ABSTRACT

METHANOGEN COMMUNITY DYNAMICS WITHIN A DRY ANAEROBIC DIGESTER

By Ryan D. Bartell

Greenhouse gasses such as methane and carbon dioxide are thought to play a major role in global climate change. Anthropogenic methane is derived mainly from energy production, agricultural sources, and waste management practices. Anaerobic digesters function to produce energy using the methane derived from organic matter which would typically be deposited in landfills. The three main types of digesters are low solids wet digesters, high solids wet digesters, and dry anaerobic digesters. The dry anaerobic digester, which is the least common type, has relatively high solids content with very few moving parts, and the bulk phase of the system is solid instead of liquid. Due to the rarity of dry anaerobic digesters, the microbial community responsible for the production of biogas is less-studied than in other types of digesters. Biogas, which is usually comprised of methane, carbon dioxide, and hydrogen sulfide, is produced as a result of four metabolic steps which include hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In hydrolysis, acidogenesis, and acetogenesis, bacteria break down organic matter, and in methanogenesis archaea use the products of acidogenesis and acetogenesis to produce methane. The main objective of this research was to determine if a fluctuation pattern in the concentrations of two orders methanogenic archaea (Methanosarcinales and Methanomicrobiales) existed over a 28-day fermentation in a dry anaerobic digester. To address this objective, taxonomic groups present within the digester were determined by 454-pyrosequencing, and the biogas composition and volume were characterized. The 16S rRNA gene copy concentrations of Methanosarcinales spp. and Methanomicrobiales spp. were determined using qPCR. Bench-scale biogas experiments were conducted with and without substrate, and biological samples were collected at regular time intervals for qPCR analysis. Pyrosequencing results indicate that within the dominate class Methanomicrobia, the orders Methanosarcinales (73.7%) and Methanomicrobiales (26.2%) were most common in the tested anaerobic digester before biogas experiments. Biogas experiments show that fermenters with substrate produce a greater volume of biogas and methane than those without substrate. qPCR analysis showed that during the 28-day fermentation, Methanosarcinales spp. were present at approximately 10^7 16S genes per gram of sample, whereas Methanomicrobiales spp. had concentrations between 10^4 and 10^6 16S genes per gram of sample. Overall, Methanosarcinales was consistently more abundant at every tested time point. The different concentrations of methanogens could indicate that the nutrients and conditions within the tested digester are more suited to the metabolism of Methanosarcinales spp. than Methanomicrobiales spp. This information could be used in the future to dictate the type of substrate used in a particular dry anaerobic digester.

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By

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INTRODUCTION

Methane in the Environment and Mitigation Strategies

Methane emission rates in the United States are a major concern to scientists because of the local and global environmental and economic effects. Although methane emissions in the past two decades has dropped 8%, the United States still emitted just less than 600 million metric tons (CO_2 equivalent) of methane in 2011 [50]. Greenhouse gasses, especially methane, are thought to play a significant role in the increase of meteorological phenomena such as flooding [36] and days of extremely high temperatures (>3sd) in the summer [20]. In 2009, 41.5% of methane emissions were attributed to energy production, while agricultural and waste management sources comprised 29.5 and 28.4%, respectively [46]. However, some of the methane emissions can be captured and used for energy and heat, which could mitigate the impact of atmospheric methane on the environment. Landfills are an attractive target for the mitigation of greenhouse gases through collection processes because some solid waste can be recycled or repurposed, thereby diverting it from landfills [46]; alternatively, some solid waste can be diverted to an anaerobic digester. Material that would otherwise end up in landfills and contribute to additional methane production could be recycled for alternative uses, which would in turn prevent some methane emissions. The United States Environmental Protection Agency started the Global Methane Initiative in 2004 with goals to "reduce global methane emissions to address climate change, enhance economic growth, strengthen energy security, and improve local environmental quality

and industrial safety" [49] by increasing the amount of methane captured from landfills and other sources. Since biogas produced by landfills can contain as much as 50% methane [47], landfills were chosen as targets in the international Methane to Markets program began by the US government. Landfills produce a high percentage of methane because much of the waste that is deposited in landfills is made of material suitable for anaerobic digestion. In the US in 2011, yard trimmings, food waste, and paper and cardboard comprised 13.5%, 14.5%, and 28.0% of municipal solid waste generated, respectively [51]. Organic wastes such as paper and yard trimmings can be diverted from the landfill by means of enhanced recycling programs, and food waste has been used effectively in anaerobic digestion.

While a small percentage of methane captured from landfills is utilized for energy, a large amount of the gas is burned or "flared" off. In digesters, however, the vast majority of the methane is utilized and converted to either heat or electricity. Diverting organic waste away from landfills and to digesters would ensure that the energy produced during the fermentation of organic waste is fully harnessed. Capturing more methane for energy production would in turn reduce the volume of methane emitted into the atmosphere. After the organic matter has been broken down in by anaerobic digestion, it can be further utilized as a fertilizer in the agricultural industry. This is in stark contrast to the organic matter in landfills, which remains in place to settle and decay for decades. While in use, landfills represent a substantial spatial footprint, and after being filled to capacity, the land can eventually be reclaimed.

Types of Anaerobic Digesters

Although several different systems are able to capture methane and convert it to heat and energy, three main types exist: low solids wet anaerobic digesters, high solids wet anaerobic digesters, and dry anaerobic digesters. Wet digesters have a total solids (TS) content of less than 20 percent, whereas high solids digesters have a higher TS percent, usually 20-42 percent. [1, 6, 9]. Low solids wet digesters and high solids wet digesters have a TS content of less than 10 percent and 10 to 20 percent, respectively. Due to the differences in TS content between the types of digesters, each one has a different system of operation. Wet anaerobic digesters are often confined to a single storage tank in which sludge is stored, although hydrolytic and acidogenic steps may take place in a separate reactor before the sludge is transferred to a final reactor for methanogenesis [45]. Feedstocks can then be added to the reactor in a batch or continuous method. In a batch-fed wet digester, the feedstock is loaded into the reactor at the beginning of digestion, and no more feedstock is added until the digestion cycle is complete. In a continuously-fed wet digester feedstock is periodically added to the reactor, which introduces more nutrients into the system. The sludge within the reactor can be mixed, as in a continuously stirred tank reactor (CSTR) or allowed to settle. Sealing the tank prevents the influx of atmospheric oxygen, which can hinder the growth and viability methanogenic archaea [22, 38] and anaerobic bacteria. During methanogenesis, methane is captured from the headspace of the reactor and piped to a combined heat and power (CHP) engine capable of converting methane to electricity and heat. Alternatively, the methane can be flared off and the by-products released back into

the environment. While both forms of combustion (CHP and flaring) release carbon dioxide as a by-product, combustion via CHP is more advantageous because heat and electricity can be harnessed.

The Dry Anaerobic Digester at the University of Wisconsin Oshkosh

The anaerobic digester at the University of Wisconsin Oshkosh (UWO digester) is a dry digester which has characteristically higher solids content than most other digesters. Because of this, the digester is considered a "high solids dry anaerobic digester." Dry anaerobic digesters operate by combining the continuous feed and batch feed methods. A large bay area is used as a reactor, and feedstocks are mixed with digestate from the previous digestion cycle in a ratio such that a TS content of about 35% is achieved [Bioferm Operations Manual]. In dry anaerobic digestion, the feedstock mix is loaded into the reactor only once in the beginning of the digestion cycle, much like in a batch-fed digester. Once the mix is inside the reactor bay, doors close to seal the mix inside, which prevents contact with outside oxygen. In UWO digester, percolate (2-3% TS) from a central storage tank is sprayed over top of the mix intermittently and drained through the floor. Like a continuously-fed system, this helps to provide nutrients and inoculate much of the feedstock with bacteria and archaea necessary to carry out methanogenesis [25]. After 28 days of fermentation, the reactor bay doors are opened, and the cycle repeats. In dry digesters, digestate has a TS content of 15-30% after fermentation [7, 48]. The average TS of digestate at UWO digester is about 25%. The

reduction in TS content from initial loading phase (35%) to the unloading phase (25%) is due to the saturation of the feedstocks by the percolate which is sprayed overtop during fermentation.

While solids content is a useful starting point during the loading process of anaerobic digestion, plant managers typically examine other important factors to determine the efficiency of digestion for the substrates that they use. For example, digestate removed from UWO digester usually contains lower levels (0-150 mg/L acetic acid equivalent) of volatile fatty acids (VFAs), than percolate (200-400 mg/L acetic acid equivalent) [Internal, Unpublished Data]. Low levels of VFAs in anaerobic digesters are an indication that most of the long chain fatty acids have been broken down by bacteria into short chain fatty acids, which were in turn used by microbes to produce methane. Additionally, the digestate that is removed from the digester is nutritionally rich in phosphorus and nitrogen, and can be used as compost after further decomposition by aerobic digestion. Digestate from a dry anaerobic digester can therefore provide a valuable yet affordable source for the agricultural and composting industries.

Biogas Constituents

In anaerobic digesters, biogas is produced when organic material is broken down by microorganisms in the absence of oxygen. Common organic materials include foodwaste, plant matter, wastewater, different types of manures, and waste products from industrial processes such as beer and wine production [2, 3, 11, 19, 31]. The three main components of biogas are methane, carbon dioxide, and water vapor. Methane and

carbon dioxide can constitute as much as 65% and 30-40% of total biogas, respectively; water vapor is maximally saturated in digester biogas at 35°C which translates into about 40 g/m^3[11].

Siloxanes and hydrogen sulfide are also commonly found in biogas. Siloxanes are linear or cyclic compounds which contain silicon. Siloxanes in biogas can be detrimental to the operation of biogas engines because they form a hard layer of abrasive silica in the engine's combustion chamber. Since siloxanes are commonly used in products such as shampoos, detergents, and pharmaceuticals, biogas derived from landfills and wastewater treatment facilities tends to have a greater concentration of siloxanes than biogas from manure-derived digesters. Siloxanes in digester biogas typically occur in concentrations <15 mg/m^3 but have been recorded in concentrations up to 140 mg/m^3 in activated sludge of wastewater treatment facilities [30, 32, 37 , 39]. Certain siloxanes are more water-soluble than others, and the concentrations of siloxanes in biogas depend on the volatility and solubility of the siloxanes used in the digester or landfill [4]. More water-soluble and less volatile siloxanes remain in the solid or liquid phase and are found in biogas in only trace amounts.

Hydrogen sulfide (H_2S) is produced when organic material which contains sulfur, such as animal tissue, is anaerobically digested. While H_2S concentrations in landfill biogas is usually below 100 ppm, raw biogas from dry anaerobic digesters can range from about 50 to 1000 milligrams per normal cubic meter [39]. Intermediate desulfurization methods in anaerobic digesters can reduce H_2S concentrations to less than 70 mg/m^3, making the biogas much less corrosive on the CHP engine [11].

Metabolic Overview of Biogas Production and Methanogenesis

The production of biogas is a process generally described in four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [30]. In hydrolysis, polymers are broken down into shorter polymers or monomers. Of particular interest, long chain fatty acids are broken down into short chain fatty acids, which are subsequently used by the same or other bacteria to produce different types of acids (eg. propionic acid, butyric acid, acetic acid) [43]. While hydrolysis, acidogenesis, and acetogenesis can be carried out by many different types of bacteria, only archaea are capable of producing methane. Methanogenic archaea use two main metabolic routes to produce methane: acetoclastic and hydrogenotrophic methanogenesis.

In acetoclastic methanogenesis, the methyl group of acetate is stripped and a hydrogen atom is added to it to produce methane. The remaining carboxyl group is converted to carbon dioxide [15]. In the hydrogenotrophic pathway, hydrogen serves as an electron donor, and carbon dioxide is reduced to methane. Not all methanogens are capable of carrying out both acetoclastic and hydrogenotrophic methanogenesis, which underscores the importance of having a diverse population of methanogens within an anaerobic digester. In fact, *Methanosarcina acetivorans* is the only known organism capable of carrying out all three methanogenic pathways (acetoclastic, hydrogenotrophic, and methylotrophic) [16].

Microbial Community Dynamics in Digesters

While many studies have examined the microbial community dynamics associated with wet anaerobic digesters and activated sludge at wastewater treatment facilities [21, 28, 41, 44], to this author's knowledge, no such studies have been conducted on a system similar to the dry digester found at the University of Wisconsin Oshkosh. Physical and chemical parameters for percolate and digestate are tested frequently at digesters in order to determine whether or not the digester is operating properly. These physical and chemical parameters, such as total and volatile solids content, hydrogen sulfide concentration, volatile fatty acid content, and pH, can offer insight into the environment surrounding a microbial community within a digester, but do nothing to explain which organisms are present at a given step within the digestion process. Until the microbiology of the dry digestion process is well-defined and understood, plant managers can only make educated guesses concerning the physical and chemical parameters which are appropriate for the microbial community contained within the system. Since certain methanogens have higher or lower affinities and utilization rates for metabolites such as acetate or carbon dioxide, an optimum methane output could be achieved by creating conditions suitable to the metabolisms of the dominant microbes [52]. For example, the family Methanosaetaceae (high acetate affinity but low maximum utilization rate) would thrive in low acetate conditions, and the family Methanosarcinaceae (low acetate affinity but high maximum utilization rate) would produce the most methane in a high acetate concentration environment.

Although UWO digester has some similarities to a wet digester, it (like other dry digesters) has several differences in operation and structure that could contribute to microbial community dynamic not present in wet digesters. In dry anaerobic digestion, percolate passes through before returning to a single storage tank. Each of the bays through which the percolate passes in dry digestion may contain different feedstocks and is at a different point in the digestion process, whereas in most wet anaerobic digesters, the central tank is where digestion takes place.

