concentrations, the microbial communities shift from acetoclastic pathway to SAO pathway for CH_4 formation. This indicates that microorganisms governing the SAO pathway are more tolerant to high NH_3 concentrations and can fill the niche when the acetoclastic methanogens are inhibited. It was hypothesized that bioaugmentation of SAO microorganisms can improve anaerobic digester performance operating under NH_3 conditions of more than 5g/L. Currently, there are no established enrichment techniques for growing a SAO mixed culture. In this study, we demonstrate a novel enrichment method for developing a mixed anaerobic culture dominated by SAOB and hydrogenotrophic methanogens. This SAOB culture was subsequently used as bioaugment for anaerobic digesters operating with high ammonia-nitrogen (3, 6, and 9 g/L NH_3-N) concentrations.

5.2 Material and Methods

5.2.1 Development of Wet and Dry Cultures

Three 15L anaerobic enrichment digesters were fed non-fat dried milk in basal nutrient solution (Speece, 2008), with a chemical oxygen demand (COD) loading rate of 1 g/L_R-day at a 10-day hydraulic retention time (HRT). These digesters included the

anaerobic control digester (ACD), limited aerated anaerobic digester (LAD), and limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE). Dried biomass from each digester was then used to seed five anaerobic batch systems with a total volume of 500mL. These batch systems were designated as the anaerobic control digester-dried (ACD-D), limited aerated digester-dried (LAD-D), and limited aerated digester with augmented with an enrichment culture-dried (LADE-D) batch systems and operated for 205 days. For anaerobic toxicity assay (ATA) analysis, biomass was used from the ACD, LAD, and LADE (wet cultures), and ACD-D, LAD-D, and LADE-D (dry cultures). For the 15L and reconstituted dried biomass digesters, the ATA test was performed on day 503 and 207 of operation, respectively. The ATA assays were monitored for methanogenic rate and compared to the wet and dry culture microbial community data gathered from Illumnia sequencing.

5.2.2 NH_3-N ATA Tests

ATA tests were performed to evaluate NH_3 inhibition of the wet and dry cultures. The ATA tests were performed at varying NH_3-N concentrations ranging from 0 to 9g/L. The NH_3-N ATA tests were conducted using 160 mL serum bottles each containing 40 mL of anaerobic biomass (1.5-2.5 g/L volatile suspended solids (VSS)). Anaerobic biomass was from the ACD, LAD, and LADE. ATA tests were also performed using biomass from each digester that was dried and subsequently rehydrated. These biomass samples were the ACD-D, LAD-D, and LADE-D. Ten mL of a stock solution to obtain calcium acetate (10 g/L) with 0 to 9 g/L NH_3-N was added to 40 mL of biomass to achieve a total volume of 50 mL. Ammonium chloride (NH_4Cl) was used to prepare the

stock solution of NH_3-N. Each of the 6 biomass samples (ACD, LAD, LADE, ACD-D, LAD-D, and LADE-D) were run in triplicate for concentrations of 0, 3, 6, and 9 g/L NH_3-N. For CH_4 rate calculations, it was assumed that CH_4 and CO_2 partitioning to the liquid phase was negligible and the CH_4 concentration in the biogas produced remained constant. The CH_4 production rate was determined by linear regression of the cumulative CH_4 volume produced versus time plot after an initial, rapid period of CO_2 production that lasted from 0 to 2 days. Each ATA was calculated by measuring the maximum CH_4 production rate divided by the total VSS in each assay bottle. The half maximal inhibitory concentration (IC_{50}) expresses the toxicant concentration that produces a 50% decrease in the activity of microorganisms, in the biomass, that are exposed to the toxicant. The IC_{50} was calculated based on average percent inhibition of each ATA relative to the average ATA of each control.

