Bioaugmentation and Correlating Anaerobic Digester Microbial Community to Process Function

Kaushik Venkiteshwaran
Marquette University

BIOAUGMENTATION AND CORRELATING ANAEROBIC DIGESTER MICROBIAL COMMUNITY TO PROCESS FUNCTION

by

Kaushik Venkiteshwaran

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ABSTRACT
BIOAUGMENTATION AND CORRELATING ANAEROBIC DIGESTER MICROBIAL COMMUNITY TO PROCESS FUNCTION

Kaushik Venkiteshwaran

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This dissertation describes two research projects on anaerobic digestion (AD) that investigated the relationship between microbial community structure 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, bioaugmentation using a methanogenic, aerotolerant propionate enrichment culture was investigated as a possible method to increase digester methane production. Nine anaerobic digesters, seeded with different biomass, were operated identically and their quasi steady state function was compared. Before bioaugmentation, different seed biomass resulted in different quasi steady state function, with digesters clustering into high, medium or low methane (CH_4) production groups. High CH_4 production correlated with neutral pH and high *Methanosarcina* abundance, whereas low CH_4 production correlated to low pH and high *Methanobacterium and DHVEG-6* family abundance. After bioaugmentation, CH_4 production from the high CH_4-producing digesters transiently increased by 11±3% relative to non-bioaugmented controls ($p < 0.05$, $n=3$), whereas no functional changes were observed for medium and low CH_4 producing digesters. The CH_4 production increase after bioaugmentation was correlated to increased relative abundance of *Methanosaeta* and *Methanospirillum* originating from the bioaugment culture. In conclusion, different anaerobic digester seed biomass can result in different quasi steady state function. The bioaugmentation employed can result in a period of increased methane production.

In the second project, a quantitative structure activity relationship (QSAR) between anaerobic microorganism relative abundance values and digester methane production rate was developed using 150 lab-scale anaerobic digesters seeded with 50 biomass samples obtained from 49 US states. Although all digesters were operated identically for a minimum of 5 retention cycles, their quasi steady-state performance varied significantly, with the average daily methane production rate ranging from 0.09±0.004 to 0.98±0.05 L-CH_4/L_R-day (average ± standard deviation). Analysis of over 4.1 million-sequence reads revealed approximately 1300 operation taxonomical units (OTUs) at the genus level across all digesters, with each digester having 158±27 OTUs (mean ± standard deviation). Using Spearman's rank correlation, 10 OTUs, which included one archaeal OTU, were found to correlate significantly to digester methane production rate. The relative abundance values of the 10 OTUs were used as descriptors to develop a multiple linear regression (MLR) equation, with good statistical prediction of the digester methane production rates. The results are encouraging and provide an initial step for further research to develop more robust QSAR models to predict the function of anaerobic and other bioprocesses using microbial community descriptors.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 Introduction

Anaerobic digestion (AD) is a biological process that converts organic compounds in an oxygen free environment to methane (Pullammanappallil et al. 1998). Anaerobic digestion is an attractive method of wastewater treatment as it provides several advantages over the more widely used aerobic treatment processes. These advantages include low operational cost due to lack of aeration requirements and low sludge production as well as energy production from methane (Suryawanshi et al. 2010).

As AD is increasingly being implemented, microbiologist and engineers have been making great efforts to maximize stable and reliable digester operation. To a large extent, both the efficiency and stability of a specific digester depends on the microbial communities and the microbe activities within that digester. Digester microbial communities can be affected by a number of environmental factors, such as temperature, pH, availability and digestibility of substrates, organic loading and types of feedstocks. A better understanding of the composition and dynamics of microbial communities within anaerobic digesters is needed to improve efficiency and stability of AD processes. As a result, this dissertation describes two research projects to determine the relationship between microbial communities and digester function. For this, high-throughput Illumina sequencing technology with universal 16S rRNA gene primers was employed to characterize the microbial communities.

In the first project, the effects of adding a methanogenic, propionate degrading enrichment culture as a bioaugment to a quasi-steady state anaerobic digester was investigated. Bioaugmentation, which is defined as adding specialized or a mixed community of microorganisms to a system to improve process function (Herrero &

Stuckey 2014), has been demonstrated to be beneficial in reducing the impacts of toxic inhibitory substances which are either present in the feedstock or produced biologically in the digester (Schauer-Gimenez et al. 2010; Tale et al. 2011; Herrero & Stuckey 2014; Bocher et al. 2015; Tale et al. 2015). Nine groups of anaerobic digesters were seeded with different starting biomass to obtain different microbial communities and operated identically at an organic loading rate (OLR) of 3 g COD/L-day and 10 days hydraulic retention time (HRT). Upon attaining quasi-steady state, the digesters were then bioaugmented with the enrichment culture that previously had been shown to improve digester recovery after organic overload (Tale et al. 2011; Tale et al. 2015). Both digester functional and microbial community parameters were monitored to understand how bioaugmentation changed the activity and composition of the digester microbial community structure.

The objective of the second study was to investigate whether a quantitative model that uses microbial community structure descriptors could be developed to predict digester function, such as methane production rate. Current anaerobic digestion mathematical models, such as the Anaerobic Digestion Model 1 (ADM1), do not include microbial diversity information and they model the complex microbial interactions as trophic groups that each contain only one taxon with defined kinetic constants (Venkiteshwaran et. al, 2016). However, microbial communities actually differ greatly from one digester to the next and trophic groups often contain multiple, competing taxa having different substrate affinities and specific growth rates. In order to improve current models, it is essential to deepen our understanding of how microbial community structure relates to process function, such as methane generation.

Up to 50 distinct biomass samples from 49 US states were used to seed triplicate lab-scale digesters operated under identical, controlled conditions. The digesters were allowed to acclimate for a minimum of 5 hydraulic retention cycles before sampling, and subsequently the microbial community data were correlated to the digester quasi-steady state methane production rate using a multiple linear regression (MLR) model. The model was determined to have good predictability and results encourage future efforts to include microbial community data in ADM1 to improve modeling.

Following the general introduction in Chapter 1, Chapter 2 contains a literature review that describes AD microbiology along with topics regarding bioaugmentation of anaerobic digesters and qualitative/quantitative relationship between microbial communities and digester function. Subsequently, Chapters 3 and 4 describe project 1 and 2, respectively. Finally, Chapter 5 contains general conclusions and recommendations for future work.

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2. Relating Anaerobic Digestion Microbial Community and Process Function

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2.1. Introduction

Anaerobic digestion (AD) is a microbial process that converts organic matter to biogas containing CH_4 and CO_2 in an anaerobic environment (Pullammanappallil et al. 1998). Although the technology has been employed for decades, it has received renewed attention as it provides a more sustainable alternative to waste treatment over energy intensive methods of the past (Angenent et al. 2004; Lettinga 2010). Compared to traditional aerobic treatment, AD has several potential advantages such as lower operational costs from lack of aeration requirements, energy production from biomethane, significantly less biomass production which reduces handling and disposal costs and ability to degrade certain pollutants which cannot be aerobically removed (Suryawanshi et al. 2010).

Anaerobic conversion of organics to biogas involves a multi-step process involving interactions among many different bacterial and archaeal species. With the increasing application of anaerobic digestion, there is a steady effort by both engineers and microbiologists working in this field to increase the existing knowledge of the complex, interacting microbial community that governs the overall AD process. New knowledge is crucial in order to develop better models and design improved AD systems.

One key area requiring new knowledge involves the quantitative relationship between microbial community structure and AD process function. The aim of this review is to provide insight into the microbiology of anaerobic digesters and recent studies describing both qualitative and quantitative relationships between microbial community and digester function.

2.2 Phases in AD Process

Conceptually, the microbial processes of AD can be described by the sequential steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Bitton 2005). Each of these steps is accomplished by a guild of microorganisms, and it is critical to maintain a "balanced" reaction rates among the steps or guilds to ensure rapid and stable digestion. As described above, "balanced" essentially means that acid- and H_2-consuming reactions are as fast or potentially faster than acid and H_2-producing steps. Buildup of H_2 partial pressure to more than 10^{-4} atm inhibits the destruction of propionate and butyrate intermediates (McCarty & Smith 1986). Accumulation of these VFA intermediates can drop the pH of the digester and slow or stop methanogenesis. In addition, the rate of one of these steps limits the overall rate of methanogenesis, and the identity of the rate-limiting step can differ among systems based on substrate chemical structure and other parameters. Most importantly, increasing the rate of the rate-limiting step will increase methane production rates, whereas improving other steps will have a little impact.

2.2.1 Hydrolysis

Hydrolysis involves the breakdown of polymeric substrates, such as polysaccharides, lipids, proteins etc., to their respective monomers or oligomers using

extracellular enzymes. These enzymes generally include amylase, cellulase, lipase, pectinase and protease (Singh & Harvey 2010). Hydrolytic bacteria are phylogenetically diverse, however two phyla *Bacteroidetes* and *Firmicutes* include most of the known species.. Compared to methanogens; hydrolytic bacteria grow rapidly and have lower sensitivity to changes in environmental factors, like pH and temperature. For relatively recalcitrant substrates, such as lignin, hydrolysis is often the rate-limiting step for CH_4 production. In addition to substrate chemical structure, hydrolysis rate depend on factors including particle size, pH, enzyme production, diffusion, and adsorption of enzymes on the substrate particles (Noike et al. 1985; Mata-Alvarez et al. 2000; Vidal 2000). Methods to increase hydrolysis rate using mechanical, chemical and biological processes have been developed (Ariunbaatar et al. 2014), but a thorough review is outside the scope of this document.

2.2.2 Acidogenesis and Syntrophic Acetogenesis

In acidogenesis products of hydrolysis are converted primarily to volatile fatty acids (VFAs), which include acetate, propionate, isobutyrate, butyrate, valerate and isovalerate. Besides VFAs, other products of acidogenesis include alcohols, lactate, formate, CO_2 and H_2. *Bacteroidetes, Chloroflexi, Firmicutes* and *Proteobacteria*, are phyla that contain most identified species of acidogenic bacteria (Stiles & Holzapfel 1997; Balk et al. 2002; Dong et al. 2000; Yamada et al. 2006). Acidogenesis is generally rapid, which can lead to accumulation of VFAs and a drop in pH when acid utilization is inhibited or too slow due to organic overload, toxicants or rapid temperature change. The pH drop can inhibit or stop methanogenesis completely.

Although methanogens can directly use acetate, formate, H_2/CO_2 and methyl

compounds, other intermediates formed via acidogenesis, such as propionate, butyrate, isobutyrate, valerate, isovalerate, and ethanol, have to be further biodegraded by other microorganisms before methanogens can utilize them to produce methane. Syntrophic acetogenesis is the process in which these intermediates are further biotransformed to form acetate, H_2 and CO_2. Fermentation of propionate via syntrophic acetogenesis is critical because approximately 30% of complex substrates in municipal wastewater solids and other wastes can be shuttled through propionate to CH_4 during AD under typical conditions (Speece et al. 2006). Most of the medium to long-chain fatty acids resulting from hydrolysis of lipids and lignocellulosic compounds are also biotransformed to acetate, H_2 and CO_2 through this process.

Under standard conditions, syntrophic acetogenesis is thermodynamically unfavorable and only proceeds if the partial pressure of H_2 is lower than 10^{-4} atm (McCarty & Smith 1986; Lowe et al. 1993). Hydrogenotrophic methanogens and/or other H_2 utilizers live in syntrophy with acetogens, consuming H_2 released from the latter (Schink 1997). The syntrophic relationship makes acetogenesis thermodynamically feasible. Formic acid (HCOOH) and formate are similar to H_2 since they are essentially H_2 associated with CO_2 (i.e, $H_2 + CO_2 = HCOOH$). Therefore, interspecies formate transfer has also been observed to play a critical syntrophic role. In addition, acetogenesis from other higher-molecular-weight organic acids also relies on syntrophy with H_2 or formate utilizers. This syntrophy is based on H_2/formate transfer from H_2-producing to H_2-consuming microorganisms, which is commonly referred to as interspecies H_2 transfer (Stams & Plugge 2009). The H_2 also can be thought of as protons (H^+) with associated electrons, and interspecies hydrogen/formate transfer is also interspecies electron

transfer. Interestingly, a recent study has shown that some microorganisms perform direct interspecies electron transfer using electrically conductive pili and electrons can be shuttled in this way from, for example, *Geobacter* to *Methanosaeta* (Rotaru et al. 2014; Shrestha et al. 2013; Morita et al. 2011; Zhao et al. 2015). This interspecies electron transfer is rapid and may prove to be an important mechanism for stable AD in the future; more research is warranted to more fully understand direct interspecies electron transfer mechanisms and how it can be encouraged in engineered systems.

Most commonly observed syntrophic acetogens in anaerobic digesters involved in propionate degradation belong to the genera *Pelotomaculum*, *Smithllela* and *Syntrophobacter* (Liu et al. 1999; de Bok et al. 2001; Imachi et al. 2007). Oxidation of butyrate and other fatty acids are performed by acetogens belonging to genera *Syntrophus* and *Syntrophomonas* (Jackson et al. 1999; Imachi et al. 2007; Sousa et al. 2007). Syntrophic acetogenesis is a critical and, often, rate limiting step to maintain rapid, stable AD operation because some of the VFAs, particularly propionate, inhibit methanogenesis at high concentrations even at neutral pH (Pullammanappallil et al. 1998; Barredo & Evison 1991; Demirel & Yenigün 2002; Nielsen et al. 2007). Syntrophic acetogens play a critical role in the overall AD process, but have not been thoroughly studied, in part, due to difficulty maintaining pure cultures and lack of primers to identify them in mixed cultures using molecular techniques (Mathai et al. 2015).

2.2.3 Methanogenesis

The final step in biomethane production, methanogenesis, is performed by a specialized group of microorganisms belonging to the domain *archaea*, called methanogens. There are three known types of methanogens: acetoclastic,

hydrogenotrophic and methylotrophic. Acetoclastic methanogens convert acetate to CH_4 and CO_2, hydrogenotrophic methanogens use H_2 or formate to reduce CO_2 to CH_4 and methylotrophic methanogens produce CH_4 from methyl compounds, such as methanol, methylamines, methylsulfides etc. (Liu & Whitman 2008). In typical municipal anaerobic digesters, about 70% of the CH_4 is produced from acetate, and the rest from H_2 and CO_2. Only a minimal amount of CH_4 is produced via methylotrophic methanogenesis (Ferry 2012).

Hydrogenotrophic methanogens are critical for AD process owing to their ability to scavenge H_2 and maintain the partial pressure low. *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanospirillum* and *Methanothermobacter* are the most commonly observed hydrogenotrophic methanogens in anaerobic digesters (Cuzin et al. 2001; Savant et al. 2002; Leclerc et al. 2004; Hori et al. 2006).

Acetoclastic methanogens belong to two genera; *Methanosaeta* and *Methanosarcina*. *Methanosaeta* are obligate acetoclastic methanogens and are only known to use acetate or direct electron transport as the substrate or electron donor. *Methanosaeta* have a relatively slow growth rate but possess a high affinity for acetate, so dominate at low acetate concentration (Liu & Whitman 2008). *Methanosarcina* are facultative acetoclastic methanogens. Most *Methanosarcina* species can use H_2/CO_2, and C-1 compounds in addition to acetate (Liu & Whitman 2008; Westermann et al. 1989; Conklin et al. 2006). In addition to its wider range of substrates; *Methanosarcina* has a higher growth rate and lower affinity for acetate so it can dominate over *Methanosaeta* in digesters where the concentration of acetate is high (Hori et al. 2006; Westermann et al. 1989; Conklin et al. 2006). The filamentous morphology of *Methanosaeta* can play an

important role in granule formation since the filaments acts as binders to help hold the granule together. Many AD configurations, such as the upflow anaerobic sludge blanket (UASB), rely on formation of these microbial agglomerations, called granules that are 1 to 5 mm particles containing microbes that settle rapidly. When granulation does not occur in these bioreactors, the process is difficult to maintain and can fail.

2.2.4 Syntrophic Acetate-Oxidizing Bacteria

Under certain conditions, an alternative pathway for CH_4 production from acetate has been observed in some anaerobic digesters. This pathway combines the conversion of acetate to H_2 and CO_2 by acetate-oxidizing bacteria that are subsequently converted to CH_4 by hydrogenotrophic methanogens (Zinder & Koch 1984). Only few species of microorganisms have been identified that perform syntrophic acetate-oxidization in conjunction with H_2 consuming methanogens, they include strain AOR (i.e., Reversibacter), *Clostridium ultunense*, *Thermacetogenium phaeum*, *Tepidanaerobacter acetatoxydans*, *Thermotoga lettingae*, *syntrophaceticus schinkii* (Hattori 2008; Fotidis et al. 2013; Westerholm et al. 2010; Westerholm et al. 2011). This pathway is not believed to be a typical AD pathway for CH_4 production because acetoclastic methanogens outcompete syntrophic acetate-oxidizing bacteria in most digesters; however, more work is required to understand the importance of the process in AD systems (Rui et al. 2011). Under conditions that might be inhibitory to acetoclastic methanogens, such as high ammonia (>3g/L NH_3-N) or sulphate concentration and/or high temperature, this pathway can be critical for biogas production (Hattori 2008; Rui et al. 2011; Schnurer et al. 1999; Schnürer & Nordberg 2008; Westerholm et al. 2012; Lü et al. 2013; Hao et al. 2011). Studies have also shown that a long hydraulic retention time along with a low abundance

of *Methanosaeta* can promote a shift towards syntrophic acetate-oxidizing pathway from acetoclastic methanogenesis (Shigematsu et al. 2004; Karakashev et al. 2006).

2.3 Environmental Parameters Affecting Digester Microbial Community

Many studies have reported the influence of environmental parameters on the microbial community structure of a digester, primarily focusing on the methanogenesis pathway since it plays a direct role in reducing the pollutant load and producing CH_4 as a renewable energy source (Yu et al. 2005). Compared to bacteria, methanogens have a lower growth rate and are sensitive to environmental disturbances, such as pH decline, high VFA or ammonia concentrations (Chen et al. 2008; Karakashev et al. 2005). Environmental parameters such as pH, temperature, substrate concentration, substrate composition and presence of toxic or inhibitory compounds can cause a shift in the methanogenic community structure and affect the overall digestion process (Chen et al. 2008).

Compared to thermophilic temperature (55 °C), the methanogenic community exhibits higher diversity at mesophilic temperature (37 °C) (Liu & Whitman 2008). Lowering the temperature further to psychrophilic values may shift the community from acetoclastic to hydrogenotrophic methanogens, but the relationship is currently unclear and requires additional research (Enright et al. 2009; Zhang et al. 2012). Substrate disturbances, which includes changes in the substrate concentration and composition can affect the methanogenic community and its activity (Boe 2006). Different substrates can lead to development of different methanogenic communities, for example; manure versus

wastewater sludge (Karakashev et al. 2005) and glucose versus whey permeate and sewage sludge (Lee et al. 2009). Higher acetate concentration can lead to *Methanosarcina* being selected as a dominant acetoclastic methanogen over *Methanosaeta* (Griffin et al. 1998; McMahon et al. 2001).

In most large-scale industrial or municipal anaerobic digesters, changes in substrate concentration or substrate overload due to the variability in wastewater streams are the most common causes of digester instability. Of the four trophic phases, hydrolysis and acidogenesis can proceed at a faster rate than acetogenesis and methanogenesis (Čater et al. 2013). During substrate overload, the rate of VFA intermediates formation is higher than that of their conversion to methane. Therefore, the VFAs accumulate to high concentrations in the digester, causing a pH decrease from the typical optimal values of pH 7-8 for efficient methanogenic activity (Čater et al. 2013). Apart from lowering the pH, VFAs can inhibit methanogenesis at high concentrations, and the inhibition is much stronger at lower pH values (Deublein & Steinhauser 2008). The pH influences the ratio of undissociated to dissociated forms of VFAs and the former is more toxic to microorganisms as the undissociated form can diffuse through cell membrane and cause damage by decreasing the intracellular pH (Kadam & Boone 1996).

Many studies have investigated a wide range of environmental and nutrient factors that might severely inhibit the methanogenic process. Comprehensive reviews published by Blum and Speece (1991) as well as Chen et al. (2008) provide detailed summaries of factors that might cause inhibition of anaerobic processes, which includes specific organic chemicals, ammonia, sulfate/sulfite, light metals ions (Na, K, Mg, Ca, and Al) and heavy metal ion (Fe, Zn, Ni, Co, Mo, Cu, etc.) toxicity. However, it is

important to note that metal ions are also essential trace elements for methanogenesis and are required at adequate concentrations, below inhibitory levels, for sustained methanogenesis (Speece 2008).

2.4 Relating Microbial Community Structure to Digester Stability and Function

Despite numerous reports describing the effect of environmental parameters on the microbial community structure, the reverse approach describing the influence of microbial community structure on digester function and its stability has been studied less. Researchers have just begun to utilize the information pertaining to microbial community structure to understand or to predict its underlying influence on digester performance.

2.4.1 Qualitative Structure-Function Relationships

Microbial diversity, specifically quantified as species richness (number of species) and evenness (relative abundance of species), has been shown to play an important role in both natural and engineered ecosystem function (Fernandez et al. 2000; Hashsham et al. 2000; Briones & Raskin 2003; Allison & Martiny 2008; Wittebolle et al. 2009; Werner et al. 2011). Ecosystems containing more than one organism capable of performing a specific function (high richness) with a relatively equal abundance (high evenness) have a higher probability of functional redundancy or functional stability. It is a form of functional "insurance" for an ecosystem to have high richness and evenness based on compensatory growth. If the population of one species within a functional group is reduced or lost due to system perturbation, then another species from the same functional group, but higher resistance to the perturbation may rapidly take its place if

originally present in enough numbers (Fernandez et al. 2000; Hashsham et al. 2000; Briones & Raskin 2003; Naeem & Li 1997).

Engineered biological systems, such as anaerobic digesters, are often prone to and criticized for functional instability; therefore, studies involving functional resistance and resilience of biological treatment systems after perturbation have focused on relating species richness and evenness to overall functional stability. Although not a methanogenic system, Wittebolle et al. (2009) working on denitrifying bacteria reported that communities with higher evenness exhibited higher rates of denitrification when exposed to salt toxicity compared to communities with low evenness. In parallel papers, Fernandez et al. (2000) and Hashsham et al. (2000) studying perturbation of methanogenic digesters using glucose overload concluded that greater functional stability was observed in communities exhibiting multiple microorganisms within the same functional group.

Apart from qualitatively linking species richness and evenness to digester stability during perturbation, studies have shown the relationship between microbial community structure and digester activity under non-perturbed conditions. Clustering analysis performed by Carballa et al. (2011) using two molecular techniques, denaturing gradient gel electrophoresis (DGGE) and Terminal restriction fragment length polymorphism (T-RFLP), showed similar results, with a clear separation between the mesophilic and thermophilic communities. The bacterial and mesophilic communities were more diverse and even than the archaeal and thermophilic communities. The study also concluded that a digester with a higher evenness and diversity in its bacterial community resulted in a higher biogas/methane production (Carballa et al. 2011). Tale et al. (2011) measured

specific methanogenic activity (SMA) against propionate for 14 different biomass samples from full-scale anaerobic digesters. Microbial community analysis was performed to elucidate only the methanogenic community structure defined by DGGE banding pattern of a gene ubiquitous to methanogens, the methyl coenzyme M reductase (*mcrA*) gene. Using the band intensities as a quantitative measure and employing principal component analysis (PCA), Tale et al. (2011) showed that the digesters with high SMA values clustered together on the PCA plot and correlated linearly with the DGGE banding patterns. Upon excising and sequencing the gel bands, the presence of hydrogenotrophic methanogens, *Methanospirillum hungatei* and *Methanobacterium beijingense*, were prominent in digesters with high propionate SMA. In another study, Werner et al. (2011) in a multi year study looked at the bacterial communities of nine full-scale digesters treating brewery wastewater by employing 454 pyrosequencing to sequence the 16S rRNA gene. Using principal coordination analysis (PCoA), they observed that digesters with higher specific methanogenic activity (SMA) against acetate correlated with high community evenness.

2.4.2 Quantitative Structure Function Relation

Though general understanding of the relationship between digester function and microbial community structure is increasing rapidly, the relationship is still difficult to quantitatively model. Current AD mathematical models consider biomass to be one independent population that is viewed as a “black box” (Lawrence & McCarty 1970) or, as in the case of models such as AD Model 1 (ADM1) and others (Chen et al. 2015), as trophic groups containing one member. The lack of microbial community descriptors that may quantify, for example, functional redundancy in models is an obvious hurdle to

improving design and operation of anaerobic digesters. The very important, but underappreciated work of Ramirez et al. (2009) began to tackle this issue by including microbial diversity concepts into an extended ADM1 model. However, more work is required to improve AD models using microbial community descriptors.

A few studies have reported a direct correlation between methanogenic activity and microbial community descriptors. Although not a anaerobic digester, Freitag and Prosser (2009) observed a linear correlation between the methanogenic activities of peat soil samples and *mcrA* gene copy numbers quantified using qPCR. The *mcrA* gene copy number has also been shown to linearly correlate with SMA values against H_2/CO_2 in four biomass samples (Morris et al. 2014). Regueiro et al. (2012) reported higher hydrolytic and methanogenic activity was correlated with higher *Bacteroidetes* and *Archaea* abundances. The linear correlation observed in these studies is encouraging; however multiple linear regression (MLR) models when performed using a small sample size and a high number of independent variable (10 DGGE bands in the case for Tale et al (2011)) are over-fitted and not predictive (Tale 2010). Therefore, a great number (i.e., >30) of different microbial communities must be analyzed to develop statistically relevant empirical correlations, and this is one thing that has limited the development of structure-function relationships.

Building on the work done by Tale et al. (2011) and Morris et al. (2014), a study by Bocher et al. (2015) utilized MLR modeling and addressed the issue of over-fitting by increasing the sample size (49 samples) and reducing the number of independent variables (5 DGGE bands) to develop the MLR equations relating community and functional descriptors. Methanogenic microbial communities were assayed for

methanogenic activity against glucose and propionate and the methanogenic community structure was quantified using *mcrA* gene DGGE band intensities. Of the 49 microbial samples, 30 were randomly selected and used as a training set to develop MLR equations relating propionate and glucose SMA values to band intensities (Equation **1** and Equation **2**). The maximum correlation coefficient (R^2) value was observed using a minimum of five bands. The MLR equations derived were then used to predict the activity of remaining 19 samples (the test set). In conclusion, the MLR equation described a regression with good quantitative predictability with the validation parameter (q^2) value higher than threshold value of 0.5 for glucose ($q^2 = 0.53$) and propionate ($q^2 = 0.52$) relationships.

Equation 1

$$SMAp = -220(X_4)-82(X_8)+340(X_{10})-52(X_{14})+180(X_{15})+50$$

Equation 2

$$SMAg = -430(X_4)-470(X_7)-76(X_{11})+170(X_{15})+89$$

SMAp and SMAg are the specific methanogenic activity values against propionate and glucose, respectively (mL CH_4/mg iATP-h), and Xn is the demeaned, normalized band intensity value for band "n" on a DGGE gel of amplified *mcrA* products (Bocher et al. 2015).

To the authors' knowledge, this is the only study that has reported a quantitative, predictive model between methanogenic community structure and anaerobic biomass activity. The model as described by equations 1 and 2 shows, for example, that the presence of methanogens represented by DGGE bands X_{10}, X_{15} for SMAp and X_{15} for SMAg positively correlates to higher SMA. This kind of information could be used in the

future to select or design microbial communities to seed or bioaugment anaerobic digesters for more rapid methane production. Similarly, methanogens represented by bands X_4, X_8, X_{14} for SMAp and bands X_4, X_7 and X_{11} for SMAg negatively correlate to higher SMA.

This is a first step, and does not describe the many ways microbial community structure relates to digester function. In the future, however, these and other, more robust quantitative structure-activity relationships (QSARs) could be used to develop specific cultures that could increase process performance via digester seeding or bioaugmentation. The recently developed next-generation sequencing technologies may provide a breakthrough, as they allow sequencing of a large number of 16S rRNA gene PCR amplicon samples and have a rapid turnover time. At the same time, instead of analyzing for a specific functional or taxonomical group, next generation sequencing can be used to thoroughly describe the digester microbial community, either by using a metagenomic approach, employing universal 16S rRNA gene primers or by other approaches (Vanwonterghem et al. 2014).

