Relating Methanogen Community Structure to Function in Anaerobic Wastewater Digesters

Rachel Morris
Marquette University

RELATING METHANOGEN COMMUNITY STRUCTURE TO FUNCTION IN ANAEROBIC WASTEWATER DIGESTERS

by

Rachel L. Morris

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ABSTRACT

RELATING METHANOGEN COMMUNITY STRUCTURE TO FUNCTION IN ANAEROBIC WASTEWATER DIGESTERS

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Anaerobic wastewater treatment is an environmentally and economically beneficial biotechnology in which the degradation of organic compounds in industrial and municipal wastewaters results in the production of methane, an alternative energy source. The degradation of organic waste is carried out by an interdependent microbial community; and the *Archaea* known as the methanogens complete one of the final steps, producing methane. However, the contribution of methanogens and the community ecology of anaerobic digesters are just beginning to be understood. Specifically, links between methanogen community structure and the successful transformation of organic pollutants to methane have not been clearly defined. In order to examine the relationship between anaerobic digester function and methanogen community structure, anaerobic biomass samples were obtained from anaerobic hydrogen/carbon dioxide enrichment cultures, lab-scale anaerobic digesters, and industrial-scale digesters. DNA and cDNA clone libraries using the methanogen-specific gene *mcrA* were generated from the enrichment cultures and two industrial digester samples, and quantitative polymerase chain reaction (qPCR) was used to quantify the *mcrA* genes and transcripts in all of the biomass samples. Phylogenetic analysis of the *mcrA* sequences found in the clone libraries showed differences in the methanogen communities from different anaerobic biomass samples, even from enrichment cultures started with the same seed sludge. Furthermore, comparison of *mcrA* genes and transcripts from the enrichment cultures revealed that some methanogens were more active than others. However, no direct links were found between methanogen diversity and digester function. Tandem qPCR and specific methanogenic activity (SMA) assays showed positive correlation between *mcrA* gene copy number and methane production rates against specific substrates. This result indicates a relationship between the number of methanogens and digester function. Positive correlation was determined between *mcrA* transcript number and SMA only under certain conditions. This study represents the establishment of a direct link between the microbial community in anaerobic biomass and digester function. The data obtained from these studies provides a better understanding of methanogen communities in digesters which can be applied to develop better assays for monitoring the function of anaerobic biomass, and to engineer better microbial communities that produce more methane for use as renewable fuel.

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Background and Significance

1.1 Introduction Methanogens are important members of anaerobic microbial communities in many natural environments, including marine (Colwell et al. 2008, Kormas et al. 2008, Nercessian et al. 2005, Wilms et al. 2007) and freshwater sediments (Banning et al. 2005, Whitby et al. 2004), microbial mats (Orphan et al. 2008), rice paddies (Chin et al. 2004, Lueders et al. 2001), peat (Freitag and Prosser 2009, Freitag et al. 2010, Galand et al. 2005a), the digestive system of ruminants (Guo et al. 2008), the hindgut of termites (37), and the human gastrointestinal tract (Scanlan et al. 2008). Methanogen communities in these environments are critical facilitators of the decomposition of organic matter, as well as global carbon cycling and climate change, because they complete the degradation of organic wastes to carbon dioxide and methane, which are important greenhouse gases.

Methanogens are also important members of communities within the engineered environment of anaerobic waste digesters. Anaerobic wastewater treatment is an established, environmentally and economically beneficial process in which the degradation of organic compounds in industrial and municipal wastewaters results in the production of methane, which can then be used as an alternative energy source. The degradation of waste is carried out by an interdependent microbial community; and methanogens complete the final step, producing methane (Figure 1.1). However, the microbial community in anaerobic digesters has been a black box throughout most of the history of anaerobic waste treatment research (Rivière et al. 2009). Although several

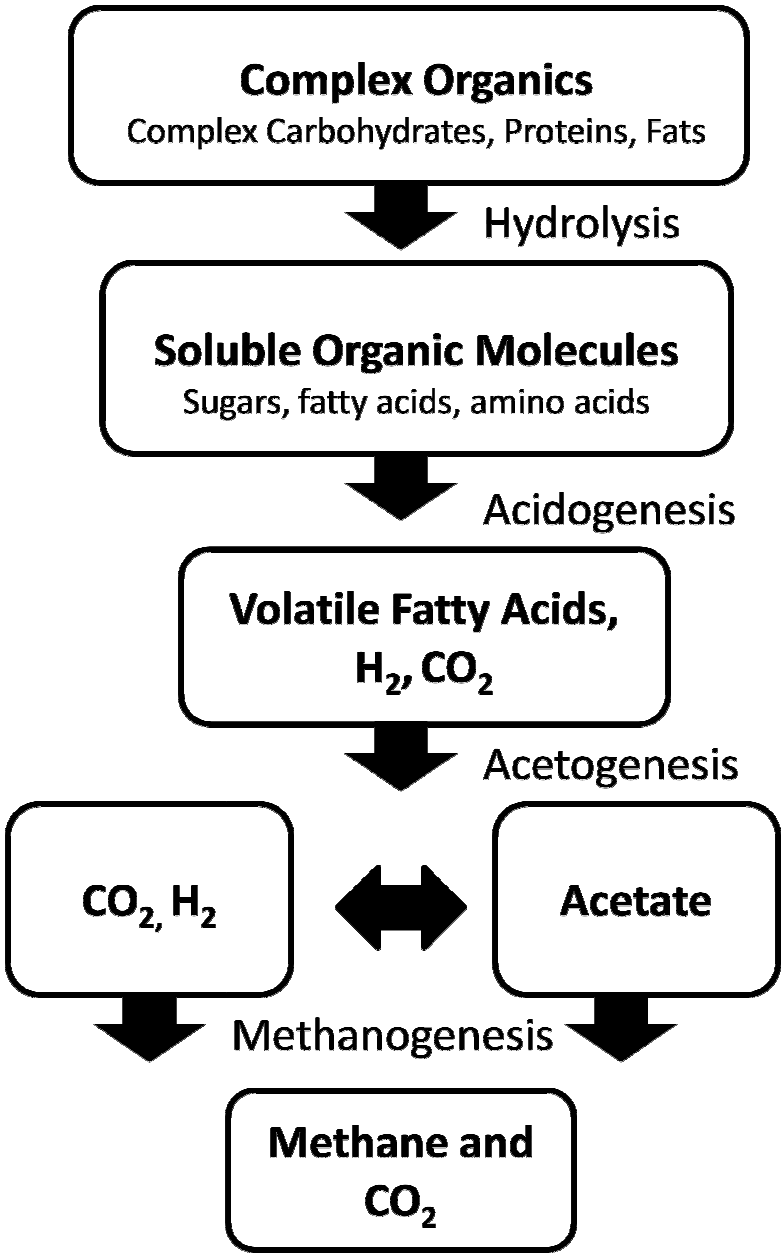


Figure 1.1. The Anaerobic Food Chain. A schematic representing the step-wise degradation of organic waste in anaerobic environments, including anaerobic digesters. Adapted from White, *Physiology and Biochemistry of Prokaryotes* (2000) and Speece, *Anaerobic Biotechnology for Industrial Wastewaters* (1996)**.**

studies have examined the microbial ecology of anaerobic digesters, the microbial communities upon which this biotechnology depends are still not fully understood.

Determining what constitutes a "healthy" methanogen community in anaerobic wastewater digesters operated under different conditions is critical when optimizing treatment systems and biogas production because methanogens play such an important role in the process. In the following chapters, the results of experiments designed to examine the structure and function of methanogen communities in hydrogen/carbon dioxide enrichment cultures, lab-scale anaerobic digesters, and industrial digesters are presented.

1.2 Anaerobic Wastewater Treatment

Recently, concerns about the effect of greenhouse gases on climate and the extent of fossil fuel reserves have generated an increased interest in research that supports the development of renewable sources of energy that have low environmental impacts. Several of the technologies that are being explored utilize microbial communities to generate energy, such as microbial fuel cells (Huang et al. 2011) and the use of cyanobacteria to produce ethanol (Luo et al. 2010). Most of these new technologies do not yet have wide-spread, real world applications. However, anaerobic wastewater treatment is an established process in which a microbial community degrades organic compounds in industrial and municipal wastewaters and produces methane, which can then be used as an alternative energy source.

According to the United Nations World Water Assessment Programme report (2003), every year approximately 1.5×10^{15} L of wastewater are produced worldwide.

As the world's population grows and industry continues to expand, clean water will become an increasingly precious resource. Thus, wastewater treatment is an essential technology, and the potential for energy production from anaerobic wastewater treatment is substantial.

Although there are numerous configurations of wastewater plants, water treatment, even in its simplest forms, can be divided into two types: aerobic and anaerobic. As the names indicate, aerobic treatment requires aeration while anaerobic treatment prevents exposure of the biomass to air. Both have specific advantages and disadvantages, and some facilities combine both types of treatment. The advantages of aerobic treatment include fast startup, rapid growth of biomass (the microbial community), and adaptability (Speece 2008). However, aerobic treatment plants can be very expensive to operate due to electricity demands from pumping and aeration equipment.

Anaerobic waste treatment has several advantages over aerobic systems. First, a large percentage of costs required by aerobic systems can be avoided when anaerobic technology is employed. Anaerobic wastewater treatment obviously does not require aeration of the biomass, which represents a considerable cost reduction (Eckenfelder et al. 2009). Higher organic loading rates are possible in anaerobic systems because physical restrictions on the transfer rate of dissolved oxygen in the biomass, a limiting factor in aerobic treatment, are not a consideration (Eckenfelder et al. 2009). Furthermore, much less excess biomass is produced, reducing disposal costs such as handling, trucking, and landfilling (Ghosh and Pohland 1974). Decreased biomass production and higher loading rates together allow design of anaerobic plants as smaller

facilities than aerobic plants, again representing a considerable cost reduction (Speece 1996) .

Another advantage of anaerobic wastewater treatment is the ability of stored biomass to remain viable for long periods of time without added substrate which is beneficial for industries, such as juice processing or wine making plants, which may only require treatment at certain times of the year (Speece 1996). Furthermore, certain toxic substances, such as tetrachloroethylene, a dry cleaning solvent, can only be degraded anaerobically (Prakash and Gupta 2000, Speece 2008).

As an added benefit, biogas is produced by the anaerobic biomass as the microorganisms degrade the waste. This biogas consists of methane and carbon dioxide, and the methane component can be burned to heat the digester to the necessary operating temperature (usually ~35°C) and to generate electricity (Speece 1996). Thus, biologically produced methane can be used as a renewable alternative to fossil fuels. Furthermore, the burning of methane produces an amount of carbon dioxide which is similar to the amount fixed from the atmosphere to produce the biomass being degraded, making anaerobic wastewater treatment a carbon neutral technology (Zitomer et al. 2008a). Therefore, anaerobic treatment is a "win-win" situation because as wastewater is economically treated, a renewable fuel with low environmental impact is created.

However, while anaerobic treatment is an established technology, it has not yet been optimized and has certain disadvantages as well. One serious disadvantage is the length of time required for the establishment of digester function. Most of the microorganisms which make up the anaerobic digester community are slow growing, and this limits the development of functioning biomass at startup. Slow growth of the organisms is also a factor in the recovery of function when systems fail, which happens because of stress on their microbial community. Both

the establishment of function and recovery from upsets take much longer in anaerobic digesters than in aerobic systems (Speece 1996).

However, despite these problems, the advantages of anaerobic treatment outweigh the disadvantages in many treatment situations. In fact, as of September 2008, there were over 3,300 anaerobic treatment plants successfully dealing with industrial wastewaters all over the world (Dennis Totzke, Applied Technologies, Inc., speaking at Anaerobic Treatment of High Strength Industrial Wastes, Milwaukee, WI, September 2008); and, according a report published in 2002, 3,450 municipal digesters are operated in the United States alone (SAIC 2002).

1.3 Microorganisms and Anaerobic Wastewater Treatment.

Understanding the microbial community is central to optimizing and expanding the use of anaerobic wastewater technology because the microorganisms are ultimately responsible for the success of treatment. Without healthy biomass, all the pipes, pumps, and holding tanks would have little positive effect on degrading pollutants in wastewater. However, although successful treatment depends on microorganisms, historically anaerobic wastewater treatment research has focused on engineering better physical plants, and much less study has been devoted to the organisms which make the process possible. Since the late 1980s, however, both greater interest in the microbial community and the advent of molecular microbiology techniques have led to numerous studies of the microbial community ecology of anaerobic wastewater systems.

Successful anaerobic treatment of organic wastes requires the stable function of a complex, interdependent microbial community (McMahon et al. 2004) (Figure 1.1). The degradation of the organic compounds to carbon dioxide and methane occurs in four discrete steps and is therefore sometimes referred to as the anaerobic food chain or series metabolism

(Speece 1996, White 2000). Each step is carried out by a different group of microorganisms. First, polymeric organic molecules such as complex carbohydrates, proteins, and lipids are hydrolyzed into their components (Speece 1996). Then, the resulting monomers and oligomers such as amino acids, simple carbohydrates, and fatty acids are fermented into organic alcohols, volatile fatty acids such as acetate, propionate, butyrate (acidogenesis), hydrogen, and carbon dioxide (White 2000). Then, the fermentation products are further degraded to acetate, hydrogen, and carbon dioxide (acetogenesis) (White 2000). Methanogenesis is the final step, typically producing methane and carbon dioxide from either acetate or hydrogen/formate and carbon dioxide (White 2000) (Figure 1.2).

The complexity and variation in metabolism found in the microbial communities in anaerobic digesters is really not as simple as a four-step food chain would suggest (Stams and Plugge 2009). In fact, after hydrolysis and fermentation, the food "chain" becomes quite complicated, as several pathways for degradation are possible depending on the products of the fermentations. For example, hydrogen and carbon dioxide may be directly converted to methane by hydrogenotrophic methanogens or converted to acetate by the homoacetogenic bacteria (White 2000). If sulfate is present in the wastewater, hydrogen may also be consumed by the sulfate-reducing bacteria (SRB) in competition with methanogens (Abram and Nedwell 1978). If acetate is produced during fermentation then two options for its degradation exist: acetate oxidation to hydrogen and carbon dioxide or conversion to methane by acetoclastic methanogensis (Karakashev et al. 2006, Schnürer et al. 1999). Some organic alcohols and other methyl-containing compounds, such as methanol and methylamine, may be directly utilized for methanogenesis (Dianou et al. 2001, Hutten et al. 1980, Tonouchi 2004) while others must be further oxidized into acetate, hydrogen, and carbon dioxide. The importance of each of these

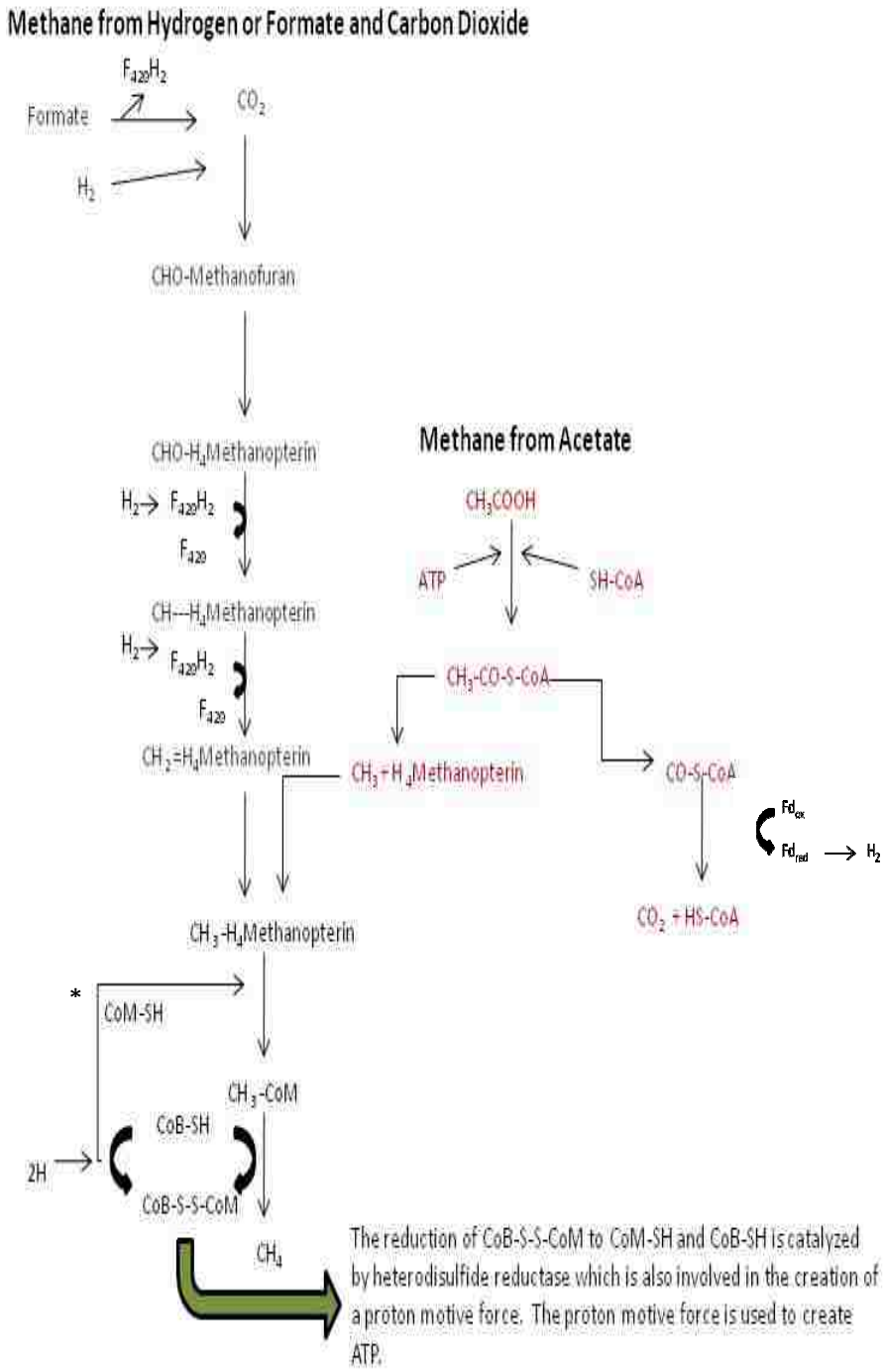


Figure 1.2 .Pathways of Methanogenesis. Methanogenesis from hydrogen/formate and carbon dioxide or acetate. Adapted from Liu and Whitman (2008) and *Brock Biology of Microorganisms* (Madigan et al. 2003). Methanol and methylamines enter the pathway just before the final step (*) as the methyl group from these compounds is transferred to CoM-SH and then to CH_3-CoM.

metabolic options is not clearly understood, although their value is probably dependent upon several factors including the type of organic waste being degraded.

However, one metabolic pathway following fermentation to volatile fatty acids (VFAs) has been shown to have important implications for the complete degradation of organic waste to methane and carbon dioxide. When propionate and butyrate are the products of fermentation, the conversion of these volatile fatty acids to acetate and hydrogen depends upon syntrophic interactions with hydrogen-utilizing organisms (Schink 1997). Unless the hydrogen produced by these organisms is continually removed so that the local concentrations remain low, the oxidation of these fatty acids is not thermodynamically favorable (Schink 1997). This process is referred to as interspecies hydrogen transfer (Bryant et al. 1967, White 2000). Without it, propionate, butyrate, and other acids accumulate in the digester and may cause the degradation of waste to slow or cease (McCarty and Smith 1986) resulting in digester failure.

1.3.a *Microbial Community Diversity***.** A microbial community is generally described as the populations of microbes which interact within a specific environment (Konopka 2009). Diversity includes the measure of the number of taxonomic units present (richness) and their relative abundance (Konopka 2009). Community structure is often used to describe richness and abundance determinations which include identification of the detected taxons (Fuhrman 2009). Although it is difficult to detect rare organisms in very diverse communities such as those in anaerobic digesters, defining community structure can provide valuable information regarding the functional potential of the community (Fuhrman 2009, Konopka 2009).

Some of the earliest studies of anaerobic digester communities utilized fluorescent polyclonal antibody probes to examine the diversity of methanogenic microorganisms (Macario and De Macario 1988, Visser et al. 1991). Using this technique, Macario et al. (1988)

demonstrated that the diversity of methanogens was greater than previously believed. Visser et al. (1991) used this type of immunological study to demonstrate that microbial diversity was reduced in a digester whose temperature was increased from 37°C to 55°C.

Soon after these studies, radioisotope and fluorescent- labeled oligonucleotide probes based on 16S rRNA sequences were developed for the quantification of methanogens and sulfate-reducing bacteria by Raskin et al. (1994a, 1994b). These probes were used to identify and quantify specific groups of methanogens and sulfate reducers in chemostats, single tank and two-stage digesters (Raskin et al. 1994a, Raskin et al. 1995). Oligonucleotide probes continue to be used to follow community dynamics in anaerobic digesters. They have the advantage of being directly applied to samples without the need for gene amplification by polymerase chain reaction (PCR) (Amann et al. 2001). However, targeted genes must be present in high copy numbers, and quantification using probes must include careful use of controls to insure accurate measurements (Amann et al. 2001, Dahllöf 2002).

In further studies of diversity, PCR and 16S rRNA or DNA specific primers have been used to examine microbial community structure within digesters. In 1997, Godon et al. (1997a, 1997b) studying both bacterial and archaeal 16S rRNA gene clone libraries from a digester treating wine distillery waste revealed that a large number of the bacterial operational taxonomic units (OTUs) found in the digester were unrelated to any cultured species. This was supported by a later study which reported that 95.6% of the bacterial OTUs found in another digester were novel phylotypes (Chouari et al. 2005). These studies illustrate the need for continued molecular analysis possibly combined with cultivation and physiological studies of organisms in anaerobic biomass so that the function of the community as a whole may be better understood.

A second study by Godon et al. (1997b) estimated that the wine distillery digester community had an *Archaea* to *Bacteria* ratio of 1 to 4. A later study using quantitative PCR (qPCR) of 16S rRNA genes in a biogas plant digester reported a ten-fold difference between the *Archaea* and *Bacteria* sequences (Nettmann et al. 2008). However, bias may have occurred in both these studies because of unequal numbers of 16S rRNA genes in different species of *Bacteria* and *Archaea* detected in these digesters. For example, the average number of 16S rRNA genes in the twenty-three sequenced methanogen genomes found in the rrnDB (a database which catalogs 16S, 23S, and 5S rRNA genes) is 2.56 (Klappenbach et al. 2001, Lee et al. 2009). However, the average number of 16S rRNA genes in other bacteria detected in the study varied widely. For example, the average 16S rRNA copy number in the sequenced genomes of the *Proteobacteria* found in rrnDB is 4.12, and the average for sequenced *Clostridium* species is 9 (Lee et al. 2009). Although some of the bacterial species detected had fewer 16S rRNA copy numbers than the methanogens (such as the *Cloroflexi*, 1.67 average 16S rRNA copy number (Lee et al. 2009)), the variation in copy numbers for these genes makes the clear determination of an *Archaea* to *Bacteria* ratio difficult at best.

Most studies of microbial communities in anaerobic digesters have focused on less than five digesters, and many have sampled only one or two digesters. This approach is limiting because anaerobic digesters treat waste of widely varying composition and different physical plant configurations exist as well, both of which may influence microbial community structure. There are two notable exceptions to this trend, however. The first attempt at gaining a wide view of the archaeal diversity found in anaerobic digesters was a study of 44 full-scale digesters from 8 countries (Leclerc et al. 2004). Using single strand conformation polymorphism and sequencing of 16S rDNA genes, Leclerc et al. (2004) found a total of twenty-three unique 16S

rRNA gene sequences from the *Archaea*. Two OTUs were found most often, and sequence analysis showed them both to be methanogens. One was determined to be *Methanosaeta concilli* and the other was related to *Methanobacterium*. The breadth of samples included in the study covered all basic physical configurations of anaerobic digesters, allowing the investigators to determine the most common microorganisms found in each type. They were able to associate particular microbial community characteristics with specific types of digesters. For instance, they observed increased diversity of the microbial community in continuously stirred tank reactors (CSTRs) when compared to other digester types. They also demonstrated that the abundance of *Methanosaeta concilii* was increased in upflow anaerobic sludge blanket (UASB) reactors. They did not, however, uncover a relationship between the type of wastewater treated and the archaeal community.

Another broad study of microorganisms in anaerobic digesters was undertaken by Riviere et al. (2009). They sequenced a total of 9, 890 bacterial and archaeal 16S rRNA genes from seven different mesophilic digesters. After phylogenetic analysis, they were able to determine that one third of the bacterial OTUs formed a core common to all digesters, another third of the OTUs were shared among a few digesters, and the final third of the bacterial OTUs were specific to certain digesters. The bacterial 16S rRNA genes that they found were primarily from uncultivated species, which agreed with an early finding by Chouari et al. (2005) which found 95.6% of the bacterial 16S rRNA gene sequences from one digester were novel phylotypes. Among all the digesters, the archaeal sequences represented fewer OTUs than the bacterial sequences, and most of them were related to the *Methanosarcinales*, the *Methanomicrobiales*, and a novel lineage which was designated the Arc I group.

These two broad studies have provided valuable information regarding the microbial communities of anaerobic digesters; however, other factors besides the type of reactor or the substrate treated have been studied to ascertain affects on digester communities.

1.3.b *Temperature***.** Temperature is an important environmental factor in the growth of most microbes, with the majority having a range of temperature in which optimal growth occurs while higher or lower temperatures are inhibitory. Most anaerobic digesters are operated at around 35 °C (mesophilic), although thermophilic digesters (~55°C) are also in use. Therefore, certain studies have explored the effects of temperature on microbial communities in digesters. As mentioned above, Visser et al. (1991) used polyclonal antibody studies to demonstrate that microbial diversity was reduced in a digester whose temperature was increased from 37°C to 55°C. Sekiguchi et al. (1998) compared mesophilic and thermophilic digesters, finding decreased diversity of 16S rRNA gene clone libraries in the thermophilic digesters. McHugh et al. (2003) used amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing to study the microbial communities in psycrophilic (10-14°C), mesophilic, and thermophilic digesters, reporting high methanogen diversity and dominance of the acetate-utilizing genus *Methanosaeta* in samples from a set of six digesters with operating temperatures varying from 10-55°C. In a further study of digesters that were operated at temperatures between 16 and 37°C, this same group reported a shift from acetoclastic to hydrogenotrophic methanogens in the microbial community as temperatures increased (see below for further discussion of methanogen community dynamics).

In summary, the above results suggest that methanogen diversity is reduced in digesters with thermophilic operating temperatures. They also suggest that acetoclastic methanogens are

able to function better than hydrogenotrophic methanogens when digester temperatures are below 37°C**.**

1.3.c *Microbial Communities and Stability of Digester Function***.** The stability of digester function (process stability) is defined as "the capacity to achieve efficient pollutant reduction under varying environmental conditions (Speece 1996)." Two terms may be used to further describe stability: resistance and resilience (Konopka 2009, Pimm 1984). Functional resistance refers to the ability of the community to continue to function (i.e., achieve efficient pollutant reduction) when environmental conditions are perturbed, and resilience describes how fast the community can return to function after a perturbation that disrupts function (Pimm 1984).

Several studies have examined how the stability of digester function is linked to the structure of the microbial community. Anaerobic digesters are sometimes subjected to overloads of substrate, and these overloads may cause digester function (i.e., organic waste degradation coupled to methane production) to slow or fail. A study by Delbes et al. (2001) showed that recovery of a digester overloaded with acetate required a community shift from hydrogenotrophic to aceteclastic methanogens. Fernandez et al. (2000) using oligonucleotide probes, ARDRA, and sequencing of 16S rDNA studied digester communities both before and after perturbation caused by an overload of glucose. Their results linked stability of function to flexibility within the digester microbial community. Digesters that had more profound changes in their community structure upon perturbation had better functional stability than those whose communities showed less change (resilience) (Fernandez et al. 2000). Concurrent observations of the degradation products generated from the glucose suggested that the shifts in community structure reflected the ability of the more stable digester communities to use multiple metabolic pathways. Similarly, Hashsham et al. (2000) further demonstrated that if microbial communities

possessed the ability to degrade substrates in multiple, parallel ways versus a single pathway for linear processing, the community with the ability to utilize parallel processing was more stable. Stability in this case was defined as the ability to continue to function with an overload of substrate, in this case of glucose (resistance (Pimm 1984)). Although these studies analyzed relatively small data sets, the results of these studies suggest that digester microbial communities with more metabolic options are more functionally stable than those with limited metabolic pathways for the degradation of organic substrates.

Other studies have also focused on the microbial community during high toxicant loads. A study of a digester with high ammonia concentrations (e.g., 3,500 mg/L) showed that the hydrogenotrophic methanogens, which use hydrogen and carbon dioxide for methanogenesis, were more abundant in digesters with high ammonia than in digesters with lower ammonia concentrations (Angenent et al. 2002). Another study in which the free ammonia nitrogen content of the digester was gradually increased from 160 mg/L to 750 mg/L showed an interesting shift in the microbial community using microscopy. At lower ammonia concentrations, filamentous *Methanosaeta* were the dominant organisms, but as ammonia concentrations increased *Methanosarcina* species were observed, with their clusters of cocci being the dominant organisms (Calli et al. 2005). The authors proposed that the formation of clusters provided protection from the higher concentrations of ammonia for the *Methanosarcina*, allowing them to thrive as the more exposed, filamentous *Methanosaeta* became less common.

Interestingly, McMahon et al. (2004) demonstrated that digesters whose communities had struggled with stability of function in the past were more tolerant of an overload or shock (resilient) than communities which had always functioned well. The comparison of microbial communities in several digesters over time led them to conclude that digester communities with

difficulties in consistent function developed microbial consortia that were able to cope with future overloads. Therefore, digesters which struggle during start up may, in fact, have better functional stability over time.

Recently, Werner et al. (2011) used pyrosequencing, generating >400,000 16S rRNA gene sequences from 112 samples, to examine the microbial communities in nine anaerobic digesters treating brewery waste over the course of twelve months. They were particularly interested in the resilience and resistance of the microbial community and the relationship between those characteristics and digester function. Using Unifrac, they showed that each of the nine digesters had a unique bacterial community (Werner et al. 2011). When they compared the structure of the bacterial communities to the digester operating conditions and digester function, they found stronger relationships between community structure and function than between structure and conditions (Werner et al. 2011). The functional parameters most closely related to community structure were methanogenic activity (reported as grams of chemical oxygen demand of methane per gram of volatile suspended solids per day) and the efficiency of the removal of the substrate (reported as %) (Werner et al. 2011). Greater community evenness was related to higher methanogenic activity, which the authors suggested was related to the existence of greater numbers of parallel metabolic pathways for the degradation of organic compounds as described previously by Hashsham (Hashsham et al. 2000). This study provided an important link between bacterial community structure and function in anaerobic digesters.

1.3.d *Physical Differences in Anaerobic Digester Configurations***.** Certain studies have examined how differences in the physical operation of anaerobic digesters may affect the structure of the microbial community. Digesters are operated under various conditions and several variations on the structure of the physical plant are utilized in full-scale operations.

For example, the biomass in continuous stirred tank reactors (CSTRs) is, as the name suggests, constantly mixed. In these reactors, mixing speed may affect the microbial community by creating an environment which is too turbulent for the formation of close interactions which are important for certain steps in the degradation of organic compounds by the community, specifically propionate degradation and methanogenesis. Hoffman et al. (2008) studied CSTRs with different mixing rates in order to examine how mixing speed might affect microbial community structure. They observed that while the bacterial community was unaffected, increasing the mixing speed in lab-scale digesters altered the structure of the archaeal community over time, with a change from *Methanosaeta*-related organisms at lower speeds to *Methanosarcina* at higher mixing intensities. Their conclusion was that mixing speed and the resulting shear of increased mixing rates could influence archaeal community structure but not bacterial. However, this shift in community structure did not result in differences in digester function as evidenced by methane production. Reasons for the observed community shift were not apparent. However, *Methanosarcina* are capable of utilizing multiple substrates for methanogenesis while the *Methanosaeta* can only utilize acetate which may begin to explain the stability of methane production under more turbulent conditions (see section 1.2.c.).

Another configuration, two-stage anaerobic digestion, compartmentalizes the biomass, aiming to create separate environments that are more amenable for acidogenesis (pH 4-5) and methanogenesis (pH 7) respectively (Ghosh and Pohland 1974). Two-stage digesters treat waste somewhat more quickly and have better organic removal rates (Speece 1996). When Raskin et al. (1995) used fluorescent and radio-labelled oligonucleotide probes based on 16S rRNA sequences to identify and quantify specific groups of methanogens and sulfate reducers in single tanks and two-stage digesters, they found methanogens in the first stage of the reactor where conditions for

methanogenesis were not optimal. However, upon comparison the first stage to the second stage, the methanogen communities in the compartments were determined to be different. This finding suggests that certain methanogens are capable of surviving in what might be considered sub-optimal conditions.

Certain types of biomass pre-treatment can also be incorporated into anaerobic treatment. Zhang et al. (2009) used pyrosequencing and qPCR to examine changes in microbial communities when pre-treating a mix of primary and waste activated sludge by exposing it to a pulsed electrical field which causes nutrients in biomass to become more available and therefore more digestible (Rittmann et al. 2008). This method, called Focused-Pulsed sludge pre-treatment, was used on anaerobic biomass, and then analyses of the sequences obtained before and after treatment showed that the dominant *Archaea* genus in the biomass changed from the hydrogenotrophic *Methanoculleus* to acetoclastic *Methanosaeta.* Higher bacterial diversity was also observed post-treatment. These findings were most likely related to the differences in availability of nutrients in the sludge.

1.3.e *Temporal Changes*. Temporal changes have also been observed in the microbial communities of anaerobic digesters. Fernandez et al. (1999) concluded that while both the dominant members and diversity of the bacteria and archaea in a lab-scale digester changed rapidly within short periods (3.3 day retention times), the dominant organisms in the bacterial community in digesters fluctuated more over time than those in the community of *Archaea*. Another study which followed the microbial community for two years supported their conclusions by demonstrating *Archaea* community structure remaining relatively stable while rapid shifts occurred in the bacterial community of a wine-distillery anaerobic digester (Zumstein et al. 2000).

