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IMPROVED ANAEROBIC DIGESTER STABILITY TO ORGANIC LOADING RATE SHOCKS WITH THE USE OF AN ENVIRONMENTALLY DERIVED INOCULUM

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ABSTRACT

Anaerobic digestion broadly describes technology that utilizes microorganisms to break down organic matter under anaerobic conditions through the coordinated efforts of several trophic groups of microorganisms. The last step is catalyzed by methanogens which produce primarily methane, carbon dioxide, and water as products of metabolism. Anaerobic digestion occurs naturally in a variety of water-saturated sediments, but is also used to treat waste in constructed reactors. There are a number of advantages to treating waste with anaerobic digestion, but perhaps the greatest is that waste treatment can be coupled to energy generation by the production of a methane-rich biogas. Despite the advantages, anaerobic digestion is severely under-utilized in waste treatment mainly due to the belief that anaerobic digesters are less stable than aerobic treatment processes. Anaerobic digesters are typically operated under warm temperatures and circumneutral pH, with operation outside of these conditions leading to instability and potential reactor failure. Reactor failure is expensive and time-consuming to recover, and has mainly been attributed to the sensitivity of the methanogens in the microbial consortium. The most often-cited reason for digester failure is acidic pH which may result from overfeeding of the reactor leading to the buildup of fermentation by-products. In contrast to constructed reactors, methanogenesis in acidic wetlands proceeds at pH values less than 4. Based on previous findings, there were two hypotheses driving this study. The first was that the methanogens present in an acidic wetland would be distinctly different than methanogens found in a constructed anaerobic reactor. The second hypothesis was that a reactor inoculated from an acidic peatland would be more tolerant of sudden increases in feeding rate, and the resultant drop in pH, than a reactor inoculated from a traditional anaerobic digester.

To investigate the first hypothesis, the methanogens in a local oligotrophic, acidic fen known as Bear Meadows Bog and a mesophilic anaerobic digester treating municipal wastewater sludge at the Pennsylvania State University Wastewater Treatment Plant were analyzed with clone libraries. Two clone libraries were created for each environment, one for the 16S rDNA and a second for the methyl coenzyme M reductase alpha subunit gene (*mcrA*). The Mcr is unique to methanogens and shows similar phylogeny as the 16S rDNA so it may be used independently of, or in conjunction with, the 16S rDNA to determine methanogen taxonomy. A quantitative framework was developed to assess the differences between these two communities by calculating the average sequence similarity for 16S rDNA and *mcrA* within a genus and family using sequences of isolated and characterized methanogens and an established methanogen taxonomy. The average sequence similarities for 16S rDNA within a genus and family were 96.0 and 93.5%, respectively, and the average sequence similarities for *mcrA* within a genus and family were 88.9 and 79%, respectively.

The clone libraries of the bog and digester environments showed no overlap at the species and genus levels, and almost no overlap at the family level. Both libraries were dominated by clones related to uncultured methanogen groups within the *Methanomicrobiales,* specifically the Fen Cluster in the bog and MCR-7 in the digester. Despite differences in the sequences detected, diversity was similar in both environments.

Based on the results of the clone libraries, real-time quantitative PCR (qPCR) methods were developed to distinguish and quantify members of different methanogen clades. Biases inherent in PCR prevent inference of an organism's environmental abundance based on end-point PCR product abundance, but qPCR overcomes some of these biases. A SYBR-Green I qPCR assay was developed to quantify total numbers of *mcrA* genes, and TaqMan probes were also designed to target several different phylogenetic groups. Groups which were targeted by TaqMan probes included *Methanosaetaceae*, *Methanosarcina*, *Methanocorpusculaceae*, *Methanospirillaceae*, *Methanobacteriaceae,* and the environmentally-defined clades Fen Cluster, MCR-7, and MCR-2 subgroups a and b. Many of these groups represent largely uncultured clades whose taxonomic standing has yet to be determined. Both SYBR-Green I and TaqMan qPCR assays showed good reproducibility and specificity for their methanogen targets. These methods were further tested by determining total *mcrA* and *mcrA* of different methanogen groups from six samples: four samples from anaerobic digesters treating either primarily cow or pig manure, and two aliquots from an acidic peat sample stored at 4°C or 20°C. The three samples obtained from cow manure digesters were dominated by members of the genus *Methanosarcina*, whereas the sample from the pig manure digester contained detectable levels of only members of the *Methanobacteriaceae*. The acidic peat samples were dominated by both *Methanosarcina* and members of the Fen cluster. In two of the manure digester samples only one methanogen group was detected, but in both of the acidic peat samples and two of the manure digester samples, multiple methanogen groups were detected.