2.4.3 Bioaugmentation as a Tool to Study Structure-Function Relationships

Bioaugmentation is the practice of adding specialized or a mixed community of microorganisms to a system to obtain a desired process function. (Deflaun & Steffan 2003) A recent review published by Herrero and Stuckey (2014) broadly covers the application of bioaugmentation in wastewater treatment. Bioaugmentation of anaerobic digesters has now been demonstrated in studies for reactor startup (Saravanane et al. n.d.), odor reduction (Duran et al. 2006) and degradation of organic compounds,

including 3-chlorobenzoate (Ahring et al. 1992), pentachlorophenol (Guiot et al. 2002), tetrachloroethene (Horber et al. 1998), benzene (Kasai et al. 2007), selenate and nitrate (Lenz et al. 2009), phenol and cresol (Hajji et al. 2000), fat, oil and grease (Cirne et al. 2006), oleate (Cavaleiro et al. 2010) and to aid in the recovery of upset digesters (Schauer-Gimenez et al. 2010; Tale et al. 2011; Tale et al. 2015).

The relevance of bioaugmentation to study structure-function relationships comes from the underlying hypothesis that addition of an exogenous culture ostensibly alters the original microbial community that may, in turn, change digester function. Hence, if the microbial community structure of the augment culture, the original digester biomass and their mixture is well characterized, then their functional activities could be used to relate function and community structure. This concept was tested in a study performed by Bocher et al. (2013), who compared the rates of propionate conversion to CH_4 before and after bioaugmentation with a propionate degrading, methanogenic augment. Nine different biomass samples, each with a different microbial community, were collected from different full-scale anaerobic reactors. Bioaugmentation was done by mixing the augment with each digester biomass sample at an iATP ratio of 1:5 (augment: biomass). Six of the nine-biomass samples assayed showed a statistically significant increase in the SMA after bioaugmentation. The bioaugmentation results were correlated with the dissimilarity (calculated as 1-pearson's correlation coefficient) between the methanogenic community structure of the augment and original digester biomass cultures (Figure 1). The results of bioaugmentation were measured as the percentage increase in SMA against propionate, before and after bioaugmentation. The dissimilarity between the methanogenic community structure of the digester biomass and the augment culture was

quantified as the distance, calculated using one minus the Pearson's correlation coefficient, of the *mcrA* DGGE banding patterns of each digester biomass sample and the augment culture.

A linear correlation was observed, and supported the argument that functional improvement (i.e. increased rate of propionate degradation) after bioaugmentation is not only a function of the augment culture community structure, but also the methanogenic community structure of the original biomass within a digester (i.e. how much different it is from the augment culture). This correlation provides a rationale to further study bioaugmentation as a tool to analyze structure-function relationship in AD process. Bioaugmentation will not improve every existing anaerobic biomass, but may improve some, and a method to quantify potential improvement based on microbial community descriptors should exist.

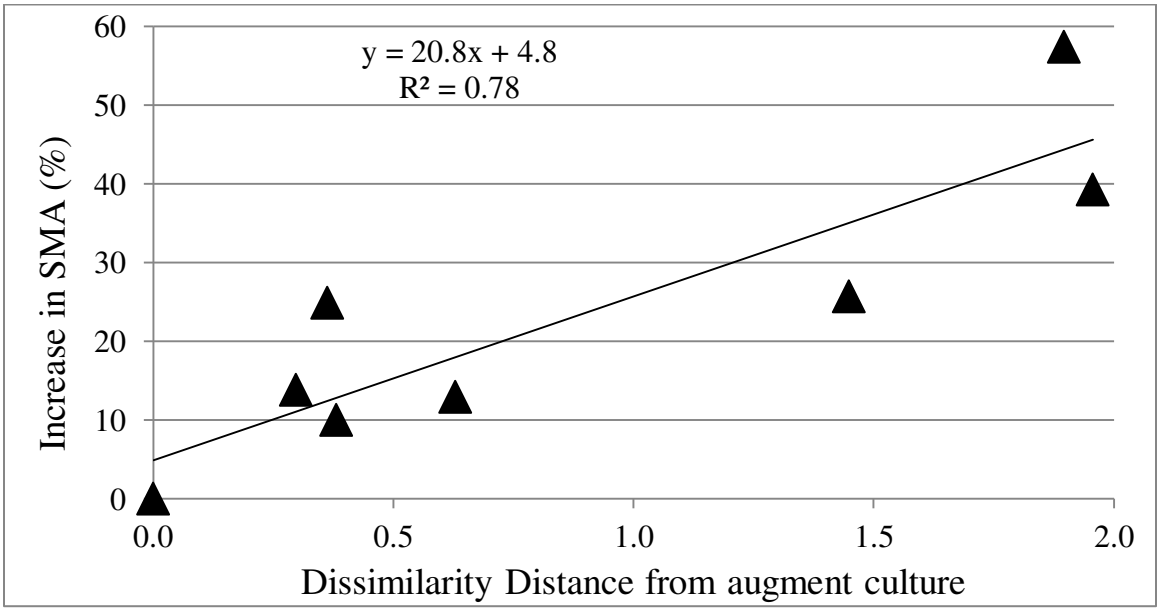

Figure 1: Difference between methanogen community structures in the augmented and biomass samples correlated with percent increase in SMA values (Bocher 2013).

Although linear models have been successful, other structure-activity models may prove to be more appropriate. This is similar to the historical development of quantitative QSARs for drug activity and chemical physiochemical parameter estimation over the last 80 years; these QSARs initially relied on linear relationships, but were later refined using nonlinear relationships such as artificial neural networks (Dearden & Rowe 2015). In any event, the initial success using empirical, linear relationships encouraged development of more robust and accurate empirical and mechanistic models. It is probable that more robust models can be developed in the near future to predict the function of engineered microbial processes using microbial community descriptors as well as environmental parameter values. These new, robust models will be very helpful to improve engineered bioprocesses.

2.5 Conclusion

As a biological treatment process, both efficiency and stability of AD technology depends fundamentally on the complex microbial communities and their activities in digesters. Owing to this, over the years scientists and engineers working in this field have focused their attention to answering the central questions: 1) which microorganisms are present, 2) how many different types of microorganisms are present, 3) which microorganisms are active and growing, 4) how do microorganisms behave under certain environmental conditions and 5) how does the microbial community structure relate to digester function.

Considerable progress has been made in the last decade to identify the key groups of microorganisms that influence the trophic phases of AD as well as how various environmental conditions affect the microbial community structure and digester function.

Yet, more work is required to realize quantitative, predictive relationships between the complex microbial community structure and the digester functional output. A robust quantitative microbial structure-function relationship would be a "holy grail" for engineers and scientists who are looking to develop new predictive models that can be used to improve the design and operation of anaerobic digesters for waste treatment and renewable energy generation. However, for a valid quantitative relationship, it is essential to analyze the microbial community structure and monitor the functional and environmental parameters for a large sample of different anaerobic digester communities, and this has limited model development. Future experimental work can be envisioned in which a large number of different microbial communities from various, controlled anaerobic digesters are analyzed using next-generation sequencing technology. The community and functional data then could be used to determine predictive, empirical or mechanistic relationships between community structure and digester function descriptors, including CH_4 production rate. It would be worthwhile endeavor and an important step forward in this field.

Other promising areas of research for improved AD processes may include (1) methods to increase hydrolysis rate, (2) direct interspecies electron transfer to methanogens via conductive pili or other mechanisms, (3) community structure and function relationships of methanogenic communities, (4) methanogenesis via acetate oxidizing bacteria, and (5) bioaugmentation to study microbial community-activity relationships or improve engineered bioprocesses.

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3. Anaerobic Digester Bioaugmentation Influences Quasi Steady State Performance and Microbial Community

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3.1 Introduction

Adding beneficial microorganisms to anaerobic digesters (i.e. bioaugmentation) has been shown to increase degradation rates of specific organics and reduce upset digester recovery time (Guiot et al. 2000; Hajji et al. 2000; Guiot et al. 2002; Cirne et al. 2006; Schauer-Gimenez et al. 2010; Tale et al. 2011). Anaerobic digester bioaugmentation may be more widely applicable if a culture was enriched to target a key, ubiquitous intermediate in existing anaerobic processes. The existing anaerobic processes typically treat readily degradable substrates, such as food production and dairy wastewater. When treating readily degradable substrates, one ubiquitous and potentially problematic intermediate is propionate (Schauer-Gimenez et al. 2010; Tale et al. 2015). Propionate accumulation is often an indicator of process imbalance in anaerobic digesters which can be caused by organic overload, nutrient deficiency, toxicant exposure or other factors (Mccarty & Smith 1986; Speece et al. 2006; Ma et al. 2009). The subsequent recovery time of upset digesters depends on the abundance of microorganisms that can biotransform an intermediate (i.e. propionate) or inhibitory compound into less harmful products (Herrero & Stuckey 2014).

Intermediates such as propionate can be biotransformed by a specific consortium

of synergistic microorganisms (McCarty & Smith 1986; Speece et al. 2006). Bioconversion of propionate to acetate and hydrogen (H_2) is thermodynamically favorable only when the partial pressure of the generated H_2 remains below 10^{-4} atm. Thus, degradation of propionate requires a synergistic relationship between H_2 producing and H_2 consuming microorganisms to maintain low H_2 concentrations (McCarty & Smith 1986). It was shown previous that adding cultures enriched to consume H_2 or propionate to anaerobic digesters can reduce recovery time after organic overload or toxicant exposure (Schauer-Gimenez et al. 2010; Tale et al. 2011). Tale et al., (2015) employed aerotolerant propionate consuming, methanogenic cultures for bioaugmentation. The aerotolerant culture may be commercially beneficial since it can be easily handled and dried in ambient air (Zitomer 2013). In addition, micro-aerated cultures outperformed a strictly anaerobic culture when used for bioaugmentation, resulting in higher specific methanogenic activity (SMA) against propionate and shorter recovery time after organic overload (Tale et al. 2015).

Despite some success, anaerobic digester bioaugmentation is still at a nascent stage. A comprehensive review published by Herrero & Stuckey (2014) reported either transient improvement in performance or a complete failure of bioaugmentation to improve anaerobic digestion, but no instances of long-term improvement. Therefore, it is still questionable whether or not adding a limited quantity of externally cultured microorganisms can increase long-term methane production (Herrero & Stuckey 2014). Microbial community analysis has often been employed to understand the relationship between microorganisms and digester function (Venkiteshwaran et al. 2016). However

changes in digester microbial communities after bioaugmentation have not been extensively studied.

In this work, bioaugmentation using a methanogenic, aerotolerant propionate enrichment culture was investigated as a possible method to improve methane production after quasi-steady operation for anaerobic digesters fed a readily degradable waste. Nine groups of anaerobic digesters were seeded with different starting biomass to obtain different microbial communities and digesters were then bioaugmented and monitored for changes in function and microbial community using high throughput Illumina sequencing.

3.2 Material and Methods

3.2.1 Anaerobic Digesters

Biomass samples were obtained from nine full-scale municipal anaerobic digesters in different US states; Delaware (Set-D), Florida (Set-B), Michigan (Set-I), Mississippi (Set-F), New Jersey (Set-H), Ohio (Set-E), South Dakota (Set-G), West Virginia (Set-C) and Wyoming (Set-A) to obtain a variety of microbial communities. With the exception of a thermophilic digester in Michigan, all other biomass samples were from mesophilic digesters. All digesters were continuous stirred-tank reactors stabilizing municipal wastewater sludge with solids retention times between 15 and 30 days. The Florida digester was also fed food waste as a co-digestate.

Each biomass sample was used to seed two sub-sets (bioaugmented and non-bioaugmented) of triplicate, 160-mL lab-scale digesters with 50-mL working volume and biomass concentration of 8g volatile solids (VS)/L. Digesters were operated at a 10-day

HRT and fed synthetic wastewater (non fat-dry milk) and basal nutrient media at an organic loading rate (OLR) of 3 g COD/L-day. The bioaugmented digesters received a daily dose of the enrichment culture from day 60 to 70. The daily dose was equivalent to 1% of the digester biomass total adenosine triphosphate (tATP) mass (this was equivalent to 1.5-2 % of the digester VS mass). Simultaneously, the non-bioaugmented digesters received a COD equivalent dose of inactivated (autoclaved) enrichment culture. Functional parameters including effluent soluble COD (SCOD) and volatile fatty acids (VFA) concentrations as well as biogas CH_4 concentration were monitored between days 60 and 80. Digester biomass samples were collected on day 71 for amplicon sequencing.

Cumulative methane volume produced was calculated by summing the daily methane production volumes (ml CH_4/day) from days 60 to 80. Biomass production rate was calculated as the product of VSS concentration (mg VSS/L) and effluent flow (L/day). Observed biomass yield was calculated as the quotient of biomass production rate and COD added to the digester per day (mg COD/day).

3.2.2 Enrichment Culture for Bioaugmentation

A moderately aerated, propionate-utilizing, methanogenic enrichment culture developed by Tale et al. (2015) having a high specific methanogenic activity (SMA) for propionate was employed for bioaugmentation. When previously used for bioaugmentation, this aerotolerant culture reduced the recovery time of transiently organically overloaded digesters more rapidly than other bioaugments (Tale et al., 2015). The enrichment was maintained in two completely mixed vessels with a volume of four liters at 35 °C at a 15 day HRT and fed 0.17 g propionate/L-day as calcium propionate with basal nutrient media. Immediately after feeding, ambient air was added directly into

the headspace of the vessel at a volume equivalent to 25 mg O_2/L-day or 10% of the OLR to provide a micro-aerated environment.

3.2.3 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]; $(NH_4)_2.HPO_4$ [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]; yeast extract [100]; $NaHCO_3$ [6000]; and resazurin [1].

3.2.4 Analytical Methods

The tATP concentration was analyzed using a commercial kit following the manufacturer instructions (BacTiter-Glo, Promega, Madison WI, USA). The inactive culture added to non-bioaugmented digesters was placed in an autoclave (Model 3870E, Tuttnauer Co., Hauppauge, NY, USA) at 15 psi and 121°C for 30 minutes prior to augmentation experiments. Daily biogas volume produced was measured by inserting a needle with a wetted glass barrel syringe through serum bottle septa. SCOD was measured by filtering the sample through a 0.45 µm pore size membrane syringe filter and determining the filtrate COD by standard methods (APHA et al., 1998). Biogas methane concentration was measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a thermal conductivity detector. VFA concentrations were measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a flame ionization detector. The VS, TSS and VSS

analyses were performed by standard methods (APHA et al., 1998).Statistical analysis such as two-sample Student's t-test with unequal variance and Pearson's coefficient were calculated on Microsoft Excel 2010 (Version 14.3.2) using built in functions.

3.2.5 Microbial Community Analysis

DNA was extracted from all bioaugmented and non-bioaugmented digesters (including all replicates, n=54) on Day 71 using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The biomass samples were subjected to bead beating on a vortex (Model 58816-121, VWR International, Radnor, PA, USA) for 10 minutes. Primer pair 515-532U and 909-928U was used (Wang & Qian 2009) including their respective linkers, to amplify the V4-V5 region of the 16S rRNA gene over 30 amplification cycles at an annealing temperature of 65 °C. The primer pairs target both archaeal and bacterial 16S rRNA genes. An index sequence was added in the second PCR reaction of 12 cycles, and the resulting products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 300 bp reads following manufacturer's instructions (v3 chemistry). Sequencing and library preparation were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). A modified version of the standard operation procedure for MiSeq data (Kozich et al. 2013) in Mothur version 1.35.0 (Schloss et al. 2009) was used to assemble forward and reverse sequences and preclustering at 4 differences in nucleotides over the length of the amplicon. Uchime was used for chimera checking (Edgar et al. 2011). Sequences that appear less than three times in the entire data set were removed. Alignment and the taxonomic affiliation for the 16S rRNA sequences were done using SILVA SSURef

NR99, release 119, as provided by Mothur (Schloss et al. 2009). Custom R scripts were used to perform dual hierarchical clustering (using R command hclust and heatmap) and nonmetric multidimensional scaling (nMDS) (using the default Bray-Curtis index), of anaerobic community sequence data gathered from Illumina sequencing (Carey et al. 2016; McNamara & Krzmarzick 2013).

3.3 Results and Discussion

3.3.1 Non-Bioaugmented Digesters Grouped Based on Function

All nine digester sets reach quasi-steady state based on less than 10% variation is daily biogas production by day 60 when bioaugmentation was initiated (Figure 2 and Figure 3). Effluent VFA concentrations for all digesters were higher than 2 g/L and methane production was below 70% of the theoretical value assuming all COD was converted to methane. Therefore, residual COD was available and could possibly be removed if system changes occurred. This challenged condition was desired so that bioaugmentation effects could be observed. During the dosage period, autoclaved propionate enrichment culture was added to the non-bioaugmented digesters, whereas live propionate enrichment culture was added to the bioaugmented digesters. Adding inactivated enrichment culture did not result in a statistical change in biogas production rate (Figure 2 and Figure 3). This was expected since the daily COD fed to the digesters from the augments was low and was less than 8% of the total synthetic wastewater COD fed.

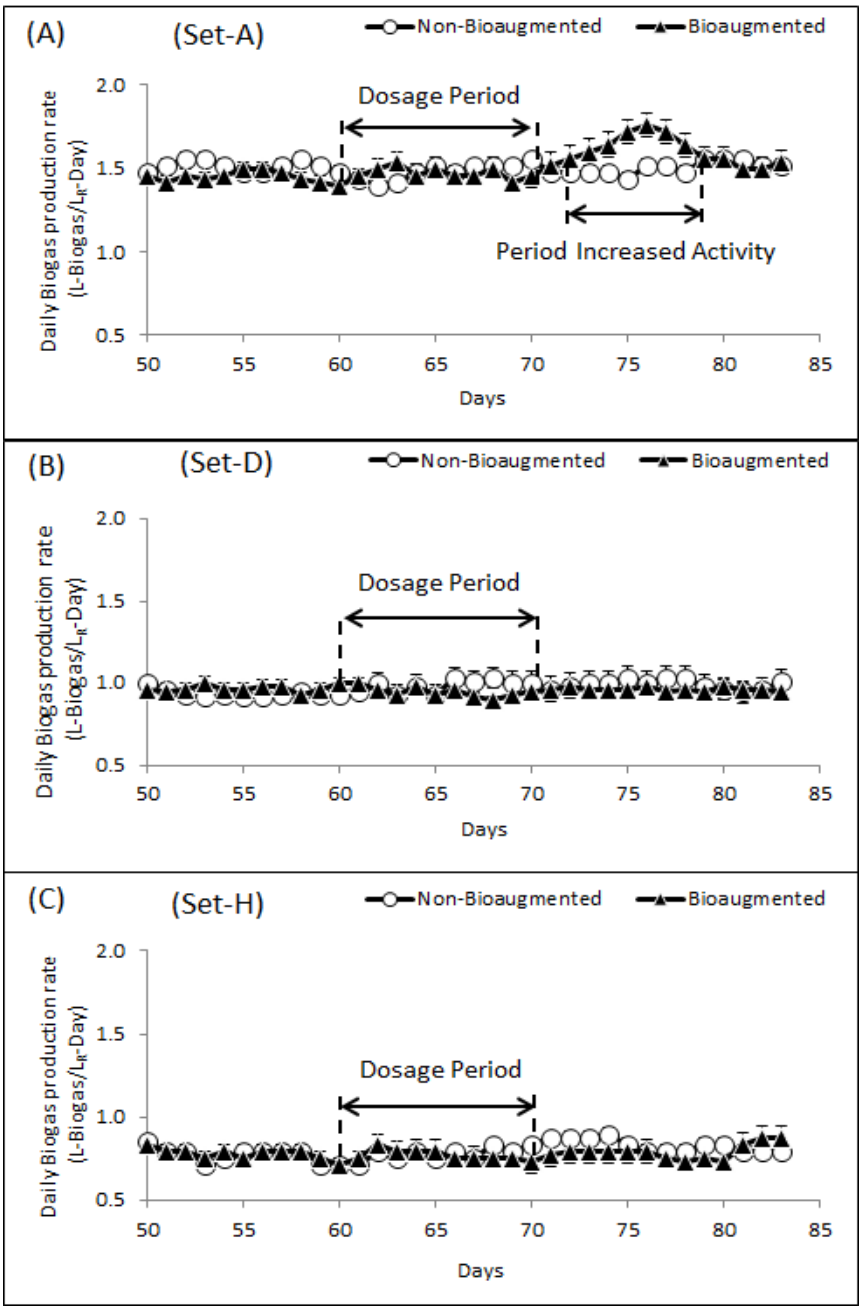


Figure 2: Daily biogas production rate observed from non-bioaugmented and bioaugmented digesters of (A) Set-A, (B) Set D and (C) Set H, respectively. The error bars represent standard deviation among triplicate digesters; some error bars are small and not visible. The dosage period represents the 10-day period during which inactivated and active enrichment cultures were added to non-bioaugmented and bioaugmented digesters, respectively. The Period of Increased Activity (period when bioaugmented digester methane production was statistically greater than that of non-bioaugmented digesters (p value <0.05, n =3)). PIA was not observed for Set-D (B) and Set-H (C) digester systems.

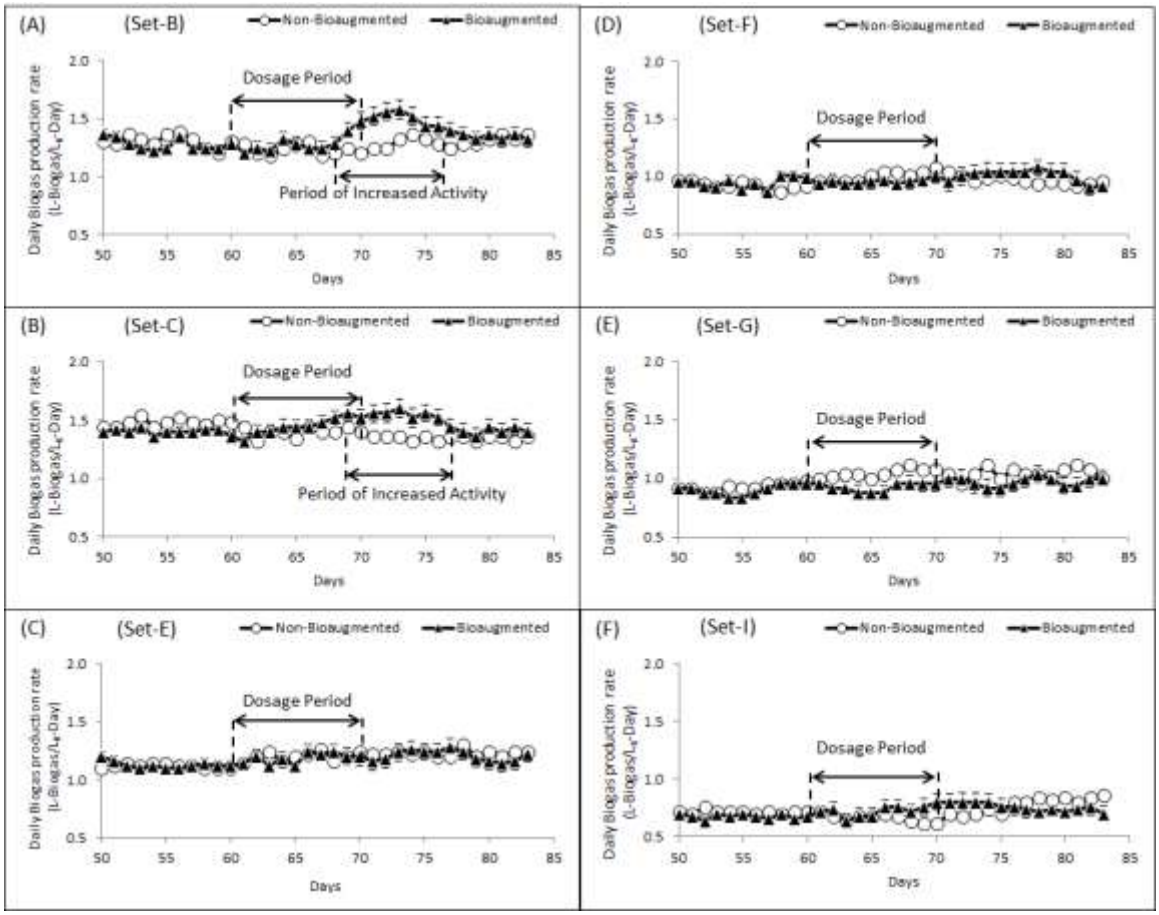


Figure 3: Daily biogas production rate for non-bioaugmented and bioaugmented digester sets B, C, E, F, G and I. Error bars represent standard deviation among triplicates. The Dosage Period represents the 10-day period during which inactivated and active enrichment cultures were added to the non-bioaugmented and bioaugmented digesters, respectively. The period of increased activity (PIA), as seen in Set-B plot (A) and Set-C digester plot (B), represents the days during which the bioaugmented digesters produced statistically higher (p value <0.05, n =3) methane than the non-bioaugmented digesters. PIA was not observed in Set-E (C), Set-F (D), Set-G (E) and Set-I (F) digesters.

Although operated identically, the digester sets did not achieve identical operational values. For example, the quasi steady state methane production rates ranged from 0.3 to 0.8 L CH_4 per L of digester per day (L CH_4/L_R-day) (Table 1). Each non-bioaugmented digester set was classified into one of three distinct groups based on statistically similar methane production rate, SCOD removal, pH and effluent acetate

concentration (p value <0.05, n=3) (Table 1). Group 1 (G1) (sets A, B and C) contained the best performing digesters with the highest methane production rate, highest SCOD removal rate, highest pH and lowest effluent acetate concentration; Group 2 (G2) (sets D, E, F and G) showed intermediary performance and Group 3 (G3) (sets H and I) contained the poorest performing digesters with the lowest methane production rate, lowest SCOD removal rate, lowest pH and highest effluent acetate concentration (Figure 4). The functional variation among digester sets can be attributed to the differences in the microbial communities the seed biomass used for each set. Future research is warranted to elucidate quantitative relationships between microbial community descriptors and digester function so that the suitability of various seed biomass samples can be estimated. This would be helpful to identify the most suitable biomass for a given process startup or re-seeding application.

Table 1: Performance parameters of digester groups.

Group->	G1	G2	G3
Digester Sets->	A, B and C	D, E, F and G	H and I
Methane production rate ($L\text{-}CH_4/L_R$-day at 35°C, 1 atm)	0.77±0.12	0.6±0.04	0.34±0.02
SCOD removal (%)	67±10	55±4	30±4
pH	7.2±0.06	6.6±0.05	6.3±0.0
Acetate (g/L)	2.4±0.6	5.4±1	7.3±2
Propionate (g/L)	2.1±1	4±1	4.3±3

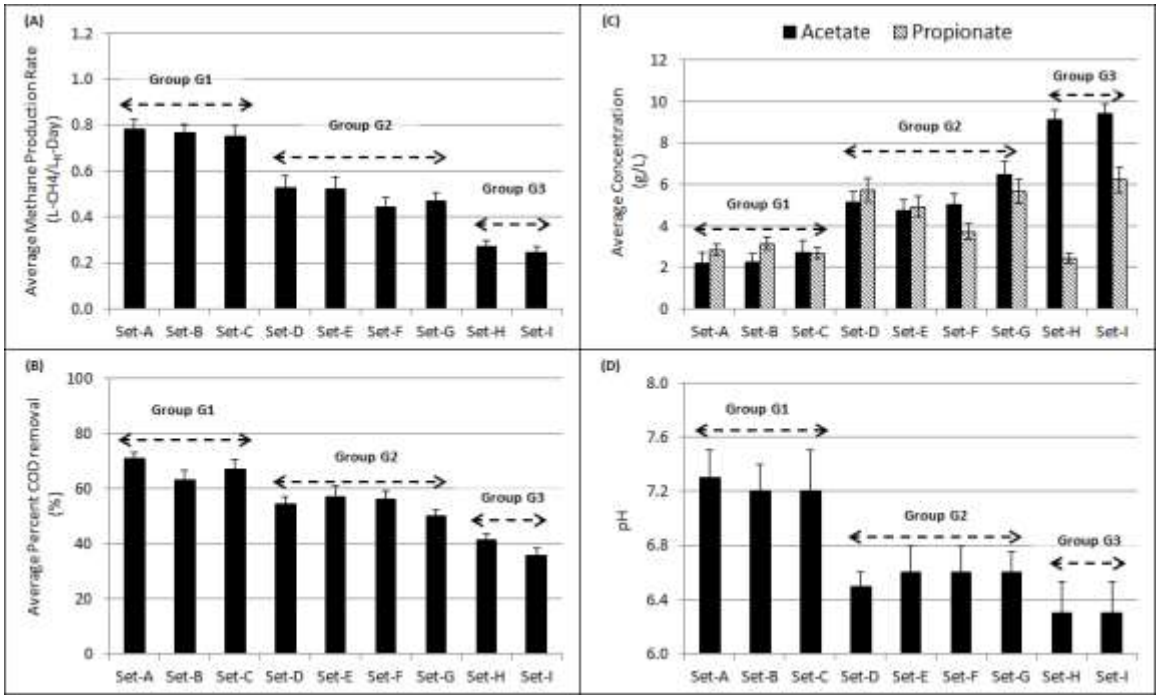


Figure 4: Digester group functional parameters. Average methane production rate (L-CH4/LR-day) (A), average percent COD removal (B), average effluent acetate and propionate concentration (C), and average effluent pH (D). Error bars represent standard deviation among triplicates. Based on average functional performance, the nine digester sets were divided into three statistically distinct groups ($p<0.05$, $n = 3$): digester groups G1 (Sets A, B and C), G2 (Sets D, E, F and G) and G3 (Sets H and I).