Fernandez et al. (1999) also observed a change in the relative abundance of certain archaeal OTUs over time using ARDRA. This was supported by the observation of methanogen succession in another study which followed a lab-scale digester from start up to day 107 (Leclerc et al. 2001). The dominant methanogen genus switched from acetoclastic (utilizing acetate for methane production) *Methanosaeta* in the early stages to hydrogenotrophic *Methanobacterium* as the biomass in their digester developed. This switch from acetoclastic to hydrogenotrophic methanogen dominance suggests that methanogen community structure may be related to the substrates which are available for methanogenesis over time during the development of anaerobic biomass. For example, hydrogenotrophic methanogens may become more important members of the community as the concentration of volatile fatty acids (VFAs) like propionate and butyrate increase. Without the removal of hydrogen by the methanogens (or other organisms), propionate and butyrate oxidation are thermodynamically unfavorable (Schink 1997) and an increase in VFA concentrations can result in digester failure (McCarty and Smith 1986). Therefore, as VFAs increase, a shift in the abundance of organisms capable of hydrogenotrophic methanogenesis would be beneficial in the development of a stable community.

1.3.f *Granulation*. The formation of granules which facilitate the syntrophic relationships between propionate-oxidizing bacteria and their hydrogen-utilizing methanogen partners within biomass is especially important for upflow anaerobic sludge blanket (UASB) reactors and similar technologies (Schmidt and Ahring 1996). Several studies have examined the microbiology of the granules that form in the biomass of this type of anaerobic digester. Diaz et al. (2006) used cloning, denaturing gradient gel electrophoresis (DGGE), fluorescent oligonucleotide probes, and electron microscopy to discover that the color of the granules (black, gray or brown) in a brewery digester were related to both their age and microbial composition. They observed that

the lighter the color, the older the granules were and that these granules had fewer living cells than the younger ones. Using oligonucleotide probes, they also observed differences among the microbial communities of granules of different colors. Black (young) granules had microbial communities dominated by Gram-positive bacteria and *Methanosaeta.* Gray (middle-aged) granules were similar to black granules in microbial community composition except there were no *Methanosarcina*, which were present in small amounts in brown and black granules. Brown (old) granules were dominated by *Proteobacteria* instead of Gram-positives; however, the dominant methanogen genus observed was also *Methanosaeta*.

Keyser et al. (2006) used DGGE to identify differences in granules from winery, brewery and peach-lye canning wastewaters. They found that granules from wastewaters treating differing substrates had different methanogen communities, leading them to the conclusion that substrate affected granule community composition. Finally, Zheng et al. (2006) used fluorescent oligonucleotide probes to follow the microbial progression in the formation of anaerobic granules, and found that *Methanosaeta concilii* was important for initiating granule formation. They also found that a layer of syntrophic bacteria form the outside of granule aggregates. These studies demonstrated that microbial community structure can be related to granule function and formation.

Taken together, the above studies provide much valuable information regarding the microbial community in anaerobic digesters. The existence of certain guilds of microorganisms can be confirmed and used to construct a simple food chain (Fernandez et al. 1999, Liu and Whitman 2008, White 2000) (Figure 1.1). While it is helpful to visualize the anaerobic degradation of organic waste in this way, this food chain is probably too simplistic to account for all the complexities of digester communities and the possible variations of microbial metabolism

(Hashsham et al. 2000). Because of the complexities of metabolism and physiology of the community of organisms found in anaerobic biomass, further studies are required to better understand the role of and the interactions between microorganisms in anaerobic wastewater treatment.

1.4. Methanogens in Anaerobic Wastewater Treatment.

The above studies have established that the microbial community in anaerobic digesters is very diverse. It is also clear that several different groups of organisms are required for the completion of waste degradation (Figure 1.1). However, the methanogens are especially important because they are the last link the anaerobic digester food chain, and because methanogenesis is often the rate limiting step in anaerobic treatment of wastes (Liu and Whitman 2008).

1.4.a *Methanogens***.** Methanogens are strict anaerobes which belong to the phylum *Euryarchaeota;* and within four classes, there are five orders of methanogens: *Methanobacteriales, Methancoccales, Methanomicrobiales, Methanosarcinales,* and *Methanopyrales* (Figure 1.3) (Liu and Whitman 2008). From these orders (except *Methanopyrales),* the genera *Methanobacterium, Methanobrevibacter, Methanoculleus, Methanogenium, Methanocorpusculum, Methanospirillum, Methanolinea, Methanothermobacter, Methanosarcina,* and *Methanosaeta* have been found in anaerobic digesters (Chaban et al. 2006, Hori et al. 2006, Imachi et al. 2008, Rastogi et al. 2008). These can be further categorized according to their substrate requirements for methanogenesis as the acetoclastic methanogens and the hydrogenotrophic methanogens, although this classification is somewhat oversimplified as some methanogens are capable of utilizing other compounds with methyl groups and *Methanosarcina* can use both hydrogen and acetate (Liu and Whitman 2008).

In general, however, members of the *Methanosaeta* and *Methanosarcina* are considered acetate-utilizing methanogens; the other genera found in digesters require hydrogen and carbon dioxide, although some are capable of using formate and certain alcohols (Liu and Whitman 2008, Madigan et al. 2003). Certain members of the *Methanosarcinales* can also utilize methanol and/or methylamine (Madigan et al. 2003). A more detailed description of the methanogenic genera detected in this study may be found in Appendix I.

1.4.b *Metabolism*. Metabolically, methanogens use a version of the acetyl CoA pathway which is similar to that of bacteria, but the electron carriers (tetrahydrofolic acid is replaced by tetrahydromethopterin) and the treatment of formate vary from the bacterial pathway (White 2000). Methanogenesis occurs as an offshoot of this pathway, linking it to an electron transport chain which provides a proton motive force for ATP production (Figure 1.2). The pathways for methanogenesis from acetate and from methanol/methylamine initially differ from the pathway used to produce methane from hydrogen and carbon dioxide. However, the pathways all converge near the end (Figure 1.2).

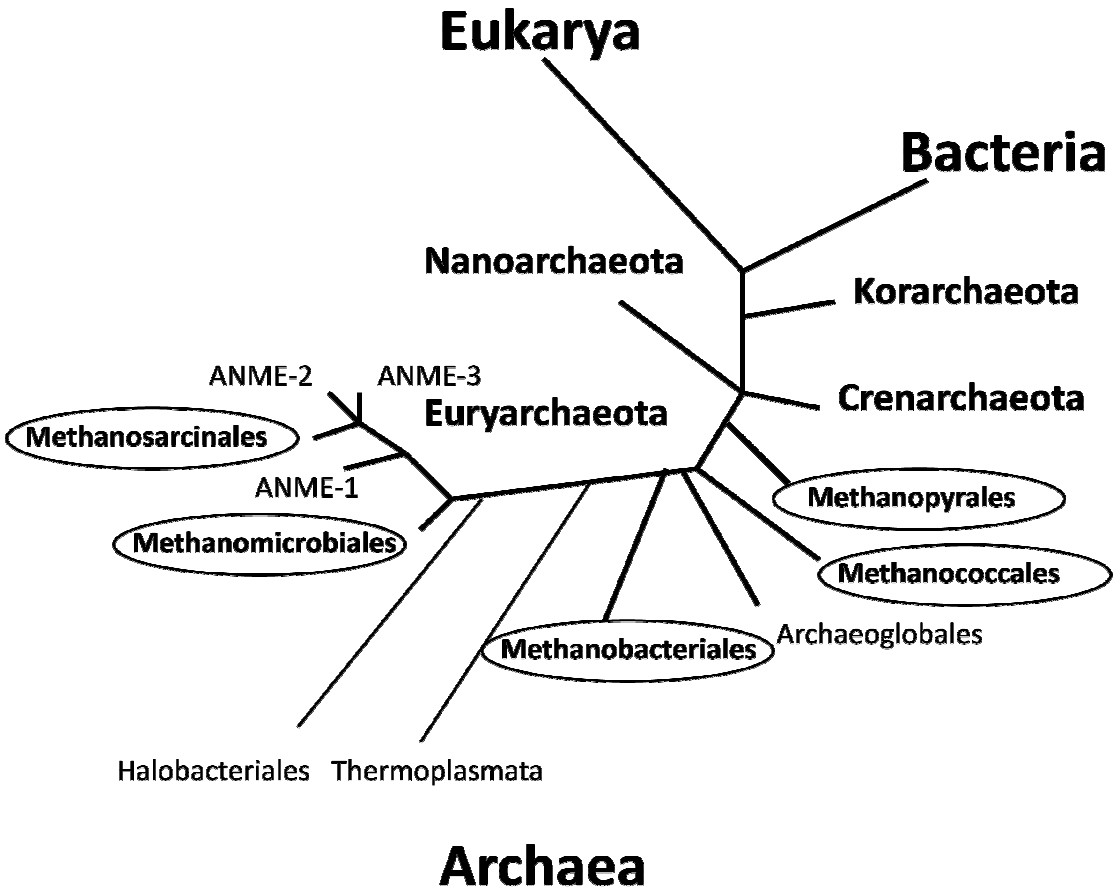


Figure 1.3. **Phylogenetic Relationships of Methanogens to Other Organisms**. Distances are estimated. Methanogenic orders are circled. Adapted from Schleper et al.(2005) and Chaban et al.(2006).

When hydrogen is required for methanogenesis, it is often provided via interspecies hydrogen transfer (Stams 1994, White 2000). This process is very important in anaerobic digestion because if methanogens fail to draw off hydrogen produced by syntrophic fatty acid-oxidizing bacteria, then fatty acid oxidation becomes energetically unfavorable for the syntrophs (Stams 1994). The break down of this important syntrophic interaction can cause digester failure characterized by the rise in concentration of volatile fatty acids such as butyrate and propionate (Hori et al. 2006). Methanogens' role in this syntrophy as well as their production of methane

which may be used as a renewable fuel make them vital members of the microbial community in anaerobic digesters.

1.4.c *mcrA.* Methanogenesis is catalyzed by a unique set of enzymes (Figure 1.2). Methyl coenzyme M reductase is the enzyme which catalyzes the final reaction in both types of methanogenesis, the reduction of CH_3-CoM to CH_4 (Figure 1.2) (Ermler et al. 1997). The operon encoding this multi-subunit enzyme is specific to the known methanogens and the anaerobic methane-oxidizing archaea (Hallam et al. 2003, Luton et al. 2002). Previous studies have established that the gene which encodes the alpha subunit (*mcrA*) can be used to detect methanogen presence in the environment and that it is a suitable gene for phylogenetic comparison of methanogen diversity (Hales et al. 1996, Luton et al. 2002, Springer et al. 1995). Amplifying an approximately 460 base pair segment of the *mcrA* sequence, the PCR primer set developed by Luton, et al. (46) has been shown to consistently amplify a wide range of methanogenic groups (Banning et al. 2005, Juottonen et al. 2005, Juottonen et al. 2006, Luton et al. 2002). Only one or two copies of *mcrA* have been found in sequenced methanogen genomes, making it a better tool for estimating the numbers of methanogens in biomass than 16S rRNA which has copy numbers ranging from 1-4 copies per genome (Lee et al. 2009).

1.4.d *Methanogen Diversity in Anaerobic Digesters.* Uncovering methanogen community diversity has been the object of several studies. Griffin et al. (1998) used family or genus-specific fluorescent oligonucleotide probes to follow the dynamics of the methanogen communities in a mesophilic digester and a thermophilic digester over time. They found a shift in abundance between *Methanosarcina* and *Methanosaeta* related to

acetate levels. They also determined that the *Methanobacteriaceae* were the most common hydrogenotrophs in the digesters they studied.

Steinberg and Regan (2008) compared the methanogen communities in an acidic fen and an anaerobic digester using both *mcrA* and 16S rRNA genes. They found almost no overlap between the sequences of either gene from the digester and the fen, and the majority of the sequences they found were unrelated to any cultured methanogen species. Similarly, 16S rRNA genes and *mcrA* were used to study the archaeal community in a biogas plant using cattle manure and corn silage as substrates (Nettmann et al. 2008). Many of the sequences in this study were also related to uncultured archaeal species; however, assignments at the genus level were possible for most.

During the development of 16S rRNA-based microarray for methanogen detection and classification called ANAEROCHIP, Franke-Whittle et al. (2009) found *Methanoculleus* to be the dominant (84.1%) species in a 16S rDNA clone library. *Methanosarcina, Methanobrevibacter, Methanobacterium, and Methanosphaera*- related clones were only detected in small quantities (<5.8%). Goberna et al. (2010) utilized the ANAEROCHIP assay along with qPCR to study the methanogen communities of anaerobic digesters treating olive mill wastes and cattle manure. In a mesophilic digester, they were able to determine that *Methanosarcina,* the dominant methanogen, was able to rapidly increase in number six-fold when the digesters substrate availability was changed from treating only cattle manure to cattle manure plus olive mill waste (Goberna et al. 2010). In a digester operated at 55°C, hydrogenotrophic methanogens *Methanobacterium, Methanoculleus,* and *Methanothermobacter* were all detected (along with a clade unrelated to known methanogens) (Goberna et al. 2010).

1.4.e *Methanogens and pH.* An increase in fatty acids (e.g., propionate, butyrate) in an anaerobic digester can lead to a decrease in pH (Liu and Whitman 2008). A pH decrease can be detrimental to methanogenesis as most methanogens have a pH tolerance range of 6-9 (Liu and Whitman 2008, Slonczewski and Foster 2009). Hori et al. (2006) used genus-specific qPCR to demonstrate that the composition of the methanogen community in a thermophilic anaerobic digester changed as the concentration of volatile fatty acids (VFAs) shifted. When VFAs were absent, *Methanoculleus* species dominated, but when the VFA concentration increased there was a 10,000 fold increase in *Methanothermobacter*-related 16S rRNA genes (Hori et al. 2006). However, known *Methanothermobacter* species have pH tolerances (6.0-8.8) similar to other methanogens (Zeikus and Wolfe 1972). For more information regarding specific pH tolerance for different methanogen genera, see Appendix I.

1.4.f *Methanogens and Temperature.* Most methanogens are mesophilic or thermophilic (See Appendix I). Using *mcrA* instead of 16S rRNA genes, Rastogi et al. (2008) found that in a digester degrading cattle manure located in a temperate climate, seasonal shifts occurred in the methanogen community and suggested that these shifts were most likely related to temperature requirements. They reported increase in the percentage of *Methanocorpusculum*-related sequences in the winter samples. This may indicate that this genus has higher tolerance for cold than others (e.g., genera found in the orders *Methanosarcinales* and *Methancoccus)* that represented higher percentages of the sequences in the summer samples, although it is reported to experience optimal growth rates at mesophilic temperatures (Zellner et al. 1989). However, O'Reilly et al. (2010) reported similar dominance of *Methanocorpusculum* in lab-scale digesters operated at

15°C using qPCR. Further information regarding the temperature requirements of methanogens can be found in Appendix I.

1.4.g *Methanogens and Oxygen Tolerance.* The sensitivity of strict anaerobes to oxygen is believed to be due to the accumulation of oxygen radicals ($OH\cdot$ and O_2^-) and hydrogen peroxide (White 2000). Aerobes and other microorganisms which can tolerate oxygen are protected by superoxide dismutase and catalase, enzymes which convert oxygen radicals and hydrogen peroxide to oxygen and water (White 2000).

Methanogens are considered strict anaerobes (Liu and Whitman 2008), unable to grow or produce methane in the presence of oxygen (Zinder 1993). There is, however, some evidence that methanogens can tolerate oxygen exposure to varying degrees. For example, previous studies have shown that methanogens in anaerobic digester sludge can cope with exposure to oxygen (Conklin et al. 2007, Zitomer and Shrout 2000, Jenicek et al. 2010, Kato et al. 1993, Stephenson et al. 1999). In fact, Zitomer and Shrout (2000) reported increased methane production in fluidized bed reactors that were exposed to air. However, the ability of methanogens to cope with air exposure is believed to be dependent upon facultative microorganisms also present in the biomass community which scavenge the oxygen before it causes damage to the methanogens (Jenicek et al. 2010, Kato et al. 1993).

Examination of all of the sequenced methanogen genomes in Genbank® reveals putative superoxide dismutase and/or catalase genes, and an active superoxide dismutase has been isolated from *Methanobacterium bryantii* (Kirby et al. 1981). However, it is unknown whether these enzymes are functional in other methanogens. Furthermore, as superoxide dismutase and catalase reactions only convert oxygen radicals and hydrogen

peroxide back to more oxygen, it is unclear how much protection active superoxide dismutase and catalase would provide.

1.4.h *Recently Described Methanogen Species.* Multiple reports of sequences which cannot be assigned to a particular methanogen species or even genus (see above) support the idea that there are other undescribed methanogens in anaerobic digesters whose physiology has not yet been studied. In the past five years, three new methanogen species isolated from anaerobic digesters have been described, *Methanobacterium beijingense*, *Methanolinea tarda,* and *Methanoregula formicica* (Imachi et al. 2008, Ma et al. 2005, Yashiro et al. 2011). Based on the number of unknown methanogens reported in the literature, the identification of other new species is likely in the future.

1.5 Introduction to Specific Aims.

Although much is known about the reactions of methanogenesis (Figure 1.2), there is still much to be learned about the role that methanogens play in anaerobic wastewater treatment. For example, questions such as "Do specific conditions contribute to the enhancement of the methane-producing ability of certain methanogens?" and "Does the number of methanogens present in digesters accurately project the ability of the biomass to make methane?" remain unanswered in the literature. The data obtained from the experiments performed in the course of this study attempt to answer these and other questions, while providing important information regarding the function and community structure of methanogens in anaerobic wastewater treatment facilities which can be used to optimize anaerobic biotechnology.

1.6 Specific Aim I: Diversity of methanogens in anaerobic enrichment cultures and industrial digesters.

1.6.a *Introduction.* Anaerobic digesters are operated under varying influent substrates and conditions. Because they complete the final transformation of organic wastes to methane, methanogen communities are essential to the proper function of digesters. Therefore, understanding how varying conditions and substrates relate to methanogen community structure is important for engineers and digester operators who wish to optimize digester function and methane production.

1.6.b *Aims and Hypothesis.* It was hypothesized that varying the conditions or available substrates would result in distinct differences in methanogen communities in anaerobic digesters. This hypothesis was tested by generating clone libraries from DNA extractions from four hydrogen- and carbon dioxide- enriched cultures (R1,R2,R3,and R4) and two industrial scale digesters (CH and CB) using the methanogen-specific gene *mcrA*. (For a complete description of the operation parameters of the cultures and digesters see Table 2.1.) Restriction fragment length polymorphism (RFLP) analysis of the clone library from each digester was used to identify unique *mcrA* clones (Chapter 3) or the clones were directly sequenced (Chapter 4). The RFLP analysis included determining the best restriction enzymes for use in RFLP with *mcrA* sequences amplified using the primers designed by Luton et al.(2002). No consensus could be found in the literature as to which or even how many restriction enzymes would provide adequate coverage of the methanogen community using RFLP analysis with *mcrA* (Chapter 3). *mcrA* clones were sequenced, and phylogenetic and statistical analyses were performed using the RFLP data and unique sequences from each clone library (Chapter 3 and 4).

The data supported the hypothesis, demonstrating that digesters and enrichment cultures (even those started at the same time with the same seed sludge) operated under varying conditions or substrates did have different methanogen communities as shown by phylogenetic and statistical analysis of *mcrA* sequences (Chapters 3 and 4). However, the data had little predictive value regarding digester function when compared to the digesters known functional parameters.

1.7 Specific Aim II: Quantification of methanogens in anaerobic enrichment cultures and digesters.

1.7.a *Introduction.* The four H_2:CO_2 enrichment cultures, several lab-scale digesters, and industrial scale digesters were observed to have differing rates of methane production against specific substrates as determined by specific methanogenic activity (SMA) assays (Coates et al. 2005, Coates et al. 1996). One explanation for the different rates of methane production was that more methanogens were present in the digesters that had greater methane production rates.

1.7.b. *Aims and Hypothesis.* It was therefore hypothesized that the abundance of methanogens in the anaerobic biomass of a digester would positively correlate to a higher rate of methane production against specific substrates. This hypothesis was tested by performing quantitative PCR (SYBR Green method (52)) on DNA extracts from each of the four cultures, the lab-scale digesters, and the industrial digesters using the Luton primer set (43) with the thermocycler program tested by Goffredi et al. (Goffredi et al. 2008, Luton et al. 2002, Ponchel et al. 2003). The results were compared to values determined for a standard curve to quantify the *mcrA* gene copy number for each

enrichment culture. The standard curve and the samples were included in the same run, as recommended by Smith et al. (2006). Gene copy number of *mcrA* was then used to estimate the numbers of methanogens present in the cultures. All twenty-nine sequenced methanogen genomes in GenBank® contained only one or two copies of *mcrA* and sequenced genomes of representatives of the genera found in the clone libraries from Specific Aim I only contained one copy of *mcrA.* The number of methanogens in each culture was compared to specific rates of methane production (SMA assays) for each culture or digester biomass (Chapter 5).

The data supported this hypothesis showing positive correlation between the number of *mcrA* genes present in biomass and the SMA results for the same biomass sample (Chapter 5).

1.8 Specific Aim III: Diversity of methanogens in anaerobic enrichment cultures which are actively transcribing *mcrA*.

1.8.a *Introduction*. Little is known about the transcription rates, mRNA half-life, and protein half-life for the enzymes necessary for methanogenesis, including methyl coenzyme M reductase. However, transcription of *mcrA* has been used to demonstrate that methanogens are metabolically active (Juottonen et al. 2008). Furthermore, the organisms in anaerobic biomass have been shown to be capable of dormancy when conditions are not optimal (Speece 1996). Thus, identifying the members of the methanogen community which are metabolically active could provide valuable insight into digester function.

1.8.b *Aims and Hypothesis*. Therefore, it was hypothesized that only certain methanogens detected in the *mcrA* clone libraries from DNA extracts were actively contributing to methane production as demonstrated by their transcription of *mcrA.* To test this hypothesis, *mcrA* clone libraries were created from RNA extracts taken from the aforementioned enrichment cultures to determine which members of the methanogen communities were actively transcribing this critical gene and compared to clone libraries created from DNA extracted from the same samples.

Cloned sequences were then compared to known sequences in GenBank® using blastn (Altschul et al. 1990, Altschul et al. 1997), and each library of clone sequences was also submitted to DOTUR for statistical analysis, including the Shannon Index, rarefaction curves, S_{chao1}, and number of OTUs (Schloss and Handelsman 2005). The percentage of clones related to different methanogen genera was calculated for each library, and phylogenetic trees (including reference sequences) were created from the sequences obtained from each digester. For each clone library, the methanogen community revealed in the sequences amplified from the DNA was compared to the community uncovered in the sequences amplified from the RNA extractions (Chapter 4). This was accomplished by using phylogenetic and statistical analyses of sequences to determine differences among the cultures' methanogen communities which were present in the biomass (i.e., DNA) versus those that were actively transcribing *mcrA* (i.e., RNA) at levels detectable by the assay.

Phylogenetic and statistical analyses showed that the methanogens that were actively transcribing *mcrA* did not represent all of the methanogen community detected within the digesters using DNA extracts to generate clone libraries (Chapter 4).

The observed differences between the transcribed *mcrA* sequences and the genomic *mcrA* sequences in this study suggest that some methanogens exist in a state of dormancy when conditions are not favorable for their particular metabolic needs while others are more involved in methane production. Overall, the examination of the diversity of the functional gene *mcrA* indicates that conditions and available substrates affect which members of the methanogen community are most active at a given time.

1.9 Specific Aim IV: Quantification of mcrA transcripts in anaerobic enrichment cultures and digesters.

1.9.a *Introduction.* Because transcription can be more closely linked to activity, the determination of *mcrA* transcript number could be a better indicator of methane production rates than *mcrA* gene copy number. This hypothesis is supported by a recently published study by Freitag and Prosser (2009) in which they found a relationship between *mcrA* transcript to gene copy ratios and methane production in peat.

1.9.b *Aims and Hypothesis.* Therefore, it was hypothesized that the abundance of *mcrA* transcripts in the anaerobic biomass of anaerobic digesters would positively correlate with methane production rates against specific substrates. In order to test this hypothesis RNA and DNA were extracted from the same sample from four enrichment cultures, six lab-scale digesters, and six industrial scale digesters. Quantitative PCR (DNA samples) and qRT-PCR (RNA samples) were performed and results were compared to a standard curve to determine *mcrA* gene copy and transcript numbers. SMA assays were also performed on each biomass sample. Regression analysis was then used to determine if a significant positive relationship existed between *mcrA* gene copy number, *mcrA*

transcript number, and the methanogenic potential of each biomass samples against specific substrates (Chapter 5).

The number of *mcrA* transcripts, when detectable, did correlate well with SMA assay results; however, transcripts were not detected from three of the six industrial digesters with low specific methane production rates (Chapter 5). Therefore, *mcrA* transcript number may be more useful in monitoring methanogens in anaerobic digesters which are functioning at or above a certain level in terms of methane production or organic waste removal, but may not discrimate between digesters which are functioning below that level.

The data obtained from these studies broadens the previous knowledge of microbial communities, especially methanogen communities, within anaerobic digesters, and will be used to better monitor and engineer microbial communities in anaerobic digesters and to design bioaugmentation mixes for digester supplementation.

Chapter Two
Materials and Methods

2.1. Sample sources: Anaerobic biomass was collected from anaerobic hydrogen enrichment cultures (R1,R2,R3,R4), lab-scale anaerobic digesters (NNR2,NNR3,NNR5 VP-0, VP-10,VP-50, M) and industrial/municipal full-scale digesters (MMBR,MMSS, JBS, CB,KI,CF). The enrichment cultures and digesters chosen for use in the study varied in substrate (Table 2.1). Enrichment cultures and lab-scale digesters were maintained at the Water Quality Center in the Civil, Construction, and Environmental Engineering Department at Marquette University, Milwaukee, WI. All cultures and lab-scale digesters were bioreactors maintained at 35°C and continuously mixed. Industrial and municipal samples were from digesters operated by municipalities and industries in the state of Wisconsin, except CH (Chapter 3) which was taken from a digester operated in California. CB is an upflow anaerobic sludge blanket reactor (UASB), JBS is an anaerobic contact process digester, and the rest are continuously-stirred tank reactors (CSTR) (Figure 2.1). Metadata for operation of industrial digesters CB, JBS, KI, and MMBR may be found in Appendix IV.

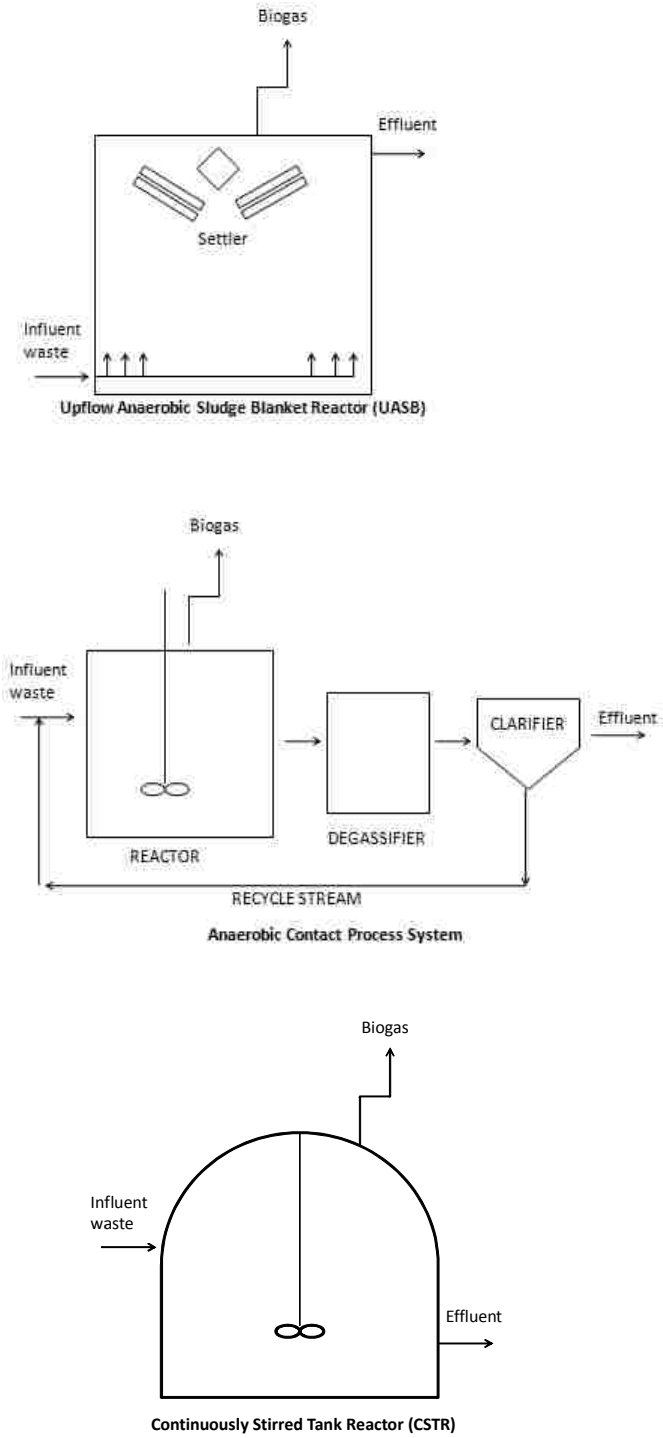


Figure 2.1. Schematic Drawings of Industrial Digesters. Drawings of the configurations of industrial digesters from which biomass was sampled in the course of this study.. (Adapted from presentation by Dennis Totzke, Applied Technologies, Inc., speaking at Anaerobic Treatment of High Strength Industrial Wastes, Milwaukee, WI, September 2008.)

Table 2.1: Descriptions of the Cultures and Digesters. Description of anaerobic cultures and digesters from which biomass was collected for this study.

Digester	Size	Substrate	Additional Amendments
R1	2L	H_2 and CO_2 (50:50) Basal media*	None
R2	2L	H_2 and CO_2 (50:50) Basal media	Glucose (84 mg/d)
R3	2L	H_2 and CO_2 (50:50) Basal media	Oxygen (75 mg/d)
R4	2L	H_2 and CO_2 (50:50) Basal media	Glucose (84 mg/d) Oxygen (75 mg/d)
NNR2	2.5L	Basal media Synthetic Sludge I[†] (4.8g TS/d)	None
NNR3	2.5L	Basal media Synthetic Sludge I (4.8g TS/d)	Flavorings yeast (0.26 gCOD/d) Float (0.52 gCOD/d) Can crushing waste (0.22 gCOD/d) Thin Stillage (0.76 g COD/d) Acid whey (0.54 g COD/d)
NNR5	2.5L	Basal media Synthetic Sludge I (4.8g TS/d)	Flavorings yeast (1.05 gCOD/d)
VP0	150ml	Calcium Propionate (0.17g/L-d) Basal media	None
VP10	150ml	Calcium Propionate (0.17g/L-d) Basal media	Oxygen (0.025mg/L)
VP50	150ml	Calcium Propionate (0.17g/L-d) Basal media	Oxygen (0.125mg/L)
M	40L	Synthetic Sludge II[‡] (30g /day) Basal media	None
CH	Hilmar Cheese	Whey	None
MMSS	Municipal	Municipal	None
MMBR	Municipal	Municipal	None
JBS	JBS Packerland	Beef slaughter plant	None
CB	City Brewery	Brewery	None
KI	Kerry Ingredients	Milk-derived food additives	None
CF	Crave Bros. Farm	Dairy cow manure	None

*Basal media for hydrogen enrichment cultures contained the following (mg L^{-1}): 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 trace metal salts $MnCl_2 \cdot 4H_2O$, NH_4VO_3, $CuCl_2 \cdot 2H_2O$, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $NaMoO_4 \cdot 2H_2O$, H_3BO_3, $NiCl_2 \cdot 6H_2O$, $NaWO_4 \cdot 2H_2O$, and Na_2SeO_3) (each at 0.5 mg L^{-1}); yeast extract (100); $NaHCO_3$ (5000); and resazurin (Schauer-Gimenez et al. 2010). [†]Synthetic sludge I consisted of ground dry dog food. [‡]Synthetic sludge II consisted of nonfat dried milk dried overnight in 103 °C oven, weighed, and then dissolved in water.

Anaerobic biomass samples from cultures and digesters operated in the lab (except lab-scale digester M samples which were collected during time course experiments) were collected using sterile syringes or pipets and placed in Dnase- and RNase- free centrifuge bottles. The samples were then kept on ice or refrigerated until the nucleic acid extraction procedure, which for RNA extractions was initiated within one half hour of collection. The samples collected from M were poured directly in Dnase- and RNase- free centrifuge bottles from a port on the digester and then were immediately stored at -80 ° C until all samples for the time course were collected. Industrial/municipal digester samples were collected in Dnase- and RNase- free (DEPC-treated) centrifuge bottles, placed on dry ice for transport, and then stored at -80 °C until centrifugation (24 – 48 hr), except samples C and B (Chapter 3) which were shipped on ice from their respective plants.

2.2. Nucleic Acid Extractions. DNA was extracted from each of the biomass samples using one of two methods (Table 2.2). The first extraction method was performed using the Powersoil™ DNA Extraction kit (MOBIO, Carlsbad, CA) according to the Alternative Lysis protocol suggested by the manufacturer's instructions for the reduction of DNA shearing (Chapter 3). DNA extractions were also performed in tandem with RNA extractions on biomass samples using the RNA Powersoil™ Total RNA Isolation kit with the DNA Elution Accessory Kit (MOBIO, Carlsbad, CA) according to the manufacturer's standard protocol (Chapters 4 and 5).

RNA extractions were performed, along with DNA extractions, on biomass samples using the RNA Powersoil™ Total RNA Isolation kit with the DNA Elution Accessory Kit (MOBIO, Carlsbad, CA) according to the manufacturer's standard

protocol. RNA samples were treated with Rnase-free Dnase (Rnase-free Dnase Set, Qiagen, Valencia, CA) and purified using the Rneasy ®Mini Kit (Qiagen, Valencia, CA).