5.2.3 Analytical Methods

Biogas production was measured periodically by inserting the needle of a glass syringe though each ATA bottle septum. Chemical oxygen demand (COD) was measured according to standard methods (APHA, 2012). Soluble COD (SCOD) was measured by filtering each sample through a 0.45 um pore size membrane syringe filter and determining filtrate COD by standard methods (APHA, 2012). CH_4 concentration was measured by gas chromatography (GC System 7890B, Agilent Technologies, Irving, TX, USA) with a thermal conductivity detector. Volatile fatty acid (VFA) concentrations were measured by gas chromatography (GC System) using a flame ionization detector. Total solids (TS), volatile solids (VS), total suspended solids (TSS), and VSS were measured by standard methods (APHA, 2012). Statistical analyses, such as the ANOVA single

factor, and post-hoc two-sample Student t-test with equal variance, were performed using Microsoft Excel 2016 and built-in macro functions. The ANOVA single factor test and Bonferroni correction were used to adjust the significance to avoid statistical error when comparing groups greater than 2 samples.

5.2.4 Microbial Analysis

DNA was extracted from the 15L digesters on day 446 and day 150 for AD systems with rehydrated biomass using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer instructions. Biomass samples were subjected to bead beating on a vortexer (Model 58816-121, VWR International, Radnor, PA, USA) for 10 minutes. Sequencing was performed by a commercial laboratory (MR DNA, Shallowater, TX, USA) using an Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA, USA) following manufacturer guidelines. Barcodes and primers were removed from Q25 filtered sequences and processed as previously described (Dowd et al., 2008). Briefly, data were refined by removing sequences <200 bp, sequences with ambiguous base calls, and sequences with homopolymers >6 bp. Denoised sequences were clustered into operational taxonomic units (OTUs) having 97% similarity. Singleton sequences and chimeras were removed. BLASTn was used to taxonomically classify OTUs against a curated data- base derived from GreenGenes, RDPII, and NCBI (CME, 2015; DeSantis et al., 2006; NCBI, 2015). A sample-based rarefaction was performed on the combination of archaea and bacteria operational taxonomic units (OTUs). Principal component analysis (PCA) was performed using R, and custom R scripts using the packages phyloseq,

FactoMineR, and factoextra.

5.3 Results and Discussion

5.3.1 NH_3-N ATA Analysis

For the NH_3-N ATA tests, VSS concentrations for LAD, LADE, and ACD biomass samples were 2.27±0.27, 2.28±0.13, and 2.11±0.15 g/L, respectively. VSS concentrations for LAD-D, LADE-D, and ACD-D biomass samples were 1.57±0.12 g/L, 1.29±0.067 g/L, and 1.79 ±0.17 g/L. Initial pH values of each biomass sample with the NH_3-N and calcium acetate amendments are shown in Table 15 and Table 16.

Table 15. Initial pH of biomass samples with calcium acetate and NH_3-N.

15 L Biomass	Calcium Acetate Concentration	NH_3-N Concentration	Average pH Measurement
LAD-1	10 g/L	0	7.08±0.03
LAD-2	10 g/L	3	7.18±0.03
LAD-3	10 g/L	6	7.24±0.02
LAD-4	10 g/L	9	7.13±0.02
LADE-1	10 g/L	0	7.23±0.02
LADE-2	10 g/L	3	7.13±0.01
LADE-3	10 g/L	6	7.13±0.01
LADE-4	10 g/L	9	7.11±0.00
ACD-1	10 g/L	0	7.07±0.03
ACD-2	10 g/L	3	7.12±0.00
ACD-3	10 g/L	6	7.16±0.01
ACD-4	10 g/L	9	7.10±0.02

Table 16. Initial pH of Biomass Samples with Calcium Acetate and NH_3-N

Dried Biomass Digesters	Calcium Acetate Concentration	NH_3-N Concentration	Average pH Measurement
LAD-D-1		0	7.05±0.01

LAD-D-2		3	7±0.01
LAD-D-3		6	7.05±0.01
LAD-D-4		9	6.94±0.01
LADE-D-1		0	7.11±0.03
LADE-D-2		3	6.98±0.01
LADE-D-3		6	6.93±0.03
LADE-D-4		9	6.93±0.03
ACD-D-1		0	7.04±0.04
ACD-D-2		3	6.94±0.01
ACD-D-3		6	7.09±0.01
ACD-D-4		9	6.96±0.01