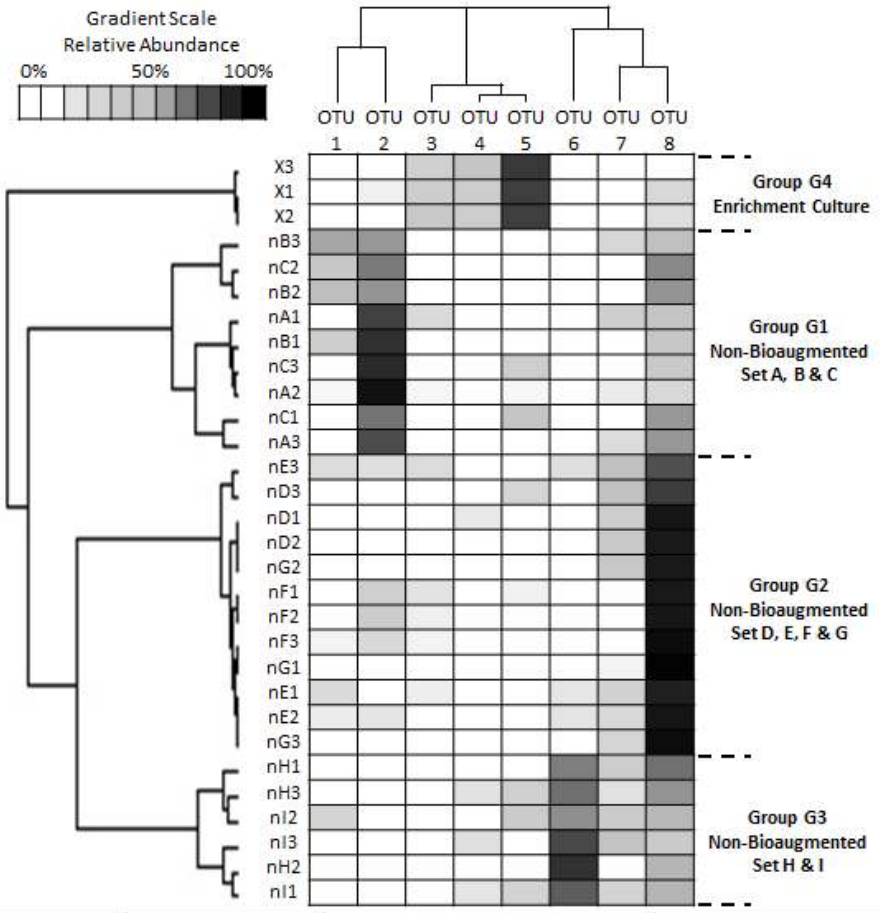
3.3.2 Non-Bioaugmented Digesters Grouped Based on Archaeal Community

A total of 32 archaeal OTUs, based on 97% similarity, were identified among all the digester samples analyzed. The relative abundance of archaeal sequences varied from 1 to 4% for G1, G2 and 0.1 to 1% for G3 digesters, respectively. Eight archaeal OTUs represented more than 99% of the archaeal abundance in all non-bioaugmented digesters (Figure 5). These eight OTUs were most similar to the genera *Methanofollis*, *Methanosarcina*, *Methanospirillum*, *Methanosaeta*, *Methanobacterium*, *Candidatus*

Methanomethylophilus and two unclassified genera in the order *WCHA1-57* and the family *Deep Sea Hydrothermal Vent Grp 6* (*DHVEG 6*) (Figure 5).

The nine non-bioaugmented digester sets clustered in the same three groups that were identified by functional data (95% confidence interval) (Figure 6). Digesters with similar functional performance contained similar archaeal communities. Non-bioaugmented G1 digester communities were distinguished from G2 and G3 communities by high relative abundance of *Methanosarcina*, which ranged from 60 to 95% of archaea in G1 digesters (Figure 5). *Methanobacterium* dominated non-bioaugmented G2 digesters, with a relative abundance that ranged from 80 to 99% of the archaeal diversity. The G3 digesters were distinguished by high relative abundance of the *DHVEG 6*family, with *Methanobacterium* also observed in high relative abundance (Figure 5).

Methanosarcina and *Methanosaeta* are the only two methanogenic genera known to consume acetate (Liu & Whitman 2008) . High relative abundance of *Methanosarcina* in the high-performing G1 digesters correlated to higher methane production and lower effluent acetate concentration. This is ostensibly because the *Methanosarcina* maximum specific substrate utilization rate is higher than that of *Methanosaeta* (Liu & Whitman 2008). Therefore, the presence of *Methanosarcina* in digesters with moderate to high VFA concentrations, such as the ones of this study, may be beneficial to maintain more rapid bioconversion of acetate to methane. Compared to G1 digesters, *Methanosarcina* relative abundance in the lesser performing, non-bioaugmented G2 digesters was lower, ranging from 0.5 to 18%. In addition, *Methanosarcina* was undetectable in the poorest performing, non-bioaugmented G3 digesters which had the highest acid concentrations and lowest pH.



OTU	Class	Order	Family	Genus
1	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
2	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
3	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
4	Thermoplasmata	WCHA1-57	unclassified	unclassified
5	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
6	Halobacteria	Halobacteriales	Deep Sea Hydrothermal Vent Gp 6	unclassified
7	Thermoplasmata	Thermoplasmatales	Thermoplasmatales Incertae Sedis	Candidatus Methanomethylophilus
8	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium

Figure 5: Dual hierarchal clustering of the archaeal communities. The eight OTUs identified based on 97% similarity, represent >99% of the total archaeal sequences observed in the enrichment culture and G1, G2, G3 digesters. The gradient scale ranges from 0 to 100% relative abundance. Sample names x1, x2 and x3 represent the enrichment culture. The sample names for digesters are denoted as follows: for example "nB1" – the prefix "n" represents "non-bioaugmented, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture is dominated by *Methanosaeta* (OTU 5), *Methanospirillum* (OTU 4) and *WCHA1-57* (OTU 3). Set-A, B & C digesters, belonging to group G1 are dominated by *Methanosarcina* (OTU 2). G2 digesters, Set-D, E, F and G, are dominated by *Methanobacterium* (OTU 8). Set H and I are dominated by sequences related to *Methanobacterium* (OTU 8) and *DHVEG6* (OTU 6).

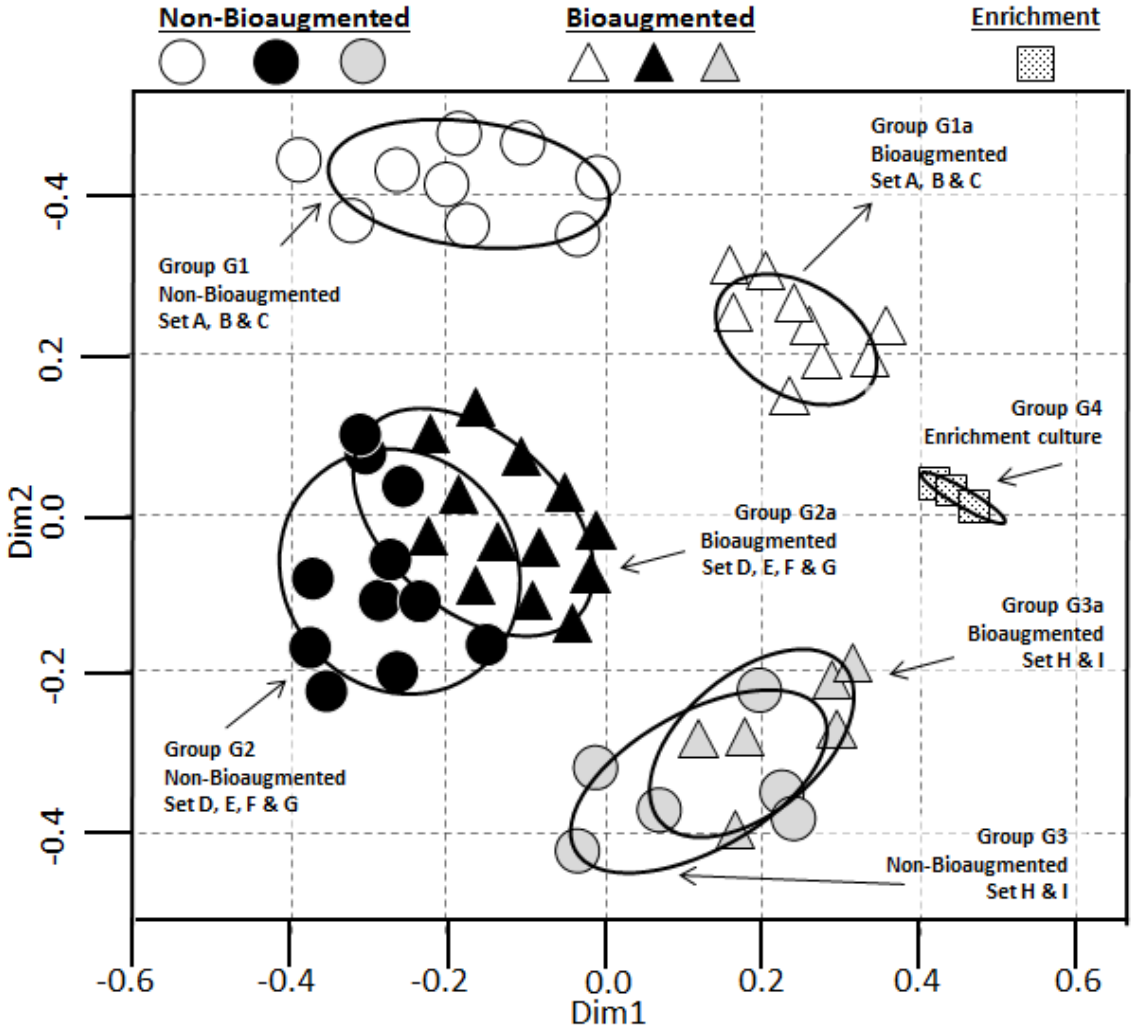


Figure 6: Archaeal sequence nMDS scaling plot. Eight archaeal OTU's, identified based on 97% similarity and representing >99% of the total archaeal sequences in all the biomass samples analyzed were used to construct the nMDS scaling plot. The non-bioaugmented digesters form three distinct clusters G1, G2 and G3. Clusters G1a, G2a and G3a represent the bioaugmented digesters. Cluster G4 represents the enrichment culture. Only the bioaugmented G1 digesters show a significant shift towards the G4 enrichment cluster in its archaeal community structure.

The poor performing G3 digesters were distinguished from the better performing G1 and G2 digesters by the low archaeal sequence abundance (≤ 1% of the total sequence) and further by high relative abundance of *DHVEG 6*, which ranged from 60-90% in G3 digesters (Figure 5). *DHVEG 6* have been observed in acidic environments,

marine environments, terrestrial soils, hydrothermal sediments, deep sea methane seep sediments, rice paddy soil and saline lakes (Casamayor et al. 2013; Nunoura et al. 2010; Nunoura et al. 2011; Hugoni et al. 2013; Grosskopf et al. 1998). Given that *DHVEG6* microorganisms have been observed in extreme environmental conditions that typically are not present in a healthy functioning digester, high abundance of *DHVEG 6* in anaerobic digesters ostensibly indicates an upset digester with low pH and low biogas production such as the G3 digesters.

3.3.3 Non-Bioaugmented Digesters Grouped Based on Bacterial Community

Approximately 1300 bacterial OTUs were identified based on 97% similarity among all the biomass samples analyzed in this study. The 29 OTUs having the highest relative abundance and prevalence among all the digesters and the enrichment culture were considered for bacterial community analysis. These 29 OTUs contributed 70-85% of the total bacterial sequences in the non-bioaugmented digesters.

The nine digesters bacterial communities formed two clusters, with G1 and G2 non-bioaugmented digesters forming one bacterial cluster and G3 non-bioaugmented digesters in the second bacterial cluster (Figure 7). Bacterial communities in all nine digester sets were dominated by OTUs most similar to fermenters belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Synergistetes* (Figure 8). The most common bacterial OTUs that were observed in the digesters were the genera *Bacteroides*, *Peptostreptococcus*, *Pyramidobacter*, *Aminobacterium*, *Atopobium* and *RC9 Gutgroup*. Non-bioaugmented G1 and G2 digesters were distinguished from G3 digesters by the higher abundance of the genera *Porphyromonas*, *Petrimonas* and unclassified *FamilyXI*,

whereas non-bioaugmented G3 digesters were dominated by OTUs most similar to RC9 Gut Group microorganisms which contributed more than 60% of the total bacterial relative abundance (Figure 8).

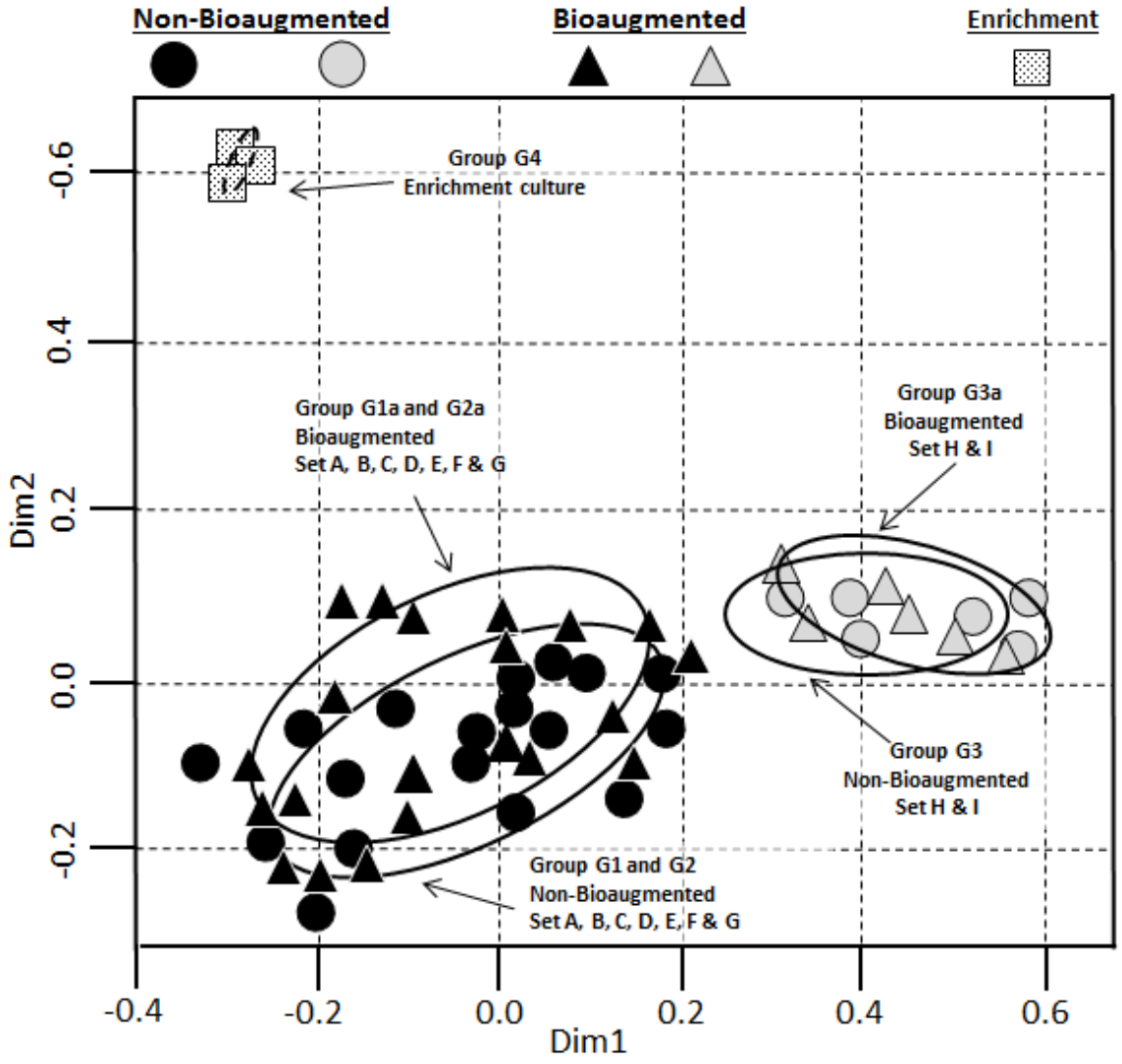
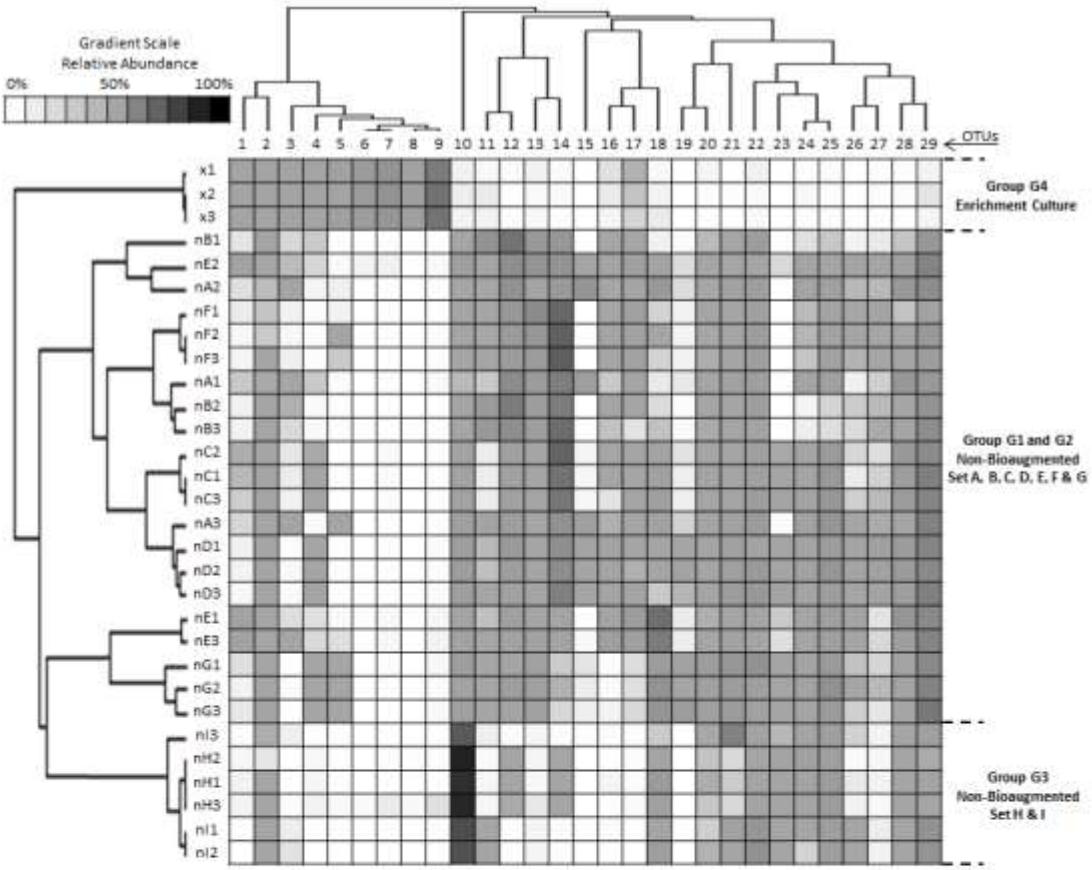


Figure 7: Nonmetric multidimensional scaling or nMDS of the bacterial sequence data. The top 29 OTUs based on their relative abundance and prevalence among all the digesters, including the enrichment culture, were considered in the nMDS scaling plot. G4 represents the bacterial community of the enrichment culture. The bacterial community of the non-bioaugmented digesters cluster in two distinct groups, (1) G1 and G2 (2) G3. The bacterial community of the bioaugmented digesters also cluster into two distinct groups, (1) G1a and G2a (2) G3a. The ellipses represent 95% confidence interval for each cluster.



OTU	Phylum	Class	Order	Family	Genus
1	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminivibrio
2	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	Spirochaeta
3	Tenericutes	Mollicutes	NB1-n	unclassified	unclassified
4	Bacteroidetes	vadinHA17	unclassified	unclassified	unclassified
5	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
6	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Desulfobulbus
7	Synergistetes	Synergistia	Synergistales	Synergistaceae	Thermovirga
8	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	unclassified	unclassified
9	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
10	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group
11	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	vadinBC27 wastewater sludge grp
12	Firmicutes	Clostridia	Clostridiales	Family XI	unclassified
13	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas
14	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas
15	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
16	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
17	Synergistetes	Synergistia	Synergistales	Synergistaceae	unclassified
18	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	unclassified
19	Firmicutes	Clostridia	Clostridiales	Family_XI	Sedimentibacter
20	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
21	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Atopobium
22	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminobacterium
23	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
24	Firmicutes	Clostridia	Clostridiales	Family_XIII	unclassified
25	Synergistetes	Synergistia	Synergistales	Synergistaceae	Pyramidobacter
26	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Fastidiosipila
27	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus
28	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus
29	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides

Figure 8: Dual hierarchal clustering of the bacterial communities. The top 29 bacterial OTUs, based on their relative abundance and prevalence among all the digesters, including the enrichment culture, were considered in the clustering

analysis. The 29 OTUs contributed to 70-85% of the total bacterial sequences in all the biomass samples analyzed. The gradient scale ranges from 0 to 100% relative abundance. Sample names x1, x2 and x3 represent the enrichment culture. The sample names for digesters are denoted as follows: for example "nB1" – the prefix "n" represents "non-bioaugmented, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture has a unique bacterial community structure as compared to the other digesters and clusters separately. Fermenters of the phyla *Firmicutes*, *Bacteroidetes* and *Synergistetes* dominated all non-bioaugmented digesters. G3 digesters (Set-H & I) were uniquely dominated by *RC9 gut group* (OTU 10), contributing 50-60% of the relative abundance.

3.3.4 Enrichment Culture

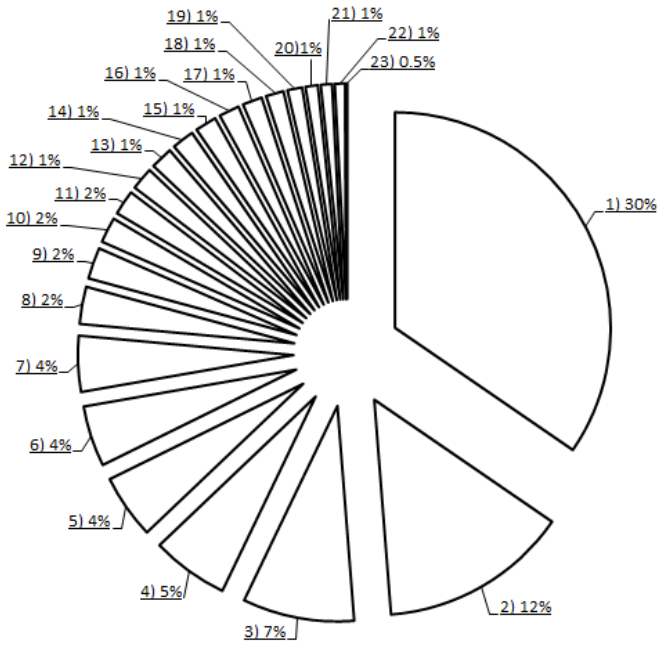
The enrichment culture functional parameters were stable between days 60 to 80, with 99% SCOD removal, no detectable VFAs, pH of 7.5±0.2, 60±3% biogas methane content, methane yield of 330±16 mLCH_4/g-COD removed and observed biomass yield of 0.08±0.01 gVSS/gCOD.

More than 700 bacterial OTUs were identified in the enrichment culture based on 97% similarity. The 25 bacterial OTUs with the highest relative abundance represented approximately 80% of total bacterial sequences and are shown in Figure 9. The two most abundant bacterial taxa were most similar to an unclassified genus within *Spirochaetaceae* (30% of the total bacterial relative abundance) and *Thermovirga* within *Synergistaceae* (12% of the total bacterial relative abundance) (Figure 9). *Thermovirga* is currently represented by a single member species *Thermovirgalienii*, which is a moderately thermophillic, amino acid degrading fermentative bacterium (Dahle 2006). Some members of the *Spirochaetaceae* family such as *Treponema* species, are reported to be abundant in iron-reducing consortia that were used by others to bioaugment anaerobic digesters (Baek et al. 2015). Iron-reducing bacteria (IRB) are commonly observed in

anaerobic systems and can utilize acetate, H_2, ethanol and other complex substrates and ferric iron as an electron acceptor (Kim et al. 2014). They are also known to form syntrophic associations and, via interspecies electron transfer, transfer electron directly to their methanogenic partner, which can facilitate CO_2reduction to CH_4 (Stams & Plugge 2009; Rotaru et al. 2014). Addition of an IRB consortium has been shown to increase the methane production rate in anaerobic digesters (Baek et al. 2015).

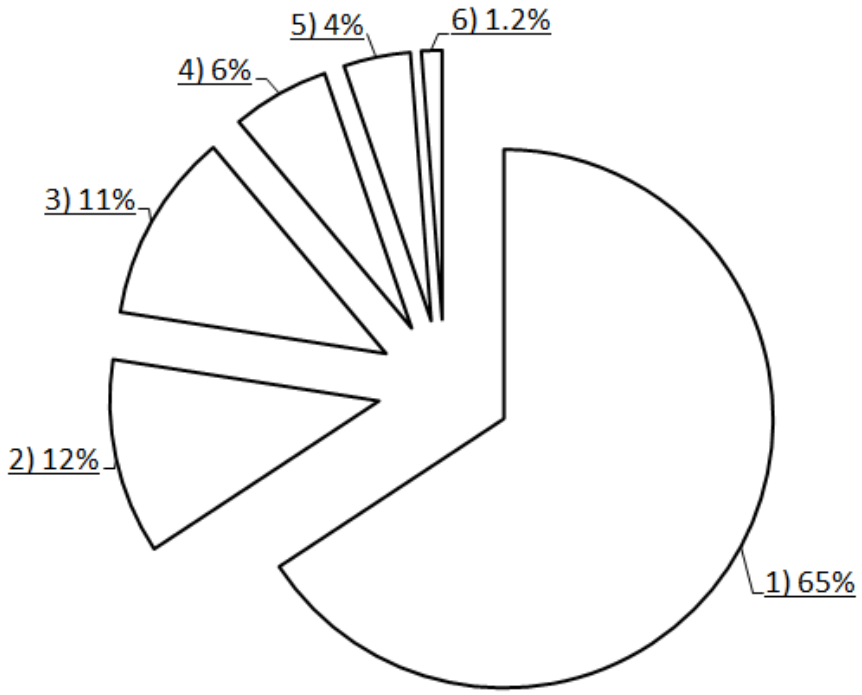
Given that the enrichment culture was fed calcium propionate, it was expected that bacteria associated with syntrophic propionate degradation would be abundant. Of the known bacterial genera with members capable of degrading propionate (de Bok et al. 2001; De Bok et al. 2004; Stams & Plugge 2009), *Smithella*, *Syntrophobacter* and *Desufobulbus* were observed with a combined relative abundance of 9%, with *Desulfobulbus* contributing 7% (Figure 9).