Table 2.2. Nucleic Acid Extraction Methods. A description of the various nucleic acid extraction methods used in this study and the data sets which were obtained using samples obtained through the use of each of them.

MO BIO Kit	Data Sets Generated from the Extraction Method
Powersoil®DNA isolation kit alternative lysis method**: replacing bead-beating with heating and brief vortexing**	RFLP study (Chapter 3)
Powersoil® RNA isolation kit with DNA elution accessory kit **chemical and physical lysis including phenol:chloroform: isoamyl alcohol Larger mass of sample**	DNA and RNA clone libraries (Chapter 4) qPCR (Chapter 5)

After purification, DNA and RNA were checked for integrity on agarose gels (1.5% w/v) stained with ethidium bromide and quantified using a spectrophotomer (Nanodrop ND-1000, ThermoScientific, Waltham, MA). Gels were visualized using a UVP Model M-20 UV transilluminator (UVP, Upland, CA).

2.3.Polymerase chain reaction amplification of ***mcrA*****:** The primer pair designed by Luton et al. (2002), mcrF 5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3' and mcrR 5'-TTCATTGCRTAGTTWGGRTAGTT-3' was used for PCR amplification. The final component concentrations per 50 µL PCR reaction were as follows: 100 nM each primer, 0.2 mM dNTPs, 1X Colorless GoTaq Reaction Buffer which contained 1.5 mM $MgCl_2$ (Promega, Madison, WI), and 1.25U GoTaq polymerase (Promega). Template concentrations were approximately 100 ng per reaction tube. The PCR

conditions were as follows: initial denaturation at 95°C (5 min), 35 cycles of 95°C (1 min), 49°C (1 min), and 72°C (3 min), and a final extension of 10 minutes at 72°C. The program included a slow ramp in temperature (0.1°C s^{-1}) between the annealing and extension steps of the first 5 cycles of the protocol to assist in the initial formation of product due to the degenerate nature of the primers, as recommended (Luton et al., 2002). The size of the expected PCR products was confirmed using a 1% (w/v, Tris-acetate-EDTA buffer, Sambrook and Russell, 2001) agarose gel and a λ(Hind III digest) ϕX174 (Hae III digest) DNA ladder stained with ethidium bromide (0.01%, v/v). Gels were visualized as described above.

2.4.Cloning: Clone libraries were constructed by ligating the *mcrA* PCR products into the pCR 2.1-TOPO® vector and then transformation into One Shot TOP10™ chemically competent *E. coli* using the TOPO TA® cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Ampicillin (25µL of 50mg/ml) and X-gal (40µL of 40mg/ml) amended Luria-Bertani agar was used for blue-white screening of the transformants (Sambrook and Russell 2001). Randomly selected white colonies were used for direct PCR with the vector-specific primers PUCF (5´-GTAAAACGACGGCCAG-3´) and PUCR (5´-CAGGAAACAGCTATGAC-3´) (Invitrogen, Carlsbad, CA). The 50-µL final volume PCR reaction component concentrations were as described above. The PCR conditions for the PUC primers were as follows: denaturing temperature of 94°C (1 min), annealing temperature of 55°C (1 min), and elongation temperature of 72°C (1 min), and a final extension of 10 minutes at 72°C. The size of the PUC-amplified PCR products were confirmed as described above.

2.5.Restriction Fragment Length Polymorphism Analysis: Four individual digests were performed on the cloned PUC-amplified PCR product containing *mcrA* using the restriction enzymes *Taq$^{\alpha}$I, RsaI, MspI* and *Sau961* (New England Biolabs, Ipswich, MA) for cultures R1 and R3 as well as digesters C and B (CB). Three individual digests were performed on the cloned PUC-amplified PCR product containing *mcrA* from cultures R2 and R4 using the restriction enzymes *Taq$^{\alpha}$I, RsaI,* and *Sau961* (New England Biolabs, Ipswich, MA). PCR product (10-100 ng) was added to each 20 µl (total) digest mixture, which included 1 µl of enzyme (5,000U ml^{-1}, *Sau961;* 10,000U ml^{-1}, *RsaI* ; 20,000U ml^{-1}, *Taq$^{\alpha}$I* and *MspI*) and 1X concentration of the buffer provided with the enzyme (New England Biolabs). *RsaI, MspI,* and *Sau961* digests were incubated at 37º C, and *Taq$^{\alpha}$I* digests were incubated at 65ºC, as indicated by the manufacturer's instructions. After digestion to completion, digests were separated on 2% (w/v, Tris-acetate-EDTA buffer, (Sambrook and Russell 2001)) agarose gels stained with ethidium bromide (0.01%, v/v) and compared to a 100-base pair ladder (Promega) to ascertain their restriction patterns. Gels were visualized as described above.

2.6.Sequence Analysis: For the clone libraries subjected to RFLP analysis, the PCR products having unique restriction patterns when the results of three restriction digests (*RsaI, Sau961, and Taq$^{\alpha}$I)* were combined (see Results) were purified using Qiaquick™ PCR Purification Kit (Qiagen, Valencia, CA), normalized to a concentration of 50 ng/µl, and sequenced with a capillary automated DNA sequencer (Applied Biosystems, Carlsbad, CA) at the University of Chicago Cancer Research Center DNA Sequencing Facility. Several clones from clone library DNA-I with replicate RFLP patterns were included in the sequencing run. All clones from libraries not subjected to RFLP analysis

were sequenced. The forward and reverse sequences were analyzed using FinchTV (Geospira Inc., Seattle, WA) and VectorNTI (Invitrogen Corporation, Carlsbad, CA) software. Consensus sequences were assembled using the ContigExpress tool in VectorNTI. Residual vector sequence was removed from the consensus sequences using a software program which utilized VecScreen in the Basic Local Alignment Search Tool (BLAST) at NCBI to flag and remove vector sequences from the sample sequence files (Altschul et al. 1997). Nucleotide-nucleotide BLAST searches were conducted with the *mcrA* sequences to determine their relationship to reference *mcrA* sequences in GenBank®.

2.7. Computer Simulation of Restriction Fragment Length Polymorphism Analysis: Computer simulations of the RFLPs with a method similar to that of Moyer et al. (1996), were conducted using the *mcrA* sequences representing an OTU from each of the four digesters. An *in silico* digest was generated using the Biopython restriction enzyme application (Cock et al. 2009) for all four enzymes used in the actual RFLP analysis. The segments resulting from *in silico* digestion of the sequences were binned into 20 base pair categories to determine the presence or absence of a band of corresponding size which was translated into an output file suitable for use with restdist, a program included in the PHYLIP package which creates distance matrices from *in silico* restriction digest data (Felsenstein 2005). Combinations of these output files were then made for each of the enzymes and all possible enzyme combinations.

2.8.Phylogenetic Analysis: *mcrA* sequences were aligned using ClustalW (Thompson et al. 2002). Further phylogenetic analysis was carried out using the PHYLIP suite of programs (Felsenstein 2005). Seqboot (PHYLIP) was used on the combined output file

for each enzyme or combination of enzymes and on each set of unique sequences from each digester to create bootstrap samples. Then the bootstrap samples were entered into dnadist (sequences) or restdist (*in silico* restriction digests) PHYLIP programs to create distance matrices. The dnadist or restdist output files were used to create bootstrapped neighbor-joining trees using neighbor and a final consensus tree for each file was created using consense (PHYLIP). The consense trees were visualized using Figtree v1.2.3. Maximum likelihood and maximum parsimony trees were also created for the sequences using dnapars and dnaml (PHYLIP). No major differences were observed among the maximum likelihood, maximum parsimony, and neighbor-joining trees from each individual set of sequences.

Treeclimber (Schloss and Handelsman 2006) was used to compare the methanogen communities as represented by the neighbor-joining phylogenetic trees.

2.9.Statistical Analysis of Clone libraries: Rarefaction curves were generated for the RFLP data from each clone library as described by (Kemp and Aller 2004) to examine the extent of coverage of the diversity of the methanogen community in each digester, and the Shannon Index was calculated from the RFLP data for each library to determine methanogen community heterogeneity (Shannon and Weaver 1964). The S_{CHAO1} value was also calculated from the RFLP data from DNA-I to estimate coverage of *mcrA* diversity within the culture by the library (Chao 1984, Chao 1987).

For the clone libraries in which all of the clones were sequenced, DOTUR (Schloss and Handelsman 2005) was used to calculate the Shannon Index and S_{CHAO1} values, as well as to determine the number of unique operational taxonomic units (OTUs)

present and to generate rarefaction curves. Although the percentage of sequence similarity among *mcrA* nucleotide sequences has not been determined, Edmonds et al. (2008) suggested using amino acid sequence similarity of 90% (0.10) and Rastogi et al. (2008) suggested using 94% (0.06). In order to determine which sequence similarity to use with *mcrA* nucleotide sequences, the DNA and cDNA sequences for each of the enrichment cultures (R1,R2,R3, and R4) were translated into amino acids using the Virtual Ribosome web-based tool (Wernersson 2006). The translated sequences were aligned using ClustalW (Thompson et al. 2002). The alignment was converted into a distance matrix by protdist from the PHYLIP suite of programs (Felsenstein 2005), and the matrices were then analyzed using DOTUR (Schloss and Handelsman 2005). DOTUR results based on 90-94% similarity were compared to DOTUR results from the original nucleotide sequences. The amino acid sequence data for 90-94% similarity was comparable to nucleotide sequence results at 97% (0.03) similarity, and therefore that value was used when reporting data from DOTUR analyses.

Evenness was calculated using the following equation:

$$J = H'/\ln S$$

where J is evenness, H' is heterogeneity (Shannon index), and S is richness (Pielou 1966).

2.10.Nucleotide Sequence Accession Numbers: All nucleotide sequences generated in the course of this study can be found in the Genbank® database under accession numbers HM800526 through HM800637 and HM80666 through HM80695 and JF460039 through JF460714.

2.11.Reverse Transcriptase (RT-) PCR. RT- PCR was performed using the iScript™ Select cDNA Synthesis Kit (Biorad, Hercules, CA) on 1400 ng of each purified RNA extract, except in cases where the concentration of the extracted RNA was too low to allow addition of the entire 1400ng. In these cases, as much RNA as possible (R1-11: 758 ng, R1-16: 589 ng, M-BR-24: 256 ng, M-2R-24: 1109 ng, VP-0: 261 ng, VP-10: 119 ng, VP-50: 150 ng, M-8R-24: 696 ng, and CF: 481 ng) was added to the RT-PCR reaction (only 12.9 µl of template could be added to each reaction). Controls included no-reverse-transcriptase controls for each sample, and no-template controls for each run. Each 20 µl reverse transcriptase reaction consisted of 1X iScript select reaction mix (reaction buffer containing dNTPs, magnesium chloride, and stabilizers), 500nM mcr-R primer 5'-TTCATTGCRTAGTTWGGRTAGTT-3'(Luton et al. 2002), 2 µl GSP enhancer solution (Biorad), 1 µl iScript reverse transcriptase (RNase H^+ MMLV reverse transcriptase and RNase inhibitor protein), and RNA as discussed above. The RT reaction conditions were as follows: 42 °C for 1hr 30 min and then 85 °C for 5 minutes. The resulting cDNA samples were stored at -20°C.

2.12.Quantitative PCR. qPCR was performed according to the guidelines suggested by Smith (Smith et al. 2006, Smith and Osborn 2009) except for the standard curve (see below) and the suggestions found in the MIQE guidelines (Bustin et al. 2009) which are applicable to environmental samples.

qPCR was performed using the primers designed by Luton et al. (2002): mcrF 5' -GGTGGTGTMGGATTCACACARTAYGCWACAGC-3' and mcrR 5'-TTCATTGCRTAGTTWGGRTAGTT-3' and previously used for qPCR (Freitag and Prosser 2009, Freitag et al. 2010, Goffredi et al. 2008, Vianna et al. 2006). The product

of these primers is ~460 bp of *mcrA*, the gene encoding the α subunit of methyl coenzyme M reductase. The final qPCR mix per 25 µl reaction was as follows: 1X iQ™ SYBR® Green Supermix reaction buffer containing dNTPS , iTaq DNA polymerase and 3 mM $MgCl_2$ (Biorad, Hercules, CA); 750 nM mcrF and mcrR; and template DNA (0.3-1 ng) or cDNA (1 µl of RT-PCR reaction, unless RT input amount was less than 1400 ng in which case the amount of RT reaction added to qPCR mix was increased to account for the difference between 1400 ng and the actual amount (see above). Each qPCR run included a no template controls and the no-RT controls from the RT reactions. Samples were kept on ice during set up of the run. The qPCR reactions were performed with the Biorad MyIQ™ Single-Color Real-Time PCR Detection System using the following program: initial denaturation at 95°C (10 min), 45 cycles of 95°C (30 sec) and 58.5°C (1 min), and a final extension of 7 minutes at 72°C. The amplification program was followed by a denaturation curve program (80 cycles 10 sec in length starting at 55°C and increasing in 0.5°C increments) to check for product specificity. Products from initial runs were also examined for specificity using 1.5% agarose gels as described above. Starting quantity amounts and threshold cycle values were calculated using the MyiQ™ optical system software version 1.0.

qPCR standards used in all runs were created using pooled *mcrA* DNA clones from anaerobic biomass samples whose sequences had been determined as part of a previous study. Care was taken to choose a broad spectrum of *mcrA* sequences representative of methanogen genera commonly seen in anaerobic digesters (*Methanospirillum, Methanobacterium, Methanosaeta, Methanoculleus, Methanobrevibacter*) as well as clones whose sequences could not be related to a specific

methanogen genera (Steinberg and Regan 2008). Nucleotide sequences for these *mcrA* clones can be found in Genbank® under accession numbers HM800527-528, HM800531, HM800534-536, HM800542, HM800547, HM800549, HM800560, HM80072, HM800574, HM800581, and HM800611. Concentrations of purified (QIAquick ® PCR Purification Kit, Qiagen) *mcrA* clones were determined by spectrophotometry (Nanodrop ND-1000, Thermo-Scientific), and then 50 ng of each was added to the standard mix. Concentration of the mix was confirmed, and the mix was diluted to 0.1ng/ µl. 5 µl aliquots of the diluted mix were stored at -80 °C. Freshly thawed aliquots were used for each qPCR run.

2.13. Denaturing Gradient Gel Electrophoresis (DGGE): DNA extracts from industrial samples were amplified with *mcrA* specific primers described above with one exception, the forward primer was modified to include a GC clamp (5'-CGCCCGCCGC GCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGTGGTGTMGGATTCACACART AYGCWACAGC-3') (Luton et al. 2002, Muyzer et al. 1993). The final component concentrations per 50 µL PCR reaction were as follows: 100 nM each primer, 0.2 mM dNTPs, 1X Colorless GoTaq Reaction Buffer which contained 1.5 mM $MgCl_2$ (Promega, Madison, WI), and 1.25U GoTaq polymerase (Promega). Template concentrations were approximately 100 ng per reaction tube. The PCR conditions were as follows: initial denaturation at 95°C (5 min), 35 cycles of 95°C (1 min), 58°C (1 min), and 72°C (3 min), and a final extension of 10 minutes at 72°C. The program included a slow ramp in temperature (0.1°C s^{-1}) between the annealing and extension steps of the first 5 cycles of the protocol to assist in the initial formation of product due to the degenerate nature of the

primers, as recommended (Luton et al., 2002). The size of the expected PCR products was confirmed using a 1% agarose gel as described above.

Analysis of the PCR products on polyacrylamide gels was performed by Dr. V.P. Tale (Department of Civil, Construction, and Environmental Engineering, Marquette University). Equal PCR product concentrations from each PCR reaction were then used for DGGE in a 1mm thick 8% polyacrylamide gel (37.5:1 acrylamide to bis-acrylamide) with 40-70% denaturant gradient (urea and formamide). Electrophoresis at 100V for 15h was performed using the Universal DCode Mutation Detection System (Biorad). The DGGE gel was stained with 1% SYBR Gold Nucleic Acid stain (Invitrogen) for 30 minutes and visualized using the GelDoc-It Imaging System (UVP).

A tree representing the relationships between the industrial samples' DGGE patterns was constructed by Dr. V.P. Tale (Department of Civil, Construction, and Environmental Engineering, Marquette University) using the optical density data collected by the Labworks™ software (Lablogics, Inc., Mission Viejo, CA). Pearson's correlation coefficient (r) was calculated using the densitometric data for each pair of samples. A distance matrix representing the relationships among the densitometric data was calculated using 1- r values. An unweighted pair group method with arithmetic mean (UPGMA) tree was plotted using the distance matrix and the PHYLIP software package (Felsenstein 2005). The obtained tree was rooted to the sample having highest SMA against propionate (i.e., CB).

2.14. Volatile Solids (VS) and Volatile Suspended Solids (VSS): VS and VSS measurements were performed by Dr. A.E. Schauer-Gimenez, Dr. V.P. Tale, Mr. N. Navareenthan, and Mr. U. Bhattad (Department of Civil, Construction, and

Environmental Engineering, Marquette University) according to standard methods (American Public Health Association (APHA 1998).

2.15. Specific Methanogenic Activity Assays. Methanogenic activity assays were conducted by Dr. A.E. Schauer-Gimenez, Dr. V.P. Tale, Mr. N. Navareenthan, and Mr. U. Bhattad (Department of Civil, Construction, and Environmental Engineering, Marquette University) in triplicate, modified from the protocol described by Coates et al. and others (Coates et al. 2005, Coates et al. 1996, Schauer-Gimenez et al. 2010, Zitomer et al. 2008b). All assays were performed under anaerobic conditions in 160-ml serum bottles with 25 ml (< 3g VSS/L) of biomass. The VSS concentration was determined at the beginning and end of activity tests and the average of the two values was employed for specific activity calculations.

For H_2/CO_2 specific activity assays, the serum bottles were sparged with gas (4:1 v/v H_2:CO_2) and closed with solid Balch-type butyl rubber stoppers and aluminum crimped seals. Immediately thereafter, 100 ml of the H_2:CO_2 gas blend at ambient pressure and temperature was injected through the stopper using a syringe and needle.

For acetate and propionate specific activity tests (Zitomer et al. 2008b), assays were supplied with 3g/L propionate in the form of calcium propionate or 10g/L calcium acetate whereas the control assays were not supplied with any substrate. All the propionate and acetate assays were then sparged with gas (7:3 v/v N_2:CO_2) to establish anaerobic conditions and solid Balch-type butyl rubber stoppers and aluminum crimped seals were used to maintain anaerobic conditions.

Immediately after the addition of substrate to the test assays, all bottles were incubated at 35°C and shaken at150 rpm using an incubator shaker (model C25KC, New

Brunswick Scientific, Edison, NJ). Bottle head space volume was measured at ambient pressure (approximately 1 atm) for 30 days by inserting the needle of a glass syringe with wetted barrel. Syringe content was re-injected into the serum bottle after volume measurement. Headspace methane content was analyzed using gas chromatography by standard methods (APHA et al. 1998). Methane produced by the control assays accounted for endogenous decay, so was subtracted from methane produced by test assays. Finally, maximum methane production rate (ml CH_4/g VSS-hr) was determined as described in the literature (Owen et al. 1979, Speece 2008, Zitomer et al. 2008b).

Chapter Three
Application of Restriction Fragment Length Polymorphism Analysis of *mcrA* for Determination of Methanogen Diversity

3.1 Introduction

Methanogenic *Archaea* are important members of the microbial community in anaerobic environments, responsible for completing one of the final steps in the degradation of organic matter (i.e., methane production) and thus, maintaining the cycling of carbon. Methanogens play an especially important role in anaerobic waste treatment digesters. Although degradation of organic waste as a whole is actually carried out by an interdependent microbial community, methanogens complete the anaerobic treatment process by creating methane from products such as acetate, carbon dioxide and hydrogen gas which are produced by other microbes. Furthermore, the methane produced by these organisms can be collected and used for energy as a renewable alternative to fossil fuels. Therefore, understanding methanogen community structure is important when attempting to optimize both waste treatment and methane production.

Diversity is an important facet of community structure which can be especially significant in studies which seek to link structure to function. In the case of anaerobic wastewater treatment, the entire microbial community has been a "black box" (Rivière et al. 2009) throughout most of the history of this technology. The relationship between structure and function of the community as a whole as well as within the guilds of organisms such as the methanogens within the community is not clear. However, because anaerobic digesters are controlled, artificial environments, the potential for engineering microbial communities with improved stability, organic waste removal, and methane

production clearly exists. Methanogens would be critical members of engineered communities of this sort because of their contributions to both waste removal and methane production. Therefore, methods for the detection of methanogen diversity used for comparisons with digester function must be thorough and comprehensive.

Although 16S ribosomal RNA (rRNA) genes are used to determine operational taxonomic units (OTUs) in most molecular studies of microbial communities, methanogens possess a unique operon which encodes the methyl coenzyme M reductase (MCR). MCR is a multi-subunit enzyme which catalyzes the final step of methanogenesis and is unique to methanogens and the anaerobic methane-oxidizing *Archaea* (Hallam et al. 2003, Springer et al. 1995). Previous studies have established that the gene which encodes the alpha subunit of MCR (*mcrA*) can be used to detect methanogen presence in the environment and is suitable for defining methanogen diversity (Luton et al. 2002, Springer et al. 1995). The PCR primer set developed by Luton, et al. (2002) has been shown to consistently amplify an approximately 460 base pair segment of the *mcrA* sequence from a wide range of methanogenic genera (Banning et al. 2005, Juottonen et al. 2005, Juottonen et al. 2006, Luton et al. 2002, Pereyra et al. 2010).

Restriction fragment length polymorphism analysis (RFLP) of *mcrA* clone libraries has often been, and continues to be, used to determine the diversity of OTUs during the examination of methanogenic communities (Castro et al. 2004, Earl et al. 2003, Galand et al. 2002, Galand et al. 2005b, Nercessian et al. 2005, Nettmann et al. 2008, Orphan et al. 2008, Pereyra et al. 2010, Scanlan et al. 2008, Smith et al. 2007, Ufnar et al. 2007). However, there is no consensus in the published literature to date as to

Table 3.1. Description of Restriction Endonucleases. Restriction endonucleases used in previous studies using RFLP and terminal restriction fragment length polymorphism (T-RFLP) analysis with the Luton et al. (2002) primers for *mcrA* clone libraries. (Source for restriction sites, New England BioLabs, Ipswich, MA)

Enzyme(s) Used **Restriction Sites**	**Publication**
TaqI **5'-T□CGA-3'** **3'-AGC□T-5'**	Luton et al. 2002, Castro et al. 2004, Nettmann et al. 2008
Sau961 **5'-G□GNCC-3'** **3'-CCNG□G-5'**	Castro et al. 2005 (T-RFLP)
RsaI **5'-GT□AC-3'** **3'-CA□TG-5'**	Smith et al. 2007
MspI* and *TaqI **5'-CC□GG-3'** **3'-GG□CC-5'** **5'-T□CGA-3'** **3'-AGC□T-5'**	Galand et al. 2005, Ufnar et al. 2007
MspI* and *HaeIII **5'-CC□GG-3'** **3'-GG□CC-5'** **5'-GG□CC-3'** **3'-CC□GG-5'**	Nercessian et al. 2005
RsaI and HaeIII **5'-GT□AC-3'** **3'-CA□TG-5'** **5'-GG□CC-3'** **3'-CC□GG-5'**	Orphan et al. 2008
MboII **5'-GAAGA(N)$_8$□-3'** **3'-CTTCT(N)$_7$□-5'**	Pereyra et al. 2010

which restriction enzymes, or even how many, should be used to obtain the most complete RFLP coverage of *mcrA* diversity. In previous RFLP studies, after PCR amplification with the Luton et al. (2002) primer set, one or two enzymes were used to examine *mcrA* diversity (see Table 3.1).

However, Moyer et al. (1994) demonstrated that the use of three or four restriction enzymes in RFLP was necessary to obtain good coverage of OTUs represented by the 16S rRNA gene. Although this study is now seventeen years old and the use of RFLP has been expanded to examine the diversity of many functional genes, very few studies have examined the choice of restriction enzymes for use with functional genes and RFLP. Poly and colleagues (2001) did show that three enzyme combinations were best for use with *nifH* genes, but the majority of studies which use RFLP on functional genes for diversity studies fail to address this topic. Based on *mcrA* sequences in GenBank®, Steinberg and Regan (2008) determined that *mcrA* sequence similarity within genera varies much more widely than among 16S rRNA sequences from the same genus and a percentage of *mcrA* sequence similarity which may be used for taxonomic resolution to the species level has not been determined. If RFLP is used to detect unique *mcrA* clones either for determination of OTUs or for selection of unique clones to be sequenced for identification and phylogenetic analysis, detection of as many of the truly unique sequences as possible is important.

This study was performed to test the hypothesis that the use of multiple restriction enzymes in RFLP is necessary to obtain sufficient coverage of OTUs when examining diversity of *mcrA.* The hypothesis was tested by digesting *mcrA* clones with *Taq$^{\alpha}$I, RsaI, MspI,* and *Sau961.* The clone libraries used were constructed using PCR products from

DNA extractions from biomass of four different methanogenic cultures: two laboratory-scale bioreactors and two full-scale digesters. The RFLP data from each restriction enzyme (and all possible enzyme combinations) were used to generate rarefaction curves and to calculate the Shannon Index and the number of OTUs for each clone library. Unique *mcrA* clones were sequenced. Phylogenetic and *in silico* RFLP analysis was also performed on the unique *mcrA* sequences. Results of these analyses were then compared to determine which enzyme or enzyme combination provided the most thorough coverage of methanogen OTUs.

3.2.Results

3.2.a Restriction Fragment Length Polymorphism (RFLP) Analysis

Four clone libraries constructed from *mcrA* sequences from the four different anaerobic cultures were used to determine the number and specific restriction enzymes necessary to achieve the most coverage of *mcrA* diversity. Analysis of the RFLP patterns indicated that more than one enzyme was required to distinguish unique *mcrA* clones (Figure 3.1). This observation held true for each of the clone libraries examined.

3.2.b Operational taxonomic units (OTUs).

When using RFLP with *mcrA* sequences to select unique OTUs, more were identified as additional restriction enzymes were used for analysis as expected (Table 3.2). Furthermore, using three enzyme combinations in RFLP the greatest number of unique *mcrA* sequences was determined using *Taq$^{\alpha}$I, RsaI,* and *Sau961* digests of clones from R1 and B, and *Taq$^{\alpha}$I, RsaI,* and *MspI* digests of clones from R3 and CH. Examination of RFLP using *Taq$^{\alpha}$I, RsaI,* and *MspI* resulted in identification of similar numbers of unique

OTUs as found using *Taq$^{\alpha}$I, RsaI,* and *Sau961* on *mcrA* from all cultures except R1, in which the use of *Sau961* resulted in the detection of nine more OTUs than *Msp*I. In contrast, in cultures CH, CB, and R3, only 1-3 OTU differences were detected when *Sau961* and *MspI* were interchanged.

Five sets of clones with replicate sets of restriction patterns were sequenced and the sequences were compared to determine whether clones with replicate patterns were truly duplicates. When aligned and compared, clones with replicate RFLP patterns showed between 94-100% sequence similarity and when compared to *mcrA* sequences found in GenBank® using blastn (Altschul et al. 1990, Altschul et al. 1997) replicates were most similar to the same stored sequences.

3.2.c Shannon Index.

When the RFLP data from the four enzymes were used sequentially to calculate the Shannon Index (Shannon and Weaver), greater heterogeneity was observed when using data obtained from three enzymes than using only one or two enzymes (Table 3.2). When RFLP data from digests using all four enzymes were used to calculate the Shannon Index, higher values were obtained than when using three enzymes. However, further analysis revealed that while statistical differences existed between using one or two enzymes and between using three or four enzymes ($p>0.05$, ANOVA, Tukey test), there was no statistical difference between Shannon Indices calculated using either three or four enzymes ($p<0.05$, ANOVA). The greatest *mcrA* gene heterogeneity was demonstrated using the three restriction enzymes *Taq$^{\alpha}$I, RsaI,* and *Sau961* in three out of the four methanogenic cultures sampled. However, analysis using *Taq$^{\alpha}$I, RsaI,* and *MspI* resulted

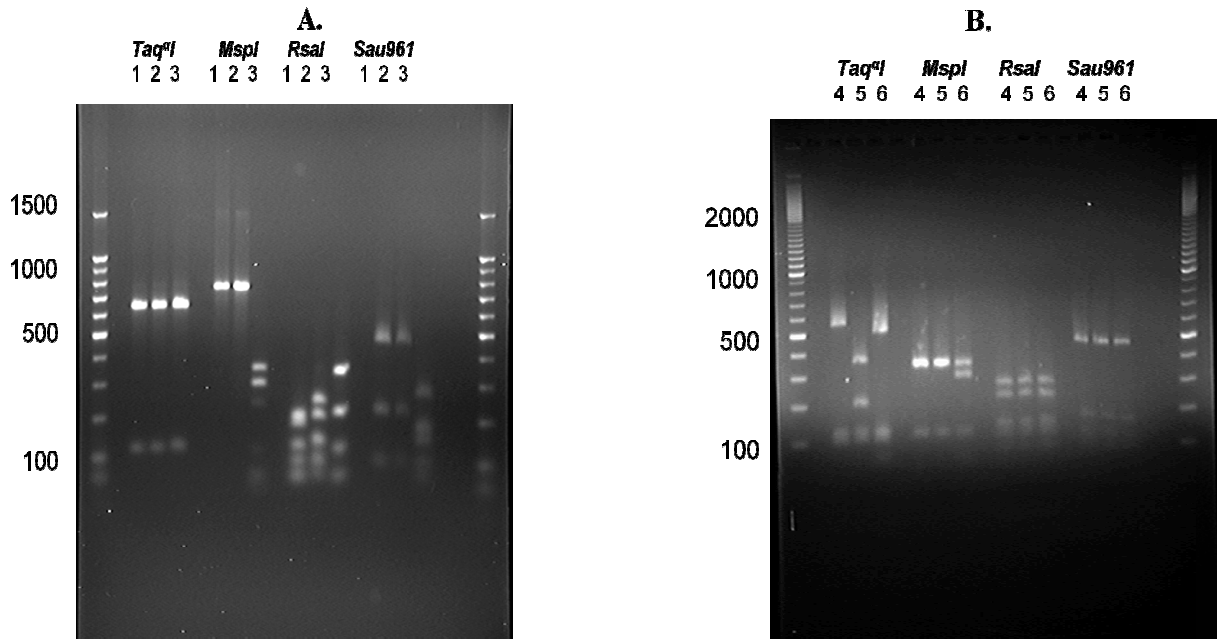


Figure 3.1: Representative Restrictioin Digest Gels. Restriction digests separated in 2% agarose gels and stained with ethidium bromide. Each gel shows 3 representative *mcrA* clones cut with four different restriction enzymes. **A.** Digests of *mcrA* clones rlm_B_58 (1), rlm_B_59 (2), and rlm_B_52 (3). In this case, digests using *RsaI* demonstrate that the three sequences are different while *Taq$^{\alpha}$I* digestion did not discriminate among the sequences at all. *MspI* and *Sau961* were able to distinguish differences in two of the three sequences **B.** Digests of *mcrA* clones rlm_R_194 (4), rlm_R_195 (5), and rlm_R_196 (6). In this case, *RsaI* and *Sau961*digestion failed to discriminate among the sequences while *Taq$^{\alpha}$I* digestion showed that all three sequences were unique.

Table 3.2. Comparison of Restriction Enzyme Combinations. Comparison of combinations of restriction enzymes using the number of unique operational taxonomic units (OTUs) and the Shannon Index.

	CULTURE							
	R1		R3		CH		CB	
Restriction enzymes	Unique OTUs	Shannon Index	Unique OTUs	Shannon index	Unique OTUs	Shannon index	Unique OTUs	Shannon Index
RsaI	13	2.19	10	1.82	11	1.81	10	1.94
Sau961	11	2.15	10	1.46	11	1.31	10	1.72
MspI	9	1.81	13	1.95	7	1.15	10	1.81
Taq$^{\alpha}$I	15	2.14	16	2.11	11	1.69	8	1.57
RsaI and Sau961	37	3.21	30	2.64	27	2.46	26	2.72
MspI and Sau961	25	2.67	31	2.68	20	2.01	23	2.42
Taq$^{\alpha I}$I and Sau961	35	3.12	37	2.96	29	2.66	21	2.36
RsaI and MspI	28	2.87	39	2.98	25	2.57	27	2.83
Taq$^{\alpha I}$I and MspI	30	2.89	44	3.22	24	2.41	20	2.40
Taq$^{\alpha I}$I and RsaI	33	3.14	44	3.31	36	3.03	25	2.66
RsaI, MspI, and Sau961	42	3.29	47	3.28	38	3.01	32	2.95
Taq$^{\alpha I}$I, MspI, and Sau961	43	3.29	53	3.54	39	3.14	27	2.67
Taq$^{\alpha I}$I, RsaI and Sau961	52	3.64	57	3.67	46	3.47	36	3.11
Taq$^{\alpha I}$I, RsaI, and MspI	43	3.35	58	3.65	49	3.41	35	3.16
All four	55	3.71	64	3.85	57	3.74	39	3.26

in similar Shannon Indices to those obtained by using *Taq$^{\alpha}$I, RsaI,* and *Sau961* in three out of four cultures (Table 3.2).

3.2.d Rarefaction curves.

The rarefaction curves for culture R1 (Figure 3.2) was representative of the rarefaction curves representing the RFLP data from each digester. All of the rarefaction curves demonstrated that the diversity in *mcrA* sequences increased as patterns from more enzymes were included in the analysis. For example, when only a single enzyme was used, most of the unique patterns present were detected after about thirty clones were analyzed. However, rarefaction curves created using combined results from two or three different enzyme digests showed that more and more unique patterns were still being detected even after 88-100 clones had been analyzed (Figure 3.2).