Another difference between most digesters and UWO digester is that plant material makes up a significant portion of feedstock loaded into UWO digester. Plant material is important in the function of UWO digester because it provides structural integrity for the feedstock mix as the digestion cycle progresses. Since structural integrity is not required in the function of wet digesters, plant material does not constitute as large a portion of the feedstock. Typically composed of straw, the plant material ensures that the feedstocks loaded into each bay do no decompose and drain into the main percolate collection tank. If feedstock were allowed to enter the drains for each bay, the drains would likely clog and require maintenance. By keeping the feedstock of one bay separate from the feedstock of the other bays, there is less risk of introducing contaminates, such as heavy metals or antibiotics, into the main percolate storage tank. Aside from providing structural integrity for each bay's feedstock, the plant material provides a means in which to inoculate the next feedstock for that bay, ensuring that microbes which were taking part in methanogenesis from the previous cycle are present in the next cycle.

Previous studies concerning microbial community dynamics in wet digesters have examined the changes in both bacterial and archaeal communities. One study examined bacterial community dynamics during hydrolytic and acidogenic stages (a period of 8 days) of biogas production and found that in the first two days when lactic and acetic acid were being formed, *Lactobacillus* spp. and *Acetobacter* spp. dominated. In the next three days, *Clostridium* spp. dominated, which corresponded to the production of butyric acid, hydrogen, and carbon dioxide. In the final three days of the study, a high rate of biogas production and formations of caproic and acetic acid were found and corresponded to increased populations of Ruminococcaceae and Lachnospiraceae [43]. The results from microbial ecology studies such as these show that biodigesters are very dynamic systems in which groups of microorganisms are commensally-dependent on the production of metabolites by other groups of microorganisms. If conditions within a digester are not conducive to the proliferation of certain groups of microorganisms, certain metabolites, such as butyric, propionic, or acetic acid, may not be produced in large enough quantities to be used in the later steps of methanogenesis.

A study concerning fluctuations in microbial communities in response to glucose influxes in a methanogenic bioreactor indicated that a less diverse community was more able to shift back to the initial community structure [14]. The study examined the resilience of two different microbial communities using eight different reactors (two different communities measured in quadruplicate). The reactors were maintained with low levels of glucose (44.4 mM/day) as the only carbon source. Next, each reactor was injected with a shock load of glucose so that the reactor liquid reached a glucose

concentration of 38 mM. Morphotype frequencies revealed that the less diverse community was able to return to about 90% similarity of its pre-perturbation community structure, and the study concluded that community flexibility may be closely related to the community's ability to effectively alter carbon and electron flow through various pathways. When the microbial community is perturbed, its ability to recover might be linked with how well established a particular metabolic pathway is, as well as which pathways are being readily used at the time of the perturbation. Given the wide variety of feedstocks used in anaerobic digesters, community stability studies can help to elucidate how a change in feedstock or an increase/decrease in feedstock concentration can affect a microbial population and consequently alter the quality and quantity of biogas output.

Quantitative real-time polymerase chain reaction (qPCR) has gained popularity in recent years as a method of determining microbial populations in methanogenic systems. Primers and probes for several different clades of methanogens have been developed and tested in previous studies [17, 34, 52, 53]. In an attempt to better understand the community dynamics involved in biogas production, Lee et al used qPCR to determine the 16S gene copy concentration of different groups of methanogens over a 60 day period in three wet anaerobic digesters [27]. Using qPCR, they found that the family Methanosarcinaceae (in the order Methanosarcinales*)* had the highest gene copy concentration midway through the 60 day digestion process, and that the order Methanomicrobiales had the highest initial and final gene copy concentrations. Other studies have also found an abundance of Methanomicrobiales present in digesters [41],

and Methanosaetaceae (also in the order Methanosarcinales) has been found to be present in many other environments [5, 8, 10, 21, 27, 29], including anaerobic digesters [18, 33]. The ubiquitous nature of Methanosaetaceae indicates that it could be a versatile group of methanogens which play an important role in microbial assemblages within anaerobic digesters and elsewhere despite their low abundance.

Metagenomic Analysis of Methanogenic Populations

Metagenomic studies from anaerobic digesters are limited and vary significantly, which may be due to the variety of types of anaerobic digesters sampled. One metagenomic study which used samples from an agricultural biogas fermenter indicated that the genus *Methanoculleus* (in the order Methanomicrobiales) was the most abundant genera of all of the prokaryotes present in the system [23]. A more comprehensive study in 2011 examined the overall microbial diversity of many different digesters using 16S rRNA gene sequences submitted to public databases [35]. Using these gene sequences (bacteria sequences n=16,519, archaeal sequences n=2,869) the researchers found that *Methanosaeta* (in the order Methanosarcinales) was the most abundant archaeal genus when all of the sequences were pooled together. Many of the datasets used in this analysis were derived from studies conducted on wastewater treatment and agricultural digesters.

While the metagenomic data derived from other studies provide a basis for the investigation of the microbial diversity in UWO digester, the applicability of the data is severely limited by the fact that UWO digester is dissimilar in structure, function, and

operation to other digesters used in previous studies. For example, in most digesters, the liquid phase constitutes the majority of the volume. In high-solids dry digesters, however, the majority of the digesters volume is solid phase, with the remaining portion being gas phase. The percolate is the only liquid phase constituent in the digester, and is sprayed periodically over the solid phase in order to seed the solid phase with a well-established culture of bacteria and archaea.

Many previous studies have been conducted to show the change in microbial community structure within digesters. These studies are specific to anaerobic digesters which have a lower total solids content that UWO digester. Previous works have shown a fluctuation in the gene copy numbers at different points in time between the orders of Methanomicrobiales and Methanosarcinales during anaerobic digestion. I propose that there is a successional pattern in methanogenic community structure during a 28-day anaerobic digestion period within UWO digester. The hypothesis of my research is: Given similar starting environments derived from UWO digester, 16S rRNA gene copy concentrations for Methanosarcinales and Methanomicrobiales spp. will fluctuate significantly during 28 days of anaerobic digestion.

PROJECT OBJECTIVES

The overarching objective of this research is to determine a fluctuation pattern in Methanomicrobiales and Methanosarcinales spp. concentrations within a dry anaerobic digester. This study addressed the following specific objectives:

1. To determine which taxonomic groups of methanogens are likely to be abundant within UWO digester.
2. To characterize biogas composition and total volume production of digestate from UWO digester.
3. To determine the 16S rRNA gene copy concentration of each tested order of methanogens.
4. To determine if a relationship exists between biogas production and/or composition, the time spent in the digestion process, and the gene copy concentration of Methanosarcinales and/or Methanomicrobiales spp.
5. To determine the fluctuation trends of the two tested orders of Archaea during a 28 day digestion cycle.

METHODS AND MATERIALS

Biogas Composition Experiments Using Eudiometers

Digestate was collected from UWO digester after 28 days of fermentation. Digestate samples were taken from the middle of the digestate pile within the fermenter. 12,032 grams of digestate were segregated for mixing with 768 grams of microcrystalline cellulose. The microcrystalline cellulose was hand-mixed into the digestate until an even distribution was established. Sixteen-hundred grams of the digestate/microcrystalline cellulose mix were added to each of eight 2-liter glass jars. Eudiometers were positioned on top of the glass jars and an airtight seal was established using high-vacuum grease. Eudiometers were filled with a buffer with a pH less than 2 in accordance with method DIN 38414-S8. Glass jars were loaded with 1,504 grams of digestate before being sealed with eudiometers as above. The samples that did not have microcrystalline cellulose added were considered to be negative controls. All eudiometers were placed in a water bath with a temperature maintained at 39 °C. Water temperature was maintained using two heaters and digital thermostat switches. Buffer levels were brought to the zero mark at the beginning of the gas recording period. Biogas volumes for each eudiometer were recorded multiple times daily, and the air temperature, date, time, and barometric pressure (in millibars).

Biogas composition (CH_4 %, CO_2 %, and O_2 %) was measured using a digital gas meter (Gas Data Ltd, UK) when biogas volumes were greater than 200 ml. The gas meter used meets MCERTS performance standards for portable emissions monitoring

systems, and has response times of 9.5, 14.5, 14.5, and 78.5 seconds for CH_4, CO_2, O_2, and H_2S (0-5000 ppm), respectively. Standard deviation for each analyte measured with the gas meter was less than 0.5%. In order to maintain an anaerobic environment, the tube of the gas reader was connected to the sampling port of the eudiometer and then turned on to create negative pressure. The stopcock of the eudiometer was then opened to allow for sampling of the gas. After each gas composition sampling event, the level of the eudiometer buffer was set to zero. Gas composition data were collected from each eudiometer until the eudiometer was destructively sampled for microbiological analysis. Two eudiometers containing the digestate/microcrystalline cellulose mix (Alfa Aesar, Ward Hill, MA) were destructively sampled on days 7, 14, 21, and 28. The two eudiometers containing only digestate were destructively sampled on day 28.

A second experiment was run using digestate collected as above on a separate occasion. In this experiment, 10,528 grams of digestate were segregated and hand-mixed with 672 grams of microcrystalline cellulose until an even distribution of microcrystalline cellulose was established. Although the mass of materials used varied from the previous eudiometer biogas experiment, the ratio of digestate to microcrystalline cellulose was the same. The mass of digestate/microcrystalline cellulose was reduced in this experiment to make gas composition reading events more manageable. Eight-hundred grams of the digestate/microcrystalline cellulose mix were loaded into each of fourteen 2-liter glass jars. Seven-hundred-fifty-two grams of digestate were loaded into each of eight 2-liter jars and used as negative controls. The eudiometers were measured and maintained for a period of 28 days as described in the previous eudiometer experiment. Destructive

sampling for microbiological analysis was conducted in duplicate on two eudiometers of digestate/microcrystalline cellulose on days 4, 8, 12, 16, 20, 24, 28. Eudiometers containing only digestate were destructively sampled on days 7, 14, 21, and 28. The negative controls were included in order to examine the composition of the biogas produced by the inoculums alone, as well as how the microbiological community progresses without any additional substrate.

Biogas Volume Experiments Using Bioprocess AMPTS-II

Digestate was collected from the UWO digester after 28 days of fermentation for these experiments. Four-thousand-five-hundred-twelve grams of digestate were segregated and hand-mixed with 288 grams of microcrystalline cellulose until an even distribution of microcrystalline cellulose was established. Four-hundred grams of the digestate/microcrystalline cellulose mix were loaded into each of twelve 600 ml glass bottles. Three-hundred-seventy-six grams of digestate were loaded into each of three 600 ml glass bottles. These three bottles were considered negative controls because no additional substrate was added. Each of the fifteen 600 ml bottles was sealed, and gas readings were collected as per AMPTS-II (Bioprocess Control, Sweden) procedure. The bottles were placed in a water bath and maintained at 39° C. Gas volumes were recorded from each bottle until the bottle was destructively sampled for microbiological analysis. Three bottles containing the digestate/microcrystalline cellulose mix were sampled at days 7, 14, 21, and 28. The three bottles containing only digestate were sampled on day 28.

A second gas volume experiment was run using digestate collected from the UWO digester. Bottles were loaded and maintained the same as above, except that three digestate/microcrystalline bottles were sampled on days 4, 8, 12, and 16. The three bottles containing only digestate were sampled on day 28.

A third gas volume experiment was run using digestate collected from the UWO digester. Twelve 600 ml bottles were loaded with 376 grams of digestate. Seventy-two grams of microcrystalline cellulose were mixed 1,128 grams of digestate until an even distribution of microcrystalline cellulose was established. Four-hundred grams of the digestate/microcrystalline cellulose mix were loaded into each of three 600 ml bottles. All 15 samples were maintained and monitored as previously described, except that three bottles containing only digestate were destructively sampled for microbiological analysis on days 7, 14, 21, and 28. The three bottles containing microcrystalline cellulose were destructively sampled on day 28.

Collection of Samples for Microbiological Community Analysis

Samples of digestate/microcrystalline cellulose and negative controls were collected from eudiometers and AMPTS-II biogas experiments at both 4 and 7 day intervals. Each vessel was opened and emptied into a plastic bin, mixed, and sampled. Flame-sterilized tweezers were used to collect duplicate samples from each vessel. Samples were placed into sterile 15 ml centrifuge tubes and frozen immediately at -80°C for later DNA extraction. Exact sampling intervals for each biogas experiment are described in the respective experiment.

An additional experiment was run using the AMPTS-II system with digestate collected from the UWO digester on a later date than the first experiment. This experiment had 12 bottles of digestate/microcrystalline cellulose and 3 bottles of digestate in the same masses described in the AMPTS-II biogas experiments. No gas data were recorded from this experiment; however a sealed system was maintained and sampled for microbiological analysis. Three digestate/microcrystalline cellulose bottles were destructively sampled on days 7, 14, 21, and 28. The three digestate samples were destructively sampled on day 28.

Collection of Metagenomic Data from UWO Digester

Samples of percolate and digestate were obtained from UWO digester. DNA from these samples was extracted in duplicate using the Powersoil DNA Extraction Kit (Mobio, Carlsbad, CA). DNA quantity was measured by determining the absorbance of the extracted DNA at 260 nm using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). Samples were diluted to approximately 30 ng/ul of DNA. Two DNA samples from percolate and two DNA samples from digestate were sent to Research and Testing Laboratories (Lubbock, Texas) for 454 pyrosequencing. One percolate and one digestate DNA sample were analyzed for bacterial diversity by targeting the variable regions V1-3 using primers 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'). One percolate and one digestate DNA sample were analyzed for archaeal diversity using primers YuiArchF (5'-CCCTAYGGGGYGCASCAG-3') and Arch958R (5'-YCCGGCGTTGAMTCCAATT-

3')[53]. Subsequent bacterial and archaeal pyrosequencing data were analyzed using Ribosomal Database Project (RDP) Pipeline. Samples were trimmed and classified using RDP Pipeline and Classifier. An archaeal rarefaction curve was constructed using RDP with genus, family, and order characterized as having 97, 95, and 90% sequence similarity, respectively.