To investigate the second hypothesis of this study, lab-scale reactors were inoculated with either bog sediments (Bog reactors,) wasted sludge from the anaerobic digester (Digester reactors), or with a mixture of these two inocula (Hybrid reactors). Reactors were maintained at 30°C and operated in semi-batch mode with feeding and wasting every four days, and gentle mixing once daily. Digester performance was monitored chemically and microbiologically using the previously designed qPCR assays. After the establishment of active methanogenic cultures in all reactors, a set of test reactors were inoculated from the control reactors and used to examine the effects of periodic doses of glucose to the consortia derived from different inocula.

A total of three sets of organic shocks were delivered. Glucose pulses were equal to 1-10 times the normal amount of volatile carbon in a feeding, and these resulted in the production of large amounts of volatile fatty acids which reduced reactor pH to less than 5. The first shock was 10 g of glucose, and this caused the Digester- and Hybrid-inoculated test reactors to fail, but the Bog-inoculated reactor recovered. After re-inoculating the Digester- and Hybrid-test reactors, three successive glucose pulses of 1, 5, and 10 g of glucose were delivered. The Hybrid-test reactor failed after receiving the 5 g glucose pulse, but the Bog- and Digester-test reactors only failed after receiving the 10 g glucose pulse. All reactors were recovered by the addition of NaOH to raise reactor pH to neutral levels. A second set of test reactors was established from the first set, and a third glucose pulse of 10 g was delivered. One set of reactors received mineral nutrients with the glucose pulse, but the second set did not. All reactors failed, but the Bog- and Digester-inoculated reactors recovered after pH was raised to only 4.8-5. The Hybrid-inoculated reactor did not show signs of recovery until the end of the testing period. Reactors with the same inoculum acted similarly, suggesting mineral nutrients did not prevent reactor failure.

Originally the Bog-inoculated test reactor was dominated by members of the Fen Cluster, but this clade only persisted through the second set of glucose shocks. The Fen Cluster was detected in the Hybrid-test reactor after glucose pulses, but was never detected in the control reactor. *Methanosarcina* and *Methanobacteriaceae* came to quickly dominate all control and test reactors, and the presence of *Methanosarcina* correlated with reactor recovery after glucose pulses. The control reactors showed less diversity than test reactors, and all control reactors were dominated by *Methanosarcina* with fewer numbers of *Methanobacteriaceae* and MCR-7.

Although distinct differences existed in the methanogen communities from the bog and digesters, these differences could not be maintained in constructed reactors. The methanogen communities in control reactors were less diverse than those in test reactors, and the acidophilic Fen Cluster was detected when reactor pH was acidic. The test reactors showed greater resilience to low pH conditions than has been previously reported in the literature, and reactors were able to recover activity after a glucose pulse from a pH as low as 4.8-5. Further research is needed to understand the role that different methanogen groups, specifically the Fen Cluster and *Methanosarcina*, play during periods of acidic operation, and how their presence may be maintained and their activity enhanced in order to provide greater stability to anaerobic digesters.

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Chapter 1

INTRODUCTION

Anaerobic digestion (AD) broadly describes technology which utilizes microbes to mineralize organic matter under anaerobic conditions. AD is capable of coupling waste treatment with energy production as organic carbon is degraded to carbon dioxide and methane. Very few wastes are resistant to anaerobic degradation, and AD technology has been successfully used to treat agricultural, municipal, and industrial waste streams including toxics such as methanol, phenol, toluene, formaldehyde, and chlorinated solvents (1, 43). Most energy worldwide derives from nonrenewable resources, and many wastes that could be treated anaerobically with co-generation of energy are instead treated aerobically incurring a net cost for waste treatment. In 2007, biomass-derived energy was 3.5% of total energy consumption in the U. S., of which only 12% came from waste materials (U.S. Department of Energy, Energy Information Administration www.eia.doe.gov).

The biggest advantage to aerobic versus anaerobic waste treatment is that microbial kinetics are faster, thus allowing faster waste treatment and smaller reactor size (220). The disadvantages to aerobic treatment include greater solids production and increased cost with aeration itself usually the most expensive part of waste treatment. Aerobic microorganisms have higher growth yields than anaerobic microbes, and the solids produced during treatment are largely composed of aerobic bacteria which must be further treated and disposed of (220). AD requires less energy to operate, reduces pathogens, and produces fewer solids as organic carbon is mineralized to a methane-rich biogas which can be used to recover some or all of the cost of treatment (1). Although anaerobic growth is generally slower than aerobic growth, the design of newer high-rate anaerobic reactor systems allows the retention of slow-growing anaerobes while still achieving rapid waste treatment and thus reducing the size of the reactor needed for treatment (1, 278).