Table 17 shows CH_4 production rate normalized to VSS (CH_4/g VSS-day) for the LAD, LADE, ACD (wet cultures) and LAD-D, LADE-D, and ACD-D (dried, rehydrated cultures) at various NH_3-N concentrations. CH_4 production rate for the wet cultures was greater than the CH_4 rate for the dry cultures for up to 9 g/L NH_3-N with 10 g/L calcium acetate (Table 17). Increased CH_4 production rate at 3 g/L NH_3-N for the LAD and ACD systems could have been a result of enhanced activity of protein break down with the coenzyme F_{420} (Dai et al., 2017). The CH_4 production rate relative to the control decreased with increasing NH_3-N at 3 g/L NH_3-N for the dry cultures and 6 g/L NH_3-N for the wet cultures. The decreased CH_4 production rate with increasing NH_3-N concentration confirmed previous studies that NH_3-N is inhibitory to methanogenic microbial communities at high concentrations (Nakakubo et al., 2008; Fotidis et al., 2013; Dai et al., 2017). For dried, rehydrated cultures, CH_4 production was not detected at 6 g/L NH_3-N or greater, and not detected for the LADE biomass at 9 g/L NH_3-N, indicating that biogas production was essentially all CO_2.

The LAD biomass was 47% and 93% greater than the LAD-D biomass at 0 and 3 g/L NH_3-N; the LADE biomass was 1% lower at 0 g/L NH_3-N and 93% greater at 3 g/L NH_3-N than the LADE-D biomass; the ACD biomass was 52% and 94% greater than the ACD-D biomass at 0 and 3 g/L NH_3-N (Table 17). At 6 and 9 g/L NH_3-N, the LAD and ACD 100% greater ATA values than the LAD-D and ACD-D as these biomass sources did not produce CH_4. At 6 g/L NH_3-N the LADE had a 100% greater ATA value than the LADE-D biomass (the LADE-D did not produce CH_4). Both the LADE and LADE-D biomass did not produce CH_4 at 9 g/L NH_3-N (Table 17). The LAD and LAD-D biomass were determined to be statistically different for NH_3-N concentrations at 0,3,6, and 9 g/L ($p<0.05$, $n=4$); the LADE and LADE-D biomass were statistically different at NH_3-N concentrations of 3 and 6 g/L ($p<0.05$, $n=4$); the ACD and ACD-D biomass were determined to be statistically different for NH_3-N concentrations at 0,3,6 and 9 g/L ($p<0.05$, $n=4$). At NH_3-N concentrations of 0 g/L and 9 g/L, the LADE and LADE-D ATA values were not statistically different ($p<0.05$, $n=4$).

Table 17. CH_4 Specific Production Rate (mL CH_4/g VS-day) at Various NH_3-N Concentrations[1]

	NH_3-N (g/L)			
Biomass Source	0	3	6	9
LAD-D	194.1±1.9	24.1±0.62	ND	ND
LADE-D	250.4±12.9	9.5±1.2	ND	ND
ACD-D	170.3±0.93	23.9±1.78	ND	ND
LAD	363.9±12.1	326.4±12.1	11.2±0.32	0.9±0.03

LADE	248.0±4.6	131.0±5.2	4.6±0.14	ND
ACD	351.4±8.8	388.0±4.1	4.6±0.25	3.5±0.2

[1] ND is not detected.

The IC_{50} concentration graphs for the LAD-D (Figure 24-a), LADE-D (Figure 24-b), ACD-D (Figure 24-c), LAD (Figure 24-d), LADE (Figure 24-e), and ACD (Figure 24-f) show the inhibitory concentration of NH_3-N that reduces the biomass activity by 50%. Figure 24 shows that the wet cultures had greater IC_{50} concentrations than the dry cultures. The average IC_{50} concentration for the LAD biomass (~4.5 g/L NH_3-N) was 67% greater than the LAD-D biomass (~1.5 g/L NH_3-N); the average IC_{50} concentration for the LADE biomass (~3.25 g/L NH_3-N) was 54% greater than the LAD-D biomass (~ 1.5 g/L NH_3-N); the average IC_{50} concentration for the ACD biomass was (~4.5 g/L NH_3-N) was 56% greater than the ACD-D biomass (~2.0 g/L NH_3-N) (Figure 24). Lower IC_{50} concentrations for the dry cultures indicate that dry cultures had lower NH_3-N tolerance than the wet cultures.