15 archaeal OTUs were detected in the enrichment culture based on 97% similarity, of which 6 OTUs contributed more than 99% of the total archaeal sequences (Figure 10). Archaeal sequences constituted approximately 5-6% of the total sequences detected in the enrichment culture. The archaeal community was dominated by sequences most similar to *Methanosaeta,* constituting 65% of the total archaeal sequences (Figure 10). *Methanosarcina* constituted only 1.2% of the total archaeal sequences in the enrichment culture (Figure 10). Unlike *Methanosarcina*, *Methanosaeta* have a high substrate affinity and a lower maximum specific substrate utilization rate. Hence, *Methanosaeta* usually dominate over *Methanosarcina* in cultures such as the enrichment culture in this study having acetate concentrations lower than 500 mg/L (Liu & Whitman 2008).



OTU	Phylum	Class	Order	Family	Genus
1	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
2	Synergistetes	Synergistia	Synergistales	Synergistaceae	Thermovirga
3	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Desulfobulbus
4	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	unclassified	unclassified
5	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Denitrovibrio
6	Hyd24-12	unclassified	unclassified	unclassified	unclassified
7	Spirochaetae	Spirochaetes	Spirochaetales	PL-11B10	unclassified
8	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum
9	Bacteroidetes	vadinHA17	unclassified	unclassified	unclassified
10	Tenericutes	Mollicutes	NB1-n	unclassified	unclassified
11	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminivibrio
12	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Smithella
13	Proteobacteria	Gammaproteobacteria	unclassified	unclassified	unclassified
14	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
15	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
16	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
17	Candidate division OP8	unclassified	unclassified	unclassified	unclassified
18	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	Spirochaeta
19	Chlorobi	Ignavibacteria	Ignavibacteriales	unclassified	unclassified
20	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	unclassified
21	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	WCHB1-69	unclassified
22	Deferribacteres	Deferribacteres	Deferribacterales	SAR406 clade (Marine group A)	unclassified
23	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Defluviimonas
24	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	WCHB1-69	unclassified
25	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Meniscus

Figure 9: Bacterial community composition of the enrichment culture based on percent relative abundance. The figure includes the 25 bacterial OTUs observed with the highest relative abundance which constituted 80% of the total bacterial sequences.



OTU	Class	Order	Family	Genus
1	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
2	Thermoplasmata	WCHA1-57	unclassified	unclassified
3	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
4	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
5	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
6	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina

Figure 10: Archaeal community composition of the enrichment culture based on percent relative abundance. The figure includes 6 archaeal OTUs identified based of 97% similarity of the sequences.

Apart from acetoclastic methanogens, the enrichment culture archaeal composition consisted of OTUs most similar to known hydrogenotrophic methanogens including *Methanospirillum, Methanobacterium, Methanolinea* and an unclassified genus in the order WCHA1-57 (Figure 10) (Liu & Whitman 2008). Conversion of propionate to methane only becomes thermodynamically favorable through H_2 utilization. Therefore,

the significant presence of hydrogenotrophic methanogens contributing 30-35% of the total archaeal sequences could have positive functional results. The presence of microorganisms classified in the genera *Methanospirillum, Methanobacterium, Methanolinea* has previously been reported to play an important role in propionate utilization during digester recovery after organic overload (Tale et al. 2011; Tale et al. 2015; Schauer-Gimenez et al. 2010).

The archaeal order *WCHA1-57* was observed at a significant relative abundance (12%) in the enrichment culture. Although many *WCHA1-57*-related 16S rRNA gene sequences have been identified in anaerobic digesters (Chouari et al. 2005; Rivière et al. 2009; Schauer-Gimenez et al. 2010), no reports were found regarding their role in propionate oxidation or methane production. In some anaerobic digesters treating municipal sewage sludge, the *WCHA1-57* phylotype population represented one of the predominant archaeal components, with relative abundance >70% in archaeal clone libraries (Chouari et al. 2005; Rivière et al. 2009). These observations indicate that *WCHA1-57* archaea represent a potentially important group in anaerobic digesters. Chouari et al. (2005) reported the enrichment of *WCHA1-57* phylotypes in cultures fed formate or H_2/CO_2. This indicates that *WCHA1-57* plays a role in reducing hydrogen concentration and, therefore, aiding in conversion of propionate to methane.

Both bacterial and archaeal enrichment culture communities were distinct from those of the nine digester sets. The nMDS scaling plots based on the top eight archaeal (Figure 6) and 29 bacterial (Figure 7) OTUs, selected based on their relative abundance and prevalence among all the biomass samples, shows distinct clustering of the

enrichment culture separate from the G1, G2 and G3 non-bioaugmented and bioaugmented digesters.

3.3.5 Bioaugmentation, Digester Function and Microbial Community Changes

Cumulative methane produced by both non-bioaugmented and bioaugmented digesters between days 60 and 80 were calculated and compared to observe any difference in performance (Figure 11). Only the three G1 digester sets A, B and C showed a statistically significant increase ($P < 0.05$, $n = 3$) in average methane production of 11± 3% after bioaugmentation, with increases of 9±1%, 12±2% and 13±2%, respectively, compared to non-bioaugmented controls (Figure 11).

The increased methane production in G1 digesters after bioaugmentation was not sustained. The period of increased methane production averaged 9 days, and was 7, 11 and 9 days for sets A, B and C, respectively (Figure 2, Figure 3 and Figure 3). Also, the increased methane production did not occur immediately after bioaugmentation was initiated. The lag between the dosage period start (Day 60) and the first day of increased methane production for set A, B and C bioaugmented digesters averaged 10 days, and was 12, 8 and 9 days, respectively (Figure 2, Figure 3 and Figure 3).

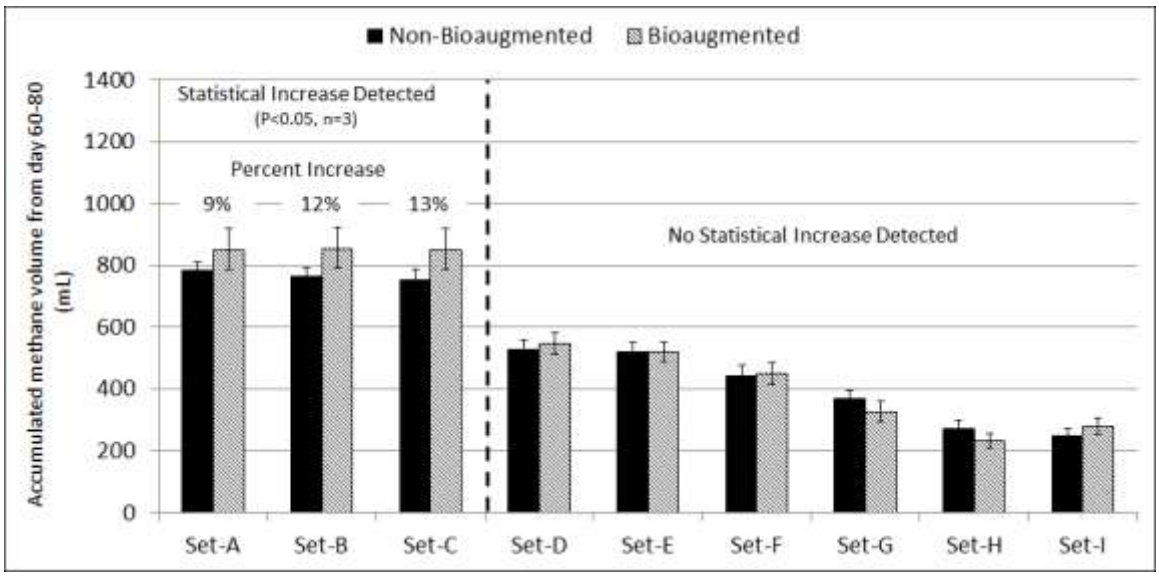
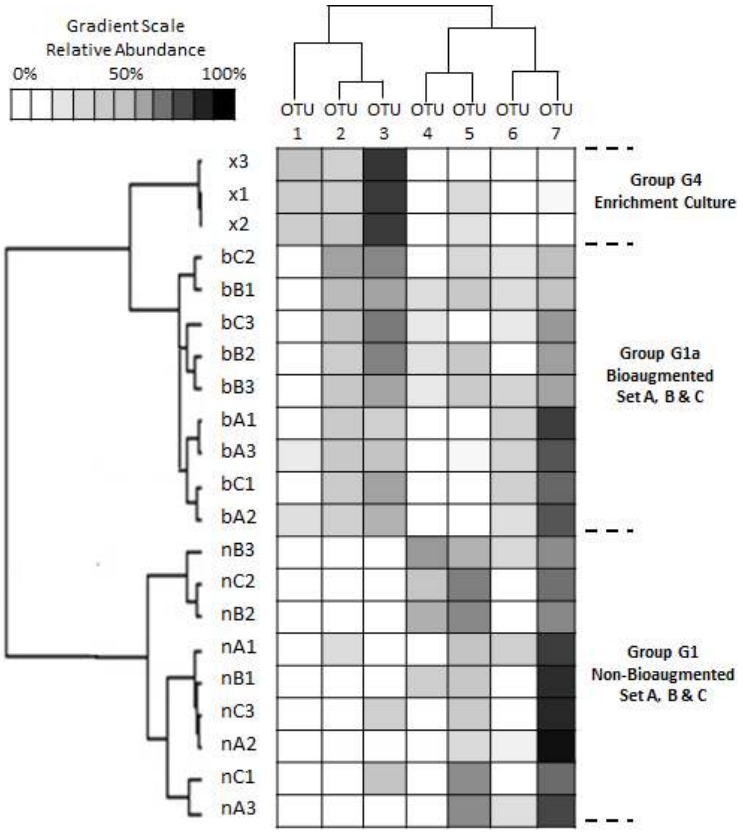


Figure 11: Cumulative CH_4 produced between day 60-80. Error bars represent standard deviation among triplicates. Bioaugmented digesters of Sets A, B and C showed statistically higher cumulative CH_4 production ($p<0.05$, $n=3$).

The archaeal communities in the bioaugmented digesters were grouped into three distinct clusters based on archaeal sequences (Figure 6). The archaeal community of the bioaugmented digesters belonging to functional groups G2a and G3a, which did not improve after bioaugmentation, did not significantly change after bioaugmentation (Figure 6). In contrast, however, the G1 bioaugmented digesters showed a statistical improvement in methane production and the archaeal community changed significantly after bioaugmentation (Figure 6). After bioaugmentation, the archaeal community of G1 digesters became more similar to that of the enrichment culture (G4).

The community structure shift in G1 digesters after bioaugmentation was primarily caused by the increased abundance of two archaeal genera: *Methanosaeta* and *Methanospirillum* (Figure 12), which are in high abundance in the enrichment culture (Figure 10). In contrast, the relative abundance values of *Methanosaeta* and *Methanospirillum* were very low (<1%) in G2 and below detection in the G3

bioaugmented digesters, respectively. The bacterial community compositions of the bioaugmented digesters did not show any significant shift after bioaugmentation (Figure 7). Sequences related to the two most abundant bacterial genera observed in the enrichment culture, unclassified *Spirochaeata* and *Thermovirga*, which were not detected in the non-bioaugmented digesters, were detected in all the bioaugmented digesters, but their relative abundance remained below 1% after bioaugmentation.



OTU	Class	Order	Family	Genus
1	Thermoplasmata	WCHA1-57	unclassified	unclassified
2	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
3	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
4	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
5	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
6	Thermoplasmata	Thermoplasmatales	Thermoplasmatales Incertae Sedis	Candidatus Methanomethylophilus
7	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina

Figure 12: Dual hierarchal clustering of the top seven archaeal OTUs observed in the enrichment culture and G1 digesters. These seven OTU's, based on 97% similarity, represent >99% of the total archaeal abundance in the enrichment

culture and G1 digesters. The gradient scale ranges from 0 to 100% relative abundance. Sample names x1, x2 and x3 represent the enrichment culture. The sample names for digesters are denoted as follows: for example "nB1" and bB1 – the prefix "n" and "b" represents "non-bioaugmented" and "bioaugmented", respectively, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture is dominated by *Methanosaeta*(OTU 3), *Methanospirillum* (OTU 2) and *WCHA1-57* (OTU 1). The non-bioaugmented digesters are dominated by *Methanosarcina* (OTU 7), followed by *Methanobacterium* (OTU 5). The bioaugmented digester of Set-A, B and C showed a significant abundance of *Methanosaeta*(OTU 3) and *Methanospirillum* (OTU 2) as compared to the non-bioaugmented digesters.

The resulting increase in methane production observed in G1 digesters from bioaugmentation was associated with a shift in the archaeal community structure. Increased relative abundance of the genera *Methanosaeta* and *Methaospirillum* was observed in digesters with improvement in the methane production rate. The relative abundance of *Methanosaeta* and *Methanospirillum* increased from below detection in the non-bioaugmented digesters to 10-40% and 10-30%, respectively, in the bioaugmented G1 digesters (Figure 12). However, it is important to note that the methane production rate increase lasted only 7 to 11 days in the bioaugmented G1 digesters. This could be due to washout of *Methanosaeta* and *Methanospirillum* once bioaugmentation ceased. It may be possible to improve the methane production further by increasing the dose concentration of the enrichment biomass, extending the duration of the dosage period or employing a membrane bioreactor to retain the bioaugment.

The enrichment culture used in this study was produced at a pH of 7.5 with no detectable VFAs present. The most abundant methanogens in the enrichment culture, *Methanosaeta* and *Methanospiririllum* (i.e., *M. hungatii*), are sensitive to low pH and high acid or propionate concentrations (Liu & Whitman 2008; Barredo & Evison 1991). It is likely that the methane production increase in G1 digesters after bioaugmentation

was due to the relatively low VFA concentration and neutral pH, which was conducive for the activity of *Methanosaeta* and *Methanospiririllum* added via the bioaugment. In contrast, the low pH, high VFA concentration environment in G2 and G3 digesters may have inhibited the enrichment culture microorganisms. Therefore, the environment the enrichment culture is being added into must be carefully considered and additional steps such as acclimating the augment culture or increasing the digester pH before bioaugmentation may be required to increase methane production and COD removal by bioaugmentation.

3.4 Conclusion

Different anaerobic digester seed biomass can result in significantly different quasi steady state functional parameters, including methane production rate, SCOD removal, pH and effluent VFA concentration. Therefore, care should be taken to select seed biomass with high activity for digester startup or re-seeding.

Identically operated digesters that contain different archaeal communities can exhibit different functional characteristics during quasi steady state operation. When operating under challenging conditions (VFA >2 g/L and theoretical methane production less than 70%), digesters with high methane production rates can be distinguished by high *Methanosarcina* relative abundance. The presence of *Methanosarcina* in digesters with moderate to high VFA concentrations is beneficial to maintain more rapid bioconversion of acetate to methane. In contrast, digesters with low methane production can be distinguished by high abundance of *Methanobacterium* and *DHVEG 6* family organisms. Since *DHVEG6* microorganisms have been found in extreme environments, including deep-sea hydrothermal vents, their high abundance in anaerobic digesters may

indicate past or current digester upset (i.e., high VFA concentration and low methane production).

Bioaugmentation with a methanogenic, propionate degrading enrichment culture resulted in a 11±3% increase in methane production when digester pH was approximately 7.2. However, methane production did not change after bioaugmenting digesters that had pH values less than 6.7. Therefore, when predicting bioaugmentation outcomes, the environment into which an augment culture is added must be carefully considered as well as the composition of the bioaugment itself. Steps such as increasing low digester pH before bioaugmentation may be necessary to improve digester function.

The methane production increase after bioaugmentation was correlated to increased relative abundance of *Methanosaeta*, *Methaospirillum,* unclassified *Spirochaeata* and *Thermovirga* that were in the bioaugment culture employed. However, the methane production rate increase lasted only 7 to 11 days. It may be possible to increase methane production for longer periods by increasing the dose concentration of the bioaugment, periodically repeating bioaugmentation, or employing a membrane bioreactor to retain the bioaugment. More research is warranted to develop sustained, steady state improvements via bioaugmentation or bioaugmentation combined with pH adjustment for challenged digesters.

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4. Predicting Anaerobic Digester Methane Production Using Microbial Community Descriptors

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4.1 Introduction

There is an increased emphasis among industries and municipalities to achieve sustainability goals by shifting from wastewater treatment to energy generation and resource recovery (Angenent et al. 2004; Novotny, Ahern, and Brown 2010). In terms of appropriation of anaerobic bioprocesses, a challenge still remains, as much is still undetermined about the distinguishing microbial factors between a healthy and unhealthy digester (Leitão et al. 2006). Current mathematical models used to understand anaerobic treatment plants such as ADM1 typically do not include microbial community information and rely on the assumption that trophic groups containing only one member (Ramirez et al. 2009). ADM1 requires input of 24 variables, of which seven relate to microbial function associated with seven trophic groups. The seven trophic groups correspond to the degradation of sugar, amino acids, long chain fatty acids, acetate, propionate, butyrate-valerate and hydrogen. One of the major reasons that microbial community parameters are not included in models is because inadequate microbiological data exist, specifically community structure-function relationships and kinetic data. Therefore, in order to improve the predictability of current models, understanding

regarding how microbial community structure relates to process function, such as methane generation, must be deepened (Curtis, Head, and Graham 2003).

Microbial community descriptors, specifically overall biomass concentration, species richness, microbial diversity and evenness have been shown in previous studies to correlate with anaerobic digester function and stability when operating under transient conditions such as variable influent organic strength (Hashsham et al. 2000; Fernandez et al. 2000). However, the relationships established are qualitative, not quantitative or predictive.

A few studies using multiple linear regression (MLR) modeling have reported quantitative linear relationships between archaeal (i.e, methanogen) descriptors and their activity (Venkiteshwaran et al., 2016). Tale et al. (2011) applied MLR to anaerobic digester data, finding similar specific methanogenic activity (SMA) values related to similar community structures as defined by DGGE banding patterns of methyl coenzyme A (mcrA) amplicons. The mcrA gene copy number was also linearly correlated with SMA values of four H_2/CO_2 enrichment cultures (Morris et al. 2014). Bocher et al. (2015) used mcrA DGGE banding pattern data from a larger set of 49 distinct biomass samples to develop MLR equations that predicted SMA values against propionate and glucose. The results observed in these previous studies were encouraging; however only archaeal community data were used to predict methanogenic activity, and batch SMA results, not steady-state digester function, was predicted. Anaerobic digestion (AD) involves both archaea and bacteria that are typically classified in four trophic groups: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Bitton 2005). Hence, it is prudent to investigate whether both archaeal and bacterial microorganism abundance

values are good descriptors in an MLR model to predict function of digesters fed a complex synthetic wastewater. And it is important to predict quasi steady state digester function, not only SMA values.

In this study we employed high throughput sequencing technology, Illumina MiSeq sequencer, along with universal 16S rRNA primers that target both archaeal and bacterial populations. To include a large data set and diversity of anaerobic microorganisms, 50 digester sets were operated under identical conditions. Digesters acclimated for a minimum of 5 hydraulic retention times (HRTs) before functional data and microbial community samples were collected. Subsequently, a predictive, quantitative structure activity relationship (QSAR) between anaerobic microbial community descriptors and digester methane production rate was developed.

4.2 Materials And Methods

4.2.1 Inocula

Biomass samples were obtained from 50 full-scale, engineered methanogenic systems in 49 states within the United States and used to inoculate laboratory digesters (Table S1). No anaerobic systems were found in Rhode Island, and two samples were obtained from different anaerobic systems (Systems A and B) in Wisconsin (WI). One sample was obtained from each of the remaining 49 states. Methanogenic biomass was from 25 anaerobic systems treating industrial waste (food, dairy and brewery industries) and 25 digesters stabilizing municipal wastewater sludge. One sample was from an AnMBR (TX) and six biomass samples were from upflow anaerobic sludge blanket (UASB) reactors (from Alabama (AL), Arkansas (AR), Colorado (CO), Idaho (ID),

Kansas (KS) and Wisconsin A (WI A) with granular biomass, whereas all other samples were flocculent biomass from continuous stirred-tank reactors (CSTRs). With the exception of a thermophilic digester in Michigan (MI), biomass samples were from mesophilic systems.

4.2.2 Laboratory Digesters

Each biomass sample was used to inoculate a set of three laboratory digesters that were 160 mL serum bottles with 50 mL of active volume incubated at 35 ± 2°C on a shaker table. A 10-day hydraulic retention time (HRT) was maintained by removing 5 mL of effluent and adding an equal volume of synthetic industrial wastewater every day. Effluent removal and feeding was done by inserting a needle with a plastic syringe through serum bottle septa. Inocula containing granular biomass from all six UASB digesters were blended using a bench-top blender for 10 sec prior to seeding laboratory digesters. All digesters were seeded at an initial volatile suspended solids (VSS) concentration of 8 g/L and operated at an organic loading rate (OLR) of 3 g COD/L-day. This OLR was identified after a preliminary investigation when five inocula were tested at OLR values of 2, 3, 4 and 5 g COD/L-day. The preliminary investigation purpose was to identify a sustainable OLR that did not result in digester failure (i.e., digester methane production less than 10% of theoretical maximum), but challenged the system with a high OLR to observe a wide range of COD removal and methane production rate values. An OLR of 2 g COD/L-day resulted in >98± 0.1 % COD removal for all digesters. In contrast, all preliminary digesters failed at OLR values of 4 and 5 g COD/L-day. Therefore, the 3 g COD/L-day OLR was used for subsequent testing since it did not cause failure, but resulted in 60 to 90% COD removal for the different inocula tested.

Biogas production volume was measured daily by inserting a needle with a wetted glass barrel syringe through serum bottle septa. After 50 days of operation, daily biogas production varied less than 20% and systems were assumed to be at quasi steady state. Digester biogas and effluent samples were then collected for quasi steady state functional analysis over seven consecutive days. Functional parameters measured included biogas methane concentration, effluent volatile fatty acids (VFAs) and soluble COD (SCOD) concentrations. Effluent total suspended solids (TSS) and VSS concentrations as well as pH were also measured on day seven of the analysis period.

Synthetic industrial wastewater was composed of non-fat dry milk (Roundy's Supermarkets, Inc., Milwaukee, WI USA) containing 52% w/w sugars and 35% w/w proteins as well as 10 g/L $NaHCO_3$ and nutrient medium. Nutrient medium, as described by Speece (2008), contained the following [mg/l]: NH_4Cl [400]; $MgSO_4 \cdot 6H_2O$ [250]; KCl [400]; $CaCl_2 \cdot 2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3 \cdot 6H_2O$ [55]; $CoCl_2 \cdot 6H_2O$ [10]; KI [10]; the salts $MnCl_2 \cdot 4H_2O$, NH_4VO_3, $CuCl_2 \cdot 2H_2O$, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, H_3BO_3, $NiCl_2 \cdot 6H_2O$, $NaWO_4 \cdot 2H_2O$, and Na_2SeO_3) [each at 0.5]; yeast extract [100]; and resazurin [1].

4.2.3 Microbial Community Analyses

Digester effluent samples (1 mL) were collected for DNA extraction on six consecutive days when digester functional analyses were performed. The effluent samples were centrifuged at 10,000 RPM for 10 min. Centrifuged solids were combined and DNA was extracted using a commercial kit according to manufacturer instructions (PowerSoil™ DNA Isolation Sample Kit, MoBio Laboratories, Inc., Carlsbad, CA).

Biomass samples were subjected to bead beating for 10 minutes on a vortexer (Model 58816-121, VWR International, Radnor, PA, USA).

Forward primer 515-532U and reverse primer 909-928U (Wang and Qian 2009) were used to amplify the V4-V5 region of the 16S rRNA gene. The DNA sample and primers with their respective linkers were amplified over 30 cycles at an annealing temperature of 65°C. The primer pairs target both bacterial and archaeal 16S rRNA genes. An index sequence was added in a second PCR reaction of 12 cycles, and the resulting products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 300 bp reads following manufacturer instructions (Reagent Kit v3, Illuminia, Inc., San Diego, CA USA). Sequencing work was performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr).

Forward and reverse sequences were assembled and quality checked using a modified version of the standard operation procedure by Kozich et al. (2013) in Mothur version 1.33.0. Sequence alignment and taxonomic outlining was accomplished using SILVA SSURef NR99, release 119, as provided by Schloss et al. (2009). Final sequence data were assembled at 97% similarity and rarified to the lowest sequence reads (~27,500 reads) observed among the samples analyzed. Extract from one digester inoculated with biomass from North Dakota (ND3) did not contain detectable DNA. Therefore, data from this digester were excluded from further analysis and data from the remaining 149 digesters were subsequently employed.

4.2.4 Initial Screening to Select Significant OTUs

Initial screening was performed to select OTUs with relative abundance values that highly correlated with average methane production rates. Initial screening was done by performing 50 iterations to calculate Spearman's rank correlation matrix. Spearman's rank was employed as a measure of statistical dependence because of its robustness since it does not require underlying assumptions regarding the frequency of distribution of variables (e.g., normal distribution, uniformly distributed etc.) or the existence of a linear relationship between variables (Zuur, Ieno, and Smith 2007). In each iteration, the Spearman's rank correlation coefficients for the relative abundance values of each of the 1300 OTUs and the average methane production rates in 75 randomly selected digesters were calculated. A total of 20 significant OTUs were selected during each initial screening iteration, with 10 OTUs resulting in the highest and 10 OTUs resulting in the lowest Spearman's rank correlation coefficient values with methane production rate. Ten OTUs were repeatedly identified in more than 75% of the initial screening iterations and, therefore, were deemed to be highly significant. The 10 highly significant OTUs were subsequently employed to develop the QSAR linear regression model.

4.2.5 Linear Model Validation and QSAR Equation

A MLR leave group out (LGO) approach was employed to validate a quantitative relationship between methane production rate and the relative abundance values of the 10 highly significant OTUs identified during initial screening. Digesters were randomly partitioned into 10 subsets of 14 or 15 digesters each. Subsequently, 9 subsets were combined and used as a training set to develop an MLR equation, whereas the remaining

subset was used as the validation set to test the equation predictability. This was repeated until all 10 digester subsets were used once for validation.

MLR equation predictability was deemed to be good if the following four criteria were met (Golbraikh and Tropsha 2000; Konovalov et al. 2008): $q^2 > 0.5$, $R^2 > 0.6$, $(R^2-R_O^2)/R^2 < 0.1$ and $0.85 \leq K \leq 1.15$, where q^2 is the chi square value calculated using the observed versus predicted methane production values described by Schüürmann et al. (2008); R^2 is the coefficient of determination for the test set linear regression equation of predicted versus observed methane production rates, and R_O^2 and K are the coefficient of determination and the slope for the test set linear regression equation of predicted versus observed methane production rates forced through the origin, respectively.

After confirming that MLR equations demonstrated good predictability, all 10 digester subsets were combined and used to determine a final, QSAR linear regression model.

4.2.6 Analytical Methods

Biogas methane concentration was quantified by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a thermal conductivity detector. SCOD was measured by filtering the sample through a 0.45 µm pore membrane syringe filter and determining the filtrate COD by standard methods (APHA et al., 1998). VFA concentrations were measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a flame ionization detector. Digester VSS concentrations were determined by standard methods (APHA et al., 1998). The pH was measured using a pH meter and probe (Orion 4 Star, Thermo, Waltham, MA, USA).

Average, standard deviation, variance and t-test calculations were performed using Excel 2010 (Version 14.3.2 – Microsoft, USA) built in functions. Richness (S), Shannon-Weaver diversity (H), and evenness (E) indices were calculated using Illumina sequence results. Richness was calculated as the number of OTUs identified at the genus level from Illumina sequencing data. Shannon-Weaver diversity index values were determined as described by Briones et al. (2007). Evenness was calculated as described by Falk et al. (2009). Principal component analysis (PCOA) was performed using R statistical freeware package custom scripts. ANOSIM using Bray-Curtis dissimilarity was performed to assess the relationship between methane production and microbial community using the vegan package in R. The Spearman rank correlation and MLR analyses were performed using Excel 2010 (Version 14.3.2 – Microsoft, USA) with the added statistical software package XLStat Pro 2014 (Addinsoft, USA).