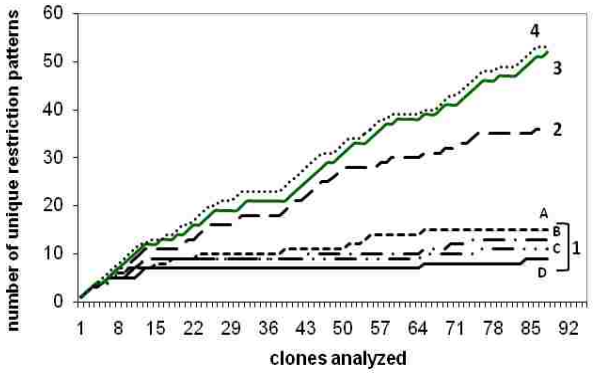


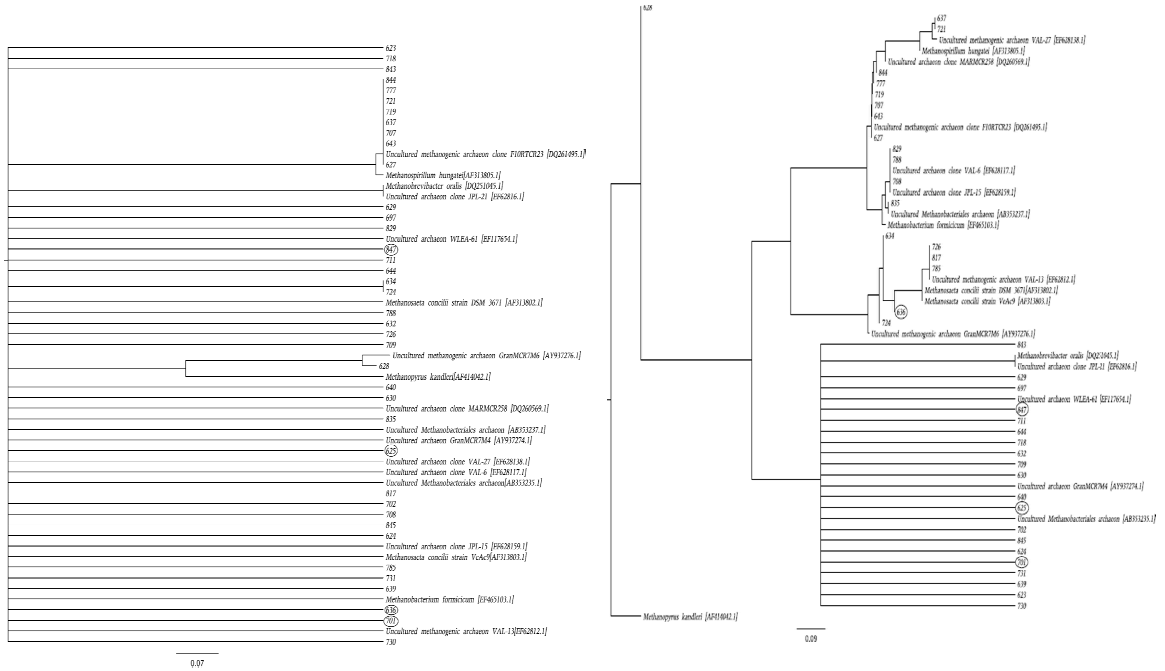
Figure 3.2. **Sample Rarefaction Curves.** Sample rarefaction curves generated from RFLP data from culture R1 showing the effect of single and multiple restriction enzymes on the slope of the curve. Similar results occurred with other digester samples. 1. Single enzymes. A = *Taq$^{\alpha}$I***;** B = *RsaI***;** C = *Sau961***;** D = *MspI***;** 2. Two enzymes**.** *RsaI* and *Sau961***;** 3. Three enzymes**.** *Taq$^{\alpha}$I, RsaI* and *Sau961***;** 4. All four enzymes.

Adding a fourth enzyme did change the rarefaction curves slightly, but not as drastically as adding a second or third enzyme. Adding a third enzyme resulted in the detection of 10-15 additional OTUs per digester while the addition of a fourth enzyme only resulted in the detection of 3-8 more OTUs (Table 3.2).

3.2.e *In silico* RFLP analysis.

When the *mcrA* sequences generated from this study which had unique RFLP patterns were used to make neighbor-joining trees from distance matrices based on *in silico* restriction digests (the output from restdist, (Felsenstein 2005), the trees created from data from one enzyme showed less branching than trees made using data from two enzymes (Figure 3.3 A & B). Using data from three different enzyme digests resulted in more branching of the trees than those created from the data using two enzymes (Figure 3.3 B & C). However, trees created using four enzyme combinations had similar branching and clades to those made from using three enzyme combinations (Figure 3.3 C & D). Trees created using the three enzyme combination *Taq$^{\alpha}$I, RsaI,* and *Sau961* resulted in branching similar to trees made directly from the sequences instead of from the *in silico* restriction digests (Figure 3.3 C & D).

Histograms depicting size and number of fragments resulting from *in silico* digests of the sequences were also compared to photographs of gels for the same samples. Although the sequences had been processed and trimmed as described in the methods and sequence data provides a higher resolution than that provided by observation of gel patterns, the number and size of major bands represented in the histograms was similar to the number of bands seen in the gels.



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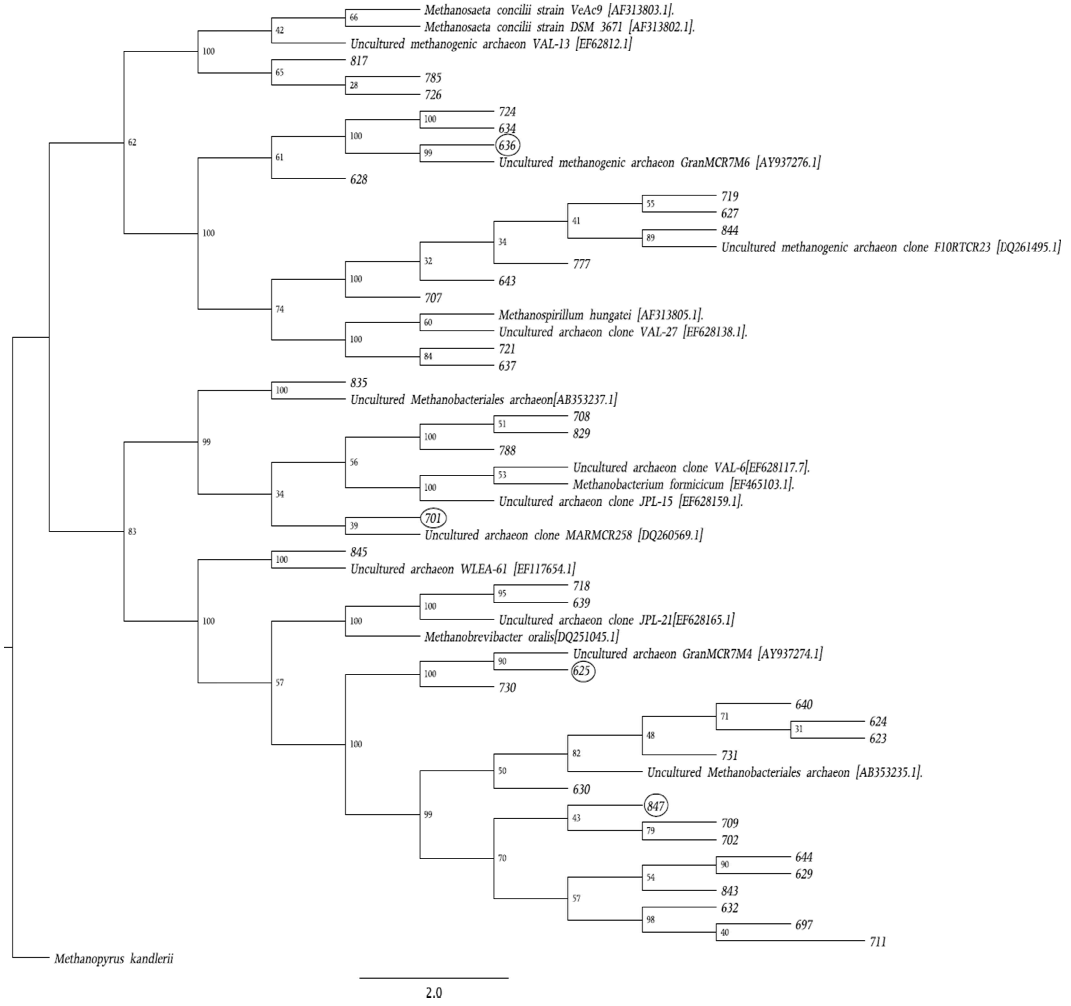
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3.2.f Methanogen Community Diversity. The sequence data from the unique clones was used to determine the assignments of OTUs to specific methanogen genera. Relative abundances of clones in each genera showed that lab-scale digesters were dominated by *Methanospirillum*-related clones while industrial digester biomass was dominated by *Methanobacterium* (Figure 3.4).

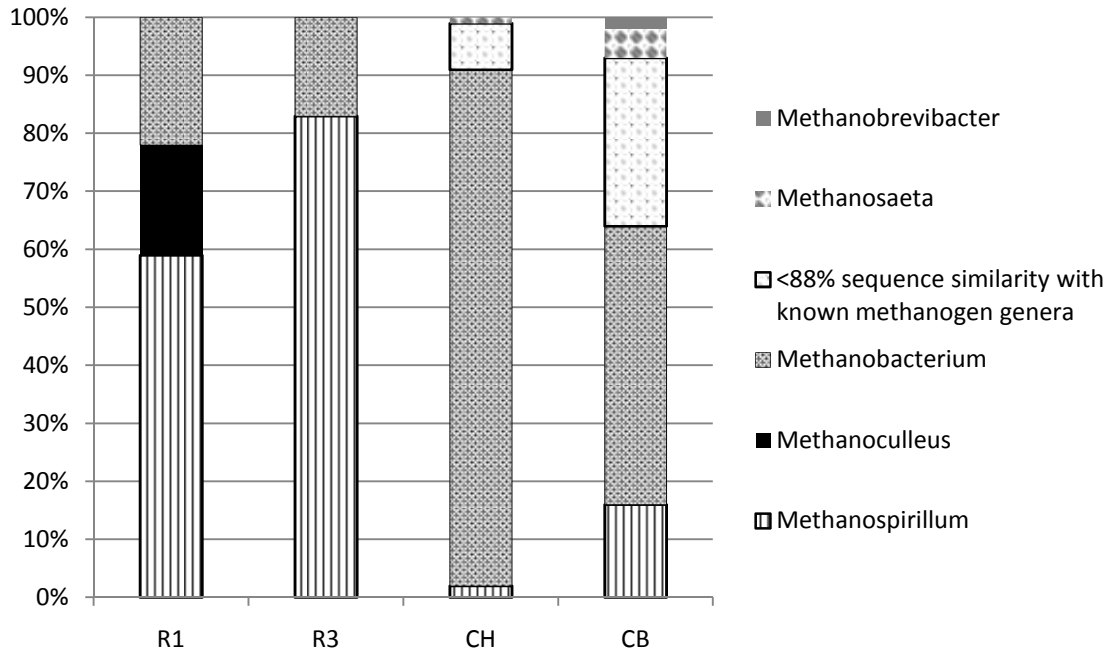


Figure 3.4 Relative Abundance of *mcrA* Clones. Abundance of clones in specific methanogen genera based on 88% sequence similarity (Steinberg and Regan 2008)**.**

3.3 Discussion

Methanogens play an important role in the global carbon cycle as well as in engineered environments such as anaerobic waste digesters. Therefore, obtaining the best data regarding their community structure is important. While *mcrA* has been demonstrated to be a valuable gene for use in the investigation of methanogens in the

environment, the data obtained from PCR-based methods using primers for *mcrA* are subject to biases inherent in the process from the extraction of DNA from environmental samples to PCR amplification efficiencies (v. Wintzingerode et al. 1997). However, the primer set designed by Luton et al. (2002) has previously been shown to consistently amplify *mcrA* from a wide range of methanogen genera (Banning et al. 2005, Juottonen et al. 2006), making the set a sound choice for the examination of methanogen OTUs in environmental samples using RFLP. On the other hand, the choice of restriction enzymes also plays a role in the number of OTUs that can be identified by such a study.

The data in this study indicated that combining the results of at least three or more restriction enzyme digests of *mcrA* clones provided better coverage of methanogen OTUs and community diversity in anaerobic biomass cultures than the use of only one or two enzymes for RFLP analysis. For example, the number of OTUs increased 57-69% when a second restriction enzyme was used in this determination and an additional 24-33% when a third enzyme was added to the analysis (Table 3.2 and Figure 3.2), and different restriction enzyme combinations detected differing numbers of OTUs (Table 3.2). The Shannon Index, which measures community heterogeneity, also increased as the data from additional restriction enzymes were used in its calculation with significant increases occurring between the use of one or two enzymes and between two or three enzymes (Table 3.2). Examination and comparison of the neighbor-joining phylogenetic trees created using the *in silico* restriction digests also showed that increased diversity was detected as more restriction enzymes were used in the analysis (Figure 3.3). This was demonstrated by the increased branching and number of clades seen in the trees as the data from additional enzymes used for *in silico* digests were included (Figure 3.3). Taken

together, these results indicated that more than two restriction enzymes were needed to be used with RFLP analysis to obtain the best coverage of the diversity of methanogen communities.

Further examination of the data demonstrated that the use of three enzymes provided sufficient coverage of the methanogen diversity. For example, when rarefaction curves were produced for each clone library, adding a fourth enzyme did not greatly change the estimate of coverage determined when using only three enzymes (Figure 3.2). Adding the data from a fourth enzyme did change the rarefaction curves slightly, but not as drastically as adding a second or third enzyme to the analysis did (Figure 3.2). Rarefaction curves produced using the data from RFLP with only one enzyme flattened after approximately 15 clones were examined, suggesting that most of the *mcrA* clone diversity was captured (Figure 3.2). However, curves plotted using the data from two, three or four enzymes for analysis continued to climb even when >85 clones were analyzed (Figure 3.2), suggesting that the diversity of the methanogen community had not been fully captured. Furthermore, there was no significant difference between the Shannon Indices calculated using the data from three and four restriction enzymes. These results suggested that three enzymes were sufficient to determine community heterogeneity, and the costs in labor and materials for more restriction digests did not provide enough additional information to be necessary.

The data further suggest that the specific combination of *Taq$^{\alpha}$I, RsaI,* and *Sau96* digests was the best choice for detecting methanogen diversity using RFLP with *mcrA.* In half of the cultures included in the study, using this combination for analysis resulted in the detection of the greatest number of OTUs, and in three out of the four cultures, this

combination resulted in the greatest Shannon Index based on the data from three enzymes (Table 3.2).

The results of this study suggest that previous investigations, which used only one or two restriction enzymes for *mcrA* RFLP analysis to determine OTUs and/or calculate the Shannon Index, may have underestimated methanogen diversity in the habitats that were investigated. For example, the Shannon Indices and number of OTUs determined using *mcrA* RFLP analysis with one enzyme on samples from the Florida Everglades by Castro et al. (2004) and Smith et al. (2007) were similar to those found in this study when using only one enzyme to collect data from anaerobic biomass (Table 3.2). In other studies, Shannon Indices or OTU quantification calculated using RFLP with two enzymes resulted in lower values than those found in this study (Galand et al. 2005a, Nercessian et al. 2005). However, the environments sampled in these studies, peatlands and deep-sea hydrothermal vents, may have lower methanogen diversity due to the extreme conditions in these ecosystems.

The data from this study also suggest that restriction enzyme choice can make a significant difference in estimates of diversity. Therefore, care should be taken when interpreting methanogen community fingerprints obtained using terminal restriction fragment length polymorphism (T-RFLP) analysis with *mcrA*, as previously discussed by Castro et al. (2005). Denaturing gradient gel electrophoresis may provide a better community fingerprint than T-RFLP when using *mcrA* to study methanogens in the environment.

When studying the methanogen community in wastewater treatment plants and other environmental samples, obtaining good coverage of the whole methanogen community is important for understanding the relationship between methane output and community structure. The additional unique sequences that are detected when using three restriction enzymes for RFLP analysis may represent organisms which play an important role in methanogen community dynamics and function. However, the use of multiple restriction digests on clone libraries is labor intensive and may require considerable amounts of supplies. On the other hand, sequencing costs have decreased rapidly in recent years, and costs are predicted to continue to decline. If multiple digests are truly necessary to obtain good coverage of diversity, either using 16S rRNA or functional genes such as *mcrA*, sequencing alone may be the most cost-effective approach, especially in very diverse habitats such as anaerobic digesters.

Finally, the connection between genetic differences and differences in MCR function is not clear. Further studies must be done before the relationship between the diversity of *mcrA* sequences and methanogen function can be closely linked.

Chapter Four
Revealing the active methanogen community: comparison of methyl coenzyme M reductase alpha subunit (*mcrA*) genes and transcripts present in anaerobic biomass.

4.1 Introduction

Methanogenic *Archaea* are important members of the microbial community in anaerobic environments, responsible for completing one of the final steps in the degradation of organic matter and thus, maintaining the cycling of carbon. Carbon dioxide and methane are the products of this process, making methanogens important sources of greenhouse gases as well. Therefore, understanding the structure and function of methanogen communities can provide important insight into mechanisms which have global impact.

Methanogens also play an especially important role in anaerobic waste treatment digesters. Anaerobic wastewater treatment is an environmentally and economically beneficial process in which the biological degradation of organic compounds found in wastewater results in the production of methane, an alternative energy source. Although treatment is carried out by a complex microbial community, methanogens play an especially important role, completing the degradation of organic wastes into methane. If the methane is captured and burned, the carbon dioxide released is approximately equal to the carbon dioxide required by the living biomass in the digester (Zitomer et al. 2008a). Therefore, anaerobic digestion has two major benefits: the removal of organic wastes from water and the production of a carbon neutral alternative fuel.

However, the microbial community in anaerobic digesters has been a black box throughout most of the history of this technology (Rivière et al. 2009), and the microbial

ecology of anaerobic digesters is just beginning to be understood. Because methanogens play such an important role, understanding the methanogen community in anaerobic environments is critical when attempting to increase the efficiency of waste removal and biogas production, especially if bioaugmentation is used to encourage digester function. Bioaugmentation has been shown to increase recovery of stressed anaerobic digesters under certain conditions (Schauer-Gimenez et al. 2010). However, the composition of microbial communities used as supplements for successful bioaugmentation has not been fully explored.

Most studies that have utilized molecular biology techniques to study the microbial community in anaerobic digesters have used analyses based on 16S rRNA genes. However, the methanogens alone may be studied using the methanogen-specific gene *mcrA* which encodes the alpha subunit of methyl coenzyme M reductase (MCR) for quantitative and phylogenetic analyses (Freitag and Prosser 2009, Luton et al. 2002). Previous studies have established that the presence and transcription of *mcrA* can be used to detect methanogen presence and activity in the environment (Juottonen et al. 2008, Luton et al. 2002, Springer et al. 1995). MCR catalyzes the final step in all known methanogenesis pathways and is required for methane production (Ermler et al. 1997).

Several previous studies have focused specifically on the methanogens in anaerobic digesters. Hori et al. (Hori et al. 2006) used genus-specific quantitative polymerase chain reaction (qPCR) to demonstrate that the composition of the methanogen community in thermophilic anaerobic digesters changed as the concentration of volatile fatty acids (VFAs) shifted. Using *mcrA* instead of 16S rRNA genes, Rastogi et al. (2008) found that in a digester degrading cattle manure, seasonal shifts occurred in the

methanogen community. Steinberg and Regan (2008) compared the methanogen communities in an acidic fen and an anaerobic digester using both *mcrA* and 16S rRNA genes. They found almost no overlap between the sequences from the digester and the fen, and the majority of the sequences they found were unrelated to any cultured methanogen species. Similarly, when 16S rRNA genes and *mcrA* were used to study the archaeal community in a biogas plant using cattle manure and corn silage as substrates (Nettmann et al. 2008), many of the sequences generated in this study were also related to uncultured archaea. However, assignments to the genus level were possible for most.

Although these previous studies provide a basis for understanding the methanogen community in anaerobic digesters, they only addressed the presence or absence of methanogen genera or species. They did not, however, examine the diversity of the active methanogen community by looking at the diversity of *mcrA* genes which are actually transcribed into mRNA. When qPCR amplification of *mcrA* genes has been used to quantify transcripts in peat, the transcript to gene copy ratio of *mcrA* has been shown to correlate positively with methane production (Freitag and Prosser 2009, Freitag et al. 2010). Therefore, determining the relative abundance of *mcrA* transcripts from methanogen genera present in anaerobic biomass may provide valuable insight which links community structure to digester function.

This study was conducted to investigate the diversity of *mcrA* in methanogen communities found in the biomass of anaerobic cultures started from the same seed sludge, enriched with a mix of hydrogen and carbon dioxide, but maintained under varying conditions or fed varying substrates. It was hypothesized that varying conditions and/or substrates would reveal changes in functional methanogen community structure.

Previous studies have shown that altering temperature and ammonia concentrations causes changes in methanogen community structure (Calli et al. 2005, McHugh et al. 2004). More importantly, it was predicted that not all methanogens present in the cultures would be contributing equally to methane production as the substrates and/or conditions varied, demonstrated by the relative abundance of their transcription of *mcrA*. The data obtained from these experiments can be used to engineer better anaerobic digester communities and bioaugmentation supplements aimed at increasing methane production or ailing digester community recovery.

4.2 Results

4.2.a Genus assignments of *mcrA* clones: Sequence similarity of >88% was used to assign the 677 *mcrA* clones to a methanogen genus using blastn to compare clone sequences to Genbank® as suggested by Steinberg and Regan (Altschul et al. 1997, Steinberg and Regan 2008) (Figure 4.1). Comparison of the DNA and cDNA libraries showed major differences in relative abundances of clones assigned to each methanogen genus (Figure 4.1). For example, in the R1 DNA library, just over 50% of the clones were related to *Methanospirillum* with the rest split fairly equally between *Methanoculleus* and *Methanobacterium.* However, the vast majority (98%) of clones from the R1 cDNA library had the greatest sequence similarity to *Methanospirillum.* Shifts in the relative number of clones related to *Methanobacterium* and *Methanoculleus* were observed when comparing the R2 DNA and cDNA libraries. Furthermore, when *Methanosaeta* –related clones were observed in a DNA library (R2, R3, and R4), the percentage of *Methanosaeta* –related clones was much reduced in the respective cDNA library.

One effect of the addition of air on the relative abundance of *mcrA* clones in differing methanogen genera could be observed in both libraries. Specifically, when cultures were briefly sparged with air on a daily basis, *Methanoculleus*-related sequences were not detected in the *mcrA* clone libraries (Figure 4.1). Interestingly, *Methanolinea* – related sequences were only observed in libraries which received glucose (Figure 4.1).

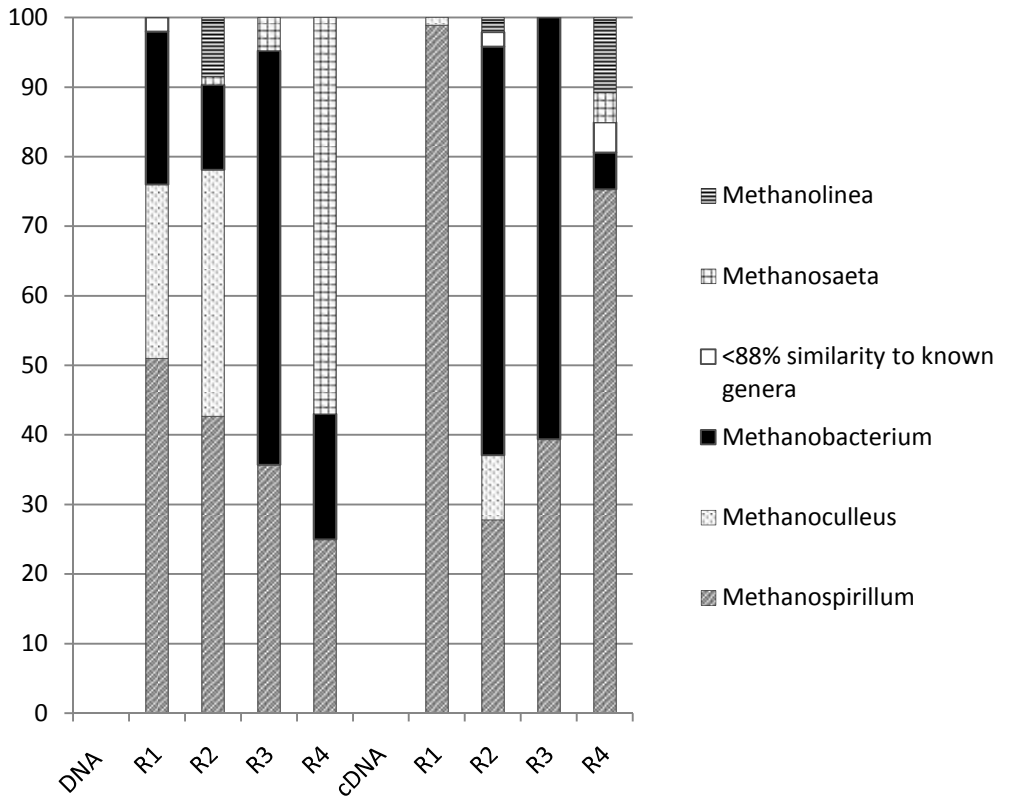


Figure 4.1. Methanogen Genus Assignments for *mcrA* Clones. Relative abundance of *mcrA* clones in each clone library to specific methanogen genera based on 88% sequence similarity (Steinberg and Regan 2008).

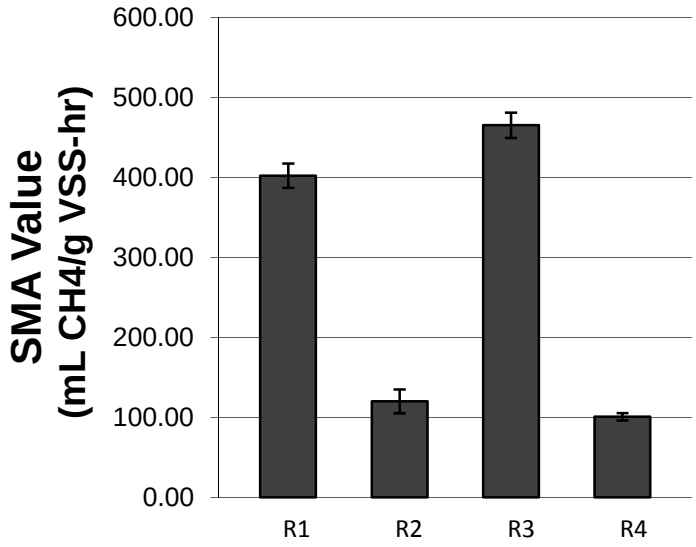


Figure 4.2.**Specific Methanogenic Activity Assays.** Specific methanogenic activity (SMA) against H2/CO2 (ml CH4/g VSS-hr) for each anaerobic enrichment culture (n=3).

4.2.b Specific Methanogenic Activity Assays against H_2/CO_2: SMA assay values (n =3) ranged from 100.67-456.1 ml CH_4 per g of volatile suspended solids per hour (Figure 4.2). SMA values for cultures R1 and R3 were significantly higher than those of R2 and R4 ($p<0.05$, ANOVA, Tukey test).

No specific abundance ratio for methanogen genera could be related to high or low specific methane production rates against H_2/CO_2 (Figures 4.1 and 4.2). However, the presence or absence of *Methanoculleus*-related sequences appeared to have no affect on SMA values when comparing the abundance of clones related to this genus in the libraries for R1 as well as R2 (Figures 4.1 and 4.2).

4.2.c Statistical analysis of clone libraries: Operational taxonomic units (OTUs) based on *mcrA* sequence were determined by DOTUR (distance =0.03, 97% similarity) for DNA and cDNA clone libraries (Schloss and Handelsman 2005). The libraries each had

between 5 (R3, DNA) and 19 (R2, cDNA) unique OTUs (Table 4.1). A relationship between the richness of cDNA OTUs and specific methanogenic activity could be determined when comparing OTU number and SMA values (Table 4.1 and Figure 4.2). The richness of cDNA libraries was reduced in biomass samples from cultures with higher SMAs, R1 and R3, (R^2=0.85, p=0.028).

Evenness values ranged from 0.12 for R1's DNA library to 0.88 for R3's DNA library (Table 4.1). Shannon indices ranged from 0.41-2.33 with the least heterogeneity seen in R1's cDNA library and the greatest heterogeneity observed in R1's DNA (Table 4.1). No relationship between evenness or heterogeneity and SMA results could be determined (Table 4.1 and Figure 4.2).

Table 4.1. Enrichment Culture OTUs, Shannon Indices, Evenness, and Coverage Estimates. Operational taxonomic units (OTUs), Shannon indices, and S_{Chao1} –based estimates of coverage for each of the clone libraries. Data was obtained using DOTUR when distance = 0.03 (97% similarity). Evenness was calculated as described by Pielou (1966).

	OTUs		**Evenness**		**Shannon Index**		**Coverage Estimate**	
	DNA	**cDNA**	**DNA**	**cDNA**	**DNA**	**cDNA**	**DNA**	**cDNA**
R1	14	5	0.88	0.25	2.33	0.41	97%	91%
R2	12	19	0.70	0.75	1.75	2.21	86%	92%
R3	5	7	0.12	0.50	1.19	0.97	100%	70%
R4	7	15	0.64	0.43	1.24	1.18	54%	19%

Rarefaction curves and S_{CHAO1} calculations were used to estimate the coverage of the diversity within each library (Table 4.1 and Figure 4.3). The rarefaction curves for both DNA and cDNA libraries from cultures R1 and R3 began to level off before 20 clones were sampled. The curves from the R2 and R4 DNA and cDNA libraries continued to climb after all clones were sampled (Figure 4.3). S_{CHAO1}-based coverage

estimates ranged from 19% for R4's cDNA to 100% for R3's DNA (Table 4.1). Only three of the eight libraries had coverage estimates under 80%: DNA and cDNA from R4, and cDNA from R3 (Table 4.1).

Assignments of clones by DOTUR were used to generate graphs representing the relative abundance of OTUs from the DNA and cDNA clone libraries (Figure 4.4 A-D). For R1, the DNA library *mcrA* OTUs that were in the greatest abundance matched most closely to *Methanospirillum* and *Methanoculleus* sequences in Genbank®. Both OTUs represented 16.7% of the total DNA sequences (Figure 4.4A). The most abundant (91%) cDNA OTU was most closely related to *Methanospirillum* (Figure 4.4A). In culture R2, the most abundant DNA OTUs were also most closely related to *Methanospirillum* and *Methanoculleus;* however, the most abundant cDNA OTU was related to *Methanobacterium* (Figure 4.4B). For culture R3, the most abundant OTU in both libraries was related to *Methanobacterium* (Figure 4.4C). Finally, in culture R4 the most abundant DNA OTU was related to *Methanosaeta* while the most abundant cDNA OTU was related to *Methanospirillum* (Figure 4.4D).