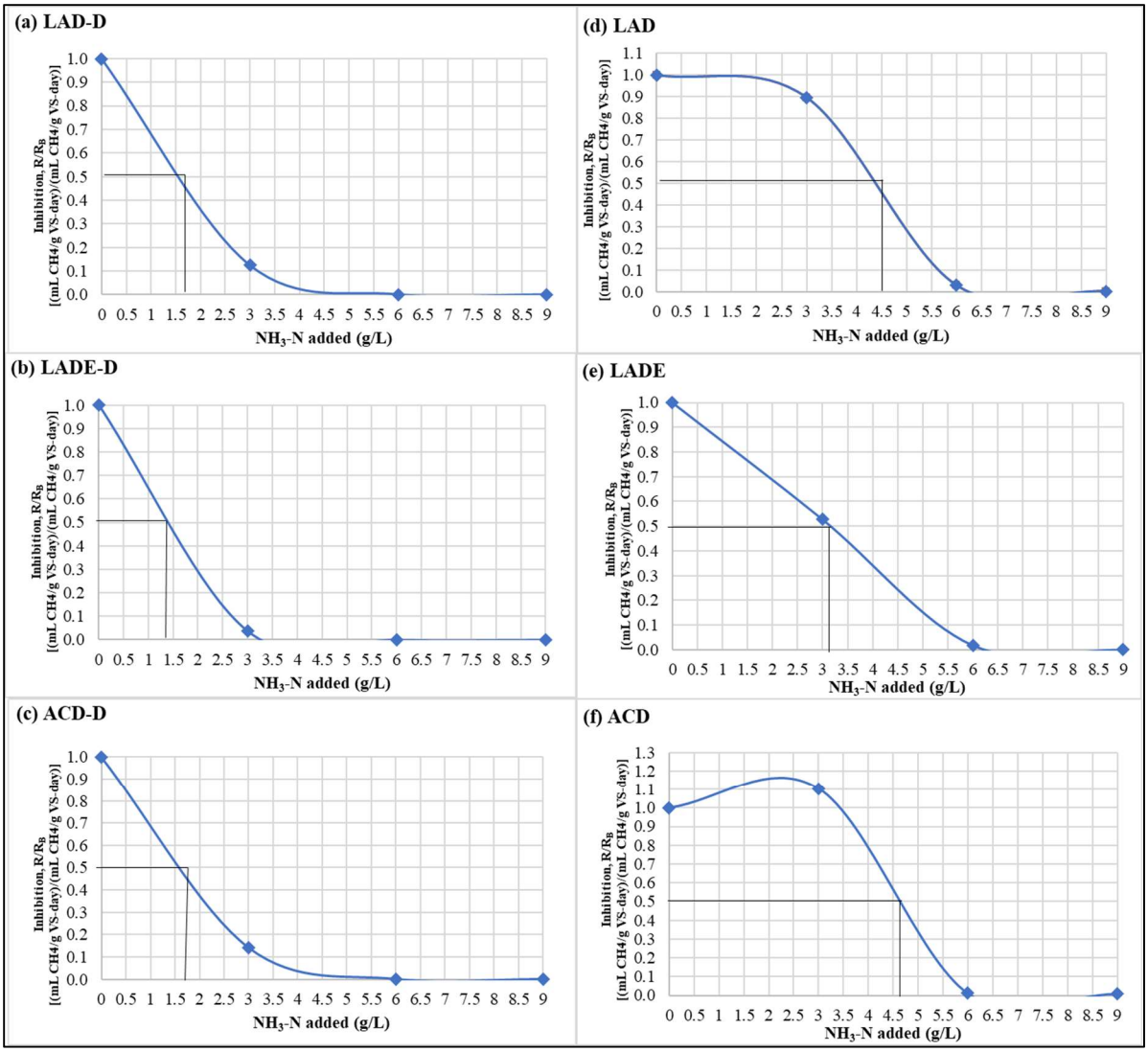

Figure 24. Average IC_{50} concentrations from the effluent of the batch systems (a) LAD-D, (b) LADE-D, and (c) ACD-D and effluent of the 15L systems (d) LAD, (e) LADE, and (f) ACD. The average IC_{50} concentration for the LAD-D, LADE-D, and ACD-D was approximately 1.5, 1.5, and 2 g/L NH_3-N, respectively. The average IC_{50} concentration for the LAD, LADE, and ACD was approximately 4.5, 3.25, and 4.5 g/L NH_3-N, respectively.