TaqMan qPCR Assay Conditions

All reactions were run on a StepOne Plus (Applied Biosystems, Carlsbad, CA). Archaea in the orders of Methanosarcinales (MSL) and Methanomicrobiales (MMB) were detected using primers and probes listed in Table 1. All reactions were run with primer and probe concentrations at 300 nM and 200 nM, respectively. One µl of genomic DNA was used per reaction, along with 10 µl of Bullseye TaqProbe 2X qPCR Master Mix (Midsci, St. Louis, MO) containing 2X dNTPs, Hot Start Taq polymerase, $MgCl_2$, and ROX reference dye. Reactions were diluted to 20 µl using PCR-grade water.

Three-step amplification (denaturation, annealing, and extension) was carried out on target DNA for each reaction. An initial ten minute period at 95°C was used to activate Hot Start Taq polymerase, followed by 40 cycles of annealing, extension, and denaturation. For samples in which MSL was the target group, annealing took place at 60°C for 60 seconds and extension was carried out at 72°C for 40 seconds. Denaturation was performed at 95°C for 40 seconds. The temperature ramp rate between steps was set at 100%. Fluorescence data were recorded during the annealing steps, and signal data obtained from reactions were processed using StepOne Software (version 2.0). For

samples in which MMB was the target group, the following annealing, extension, and denaturation conditions were used: annealing at 58°C for 60 seconds, extension at 72°C for 40 seconds, and denaturation at 95°C for 40 seconds [52]. Positive standards of 10^7 or 10^6 16S rRNA gene copies per microliter were included in each qPCR analysis, along with DNA/RNA free water as a negative control.

Table 1. Primer and probes sets used for qPCR. The two target orders were Methanosarcinales (MSL) and Methanomicrobiales (MMB). Annealing steps for MSL and MMB were conducted at 58°C and 60°C, respectively.

Name	Function	Target Group	Sequence (5'--->3')	*E. coli* Numbering	T_m C (°C)	GC (%)	Amplicon Size (bp)
MSL812	F Primer	*MSL*	GTAAA CGATR YTCGC TAGGT	812-831	61.3	45	354
MSL860F	Probe		AGGGA AGCCG TGAAG CGARC C	860-880	71.2	64.3	
MSL1159R	R Primer		GGTCC CCACA GWGTA CC	1143-1159	62.3	64.7	
MMB282F	F Primer	*MMB*	ATCGR TACGG GTTGT GGG	282-299	63.8	58	506
MMB749F	Probe		TYCGA CAGTG AGGRA CGAAA GCTG	749-772	70.2	54.2	
MMB832R	R Primer		CACCT AACGC RCATH GTTTA C	812-832	61.5	45.4	

PCR Cloning of 16S rRNA Gene Sequences

qPCR was run on percolate and digestate DNA samples collected from UWO digester using MSL and MMB primer/probe sets and conditions previously listed for each target group. To check for amplification, one reaction for each of the replicates of each target group contained the target group's respective probe. Within two hours of completion of the reaction, a 1:20 dilution of the PCR product was made, and one microliter of the dilution from a reaction not containing a probe was inserted into a pCR2.1 plasmid vector from a TA TOPO10 Cloning Kit (Invitrogen, Madison, WI). The plasmid vector was then transformed into chemically competent *E. coli* cells included in the kit following the recommended steps prescribed in the kit.

Transformed *E. coli* cells were plated onto warm LB plates containing 50 µg/ml ampicillin and 50 µl of 20 µg/ml Xgal. Cells were incubated overnight at 37°C and examined for white colonies. Seven white colonies were selected, aseptically picked from the plate, and inoculated into 5 ml of LB containing 100 ug/ml ampicillin. The transformed cells were incubated at 37°C for 48-72 hours. After 48-72 hours, the plasmid vector was extracted from each of the seven clones using a Purelink Quick Plasmid DNA Miniprep Kit (Invitrogen, Madison, WI) and stored at -20°C for later use in constructing a standard curve for qPCR.

DNA concentration of the solution containing the extracted plasmids was measured, and the solution was diluted in order to make standards containing 10^3, 10^4, 10^5, 5.0×10^5, 10^6 MSL 16S rRNA gene copies. For the MMB standard curve, solutions containing 10^8, 5×10^7, 10^7, 5.0×10^6, 10^6, 5.0×10^5, 10^5, 10^4, and 10^3 copies of the 16S

rRNA gene were made using serial dilutions from the top standard. Each standard curve qPCR assay was run using the previously describe parameters. Each gene concentration was tested in triplicate, and the log value of each concentration was plotted against the average cycle threshold value in order to create a standard curve. Standard curve assay results can be found in the Appendixes C and D.

RESULTS

Metagenomic Analysis of Biodigester Samples

The 454 pyrosequencing analysis using bacterial primers showed that 4,206 sequences were obtained, and 47.6%, 24.3%, and 15.7% of the sequences belonged to phyla Firmicutes, Bacteroidetes, and Tenericutes, respectively. The most prevalent bacterial classes were Bacilli, Clostridia, and Bacteroidia (28.0%, 19.3%, and 18.0%, respectively), and the most prevalent orders were Bacteroidales, Bacillales, Clostridiales (18.0%, 16.8%, and 16.8%, respectively).

The 454 pyrosequencing of percolate samples from the biodigester showed that of the roughly 11,000 archaeal 16S rRNA sequences obtained, 99.5% were from the phylum Euryarchaeota. The majority (87.9%) of Euryarchaeota sequences fell within the class Methanomicrobia, and two main orders of Archaea were present within the class Methanomicrobia: Methanosarcinales (26.2%) and Methanomicrobiales (73.7%) **(Figure 1)**. Family-level analysis for each order is shown in Appendix F.

Rarefaction analysis of sequencing data for archaea shows that the archaeal diversity within percolate was well-characterized at the order level ($\geq$90% sequence similarity) (**Figure 2**). Family and genus ($\geq$95% and $\geq$97% sequence similarity, respectively) level taxa were not as well characterized, as the number of new operational taxonomic units (OTUs) continued to increase significantly as more sequences were analyzed.

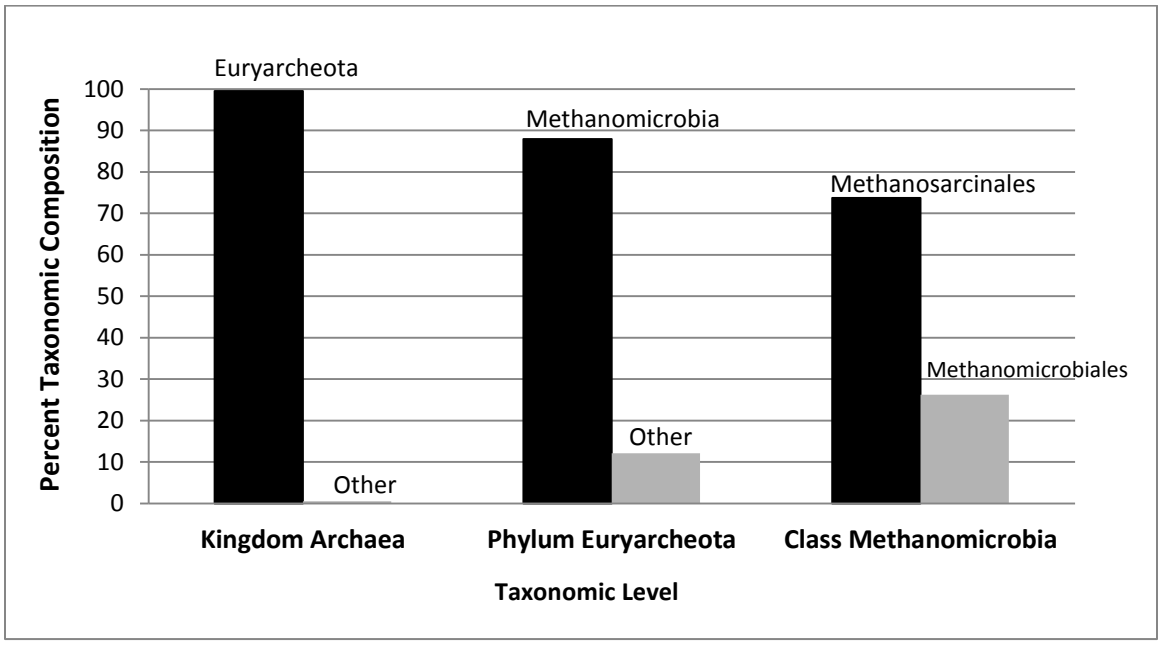

Figure 1. Taxonomic diversity within percolate samples. Within the phylum Euryarcheota, the class Methanomicrobia was most common. Two orders, Methanosarcinales and Methanomicrobiales, were most common within Methanomicrobia.

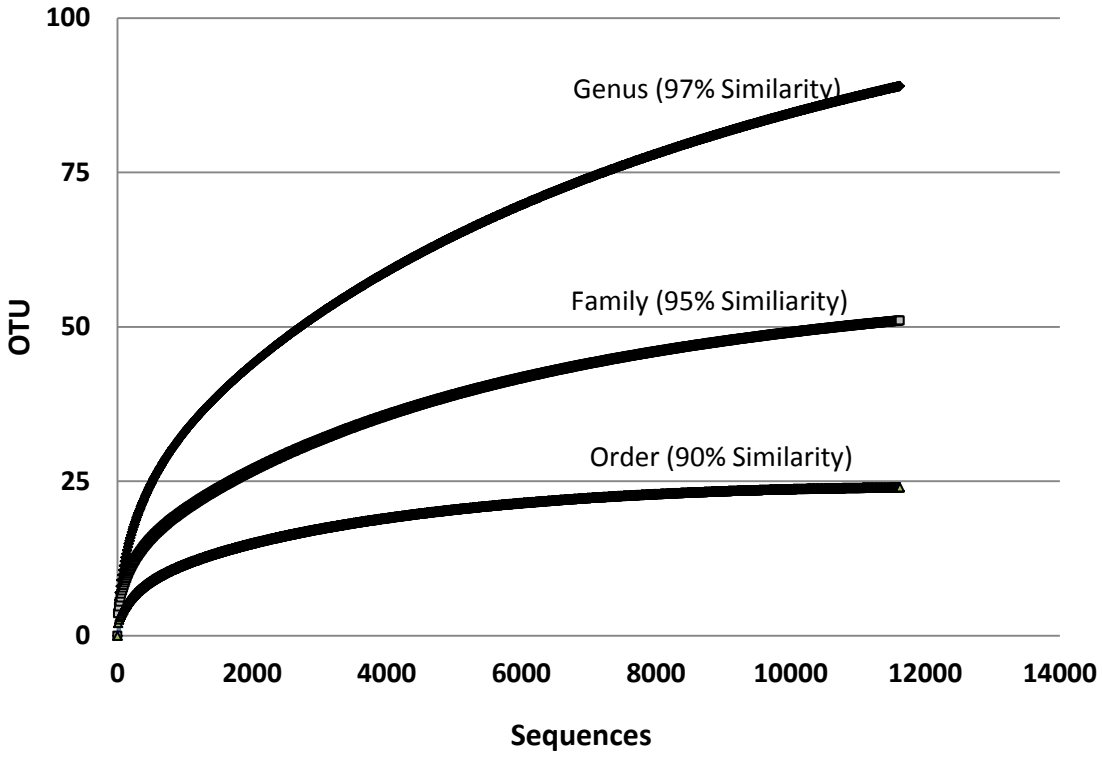

Figure 2. Rarefaction curve for pyrosequencing analysis of percolate. With over 10,000 sequences sampled, order was the most completely sampled taxonomic level for archaea. Lines for genus and family have a larger slope than that found in order after 10,000 sequences.

Biogas Volume and Production Rate Results from AMPTS-II System

Experiments utilizing the AMPTS-II showed a total production of 13.89 normal liters (NL) of biogas from vessels loaded with digestate and microcrystalline cellulose (positive vessels, n=3). Normal liters refers to the volume of the gas at standard temperature and pressure (20°C, 1 atm), as calculated by the AMPTS-II software. Vessels containing only digestate (negative vessels, n=12) produced an average of only 1.66 NL over the 28-day testing period (**Figure 3a**). Subtracting the average total production of negative vessels from the average total production of positive vessels yields a difference of 12.23 NL. Subtracting the negative average from the overall gas production results in the net biogas production, which came from the nutrients remaining in the digestate at the beginning of the experiment. Digestate inoculated with 24 grams of microcrystalline cellulose produced 12.23 NL of gas, yielding an average of 0.51 NL of biogas produced per gram of microcrystalline cellulose.

Biogas production rates from AMPTS-II experiments showed that vessels loaded with microcrystalline cellulose had consistently higher production rates (**Figure 3b**). Positive vessels had an average rate of biogas production of 479 normal milliliters (Nml) per day, while negative vessels produced an average of 57 Nml of biogas per day. Biogas production rates from positive vessels increased twice periodically before returning to about 400 Nml (**Figure 3b**). Production rates reached 718 and 510 Nml per day respectively, during the two periods of increased production (days 10 and 26). Gas production in negative vessels peaked at 99 Nml per day on day 6.