The limited use of AD for waste treatment is largely due to the reputation of this technology for being more unstable than comparable aerobic processes, resulting in reactor failure (1). Process failure is seen as a cessation of methane production, an accumulation of fermentation products, and an eventual end to all microbial activity. When this occurs, the reactor is referred to as “stuck” or “sour”, and activity is difficult, costly, and time-consuming to recover. Anaerobic reactors may fail for many reasons, including a sudden change in the operating pH or temperature, a sudden influx of toxic or inhibitory substances, or a rapid change in the quantity or type of waste being treated (43, 137, 173). Even when anaerobic reactors are

producing methane, the biogas may be methane-poor which limits energy recapture and reduces the economic advantages of using AD (59). Other factors that have led to a general reluctance to accept AD technology include poor reactor planning and design, lack of monitoring and maintenance, and lack of technology transfer and technical support for AD operators (59, 132, 238). This last reason is especially important for small, decentralized reactors in agricultural settings where the farmer must also function as the AD operator.

The most commonly cited reason for anaerobic reactor failure is overfeeding, which results in the accumulation of fermentation products such as volatile fatty acids (VFAs), and causes the reactor to become acidic (43). The optimal pH for AD has been reported as ranging from approximately 6.3 to 7.8 with operation outside of this range resulting in reactor instability and potential failure (1, 74). AD requires several different trophic groups of microorganisms to completely mineralize organic matter, and maintenance of neutral reactor pH depends on the coordination of these groups (79). The first step of AD is the hydrolysis of particulate matter to soluble molecules which are then fermented to VFAs, alcohols, carbon dioxide and hydrogen by primary fermenters. Secondary fermenters further metabolize VFAs and alcohols to acetate, hydrogen, carbon dioxide, and formate, and methanogens then convert these products to methane and carbon dioxide (79). Primary fermenters can grow and metabolize faster than secondary fermenters and methanogens, but typically hydrolysis, which is catalyzed by extracellular enzymes, is the rate-limiting step so that methanogenesis consumes fermentation products as quickly as they are produced (55, 144). If there is a sudden influx of non-particulate, easily fermented substrate into the reactor, fermentation can exceed methanogenesis thus causing the accumulation of VFAs and a drop in pH. Methanogens are considered the most sensitive members of the AD consortium to a sudden drop in pH as VFAs continue to accumulate even as pH drops and methanogenesis ceases (13, 18, 137).

Although the optimal pH for anaerobic reactors is considered to be circumneutral, methanogenic communities are found in a wide range of acidic environments, including temperate peatlands, acidic lakes, and acidic tundra and permafrost soils (30, 40, 62, 114). Few researchers have attempted to obtain an acidotolerant methanogenic consortium for AD, but there would be several potential advantages to such a community. Wastes with high carbon-to-nitrogen ratios have little buffering capacity from the release of excess nitrogen as ammonium, and anaerobic treatment tends to result in a rapid acidification of the reactor (13, 102). Crop residues, food wastes, the organic fraction of municipal solid waste, and wastes from pulp and paper industries all fall into this category. An acidotolerant consortium would also be valuable for direct treatment of municipal or industrial wastewater in which organic concentrations and

compositions may change rapidly (155). In addition, a reactor that is more resistant to changes in substrate composition or pH would reduce the time needed for monitoring and operation, an aspect important for farm-based systems (59, 132, 238).

The inoculum for most anaerobic reactors derives from existing reactors treating similar waste, or started with a methanogen-rich substrate such as manure or municipal wastewater (3, 37, 179). It has been taken for granted that the same organisms exist everywhere, and that appropriate operation of a reactor will allow the desired microbes to be retained (1, 240). Methanogen communities in animal digestive tracts live under circumneutral pH (89, 247) and it is thus reasonable that anaerobic digesters utilizing these methanogenic consortia would also have to be operated at neutral pH. To date, no study has explored the use of an environmentally-derived inoculum from an acidic environment for an anaerobic digester to improve stability under low pH or fluctuating pH conditions.