4.3 Results and Discussion

4.3.1 Digester Function

All digester sets were operated identically, but were seeded with different biomass. The seed biomass identity had a significant influence on functional performance, both initially as well as after 80 days (Figure 13). For example, digester set biogas production rates varied greatly, ranging from 0.4±0.02 to 1.6±0.09 L-biogas/L_R-day (average ± standard deviation) (Figure 13). Significant variability was also observed in methane production rate (0.09±0.004 to 0.98±0.05 L CH_4/L_R-day) as well as effluent SCOD concentration (2.6±0.3 to 25±1.1 g/L), total VFA concentration (1.6±3.8 to 19±1.3

mg/L as acetic acid) and pH (5.8 to 7.6) (Figure 14). VFAs constituted 56±12 % of the effluent SCOD.

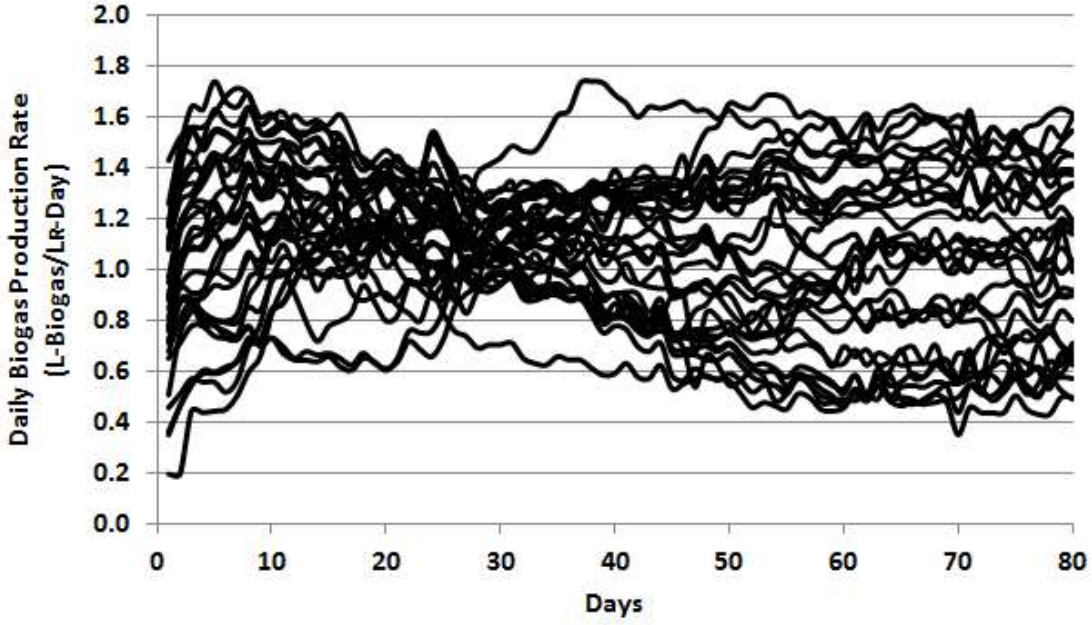

Figure 13: Digester average daily biogas production rates. A subset of 30 of the 149 digesters is presented for clarity.

Relationships between digester methane production rate and effluent parameters were as expected; methane production was inversely correlated to effluent SCOD concentration, effluent total VFA concentration and pH values (Figure 14). Acetic and propionic acids were the VFA observed at the highest concentration in most digester effluent, with acetic acid contributing 55±18% of the total VFA equivalents and propionic acid contributing 40±20% (Figure 15). Effluent acetic acid concentration also showed the strongest linear correlation of all the VFAs with methane production rate (Figure 15).

4.3.2 Microbial Community Analysis

A total of 4.1 million sequence reads from all 149 digesters were analyzed with 27,500 rarified sequence reads per sample. Based on 97% similarity, 1300 microbial OTUs were observed with an average of 158±27 OTUs in each digester. These results were similar to those of Vanwonterghem et al. (2014) who also operated digesters under controlled laboratory conditions and fed a consistent, synthetic feed for almost a year. They reported 90 to 200 OTUs in each digester using universal 16S rRNA primers and Roche 454 high throughput sequencing. However, the number of OTUs identified herein and by Vanwonterghem et al. (2014) was significantly lower than the 1200 to 3600 OTUs reported by Rivière et al. (2009) in full-scale anaerobic digesters using 16S rRNA gene clone libraries. It is possible that the lower number of OTUs resulted from the consistent and controlled feeding operation, leading to enrichment for microorganisms adapted to the relatively consistent conditions studied. At the same time, the number of OTUs observed across all digesters was an order-of-magnitude higher than the number observed in a single digester alone. The relatively high microbial diversity among all digesters was ostensibly a result of significant differences among the different seed cultures employed.

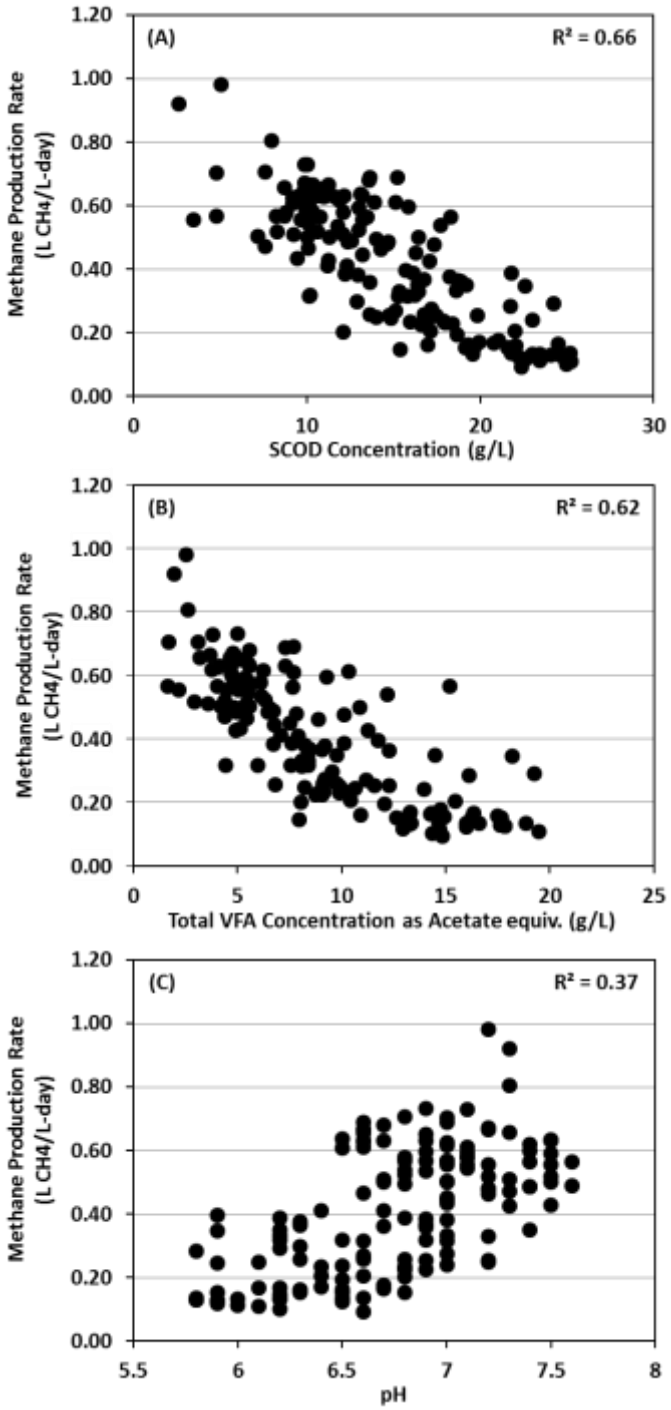

Figure 14: Average daily methane production versus effluent parameters. Average daily methane production (L-CH_4/L_R-day) versus (A) SCOD concentration (g/L), (B) total VFA concentration as acetic acid (g/L) and (C) pH.

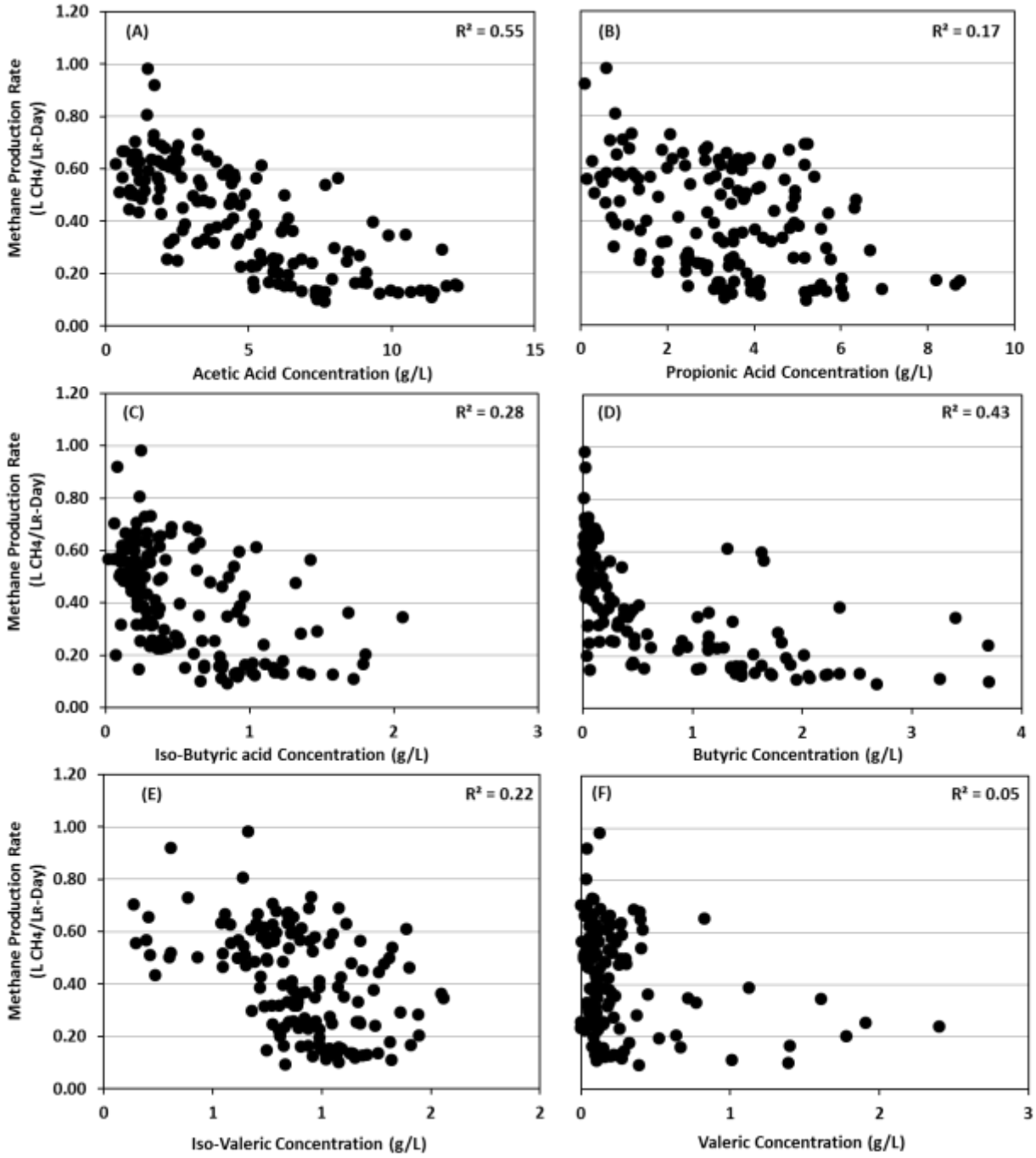

Figure 15: Average daily methane production versus individual VFA concentrations. Average daily methane production ($L\text{-}CH_4/L_R\text{-day}$) versus (A) acetic acid, (B) propionic acid, (C) iso-butyric acid, (D) butyric acid, (E) iso-valeric acid and (F) valeric acid concentration (g/L). Error bars are not included.

4.3.3 Archaeal Community

Archaeal sequence relative abundance ranged from <0.01% to 3%. The most dominant (highest average relative abundance) and prevalent (detected in the greatest number of digesters at >0.01% relative abundance) archaeal OTUs were most similar to *Methanosarcina* and *Methanobacterium*,. These two archaeal OTUs were detected in 67% and 81% of the digesters, respectively, and their combined relative abundance was 81±20 % of the total archaeal OTUs observed. *Methanosarcina* and *Methanobacterium* are aceticlastic (acetate utilizing) and hydrogenotrophic (hydrogen utilizing) methanogens, respectively (Liu and Whitman 2008).

Methanosarcina have a higher growth rate and lower affinity for acetate than the only other known aceticlastic methanogen (*Methanosaeta*), and typically outcompete *Methanosaeta* in digesters with high acetate concentration (>500 mg/L) such as the ones in this study (Hori et al. 2006; Westermann, Ahring, and Mah 1989; Conklin, Stensel, and Ferguson 2006). Since 99% of the digesters had an acetic acid concentration >500 mg/L, the presence of *Methanosarcina* as the dominant aceticlastic methanogen is reasonable.

Hydrogenotrophic methanogens including *Methanobacterium* are typically more tolerant than aceticlastic methaogens to stress conditions such as low pH and high VFA concentrations (Liu and Whitman 2008). *Methanobacterium* relative abundance was higher than that of *Methanosarcina* in 66% of the digesters (Figure 16) and the average pH of these digesters was 6.4±0.3. Therefore, the higher relative abundance of *Methanobacterium* in most digesters was probably due to the inhibition of

Methanosarcina by the low pH and high VFA concentration conditions that predominated.

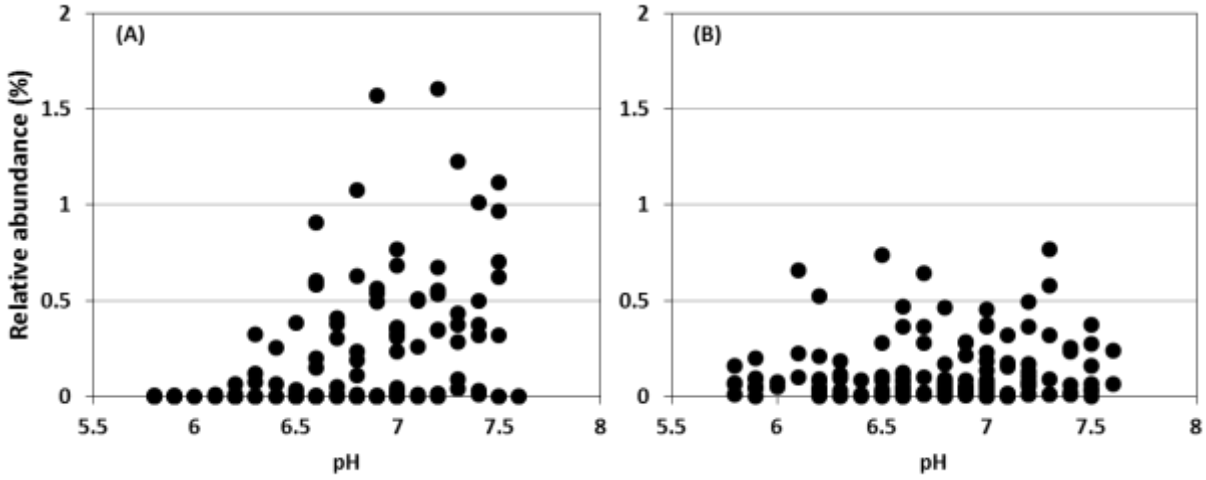

Figure 16: Percent relative abundance of dominant methanogens versus digester pH. Percent relative abundance of (A) *Methanosarcina* and (B) *Methanobacterium* versus digester pH.

4.3.4 Bacterial Community

Bacterial communities were dominated by the phyla *Bacteroidetes*, *Firmicutes* and *Synergistes*, contributing 59±17%, 22±17% and 9±5% of the total bacterial sequences, respectively. The combined relative abundance of these three phyla was 91±8% of the total bacterial sequences. Members of the phyla *Bacteroidetes* and *Firmicutes* are functionally diverse, and the phyla contains mostly known species of hydrolytic bacteria as well as acidogenic, fermentative bacteria (Noike et al. 1985; Mata-Alvarez, Macé, and Llabrés 2000; Vidal 2000; Ariunbaatar et al. 2014; Stiles and Holzapfel 1997).

The most abundant genera members of phyla *Bacteroidetes* observed across digesters were *Bacteroides*, *Petrimonas*, *Paludibacter*, *Porphyromonas*, *VadinBC27 wastewater sludge group*, *unclassified M2PB4-65 termite group* and *unclassified Prevotellaceae* (present in >95% digesters; combined abundance = 87±15% of total *Bacteroidetes*). Similarly for *Firmicutes*, unclassified *Family XI*, *Family XIII* and *Ruminococcaceae* were prevalent in >95% of the digesters, contributing 40±17% of the total *Firmicutes* sequences. *Synergistes* were dominated by the genus *Aminobacterium*. The synthetic wastewater carbon source was non-fat dry milk that contained 16% proteins by mass. *Aminobacterium* is a amino acid fermenting bacterium; thus its detection in systems fed protein is reasonable (Baena et al. 2000).

Digester methane production rates did not correlate with total biomass concentration (measured as VSS concentration), microbial richness, Shannon-Weaver diversity nor evenness indices (Figure 17). Although it is generally assumed that digesters with higher biomass VSS concentration achieve higher biogas production rates compared to similar digesters with lower biomass concentration, results indicate that having a higher VSS concentration cannot be universally considered to yield better performance.

Others have reported that microbial community descriptors such as diversity and evenness indices relate to anaerobic digester function (Fernandez et al. 2000; Hashsham et al. 2000; Carballa et al. 2011; Werner et al. 2011). Increased microbial diversity and evenness increase functional resistance and resilience when conditions are not steady and influent characteristics such as flow rate, organic strength, feedstock composition and temperature vary and cause perturbations. Higher diversity results in a higher probability

of functional redundancy and, thus, functional stability during and after perturbation. If the activity of one taxon within a functional group is reduced or lost due to system perturbation, then another taxon from the same functional group, but with higher resistance to the perturbation, may take its place (Fernandez et al. 2000; Hashsham et al. 2000; Carballa et al. 2011; Werner et al. 2011).

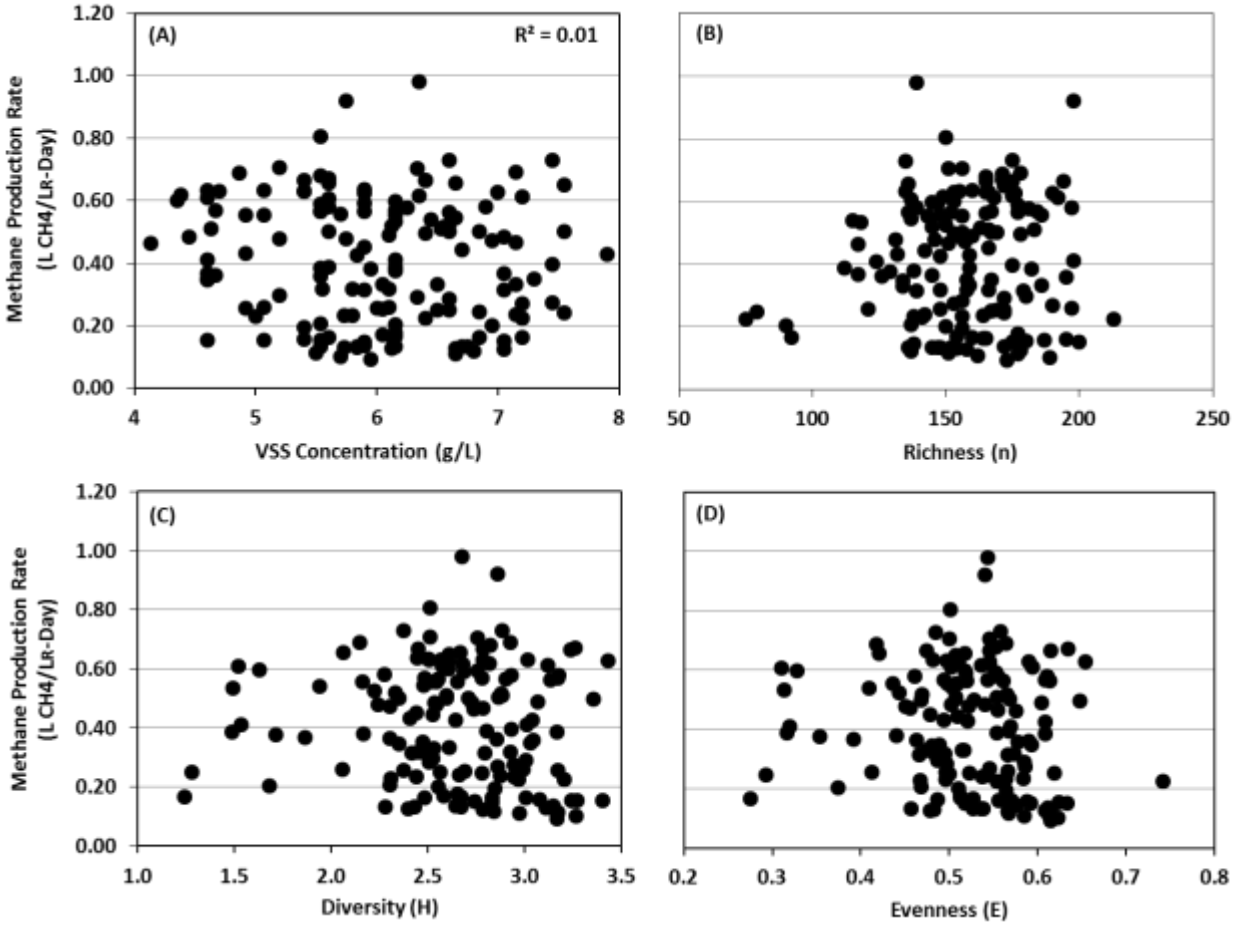

Figure 17: Average methane production (L-CH_4/L_R-day) versus microbial community descriptors. (A) VSS Concentration (B) Richness (C) Diversity (D) Evenness.

In contrast, when conditions are steady and influent characteristics do not vary, high diversity and high evenness are not critical for consistent function. For example, under the controlled bioreactor conditions of commercial bioethanol production, low diversity was beneficial; high abundance of *Saccharomyces cerevisiae* and low abundance of infecting yeast (De Souza Liberal et al. 2006) and bacteria, such as *Lactobacillus* (Skinner and Leathers 2004) resulted in higher ethanol production. If biological wastewater recovery processes become more controlled, then lower diversity may be beneficial in full scale processes.

Typically, it is assumed that higher overall biomass concentration, species richness, microbial diversity and evenness values in functioning engineered bioprocesses are correlated with increased methane production irrespective of the exact methanogenic microbial community composition. However, methane production rate did not correlate with these parameters under the conditions studied. But digester function did vary greatly among the digesters that were identically operated and that contained different microbial communities. Therefore, it was hypothesized that digester function was correlated to other descriptors of microbial community composition, such as OTU relative abundance values.

4.3.5 Initial Screening and QSAR

Initial screening of all OTUs using Spearman's rank analysis was performed to identify the 10 highly significant OTUs with relative abundance values that most significantly correlated to methane production rate (Table 2).

Digesters were sorted in the order of highest to lowest average methane production rate; the 50 digesters with the highest methane production rates were

classified as "high", while the 49 digesters having the lowest methane production rates were classified as "low" and the remaining 50 digesters were classified as "medium". The methane production rates in high, medium and low categories were 0.63±0.09, 0.41±0.08 and 0.18±0.05 L-CH_4/LR-day, respectively. Microbial communities associated with high methane production digesters were different from those associated with low (ANOSIM, p = 0.001, R^2 = 0.368) and medium (ANOSIM, p =0.002, R^2 = 0.072) methane production (Figure 18). Reducing the number of descriptors from 1300 to the 10 highly significant OTUs resulted in greater observable differences among microbial communities, increasing the observed community variation by PCOA axis-1 from 21.4 to 42.3% (Figure 18). In addition, using only the 10 highly significant OTUs resulted in greater observable variation between high and medium (ANOSIM, p = 0.001, R^2 = 0.138) as well as high and low (ANOSIM, p= 0.001, R^2 =0.493) digester groups (Figure 18).

Table 2: Highly significant OTUs determined by initial screening. 50 iterations of spearman's rank analysis were performed, where 75 out of 149 digesters where randomly selected and correlated with digester methane production rate. The value column "*N*" represents the number of times the OTU was observed as the top ten positively or negatively correlated out of the total 50 iterations. *N* = 50 represents 100% observation and *N* = 25 equals 50% OTUs and so on. The 7 OTUs in Table A and 3 OTUs in Table B were observed to be positively and negatively correlated to methane production rate in 38 out of the 50 iterations (>75% of the iterations). These 10 OTUs were selected for the subsequent MLR analysis.

(A) Relative abundance positively correlated to methane production				
Class	**Order**	**Family**	**Genus**	***N***
Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas	50
Bacteroidia	Bacteroidales	Marinilabiaceae	unclassified	50
Spirochaetes	Spirochaetales	PL-11B10	unclassified	50
Bacteroidia	Bacteroidales	M2PB4-65 termite group	unclassified	46
Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas	46
Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	46
Clostridia	Clostridiales	Gracilibacteraceae	Lutispora	40

(B) Relative abundance negatively correlated to methane production				
Class	**Order**	**Family**	**Genus**	**N**
Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	50
Clostridia	Clostridiales	Ruminococcaceae	unclassified	50
Clostridia	Clostridiales	Lachnospiraceae	unclassified	50

The statistical validity of any MLR correlation was tested by screening highly significant OTU relative abundance values for intercorrelation. As described by Nirmalakhandan and Speece (1988), "it is known that, in MLR analysis, intercorrelation between any two variables (collinearity) or among many variables (polycollinearity) can lead to false models." However, collinearity problems did not exist among the 10 highly significant OTU relative abundance values based on the intercorrelation matrix values (Table 3). For example, intercorrelation R^2 values were relatively low, averaging 0.07±0.07 (n=45) and ranging from 0.001 to 0.34. Therefore, relative abundance values of the 10 highly significant OTUs in each digester (Table 2) were used as descriptor variables for subsequent MLR analysis.

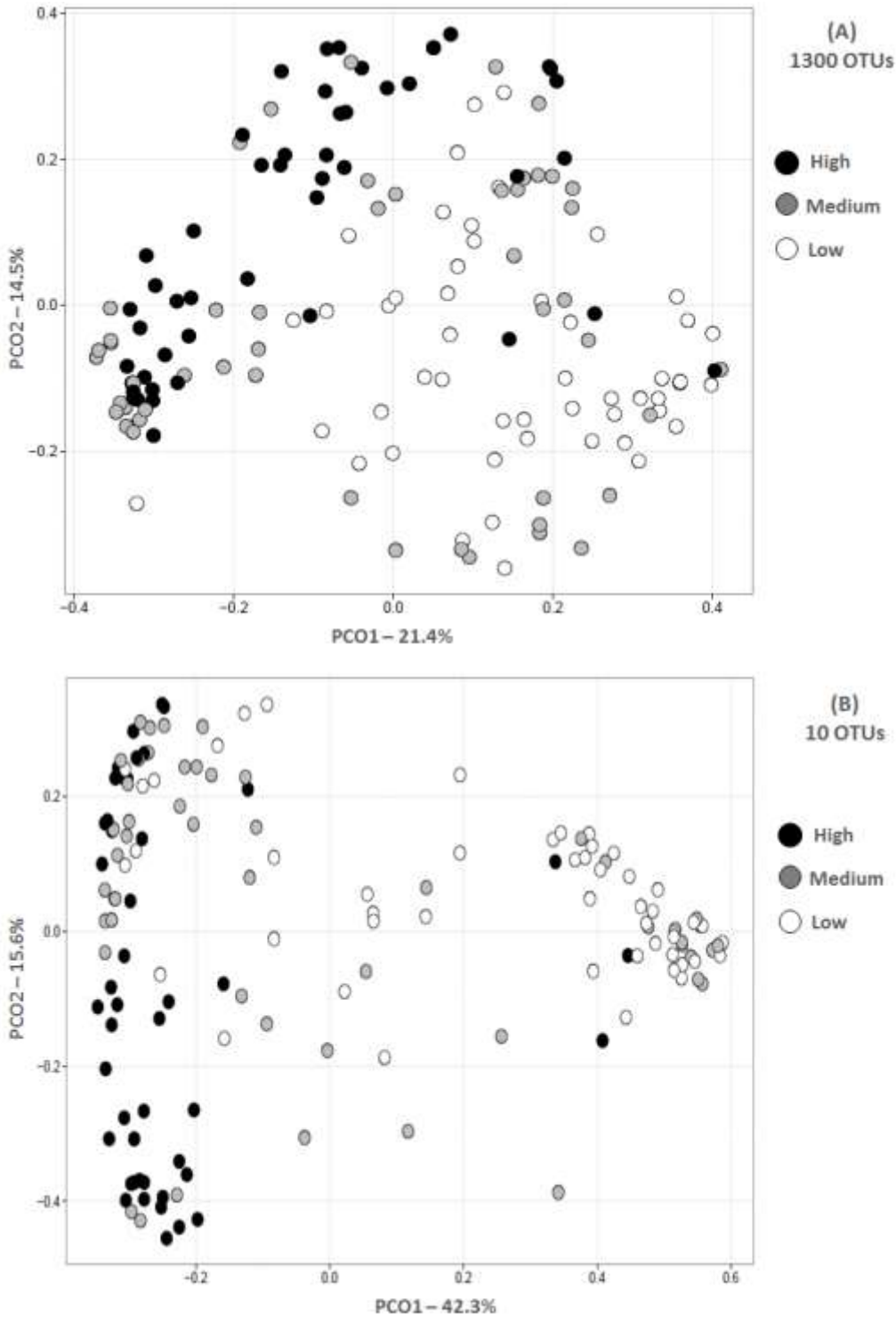

Figure 18: Microbial community principal component analysis (PCOA).PCOA plots using (A) all 1300 OTUs and (B) 10 highly significant OTUs. Methane production rate classifications are shown as high (black), medium (grey) and low (white) symbols.