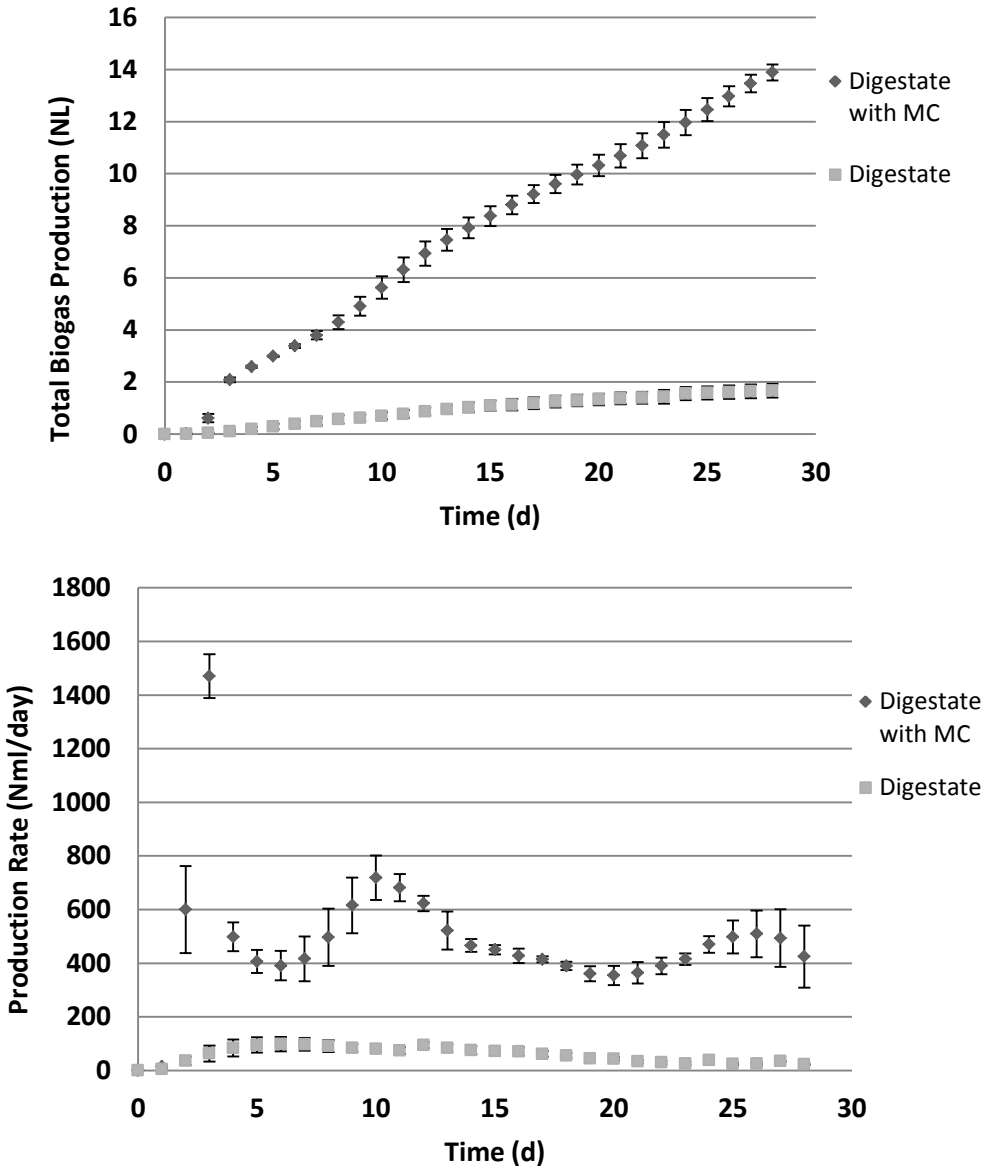

Figure 3. Total biogas production and rate of production from AMPTS-II experiments. Positive vessels (those containing microcrystalline cellulose as a substrate) had greater biogas production (a) and rates of production (b). Standard deviations are shown for every point.

Biogas Volume and Production Rate Results from Eudiometers

Biogas production from two different eudiometer experiments was experimentally determined. In the first trial, digestate with microcrystalline cellulose produced 30.0 L of biogas, and digestate with no additional substrate produced a total of 12.7 L of biogas (**Figure 4a**). Digestate with microcrystalline cellulose produced 18.75 ml biogas/gram of biomass, while digestate alone produced 7.94 ml biogas/gram of biomass (wet weight). The difference between the two production volumes can be attributed to the addition of microcrystalline cellulose to digestate. Total gas production between the two treatments was very similar in the first 11 days of fermentation. At 11 days, digestate with microcrystalline cellulose had produced 7.74 L of biogas, and digestate alone produce 6.64 L of biogas. In the following 17 days, digestate with microcrystalline cellulose produced 22.26 L and digestate alone only produced 6.06L of biogas. In the same trial, digestate with microcrystalline cellulose had an average gas production rate of 1603.5 ml/hr, and the average gas production for digestate alone was 463.0 ml/hr. Gas production rates of digestate with microcrystalline cellulose were highlighted by sporadic distribution of greatly increased periods of production, but generally increased after day 11. Digestate alone had more consistent gas production rates with no periods of production surpassing 1200 ml of biogas/hour (**Figure 4b**).

In the second eudiometer trial, digestate with microcrystalline cellulose and digestate alone produced 27.0 L of biogas and 5.73 L of biogas respectively (**Figure 5a**). Based on the total biogas produced, digestate with microcrystalline cellulose produced 33.75 ml biogas/gram biomass (wet weight) and digestate alone produced 7.16 ml

biogas/gram of biomass (wet weight). Average gas production rates for digestate with microcrystalline cellulose and digestate alone were 1003.5 ml/hr and 270.9 ml/hr, respectively (**Figure 5b**).

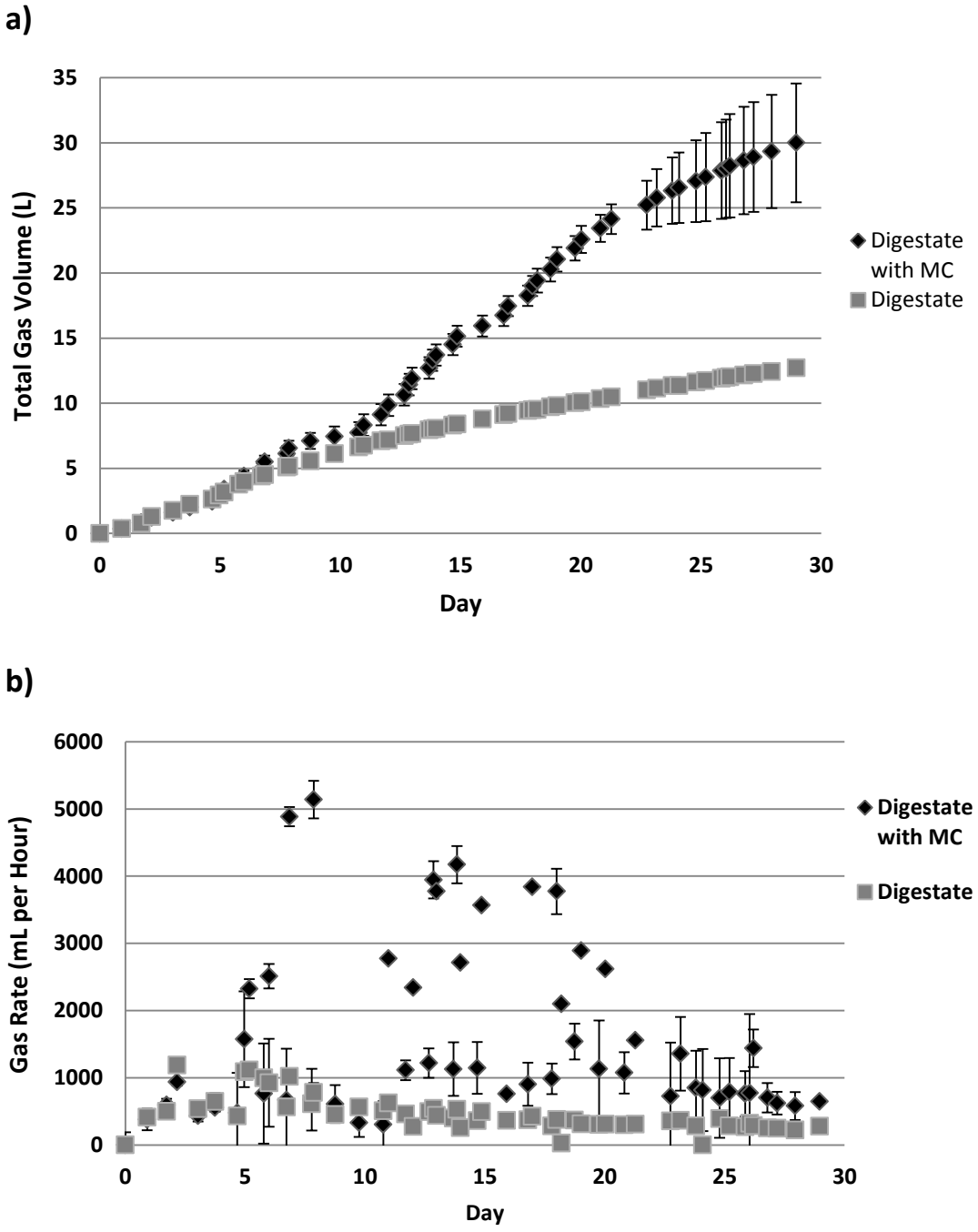

Figure 4. Total gas production volumes and rates of production from eudiometer trial #1. After day 7, Digestate with Microcrystalline Cellulose (MC) produced more biogas (a) and had consistently higher production rates (b). Standard deviations are shown for each point.

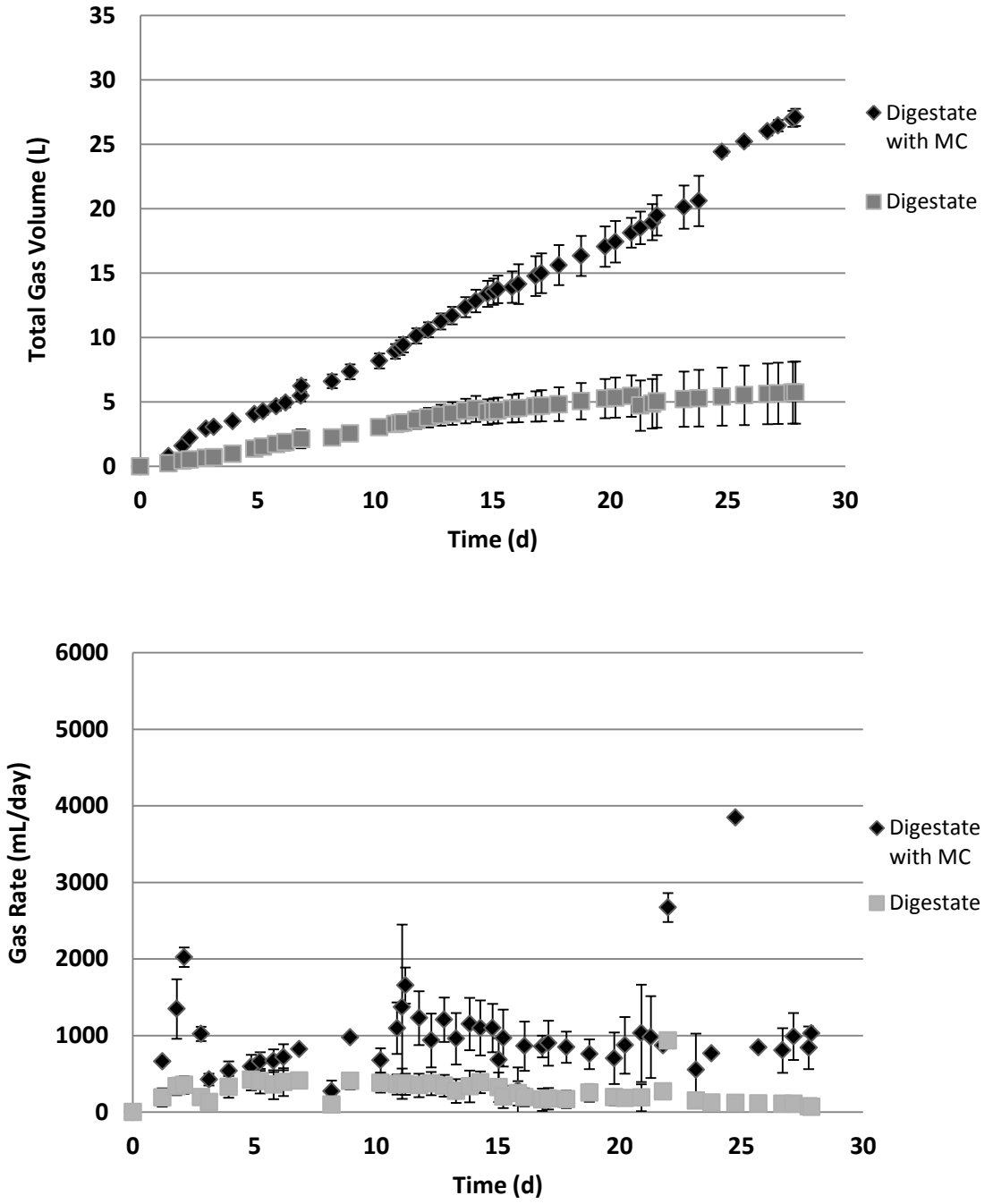

Figure 5. Total gas production volumes and rates of production from eudiometer trial #2. Digestate with Microcrystalline Cellulose (MC) consistently produced more biogas (a) and had a higher rate of production (b). After day 10, production was usually close to 1000 ml/day. Standard deviations are shown for each point.