Methanogen communities may be studied with culture-based techniques, but there are several advantages to the use of molecular tools to examine microbial communities. Most microorganisms are difficult or impossible to culture with current techniques, and molecular monitoring can capture a more complete picture of the methanogen community. In addition, molecular monitoring is much faster than culturing, allowing more sampling to take place in a given amount of time (267). Real-time quantitative PCR (qPCR) is capable of determining the number and type of gene sequences present in the DNA extracted from a sample (125). Currently most qPCR primers and probes for methanogens target the 16S rRNA gene (215, 256, 293), but the gene for the alpha subunit of the methyl-coenzyme M reductase (*mcrA*) has also been used to quantify methanogens (100, 195). This gene is exclusive to methanogens and shows similar phylogeny to the 16S rDNA allowing its use as a molecular marker alone, or in conjunction with, the 16S rDNA (15, 168, 257). The greatest advantage to targeting the *mcrA* is that there is no interference from non-methanogen DNA. Currently the only probes targeting the *mcrA* that allow distinction between different methanogen groups have only been designed for the Anaerobic Methane-Oxidizing *Archaea* (195).

The research presented here is intended to bridge the gap between microbial ecologists studying environmental methanogens and environmental engineers studying improvements in waste treatment coupled with energy production. There are two hypotheses driving this work:

- the methanogens present in an acidic peatland are phylogenetically different than the methanogens in a traditional anaerobic digester.

- a lab-scale reactor inoculated from an acidic peatland would be more stable under periodic substrate overloadings than a reactor inoculated from a municipal sludge digester.

To test the first hypothesis, samples were taken from a central-Pennsylvanian transitional fen named Bear Meadows Bog that has a sediment pH of approximately 4.5, and the anaerobic digester of the Pennsylvania State University municipal wastewater treatment plant digesting primary settled materials and waste-activated sludge. Phylogenetic diversity was assessed with clone libraries for the 16S rDNA and *mcrA* genes. These results are presented in chapter 3 which is adapted from Steinberg and Regan (2008). Analysis of the current methanogen taxonomy and gene sequences for the 16S rDNA and *mcrA* of cultured methanogens was used to determine the degree of phylogenetic distinction between each methanogen community. Based on the results of the clone libraries, probes specific to different methanogen groups were developed for use in qPCR TaqMan assays and were tested with six different methanogenic samples. Results of this study are described in chapter 4, which is adapted from Steinberg and Regan (2009). Samples were taken from Bear Meadows Bog stored at two different temperatures for approximately two years, as well as from four animal manure digesters. One sample was taken from a full-scale digester treating swine manure, and the three other manure samples were taken from full-scale digesters treating dairy manure co-digested with other wastes. The swine manure sample and details of the digester operation and maintenance were obtained with the assistance of Robb Meinen, a senior extension associate in the Department of Dairy and Animal Sciences, Penn State University. The three cattle manure samples were obtained with assistance of Pat and Deb Topper of the Biogas and Anaerobic Extension Program in the Department of Biological and Agricultural Engineering, Penn State University (www.biogas.psu.edu).

The final part of the research project tested the second hypothesis through the operation of lab-scale reactors for approximately 1.5 years. Lab-scale reactors were inoculated from the acidic peatland, the Pennsylvania State University municipal digester, or from both sources. Reactor stability was tested with periodic increases in fermentable substrate delivered as glucose pulses which caused acidic, high-VFA conditions. Reactor response was monitored chemically and microbiologically as changes in methanogen community were followed by qPCR utilizing the previously designed methods. The results of this study are presented in chapter 5 which is in preparation for submission to Water Research. Final conclusions are described in chapter 6, and suggestions for future research based on the observations of this study are outlined in chapter 7.

Chapter 2

LITERATURE REVIEW

I. Introduction to methanogenesis

The Italian physicist Alessandro Volta is credited with discovering biologically-produced methane, which he termed "combustible air" (79). The "Volta experiment", which illustrated the production of methane in saturated sediments, was first demonstrated at Lake Como in northern Italy. Volta gathered methane in a partially submerged inverted funnel from the underlying sediments which were disturbed with a pole, and then set the collected gas on fire. Two hundred years later, the microbiologist Carl Woese identified methanogens as the microorganisms responsible for methane formation. Methanogens not only became the founding members of the newly described *Archaea*, but were the basis of Woese's theory on the three domains of life (79).