Table 3: **R square values from the cross correlation of the 10 highly significant OTUs.**

	OTU1	OTU2	OTU3	OTU4	OTU5	OTU6	OTU7	OTU8	OTU9	OTU10
OTU1	**1**	0.19	0.06	0.096	0.087	0.23	0.02	0.18	0.077	0.093
OTU2	0.19	**1**	0.04	0.003	0.03	0.17	0.012	0.069	0.033	0.044
OTU3	0.06	0.04	**1**	0.022	0.059	0.17	0.038	0.078	0.042	0.045
OTU4	0.096	0.003	0.022	**1**	0.03	0.02	0.001	0.07	0.047	0.03
OTU5	0.087	0.03	0.059	0.03	**1**	0.17	0.34	0.044	0.03	0.05
OTU6	0.23	0.17	0.17	0.02	0.17	**1**	0.012	0.13	0.07	0.07
OTU7	0.02	0.012	0.038	0.001	0.34	0.012	**1**	0.013	0.007	0.0067
OTU8	0.18	0.069	0.078	0.07	0.044	0.13	0.013	**1**	0.047	0.003
OTU9	0.077	0.033	0.042	0.047	0.03	0.07	0.007	0.047	**1**	0.088
OTU10	0.093	0.044	0.045	0.03	0.05	0.07	0.0067	0.003	0.088	**1**

Ten MLR validation tests using the 10 highly significant OTUs were conducted by randomly dividing the 149 digesters into 10 groups (Table 4, Figure 19). All four criteria for good predictability were satisfied in nine of the 10 validation iterations, indicating that the MLR approach resulted in equations with good predictability (Table 5). Therefore, the final QSAR MLR equation was generated by combining data from all 149 digesters:

$$MPR = 0.4+2^{-04}*\boldsymbol{OTU1}+1.3^{-01}*\boldsymbol{OTU2}+2.6^{-01}*\boldsymbol{OTU3}+6.0^{-03}*\boldsymbol{OTU4}+4.5^{-04}*\boldsymbol{OTU5}$$

$$+2.1^{-01}*\boldsymbol{OTU6}+9.1^{-03}*\boldsymbol{OTU7}-1.5^{-03}*\boldsymbol{OTU8}-5.8^{-02}*\boldsymbol{OTU9}-2.5^{-01}\boldsymbol{OTU10}\ldots\ldots\text{Equation 3}$$

$$n= 149;\ R^2 = 0.66;\ SE = 0.12\ L\text{-}CH_4/L_R\text{-day}$$

where MPR is the methane production rate ($L\text{-}CH_4/L_R$-day), OTUn is the relative abundance for taxon n (%) and SE is the standard error. A plot of observed methane production rates versus rates predicted using Equation 1 for all 149 digesters is shown in Figure 20.

Table 4: Test and training groups for the 10 validation tests. Validation tests indicating (A) test and training groups employed and (B) identities of digesters employed for each iteration.

(A)		
Validation test number	**Test set group number**	**Training set group numbers**
1	10	1 to 9
2	9	1 to 8 & 10
3	8	1 to 7,9 & 10
4	7	1 to 6 & 8 to 10
5	6	1 to 5 & 7 to 10
6	5	1 to 4 & 6 to 10
7	4	1 to 3 & 5 to 10
8	3	1, 2 & 4 to 10
9	2	1 & 3 to 10
10	1	2 to 10

(B)									
Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**	**Group 10**
Digester ID									
AL3	ID2	AL1	CT1	AK2	AK1	CO2	AL2	NJ1	AR1
CT2	ID3	FL2	GA3	CT3	AK3	HI3	AR3	NY3	AR2
FL1	MD3	ID1	HI2	DE2	AZ2	IL3	IN3	OH2	AZ1
IL1	MO1	KS3	IA3	IN1	CA2	KY2	KS2	OH3	AZ3
IL2	MO2	KY1	KS1	IN2	CA3	LA3	KY3	OK1	CA1
MI3	NE2	ME2	LA1	LA2	CO1	MD1	MA1	OK3	DE1
NC2	NE3	MI2	MI1	MA3	CO3	MS3	ME1	OR1	DE3
NE1	NH1	MT3	MN2	MD2	GA1	NC1	MT1	SD2	FL3
OH1	OK2	NJ3	NC3	MN1	GA2	ND2	MT2	VA2	HI1
SC2	OR2	NY1	ND3	MO3	MA2	NV2	NM3	VA3	IA1
TX3	TNS1	SC3	NH3	MS1	MS2	NY2	PA2	VT2	IA2
UT3	TNS3	TNS2	NM2	NJ2	NM1	PA1	SD1	WA1	ME3
VT1	UT1	WI2	NV3	NV1	TX1	PA3	SD3	WI4	MN3
WV2	WA2	WI6	SC1	OR3	TX2	UT2	VA1	WV1	NH2
WY3	WI5	WY1	WY2	WI3	VT3	WA3	WI1	WV3	-

Table 5: **Summary table of the 10 validation tests with the results of the four validation criteria tested.**

Validation Test no.	q^2	R^2	$(R^2-R_0^2)/R^2$	K
1	0.65	0.68	0.04	1.0
2	0.69	0.83	0.02	0.86
3	0.68	0.68	0.0	1.03
4	0.52	0.62	0.05	1.11
5	0.58	0.67	0.01	0.90
6	0.22	0.35	0.26	1.09
7	0.57	0.68	0.01	1.01
8	0.64	0.74	0.09	0.93
9	0.54	0.65	0.09	1.09
10	0.65	0.66	0.0	0.97

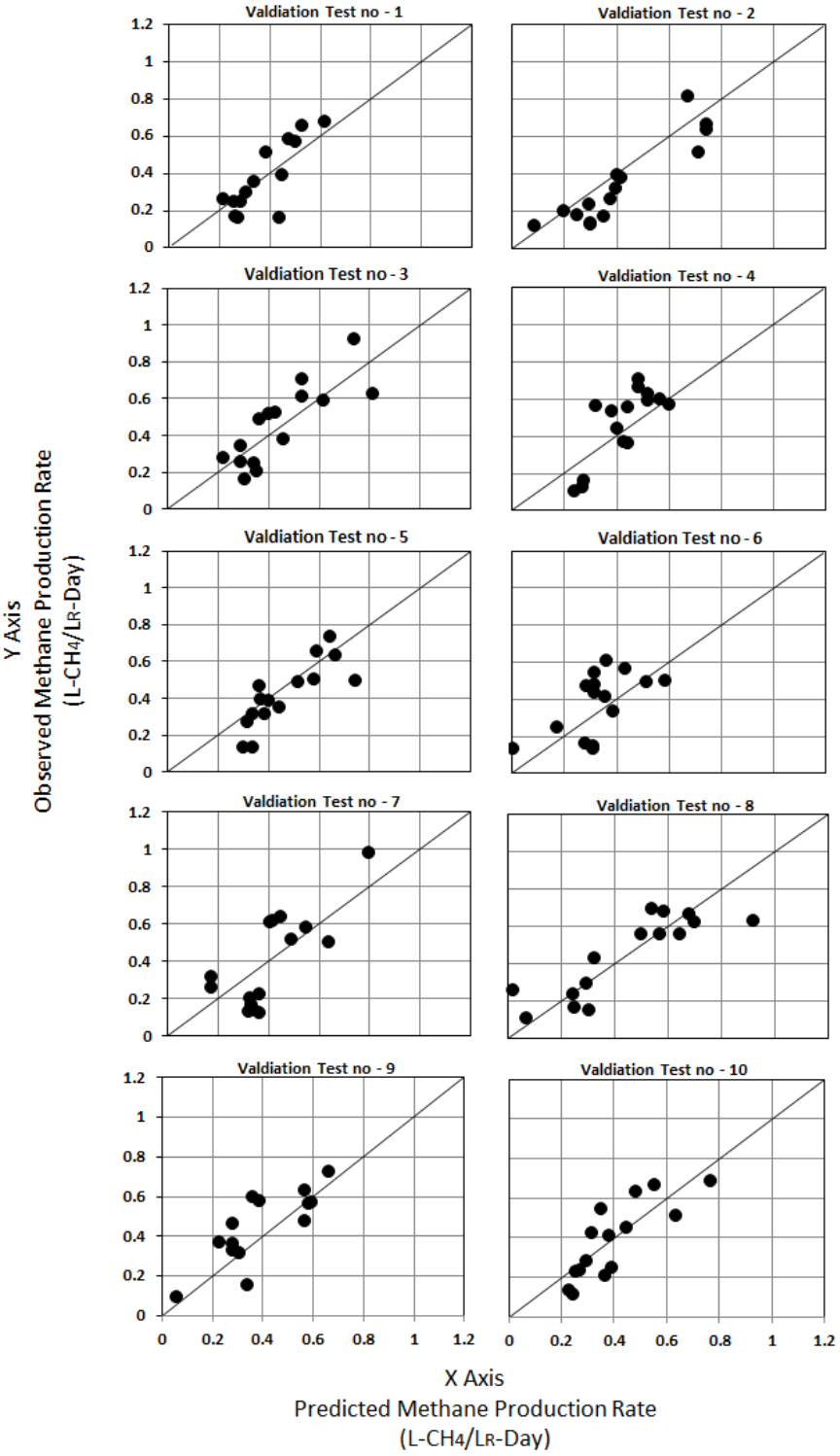

Figure 19: Summary plots of the 10 validation tests. Results of validation tests using the highly significant OTUs. The data points are for digesters in the test set for each validation test. Values of the validation criteria (q^2, R^2, R^2-R_0^2/R^2 and K) are shown in Table 5. The line in each plot represents the regression line with slope equal to one and intercept equal to zero.

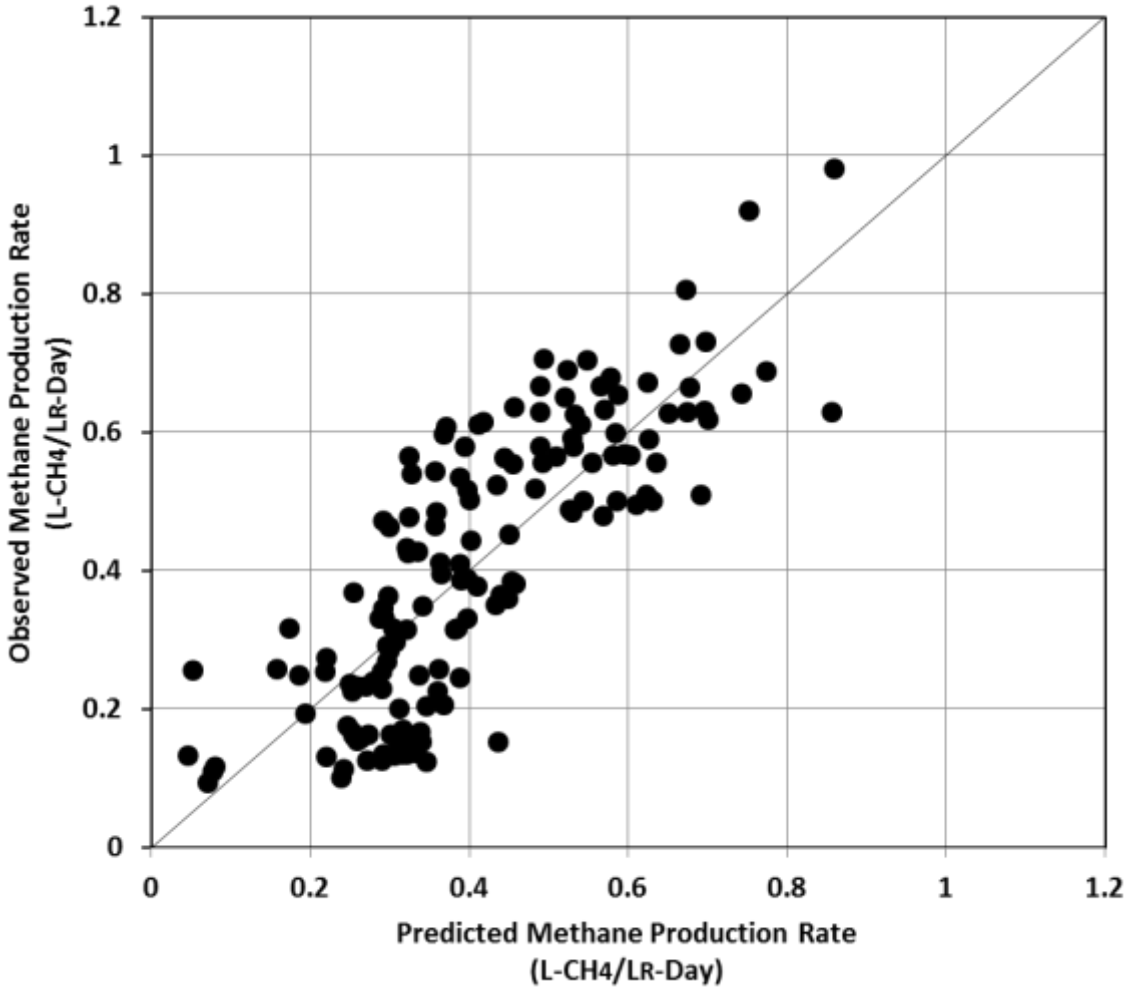

Figure 20: Observed versus predicted methane production rate. The predicted rate was calculated using Equation 3.

The prevalence and range of relative abundance values for the 10 highly significant OTUs varied across all the digesters. The OTU prevalence ranged from 52% (OTU7) to 100% (OTU1), whereas the average relative abundance across all the digesters ranged from 0.2% (OTU6) to 8.9% (OTU8) (Table 6). Also, the sum of all 10 OTU relative abundance values ranged from 0.2 to 68%.

Table 6: Highly significant OTU results.

OTU	Order	Prevalence (%)	Relative abundance range & (average) (%)	QSAR coefficient	Average contribution value (Absolute value (Coefficients*average relative abundance)*100
1	Bacteroidales	100	<0.01 to 36 (6.5)	2^{-04}	0.13
2	Bacteroidales	77	<0.01 to 1.3 (0.09)	1.3^{-01}	1.1
3	Spirochaetales	67	<0.01 to 0.7 (0.05)	2.6^{-01}	1.3
4	Bacteroidales	98	<0.01 to 54 (6.1)	6^{-03}	3.7
5	Clostridiales	97	<0.01 to 3.7 (0.3)	4.5^{-04}	0.013
6	Methanosarcinales	54	<0.01 to 2.6 (0.2)	2.1^{-01}	4.2
7	Clostridiales	52	<0.01 to 5.2 (0.08)	9.1^{-03}	0.072
8	Bacteroidales	99	<0.01 to 58 (8.9)	-1.5^{-03}	1.4
9	Clostridiales	97	<0.01 to 4.4 (0.36)	-5.8^{-02}	2.1
10	Clostridiales	78	<0.01 to 1.0 (0.08)	-2.5^{-01}	2.0

The coefficient values of the MLR equation could not be used directly as indicators of the relative contribution of independent variables since the OTU average relative abundance values were different. Therefore, an average contribution value was calculated for each OTU as the absolute value of the product of the MLR coefficient and the corresponding average relative abundance (Table 6). Based on average contribution values, OTU6 was the independent variable that most significantly contributed to the predicted methane production rate, followed by OTU4 and OTU9.

A blast search was conducted using the reference sequences of the 10 highly significant OTUs (Table 7). OTUs 6, 4 and 9 were most similar to *Methanosarcina*, unclassified *Bacteroidales* and unclassified *Intestinimonas sp.*, respectively. Therefore,

high *Methanosarcina* and unclassified *Bacteroidales* relative abundance and low relative abundance of unclassified *Intestinimonas sp.* are ostensibly beneficial when high methane production rate is desired under the conditions studied. In this way, QSAR models can be employed as research tools to identify desirable and undesirable taxa for further consideration. For example, bioaugmenting low methane producing digesters with taxa identified as beneficial by QSAR modeling may be promising, but more research is necessary to explore this approach.

Apart from a methanogen (*Methanosarcina*) and a syntrophic acetogen (*Syntrophomonas*), the 10 descriptors also include fermenters (acidogens) (Table 7). Also, the fermenters were both positively and negatively correlated with digester methane production rate. Of the fermenters identified at the genus level, members of *Petrimonas* and *Porphyromonas* are known to ferment sugars whereas *Lutispora* are amino acid fermenters (Grabowski et al. 2005; Shah & Collins 1988; Shiratori et al. 2008). The positive correlation of higher methane production with high relative abundance of OTU7 (most similar to *Lutispora*) is reasonable since the synthetic wastewater contained protein.

Table 7: BLAST search results for 10 highly significant OTUs.

OTU	Accession #	Name	Similarity %
1	AY570690	*Petrimonas sulfuriphila strain BN3*	97
2	KF282390	*Cytophagaceae bacterium GUDS1294*	89
3	NR_102767	*Syntrophothermus lipocalidus strain DSM 12680*	86
4	LC049960	*Bacteroidales bacterium TBC1*	86
5	NR_122058	*Syntrophomonas wolfei strain Goettingen G311*	97
6	CP008746	*Methanosarcina barkeri CM1*	99
7	NR_041236	*Lutispora thermophila*	95
8	FJ848568	*Porphyromonas sp. 2192 16S ribosomal RNA gene*	93
9	KP114242	*Intestinimonas sp. FSAA-17*	99
10	AB910747	*Clostridium scindens*	100

Digesters exhibiting high methane production also had higher pH values (Figure 14). Also, it is probable that different digester pH values selected for different fermenters. Studies have reported fluctuation in anaerobic digester bacterial populations in response to variations in environmental parameters including pH (Lü et al. 2009; Ogbonna, Berebon, and Onwuegbu 2015). Digesters with near-neutral pH may have supported higher growth rates of *Petrimonas* and *Lutispora* that exhibited relative abundance values positively correlated with methane production (Table 7).

Representative species of these genra (i.e., *Petrimonas sulfuriphila* and *Lutispora thermophile*) show optimal growth rates at neutral pH (Grabowski et al. 2005; Shiratori et al. 2008). Relative abundance of *Ruminococcaceae*, which was negatively correlated with methane production, has been observed to increase in digesters undergoing perturbation and in low pH (Tian et al. 2014).

Given the many factors influencing microbial community, including wastewater composition, digester operation, environmental parameters (pH, temperature, salt, VFA concentration etc.) and optimal growth range of various archaeal and bacterial microorganisms, developing a more general, robust QSAR may require extensive research using a large number of environmental conditions. It is hoped that the results of this study encourage future research to develop more robust QSAR models. This would be a worthwhile endeavor to help improve modeling and performance of anaerobic digesters and other engineered bioprocesses.

4.4 Conclusions

The study investigated whether microbial community composition data can be used as descriptors in a quantitative structure activity relationship (QSAR) model to predict digester methane production rate. Although all digesters were operated identically for a minimum of 5 retention cycles, their quasi steady-state performance varied significantly. The microbial community was dominated by bacterial OTUs, with total relative abundance of archaeal sequences ranging from <0.01% to 3%. The most dominant and prevalent archaeal OTUs were *Methanosarcina* and *Methanobacterium*. The bacterial community was dominated by the phyla *Bacteroidetes*, *Firmicutes* and *Synergistes*.

No correlation was observed between methane production rate and the common biomass descriptors, digester biomass concentrations (VSS), microbial richness, Shannon Weaver diversity and evenness indices. However, the relative abundance values of 10 OTUs including one archaeal and nine bacterial taxa were correlated with digester methane production rate. Seven OTUs positively correlated and the remaining three negatively correlated to digester methane production rate. The relative abundance values of the 10 OTUs were used as descriptors to develop a MLR equation demonstrating good predictability of digester methane production rate. Apart from a methanogen (*Methanosarcina*) and a syntrophic acetogen (*Syntrophomonas*), the 10 descriptors also included fermenters (acidogens). To the author's knowledge, this is the first report of a quantitative, predictive correlation between digester methane production rate and microbial community descriptors.

A future research project is envisioned where multiple biomass samples from different sources would be used to seed operating digesters for a period of one to two years. This in combination with high-throughput microbiological methods and conventional approaches for kinetic study can pave the way towards developing more robust QSAR structure-activity models that can be incorporated within the existing AD models to improve their predictability.

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5. General Conclusions and Recommendations

Technical advances in microbial analysis technology have been used to discover remarkable capabilities and adaptability of microorganisms and their communities. These advances have also increased our understanding of the microbial communities that perform anaerobic digestion. This dissertation utilized high-throughput Illumina sequencing technology along with universal primers to correlate microbial community structure to digester function in two experimental projects.

The objective of the first project was to investigate a novel approach to improve quasi-steady digester function by bioaugmenting digesters with a methanogenic, aerotolerant propionate enrichment culture. Nine groups of anaerobic digesters were seeded with different starting biomass to obtain different microbial communities and operated identically. The results of the study showed that different anaerobic digester seed biomass result in significantly different methane production rate, SCOD removal, pH and effluent VFA concentration. Therefore, careful consideration should be taken to select seed biomass with high activity for digester startup or re-seeding.

Digesters with different quasi-steady state function exhibited different archaeal communities. Digesters with high methane production rate, when operating under challenging conditions (VFA >2 g/L and theoretical methane production less than 70%), showed a high *Methanosarcina* relative abundance. The presence of *Methanosarcina* is beneficial and can lead to a more rapid bioconversion of acetate to methane. Digesters with low methane production can be distinguished by high abundance of

Methanobacterium and *DHVEG 6* family organisms. Since *DHVEG6* microorganisms have been found in extreme environments, including deep-sea hydrothermal vents, their high abundance in anaerobic digesters may indicate past or current digester upset (i.e., high VFA concentration and low methane production).

Bioaugmentation of the digesters with a methanogenic, propionate degrading enrichment culture resulted in an increase in methane production; however only in digesters with neutral pH. The methane production increase after bioaugmentation correlated with increased relative abundance of *Methanosaeta*, *Methaospirillum,* unclassified *Spirochaeata* and *Thermovirga* that were in the bioaugment culture employed. The increase in the methane production rate was only temporary. Digesters with less than neutral pH did not show any change in methanogenic community structure or the methane production rate. This indicates that the environment into which an augment culture is added must be carefully considered as well as considering the composition of the bioaugment itself. Steps such as increasing low digester pH before bioaugmentation may be necessary to improve digester function. Also by increasing the dose concentration of the bioaugment, periodically repeating bioaugmentation, or employing a membrane bioreactor to retain the bioaugment, it may be possible to increase methane production for longer periods. Further research is necessary to develop and optimize both the bioaugment culture and bioaugmentation process for a sustained, steady state improvement in function for challenged digesters.

The second project in this dissertation investigated whether the relative abundance of microorganisms can be used as descriptors in a quantitative structure activity relationship (QSAR) model to predict digester methane production rate. Traditional

mathematical models that are used for designing anaerobic treatment plants, such as ADM1, do not include microbial diversity information input. Therefore in order to improve the predictability of current models, it is essential is to deepen our understanding of how microbial community structure relates to process function, such as methane generation rate.

To include a large data set and diversity of anaerobic microorganisms, 50 distinct biomass samples from 49 US states were used to seed triplicate lab-scale digesters and operated under similar controlled conditions. Although all digesters were operated identically for a minimum of 5 retention cycles, their quasi steady-state performance varied significantly.

Over 4.1 million sequence reads were obtained through high throughput Illumina Miseq sequencer and approximately 1300 OTUs were observed at genus level across all digesters; with each digester having 158±27 OTUs (mean ± standard deviation). The microbial community was dominated by bacterial OTUs, with total relative abundance of archaeal sequences ranging from <0.01% to 3%. The most dominant (based on relative abundance) and prevalent (number of the digesters in which a OTU was observed at >0.01% relative abundance) archaeal OTUs were *Methanosarcina* and *Methanobacterium*. The bacterial community was dominated by the phyla *Bacteroidetes*, *Firmicutes* and *Synergistes*.

Using Spearman's rank correlation analysis, 10 OTUs which included one archaeal and nine bacterial taxa were identified to have a strong correlation with digester methane production rate. Seven OTUs positively correlated and the remaining three negatively correlated to digester methane production rate. The descriptor OTUs with

relative abundance values that were positively correlated to methane production included *Petrimonas*, *Marinilabiaceae* (Family), *PL-11B10* (Family), *M2PB4 65 termite group* (Family), *Syntrophomonas*, *Methanosarcina* and *Lutispora*. The remaining three, *Porphyromonas*, *Ruminococcaceae* (Family) and *Lachnospiraceae* (Family) had relative abundance values that were negatively correlated with methane production.

The relative abundance values of the 10 OTUs were used as descriptors to develop a MLR equation that showed a statistically good prediction of the digester methane production rates. Apart from a methanogen (*Methanosarcina*) and a syntrophic acetogen (*Syntrophomonas*), the 10 descriptors also included fermenters (acidogens). To the author's knowledge, this is the only study that showed a quantitative correlation between digester methane production rate and both bacterial and archaeal descriptor OTUs. This is an important finding because it shows that factors such as presence, absence and relative abundance of individual OTUs do possess the information to discern and model digester activity.

Traditional mathematical models such as ADM1 currently include general microbial information. ADM1 requires input of 24 variables, of which seven relate to microbial function associated with seven trophic groups. The seven trophic groups correspond to the degradation of sugar, amino acids, long chain fatty acids, acetate, propionate, butyrate-valerate and hydrogen. One of the major reasons for not including extensive microbial parameters has been inadequate microbiological data, specifically community structure-function relationships and kinetic data. However, as researchers strive to provide deeper understanding using rapidly developing molecular

microbiological methods, it is prudent that information is continuously augmented and tested against the current models.

Based on the experience and the results obtained from this study, a future research project is envisioned where multiple biomass samples from different sources are used to seed operating digesters for a period of one to two years. Unlike the current study, this long term project would include periodic monitoring of digester microbial and functional parameters beginning at the start-up stage. This would relate the shift observed in the microbial community structure with the changes in the functional output within the same digester. Monitoring the lag time between the observed changes in digester community and functional activity can assist to answer microbiological question such as whether certain microorganisms (such as fermenters) are needed for a healthy functioning digester or if they start to proliferate due to environmental selection. Additional factors that are known to influence digester microbial community and their activity such as different wastewater composition, pH, temperature, digester configuration and OLR can also be included to incorporate the diversity in conditions observed in large-scale systems.

This comprehensive research plan, in combination with high-throughput microbiological methods and conventional approaches for kinetic study can pave the way towards developing more robust QSAR structure-activity models that can be incorporated within the existing AD models to improve their predictability.