Gas Quality

Biogas from eudiometers containing digestate with microcrystalline cellulose had averages of 38.0, 53.0, 50.1, and 64.2% methane at 7, 14, 21, and 28-day time points, respectively. Biogas from negative control eudiometers averaged 39.9, 59.7, 63.0, and 59.8% methane at 7, 14, 21, and 28-day time points, respectively (**Figure 6**). Biogas from eudiometers containing digestate with microcrystalline cellulose had averages of 62.0, 46.4, 49.8, 35.8% carbon dioxide at 7, 14, 21, and 28-day time points, respectively. Biogas from eudiometers with only digestate averaged 33.2, 33.9, 35.0, 37.7% carbon dioxide at 7, 14, 21, and 28-day time points, respectively (**Figure 7**).

In eudiometers containing digestate with microcrystalline cellulose, the biogas started with lower percent methane content and trended upward, whereas the percent carbon dioxide started out high and declined over time. Biogas from digestate alone started out with low methane and carbon dioxide content (at or below 20%) and increased every day until levelling off around day 14 (**Figures 8 and 9**).

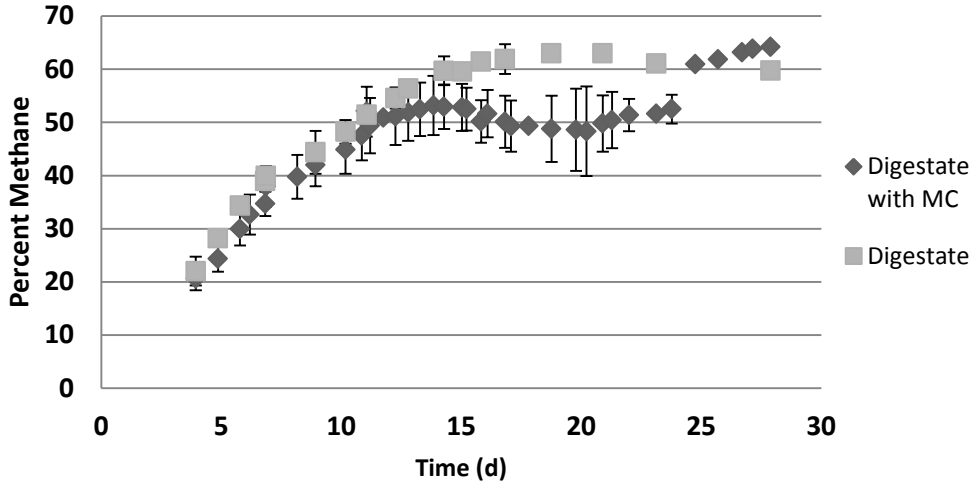

Figure 6. Average percent methane of biogas produced from digestate. Digestate loaded with microcrystalline cellulose (MC) initially produced biogas with lower percent methane than that produced from digestate alone. Standard deviations are shown for each point.

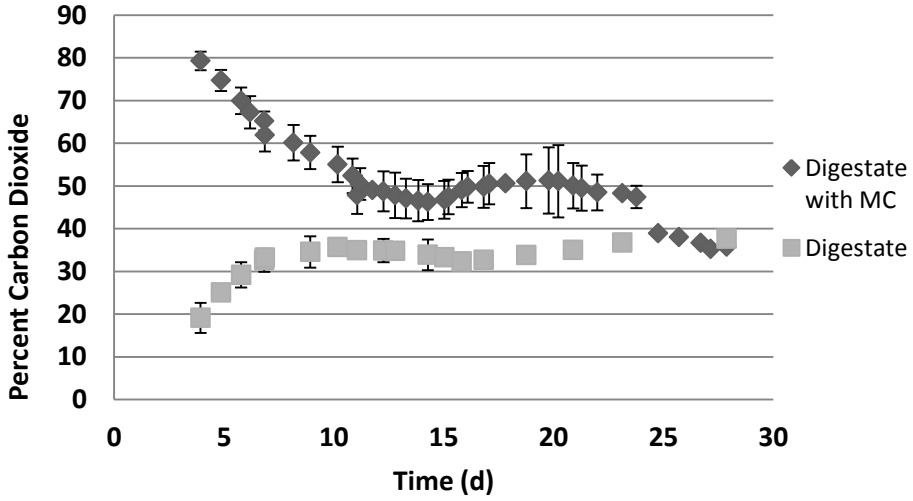

Figure 7. Average percent carbon dioxide of biogas produced from digestate. Digestate loaded with microcrystalline cellulose (MC) had higher initial carbon dioxide production than the negative control digestate. Standard deviations are shown for each point.

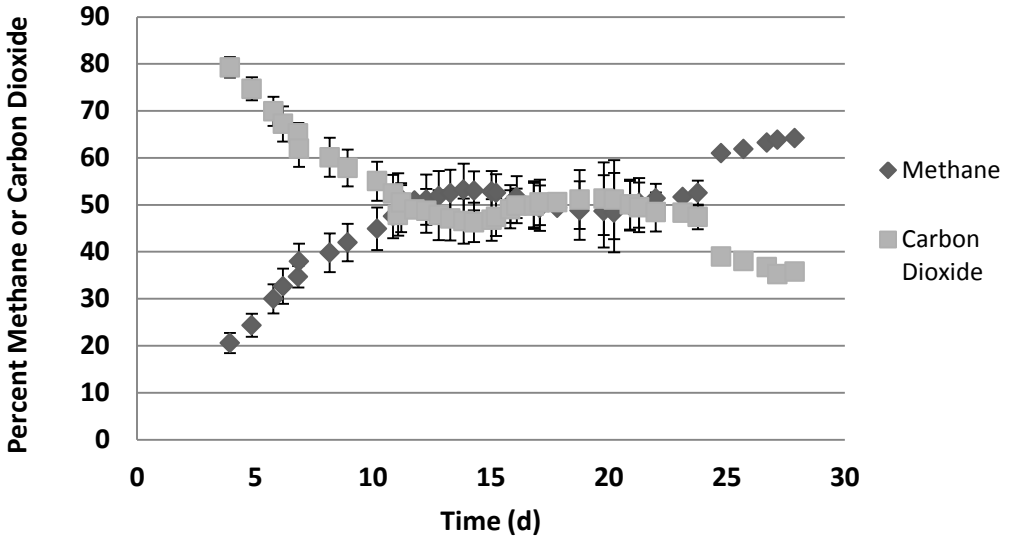

Figure 8. Methane and carbon dioxide production from digestate loaded with microcrystalline cellulose. Initial methane composition was at about 20 percent, but increased to roughly 65 percent by day 28. Standard deviations are shown for each point.

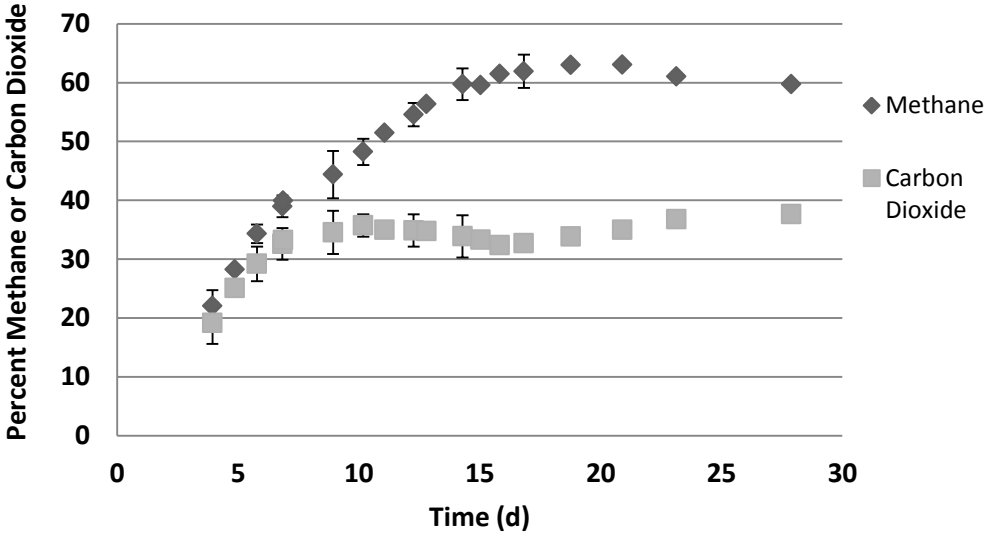

Figure 9. Methane and carbon dioxide production from digestate only. Methane and carbon dioxide both made up 20 percent of the total gas before day 5. After day 7, carbon dioxide leveled off at 35 percent, while methane percentages continued to increase. Standard deviations are shown for each point.

Dry Matter, Organic Dry Matter, and pH Measurements

The dry matter (DM) and organic dry matter (oDM) of digestate with microcrystalline cellulose from biogas experiments was measured at four-day intervals. DM remained consistent around 30%, while oDM content varied slightly over the 28-day period. oDM began at approximately 52%, and declined slowly but consistently until reaching about 44%. The pH of the digestate began fairly basic, at about 8.5, and increased to 8.9 by day 28 of the experiment.

DM in digestate alone samples was measured every seven days. While DM from digestate alone began roughly 10% lower than that found in digestate with microcrystalline cellulose, DM increased to about 30% by the end of the experiment, indicating that evaporation could be occurring as biogas is being produced. oDM began around 47% and ended at roughly 54% by the end of the trial, which is comparable to the oDM found in the digestate with MC samples (**Table 2**).

Table 2. Dry matter (DM) and organic dry matter (oDM) percent total mass of digestate with and without microcrystalline cellulose (MC). – Indicates samples not processed.

	Digestate with MC		Digestate Only	
Day	**DM**	**oDM**	**DM**	**oDM**
4	29.828	51.865	-	-
7	-	-	18.22	47.381
8	29.135	50.872	-	-
12	32.131	49.786	-	-
14	-	-	24.011	54.084
15	30.048	49.164	-	-
20	29.195	46.345	-	-
21	-	-	23.098	54.164
24	26.779	49.465	-	-
28	29.118	44.177	30.699	39.098

Real-time PCR Methanogen Analyses

Two different orders of methanogens were quantified using real-time polymerase chain reaction (qPCR). For each sample, DNA from 0.25 grams of digestate was extracted, and gene copies were quantified for the 16S ribosomal RNA (rRNA) genes in Methanomicrobiales (MMB) and Methanosarcinales (MSL) over a 28-day period. Gene copy concentration data was normalized to reflect gene copies per wet weight grams of digestate. A one-way ANOVA was conducted using Minitab software on the entire data set, showing that differences between means existed ($p<0.001$). Tukey's HSD (95% confidence interval) was used to determine which individual means were different or similar to one another. In eudiometers loaded with digestate with microcrystalline cellulose, MSL was present in consistently higher densities than MMB. MSL concentrations began about 10^7 gene copies per gram digestate, dropped to 3.2×10^6 after 14 days, and then increased in concentration until reaching 10^7 gene copies per gram digestate at day 28. MMB began the experiment at 2.5×10^4 copies per gram digestate and increased at day 7 to 10^5 copies per gram digestate. MMB copy concentrations decreased at days 14 and 21, and then increased again at day 28 (**Figure 10**).

In biogas experiments where there was no substrate mixed into the digestate, MSL gene copies stayed consistent at about 10^7 gene copies per gram digestate throughout the entire experiment. MMB copies began the 28-day experiment at 3.2×10^5 copies per gram digestate and fluctuated around 3.2×10^4 and 10^5 copies at each time point measured (**Figure 11**).

MMB 16S rRNA gene copy concentrations differed minimally between digestate with microcrystalline cellulose and digestate alone samples. Samples containing digestate alone began with higher MMB concentrations (**Figure 12**). The fluctuation in 16S rRNA gene concentration between the two types of samples was the same, however. There was a decrease from day 7 to day 21, and an increase from days 21 to 28. Aside from the order of magnitude difference between the two samples at day 0, the gene copy concentrations were statistically similar.

MSL copies began above 10^7 copies per gram digestate in both digestate with microcrystalline cellulose and digestate alone samples. At day 7, there was a one-half order of magnitude difference between the two types of samples, with digestate alone decreasing to about 3.2×10^6 copies per gram digestate and digestate with microcrystalline cellulose increasing to 1.6×10^7 copies per gram digestate. From days 14-28, MSL 16S rRNA copy numbers remained within one order of magnitude of each other (**Figure 13**).

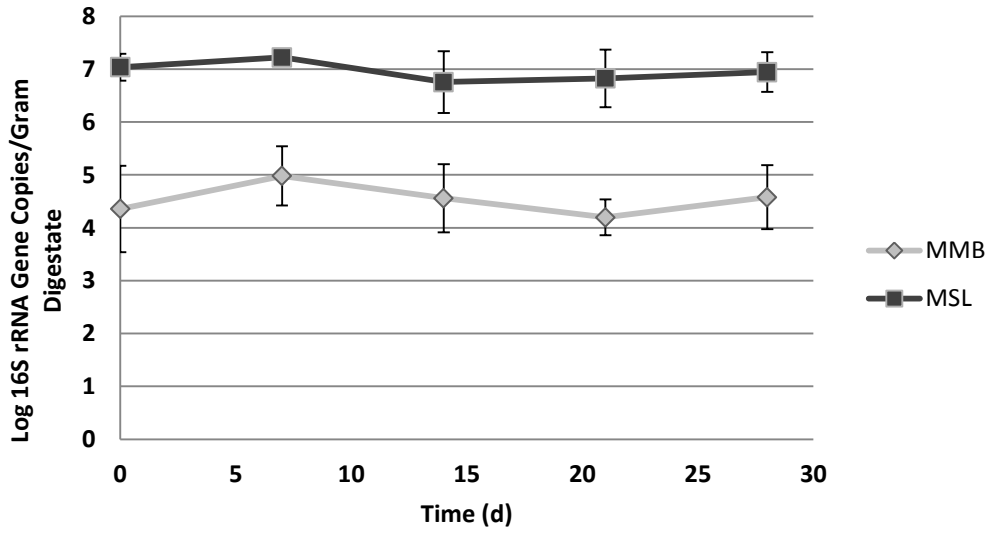

Figure 10. Methanomicrobiales and Methanosarcinales 16S rRNA gene copy concentrations in digestate with microcrystalline cellulose eudiometers. Methanosarcinales (MSL) was consistently more present over the tested period than Methanomicrobiales (MMB). Standard deviations are shown for each point. Tukey's HSD test showed that each time-point had significantly different means.