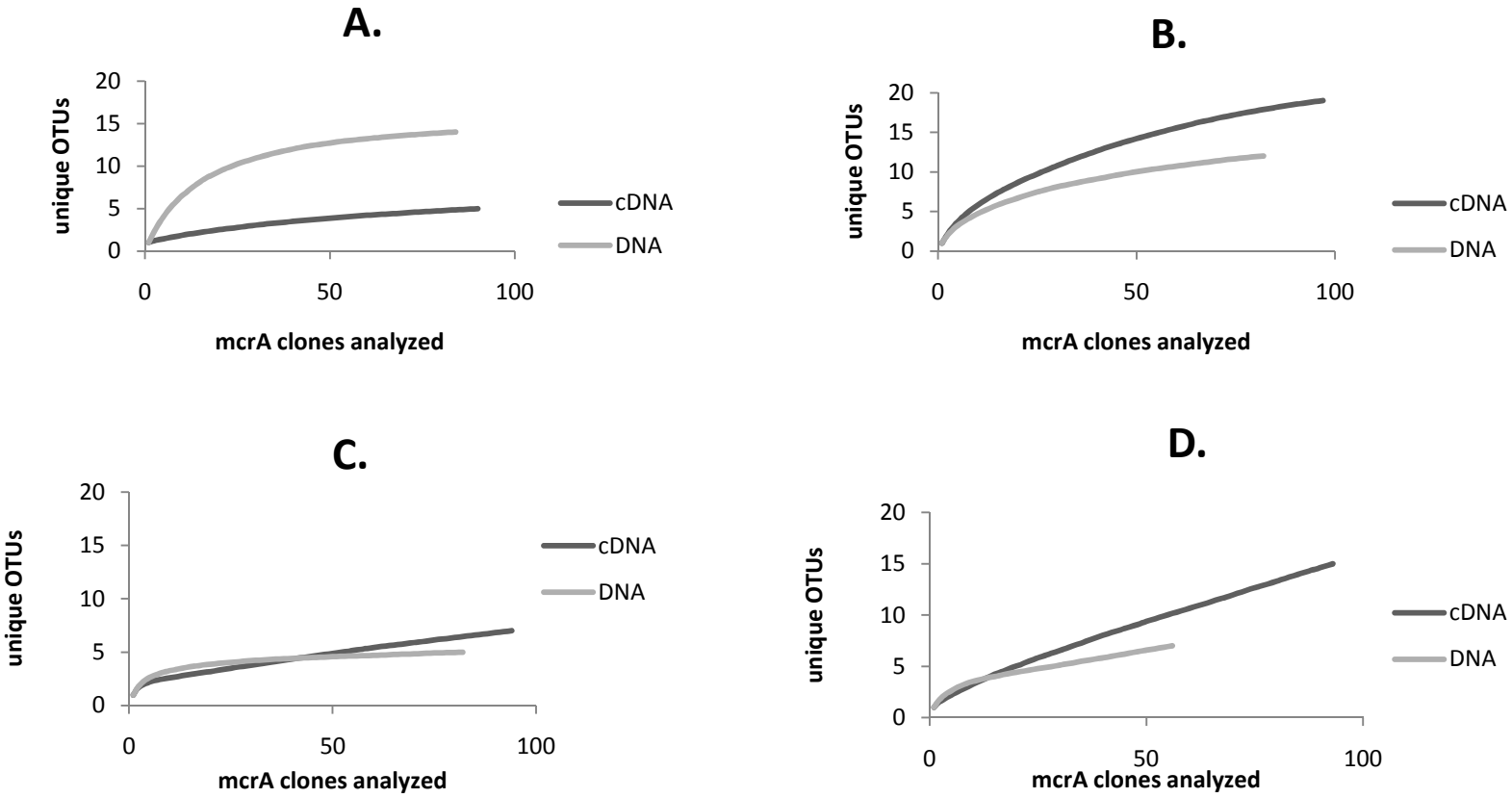


Figure 4.3 A-D. Rarefaction Curves for DNA and cDNA Clone Libraries. Rarefaction curves demonstrating the collection of unique *mcrA* clones for each clone library from each digester. A. R1. B. R2 C. R3. D. R4

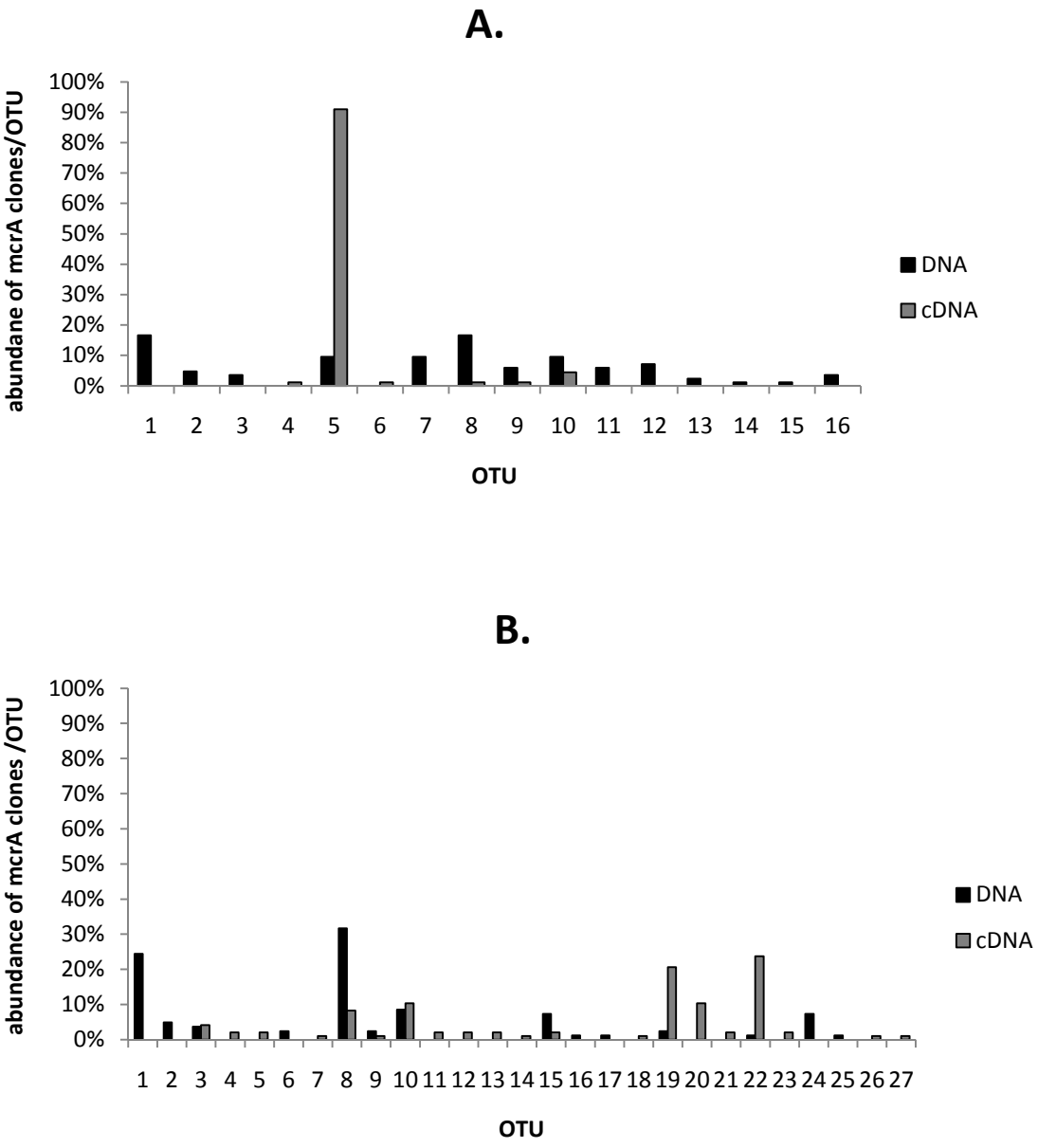
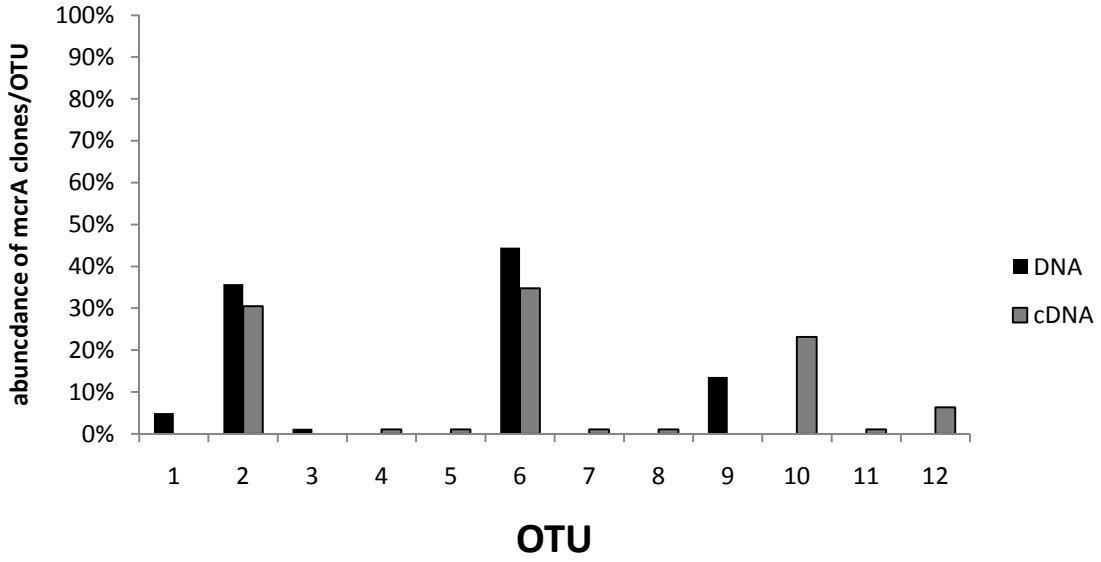
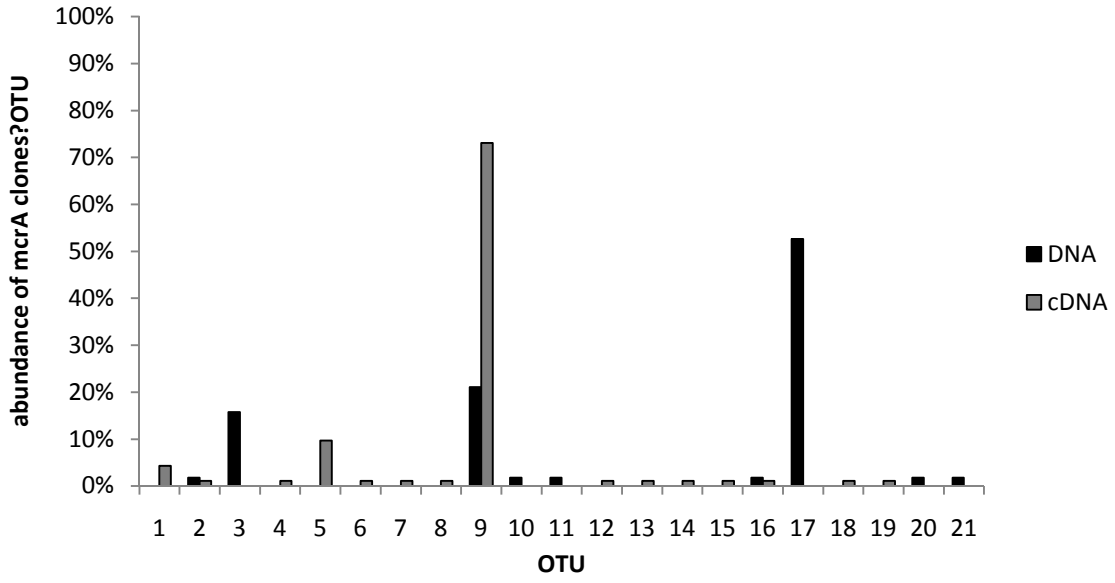


Figure 4.4 A-D. Comparison of OTUs from DNA and cDNA *mcrA* clones. OTUs were determined using DOTUR with distance=0.03 (97% similarity). A. R1. B. R2 C. R3. D. R4

C.


abuncdance of mcrA clones/OTU
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12
OTU
DNA
cDNA

D.


abundance of mcrA clones?OTU
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
OTU
DNA
cDNA

4.2.d Phylogenetic analysis: When neighbor-joining trees constructed from the unique DNA and cDNA sequences were compared, distinct differences in the methanogen communities were observed (Figure 4.5 A-H). When comparing the trees generated using sequences from R1 biomass, differences between the two major clades for each tree were observed (Figures 4.5A and 4.5B). In the tree constructed from DNA sequences, one major clade was related to *Methanobacterium* and two smaller clades grouped with reference sequences from the genera *Methanospirillum* and *Methanoculleus*. However, in the tree generated using *mcrA* cDNA sequences from the same culture with the same reference sequences included, none of the clones grouped with *Methanobacterium.* Instead, most of the cDNA sequences grouped with an uncultured clone (mcrA_dig_D46) related to *Methanospirillum* (Figure 4.5B) and the rest with *Methanoculleus.*

The major difference observed when comparing the trees constructed using sequences obtained from R2 biomass was the relative number of clones which were associated with each major clade (Figures 4.5C and 4.5D). More sequences from the cDNA library were associated with *Methanobacterium* reference sequences than in the tree constructed from DNA library. In the latter, more sequences were associated with *Methanoculleus* and *Methanospirillum* reference *mcrA* sequences. Both trees had small clades associated with *Methanolinea,* and a single *Methanosaeta*-related sequence was found in each library.

4.5A.

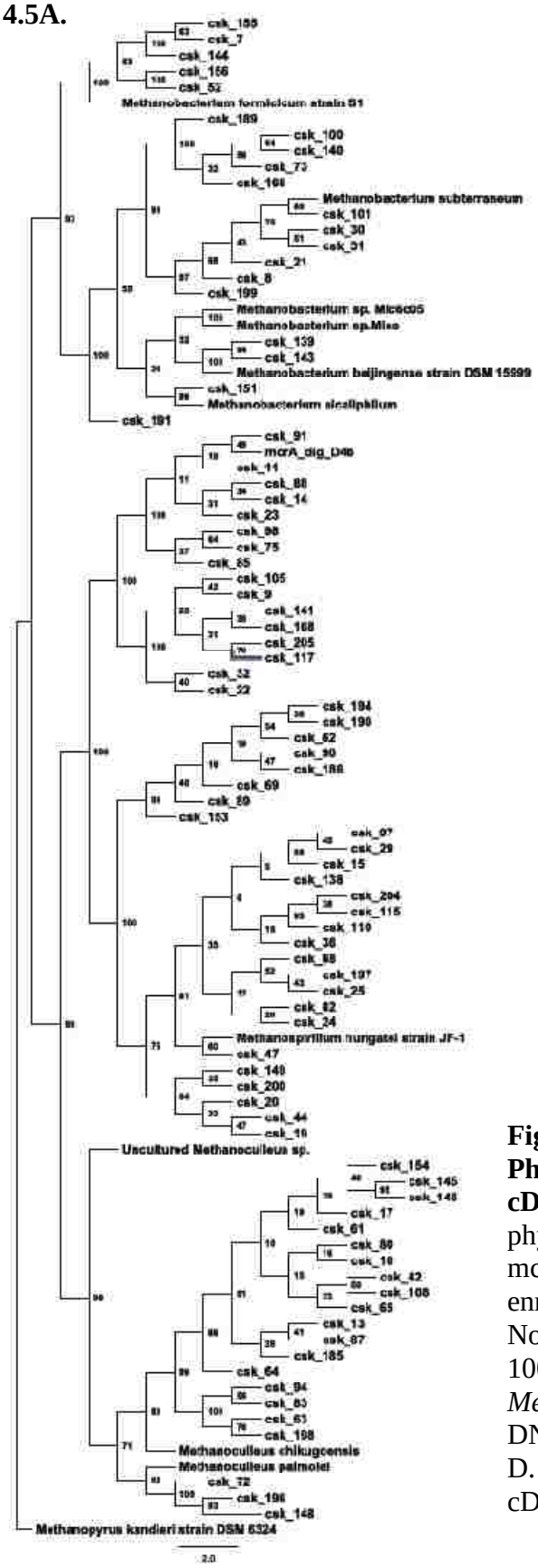
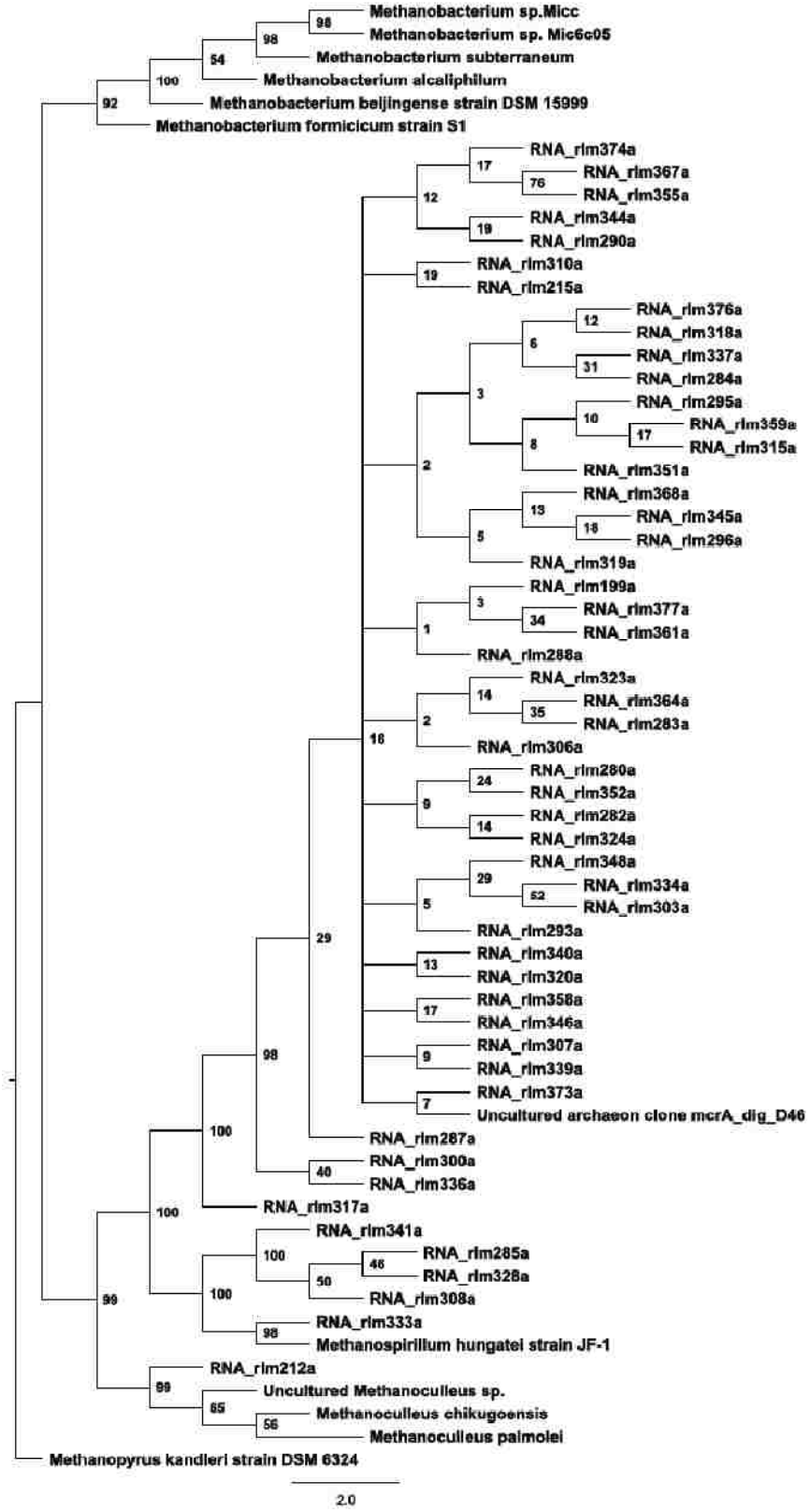


Figure 4.5. Neighbor-joinging Phylogenetic Trees from DNA and cDNA Sequences. Neighbor-joining phylogenetic trees created using unique mcrA clone sequences from each enrichment culture biomass sample. Node labels are bootstrap values from 100 analyses, and the tree is rooted with *Methanopyrus kandlerii mcrA*. A. R1 DNA . B. R1 cDNA . C. R2 DNA . D. R2 cDNA . E. R3 DNA . F. R3 cDNA . G. R4 DNA. H. R4 cDNA**.**

4B.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Methanobacterium sp. Mic6c05
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Methanobacterium alcaliphilum
Methanobacterium beijingense strain DSM 15999
Methanobacterium formicicum strain S1
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RNA_rim355a
RNA_rim344a
RNA_rim290a
RNA_rim310a
RNA_rim215a
RNA_rim376a
RNA_rim318a
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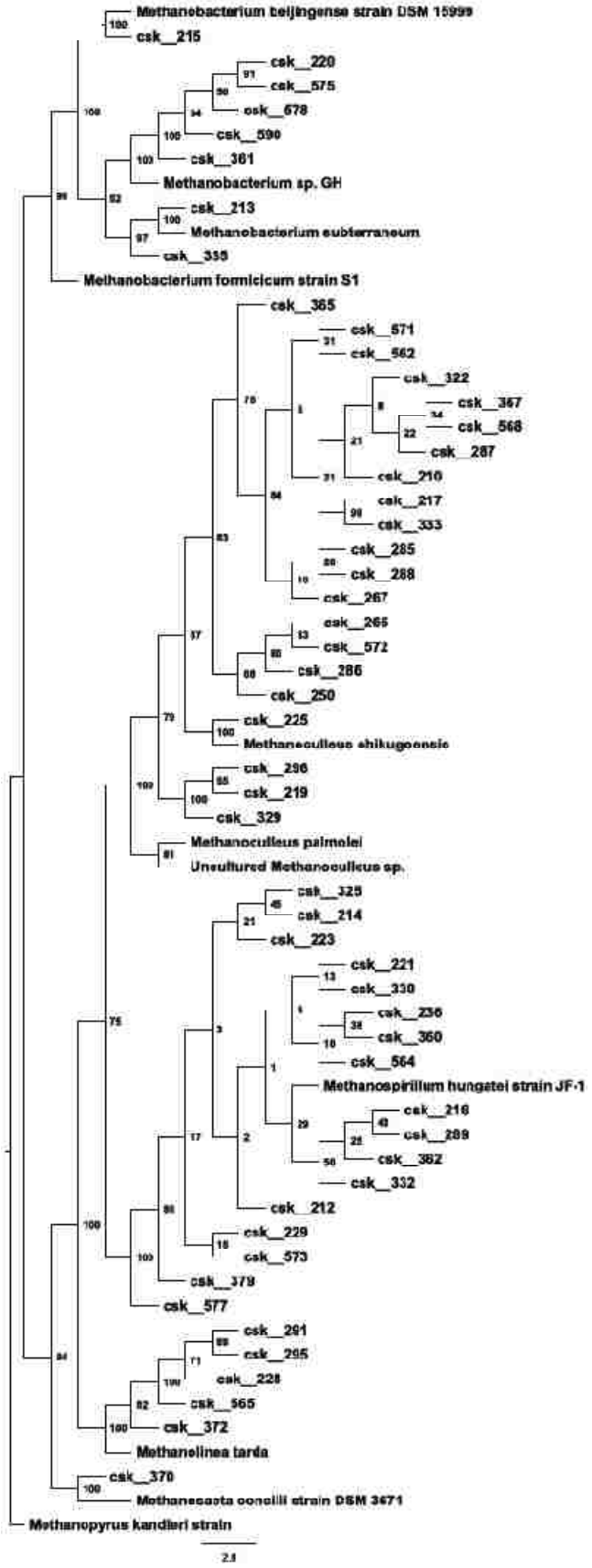
RNA_rim303a
RNA_rim293a
RNA_rim340a
RNA_rim320a
RNA_rim358a
RNA_rim346a
RNA_rim307a
RNA_rim339a
RNA_rim373a
Uncultured archaeon clone mcrA_dig_D46
RNA_rim287a
RNA_rim300a
RNA_rim336a
RNA_rim317a
RNA_rim341a
RNA_rim285a
RNA_rim328a
RNA_rim308a
RNA_rim333a
Methanospirillum hungatei strain JF-1
RNA_rim212a
Uncultured Methanoculleus sp.
Methanoculleus chikugoensis
Methanoculleus palmolei
Methanopyrus kandleri strain DSM 6324
2.0

4C.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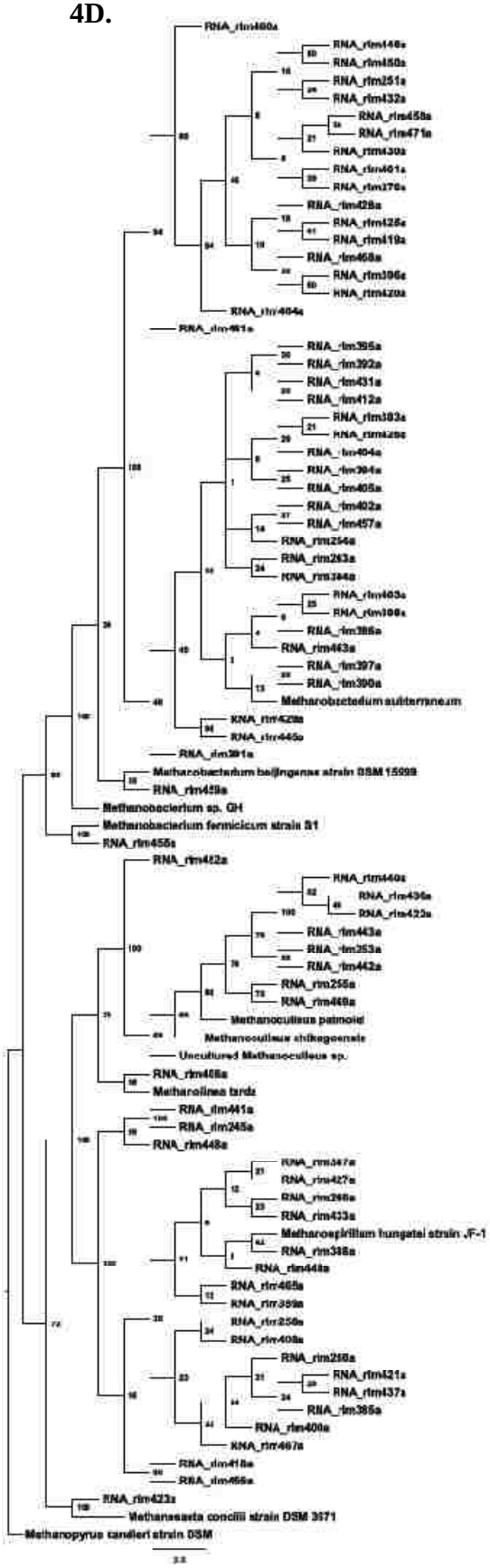
csk__220
csk__575
csk__678
csk__590
csk__361
Methanobacterium sp. GH
csk__213
Methanobacterium subterraneum
csk__335
Methanobacterium formicicum strain S1
csk__365
csk__571
csk__562
csk__322
csk__367
csk__568
csk__287
csk__210
csk__217
csk__333
csk__285
csk__288
csk__267
csk__265
csk__572
csk__286
csk__250
csk__225
Methanoculleus shikugoensis
csk__296
csk__219
csk__329
Methanoculleus palmolei
Unsultured Methanoculleus sp.
csk__325
csk__214
csk__223
csk__221
csk__330
csk__236
csk__360
csk__564
Methanospirillum hungatei strain JF-1
csk__216
csk__289
csk__362
csk__332
csk__212
csk__229
csk__573
csk__379
csk__577
csk__291
csk__295
csk__228
csk__565
csk__372
Methanolinea tarda
csk__370
Methanosaeta concilii strain DSM 3671
Methanopyrus kandleri strain
2.0

4D.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RNA_rim256a
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RNA_rim463a
RNA_rim397a
RNA_rim390a
Methanobrevibacter ruminantium
RNA_rim428a
RNA_rim445a
RNA_rim391a
Methanobacterium beijingense strain DSM 15999
RNA_rim459a
Methanobacterium sp. GH
Methanobacterium formicicum strain 31
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RNA_rim462a
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RNA_rim436a
RNA_rim422a
RNA_rim443a
RNA_rim253a
RNA_rim442a
RNA_rim255a
RNA_rim446a
Methanoculleus palmolei
Methanoculleus chikugoensis
Uncultured Methanoculleus sp.
RNA_rim466a
Methanolinea tarda
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RNA_rim245a
RNA_rim448a
RNA_rim427a
RNA_rim266a
RNA_rim433a
Methanospirillum hungatei strain JF-1
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RNA_rim444a
RNA_rim465a
RNA_rim389a
RNA_rim258a
RNA_rim408a
RNA_rim256a
RNA_rim421a
RNA_rim437a
RNA_rim385a
RNA_rim409a
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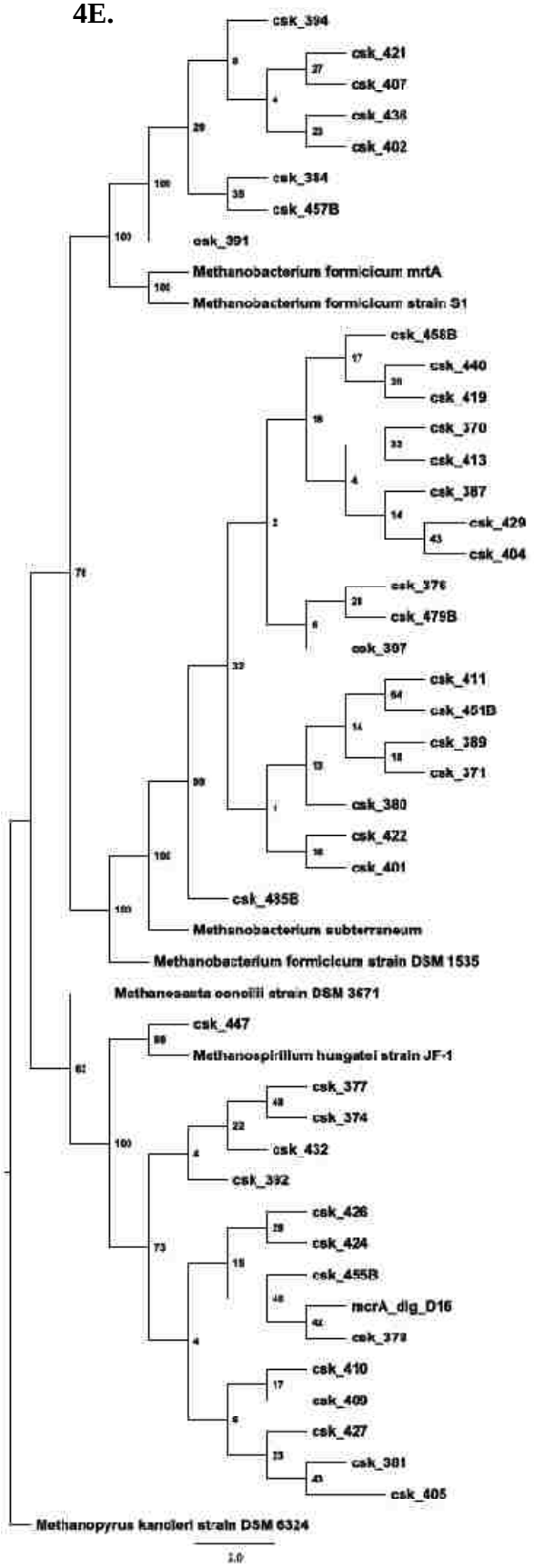
Methanopyrus kandleri strain DSM

4E.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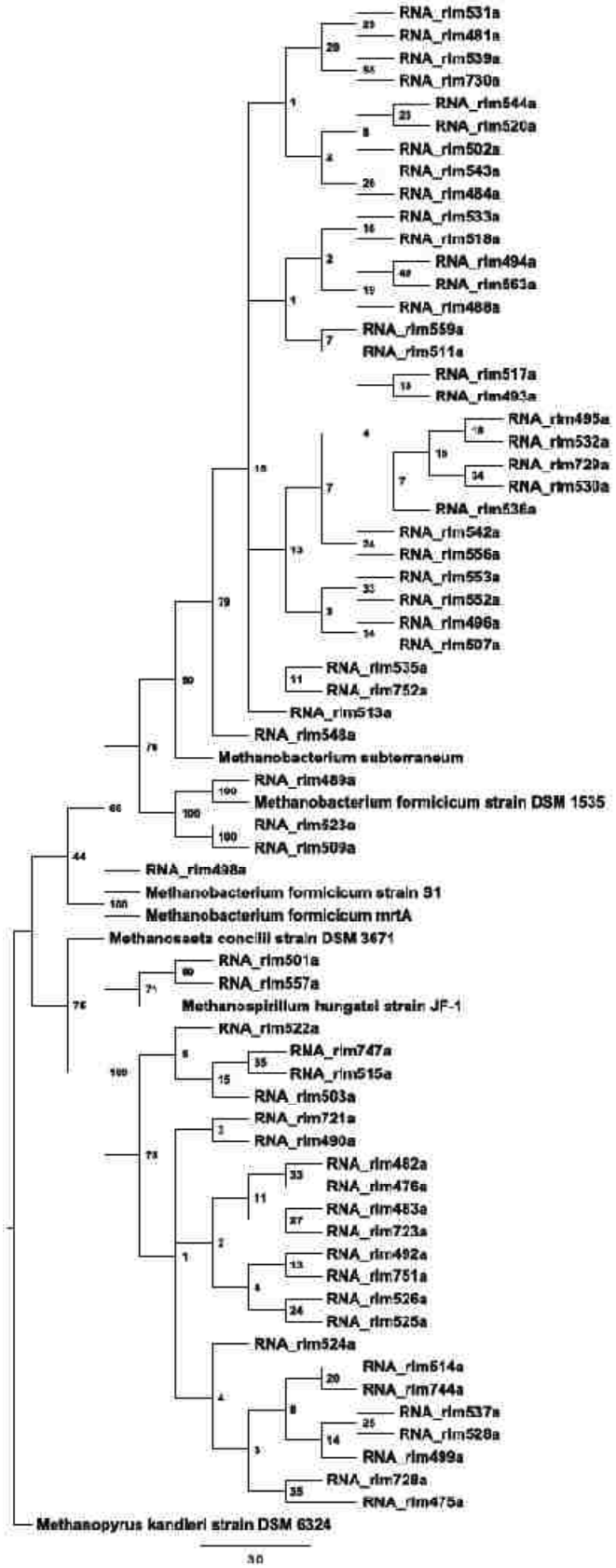
csk_421
csk_407
csk_438
csk_402
csk_384
csk_457B
csk_391
Methanobacterium formicicum mrtA
Methanobacterium formicicum strain S1
csk_458B
csk_440
csk_419
csk_370
csk_413
csk_387
csk_429
csk_404
csk_376
csk_479B
csk_397
csk_411
csk_451B
csk_389
csk_371
csk_380
csk_422
csk_401
csk_435B
Methanobacterium subterraneum
Methanobacterium formicicum strain DSM 1535
Methanosaeta concilii strain DSM 3671
csk_447
Methanospirillum hungatei strain JF-1
csk_377
csk_374
csk_432
csk_392
csk_426
csk_424
csk_455B
mcrA_dig_D16
csk_379
csk_410
csk_409
csk_427
csk_381
csk_405
Methanopyrus kandleri strain DSM 6324
2.0