5.3.2 NH_3-N Inhibition Compared to Archaeal and Bacterial Communities

The PCA graph in Figure 25 shows the dominant archaea present in the varying digester biomass. In the graph, the 15L digesters LAD, LADE, and ACD are represented

by SMA.A, SMA.T, and SMA. C (Wet Samples). The 15L digester biomass that was dried at 104°C the LAD-DS, LADE-DS, and ACD-DS are represented by DS. A, DS. T, and DS.C, respectively (Dry Samples). The triplicate samples of the LAD-D, LADE-D, and ACD-D are represented by A.1-3, T.1-3, C.1-3 (Reconstituted LAD, LADE, and ACD) (Figure 25). For archaea, vectors aligned with the OTUs that had the highest relative abundance in each sample, indicating the archaeal community difference across each sample was due to specific dominant OTUs in each sample (Figure 23). The dried, rehydrated cultures exhibited an increased relative abundance of *Methanosarcina* and *Methanosarcina* which are reported to be either sensitive (Garcia and Angenent, 2009) or tolerant to ammonia stress (Fotidis et al., 2013). In the dry cultures, the *Methanosarcina* OTU was most similar to *Methanosarcina siciliae* which is a methylotrophic methanogen that reportedly uses methanol, trimethylamine, and dimethyl sulfide, but not H_2/CO_2, formate, or acetate (Hi et al., 1999). The increased relative abundance of *Methanosarcina siciliae* in the dried biomass is unique as previous research has shown optimal growth at 35°C (Elberson and Sowers, 1997). *Methanosarcina siciliae* is notable since most *Methanosarcina* species utilize acetate and, possibly, H_2/CO_2 (Hi et al., 1999). Generally, methylotrophic methanogens have low tolerance to NH_3 (Kadam and Boone, 1996), which could potentially explain why dried, rehydrated biomass samples were more sensitive than wet cultures to increasing NH_3-N concentrations.

Hydrogenotrophic methanogens have been shown to be more tolerant to NH_3 inhibition than acetoclastic methanogens (Sprott & Patel, 1986; Schnürer *et al*., 1994). Because of the suppression of acetoclastic methanogens and accumulation of acetate, high NH_3-N conditions favor development of SAOBs. It was expected that digesters with higher hydrogenotrophic methanogen relative abundance would promote SAOB growth

and perform better in higher NH_3 conditions (Westerholm et al., 2011, 2010). However, the dry cultures had lower CH_4 production at NH_3-N concentrations from 0-9 g/L than the wet cultures (see Table 17). Potential reasons for this were that dry cultures did not have a significant increase in relative abundance of known SAOBs in comparison to the wet cultures, and dry cultures showed a significant increase in the Methanosarcina species, *Methanosarcina siciliae*, which does not eat acetate and has low tolerance to NH_3 concentrations.