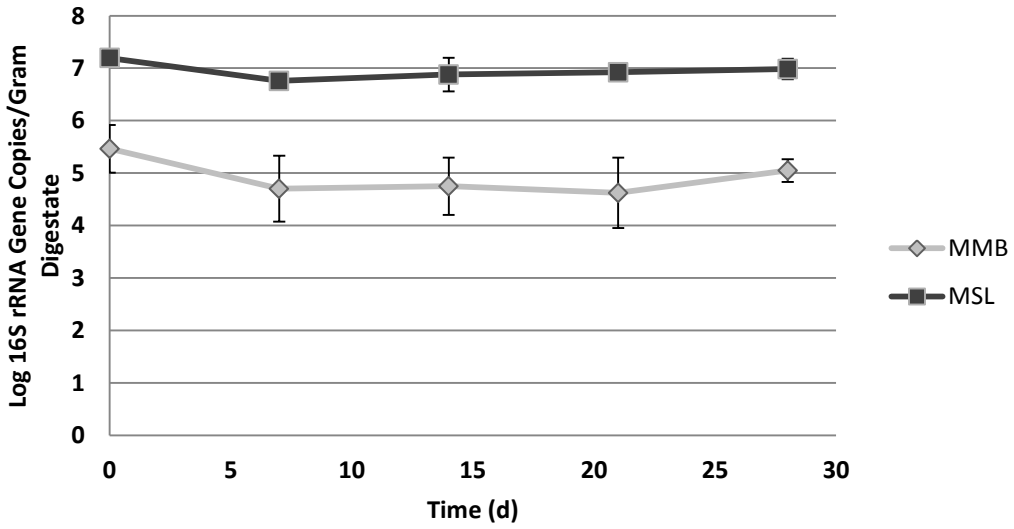

Figure 11. Methanomicrobiales and Methanosarcinales 16S rRNA gene copy concentrations in digestate with no substrate. Methanosarcinales (MSL) was consistently more present over the tested period than Methanomicrobiales (MMB). Standard deviations are shown for each point. Tukey's HSD test showed that each time-point had significantly different means.

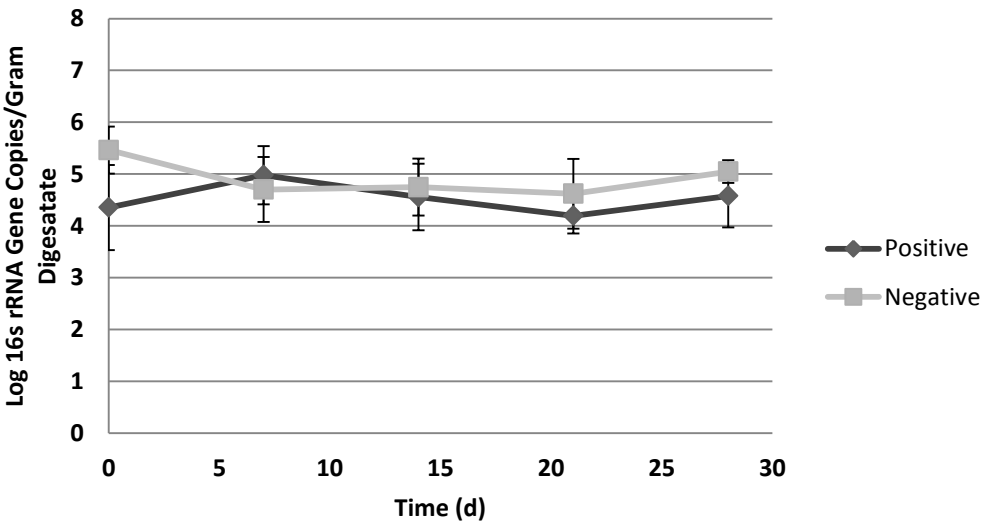

Figure 12. 16S rRNA gene copy numbers of Methanomicrobiales in digestate. 16S rRNA gene copy numbers were quantified in digestate with microcrystalline cellulose samples (Positive) and digestate only samples (Negative). Standard deviations are shown for each point. Tukey's HSD test showed that only the data points at day 0 had significantly different means.

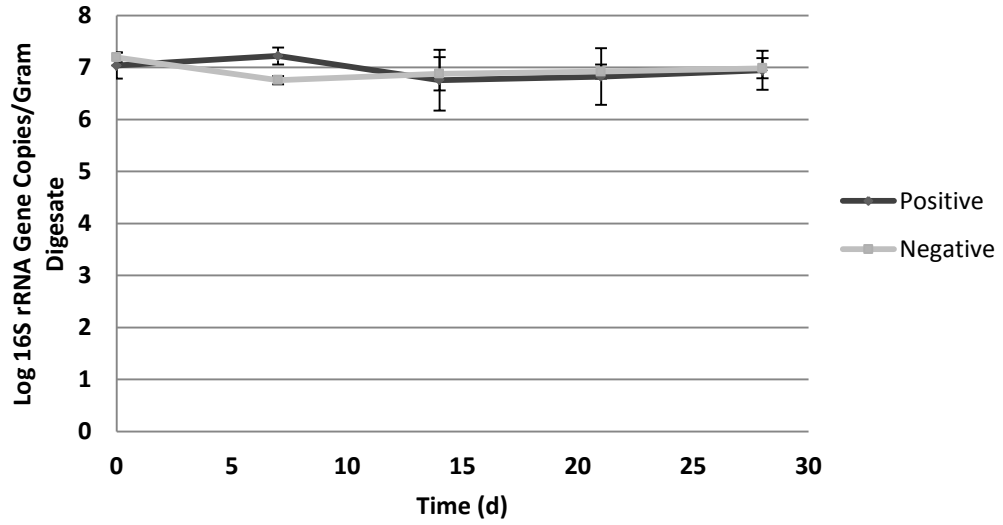

Figure 13. 16S rRNA gene copy concentrations of Methanosarcinales in digestate. 16S rRNA gene copy numbers were quantified in digestate with microcrystalline cellulose samples (Positive) and digestate only samples (Negative). Standard deviations are shown for each point. Tukey's HSD test showed that only the data points at day 14 had significantly different means.

DISCUSSION

Microbial Characterization of UWO Digester

Analysis of pyrosequencing data indicates a large presence of phyla Firmicutes, Bacteroidetes, Tenericutes, and Euryarcheota. The dominance of anaerobic or facultative anaerobic bacteria was an expected outcome of the metagenomic analysis, as the majority of a fermentation cycle is spent in anoxic conditions. The change-out portion of the digestion process is an operationally unique aspect of dry anaerobic digesters, and it could be that aerobic or facultative aerobic bacteria are able to replicate during this time and the following few days when oxygen is still present within the digester. Depending on the timing of sampling, metagenomic data may shift slightly towards bacteria capable mainly of oxygen-respiration; however it is likely, given their overwhelming dominance, that obligatory anaerobic families such as Clostridiales consistently outnumber the other types of bacteria. These bacterial and archaeal metagenomic results are consistent with previous culture-independent studies looking into bovine intestinal microbial communities [12, 13, 31, 39].

Within Euryarcheota, there was a large constituency of the orders Methanosarcinales and Methanomicrobiales. Organisms within Methanosarcinales are metabolically diverse, and are able to produce methane using acetoclastic and/or carbon dioxide reducing pathways, and organisms within Methanomicrobiales are only capable of producing methane by reducing carbon dioxide or utilizing formate or alcohols.

The notable presence of Methanomicrobiales organisms, which have very specific metabolic needs, reflects the fact that biodigesters often contain a large percentage of carbon dioxide. Methanomicrobiales spp. are able to subsist during fermentation by reducing carbon dioxide, and it is hypothesized that they are able to survive periods of oxygen-exposure due to their ability to produce super oxide dismutase [24]. Since dry anaerobic digesters are less common than wet anaerobic digesters, little data exists on this type of digester, and long-term studies should be considered to determine if the taxonomic profile is specific to the manure-source used in digester start-up, how the profile changes and each digestion cycle, and the effect of different feedstocks on the taxonomy and biogas production of a digester.

Similar to bacterial taxonomy, archaeal populations may shift significantly at different points in the digestion process. Although this study probed the gene concentrations for two orders of Archaea in a bench-scale digestion, a broader view of Archaea at all taxonomic levels during different time-points of digester operation (ie, change-out and daily sampling during fermentation) could help to elucidate better how operational practices and feedstock variation affects microbial population shifts. Due to the cost and operational restraint to sampling, these presented metagenomic data were used strictly as a screening tool to determine the best possible methanogenic targets for qPCR analysis. Ideally, multiple samples would have been taken, and a molecular target would have been established base on that dataset as a whole.

Although similar data exist for metagenomic studies of wet anaerobic digesters, the physical and operational differences between types of digesters may have significant

impacts on the taxonomic profile of microbial populations, which could be elucidated by long-term and comparative studies. Aside from this study, I am not aware of any data detailing the taxonomic differences between wet and dry digesters, nor any data showing a complete taxonomic profile of multiple points in time in a dry anaerobic digester. These data may be useful in the future to tailor coculture, chemical, or feedstock amendments to a specific digester, when used in conjunction with the current methodologies for determining digester functionality or efficiency.

Biogas Volume and Quality Analysis

Biogas volume differences between the digestate with microcrystalline cellulose and the negative control digestate were notable. Digestate which was not supplemented with microcrystalline cellulose had much lower overall gas production than digestate with microcrystalline cellulose. This is expected because microbes are able to use the microcrystalline cellulose (when present) as a source of carbon. When there is no substrate present, the microbes are forced to use the minimal amount of nutrients that remain in the digestate. If bacteria do not have an abundant source of carbon to metabolize, they will not replicate to greater cell concentrations, and the hydrolysis is thusly inhibited. During normal metabolic processes, bacteria (especially *Clostridium* spp. and *Acetobacter* spp.) produce the long and short chain fatty acids needed for acetogenic and methanogenic steps of methanogenesis. In anaerobic digestion, fatty acids are broken down by bacteria and used by archaea to make methane, so it stands to reason that providing a carbon source for the microbes will increase hydrolytic activity

and fatty acid production within the digestate which provides methanogens the metabolic precursors needed to produce methane. The effects of the limitation on the hydrolytic step of methanogenesis are apparent when examining the volume of biogas production along with the quality (ie methane and carbon dioxide content) of the gas produced.

Biogas from digestate with microcrystalline cellulose had lower initial methane percentages than biogas produced from negative control digestate. Digestate with microcrystalline cellulose had higher initial carbon dioxide content. In the first seven days, the gas composition of digestate with microcrystalline cellulose biogas may have been higher in carbon dioxide because bacteria may be using the microcrystalline cellulose as an energy source and producing carbon dioxide as a byproduct. The limited initial methane production may be due to the time it takes bacteria to proliferate to a level that provides methanogens the substrate needed to produce methane. This means that for the first seven days, nutrients are limited for methanogens which need either carbon dioxide or acetic acid as an energy source to make methane. Also, in the initial days of the experiment, oxygen is present in trace amounts, which prevents strict anaerobes (such as methanogens) from growing. While the presence of oxygen in this experiment likely contributed to initial anaerobic activity, this artifact of the method is essential because it closely reflects the conditions of the industrial-scale digester.

Although methane was a main constituent in biogas from negative control digestate, the digestate did not produce a high volume of biogas. Digestate with no added substrate produced only a small amount of gas high in methane content, which underlines the importance of the addition of feedstock to a digester. If a full-scale digester did not

have feedstock added to it, the digester would likely not produce a high volume of biogas, even though the relatively small volume of biogas produced per unit of substrate would have high methane content.

Methanogen Abundance and Relationship to Methane Production

qPCR analysis showed that Methanosarcinales (MSL) was more abundant than Methanomicrobiales (MMB) in both the digestate with microcrystalline cellulose and the negative control digestate. In digestate with microcrystalline cellulose, MSL gene copies started day seven at about 10^7 and then decreased before increasing as the experiment progressed. This increase in abundance parallels the increase in methane content and total biogas production.