4F.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RNA_rim493a
RNA_rim495a
RNA_rim532a
RNA_rim729a
RNA_rim530a
RNA_rim536a
RNA_rim542a
RNA_rim556a
RNA_rim553a
RNA_rim552a
RNA_rim496a
RNA_rim507a
RNA_rim535a
RNA_rim752a
RNA_rim513a
RNA_rim548a
Methanobacterium subterraneum
RNA_rim489a
Methanobacterium formicicum strain DSM 1535
RNA_rim523a
RNA_rim509a
RNA_rim498a
Methanobacterium formicicum strain 91
Methanobacterium formicicum mrtA
Methanosaeta concilii strain DSM 3671
RNA_rim501a
RNA_rim557a
Methanospirillum hungatei strain JF-1
RNA_rim522a
RNA_rim747a
RNA_rim515a
RNA_rim503a
RNA_rim721a
RNA_rim490a
RNA_rim482a
RNA_rim476a
RNA_rim483a
RNA_rim723a
RNA_rim492a
RNA_rim751a
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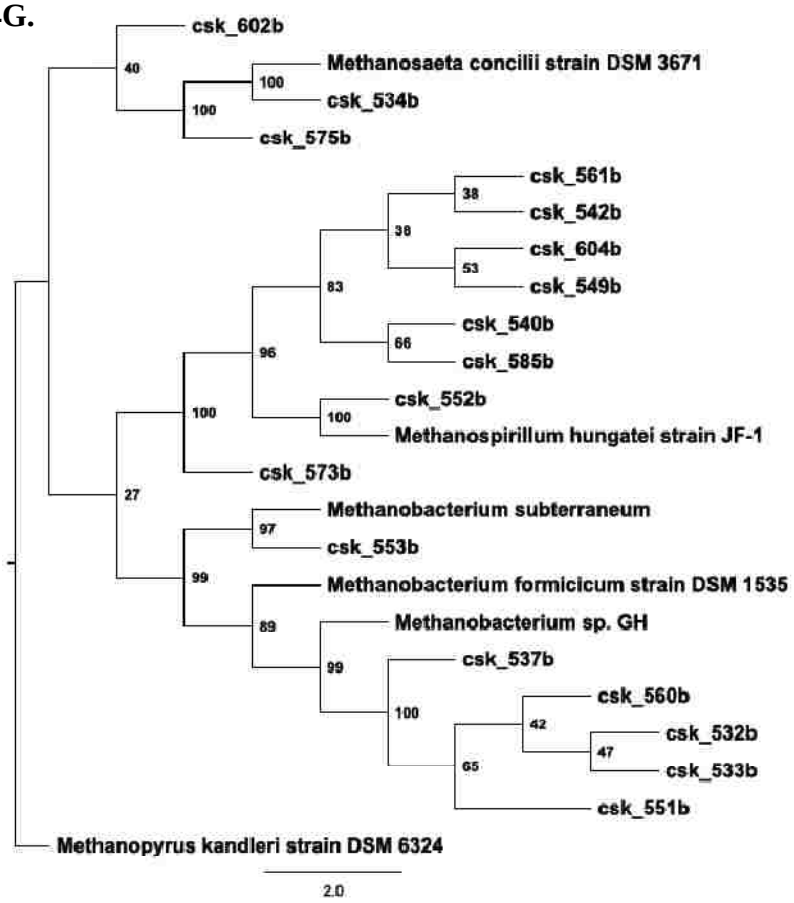
Methanopyrus kandleri strain DSM 6324

4G.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40
Methanosaeta concilii strain DSM 3671
100
csk_534b
100
csk_575b
csk_561b
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csk_542b
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csk_604b
53
csk_549b
83
csk_540b
66
csk_585b
96
csk_552b
100
Methanospirillum hungatei strain JF-1
100
csk_573b
27
Methanobacterium subterraneum
97
csk_553b
99
Methanobacterium formicicum strain DSM 1535
89
Methanobacterium sp. GH
99
csk_537b
csk_560b
100
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csk_532b
47
csk_533b
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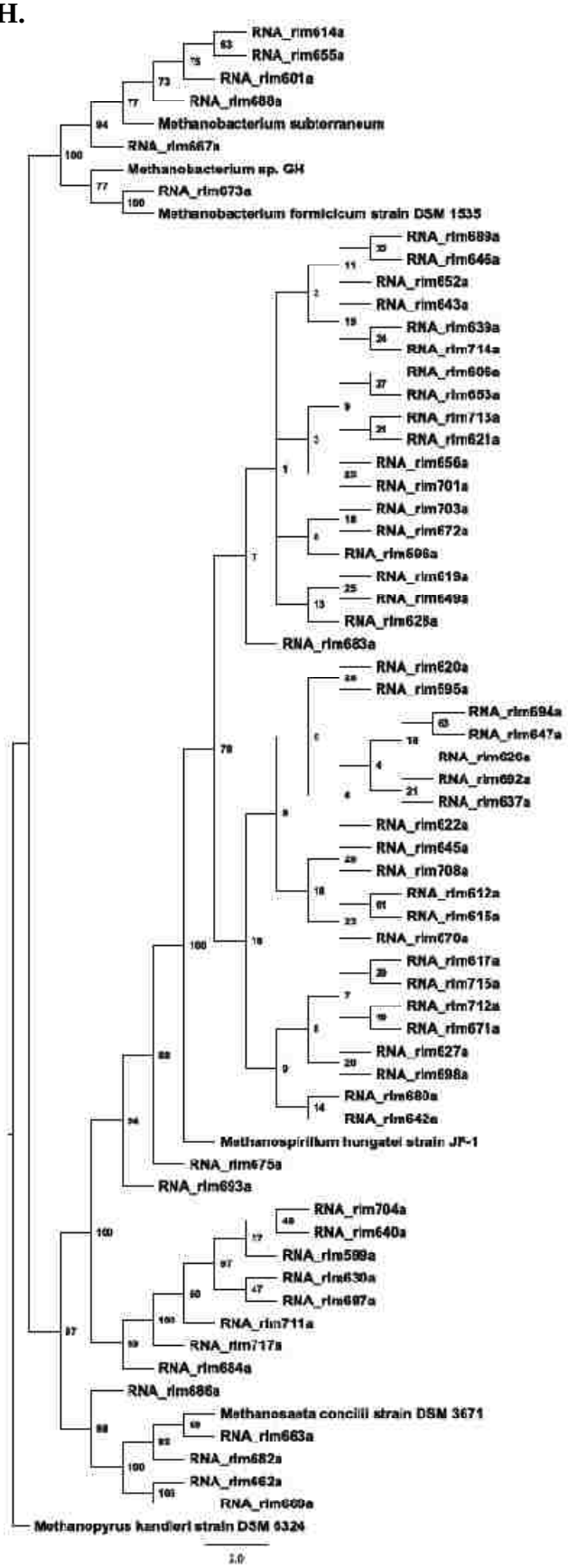
csk_551b
Methanopyrus kandleri strain DSM 6324
2.0

4H.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RNA_rim683a
RNA_rim620a
RNA_rim695a
RNA_rim694a
RNA_rim647a
RNA_rim626a
RNA_rim692a
RNA_rim637a
RNA_rim622a
RNA_rim645a
RNA_rim708a
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RNA_rim615a
RNA_rim670a
RNA_rim617a
RNA_rim715a
RNA_rim712a
RNA_rim671a
RNA_rim627a
RNA_rim698a
RNA_rim680a
RNA_rim642a
Methanospirillum hungatei strain JF-1
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RNA_rim693a
RNA_rim704a
RNA_rim640a
RNA_rim599a
RNA_rim630a
RNA_rim697a
RNA_rim711a
RNA_rim717a
RNA_rim684a
RNA_rim686a
Methanosaeta concilii strain DSM 3671
RNA_rim663a
RNA_rim682a
RNA_rim662a
RNA_rim669a
Methanopyrus kandleri strain DSM 6324
2.0

The major clades of the R3 DNA and cDNA sequence trees were similar (Figures 4.5E and 4.5F). Both had two large clades, one which clustered around *Methanobacterium* reference sequences, and one which grouped with *Methanospirillum.* However, the organization of the smaller clades within the *Methanobacterium* differed slightly. In the tree constructed from cDNA sequences (Figure 4.5F), most of the *Methanobacterium* –related sequences clustered with the reference *mcrA* from *Methanobacterium subterraneum,* and the few sequences which clustered with *Methanobacterium formicicum* were related to *strain DSM 1535.* The majority of sequences in the DNA tree also clustered to *Methanobacterium subterraneum* while those that clustered with *Methanobacterium formicicum* were related to strain *S1* (Figure 4.5E).

Finally, the comparison of the R4 neighbor-joining trees revealed differences in the abundance of sequences which clustered with *Methanobacterium* reference sequences (Figures 4.5G and 4.5H). The tree which consisted of DNA sequences (Figure 4.5G) showed a fairly even division between those that clustered with *Methanobacterium* (6 clones) and *Methanospirillum* (8 clones) reference sequences. However, the tree made from the library of cDNA sequences (Figure 4.5H) primarily consisted of a very large clade of *Methanospirillum*-related sequences with only a small clade which grouped with *Methanobacterium* reference *mcrA.* Both trees contained a very small clade related to *Methanosaeta mcrA.*

When Treeclimber was used to compare phylogenetic trees generated from the *mcrA* sequences found in each enrichment cultures, all of the communities were determined to be different from one another ($p<0.01$)(Schloss and Handelsman 2006).

4.3.Discussion

Understanding how methanogens function as part of microbial consortia is important because of their role in global carbon cycling. In anaerobic wastewater treatment, methanogens are also very important, serving as both the end of the degradation chain and the source of a renewable fuel. Therefore, studies of methanogen structural and functional community dynamics can provide valuable information for the development of this form of biotechnology.

4.3.a Genomic *mcrA* compared to transcribed *mcrA* Previously, Delbes et al. (2000), examining microbial communities in anaerobic digester biomass before and after perturbation with acetate using single strand polymorphism analysis, demonstrated that using 16S rRNA gene alone was not sufficient to detect all the organisms. Additional organisms were detected when 16S rRNA was compared to 16S rDNA. The data from this study, generated from extractions of the same biomass samples, also indicated that there were differences between the DNA and cDNA libraries. Clearly, the *mcrA* found in the DNA was not all transcribed into mRNA at levels detectable by the methods used. Differences in the relative abundance of methanogen genera (Figure 4.1), and Treeclimber analysis of phylogenetic trees also showed that the methanogens that were present and the methanogens that were actively transcribing *mcrA* in large abundance were not the same

Taken together, these results indicated that the active methanogen community was a subset of the methanogen diversity that was present in anaerobic biomass. The fraction of the community which became metabolically active was influenced by available substrates and conditions. The data also suggest that certain methanogens may exist in a state of dormancy when conditions or substrate availability are not favorable for their particular metabolic needs.

These organisms should provide functional diversity so that the methane production of the community can be maintained when conditions or substrates change. This finding is supported by a previous study which showed that methanogens in drained rice paddy soil can survive unfavorable conditions and then recover from a dormant state to produce methane when conditions become anaerobic once more (Watanabe et al. 2007). Overall, the structural and functional diversity of the functional gene *mcrA* in the present study indicated that conditions and available substrates affected which members of the methanogen community were most active at a given time.

4.3.b Community structure and function. Using pyrosequencing of 16S rRNA variable regions to examine the diversity of the bacterial community in anaerobic digesters, Werner et al. (2011) detected a relationship between bacterial community structure and methanogenic activity. Specifically, they found that as evenness of the bacterial community increased methanogenic activity increased as well. The current study was performed to discover if similar determinations could be made between the diversity of methanogen communities maintained under varying conditions and community function. However, neither the evenness nor the heterogeneity of the methanogen community were related to methanogenic activity in the cultures tested (Table1 and Figure 2). The phylogenetic identity of the dominant transcript in the biomass was not related to SMA values, either. However, a decrease in cDNA richness was related to higher methanogenic activity. This finding suggests that when conditions allow more a greater diversity of methanogens to be metabolically active, overall methanogenic activity is reduced.

4.3.c Implications for bioaugmentation. Bioaugmentation is the addition of microorganism to biological systems to aid or improve performance. For anaerobic digesters, bioaugmentation could be used to shorten start up times or reduce the length of recovery of distressed digesters

(Schauer-Gimenez et al. 2010, Tale and Zitomer 2010). Comparison of the various percentages of methanogen clones from each genus for each library revealed information that may be used in the design of bioaugmentation mixes. First, *Methanoculleus* – related *mcrA* sequences were not observed in the clone libraries generated from biomass of cultures which were sparged with air (Figure 4.1). Furthermore, the absence of *Methanoculleus* from these cultures did not negatively affect methane production rates (Figures 4.1 and 4.2). These results suggest a limited contribution for *Methanoculleus* to methane production under these conditions and a greater sensitivity to air than other methanogen genera which were observed in this study. Therefore, the use of cultures dominated by *Methanoculleus* for bioaugmentation may not be recommended if there is exposure to air.

Methanolinea –related sequences were only found in the clone libraries of cultures which received glucose. This was an interesting finding considering the fact that the *Methanolinea* are hydrogenotrophs, and therefore it is difficult to predict how the addition of glucose might influence their abundance. However, it has also been shown that acetate is required for their growth (Imachi et al. 2008) even though they do not utilize it for methanogenesis, and perhaps the addition of glucose makes acetate more available in these cultures than in those which received only hydrogen and carbon dioxide. Another possibility is that the addition of glucose stimulated the growth of bacteria which exist in syntrophy with *Methanolinea*, creating conditions which were more favorable for methanogens in that genus.

Methanobacterium- and *Methanospirillum*- related sequences were found in all the clone libraries generated from DNA extractions (Figure 4.1). Cultures with a higher percentage of *Methanobacterium*- related *mcrA* sequences had higher SMA values (R1 and R3) (Figures 4.1 and 4.2). However, the cDNA libraries did not follow the same pattern. Even accounting for

coverage estimates (Table 4.1), the transcription of *Methanobacterium*-related *mcrA* was not related to higher specific methane production rates (Figures 4.1 and 4.2).

However, analysis of the phylogenetic trees created from the cDNA *mcrA* sequences suggests that *Methanobacterium* and *Methanospirillum* were important, at least in these cultures (Figures 4.5). While there was no apparent connection between one genus or another and SMA values, both genera utilize hydrogen or formate and carbon dioxide for methanogenesis and have similar temperature requirements (Liu and Whitman 2008). Therefore, their presence in the community could add functional redundancy, and both genera could be further investigated for use in bioaugmentation cultures.

The observed differences between the transcribed *mcrA* sequences and the genomic *mcrA* sequences in this study indicate that certain methanogens were more valuable players in microbial communities than others, especially under specific conditions. Variation among methanogen transcription and translation rates for *mcrA*, as well as the half-life and stability of the mRNA and the protein itself, may all affect the outcome; however, very little of this data is available for methanogen genera. Furthermore, while *mcrA* has been demonstrated to be a valuable gene for use in the investigation of methanogens in the environment, the data obtained from PCR-based methods using primers for *mcrA* are subject to biases inherent in the process from the extraction of DNA from environmental samples to PCR amplification efficiencies (v. Wintzingerode et al. 1997). However, the primer set designed by Luton et al. has previously been shown to consistently amplify *mcrA* from a wide range of methanogen genera, making the set a sound choice for the examination of methanogen OTUs in environmental samples (Banning et al. 2005, Juottonen et al. 2006, Luton et al. 2002). Further information about MCR and *mcrA* for

specific methanogen genera would be useful for interpreting this data as the link between MCR genetic and functional differences is not clear at this time.

These results may also have been affected by the conditions to which the cultures were subjected. The daily addition of hydrogen and carbon dioxide specifically enriched for hydrogenotrophic methanogens in these cultures, bypassing the earlier steps (such as fermentation and acidogenesis) in the degradation of more complex organic compounds which normally precede methanogenesis in anaerobic environments. Important syntrophic relationships are known to exist between methanogens and bacterial partners which degrade volatile fatty acids such as propionate and butyrate (Stams 1994). The structure of the methanogenic community may have been affected by the bypass of this syntrophy. However, even though this study utilized hydrogen and carbon dioxide enriched cultures, there is no reason to believe that similar results would not be found in mixed cultures or cultures enriched for acetoclastic methanogens.

In summary, the data from this study provide insight into the effect changing conditions and available substrates can have on the structural diversity and functional activity of methanogens by examining the differences in the presence and transcription of a functional gene unique to these *Archaea*. These findings, especially the relationship between the decrease in *mcrA* cDNA richness and specific methanogenic activity, may be used to better understand relationships between methanogen community structure and anaerobic digester function, and this information may be also be used in the development of bioaugmentation supplements for digesters.

Chapter Five
Linking *mcrA* Gene Copy and Transcript Numbers and Methane Production in Anaerobic Biomass

5.1.Introduction

Anaerobic digestion is an under-utilized technology. One reason for the decision against installing anaerobic treatment plants is that digesters which fail can take months to recover (Speece 1996). This failure occurs when the complex microbial community upon which digester function depends is sufficiently stressed by organic overload or toxicants or other abrupt environmental changes (Castellano et al. 2007). Prudent use of bioaugmentation or system control may be used to prevent digester failure or encourage faster recovery of stressed digesters (Castellano et al. 2007, Schauer-Gimenez et al. 2010). Therefore, the results of assays which rapidly and directly monitor the microorganisms in anaerobic biomass could provide useful information to operators seeking to manage digester function.

In practice, however, the organisms in the anaerobic microbial community are not monitored directly. SMA assays, methane production rates, biogas composition, chemical oxygen demand (COD) removal, pH, granule morphology, acetate utilization rates, methanethiol concentration, and quantification of volatile fatty acids have all been suggested or used to evaluate digester function (Castellano et al. 2007, Coates JD et al. 1996, Conklin et al. 2008, DH Zitomer et al. 2000, Molina et al. 2009). Although these parameters are closely related to the metabolic functions of the microbial community, they do not directly assay microorganisms. Digester communities are complex, with multiple trophic levels in which different groups of organisms carry out waste degradation in a series of steps (Fernandez et al. 1999, Leclerc et al. 2004, Liu and Whitman 2008, Rivière et al. 2009, Schink 1997, White 2000). Therefore, direct

monitoring of the microorganisms that are involved in each level may provide better insight for improving digester function, especially if direct manipulation of communities, such as bioaugmentation, is to be employed to aid recovery of stressed digester communities.

Although digester microbial communities are very diverse and different groups of organisms perform the various steps of anaerobic digestion, the methanogens are especially important as the last link in the food chain, performing the final step in the degradation of organic waste to methane (McCarty and Smith 1986). Additionally, their function is closely tied to that of the propionate- and butyrate- reducing bacteria whose metabolism of these compounds is dependent upon removal of hydrogen (Conrad and Klose 1999, Schink 1997). Methanogenesis is often considered to be the rate-limiting step in the anaerobic treatment of wastes (Liu and Whitman 2008). Therefore, methanogens are important members of the digester community, and they are critical to digester functional stability. Monitoring this specific group of organisms could provide an important link between digester function and microbial community structure.

Methanogens possess a unique operon which encodes the methyl coenzyme M reductase (MCR). MCR is a multi-subunit enzyme which catalyzes the final step of methanogenesis and is unique to methanogens and the anaerobic methane-oxidizing *Archaea* (Hallam et al. 2003, Springer et al. 1995). Previous studies have established that the presence and transcription of the gene which encodes the alpha subunit of MCR (*mcrA*) can be used to detect methanogen presence and activity in the environment (Juottonen et al. 2008, Luton et al. 2002, Springer et al. 1995). Furthermore, quantitative polymerase chain reaction (qPCR) amplification of *mcrA* genes has been used to estimate methanogen abundance in the environment, and the transcript to gene

copy ratio has been shown to correlate positively with methane production in peat (Freitag and Prosser 2009, Freitag et al. 2010, Goffredi et al. 2008).

This study was performed to test the hypothesis that the number of *mcrA* gene copies and/or transcripts would correlate with methane production rates for anaerobic biomass given specific substrates. This hypothesis was tested by performing qPCR using *mcrA*- specific primers on DNA and cDNA from anaerobic biomass collected from hydrogen enrichment cultures, lab-scale anaerobic digesters, and full-scale anaerobic treatment plants. SMA assays, standard methods for determining methane production activity of anaerobic biomass given specific substrates (Coates et al. 2005, Coates et al. 1996), were performed on the biomass samples in tandem with qPCR analyses. The qPCR results were then compared with SMA assay data to determine if a relationship existed between *mcrA* gene or transcript number and the standard indicators of anaerobic digester biomass activity.

5.2.Results

5.2.a Quantitative PCR: qPCR was performed in five separate runs: enrichment cultures (Figures 5.1A and 5.1B), two M time courses (Figure 5.2A and 5.2B), lab-scale digesters (Figure 5.3A and 5.3B), and full-scale digesters (Figures 5.4A and5. 4B). Descriptions of the standard curves for each run can be found in Table 5.3. Transcripts for *mcrA* from biomass from three lab-scale digesters (VP-0, VP-10, and VP-50) and three industrial digesters (MMBR, MMSS, and CF) were not detectable within the limits of the assay. Therefore, gene:transcript ratios could not be calculated for these samples as a measure of activity as has been reported by others (Freitag and Prosser 2009, Freitag et al. 2010).

When the data from each date were compared, biomass from cultures R1 and R3 had greater *mcrA* gene copy and transcript numbers than did biomass from cultures R2 and R4 which received glucose (Figures 5.1A -5.1F and Table 2.1). Variations in *mcrA* copy number and transcript number were observed among the three samples of biomass taken from each of the enrichment cultures on different dates (Figures 5.1A - 5.1F). However, in spite of the variation between sampling dates, the trend of greater copy and transcript numbers in R1 and R3 remained the same.

Table 5.1. Critical Parameters for qPCR Standard Curves. NTC is the no template control, and NO-RTs are no reverse transcriptase controls.

qPCR run	PCR efficiency	Slope of standard curve	y-intercept of standard curve	Correlation coefficient	C_t of negative control
Enrichment cultures	110.5%	-3.093	5.134	0.949	NTC: 24.03 NO-RTs: ≥ 26.6
Lab-scale digesters	97%	-3.396	5.273	0.989	NTC: 30.42 NO-RTs: ≥ 22.77
Full-scale digesters	87.5%	-3.662	5.465	0.993	NTC: 26.45
Monster Time Course- 6h	90%	-3.588	6.263	0.993	NTC: 33.34 NO-RTs: ≥ 28.22
Monster Time Course- 24h	89.1%	-3.615	4.323	0.995	NTC: 33.49 NO-RTs: ≥ 30.47

The enrichment culture biomass samples were not collected at any specific time of day, especially in reference to the daily pulse feeding of the digesters. To test whether the variations among the data from the three sampling dates might be linked to the feeding regimen of lab-scale digesters, the *mcrA* gene and transcript levels were measured in the biomass of M, a large lab-scale digester (Table 2.1), over a six hour period and a twenty-four hour period (Figures 5.2A

and 5.2B). Digester M was chosen for this experiment because up to a liter of biomass (as opposed to 133 ml for the enrichment cultures) could be used over a twenty-four hour period without changing its maintenance regimen, providing the necessary amount of sample (40 ml/extraction) for multiple time points. The results of these experiments showed that a variation in copy number of the observed magnitude could have occurred when sampling the enrichment cultures at differing times post-feeding (Figures 5.2A and 5.2B). Thereafter, care was taken to sample the pulse-fed digesters exactly 24 h after a feeding. This method was not possible with the industrial digesters due to their being constantly fed a waste stream.

When comparing the lab-scale digesters NN-R2, NN-R3, and NN-R5, *mcrA* gene copy and transcript number were highest in biomass from digester NN-R3 which was fed the most varied substrates (Figure 5.3A, 5.3B, and Table 2.1). Although transcripts of *mcrA* were not detectable in VP-0, VP-10, VP-50, highest gene copy numbers were observed in VP-10 which received 0.025 mg/L of O_2 per day and lowest in VP-50 which received a higher daily oxygen dose (0.125 mg/L) (Figure 5.3A and Table 2.1). However, when the standard deviation within each sample's replicates was taken into consideration, there was very little difference among the digesters in either set.

Figure 5.1. Results of qPCR Experiments for Enrichment Cultures. Each bar represents results from three technical replicates. Error bars show standard deviation from the mean. A. *mcrA* gene copy number/ng of DNA from sample taken December 5. B. *mcrA* gene copy number/ng of DNA from sample taken December 16.C. *mcrA* gene copy number/ng of DNA from sample taken January 11. D. *mcrA* transcripts/ng of RNA from sample taken December 5. E. *mcrA* transcripts/ng of RNA from sample taken December 16. and F. *mcrA* transcripts/ng of RNA from sample taken January 11.

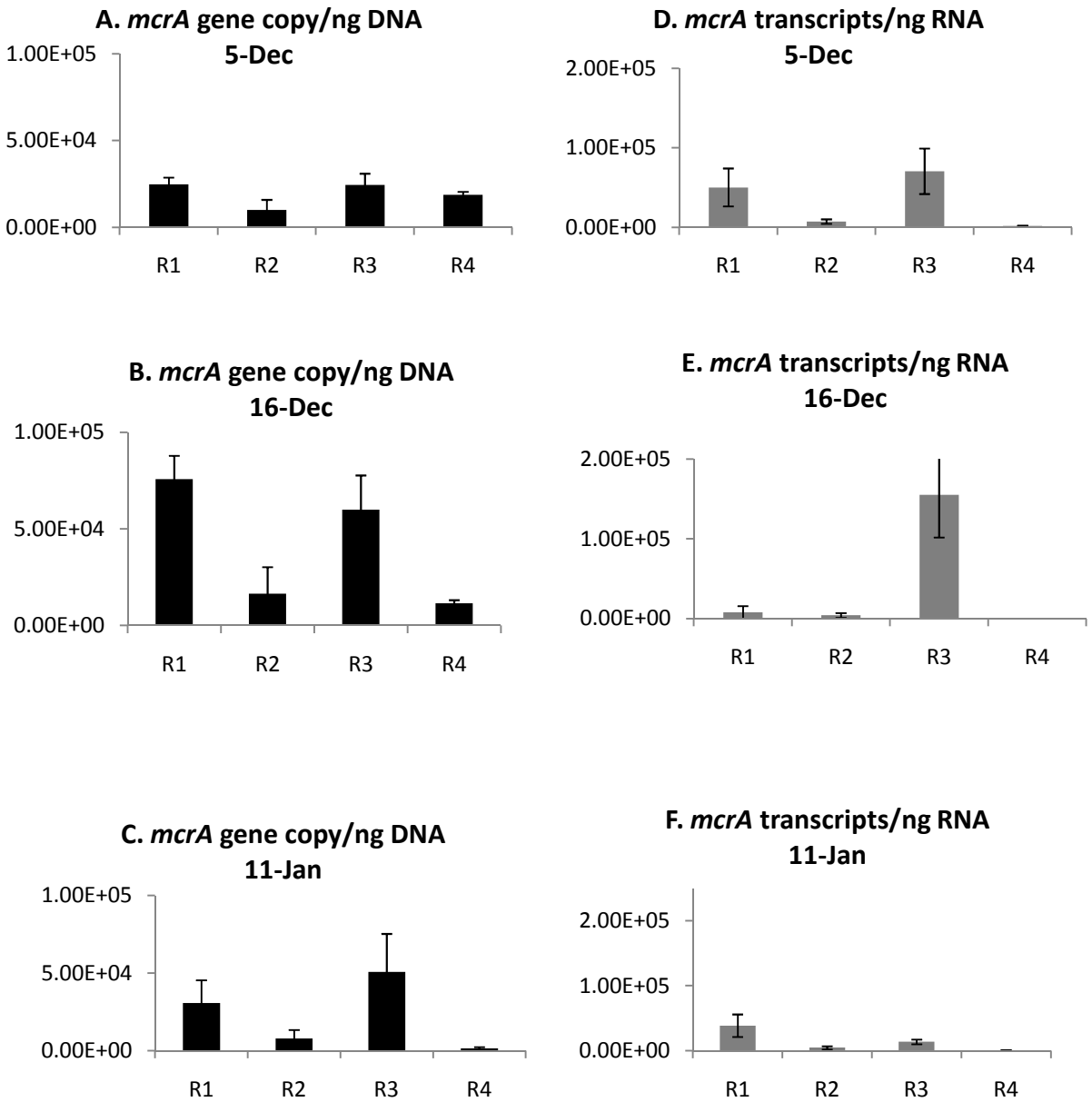
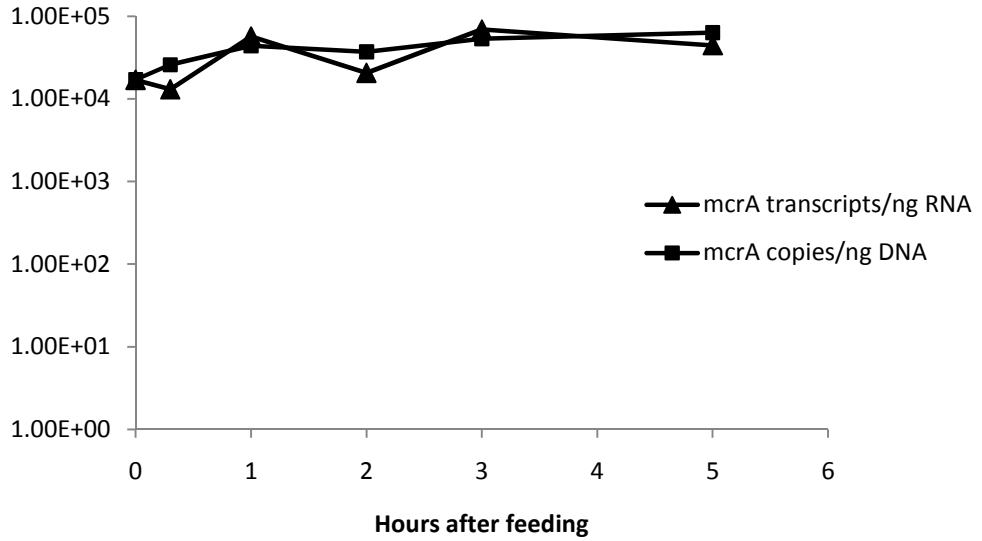
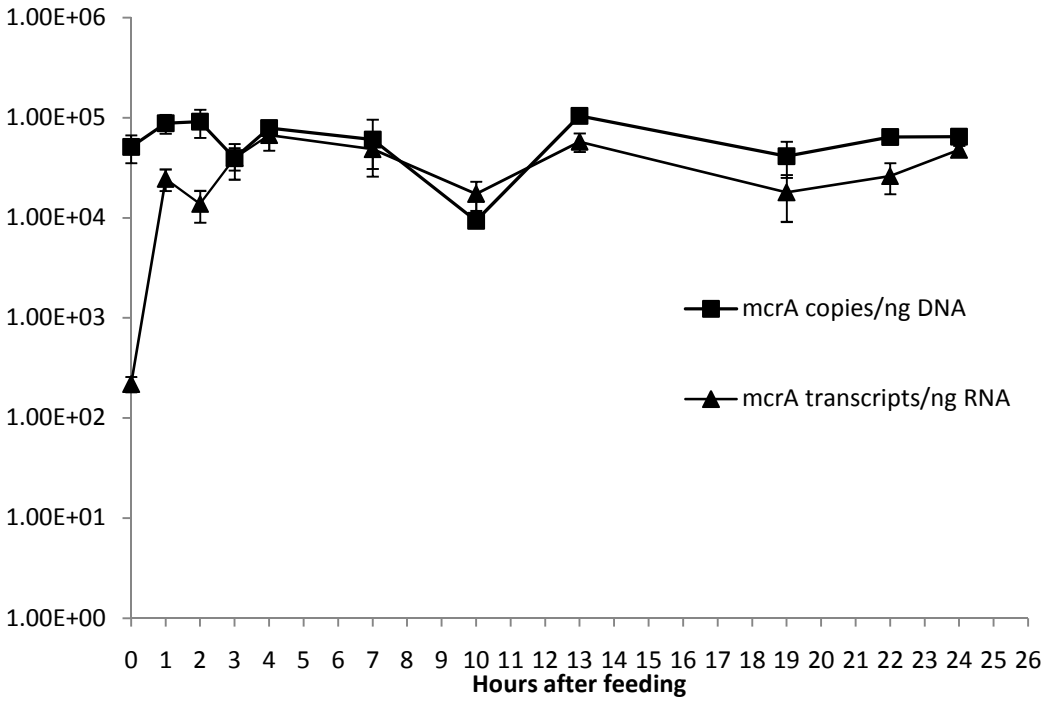


Figure 5.2. Time-course qPCR Experiments for M. A. Six hour time course. B. Twenty-four hour time course.

A. Six hour time course.



B. Twenty-four hour time course.



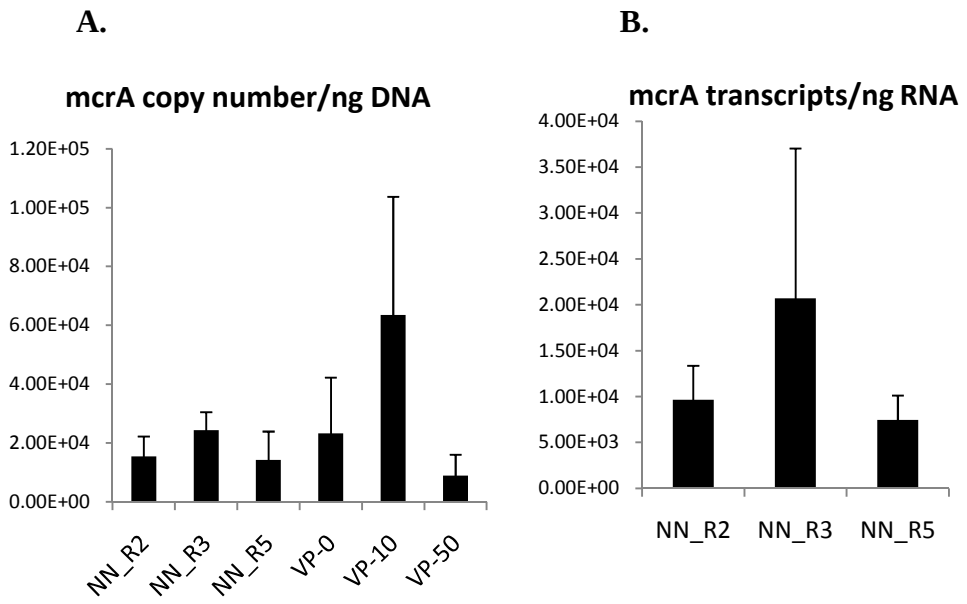


Figure 5.3. Results of qPCR for Lab-Scale Digesters. Each bar represents results from three technical replicates. Error bars show standard deviation from the mean. A. gene copy number. B. transcripts.

Total *mcrA* copy number per ng DNA and per g pellet were calculated from qPCR results for the industrial samples (Figure 5.4A and 5.4B). The "per g of pellet" normalization was calculated using the g of the biomass pellet after centrifugation and supernatant removal. Results were normalized to this parameter to account for differences in total solids among the digesters. When the qPCR results were normalized in this way, the digester with the highest *mcrA* gene copy number switched from JBS to CB (Figures 5.4A and 5.4B). Transcripts of *mcrA* were detected in biomass from CB, KI, and JBS (data not shown).