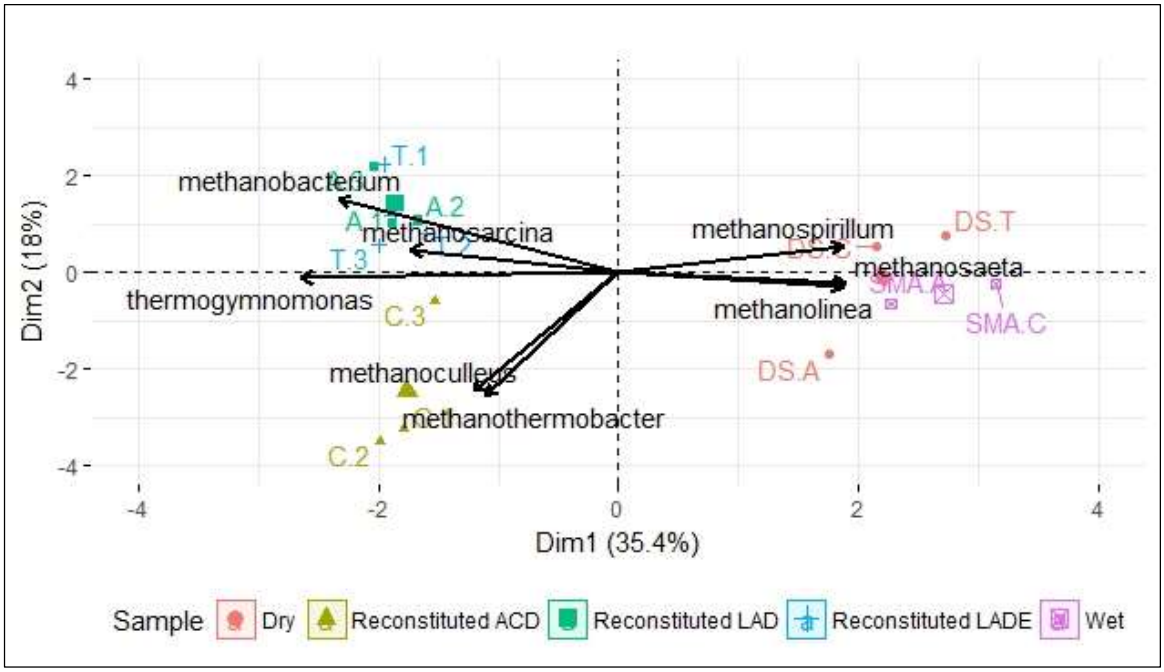

Figure 25. PCA analysis of Archaea 16S rRNA sequencing profiles for each AD biomass sample.

5.4 Conclusion

The NH_3-N inhibition was studied for both wet and dried, rehydrated methanogenic cultures. ATA bottles were operated to determine differences in NH_3-N

tolerance in wet and dry cultures. The CH_4 production rate for the wet cultures was greater than the CH_4 rate for the dry cultures for up to 9 g/L NH_3-N with 10 g/L calcium acetate. The CH_4 production relative to the CH_4 production of the biomass that received 0 g/L NH_3-N and 10 g/L calcium acetate, decreased at NH_3-N concentration greater or equal to 3 g/L for dried, rehydrated cultures and greater or equal to 6 g/L NH_3-N for the wet cultures. Based on biogas production and CH_4 percentage, wet cultures were determined to have greater CH_4 production than dry cultures at tested NH_3-N concentrations.

The hypothesis that dry cultures would have grater CH_4 yield at NH_3-N concentrations was not met as heat dried cultures from the LAD and LADE did not produce more NH_3 tolerant cultures. However, heat drying of digester biomass did induce higher H_2/CO_2 activity. Greater inhibition from higher NH_3-N concentrations in dry cultures can be potentially explained microbially. Dry cultures saw an increased relative abundance to *Methanosarcina,* which was most similar to the species *Methanosarcina siciliae*, which favors the metabolism of methanol species and is not tolerant to high heat and NH_3-N conditions. Based on the increased relative abundance of *Methanosarcina siciliae* in the dry cultures in comparison to the wet cultures, further research is needed to investigate enrichment and survivability of *Methanosarcina siciliae* at higher temperatures, specifically 104°C (which was the temperature used in this study), and use as a bioaugment to AD systems with high concentrations of methanol, trimethylamine, and dimethyl sulfide (DMS).

6. General Conclusions and Recommendations

Anaerobic digestion (AD) is the process of converting organic matter to methane (CH_4) in oxygen depleted zones. The addition of limited quantities of oxygen (O_2) has been shown to increase CH_4 yield and improve chemical oxygen demand (COD) and volatile fatty acid (VFA) removal. Advances in molecular techniques such as high throughput Illumnia sequencing have increased understanding of microbial communities involved in anaerobic digestion and O_2 tolerance of anaerobic communities.

The objective of the first project was to determine if limited aeration, and limited aeration with an aerotolerant enrichment culture could increase digester functional performance in comparison to an anaerobic control digester. Three 15L digesters: the limited aerated digester (LAD), limited aerated anaerobic digester augmented with an aerotolerant enrichment culture (LADE), and anaerobic control digester (ACD) were operated for over 500 days to obtain differences in digester function and microbial communities. The results showed that digesters that were operated differently displayed similar functions in CH_4 yield, and COD and VFA removal.