6. Appendices

6.1. Supplementary Data

Table S1: Seed biomass source data

Digester ID	Waste treated at seed source	Digester configuration
Alaska (AK)	Municipal sludge	CSTR
Alabama (AL)	Petrochemical	UASB
Arkansas (AR)	Food waste	UASB
Arizona (AZ)	Municipal sludge	CSTR
California (CA)	Winery	CSTR
Colorado (CO)	Brewery	UASB
Connecticut (CT)	Municipal sludge	CSTR
Delaware (DE)	Municipal & industrial WW mix	CSTR
Florida (FL)	Municipal sludge	CSTR
Georgia (GA)	Municipal sludge	CSTR
Hawaii (HI)	Municipal sludge	CSTR
Iowa (IA)	Confections manufacture	CSTR
Idaho (ID)	Ethanol	UASB
Illinois (IL)	Food & beverage	CSTR
Indiana (IN)	Corn mill	CSTR
Kansas (KS)	Soda bottling	UASB
Kentucky (KY)	Cracker & cereal	CSTR
Louisiana (LA)	Food waste	CSTR
Massachusetts (MA)	Food waste	CSTR
Maryland (MD)	Yeast	CSTR
Maine (ME)	Municipal sludge & industrial WW mix	CSTR
Michigan (MI)	Municipal sludge & paper	CSTR
Minnesota (MN)	Paper	CSTR
Missouri (MO)	Food waste	CSTR
Mississippi (MS)	Municipal sludge	CSTR
Montana (MT)	Municipal sludge	CSTR
North Carolina (NC)	Municipal sludge	CSTR
North Dakota (ND)	Beet sugar & yeast	CSTR
Nebraska (NE)	Municipal sludge	CSTR
New Hampshire (NH)	Dairy	CSTR
New Jersey (NJ)	Food waste	CSTR

New Mexico (NM)	Dairy	CSTR
Nevada (NV)	Municipal sludge	CSTR
New York (NY)	Dairy	CSTR
Ohio (OH)	Municipal sludge	CSTR
Oklahoma (OK)	Soybean process waste	CSTR
Oregon (OR)	Municipal sludge	CSTR
Pennsylvania (PA)	Dairy	CSTR
South Carolina (SC)	Municipal sludge & fruit juice	CSTR
South Dakota (SD)	Municipal sludge	CSTR
Tennessee (TN)	Municipal sludge	CSTR
Texas (TX)	Cheese whey	AnMBR
Utah (UT)	Municipal sludge	CSTR
Virginia (VA)	Municipal sludge	CSTR
Vermont (VT)	Brewery	CSTR
Washington (WA)	Municipal sludge	CSTR
Wisconsin A (WI A)	Brewery	UASB
Wisconsin B (WI B)	Municipal sludge	CSTR
West Virginia (WV)	Municipal sludge	CSTR
Wyoming (WY)	Municipal sludge	CSTR

*CSTR – Completely stirred type reactor, UASB – Upflow anaerobic sludge blanket, AnMBR – Anaerobic membrane reactor

Table S2: Average and standard deviation values for the daily biogas production rate (L-Biogas/L_R-day), daily methane production rate (L-CH_4/L_R-day) and average pH and VSS concentration (g/L) for 149 digesters. The first two letter of the digester ID represents the two digit state code (AK = Alaska, WI = Wisconsin etc.) followed by the replicate number.

Sample ID	Biogas (L Biogas/LR-Day)		Methane (L CH4/LR-Day)		Effluent SCOD. (mg/L)		TVFA Conc. (mg/L)		VSS (g/L)	pH
	Avg	Stdev	Avg	Stdev	Avg	Stdev	Avg	Stdev		
AK1	1.02	0.06	0.32	0.02	16	1.2	8	0.5	6.1	6.9
AK2	1.01	0.10	0.33	0.03	16	0.5	8	1.0	6.5	7.2
AK3	1.16	0.08	0.39	0.03	22	0.7	10	0.7	6.1	6.8
AL1	1.01	0.06	0.43	0.02	11	0.4	5	0.1	7.9	7.5
AL2	1.12	0.06	0.49	0.03	15	0.5	6	0.3	7.1	7.4
AL3	1.18	0.06	0.54	0.03	10	1.0	5	0.1	6.6	7.1
AR1	0.72	0.08	0.24	0.03	23	0.9	14	0.6	7.6	7
AR2	0.73	0.08	0.24	0.03	18	1.0	10	0.4	7.2	6.5
AR3	0.81	0.08	0.27	0.03	17	1.6	9	0.7	7.5	7
AZ1	1.09	0.07	0.50	0.03	7	1.3	6	0.9	7.6	7
AZ2	0.97	0.10	0.40	0.04	16	1.1	12	0.9	7.5	5.9
AZ3	0.90	0.12	0.35	0.05	19	1.5	14	1.1	7.3	6.2
CA1	1.41	0.13	0.65	0.06	10	2.2	5	1.6	7.6	6.9
CA2	1.46	0.11	0.73	0.06	10	1.2	5	1.6	7.5	6.9
CA3	1.23	0.12	0.66	0.06	11	2.5	5	3.9	6.6	6.6
CO1	1.21	0.09	0.63	0.05	13	0.5	7	0.6	4.7	7.5
CO2	1.18	0.08	0.60	0.04	10	0.7	5	0.4	4.3	7.4
CO3	1.10	0.09	0.48	0.04	12	0.7	5	1.8	4.4	7.4
CT1	0.65	0.07	0.17	0.02	20	0.9	13	2.8	6.1	6.4
CT2	0.93	0.40	0.28	0.12	22	0.6	16	1.8	6.6	5.8
CT3	1.09	0.26	0.48	0.12	17	2.1	10	6.0	5.7	7.2
DE1	0.54	0.02	0.15	0.01	22	0.4	15	0.2	5.5	5.9
DE2	0.55	0.04	0.16	0.01	22	1.0	17	0.3	5.4	6.3
DE3	0.83	0.06	0.29	0.02	24	0.4	19	0.2	6.3	6.2
FL1	1.35	0.11	0.69	0.05	15	0.2	7	0.3	4.9	7
FL2	1.26	0.01	0.67	0.01	10	0.1	5	0.1	5.4	7.2
FL3	1.29	0.02	0.67	0.01	10	0.1	5	0.0	5.6	7.2
GA1	0.88	0.10	0.32	0.04	10	2.8	4	1.1	5.9	7
GA2	0.51	0.07	0.13	0.02	25	0.7	19	0.7	6.2	6.5
GA3	0.57	0.05	0.12	0.01	22	1.1	16	2.7	7.1	6.5
HI1	0.95	0.06	0.38	0.02	12	0.4	7	0.2	5.5	6.9
HI2	1.21	0.08	0.61	0.04	14	0.8	8	1.3	4.6	7.1
HI3	0.92	0.06	0.36	0.02	14	0.3	8	0.2	5.5	6.9

IA1	1.18	0.09	0.57	0.04	11	0.2	6	0.5	5.9	7.6
IA2	1.29	0.09	0.58	0.04	12	1.7	6	0.6	6.3	7.1
IA3	1.32	0.12	0.62	0.06	12	0.3	6	1.1	6.4	7
ID1	0.92	0.08	0.30	0.02	13	0.8	10	0.4	5.2	6.3
ID2	0.95	0.09	0.33	0.03	15	0.7	8	0.6	6.1	6.2
ID3	0.99	0.06	0.32	0.02	16	0.7	8	0.8	5.5	6.2
IL1	1.25	0.14	0.63	0.07	12	0.6	6	0.5	5.4	6.7
IL2	1.26	0.14	0.67	0.07	10	0.5	4	2.2	6.4	6.6
IL3	1.28	0.12	0.67	0.06	11	0.7	5	2.2	6.4	6.6
IN1	1.34	0.09	0.56	0.04	13	0.4	8	0.4	6.6	7.4
IN2	1.21	0.09	0.49	0.04	13	0.4	7	0.2	6.1	7.6
IN3	1.27	0.09	0.52	0.04	8	0.4	3	0.3	6.6	7.5
KS1	1.58	0.09	0.98	0.05	5	0.3	3	0.4	6.3	7.2
KS2	1.53	0.11	0.92	0.06	3	0.3	2	0.3	5.7	7.3
KS3	1.30	0.09	0.56	0.04	10	0.3	4	0.2	6.2	7.5
KY1	1.41	0.15	0.69	0.07	14	1.3	8	0.7	7.2	6.6
KY2	1.28	0.15	0.57	0.07	8	0.7	4	0.3	6.2	6.8
KY3	1.35	0.11	0.61	0.05	15	0.5	10	0.7	7.2	6.6
LA1	0.82	0.09	0.20	0.02	12	1.0	8	0.6	7.0	6.8
LA2	1.18	0.06	0.43	0.02	9	0.7	5	2.6	4.9	7
LA3	1.28	0.03	0.57	0.01	18	0.3	15	0.3	5.5	7
MA1	1.13	0.13	0.35	0.04	23	0.7	18	2.6	4.6	5.9
MA2	0.60	0.11	0.13	0.03	23	1.1	17	0.8	5.5	6
MA3	0.61	0.09	0.15	0.02	15	1.3	8	0.6	5.9	6.2
MD1	1.25	0.16	0.54	0.07	12	1.0	6	1.2	6.2	6.9
MD2	1.35	0.18	0.61	0.08	9	0.8	5	1.4	5.6	6.5
MD3	1.31	0.10	0.60	0.05	16	0.7	9	1.4	6.1	6.9
ME1	0.66	0.07	0.16	0.02	19	1.6	14	1.4	6.9	6.7
ME2	0.63	0.06	0.15	0.01	20	1.5	16	1.5	7.1	6.8
ME3	0.63	0.06	0.16	0.02	17	1.5	11	1.9	7.2	6.3
MI1	0.81	0.05	0.23	0.02	17	0.6	9	0.6	6.4	6.8
MI2	0.71	0.08	0.23	0.03	16	0.2	9	0.6	5.8	6.8
MI3	0.71	0.07	0.23	0.02	17	0.6	9	0.6	7.2	6.9
MN1	0.74	0.08	0.25	0.03	17	0.7	10	0.5	6.5	7.2
MN2	0.75	0.08	0.26	0.03	17	1.0	9	0.5	6.1	6.3
MN3	0.79	0.05	0.25	0.02	20	0.6	12	0.4	6.1	7.2
MO1	1.06	0.15	0.37	0.05	17	0.7	8	0.5	7.1	6.9
MO2	0.93	0.06	0.33	0.02	19	1.0	8	0.9	7.1	7
MO3	1.08	0.11	0.47	0.05	8	1.5	4	0.5	7.0	7.3
MS1	1.26	0.12	0.54	0.05	18	0.3	12	0.1	6.4	6.8
MS2	1.08	0.29	0.47	0.12	10	0.8	5	1.2	7.2	6.6

MS3	1.05	0.07	0.44	0.03	13	1.3	7	2.6	6.7	7
MT1	1.30	0.03	0.59	0.01	10	1.5	5	3.0	5.5	7.5
MT2	1.31	0.14	0.70	0.08	5	0.4	2	1.0	6.3	7
MT3	1.17	0.07	0.56	0.03	3	0.5	2	0.6	5.1	7.1
NC1	1.06	0.05	0.37	0.02	19	0.2	9	0.6	4.6	6.3
NC2	0.65	0.02	0.21	0.01	17	0.4	10	0.5	5.5	6.4
NC3	1.37	0.07	0.64	0.03	13	0.4	6	0.6	5.9	6.5
ND2	1.27	0.05	0.55	0.02	13	1.7	5	1.1	4.9	7
ND3	1.16	0.05	0.52	0.02	11	0.5	4	2.5	6.1	7.2
NE1	1.14	0.03	0.43	0.01	17	0.3	11	1.0	5.8	7.3
NE2	1.15	0.04	0.46	0.02	14	1.0	9	0.4	4.1	7.2
NE3	1.09	0.02	0.36	0.01	19	0.3	12	0.3	4.7	6.7
NH1	0.63	0.05	0.15	0.01	22	2.1	18	1.3	4.6	6.5
NH2	0.63	0.03	0.15	0.01	19	0.9	13	0.4	5.1	6.3
NH3	0.58	0.09	0.14	0.02	22	1.9	16	1.3	5.9	6.6
NJ1	0.50	0.04	0.12	0.01	23	0.8	13	1.4	6.8	5.9
NJ2	0.57	0.03	0.13	0.01	23	0.9	13	0.9	6.8	6.2
NJ3	0.45	0.05	0.11	0.01	25	1.1	19	1.3	6.7	6.1
NM1	1.11	0.15	0.50	0.07	14	0.5	7	0.2	6.4	6.8
NM2	1.29	0.08	0.58	0.03	9	0.7	6	0.2	6.9	6.8
NM3	1.33	0.12	0.63	0.05	11	0.9	4	0.4	7.0	6.6
NV1	0.49	0.06	0.13	0.02	22	0.5	15	1.2	6.7	5.8
NV2	0.47	0.03	0.13	0.01	25	0.5	18	0.1	6.7	5.9
NV3	0.57	0.02	0.13	0.01	20	1.3	13	0.5	5.8	6.2
NY1	0.64	0.08	0.17	0.02	24	0.6	16	1.5	6.2	6.1
NY2	0.69	0.10	0.16	0.02	19	1.0	13	1.4	5.6	6.5
NY3	0.72	0.05	0.18	0.01	21	0.5	15	1.5	6.1	6.7
OH1	1.15	0.09	0.41	0.03	12	0.7	8	0.5	6.2	6.4
OH2	1.06	0.13	0.39	0.05	16	1.0	8	1.8	5.6	6.2
OH3	1.11	0.13	0.38	0.04	18	1.0	9	1.3	6.1	6.3
OK1	1.35	0.08	0.66	0.04	9	0.7	3	1.4	5.6	7.3
OK2	1.38	0.11	0.73	0.06	10	1.1	4	1.4	6.6	7.1
OK3	1.40	0.09	0.81	0.05	8	0.9	3	0.9	5.5	7.3
OR1	0.82	0.06	0.26	0.02	14	0.4	10	0.5	5.1	6.8
OR2	1.24	0.08	0.58	0.04	10	0.4	6	0.2	5.6	7.1
OR3	1.06	0.07	0.41	0.03	11	0.6	7	0.3	4.6	6.7
PA1	1.28	0.16	0.63	0.08	9	1.2	5	0.6	5.9	7
PA2	1.19	0.07	0.52	0.03	13	1.2	6	0.9	6.6	6.8
PA3	1.34	0.18	0.71	0.10	8	0.9	3	1.7	5.2	6.8
SC1	1.16	0.20	0.50	0.09	16	1.3	11	1.3	6.6	6.7
SC2	1.17	0.05	0.51	0.02	9	0.6	4	1.6	6.5	6.7

SC3	1.32	0.12	0.68	0.06	14	0.6	6	0.7	5.5	6.7
SD1	0.71	0.09	0.20	0.03	22	0.5	15	0.9	6.2	6.6
SD2	0.62	0.08	0.17	0.02	21	0.3	14	1.0	6.1	6.2
SD3	0.81	0.06	0.25	0.02	14	0.8	8	1.1	6.6	6.1
TNS1	1.14	0.12	0.48	0.05	15	0.5	8	0.3	5.2	7.2
TNS2	1.28	0.24	0.63	0.12	11	2.9	4	1.0	5.4	6.9
TNS3	1.28	0.08	0.63	0.04	10	1.3	5	0.3	5.1	7.5
TX1	1.16	0.09	0.50	0.04	11	1.0	5	2.7	5.6	7.5
TX2	0.98	0.03	0.35	0.01	16	0.7	10	0.3	4.6	7.4
TX3	1.11	0.06	0.45	0.02	16	0.5	7	0.3	5.9	7
UT1	0.40	0.02	0.09	0.00	22	0.6	15	0.5	6.0	6.6
UT2	0.43	0.04	0.10	0.01	25	1.2	14	1.2	5.7	6.2
UT3	0.46	0.02	0.11	0.01	23	0.9	15	0.4	5.5	6
VA1	0.82	0.05	0.25	0.01	17	1.5	12	0.8	4.9	6.9
VA2	0.50	0.06	0.13	0.01	25	1.3	18	0.6	6.1	5.9
VA3	0.51	0.04	0.13	0.01	24	0.7	18	0.6	5.8	5.8
VT1	0.80	0.09	0.25	0.03	15	0.4	11	0.9	6.9	5.9
VT2	0.92	0.13	0.31	0.05	15	0.7	8	1.4	7.1	6.6
VT3	0.85	0.08	0.27	0.03	15	1.1	11	0.9	7.2	6.6
WA1	1.28	0.17	0.63	0.08	12	1.5	5	5.4	4.6	6.6
WA2	1.23	0.18	0.57	0.08	5	1.6	2	3.8	5.5	7
WA3	1.25	0.12	0.59	0.06	13	1.2	5	3.9	5.9	7.1
WIA1	1.02	0.06	0.38	0.02	13	0.5	8	0.1	6.0	7
WIA2	1.27	0.09	0.56	0.04	10	0.3	5	0.2	5.7	7.2
WIA3	1.11	0.08	0.50	0.04	10	0.3	4	0.7	6.9	7
WIB1	1.17	0.04	0.51	0.02	12	0.9	5	1.6	4.6	7.3
WIB2	1.23	0.03	0.57	0.01	9	0.6	5	0.8	4.7	6.9
WIB3	1.27	0.08	0.62	0.04	10	0.8	4	3.8	4.4	7.4
WV1	0.77	0.06	0.23	0.02	18	1.5	10	0.7	5.0	6.8
WV2	0.77	0.06	0.23	0.02	18	0.9	9	0.8	5.7	6.4
WV3	0.69	0.04	0.19	0.01	19	0.8	12	0.6	5.4	6.5
WY1	0.82	0.02	0.26	0.01	15	0.8	7	1.7	6.0	6.6
WY2	0.93	0.13	0.32	0.04	10	1.2	6	0.7	5.8	6.5
WY3	0.52	0.03	0.13	0.01	23	0.9	13	0.4	5.7	5.8

Table S3: Average and standard deviation values for the acetic, propionic, iso-butyric, butyric , iso-valeric and valeric acid concentration (g/L) for 149 digesters. The first two letters of the digester ID represents the two digit state code (AK = Alaska, WI = Wisconsin, etc.) followed by the replicate number.

Sample ID	Acetic (mg/L)		Propionic (mg/L)		Iso-Butyric (mg/L)		Butyric (mg/L)		Iso-Valeric (mg/L)		Valeric (mg/L)	
	Avg	Std dev	Avg	Std dev	Avg	Std. dev	Avg	Std. dev	Avg	Std. dev	Avg	Std. dev
AK1	4.6	0.3	3.5	0.5	0.3	0.0	0.32	0.03	0.89	0.08	0.04	0.02
AK2	3.5	0.5	4.7	0.4	0.3	0.0	0.18	0.04	0.89	0.09	0.19	0.02
AK3	4.2	0.3	3.1	0.1	0.9	0.0	2.34	1.35	0.99	0.05	1.12	0.01
AL1	1.9	0.0	2.9	0.1	0.2	0.0	0.03	0.02	0.72	0.01	0.12	0.01
AL2	1.8	0.1	4.9	0.2	0.2	0.0	0.16	0.03	0.69	0.02	0.05	0.01
AL3	4.4	0.1	0.5	0.2	0.3	0.0	0.17	0.01	0.64	0.03	0.04	0.01
AR1	7.2	0.3	1.8	0.4	1.1	0.0	3.70	0.19	1.25	0.06	2.40	0.01
AR2	6.6	0.3	2.7	0.2	0.3	0.0	0.95	0.23	0.93	0.05	0.00	0.01
AR3	5.4	0.4	2.5	0.1	0.5	0.0	1.15	0.38	1.04	0.07	0.22	0.01
AZ1	4.9	0.9	0.3	0.1	0.1	0.1	0.10	0.04	0.43	0.10	0.08	0.01
AZ2	9.3	0.7	1.5	0.4	0.5	0.1	0.51	0.16	0.82	0.07	0.10	0.01
AZ3	10.5	0.9	2.7	0.5	0.8	0.3	1.05	0.42	0.97	0.08	0.11	0.01
CA1	3.6	1.2	0.8	0.4	0.4	0.3	0.14	0.43	0.86	0.08	0.40	0.09
CA2	3.2	1.1	1.2	0.3	0.3	0.0	0.05	0.09	0.95	0.06	0.07	0.06
CA3	1.1	1.0	3.4	0.3	0.4	0.1	0.03	0.03	0.87	0.08	0.83	0.19
CO1	2.6	0.2	4.4	0.3	0.6	0.1	0.07	0.01	1.11	0.06	0.20	0.01
CO2	2.4	0.2	2.0	1.1	0.2	0.0	0.03	0.00	0.89	0.05	0.11	0.01
CO3	1.3	0.5	3.8	0.2	0.1	0.0	0.02	0.01	0.82	0.05	0.17	0.05
CT1	5.2	1.1	8.2	0.7	1.0	0.1	0.46	0.13	0.99	0.09	0.10	0.02
CT2	8.5	1.0	6.7	0.6	1.4	0.1	0.58	0.12	1.44	0.11	0.37	0.02
CT3	3.2	2.0	6.3	0.7	1.3	0.4	0.11	0.09	1.28	0.15	0.25	0.12
DE1	6.3	0.1	8.6	0.2	1.2	0.0	0.56	0.15	1.03	0.02	0.10	0.01
DE2	12.2	0.2	3.9	0.1	0.8	0.0	1.35	0.13	1.08	0.03	0.15	0.01
DE3	11.7	0.1	5.7	0.2	1.5	0.0	1.78	0.20	1.36	0.01	0.10	0.02
FL1	1.9	0.1	5.2	0.2	0.5	0.0	0.11	0.01	1.08	0.01	0.35	0.01
FL2	2.5	0.0	1.9	0.1	0.3	0.0	0.14	0.01	0.84	0.06	0.19	0.01
FL3	3.2	0.0	1.1	0.1	0.2	0.0	0.04	0.01	0.85	0.13	0.08	0.01
GA1	2.2	0.6	1.9	0.9	0.2	0.2	0.04	0.01	0.87	0.11	0.10	0.02
GA2	10.0	0.4	6.9	0.2	1.2	0.2	2.52	0.01	1.26	0.05	0.27	0.01
GA3	9.6	1.7	5.2	0.2	1.0	0.0	1.44	0.17	0.96	0.03	0.16	0.01
HI1	5.3	0.2	0.8	0.1	0.2	0.0	0.23	0.01	0.72	0.03	0.17	0.01
HI2	2.2	0.4	5.2	0.1	0.6	0.0	0.06	0.01	1.39	0.04	0.17	0.01
HI3	6.2	0.2	1.4	0.1	0.4	0.0	0.38	0.01	0.88	0.04	0.23	0.01
IA1	4.5	0.4	0.5	0.2	0.2	0.0	0.10	0.04	0.78	0.03	0.10	0.01

IA2	4.2	0.5	1.2	0.1	0.3	0.0	0.11	0.03	0.97	0.01	0.20	0.01
IA3	2.0	0.4	4.3	0.2	0.4	0.0	0.04	0.12	0.74	0.02	0.12	0.02
ID1	8.0	0.3	0.8	0.2	0.4	0.0	0.40	0.01	0.68	0.05	0.05	0.01
ID2	4.7	0.4	3.2	0.1	0.3	0.1	0.32	0.01	0.84	0.04	0.04	0.01
ID3	3.2	0.4	4.4	0.1	0.3	0.0	0.15	0.01	0.80	0.05	0.18	0.01
IL1	1.8	0.2	3.8	0.1	0.2	0.0	0.02	0.07	0.85	0.03	0.10	0.01
IL2	0.7	0.4	2.9	0.3	0.5	0.0	0.09	0.00	0.71	0.08	0.08	0.01
IL3	0.6	0.3	4.8	0.1	0.1	0.0	0.10	0.00	0.56	0.05	0.02	0.01
IN1	5.3	0.3	1.6	0.1	0.4	0.0	0.25	0.26	0.94	0.05	0.23	0.01
IN2	4.4	0.1	1.8	0.2	0.4	0.0	0.11	0.02	0.75	0.02	0.15	0.01
IN3	1.4	0.1	1.3	0.2	0.2	0.0	0.02	0.01	0.55	0.01	0.10	0.01
KS1	1.5	0.3	0.6	0.5	0.2	0.0	0.01	0.01	0.66	0.27	0.12	0.04
KS2	1.7	0.2	0.1	0.1	0.1	0.0	0.03	0.01	0.31	0.14	0.04	0.01
KS3	1.4	0.1	3.0	0.3	0.1	0.0	0.04	0.01	1.03	0.30	0.10	0.01
KY1	2.5	0.3	5.2	0.6	0.6	0.0	0.05	0.01	0.94	0.17	0.13	0.04
KY2	2.6	0.2	1.0	0.4	0.1	0.0	0.04	0.01	0.90	0.05	0.08	0.02
KY3	5.4	0.4	3.2	0.3	1.0	0.0	1.32	0.02	0.91	0.07	0.41	0.11
LA1	6.0	0.4	1.8	0.2	0.1	0.0	0.03	0.03	0.81	0.05	0.08	0.09
LA2	1.2	0.6	4.5	0.0	0.3	0.0	0.04	0.01	0.24	0.03	0.10	0.04
LA3	8.1	0.1	5.4	0.1	1.4	0.0	1.65	0.01	1.18	0.02	0.12	0.02
MA1	9.9	1.5	3.5	1.1	2.1	0.0	3.39	0.01	1.56	0.06	1.61	0.01
MA2	11.3	0.5	3.4	1.3	1.4	0.0	1.44	0.01	1.13	0.05	0.12	0.01
MA3	5.2	0.4	2.5	0.6	0.2	0.0	0.07	0.01	0.75	0.04	0.29	0.01
MD1	3.3	0.7	2.5	0.5	0.2	0.1	0.06	0.01	0.85	0.05	0.18	0.01
MD2	2.2	0.7	2.4	0.5	0.2	0.1	0.03	0.01	0.67	0.07	0.09	0.01
MD3	4.3	0.7	3.5	0.4	0.9	0.1	1.63	0.01	0.79	0.03	0.08	0.01
ME1	9.1	0.9	3.5	0.1	1.0	0.1	1.63	0.14	0.94	0.03	0.09	0.01
ME2	11.9	1.2	3.2	0.1	0.7	0.1	1.04	0.18	1.00	0.07	0.13	0.01
ME3	6.0	1.1	3.2	0.1	0.7	0.1	1.45	0.12	0.90	0.07	0.67	0.01
MI1	4.7	0.4	3.4	0.1	0.4	0.1	0.87	0.07	0.80	0.01	0.02	0.01
MI2	5.9	0.4	2.4	0.2	0.3	0.1	0.62	0.08	0.83	0.01	0.00	0.01
MI3	5.1	0.4	2.9	0.2	0.4	0.0	1.14	0.10	0.99	0.02	0.11	0.01
MN1	5.5	0.3	3.4	0.2	0.4	0.0	1.14	0.30	1.05	0.06	0.06	0.01
MN2	5.9	0.3	2.4	0.1	0.3	0.1	0.90	0.30	0.87	0.06	0.00	0.09
MN3	6.9	0.3	2.5	0.2	0.7	0.1	1.81	0.36	1.16	0.05	1.91	0.09
MO1	3.6	0.2	4.8	0.2	0.3	0.0	0.19	0.22	0.92	0.05	0.21	0.08
MO2	2.4	0.3	4.2	0.1	1.0	0.0	1.36	0.30	1.17	0.08	0.77	0.05
MO3	3.6	0.4	0.9	0.2	0.2	0.2	0.07	0.21	0.65	0.06	0.04	0.09
MS1	7.7	0.1	3.4	0.1	0.9	0.2	0.36	0.18	1.32	0.37	0.40	0.01
MS2	4.3	1.1	0.6	0.1	0.2	0.1	0.21	0.23	0.55	0.11	0.05	0.12
MS3	0.8	0.3	6.3	0.3	0.2	0.0	0.03	0.69	1.26	0.13	0.11	0.05