Methanogenesis takes place in a variety of diverse oxygen-free habitats throughout the planet, including freshwater lakes, ponds, and rivers, sewage digesters, rice paddies, landfills, estuaries, coastal and deep marine sediments, hydrothermal vents, the intestinal tracts of animals, and as symbionts of protozoa (285). Methanogenesis is the final step in the breakdown of complex organic material to methane and carbon dioxide, a vital link in global carbon cycling (79). Complete mineralization of organic matter requires the coordinated efforts of different trophic groups of microorganisms (Figure 2-1). The first step is the hydrolysis of particulate matter into smaller soluble molecules which can pass microbial cell membranes (149). Hydrolysis converts polymers such as polysaccharides, proteins, nucleic acids, and lipids to sugars, amino acids, purines, pyrimidines, fatty acids and glycerol by the action of extracellular enzymes produced primarily by *Bacteria* (233). Acidogenesis is the fermentation of hydrolysis products to VFAs, alcohols, carbon dioxide, and hydrogen. Primary fermenters catalyze hydrolysis and acidogenesis, and may be facultative or strictly anaerobic in nature (149). Some products of acidogenesis, such as acetate, formate, hydrogen, and carbon dioxide, may be used directly by methanogens to produce methane and carbon dioxide (233). Secondary fermentation, also referred to as acetogenesis, results in the further breakdown of fatty acids, alcohols, and aromatic compounds to acetate, carbon dioxide, hydrogen, and formate (149, 233). Acetate may also be produced from hydrogen and carbon dioxide through homoacetogenesis (149). These secondary fermentation processes are also catalyzed by members of the domain *Bacteria*, many of which are obligate syntrophic partners of methanogens (149, 233). Methanogenesis is

catalyzed exclusively by methanogens from a limited number of substrates including acetate, formate, carbon dioxide and hydrogen, some alcohols, and some methylated compounds (149).

II. Methanogens

Methanogens are members of the domain *Archaea* and may be further classified as belonging to the subdomain *Euryarchaeota*. Methanogens differ from other members of the *Archaea* and *Bacteria* not only in phylogeny, but also structurally and biochemically. Methanogens may be motile or non-motile, and cells exhibit a wide variety of morphologies including long and short rods occurring singly or in chains, regular and irregular cocci, spirilla, and even flattened plate-like structures (89). Cell wall structure is different from other prokaryotes, and many methanogens contain an external protein layer to the cell wall called an S-layer (285). The cell walls of methanogens do not contain murein, which is typical of the domain *Bacteria* (89). Instead, methanogen cell walls contain pseudomurein, proteins, or glycoproteins, thus rendering methanogens insensitive to the antibiotics that inhibit cell-wall formation in *Bacteria* (89). The protein structure of this S-layer varies among methanogen families, and some methanogens possess cell walls composed completely of proteins (285). The lipid structure of methanogen cell membranes also differs from that of the *Bacteria* in that lipids are connected to a glycerol backbone in either diether or tetraether conformations (89). Although methanogens themselves are a very diverse group, they are united in that they exclusively produce methane as the major product of energy metabolism and are strict anaerobes (285).

A. Phylogeny

Classically, methanogens are divided into five orders, the *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanopyrales*. More recently, it has been shown that methanogens are not monophyletic, but instead can be broadly divided into two classes (15). Class I methanogens include members of the orders *Methanococcales*, *Methanobacteriales*, and *Methanopyrales*, whereas Class II methanogens include members of the orders *Methanosarcinales* and *Methanomicrobiales*. Classification was determined by examining phylogenetic relationships for 53 ribosomal proteins and 20 proteins of the methanogenesis pathway. Although the monophyly of all methanogens was rejected, Class I and Class II methanogens were both found to be monophyletic (15).

i. Order *Methanobacteriales*

The *Methanobacteriales* is composed of two families, *Methanobacteriaceae* and *Methanothermaceae*. Members of the *Methanobacteriales* are nonmotile and typically rod-shaped, and quite widespread in the environment (89).

a. Family *Methanobacteriaceae*

The family *Methanobacteriaceae* contains the genera *Methanobacterium*, *Methanothermobacter*, *Methanobrevibacter*, and *Methanosphaera*. Many of the cultured members of this family are enteric organisms isolated from the digestive tracts of animals and sewage sludge (79). Nearly all members of this family produce energy from the reduction of carbon dioxide to methane, usually with electrons generated from the oxidation of hydrogen (79). This type of metabolism is classified as hydrogenotrophic methanogenesis. The exception is the *Methanosphaera* whose members reduce the methyl group of methanol with hydrogen (79). Many species of the *Methanobacteriaceae* can also produce energy and methane from formate, and some species are able to use electrons from secondary alcohols such as 2-propanol or 2-butanol as the reductant for carbon dioxide (79, 285). With the exception of the *Methanosphaera*, all species are autotrophic, but addition of complex amendments such as acetate, yeast extract, and rumen fluid are often necessary (285). Nearly all species are able to use ammonium, sulfide, and elemental sulfur for nitrogen and sulfur growth needs, and some are also able to use glutamine, urea, cysteine, methionine, sulfite, thiosulfite, or even fix dinitrogen (79, 285).