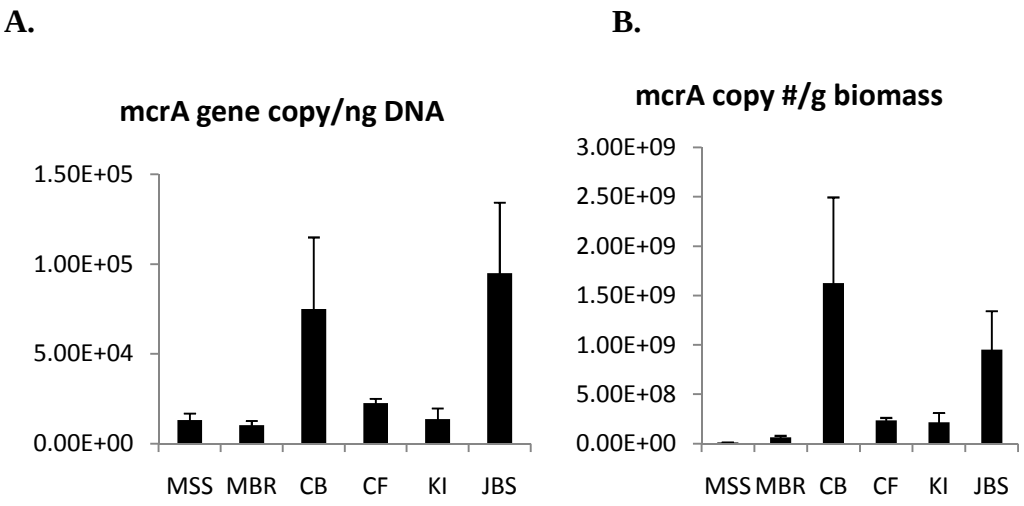


Figure 5.4. Results of qPCR for Industrial Digesters. Each bar represents results from three technical replicates. Error bars show standard deviation from the mean. A. *mcrA* gen copies/ng DNA . B. *mcrA* gene copies/g pellet of centrifuged biomass with the supernatant removed.

5.2.b Specific Methanogenic Activity Assays: SMA assays were used to compare the biomass samples' ability to produce methane given a particular substrate (Table 5.2) (Coates et al. 2005, Coates et al. 1996). The SMA assays for the enrichment cultures (R1-R4) showed that the cultures had higher (one to two orders of magnitude) methane production rates against $H_2 : CO_2$ than either the lab-scale or industrial digesters assayed, and that R1 and R3 had higher methane production rates against $H_2 : CO_2$ than R2 and R4 ($p<0.05$, ANOVA, Tukey test).

SMA values for the lab-scale digesters NN-R2, NNR3, and NNR5 were similar both against calcium acetate and $H_2 : CO_2$ (Table 5.2). The SMAs for the VP set of digesters were only measured against propionate. Among the three digesters in this set, VP-10 had the highest SMA at 13.8 ml CH_4/ g VSS-h, and VP-50 had the lowest at 6.7 ml CH_4/ g VSS-h (Table 5.2).

Table 5.2. Specific Methanogenic Activity Assay Results. SMA values for enrichment culture, lab-scale, and full-scale anaerobic digester biomass. Cv (%) is coefficient of variation for the triplicates from each sample.

Sample Name	SMA against Calcium Propionate, $mlCH_4/gVSS\text{-}hr$		SMA against Calcium Acetate, $mlCH_4/gVSS\text{-}hr$		SMA against $H_2{:}CO_2$, $mlCH_4/gVSS\text{-}hr$	
	Average	Cv (%)	Average	Cv (%)	Average	Cv (%)
CB	14.9	6.7	21.7	2.5	31.4	0.0
KI	3.6	6.3	11.0	6.3	8.8	3.7
CF	1.1	97.0	10.1	9.4	8.6	16.0
JBS-SH	0.8	29.6	4.7	48.5	12.1	0.0
MMBR	0.0	0.0	5.6	66.3	10.2	26.0
MMSS	0.0	0.0	6.6	97.4	15.0	17.0
VP-0	10.7	31.0				
VP-10	13.8	13.0				
V0-50	6.7	45.0				
NN_R2			5.3	4.2	5.8	7.1
NN_R3			6.6	5.3	7.6	7.2
NN_R5			6.2	3.1	7.2	18.6
R1					402.0	3.8
R2					120.0	12.5
R3					465.1	3.4
R4					100.7	4.6

When comparing the industrial digesters SMA results, the brewery biomass sample (CB) had the highest SMA values for all three substrates tested (Table 5.2). Comparison of SMA values for the other five samples showed that KI (milk-derived additive waste) had the next highest activity against propionate and acetate, followed by CF (manure digester). However, the municipal and slaughterhouse samples (MMBR, MMSS, and JBS) had higher activities against $H_2 : CO_2$ than KI and CF.

5.2.c Comparison of qPCR and SMA: For the hydrogen enrichment cultures (R1-R4), qPCR results (*mcrA* gene copy number/ ng DNA) positively correlated with SMA results against $H_2 : CO_2$ (Table 5.3). The propionate-enriched lab-scale digesters VP-0, VP-10, and VP-50 biomass

had no significant ($p>0.05$) correlation between the *mcrA* gene copy number/ ng DNA extracted and the SMA values against propionate (Table 5.3). Furthermore, there was no significant correlation between gene copy or transcript number and SMA for either $H_2 : CO_2$ or acetate as a substrate for the NN digester set (Table 5.3). The values for the lab-scale digester data sets from qPCR and SMA had very little variation, and the lack of significant correlation of the data sets is likely due to their similarity. Industrial digester biomass qPCR results normalized to g of biomass extracted correlated well with SMAs against $H_2 : CO_2$ (R^2= 0.67, Table 5.3) and propionate (R^2= 0.70, Table 5.3), but not acetate (R^2= 0.49, Table 5.3).

Table 5.3. Specific Methanogenic Activity Assay and qPCR Correlations. R^2 values are the result of linear regression analysis. P values are for slopes (F-ratio test of ANOVA against a slope of 0).

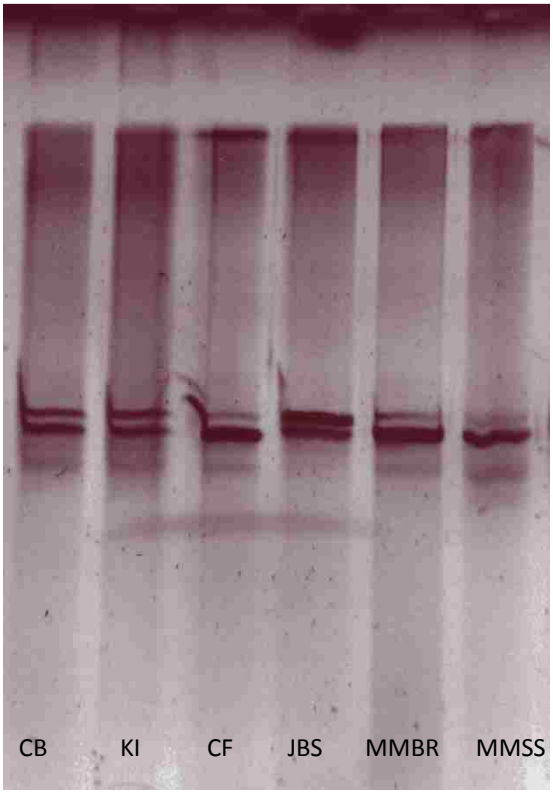
Culture or Digester Set	*mcrA*/SMA substrate	R^2	p
Enrichment cultures	**Gene copy / H_2:CO_2**	**0.99**	**0.007**
	Transcript/ H_2:CO_2	**0.83**	**0.09**
NN lab-scale digester set	**Gene copy /H_2:CO_2**	**0.35**	**0.60**
	Transcript/ H_2:CO_2	**0.31**	**0.62**
	Gene copy /acetate	**0.43**	**0.54**
	Transcript/ acetate	**0.38**	**0.57**
VP lab-scale digester set	**Gene copy/propionate**	**0.89**	**0.21**
Industrial	**Gene copy / H_2:CO_2**	**0.67**	**0.046**
	Gene copy/propionate	**0.70**	**0.038**
	Gene copy /acetate	**0.49**	**0.12**

5.2.d Denaturing Gradient Gel Electrophoresis: Previous studies of clone libraries generated from biomass sampled from the hydrogen enrichment cultures demonstrated that the methanogen communities for each culture were different (see Chapter Four, (Schauer-Gimenez et al. 2010)). The use of DGGE to obtain a methanogen community fingerprint in the industrial biomass samples also revealed a unique methanogen community within each full-scale digester (Figure 5.5A). A dendrogram constructed from the densitometric data from the gel confirmed that the communities were different even when substrates were similar (Figure 5.5B). For example,

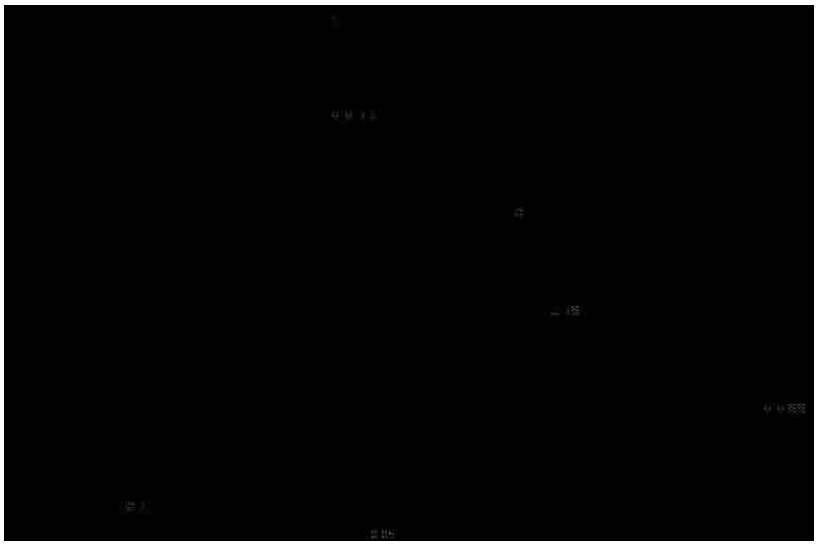
MMBR and MMSS, which were both biomass samples from municipal plants, appeared in different clades.

Figure 5.5

A. DGGE of *mcrA* from Industrial Digesters. DGGE fingerprint of *mcrA* genes present in the industrial biomass samples. Lanes are labeled with sample names. See Table 2.1 and Table 5.2 for more information regarding each sample.



B. Dendrogram showing relationships between the methanogen communities in the industrial digesters based on optical density data from the DGGE gel.



5.2.e Nucleic Acids and Volatile Suspended Solids: Yields of DNA and RNA extracted from biomass obtained from all enrichment cultures and anaerobic digesters (Table 5.4) did not positively correlate with volatile suspended solids yields per liter of biomass (R^2=0.1 and 0.08 respectively).

Table 5.4. Total DNA, RNA, and VSS Yields. Total DNA, RNA and VSS yields per L of anaerobic biomass.

Sample Name	Nucleic Acids (ng/L biomass) DNA	RNA	Volatile Suspended Solids (g/L of biomass)
CB	2.28×10^5	3.96×10^5	47.2
KI	8.99×10^5	33.58×10^5	40.4
CF	0.66×10^5	0.77×10^5	61.5
JBS-SH	2.89×10^5	4.15×10^5	6.5
MMBR	10.5×10^5	34.03×10^5	16.9
MMSS	10.1×10^5	5.67×10^5	27.4
VP-0	1.35×10^5	0.8×10^5	0.38
VP-10	1.25×10^5	0.36×10^5	0.22
V0-50	1.34×10^5	0.45×10^5	0.40
NN_R2	1.31×10^5	0.79×10^5	8.4
NN_R3	1.31×10^5	0.82×10^5	11.0
NN_R5	1.31×10^5	0.68×10^5	8.4
R1	1.21×10^5*	2.03×10^5*	0.15
R2	1.46×10^5*	3.20×10^5*	0.47
R3	1.09×10^5*	3.15×10^5*	0.16
R4	1.97×10^5*	6.62×10^5*	0.52

*mean of three extractions.

5.3.Discussion

Although variations occurred between the values obtained from three different sample collection dates, the mean *mcrA* gene copy numbers from the hydrogen enrichment cultures showed excellent correlation with specific methanogenic activity tests against H_2:CO_2 (Table 5.3). Data obtained from SMA assays and qPCR performed on industrial biomass samples also confirmed that a relationship existed between SMA and *mcrA* gene copy number, although the association between specific methanogenic activity against acetate and *mcrA* was weak (Table 5.3). This weak correlation is possibly related to the fact that several groups of microorganisms

in digesters are able to utilize acetate, and the relative abundance of these organisms could vary among the biomass samples tested. Furthermore, acetate oxidation to H_2 and CO_2 may also occur under certain conditions, which could further reduce the acetate available to the methanogens for methane production (Karakashev et al. 2006, Schnürer et al. 1997, Schnürer et al. 1999).

Transcript numbers for *mcrA* in the enrichment culture biomass also correlated with specific methanogenic activity against H_2:CO_2 (R^2= 0.83) although the p value was not significant (Table 5.3). Transcripts were also below the limits of detection in digesters which treated municipal and cattle waste (MMBR, MMSS, and CF). The exact reasons for this observation are unknown at this time; however, the presence of inhibitory compounds and low transcriptional activity within these biomass samples are possible explanations (Smith and Osborn 2009, Stults et al. 2001). Transcription of the *mcrA* gene may be more closely linked to metabolic activity of methanogens within the biomass than the presence of *mcrA* in genomic DNA. Furthermore, transcript-to-gene copy ratios showed the best correlation with methane flux in samples from peat (Freitag and Prosser 2009, Freitag et al. 2010). MMSS, MMBR, and CF did not have consistently lower SMAs compared to the other digesters (Table 5.2), but methane flux measures methane produced while SMA is a measure of methane production rate against a specific substrate. Attempts to establish a relationship between transcript-to-gene copy ratios and SMA using the data from this study were unsuccessful (data not shown). However, the data from this study supports using *mcrA* gene copy number as a more consistently obtainable measure when sampling from anaerobic biomass.

Clone libraries generated from the hydrogen enrichment culture biomass (Chapter 4) and DGGE of the *mcrA* sequences from DNA extracted from the industrial samples (Figure 5.5A and

5.5B) showed that the methanogen communities for each digester were different, demonstrating that the relationship between SMA and *mcrA* gene copy number was not dependent on the structure of a particular methanogen community or the composition of the waste stream.

The time course experiments revealed variations in the *mcrA* gene copy and transcript numbers similar to those observed in the enrichment cultures among sampling dates (Figures 5.1A-F and 5.2A-B). Variations in transcript levels were expected as a result of changing need for MCR over time, but the changes in *mcrA* gene copy number were not what would be expected during normal growth. This was especially clear at hour 10 after feeding when *mcrA* gene copy levels fell far below the initial reading from before feeding (Figure 5.2B) suggesting that there were less methanogens present at hour 10 than hour 0. The reason for this variation is unknown, but it is likely due to the heterogeneity of the biomass itself. Although care was taken to collect the same volume of biomass each time and the extractions were performed together, physical differences during the extraction process could also have contributed to the observed variation in transcript and gene copy numbers. However, Frietag et al.(2010) reported similar variations in *mcrA* gene copy (0.13-0.59 standard error in log abundance of *mcrA* template abundance, n=4-5) and transcript numbers (0.08-0.48 standard error) of in multiple samples from peat. Furthermore, variations observed in the results of oligonucleotide hybridization studies of microbial communities in anaerobic digester biomass by Raskin et al. (1994b, 1995) also suggest that biomass may be heterogeneous.

Accurate determination of the metabolically active portion of anaerobic biomass, referred to as active biomass and representing the fraction which is made up of living cells, is useful when monitoring digester function. Typically, VS or VSS per L of biomass are used as an estimation of the fraction of biomass which is metabolically active (APHA 1998). However, VS

and VSS are simple measures of organic materials in the waste, and they cannot discriminate between members of the microbial community and other organic matter. In this study, measurements of VSS did not positively correlate with DNA or RNA concentrations (Table 5.4). Thus, further research should be undertaken to establish a better means of determining the active portion of anaerobic biomass.

Taken together, the results of this study support the hypothesis that *mcrA* gene copy number can be used to monitor and compare methanogen communities in anaerobic digesters. Useful methods of monitoring digesters must be sensitive and fast (Castellano et al. 2007, Molina et al. 2009). The ability of this methodology to rapidly produce results that correlate well with SMA assays (which take weeks to complete) satisfies both these parameters, suggesting that qPCR of *mcrA* may be a very useful technique for comparing biomass from different sources.

Assays of other genes representing other members of the anaerobic food chain such as acidogens and syntrophic propionate-reducing bacteria should also be developed to monitor other important metabolic activities within digesters. Although methanogenesis is often proposed to be the rate-limiting step in anaerobic digestion, the actual metabolic process which limits the rate of methane production may depend upon substrate composition (Liu and Whitman 2008). Therefore, monitoring microorgansisms at all trophic levels in digesters could provide valuable diagnostic information. However, this study represents the establishment of a direct link between the microbial community in anaerobic biomass and digester function.

Concluding Remarks

Anaerobic wastewater treatment is an environmentally and economically beneficial biotechnology. It is an established, cost-effective method of removing organic wastes, and it has the added benefit of producing methane, an alternative energy source. However, anaerobic treatment is an underutilized technology because of historical doubts about the stability of the process (Dupla et al. 2004), as well as slow startup and recovery of stressed digesters. Therefore, research which supports the optimization of this technology can be extremely beneficial.

The degradation of organic waste in anaerobic digesters is carried out by a complex, interdependent microbial community; and the methanogens are an especially important group as they complete one of the final steps in waste degradation and produce the methane. Therefore, understanding the relationship between methanogen community structure in anaerobic biomass and digester function could provide important insight which may be used to improve this form of biotechnology. However, the contribution of methanogens to digester function and the community ecology of anaerobic digesters have not previously been clearly defined. Specifically, clear links between methanogen diversity and abundance and the successful removal of wastes have not previously been determined.

This study was performed in order to examine the relationship between anaerobic digester function and methanogen community structure. Analysis of DNA and cDNA clone libraries generated from biomass samples obtained from anaerobic hydrogen/carbon dioxide enrichment cultures and two industrial-scale digesters using the methanogen-specific gene *mcrA*, showed differences in the diversity of the methanogen communities from different anaerobic biomass samples, even from the enrichment cultures that where started with the same seed sludge. Furthermore, comparison of *mcrA* genes and transcripts from the enrichment cultures

revealed that some methanogens were more active than others under certain conditions. While no direct links were found between methanogen diversity and digester function, the data from the clone libraries may be used to engineer microbial mixes used for bioaugmentation of digesters with unstable function.

Tandem qPCR and specific methanogenic activity (SMA) assays performed on biomass samples showed significant positive correlation between *mcrA* gene copy number and methane production rates against specific substrates in the enrichment cultures and, more importantly, in the real world digesters. This result indicates a previously undiscovered relationship between the number of methanogens in anaerobic biomass and digester function, and represents the establishment of a direct link between the microbial community in anaerobic biomass and digester function. It also suggests the possibility of a new assay for monitoring digester function, qPCR of *mcrA,* which is both fast and cost-effective.

Overall, the data obtained from these studies provide new insight into methanogen communities in digesters which may be applied to develop better monitoring methods for anaerobic biomass, to engineer better microbial mixes for bioaugmentation of struggling or failing digesters, and to improve microbial communities in digesters which will aid in the production of more methane for use as renewable fuel.

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Appendix I.

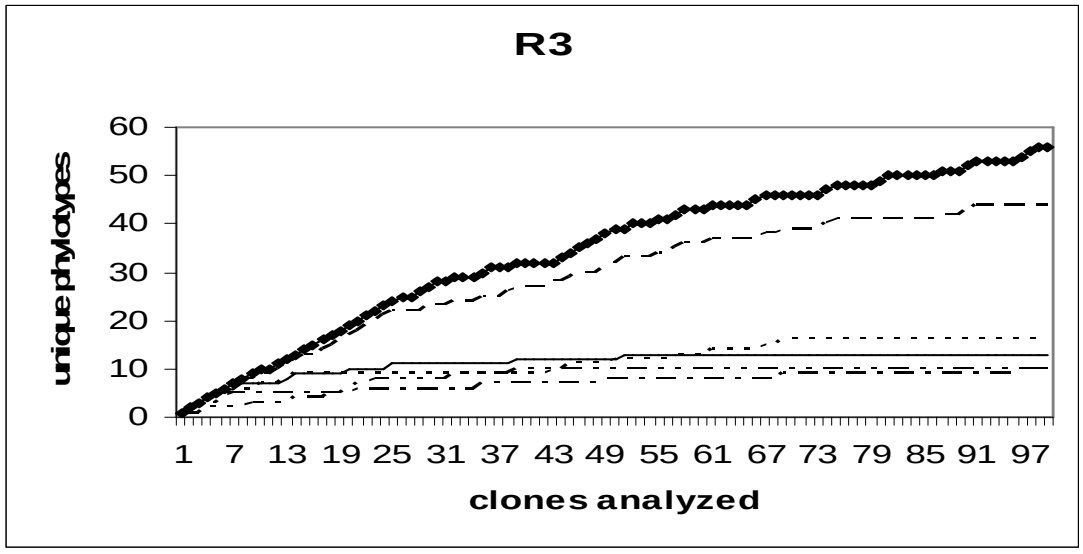
A brief description of the five orders of methanogens with specific information about genera and species that were detected in this study by blastn (Altschul et al. 1990, Altschul et al. 1997) searches using *mcrA* sequences. Adapted from Lui and Whitman (Liu and Whitman 2008) and Boone et al. (Boone et al. 1993) and references contained therein, as well as Imachi et al.(Imachi et al. 2008), Ma et al.(Ma et al. 2005), Schauer and Ferry (Schauer and Ferry 1980), Ferry and Wolfe (J.G. and R.S. 1977), Dianou et al. (Dianou et al. 2001), Maestrojuan (Maestrojuan et al. 1990), Patel (Patel 1984), and Zellner(Zellner et al. 1998). (*=Not detected in this study).

Taxonomy	Methanogenesis Substrate(s)	Morphology	Gram Stain	pH	Temper -ature [°C]	Growth rate [h^{-1}]	GC content [%]
Methanopyrales*	H2+CO2	Rods	+	5.5-7.0	84-110	NA	59
Methanococcales*	H2+CO2 Formate+CO2	Cocci	_			NA	
Mesophilic genera				5.8-8.2	18-47		30-33
Thermophilic genera				5-7.6	30-94		31-34
Methanobacteriales	H2+CO2 Formate+CO2	Rods					
Methanobacterium							
formicicum			Var.	7.0-7.5	25-50	0.006	41-42
beijingense			_	6.5-8.6	25-50	0.049	38.9
subterraneum			+	6.5-9.2	36-45	0.2-0.3	54.4 ±0.5
Methanobrevibacter			+	6-8.5	20-45		28-31
Methanomicrobiales	H2+CO2 Formate+CO2	Cocci	_				
Methanoculleus							49-61
palmolei	Secondary alcohols			4.9-7.5	22-50	0.074	
marisnigri				6.2-7.9	15-37+	0.128	
chikugoensis	Secondary alcohols			6.7-7.2	35-30	0.015	
Methanospirillum hungatei		Curved rods		6.6-7.4	30-37	0.059	45
Methanolinea tarda		rods		6.7-8.0	30-60	0.007	56.3
Methanosarcinales	Acetate						
Methanosaeta concillii		Rods	_	6.0-8.0	10-45	0.042	49
Methanosarcina*	H2+CO2 Methylamines Methanol	cocci	Var.	7.0-7.0	20-50	NA	36-44

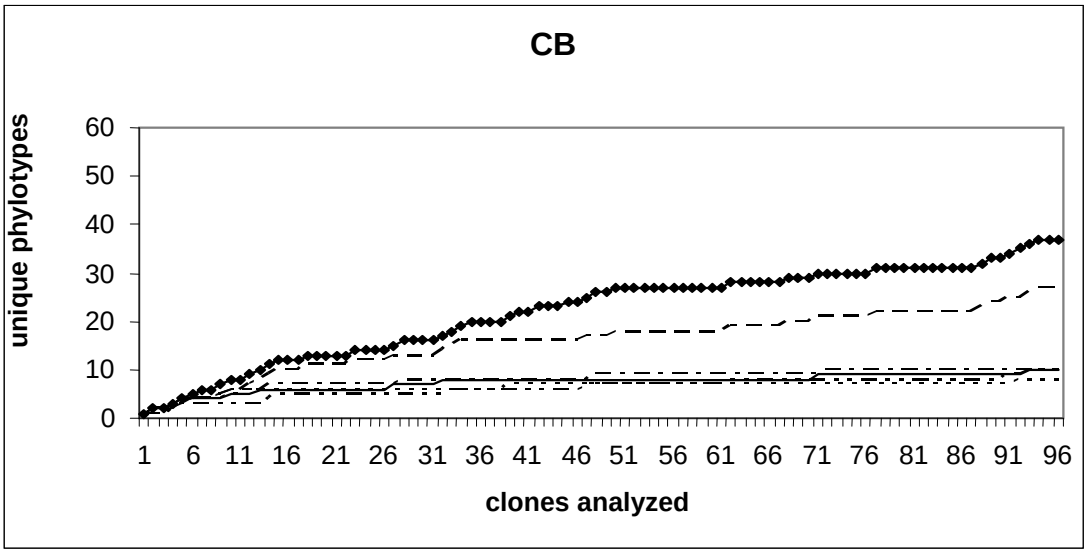
Appendix II.

Rarefaction curves (Figures A. B. and C.) from the restriction enzyme study discussed in Chapter 3 but not included in the text. Legend for graphs: - - - *Taq$^{\alpha}$I;* — - — *RsaI;* ——— *MspI;* — - - — *Sau961;* — — — *RsaI* and *Sau961;* ◆◆◆ *Taq$^{\alpha}$I, RsaI* and *Sau961*

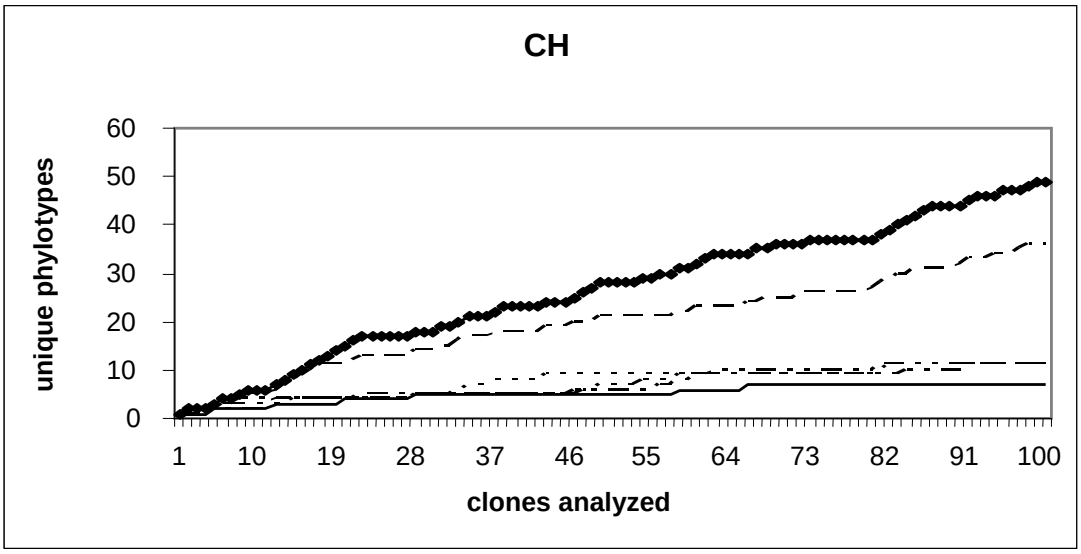
A.



B.



C.



Appendix III

Phylogenetic Trees (Figures 1, 2, and 3) from the restriction enzyme study discussed in Chapter 3 but not included in the text. Neighbor-joining phylogenetic trees made from *in silico* restriction digests of sequences and from the sequences themselves. Trees for each possible combination of enzymes were created for each biomass sample included in this part of the study: C, B, R1, and R3. Trees shown above are from C (Figure 1), R1 (Figure 2), and R3 (Figure 3). Each figure is divided into five parts (**A-E)** as follows: **A.** Phylogenetic tree using the output from *Taq$^{\alpha}$I,* single *in silico* digests. **B.** Tree made from combining the output from *Taq$^{\alpha}$I* and *MspI* digests. **C**. Tree made from combining the output from *Taq$^{\alpha}$I, RsaI,* and *Sau961* digests**.** **D.** Tree made from combining output from all four *in silico* digests, *Taq$^{\alpha}$I, RsaI, MspI,* and *Sau961*. **E.** Neighbor-joining phylogenetic tree of *mcrA sequences* obtained from industrial digester C biomass. Node labels are bootstrap values from 100 analyses, and the tree is rooted with *Methanopyrus kandlerii mcrA.*

Figure 1A.

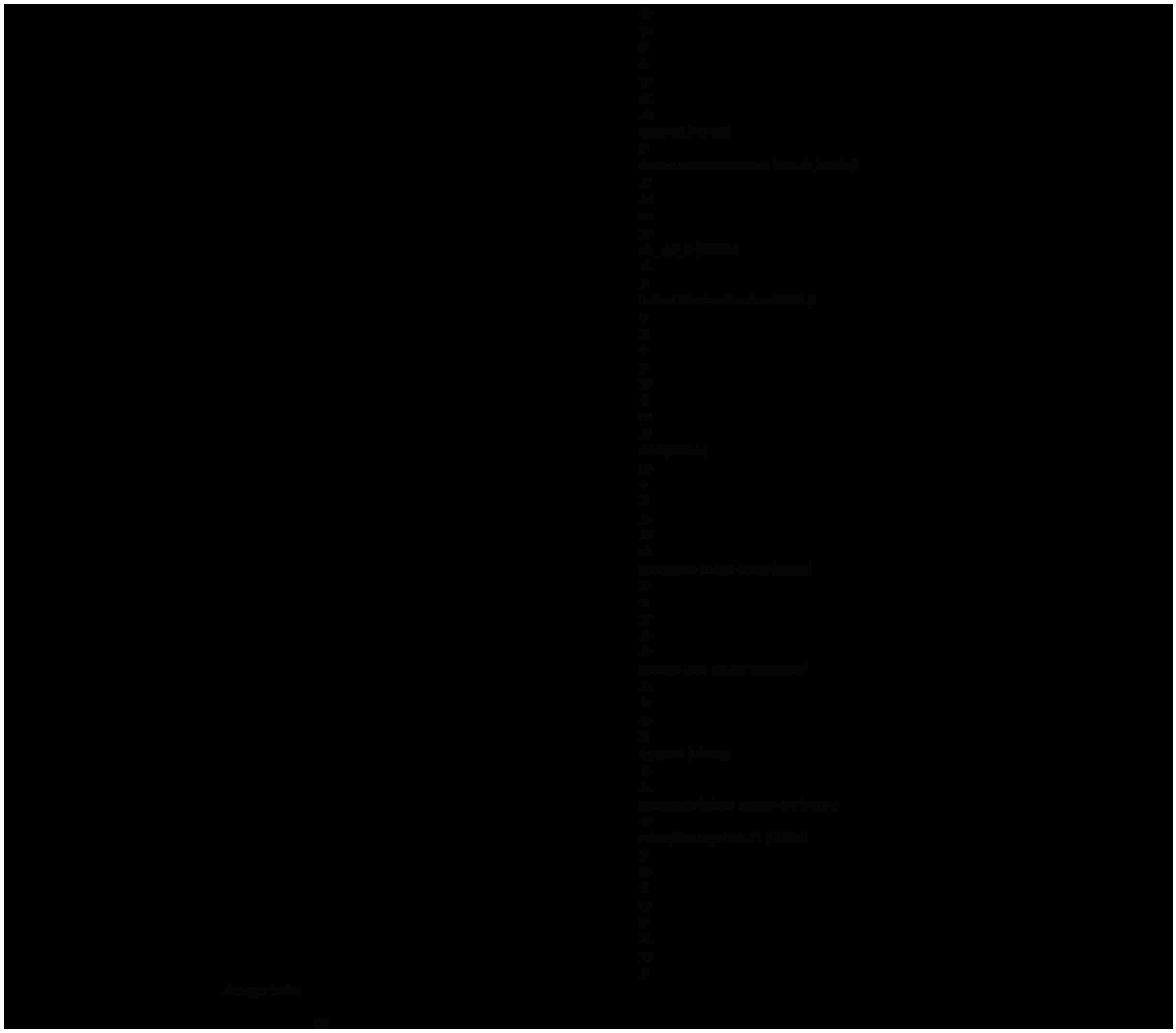


Figure1B.