MT1	1.1	0.7	3.7	0.2	0.1	0.1	0.03	0.03	1.05	0.06	0.10	0.05
MT2	1.0	0.6	0.7	0.1	0.1	0.0	0.02	0.06	0.14	0.03	0.00	0.01
MT3	1.8	0.6	0.1	0.0	0.1	0.0	0.02	0.05	0.15	0.01	0.04	0.03
NC1	2.7	0.2	5.5	0.1	0.9	0.1	1.15	0.01	0.91	0.02	0.10	0.01
NC2	5.8	0.3	2.9	0.2	0.6	0.1	1.56	0.01	0.81	0.03	0.63	0.01
NC3	1.6	0.2	3.9	0.2	0.2	0.0	0.04	0.01	0.85	0.02	0.27	0.01
ND2	1.2	0.2	4.7	0.2	0.2	0.1	0.04	0.01	0.58	0.05	0.04	0.01
ND3	0.8	0.5	4.1	0.3	0.2	0.0	0.01	0.01	0.31	0.10	0.02	0.01
NE1	5.2	0.5	5.7	0.0	1.0	0.0	0.24	0.01	1.09	0.06	0.18	0.01
NE2	4.7	0.2	3.5	0.3	0.8	0.0	0.06	0.01	1.40	0.03	0.13	0.02
NE3	6.5	0.2	4.0	0.4	1.7	0.0	0.43	0.01	1.55	0.05	0.45	0.03
NH1	12.3	1.0	4.1	0.4	0.8	0.5	1.35	0.03	1.04	0.09	0.13	0.09
NH2	6.5	0.2	5.5	0.2	0.6	0.5	1.07	0.02	1.09	0.08	0.09	0.03
NH3	11.0	0.9	3.1	0.7	1.2	0.3	1.57	0.47	1.02	0.07	0.11	0.30
NJ1	7.3	0.9	3.5	0.7	0.9	0.3	2.07	0.27	1.15	0.10	0.27	0.03
NJ2	7.5	0.5	3.2	0.6	0.9	0.3	2.34	0.22	1.15	0.09	0.22	0.01
NJ3	11.4	0.8	6.1	0.7	1.7	0.4	1.95	0.28	1.32	0.10	0.11	0.02
NM1	3.0	0.1	3.2	0.1	0.4	0.0	0.06	0.08	0.75	0.02	0.30	0.09
NM2	4.5	0.1	0.6	0.1	0.1	0.0	0.07	0.16	0.72	0.04	0.05	0.06
NM3	1.2	0.1	2.9	0.1	0.2	0.1	0.01	0.18	0.78	0.06	0.07	0.05
NV1	7.4	0.6	6.0	0.1	1.0	0.0	1.71	0.05	1.14	0.02	0.12	0.07
NV2	11.5	0.1	3.9	0.1	1.6	0.0	2.21	0.01	1.07	0.02	0.13	0.01
NV3	6.8	0.2	5.4	0.3	0.9	0.2	1.39	0.14	1.03	0.14	0.09	0.27
NY1	9.0	0.8	4.1	0.5	1.8	0.3	1.89	0.29	1.41	0.06	1.40	0.01
NY2	8.7	1.0	3.2	0.3	0.8	0.2	1.40	0.18	0.83	0.05	0.08	0.01
NY3	7.9	0.8	6.0	0.4	1.2	0.2	0.46	0.71	1.31	0.18	0.32	0.01
OH1	6.4	0.4	0.7	0.4	0.3	0.2	0.28	0.29	0.86	0.09	0.11	0.01
OH2	2.8	0.7	4.9	0.4	0.3	0.1	0.13	0.31	1.07	0.08	0.06	0.01
OH3	3.9	0.6	5.0	0.4	0.3	0.1	0.46	0.31	1.24	0.30	0.11	0.01
OK1	1.0	0.4	2.4	0.1	0.2	0.0	0.01	0.09	0.20	0.03	0.17	0.03
OK2	1.7	0.7	2.1	0.1	0.3	0.0	0.02	0.10	0.38	0.04	0.08	0.03
OK3	1.4	0.5	0.8	0.1	0.2	0.0	0.01	0.11	0.64	0.06	0.04	0.06
OR1	5.9	0.3	3.5	0.2	0.4	0.0	0.26	0.01	0.97	0.02	0.15	0.05
OR2	4.1	0.2	0.9	0.2	0.3	0.0	0.10	0.01	0.75	0.05	0.08	0.01
OR3	4.5	0.2	2.3	0.1	0.2	0.0	0.09	0.01	0.99	0.06	0.10	0.01
PA1	3.9	0.6	0.3	0.1	0.2	0.1	0.08	0.06	0.58	0.01	0.05	0.01
PA2	1.9	0.3	4.1	0.1	0.6	0.0	0.03	0.01	0.96	0.04	0.21	0.01
PA3	1.7	1.0	1.0	0.2	0.2	0.0	0.05	0.03	0.77	0.05	0.04	0.01
SC1	6.3	0.8	3.7	0.2	0.9	0.1	0.17	0.18	1.31	0.06	0.30	0.55
SC2	0.5	0.2	3.6	0.2	0.1	0.0	0.00	0.01	0.21	0.30	0.02	0.04
SC3	2.1	0.3	2.9	0.2	0.6	0.1	0.12	0.01	0.79	0.08	0.39	0.09

SD1	9.1	0.6	2.4	0.1	1.8	0.1	2.02	0.07	1.45	0.09	1.78	0.01
SD2	5.7	0.4	8.7	0.1	1.1	0.0	0.45	0.02	0.98	0.07	0.10	0.01
SD3	2.5	0.4	5.8	0.1	0.5	0.0	0.05	0.01	1.18	0.07	0.15	0.01
TNS1	3.4	0.1	3.8	0.2	0.7	0.0	0.10	0.03	1.13	0.04	0.30	0.09
TNS2	0.9	0.2	3.2	0.0	0.3	0.0	0.01	0.10	0.70	0.02	0.09	0.08
TNS3	2.3	0.2	2.1	0.1	0.2	0.0	0.02	0.20	0.54	0.04	0.09	0.09
TX1	1.0	0.6	3.7	0.2	0.3	0.0	0.18	0.02	0.61	0.05	0.27	0.04
TX2	5.1	0.2	3.7	0.2	0.6	0.0	0.41	0.01	1.10	0.05	0.72	0.02
TX3	2.7	0.1	4.9	0.3	0.2	0.0	0.06	0.01	1.19	0.04	0.09	0.04
UT1	7.6	0.3	5.2	0.2	0.8	0.1	2.68	0.01	0.83	0.04	0.39	0.01
UT2	7.4	0.6	3.3	0.1	0.7	0.0	3.70	0.16	1.08	0.05	1.39	0.10
UT3	7.5	0.2	4.1	0.1	0.8	0.0	3.25	0.02	1.02	0.04	1.01	0.04
VA1	6.0	0.4	5.2	1.5	0.8	0.0	0.29	0.00	1.17	0.07	0.15	0.01
VA2	10.2	0.4	5.7	1.3	1.4	0.0	2.06	0.04	1.19	0.06	0.20	0.01
VA3	10.7	0.4	5.3	0.4	1.2	0.0	1.73	0.00	1.05	0.10	0.16	0.02
VT1	8.4	0.8	1.4	0.4	0.5	0.1	0.47	0.15	0.77	0.05	0.08	0.02
VT2	4.6	0.8	3.3	0.4	0.3	0.2	0.33	0.33	0.74	0.07	0.06	0.03
VT3	8.9	0.8	1.4	0.4	0.5	0.2	0.47	0.21	0.92	0.07	0.08	0.03
WA1	1.0	1.2	3.6	0.4	0.2	0.1	0.02	0.21	0.84	0.16	0.26	0.02
WA2	0.6	1.4	1.3	0.3	0.0	0.1	0.00	0.20	0.19	0.06	0.01	0.01
WA3	1.5	1.1	3.7	0.5	0.3	0.1	0.04	0.22	0.86	0.22	0.27	0.02
WIA1	6.2	0.1	1.1	0.2	0.4	0.0	0.38	0.00	0.87	0.06	0.19	0.21
WIA2	3.3	0.2	1.3	0.4	0.3	0.0	0.03	0.01	0.75	0.08	0.09	0.05
WIA3	0.9	0.2	3.8	0.2	0.2	0.0	0.00	0.01	0.30	0.05	0.02	0.02
WIB1	0.9	0.3	4.9	0.1	0.2	0.0	0.00	0.01	0.65	0.03	0.03	0.04
WIB2	1.8	0.3	3.1	0.1	0.1	0.0	0.03	0.02	0.62	0.07	0.10	0.01
WIB3	0.4	0.4	3.5	0.1	0.1	0.0	0.02	0.01	0.72	0.06	0.18	0.01
WV1	5.3	0.4	3.7	0.0	0.4	0.1	1.23	0.18	0.94	0.02	0.11	0.05
WV2	5.1	0.5	2.8	0.1	0.4	0.1	1.28	0.19	0.89	0.02	0.26	0.08
WV3	6.4	0.4	3.8	0.0	0.8	0.1	1.85	0.28	0.99	0.03	0.52	0.07
WY1	2.1	0.6	4.9	0.1	0.2	0.0	0.15	0.07	0.85	0.02	0.03	0.01
WY2	3.8	0.5	2.0	0.1	0.1	0.2	0.05	0.04	0.77	0.02	0.09	0.04
WY3	7.7	0.3	3.4	0.3	0.9	0.0	2.23	0.23	1.21	0.02	0.21	0.04

Table S4: Relative abundance, in fraction of the total sequences (no. of OTU sequence/Total no. of sequences observed in a digester), of the 10 OTUs selected out the Spearman' rank analysis. Identity of OTU1 to OTU10 are shown in Figure 5C. The first two letter of the digester ID represents the two digit state code (AK = Alaska, WI = Wisconsin etc.) followed by the replicate number.

Digester ID	*OTU1*	*OTU2*	*OTU3*	*OTU4*	*OTU5*	*OTU6*	*OTU7*	*OTU8*	*OTU9*	*OTU10*
AK1	3E-02	0E+00	3E-04	3E-02	5E-04	0E+00	0E+00	7E-04	4E-05	7E-05
AK2	3E-02	1E-04	5E-04	4E-02	7E-04	0E+00	0E+00	1E-04	0E+00	1E-04
AK3	3E-02	0E+00	5E-04	4E-02	1E-03	7E-05	4E-04	2E-04	7E-05	1E-04
AL1	8E-03	5E-04	1E-04	4E-02	4E-04	0E+00	0E+00	1E-03	9E-04	2E-03
AL2	7E-03	4E-05	4E-05	3E-02	7E-04	3E-04	0E+00	7E-04	7E-04	1E-03
AL3	1E-02	5E-04	7E-05	3E-02	5E-04	1E-04	0E+00	9E-04	1E-03	1E-03
AR1	1E-02	0E+00	1E-04	4E-03	8E-04	0E+00	0E+00	1E-01	8E-03	1E-03
AR2	2E-02	0E+00	7E-05	4E-03	1E-03	0E+00	0E+00	1E-01	4E-03	3E-03
AR3	2E-02	2E-04	1E-04	8E-03	1E-03	0E+00	0E+00	1E-01	1E-02	2E-03
AZ1	2E-01	7E-05	5E-04	5E-02	6E-03	0E+00	4E-05	6E-02	3E-04	0E+00
AZ2	3E-01	7E-05	4E-04	6E-02	4E-03	4E-05	0E+00	9E-02	5E-03	1E-04
AZ3	1E-01	4E-05	1E-04	5E-02	3E-03	0E+00	7E-05	1E-01	5E-03	5E-04
CA1	7E-02	8E-04	1E-03	3E-02	8E-04	5E-03	9E-04	5E-04	1E-03	2E-04
CA2	1E-01	1E-02	2E-04	1E-01	4E-04	6E-03	4E-04	1E-03	1E-03	1E-04
CA3	6E-02	7E-04	1E-03	2E-02	7E-04	9E-03	5E-04	9E-04	1E-03	3E-04
CO1	7E-02	3E-03	1E-03	1E-02	3E-04	1E-02	5E-04	6E-04	1E-03	1E-04
CO2	8E-02	2E-04	2E-04	5E-03	6E-04	1E-02	4E-04	3E-04	6E-04	0E+00
CO3	7E-02	4E-03	1E-04	1E-02	9E-04	5E-03	5E-04	4E-03	5E-04	0E+00
CT1	2E-02	7E-05	1E-04	5E-03	2E-03	0E+00	0E+00	3E-01	1E-03	7E-05
CT2	9E-03	3E-04	0E+00	2E-03	2E-03	0E+00	0E+00	3E-01	3E-03	2E-04
CT3	1E-02	1E-04	0E+00	2E-03	1E-03	0E+00	0E+00	2E-01	9E-04	4E-05
DE1	2E-02	0E+00	4E-05	7E-02	5E-04	0E+00	0E+00	2E-02	3E-03	5E-03
DE2	4E-02	0E+00	4E-05	1E-01	3E-04	0E+00	0E+00	2E-02	3E-03	6E-03
DE3	4E-02	0E+00	0E+00	4E-02	4E-05	0E+00	0E+00	9E-02	2E-03	3E-03
FL1	1E-01	2E-04	3E-04	5E-01	3E-03	4E-03	2E-04	3E-03	1E-04	4E-05
FL2	1E-01	9E-04	3E-03	2E-01	5E-03	3E-03	2E-04	1E-02	1E-04	1E-04
FL3	2E-01	1E-03	1E-03	2E-01	8E-03	5E-03	2E-04	3E-02	0E+00	3E-04
GA1	1E-02	1E-04	0E+00	1E-02	2E-03	2E-04	0E+00	3E-01	2E-03	4E-05
GA2	8E-03	0E+00	0E+00	2E-02	2E-03	3E-04	0E+00	4E-01	4E-04	7E-05
GA3	8E-03	1E-03	0E+00	2E-02	2E-03	1E-04	0E+00	3E-01	7E-05	0E+00
HI1	2E-02	6E-04	7E-04	1E-01	9E-03	0E+00	0E+00	8E-02	3E-04	2E-04
HI2	9E-03	3E-04	2E-03	6E-02	7E-03	0E+00	4E-05	2E-01	4E-04	7E-05
HI3	2E-02	8E-04	5E-04	1E-01	3E-03	0E+00	0E+00	3E-02	1E-03	3E-04
IA1	1E-01	9E-04	0E+00	2E-01	6E-03	0E+00	1E-04	3E-02	2E-04	1E-04
IA2	1E-01	4E-03	0E+00	2E-01	1E-02	0E+00	5E-04	3E-02	1E-03	5E-04

IA3	9E-02	1E-03	0E+00	9E-02	8E-03	0E+00	4E-04	5E-03	5E-04	6E-04
ID1	3E-02	0E+00	1E-04	3E-02	3E-03	0E+00	5E-04	1E-03	5E-03	2E-03
ID2	3E-02	0E+00	0E+00	2E-02	3E-03	1E-04	0E+00	2E-03	3E-03	3E-03
ID3	3E-02	3E-04	0E+00	9E-02	2E-03	0E+00	4E-04	1E-02	1E-02	1E-03
IL1	6E-02	1E-04	4E-05	2E-01	3E-03	0E+00	2E-03	5E-04	4E-05	0E+00
IL2	4E-02	3E-04	7E-05	3E-01	1E-03	0E+00	2E-03	2E-03	4E-05	7E-05
IL3	6E-02	1E-04	4E-05	2E-01	3E-03	0E+00	2E-03	5E-04	4E-05	0E+00
IN1	2E-02	2E-03	8E-04	8E-02	3E-03	7E-05	0E+00	8E-03	3E-03	1E-04
IN2	2E-02	3E-03	5E-03	3E-02	1E-03	0E+00	0E+00	7E-03	3E-03	3E-04
IN3	3E-02	1E-03	4E-04	3E-02	1E-04	0E+00	0E+00	4E-03	4E-04	1E-04
KS1	5E-02	1E-04	3E-03	2E-01	2E-02	2E-02	1E-04	0E+00	1E-04	0E+00
KS2	1E-01	3E-03	4E-04	2E-01	1E-02	1E-02	0E+00	9E-04	7E-04	4E-05
KS3	1E-01	3E-03	7E-05	3E-01	1E-02	3E-03	4E-04	4E-03	9E-04	0E+00
KY1	1E-01	4E-03	8E-04	7E-02	7E-04	2E-03	7E-05	4E-03	8E-04	0E+00
KY2	9E-02	2E-03	1E-03	3E-01	1E-03	1E-03	5E-04	2E-04	8E-04	0E+00
KY3	3E-01	7E-04	1E-03	2E-01	5E-03	1E-03	5E-03	2E-04	4E-04	0E+00
LA1	2E-02	1E-04	4E-05	1E-02	2E-03	0E+00	0E+00	3E-01	5E-04	4E-04
LA2	3E-02	7E-05	4E-05	5E-03	9E-04	2E-04	0E+00	3E-01	3E-04	4E-05
LA3	3E-02	1E-04	0E+00	2E-02	3E-03	1E-04	4E-05	3E-01	6E-04	7E-05
MA1	2E-02	2E-04	0E+00	4E-03	2E-04	0E+00	0E+00	4E-01	2E-03	4E-05
MA2	8E-03	1E-04	0E+00	3E-03	1E-04	0E+00	0E+00	3E-01	3E-03	1E-04
MA3	1E-02	1E-04	0E+00	3E-03	2E-04	0E+00	0E+00	2E-01	1E-03	1E-04
MD1	6E-02	3E-04	1E-03	5E-03	9E-04	0E+00	0E+00	3E-04	1E-04	4E-04
MD2	5E-02	0E+00	4E-04	7E-03	2E-03	0E+00	2E-04	4E-04	4E-05	3E-04
MD3	5E-02	2E-04	3E-04	9E-03	3E-04	0E+00	4E-05	2E-03	4E-05	4E-04
ME1	9E-02	0E+00	0E+00	5E-04	4E-05	0E+00	7E-04	7E-03	7E-04	2E-03
ME2	6E-02	0E+00	0E+00	2E-04	7E-05	0E+00	1E-03	2E-02	2E-03	2E-03
ME3	5E-02	0E+00	0E+00	3E-04	0E+00	0E+00	5E-04	2E-02	4E-03	3E-03
MI1	4E-02	0E+00	0E+00	9E-02	9E-04	0E+00	0E+00	6E-02	5E-03	9E-04
MI2	2E-02	0E+00	4E-05	1E-01	3E-03	0E+00	2E-04	6E-02	1E-02	3E-03
MI3	3E-02	4E-05	0E+00	5E-02	2E-03	0E+00	1E-04	7E-02	1E-02	2E-03
MN1	1E-02	7E-05	2E-04	2E-02	2E-03	7E-05	4E-05	4E-01	2E-02	2E-03
MN2	1E-02	0E+00	0E+00	6E-03	9E-04	0E+00	0E+00	5E-01	2E-02	7E-04
MN3	2E-02	4E-04	0E+00	3E-02	2E-03	7E-05	0E+00	3E-01	2E-02	8E-04
MO1	2E-03	0E+00	0E+00	1E-03	0E+00	4E-05	0E+00	6E-01	5E-04	8E-04
MO2	3E-03	0E+00	4E-05	6E-03	1E-04	4E-04	0E+00	8E-02	7E-03	1E-03
MO3	3E-03	4E-05	4E-05	2E-02	3E-04	4E-04	0E+00	4E-01	2E-03	5E-04
MS1	2E-02	4E-05	0E+00	2E-02	6E-04	0E+00	0E+00	1E-01	3E-03	4E-04
MS2	3E-02	0E+00	7E-05	8E-02	2E-03	0E+00	0E+00	2E-01	3E-03	6E-04
MS3	2E-02	3E-04	0E+00	2E-01	4E-03	7E-05	0E+00	3E-01	4E-03	1E-04
MT1	1E-01	1E-03	2E-03	6E-03	9E-04	1E-02	1E-04	1E-04	2E-04	0E+00

MT2	2E-01	3E-03	2E-03	1E-02	3E-03	4E-03	1E-04	7E-05	1E-03	0E+00
MT3	7E-02	5E-04	8E-04	4E-03	2E-03	5E-03	0E+00	3E-04	1E-03	4E-05
NC1	9E-02	1E-04	4E-04	1E-02	5E-03	3E-03	4E-04	6E-03	2E-03	2E-04
NC2	2E-02	0E+00	0E+00	7E-04	6E-03	3E-03	0E+00	2E-03	6E-03	5E-04
NC3	1E-01	2E-04	3E-04	2E-02	5E-03	4E-03	8E-04	3E-03	1E-03	7E-05
ND2	7E-02	4E-05	4E-04	1E-02	7E-04	4E-03	4E-04	5E-04	6E-04	0E+00
ND3	1E-01	3E-03	4E-04	1E-02	7E-04	3E-03	2E-03	3E-03	1E-03	4E-05
NE1	9E-03	1E-04	0E+00	4E-03	4E-05	9E-04	0E+00	2E-01	2E-03	8E-04
NE2	2E-02	0E+00	0E+00	5E-03	1E-04	1E-04	0E+00	3E-01	1E-03	5E-04
NE3	1E-02	7E-05	0E+00	1E-02	2E-03	5E-04	0E+00	3E-01	5E-03	2E-04
NH1	6E-02	1E-03	0E+00	3E-02	7E-04	0E+00	4E-05	4E-03	8E-03	4E-04
NH2	6E-02	4E-04	8E-04	9E-02	1E-03	1E-03	7E-05	1E-02	4E-03	3E-04
NH3	3E-02	3E-04	7E-05	5E-02	2E-04	0E+00	0E+00	2E-02	8E-03	5E-04
NJ1	8E-03	0E+00	0E+00	6E-04	5E-04	0E+00	0E+00	1E-01	3E-02	3E-03
NJ2	8E-03	0E+00	7E-05	3E-04	4E-04	0E+00	0E+00	7E-02	4E-02	2E-03
NJ3	8E-03	0E+00	4E-05	6E-04	4E-04	0E+00	0E+00	7E-02	4E-02	3E-03
NM1	9E-02	2E-04	3E-04	2E-02	3E-02	1E-02	1E-03	9E-04	1E-03	0E+00
NM2	2E-01	2E-03	9E-04	1E-01	6E-03	2E-03	2E-02	8E-04	2E-04	0E+00
NM3	8E-02	3E-03	2E-03	3E-02	4E-02	6E-03	5E-02	3E-04	1E-04	0E+00
NV1	4E-02	2E-04	0E+00	3E-02	6E-03	0E+00	0E+00	4E-01	2E-03	2E-04
NV2	2E-02	7E-05	0E+00	1E-02	3E-03	0E+00	0E+00	4E-01	6E-03	1E-04
NV3	2E-02	4E-04	0E+00	5E-02	5E-03	0E+00	0E+00	4E-01	4E-03	0E+00
NY1	2E-02	3E-04	0E+00	1E-02	3E-04	7E-05	4E-05	2E-01	8E-03	2E-03
NY2	3E-02	4E-05	0E+00	2E-02	4E-04	7E-05	1E-04	2E-01	8E-03	1E-03
NY3	3E-02	3E-04	4E-05	8E-03	4E-04	7E-05	1E-04	3E-01	1E-02	1E-03
OH1	5E-02	7E-05	1E-04	2E-02	3E-03	7E-04	2E-03	1E-03	1E-03	0E+00
OH2	5E-02	1E-04	3E-04	1E-02	3E-03	6E-04	2E-03	3E-03	1E-03	0E+00
OH3	1E-01	1E-03	1E-04	2E-02	6E-03	7E-04	5E-03	3E-04	1E-03	0E+00
OK1	2E-01	7E-04	1E-04	5E-01	5E-03	4E-03	9E-04	2E-03	1E-04	0E+00
OK2	9E-02	1E-04	3E-04	4E-01	2E-03	3E-03	4E-04	1E-03	0E+00	0E+00
OK3	6E-02	8E-04	2E-04	3E-01	2E-03	4E-03	5E-04	1E-04	0E+00	4E-05
OR1	7E-02	0E+00	2E-04	3E-03	8E-03	0E+00	0E+00	1E-03	1E-03	1E-04
OR2	7E-02	3E-03	2E-04	6E-03	6E-03	4E-05	0E+00	3E-03	3E-03	4E-05
OR3	7E-02	9E-04	5E-04	7E-03	5E-03	7E-05	0E+00	2E-03	4E-03	2E-04
PA1	6E-02	8E-03	5E-04	1E-02	6E-03	3E-03	1E-04	1E-03	8E-04	5E-04
PA2	1E-01	3E-04	4E-04	3E-02	6E-03	2E-03	0E+00	7E-04	4E-04	4E-04
PA3	9E-02	7E-04	1E-04	9E-03	7E-03	6E-03	5E-04	3E-04	5E-04	7E-04
SC1	2E-01	3E-03	5E-04	2E-01	4E-03	4E-03	1E-03	2E-04	3E-04	0E+00
SC2	3E-01	2E-03	7E-04	2E-01	6E-03	4E-03	2E-03	1E-04	4E-04	0E+00
SC3	2E-01	7E-04	6E-04	2E-01	6E-03	3E-03	1E-03	2E-04	3E-04	4E-05
SD1	7E-04	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00	5E-04	1E-03	4E-04

SD2	7E-05	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00	9E-04	3E-04	9E-04
SD3	2E-04	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00	3E-04	2E-04	1E-03
TNS1	2E-01	4E-03	3E-04	4E-03	2E-03	7E-03	4E-05	2E-03	5E-04	0E+00
TNS2	4E-01	9E-03	2E-03	5E-04	7E-03	2E-02	7E-05	7E-05	2E-04	0E+00
TNS3	4E-01	2E-03	4E-04	3E-02	5E-03	7E-03	4E-05	9E-04	4E-05	7E-05
TX1	5E-02	1E-04	2E-03	4E-03	9E-03	6E-03	1E-04	1E-04	9E-04	0E+00
TX2	6E-02	5E-04	1E-04	2E-03	3E-03	3E-03	4E-05	5E-04	9E-04	1E-04
TX3	7E-02	1E-03	1E-04	6E-03	2E-03	3E-03	4E-05	3E-04	8E-04	4E-05
UT1	8E-03	0E+00	0E+00	1E-04	4E-04	0E+00	0E+00	5E-02	4E-03	1E-02
UT2	3E-03	0E+00	0E+00	2E-04	5E-04	0E+00	0E+00	7E-02	4E-03	4E-03
UT3	4E-03	0E+00	0E+00	3E-04	4E-04	0E+00	0E+00	8E-02	5E-03	3E-03
VA1	3E-02	2E-04	4E-05	4E-04	2E-03	0E+00	0E+00	1E-01	9E-03	2E-04
VA2	4E-02	1E-04	0E+00	5E-04	2E-03	0E+00	0E+00	7E-02	8E-03	7E-04
VA3	3E-02	0E+00	0E+00	2E-03	2E-03	0E+00	0E+00	1E-01	8E-03	1E-04
VT1	1E-01	3E-03	7E-05	7E-03	5E-03	0E+00	3E-04	3E-03	4E-03	4E-05
VT2	1E-01	1E-03	3E-04	8E-03	3E-03	4E-05	1E-03	5E-03	2E-03	4E-05
VT3	1E-01	7E-05	1E-04	3E-03	8E-03	0E+00	2E-03	3E-03	1E-02	2E-04
WA1	8E-02	1E-03	7E-03	4E-02	2E-03	6E-03	2E-04	7E-05	2E-04	7E-05
WA2	5E-02	4E-04	9E-04	8E-02	5E-03	7E-03	4E-05	1E-04	6E-04	0E+00
WA3	5E-02	9E-04	1E-03	4E-02	2E-03	5E-03	4E-04	2E-03	4E-04	0E+00
WIA1	7E-02	9E-04	0E+00	6E-02	2E-03	2E-03	4E-05	9E-04	9E-04	0E+00
WIA2	1E-01	2E-03	2E-04	8E-02	3E-03	6E-03	1E-04	4E-04	1E-03	0E+00
WIA3	2E-01	3E-03	2E-04	4E-02	4E-03	8E-03	1E-04	2E-03	4E-04	0E+00
WIB1	7E-02	5E-04	2E-03	4E-01	9E-03	3E-03	2E-03	9E-03	1E-04	2E-04
WIB2	1E-01	3E-04	3E-03	6E-02	4E-03	5E-03	2E-03	5E-03	7E-05	7E-05
WIB3	9E-02	3E-04	2E-03	3E-01	3E-03	4E-03	1E-03	1E-04	2E-04	2E-04
WV1	3E-02	3E-04	4E-05	4E-03	1E-04	0E+00	0E+00	3E-01	4E-03	2E-04
WV2	2E-02	3E-04	0E+00	3E-03	2E-04	0E+00	0E+00	4E-01	6E-03	2E-04
WV3	2E-02	4E-05	1E-04	1E-03	1E-04	0E+00	0E+00	3E-01	2E-02	2E-03
WY1	6E-03	0E+00	0E+00	4E-03	4E-05	0E+00	0E+00	3E-01	9E-03	9E-03
WY2	6E-03	0E+00	0E+00	6E-03	1E-04	0E+00	0E+00	3E-01	6E-03	5E-03
WY3	2E-02	1E-04	0E+00	3E-02	2E-04	4E-05	0E+00	7E-02	4E-04	6E-03