Digesters that were operated differently exhibited different CH_4 rate with specific methanogenic activity (SMA) tests with acetate, propionate, hydrogen (H_2)/carbon dioxide (CO_2), non-fat dried milk, and non-fat dried milk and O_2 (i.e. air) addition. SMA tests determined that aeration did not increase SMA with each substrate. Aeration and augmentation with an aerotolerant enrichment culture did result in a statistically greater SMA with propionate. Microbially, this was represented by increases in *Methanospirillium* and propionate oxidizing bacteria. The LAD, LADE, and ACD were

distinguished by a high relative abundance (relative abundance) of *Methanosaeta*, an acetoclastic methanogen, which ranged from 58 to 77% in each digester.

The second project of this thesis investigated whether rehydrated dried biomass could function and produce CH_4 through endogenous decay. In comparison to previous studies that have only initially augmented with an enrichment culture, this study used a continuous novel enrichment technique that involve exposing methanogenic biomass to low oxygen concentrations and to drying at >100°C for 24 hours were used to develop a methanogenic culture resilient to heat and air drying. Biomass was used from each 15L biomass, which was concentrated, then dried in a 104°C oven and stored in desiccators for 2 months. Dried biomass was then reconstituted in a nutrient solution and monitored for performance, changes in function, and changes in microbial communities using high throughput Illumnia sequencing.

Results showed that biomass that had been dried and stored for 2 months can produce biogas from soluble COD (SCOD) and VFAs utilization through endogenous decay. CH_4 production curves showed that the aerated dried biomass had a significantly shorter lag time (the amount of days till maximum CH_4 production or maximum CH_4) compared to the anaerobic dried biomass. The combination of aeration and drying was shown to greatly reduce lag time to CH_4 max compared to the rehydrated anaerobic control biomass. Aeration and drying also reduced time to initial CH_4 detection.

SMA tests determined that dry cultures had statically lower SMA with acetate and non-fat dried milk than wet cultures. Biomass that had been aerated and aerated with an aerotolerant enrichment culture did result in a statistically greater SMA with propionate. Microbially, this was represented by an increase in relative abundance of

hydrogenotrophic methanogens. The non-metric multidimensional scaling (nMDS) and principal component analysis (PCA) plots of archaea and bacteria showed significant shifts in microbial communities from wet versus dry cultures. This is an important research finding as this study showed that long-term continuous aeration and aeration with an aerotolerant enrichment cultured resulted in a significant increase in relative abundance of hydrogenotrophic methanogens and SMA with H_2/CO_2.

The third project of this thesis investigated CH_4 production for wet and dry cultures operating with high ammonia-nitrogen (NH_3-N) (3 to 9 g/L NH_3-N) concentrations. Biomass was used from operating 15L digesters and rehydrated dried biomass digesters for anaerobic toxicity assay (ATA) analysis. The NH_3-N ATA tests were conducted using 160 mL serum bottles each containing 40 mL of anaerobic biomass and 10mL of a stock solution to obtain calcium acetate (10 g/L) with 0 to 9 g/L NH_3-N to achieve a total volume of 50 mL.

Results showed that wet cultures had greater methane production than dry cultures at tested NH_3-N concentrations. Therefore, the hypothesis that dry cultures would have grater CH_4 yield at NH_3-N concentrations was not met. Inhibition from higher NH_3-N concentrations can potentially be explained microbially. The genera of Methanosarcina that was detected was most similar to the species *Methanosarcina siciliae* which favors metabolism of methanol species over acetate and H_2/CO_2. The species *Methanosarcina siciliae* also has low tolerance to high NH_3-N concentrations.

Enrichment for *Methanosarcina siciliae* is an important research finding as this species has been previously shown to have low survivability at temperatures greater than 35°C. Based on results from this study, a future research project could potentially involve

dried biomass that have been enriched for *Methanosarcina siciliae* used to bioaugment AD systems that have methanol. The long-term study would compare digester function to a control digester operating with methanol as a carbon source to enriched digesters. Microbial difference would also be compared using high throughput Illumnia sequencing. The study would further the understanding of the role of *Methanosarcina siciliae* in methanol metabolism and develop a more robust knowledge of anaerobic microbial function in an AD system with methanol.

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