There are three families within the order of MSL, and methanogens within the three families are capable of using carbon dioxide and/or acetic acid to make methane. The wider range of molecules that MSL can use may account for its increased ability to survive and proliferate within digestate at higher concentrations than MMB. Families within the order MMB, however, are only capable of using carbon dioxide or formate as an electron acceptor to produce methane. Since members of MSL are able to use multiple metabolic pathways to produce methane, it is expected that this order has an advantage in growing versus members of MMB, which can use comparatively fewer electron acceptors during methane production

Since methanogens are strict anaerobes and require very specific growth conditions, the study of methanogens using culture-based methods is notoriously

difficult. Due to the difficulty presented by culture-dependent methods of study, culture-independent methods of determining methanogen community dynamics have come into favor recently. While molecular methods have their advantages, there are also several limitations to the techniques used in this study. For example, although real-time PCR is a useful tool for measuring the amount of DNA in a sample, it cannot determine the number of live cells within a sample. A portion of the DNA detected may be naked DNA, which was released into the environment from once-living methanogens after lysis. Also, the real-time PCR method used cannot determine how the cells present are contributing to biogas production. Real-time PCR targeting the 16S rRNA region can be used to determine the number of gene copies present, but it cannot determine how often functional genes are being transcribed or translated. In other words, some cells may not be actively metabolizing substrates to produce methane even though they possess the necessary genes. Lastly, gene copy measurements do not indicate the number of viable cells in the substrate. Some species have multiple copies of their genome in their cells, and the number of genomes per cell can depend on the phase of growth that the organism is in. Cells in exponential phase, for example, may contain more copies of their genome because the DNA is replicated faster than cell division can take place. So, for cells in exponential phase, gene copy number may overestimate the number of cells present in the culture.

Future studies may be able to better elucidate the relationship methanogens and biogas production by using techniques such as reverse transcriptase real-time PCR (RT-qPCR). Reverse transcriptase PCR can be used to determine how often a gene is

transcribed because mRNA is used as the initial template for amplification instead of DNA. Determining the rate of transcription of a functional gene, such as the methyl coenzyme M reductase gene (*mcr*A), would be important to determine whether increases in biogas production are related to the increased translation rates or increased numbers of methanogens. Also of note, *mcr*A appears to vary enough in sequence between taxa that it can be used as a taxa specific marker, much in the same way that 16S rRNA gene can be used [41]. So, it may be possible to use *mcr*A in future studies to determine which methanogens are transcribing *mcr*A, and are therefore contributing to methanogenesis.

FUTURE WORKS

In order to fully understand the dynamics of methanogenesis in anaerobic digestion, especially within dry anaerobic digesters, the following efforts should be considered:

- Obtain a comprehensive picture of the microbial ecology changes that occur during digestion by using metagenomic studies to determine all of the different types and abundances of methanogens within the system.
- Use qPCR or other molecular methods to determine the change over time in microbial ecology for groups not tested in this study.
- Determine the impact of different feedstocks on microbial populations, gas production, and chemical characteristics within digestate.
- Explore methods to increase gas quantity and quality, such as inoculating digestate with a defined culture of methanogens or using amendments to alter redox potential or pH.
- Determine the impact of different bacteria on the production of methane from various feedstocks.
- Investigate procedures which reduce the amount of time it takes bacteria to hydrolyze long chain fatty acids. These should include, but are not limited to, mechanical or chemical digestion of feedstocks.

APPENDIX A

Eudiometer Setup

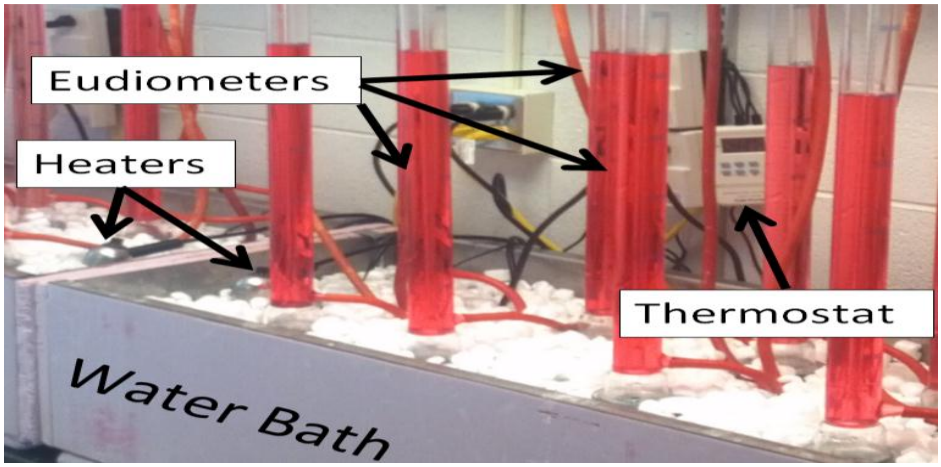

Figure A-1. Eudiometer setup. Eudiometers were placed in a 39°C water bath. Water water circulated using submersible pumps (not shown), and temperature was maintain using heaters and thermostat switches.

APPENDIX B

Eudiometer and AMPTS-II Experimental Schematics

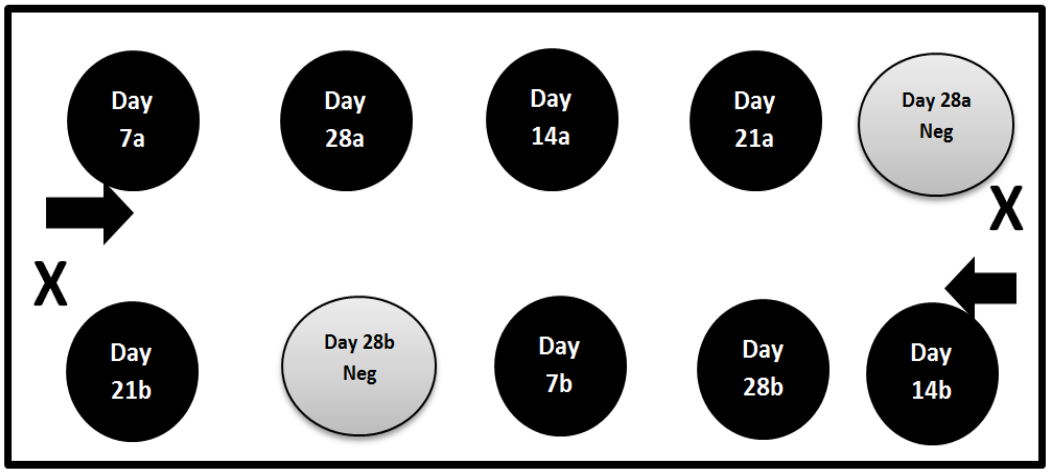

Figure B-1. Schematic of a typical layout of eudiometers for bench-scale gas production and qPCR analysis. Water baths for gas and microbial ecology analysis contained duplicate positive (●) or negative (●) control eudiometers. The duplicate eudiometers for each time point were placed randomly in the bath. "X" denotes the placement of the heaters, and the arrows indicate the placement and flow direction of water circulation pumps. Time points 7-28 were captured for negative control eudiometers using this scheme as well.

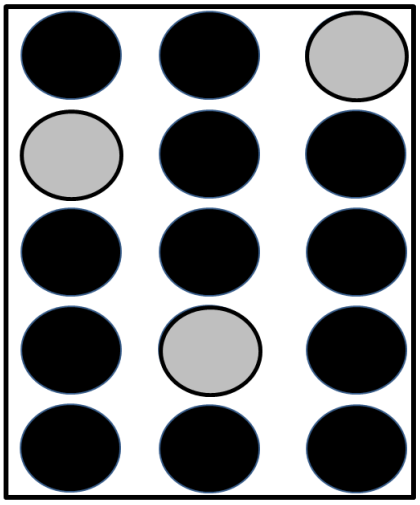

Figure B-2. Experimental layout of samples used in AMPTS-II testing. Digestate samples in the water bath containing microcrystalline cellulose are shown as black circles (●), and digestate samples not containing microcrystalline cellulose are denoted by grey circles (●).

APPENDIX C

Standard Curve for Methanomicrobiales qPCR

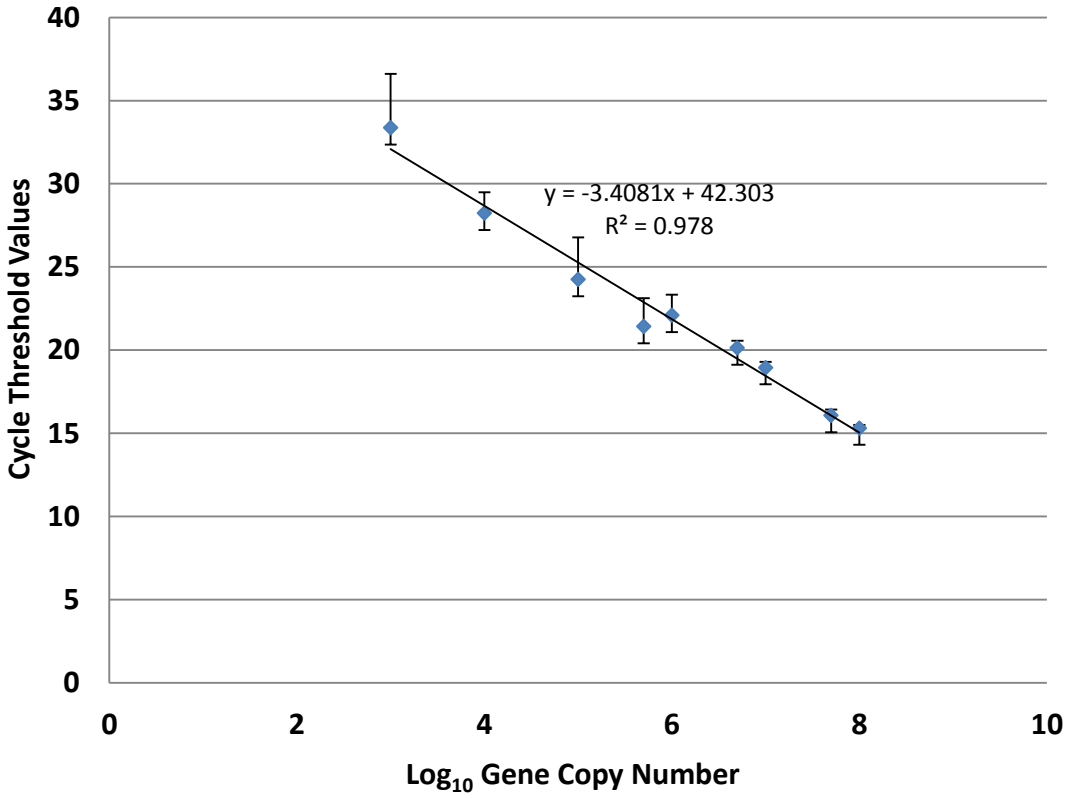

Figure C-1. Standard curve for Methanomicrobiales 16S rRNA. Cycle threshold values for 10^3 through 10^8 16S rRNA gene copies are shown. Standard deviation is shown for each point, and the R^2 value was 0.9780. No signal was detected for gene copy numbers less than 10^3.

APPENDIX D

Standard Curve for Methanosarcinales qPCR

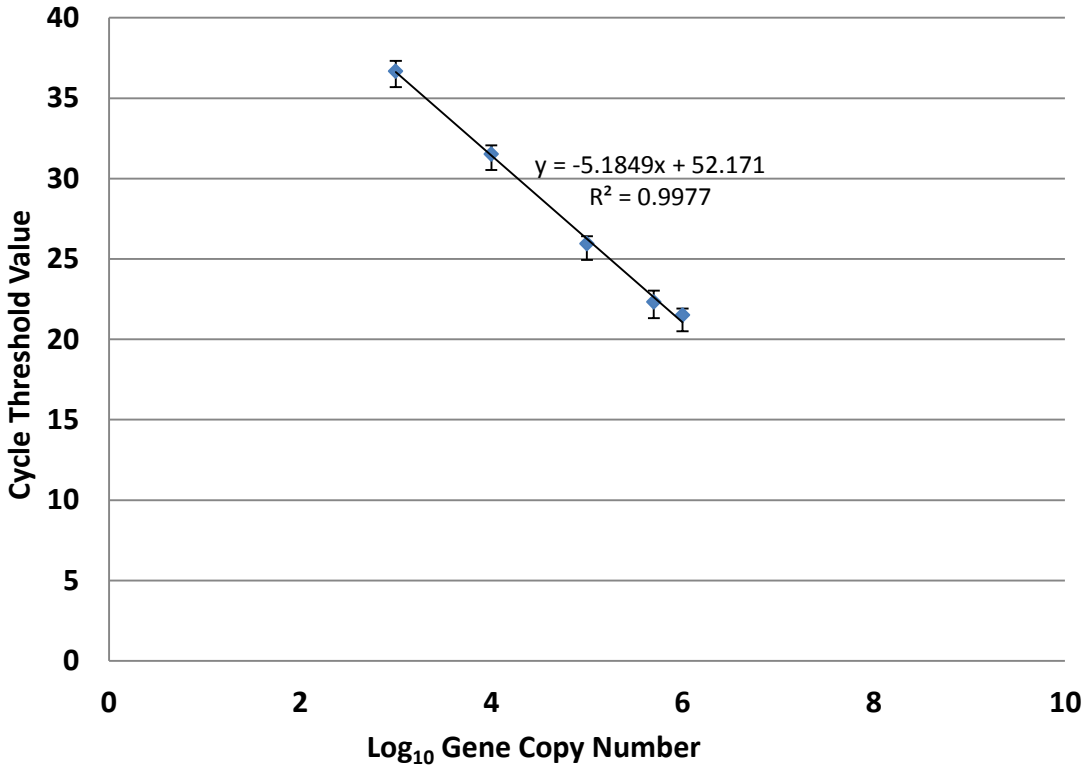

Figure D-1. Standard curve for Methanosarcinales 16S rRNA. Cycle threshold values for 10^3 through 10^6 16S rRNA gene copies are shown. Standard deviation is shown for each point, and the R^2 value was 0.9977. No signal was detected for gene copy numbers less than 10^3.