Members of the genera *Methanobacterium* and *Methanothermobacter* have been isolated from anaerobic digesters, sewage sludge, manure, groundwater, and oil-bearing shale (79, 285). These two genera are closely related and members were originally all classified as *Methanobacterium*, but eventually the thermophilic species were placed together in the genus *Methanothermobacter*. Species of *Methanobacterium* are primarily neutrophilic and mesophilic, with optimal salinity for growth below 0.2 M NaCl (285). Exceptions include one acidophilic species and another species which is both alkalophilic and halotolerant (79). *Methanosphaera* are spherical in shape and were isolated from the digestive tracts of mammals. Members of this genus have not been identified outside of the mammalian colon, and are resistant to bile salts (285). The *Methanobrevibacter* are composed primarily of neutrophilic short rods with one exception, *Methanobrevibacter acididurans*, with a growth range of pH 5-7.5 (230). Most species of *Methanobrevibacter* have been isolated from the intestinal tracts of mammals, birds, and insects and it is common for a species or strain to be associated with only one host species (89, 223, 273, 285).

b. Family *Methanothermaceae*

Family *Methanothermaceae* contains only two species and both belong to the genus *Methanothermus*. *Methanothermus* are rod shaped, motile, neutrophilic, and autotrophic with energy production exclusively through hydrogenotrophic methanogenesis (79). The two species in this family are extreme thermophiles isolated from volcanic springs with growth temperature optima of greater than 80°C (89). *M. sociabilis* forms pili-like appendages which leads to growth in aggregates up to 3 mm in diameter whereas *M. fervidus* grows as dispersed cells and never forms filaments (285).

ii. Order *Methanococcales*

The order *Methanococcales* contains the families *Methanococcaceae* and *Methanocaldococcaceae*. All members of this order are hydrogenotrophic methanogens isolated from marine and coastal environments (89). Members are irregular, motile cocci which can use both hydrogen and sometimes formate as electron donors during methanogenesis (89, 285). Nearly all members are autotrophic, and most use ammonium for growth (285). Sulfur sources for growth are typically dihydrogen sulfide, sulfide, and elemental sulfur (79, 285).

a. Family *Methanococcaceae*

This family contains the genera *Methanococcus* and *Methanothermococcus*. All species of this family were isolated from marine or estuarine environments, yet most members are only halotolerant, preferring to grow at lower salinities than those found in marine environments (79). Members of this family may be mesophilic or thermophilic, but most species fall into the latter category (79, 285). *Methanococcus maripaludis* and *Methanococcus thermolithotrophicus* are capable of fixing dinitrogen, and *Methanothermococcus thermolithotrophicus* is unique among methanogens in that it is able to grow on almost any sulfur or nitrogen source including sulfide, elemental sulfur, thiosulfate, sulfite, sulfate, ammonium, nitrate, and dinitrogen (79, 285).

b. Family *Methanocaldococcaceae*

Family *Methanocaldococcaceae* contains the genera *Methanocaldococcus* and *Methanoignis*. All members of this family were isolated from deep-sea hydrothermal habitats, and all are thermophiles or extreme thermophiles (89). Unlike the *Methanococcaceae*, members of *Methanocaldococcaceae* prefer seawater salinities for growth (79). This family also contains the fastest growing methanogen to date, *Methanocaldococcus jannaschii*, with a doubling time of

30 minutes (89). Genus *Methanotorris* only contains two species, and is differentiated from the *Methanocaldococcus* by the absence of motility and greater halotolerance (79).

iii. Order *Methanopyrales*

The Order *Methanopyrales* contains one family, *Methanopyraceae* with one genus, *Methanopyrales* containing the sole species *Methanopyrus kandleri. M. kandleri* is a hydrogenotrophic, hyperthermophilic methanogen with an optimal growth temperature of 98°C isolated from marine hydrothermal vent sediments (89). Originally placed in a deep branch of the *Euryarchaeota* based on its 16S rDNA sequence, *M. kandleri* is the most distant to other methanogens phylogenetically. The complete genome of *M. kandleri* has since been sequenced, and analysis of transcription and translation machinery resulted in this order being grouped with the Class I methanogens *Methanococcales* and *Methanobacteriales* (79).