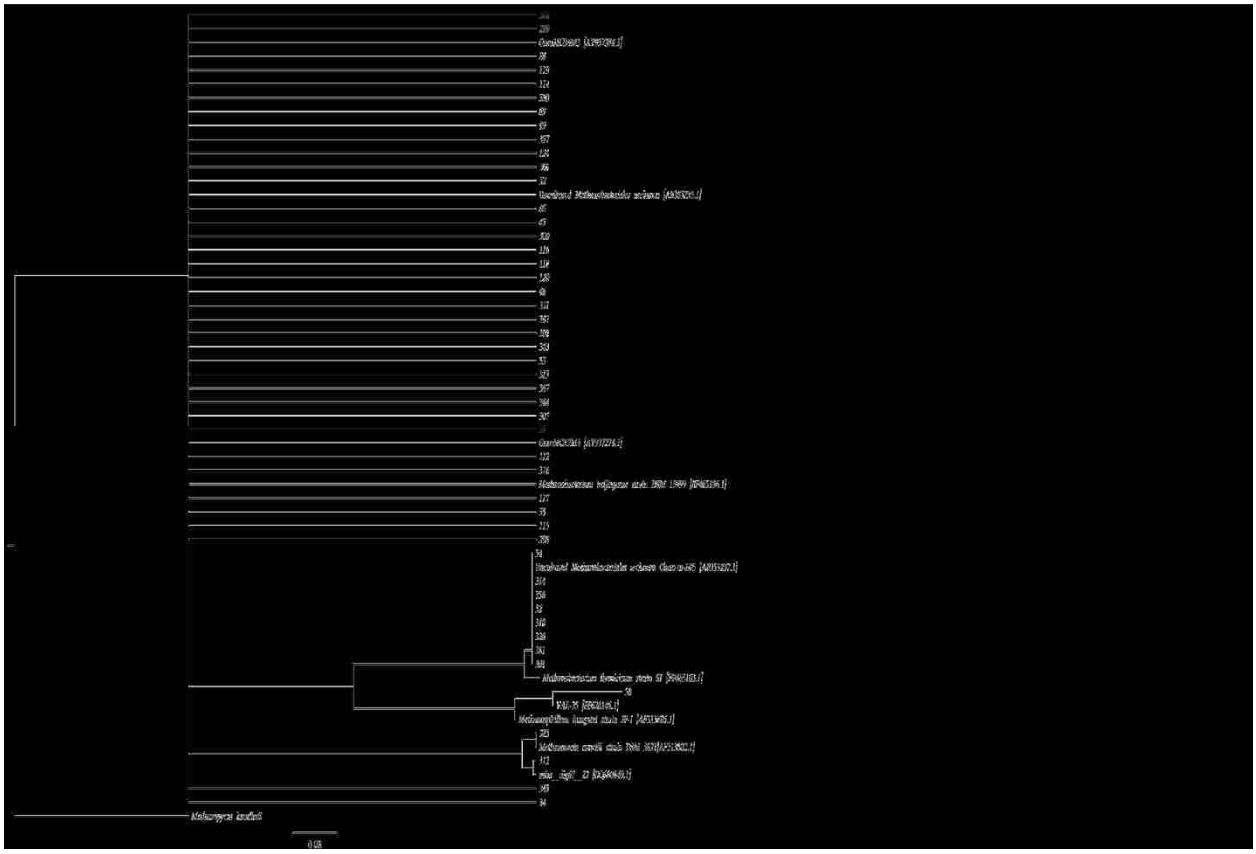


Figure 1C.

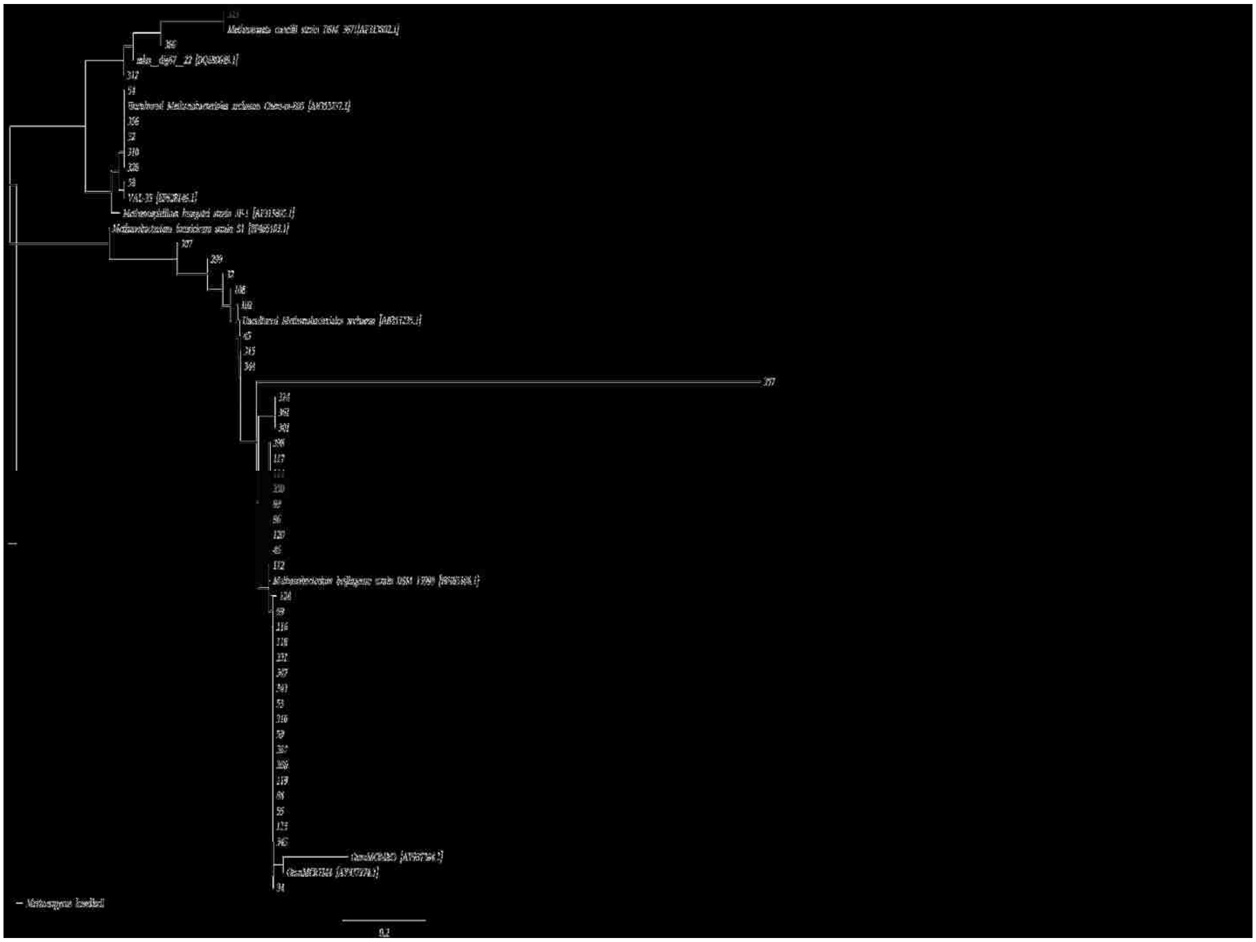


Figure 1D.

Figure 1E.

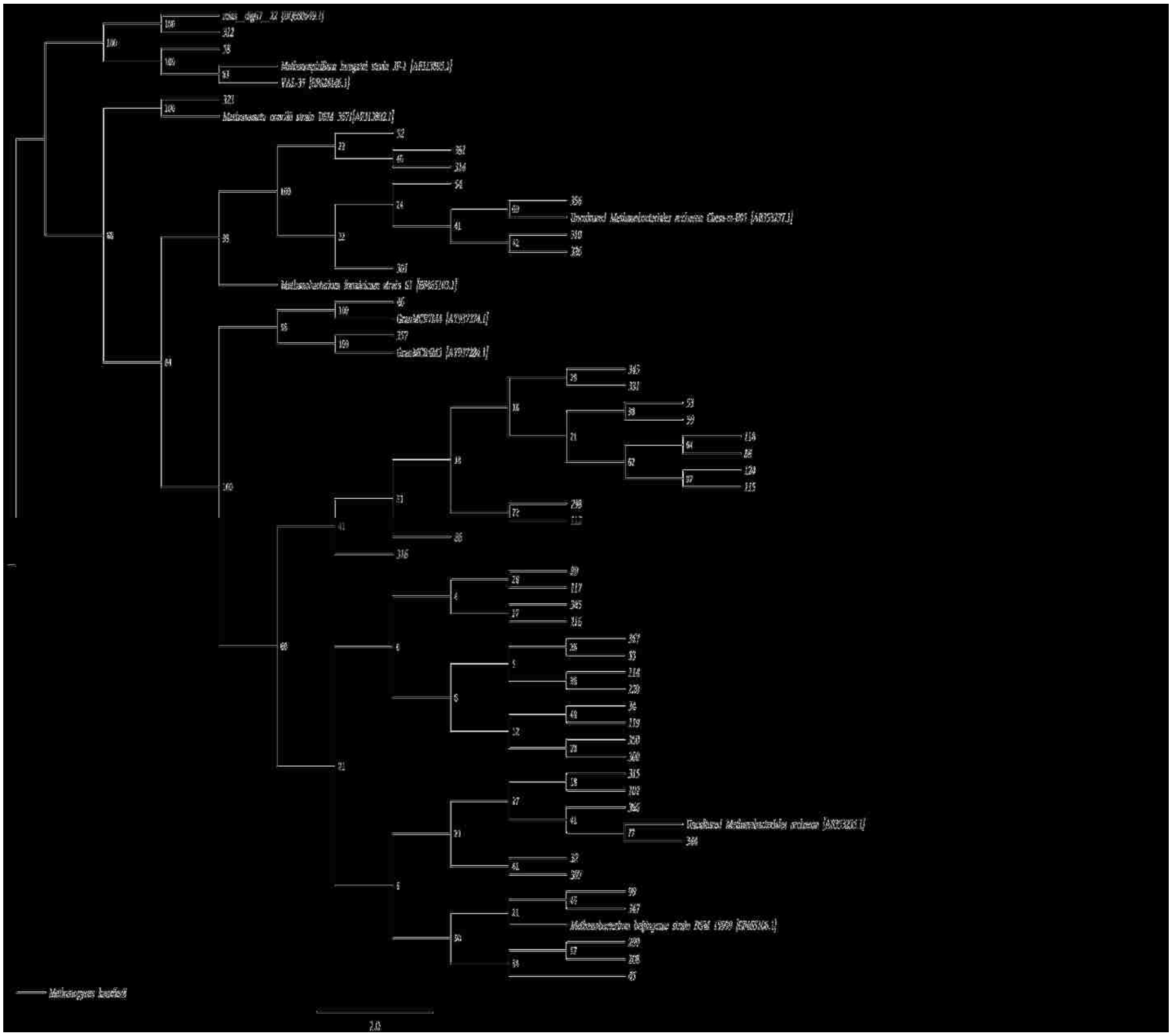


Figure 2A.

Figure 2B.

Figure 2C.

Figure 2D.

Figure 2E.

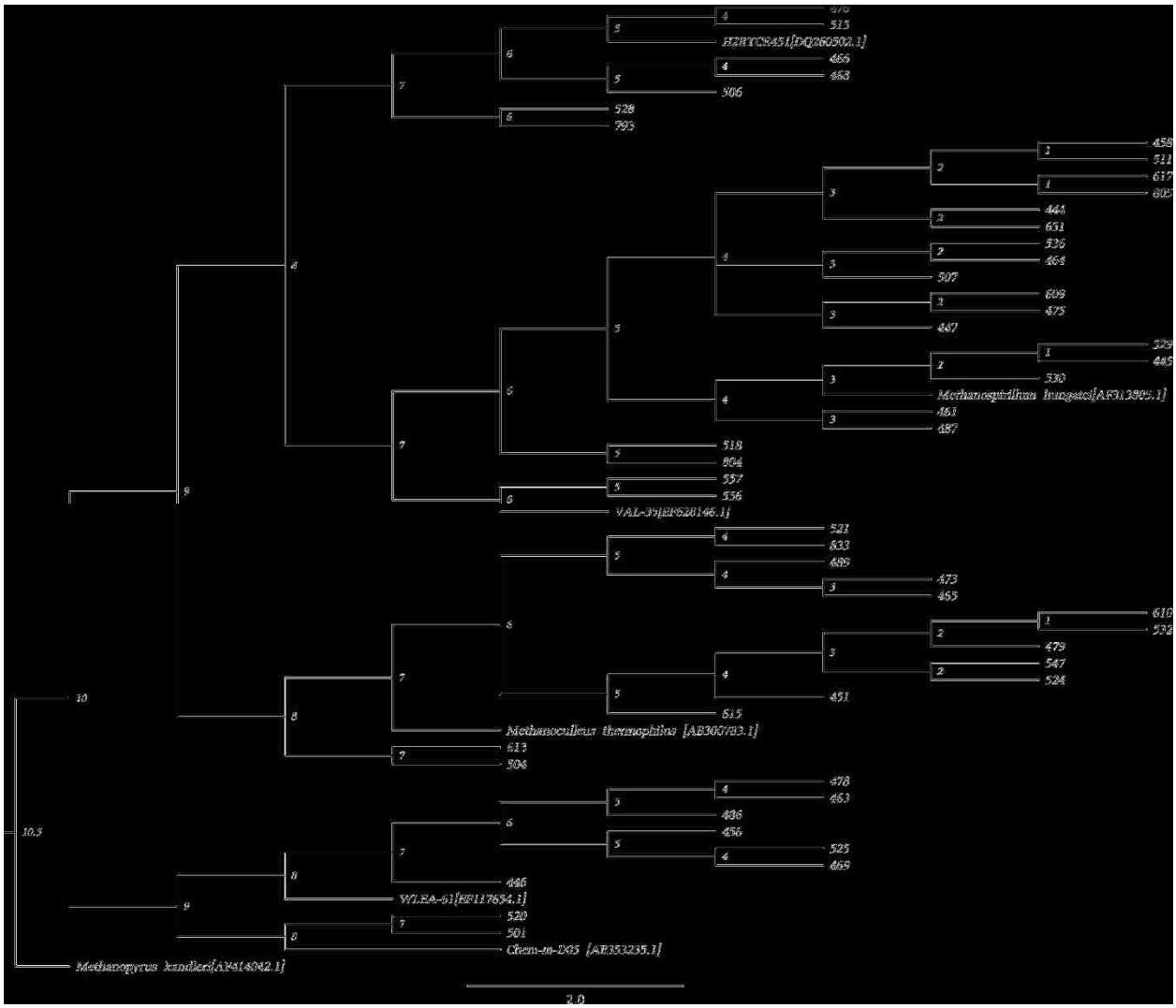

HQ260502.1]
Methanosphaera stadtmanae [AF150905.1]
VAL-39[EF528146.1]
Methanobrevibacter thermophila [AB300783.1]
WLEA-61[EF117654.1]
Chem-m-D05 [AF353235.1]
Methanopyrus kandleri[AE010442.1]
3.0

Figure 3A.

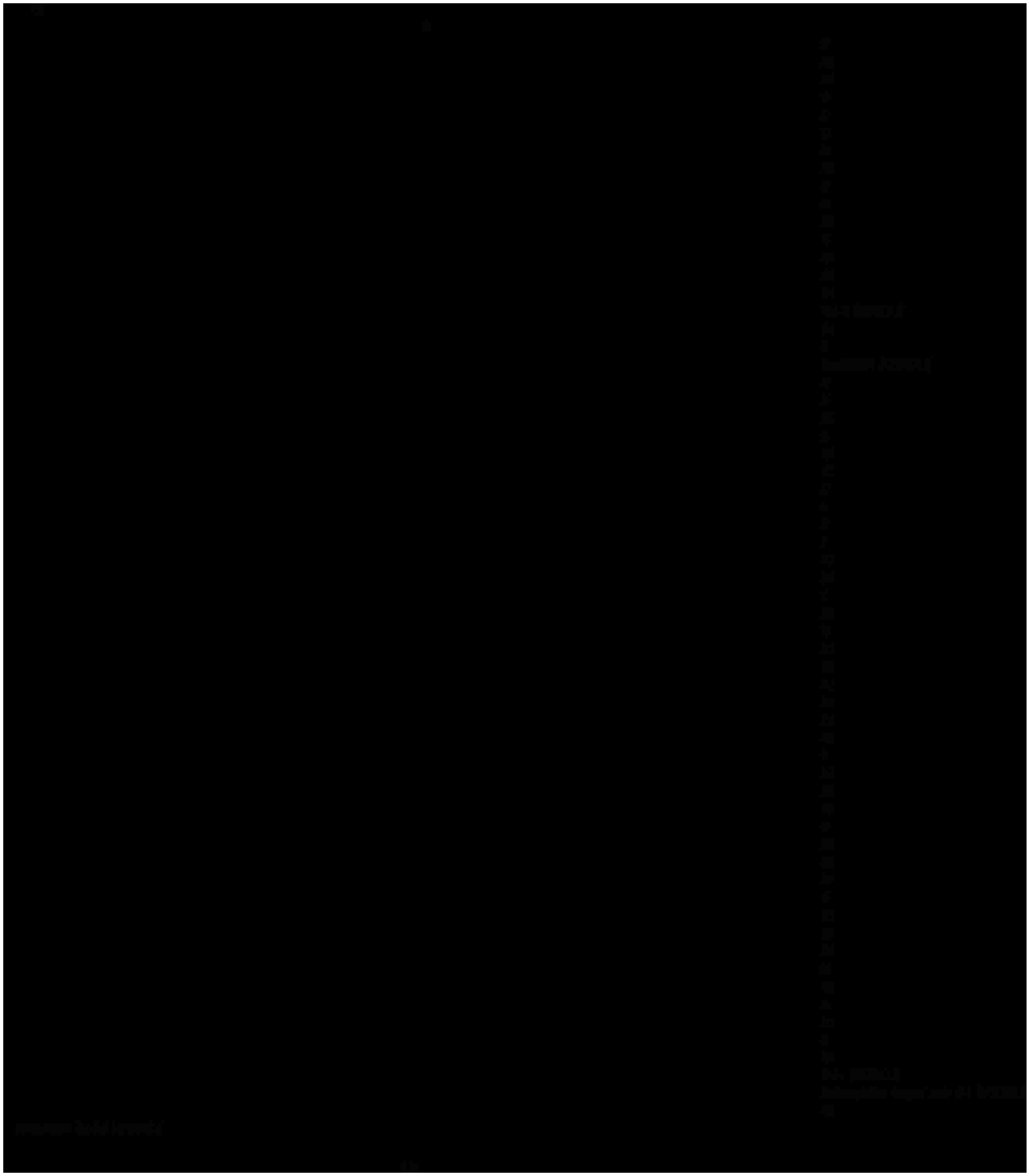


Figure 3B.

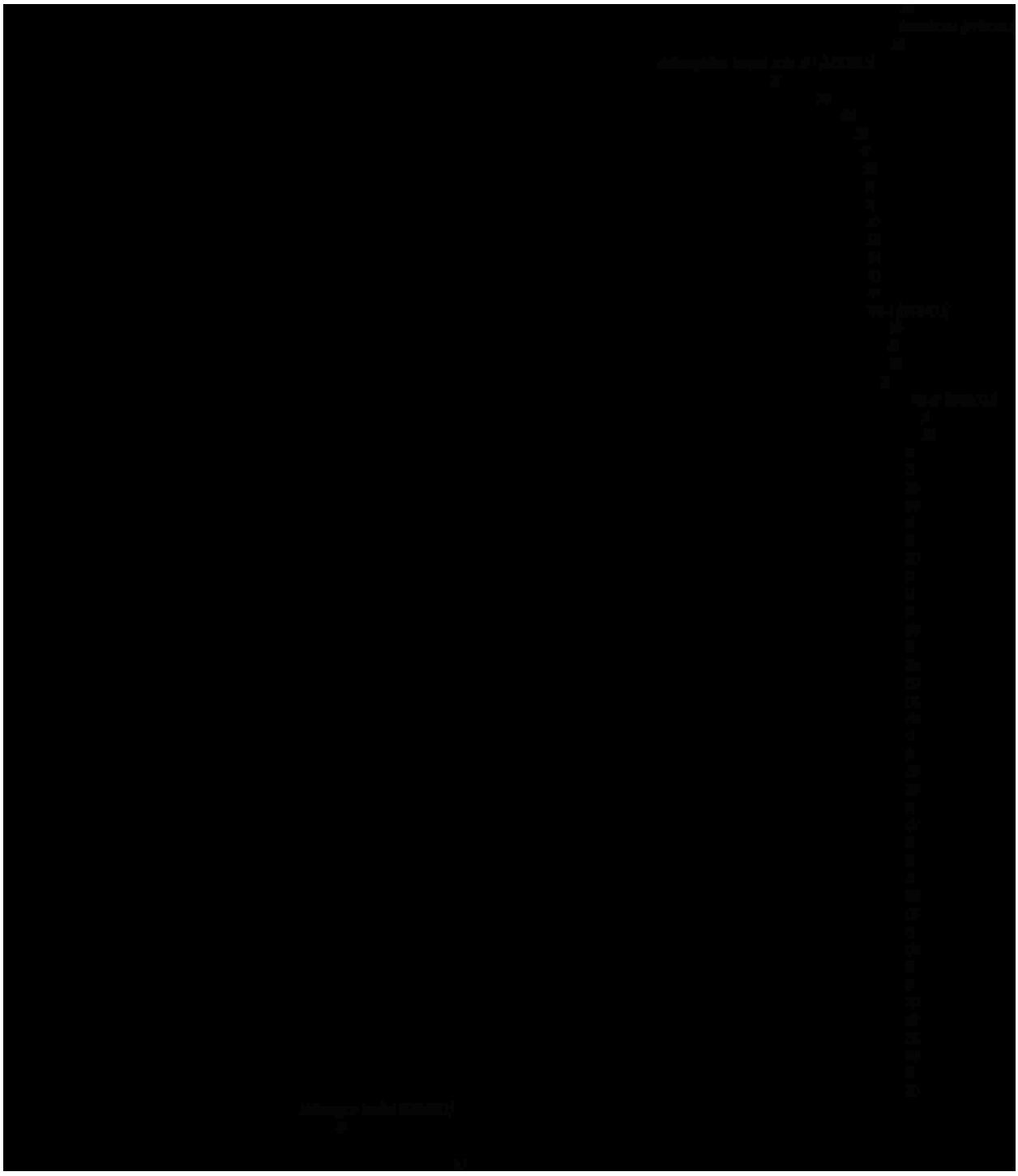


Figure 3C.

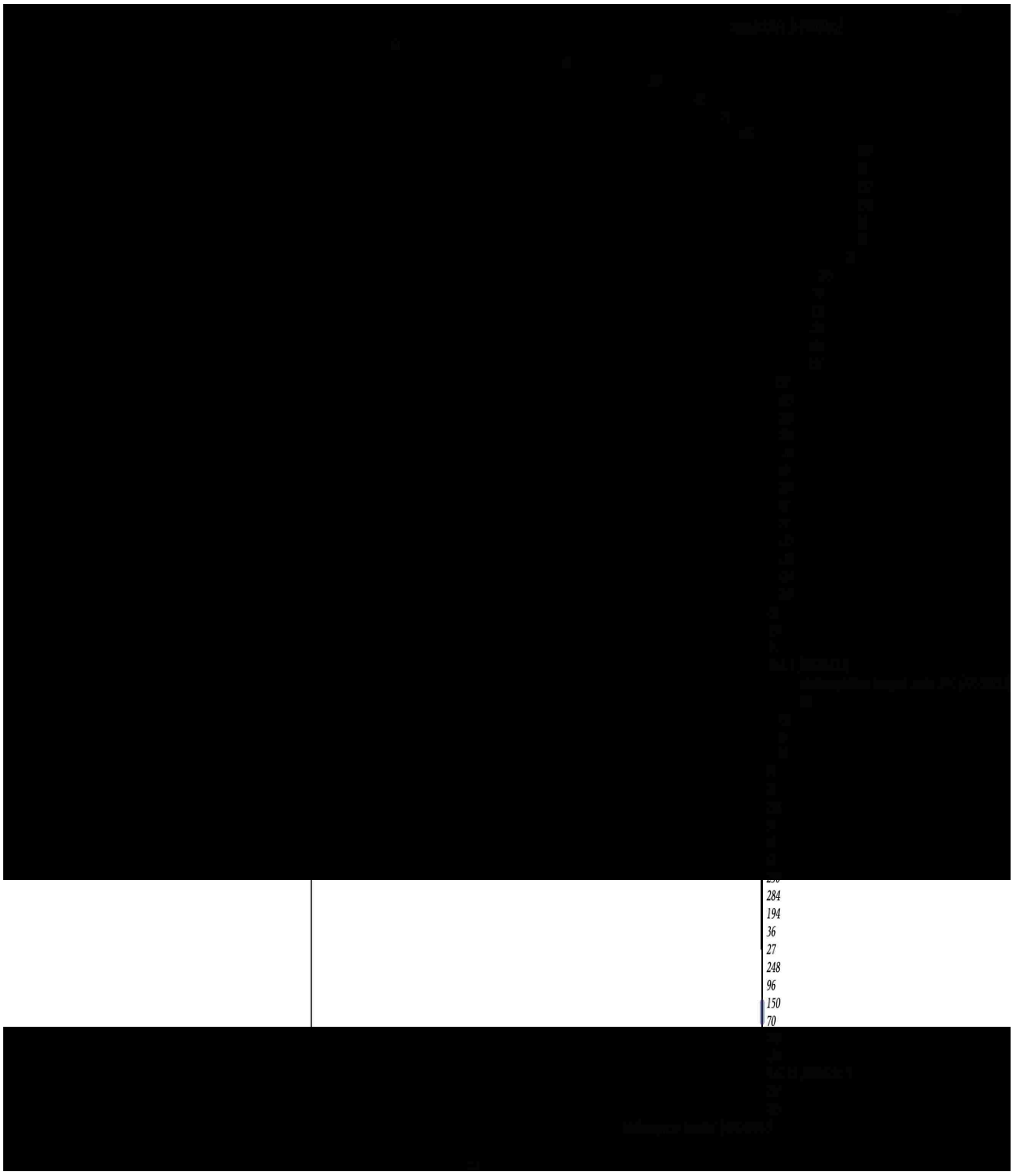
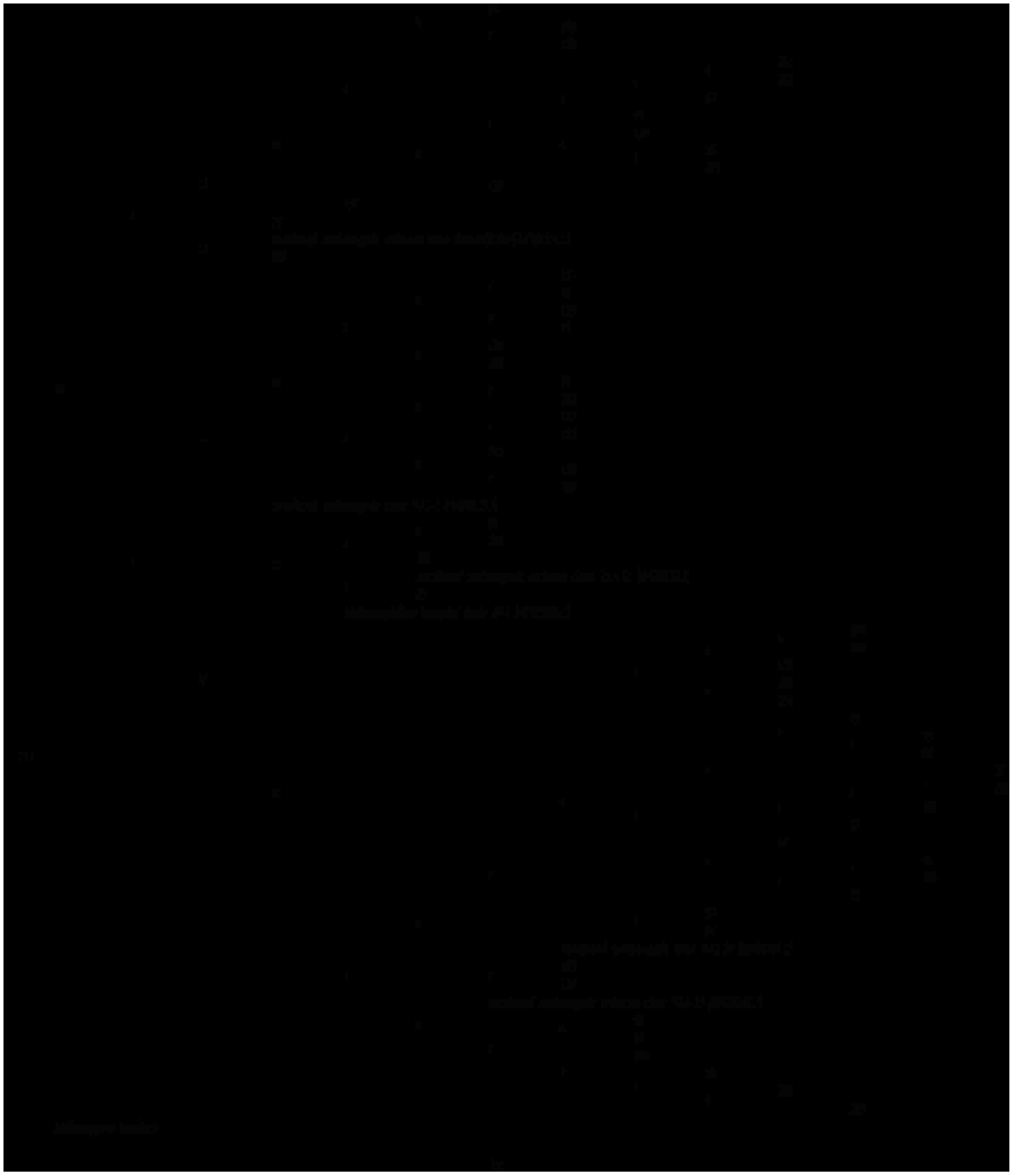


Figure 3D.

Figure 3E.



Appendix IV.

Industrial Digester Metadata

This appendix contains information obtained from digester operators regarding the physical and chemical characteristics of the industrial digesters sampled, except MMSS and CF for which metadata was not provided. The following is a summation of results provided by the operators in response to a questionnaire.

Digester CB

Wastewater Characteristics:

- **COD or TS of the waste:** avg. 6000 mg/L (1500-10000)
- **TSS/VSS of the incoming waste:** TSS avg 650 mg/L (100-2200)
- **BOD or VS of the waste** Not given (NG)

Digester Characteristics:

- **Type:** UASB
- **Operating Temperature:** 80-85 °F (26.7-29.4°C)
- **SRT:** NG
- **HRT:** 4.4 hrs
- **Biogas production:** 5.62 cubic feet/ pound COD removed
- **Methane/H_2S concentration in the biogas:** H_2S =2,000-10,000 ppm; CH_4= 60-65%
- **Organic Loading Rate:** NG
- **VSS/VS content of the digester:** 6%: 30000-50000 2.0-10.0
- **How long has the digester been running:** since 1980
- **Reseed? If so, how often and from what source do you reseed?** no

Biomass characteristics:

- **pH:** 6.5-8.0
- **ammonia-N and TKN:**0.1-2.0 and 5.6%
- **Sulfide:** dissolved 3.0-9.0 ppm
- **Metals:** Ni-5.9 ppm; Fe-1926 ppm; Mo-1.817 ppm
- **Addition of nutrients:** Ferric chloride 5-10 gal/week;Urea 50-150 pounds /week;15-20 mEq/L alkalinity

Note: Operator reports failure of the system due to overload of ethanol (17%) at the beginning of September. System flatlined for 4 days with no gas and only 6-40% COD removal. However, by the time we arrived (10/10/10) the digester was functioning normally and fully recovered in COD removal and gas production.

Digester JBS

Wastewater Characteristics:

- **COD or TS of the waste:** 4,614-6,000 mgO_2/L
- **TSS/VSS of the incoming waste:** 2,000-3,000 mg/L
- **BOD or VS of the waste:** BOD= 1/3 of the COD

Digester Characteristics:

- **Type:** Contact process (however, stirring is reported as weak)
- **Operating Temperature:** 36.1°C
- **SRT:** 10 days (design)
- **HRT:** 3-4 days
- **Biogas production:** avg. 3.2 CFM (high 5.2) weekly
- **Methane concentration in the biogas:** methane 70%
- **Organic Loading Rate:** 1.6 kg COD/m^3 - day
- **TS/VS content of the digester:** 80% (4600 mg TSS/L)
- **How long has the digester been running:**The digester is 27 years old. However, it failed last year and was cleaned out and reseeded with potato plant sludge.

Biomass characteristics:

- **pH and alkalinity?** 7.0 (VFAs <200) / add MgOH when needed
- **ammonia-N and TKN?** Effluent TKN = 260 mg/L sludge TKN=55543mg /kg ammonia nitrogen of sludge=6467 mg/kg
- **Sulfide?** ~ 5 ppm
- **Metals (mg/kg):**
    - Cd <1.4
    - Cl 2135
    - Cu 65
    - Pb 9.8
    - Ni 13
    - Nitrite/nitrate <13
    - Organic nitrogen 49076
    - Total phosphorus 15978
    - K 2195
    - Zn 413

- **Additional nutrients:** ferric chloride as coagulant

Digester KI

Wastewater Characteristics:

- **COD or TS of the waste:** 10,000-30,000 mg/l (high of 60000)
- **TSS/VSS of the incoming waste:** TSS 3,000-6,000 mg/l
- **VS of the waste:** 171 mg/L

Digester Characteristics:

- **Type:** CSTR
- **Operating Temperature:** 96-98 °F (35.6-36.7°C)
- **SRT:** 3000-6000 mg/l @ 25000- 35000 gpd
- **HRT:**25000-35000 gpd capacity 250000 gal (7-10 days)
- **Biogas production:** 33100 scfm/day
- **Methane/H_2S concentration in the biogas:** NG
- **Organic Loading Rate:** NG
- **VSS/VS content of the digester:** NG
- **How long has the digester been running:** 1991
- **Reseed:** once from a pig farm

Biomass characteristics:

- **pH and alkalinity:** 7.0 (equalization tank)
- **ammonia-N and TKN:** ammonia-N 1.375 mg/l (avg.)
- **Sulfide:** NG
- **Metals (Ni, Co, Fe, Mo, ect):** NG
- **Additional nutrients** 36,000 lbs per year

Digester MMBR

Wastewater Characteristics:

- **TS of the waste:** 169-364 mg/L
- **TSS/VSS of the incoming waste:** 145-228 mg/L VSS
- **BOD of the waste:** 123-212 mg/L

Digester Characteristics:

- **Type:** CSTR
- **Operating Temperature:** NG
- **SRT:** NG
- **HRT:** NG
- **Gas production:** NG
- **Methane/H_2S concentration in the biogas:** CH_4=62.5%
- **Organic Loading Rate:** NG
- **VSS/VS content of the digester:** 68 mg/L
- **How long has the digester been running:** NG
- **Reseed:** no

Biomass characteristics:

- **pH:** 7.29
- **ammonia-N and TKN:** ammonia-N=2.73 %; TKN=6.55%
- **Sulfide:** NG
    - **Metals (mg/kg):** s 7.82Cd 2.29
    - Cu 1,164
    - Pb 31.6
    - Hg 1.53
    - Mo 11.8
    - Ni <29.6
    - Se 5.97
    - Zn 985
- **Additional nutrients:** none