APPENDIX E

Metabolic Overview of Methanogenesis

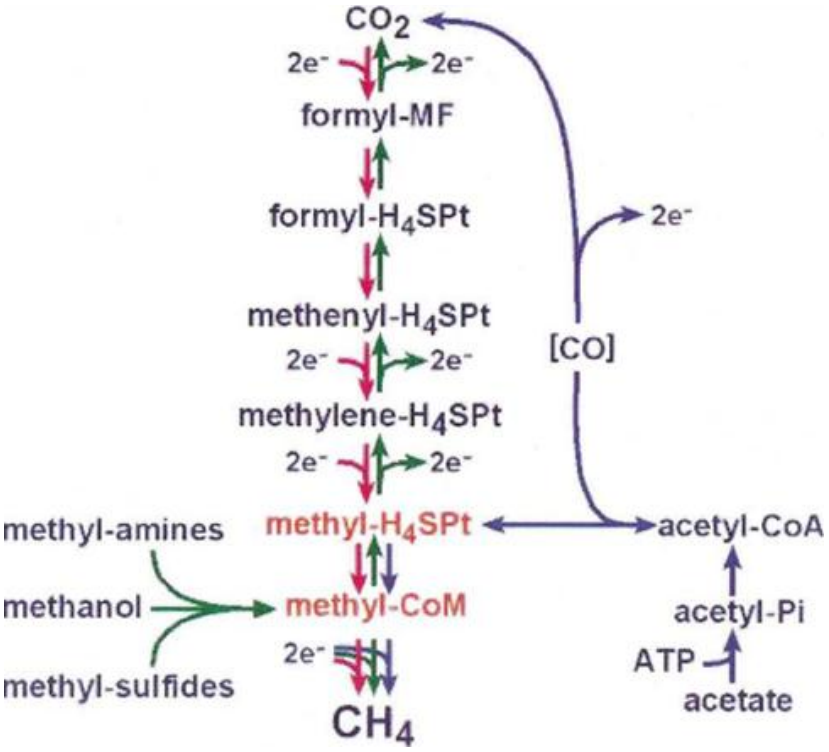

Figure E-1. Metabolic overview of methanogenesis. Three main pathways are known for methanogenesis: methylotrophic, hydrogenotrophic, and acetoclastic.

APPENDIX F

Family-Level Analysis of Archaeal Metagenomic Data

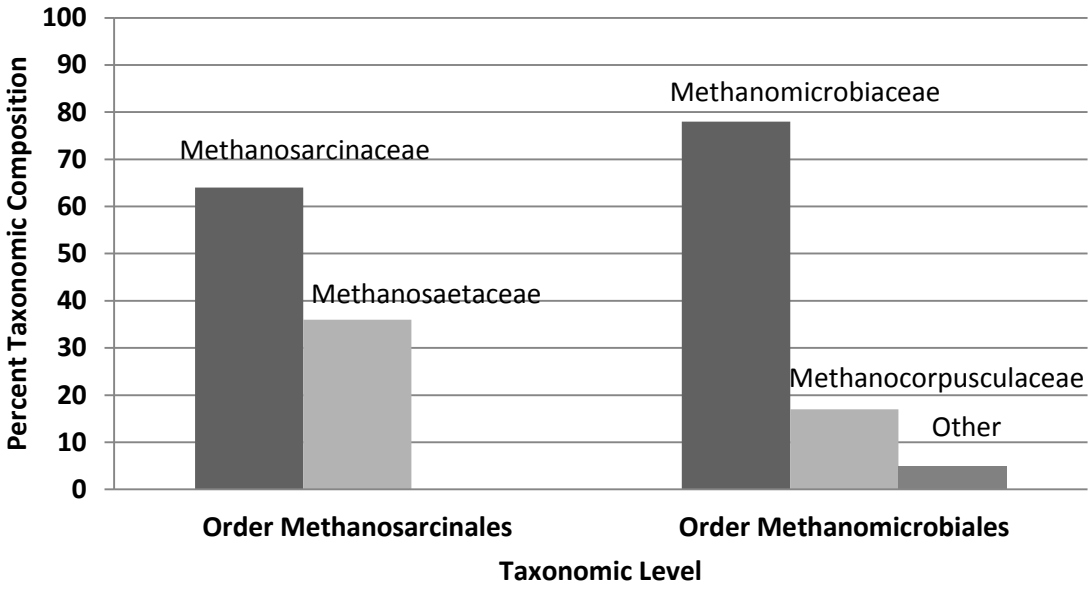

Figure F-1. Family-level analysis of archaeal metagenomic data. Analysis of pyrosequencing data showed that the main families within Methanosarcinales were Methanosarcinaceae and Methanosaetaceae, and the main families within Methanomicrobiales were Methanomicrobiaceae and Methanocorpusculaceae.

APPENDIX G

Statistical Data from ANOVA Tests

Table G-1. Statistical Data from ANOVA Tests. One-way ANOVA tests conducted for qPCR data from each time point yielded at p-value less than 0.001. For each test, α=0.05, and the null hypothesis stated that the means within each group were statistically similar. Further analysis using Tukey's HSD test showed which means were different.

	DF1	DF2	F-Statistic$_{Test}$	F-Statistic$_{0.05;\ DF1,\ DF2}$	Accept/Reject H_0
Day 0	3	15	50.87	3.29	Reject
Day 7	3	58	124.55	2.79	Reject
Day 14	3	54	78.93	2.79	Reject
Day 21	3	67	136.55	2.76	Reject
Day 28	3	82	226.82	2.72	Reject

REFERENCES

1. **American Biogas Council** What is anaerobic digestion? https://www.americanbiogascouncil.org. Accessed 25 September 2014

2. Amon T, Amon B, Kryvoruchko V, Machmüller A, Hopfner-Sixt K (2007) Methane production through anaerobic digestion of various energy crops grown in sustainable crop rotations. Bioresour Technol 98:3204-3212

3. Appels L, Baeyens J, Degrève J, Dewil R (2008) Principles and potential of the anaerobic digestion of waste-activated sludge. Prog Energ Combust 34:755-781

4. Arnold M (2009) Reduction and monitoring of biogas trace compounds. VTT Research Notes 2496 ISBN 978-951-38-7314-1

5. Basiliko N, Yavitt JB, Dees PM, and Merkel SM (2003) Methane biogeochemistry and methanogen communities in two northern peatland ecosystems, New York state. Geomicrobiol J 20:563-577

6. California Integrated Waste Management Board (2014) Renewable bioplastics and biocomposites from biogas methane and waste-derived feedstock: development of enabling technology, life cycle assessment, and analysis of costs. Publication # DRRR-2014-01502. California Environmental Protection Agency, Sacramento, California

7. California Integrated Waste Management Board (2008) Current anaerobic digestion technologies used for treatment of municipal organic solid waste. Publication #IWMB-2008-011. California Environmental Protection Agency, Sacramento, California

8. Castro H, Ogram A, Reddy KR (2004) Phylogenetic characterization of methanogenic assemblages in eutrophic and oligotrophic areas of the Florida Everglades. Appl Environ Microbiol 70:6559-6568

9. DeBruyn J, Hilborn D (2007) Anaerobic digestion basics. Order No. 07-057. Ministry of Agriculture, Food, and Rural Affairs, Ontario, Canada

10. DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685-5689

11. Deublein D, Steinhauser A (2011) Biogas from waste and renewable resources, 2nd ed. Wiley-VCH Verlag GmbH & Co, Weinheim

12. Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, Edrington TS (2008) Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol 8: 125

13. Durso LM, Harhay GP, Smith TPL, Bono JL, DeSantis TZ, Harhay DM, Andersen GM, Keen JE, Laegreid WW, Clawson ML (2010) Animal-to-animal variation in fecal microbial diversity among beef cattle. Appl Environ Microbiol 14:4858-4862

14. Fernandez AS, Hashsham S, Dollhopf SL, Raskin L, Glagoleva O et al (2000) Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microbiol 66:4058-4067

15. Ferry JG (2010) The chemical biology of methanogenesis. Planet Space Sci 58:1775-1783

16. Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P et al. (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. Genome Res 12:532–542

17. Gantner S, Andersson AF, Alonso-Sáez L, Bertilsson S (2011) Novel primers for 16S rRNA-based archaeal community analysis community analysis in environmental samples. J Microbiol Meth 84:12-18

18. Griffin ME, McMahon KD, Mackie RI, Raskin L (1998) Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol Bioeng 57:342-355

19. Gunaseelan VN (2004) Biochemical methane potential of fruits and vegetable solid waste feedstocks. Biomass and Bioenergy 26:389-399

20. Hansen J, Sato M, and Ruedy R (2012) Perception of climate change. Proc Natl Acad Sci USA 109:E2415-E2423

21. Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y (2006) Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. Appl Environ Microbiol 72:1623-30

22. Horne AJ, Lessner DJ (2013) Assessment of the oxidant tolerance of *Methanosarcina acetivorans*. FEMS Microbiol Lett 343:13-19

23. Jaenicke S, Ander C, Bekel T, Bisdorf R, Dröge M et al (2011) Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing. PLoS One 6:e14519

24. Kato, MT, Field JA, Lettinga G (1997) Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. Braz J Chem Eng 14:4

25. Kurian R, Slade J, Holliday M, Liver S, Derjugin W (2012) Avoiding indigestion: start-up of anaerobic digesters. WEAO 2012 Technical Conference, Ottawa, Ontario

26. Lee C, Kim J, Hwang K, O'Flaherty V, Hwang S (2009) Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. Water Res 43:157-165

27. Liu WT, Chan OC, Fang HHP (2002) Microbial community dynamics during start-up of acidogenic anaerobic reactors. Water Res 36:3203-3210

28. Lono-Batura M, Qi Y, Beecher N (2012) Biogas production and potential from U.S. wastewater treatment. BioCycle (53) 12:46

29. Lueders T, Chin KJ, Conrad R, Friedrich M (2001) Molecular analyses of methyl-coenzyme M reductase alpha-subunit (*mcr*A) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ Microbiol 3:194-204

30. Lynd LR, Weimer PJ, van Zyl WH, and Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506-577

31. Mata-Alvarez J, Mace S, Llabres P (2000) Anaerobic digestion of organic solid wastes: An overview of research achievements and perspectives. Bioresour Technol 74:3-16

32. McGarvey JA, Miller WG, Sanchez S, Stanker L (2004) Identification of bacterial populations in dairy wastewaters by use of 16S rRNA gene sequences and other genetic markers. Appl Environ Microbiol 7:4267-4275

33. McMahon KD, Zheng DD, Stams AJM, Mackie RI, Raskin L (2004) Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. Biotechnol Bioeng 87:823-834

34. Narihiro T, Sekiguchi Y (2011) Oligonucleotide primers, probes, and molecular methods for the environmental monitoring of methanogenic archaea. Microbial Biotechnol 4:585–602

35. Nelson MC, Morrison M, Yu Z (2011) A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour Technol 102:3730-3739

36. Pardeep P, Aina T, Stone DA, Stott PA, Nozawa T, Hilberts AGJ, Lohmann D, Allen MR (2011) Anthropogenic greenhouse gas contribution to flood risk in England and Wales in autumn 2000. Nature 470: 382–385

37. Popat SC, Deshusses MA (2008) Biological removal of siloxanes from landfill and digester gases: opportunities and challenges. Environ Sci Technol 42:8510-8515

38. Ramos I, Fdz-Polanco M (2013) The potential of oxygen to improve the stability of anaerobic reactors during unbalanced conditions: results from a pilot-scale digester treating sewage sludge. Bioresour Technol 140:80-85

39. Rasi S, Veijanen A, Rintala J (2007) Trace compounds of biogas from different biogas production plants. Energy 32:1375-1380

40. Shin EC, Choi BR, Lim WJ, Hong SY, An CL, Cho KM, Kim YK, An JM, Kang JM, Lee SS, Kim H, Yun HD (2004) Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. Anaerobe 6:313-319

41. Steinberg L, Regan J (2008) Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. Appl Environ Microbiol 74:6663-6671

42. Steinberg L, Regan J (2009) mcrA-targeted real-time quantitative PCR method to examine methanogen communities. Appl Environ Microbiol 75:4435-4442

43. Strauber H, Schroder M, Kleinsteuber S (2012) Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. Energy, Sustainability, and Society 2:13

44. Traversi, D, Villa S, Acri M, Pietangeli B, Degan R, Gilli G (2011) The role of different methanogen groups evaluated by real-time qPCR as high-efficiency bioindicators if wet anaerobic co-digestion of organic waste. AMB Express 1:28

45. US Environmental Protection Agency (2006) Biosolids technology fact sheet: multistage anaerobic digestion. Publication EPA 832-F-06-031. US Environmental Protection Agency, Washington, DC

46. US Energy Information Administration (2009) Emissions of greenhouse gases in the United States 2009. Report Number: DOE/EIA-0573(2009). US Department of Energy, Washington, DC

47. US Environmental Protection Agency (2009) Methane to markets: the US government's methane to markets partnership accomplishments fourth annual report October 2009. US Environmental Protection Agency, Washington, DC

48. US Environmental Protection Agency (2011) Recovering value from waste: anaerobic digester system basics December 2011. US Environmental Protection Agency, Washington, DC

49. US Environmental Protection Agency (2012) US government's global methane initiative accomplishments report December 2012: annual report. US Environmental Protection Agency, Washington, DC

50. US Environmental Protection Agency (2013) Inventory of US greenhouse gas emissions and sinks: 1990-2011. Publication EPA 430-R-13-001. US Environmental Protection Agency, Washington, DC

51. US Environmental Protection Agency (2013) Municipal solid waste in the United States: facts and figures 2011. Publication EPA530-R-13-001. US Environmental Protection Agency, Washington, DC

52. Yu Y, Lee C, Hwang S (2005) Analysis of community structures in anaerobic processes using a quantitative real-time PCR method. Wat Sci Tech 52:85-91

53. Yu Y, Lee C, Kim J, Hwang S (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 89:670-679