iv. Order *Methanomicrobiales*

The Order *Methanomicrobiales* is quite diverse containing three families, the *Methanomicrobiaceae*, *Methanocorpusculaceae*, and *Methanospirillaceae*, and nine genera (89). All members are hydrogenotrophic, and many species are also able to use formate or some secondary alcohols as electron donors. Members of the *Methanomicrobiales* are found in a wide variety of environments, and growth in culture typically requires the addition of organic carbon supplements such as trypticase and yeast extract (285).

a. Family *Methanomicrobiaceae*

The family *Methanomicrobiaceae* contains the genera *Methanomicrobium*, *Methanolacinia*, *Methanoculleus*, *Methanogenium*, *Methanoplanus*, *Methanofollis*, and *Methanocalculus*. This family contains species exhibiting a wide range of morphologies including irregular cocci, rods, and plate- or disc-shaped cells (285). Most species are mesophilic, but there are thermophilic and psychrophilic members as well. This family also contains two halophilic and three halotolerant species (79). Members of this family have been isolated from a diverse array of environments including bovine rumen fluid, marine sediments, oil wells, and a frozen lake in Antarctica (89).

The genera *Methanomicrobium* and *Methanolacinia* each contain only one species, isolated from bovine rumen fluid and marine sediments, respectively (89). The genus *Methanogenium* is relatively diverse, with two species capable of methanogenesis with secondary alcohols and carbon dioxide, one thermophilic species, *M. frittonii*, and one psychrophilic

species, *M. frigidum*. *M. frigidum* was isolated from Ace Lake sediments in Antarctica and has an optimum growth temperature of 15°C (79). The genus *Methanoculleus* is composed mainly of mesophilic, irregular, non-motile cocci, although one species is thermophilic (89). The genus *Methanoplanus* is unique in that cells are plate-shaped (89, 285). One species of *Methanoplanus* was isolated as an endosymbiont in marine ciliates where it is thought the methanogen serves as an electron sink for the host, using hydrogen from the host to reduce carbon dioxide to methane (285). *Methanocalculus* is a relatively new genus which contains one species of halotolerant irregular coccoid with a salinity growth range of 0 to 12% NaCl, the widest reported for any methanogen (89).

b. Family *Methanocorpusculaceae*

The family *Methanocorpusculaceae* contains one genus, *Methanocorpusculum*, composed of mesophilic, irregularly coccoid methanogens which are motile by flagella. Members of this family are methanogenic with hydrogen and carbon dioxide, formate, or with 2-propanol as an electron donor for carbon dioxide (89). Members of *Methanocorpusculum* have been isolated from a variety of digesters treating food waste and human sewage, with only one species isolated from California lake sediments (285). As such, all species of *Methanocorpusculum* require complex amendments to growth media (285).

c. Family *Methanospirillaceae*

The family *Methanospirillaceae* also contains only a single genus, *Methanospirillum*, with only one species, *Methanospirillum hungatei*, described (89). Cells are spiral-shaped and motile, and may form long chains or filaments encased in a continuous sheath (285). *M. spirillum* uses hydrogen and carbon dioxide or formate to produce methane, and some strains can also use 2-propanol and 2-butanol as electron donors (285). Acetate is required for growth, and some strains also require peptones and vitamins (285). *M. hungatei* has the highest affinity for hydrogen of all methanogens and is often utilized for isolation of syntrophic hydrogen-producing bacteria (89).

5. Order *Methanosarcinales*

The Order *Methanosarcinales* contains all the acetotrophic and/or methylotrophic methanogens. This order contains the families *Methanosarcinaceae* and *Methanosaetaceae*, and seven genera (89).

a. Family *Methanosarcinaceae*

The family *Methanosarcinaceae* contains the genera *Methanosarcina*, *Methanolobus*, *Methanococcoides*, *Methanohalophilus*, *Methanosalsus*, and *Methanohalobium*. All members of this family are able to produce methane from methylated compounds such as methanol, methyl amines, and methyl sulfides (79). *Methanosarcinaceae* have been isolated from anaerobic digesters, estuarine and marine sediments, and permanently cold sediments of Antarctic lakes (79). As many species have been isolated from saline environments, most species are halotolerant or halophilic and some members of *Methanohalobium* and *Methanohalophilus* are even classified as hyperhalophilic (79, 89).

The *Methanosarcina* are the most metabolically diverse methanogens, able to use hydrogen and carbon dioxide, acetate, and methyl compounds (89). Most species can grow without organic carbon amendments, although addition of these compounds may help stimulate growth (285). *M. barkeri* is especially diverse in that it may use methionine, cysteine, thiosulfate, elemental sulfur and dinitrogen as sulfur and nitrogen sources for growth (285). Most *Methanosarcina* are non-motile and mesophilic, but there is one thermophilic species, *Methanosarcina thermophila*, and one psychrophilic species, *Methanosarcina baltica* (79). *Methanosarcina* have been found in freshwater and marine sediments, animal-waste lagoons, the rumen of mammals, and anaerobic digesters (79, 89, 285). The remaining genera in the family *Methanosarcinaceae* are obligatory methylotrophic, growing on methanol, methylamines, and other C-1 compounds (285). Most species are neutralophilic but three species, isolated from marine environments, are alkaliphilic with pH optima for growth greater than 8 (79). Some species are even true hyperhalophiles, such as *Methanohalobium evestigatum* which grows only at salinities higher than 15.2% and has an optimum growth at a salinity of 25.1% (79). The genus *Methanococcoides* contains two species, of which one species, *Methanococcoides burtonii*, was isolated from Ace Lake in Antarctica and has an optimal growth temperature of 23°C (89). Recently, a psychrophilic member of the genus *Methanolobus* was isolated from the Zoige Wetland of the Tibetan plateau. *Candidatus* Methanolobus psychrophilus demonstrated methanogenesis from methanol, trimethylamine, and methyl sulfide at temperatures as low as 0°C and with optimal growth at 18°C (Zhang 2008).

b. Family *Methanosaetaceae*

Family *Methanosaetaceae* is composed of one genus, *Methanosaeta*, that contains two species of obligatory acetotrophic methanogens, *Methanosaeta concilli* and *Methanosaeta thermophila*. Cells are nonmotile, sheathed rods that grow in chains and may form filaments

which allow aggregation of cells (79, 89). Growth is typically slow with doubling times of 4-7 days at 37°C (79, 89). *M. concilli* was isolated from sewage sludge and *M. thermophila* was isolated from a thermophilic anaerobic digester (79). Despite the sources of these two species, *Methanosaeta* are quite widespread in the environment and have been found in a variety of habitats including lake sediments, acidic peatlands, and estuarine sediments (285). Their presence in granulated sludge and fixed-film anaerobic digesters is desirable because their filamentous nature helps to add structure to the reactor biomass (221, 243).

B. Biochemistry and metabolism

Methanogens are the only organisms that produce methane as the primary product of energy metabolism (285). Methane is produced from a relatively small number of substrates along three main metabolic pathways (Figure 2-2). The first pathway, hydrogenotrophic methanogenesis, reduces carbon dioxide to methane with hydrogen or formate as an electron donor. In the second pathway, aceticlastic methanogenesis disproportionates acetate to carbon dioxide and methane (89, 285). In the third pathway, methylotrophic methanogenesis disproportionates methylated compounds such that some molecules of substrate are oxidized to carbon dioxide to provide the electrons to reduce other molecules of substrate to methane. Methylated compounds used for methanogenesis include methanol, methylamines, and methylsulfides. Other pathways do not neatly fit into these three categories. Some methanogens can use alcohols or even carbon monoxide as an electron donor in the reduction of carbon dioxide to methane (285). Two species of methanogens, both in the genus *Methanosphaera*, reduce the methyl group of methanol with hydrogen as an electron donor (79, 89). The standard changes of free energy for methanogenesis are highest for the hydrogenotrophic pathway, followed by methylotrophic reactions, and least for aceticlastic methanogenesis (89, 285). These thermodynamic constraints have led to the predominance of hydrogenotrophic methanogen species. Not all methanogens can utilize all methanogenesis pathways, and the dominant pathway by which methanogenesis proceeds varies from environment to environment (285).

Methanogens possess a number of unique coenzymes including formate- and hydrogen-carriers, Coenzyme M-SH , and Coenzyme B-SH (89). A low-potential electron carrier, factor F_{420}, fluoresces when exposed to ultraviolet light of wavelength 420 nM. This unique feature of methanogens allows colonies or cells to be identified with UV fluorescence (89). Cytochromes b and c, catalases, benzoquinones, NADPH, NADH, iron-sulfur proteins, and ferredoxins have also been identified in some methanogens (89). No matter the substrate used for methanogenesis, all methanogenic pathways converge at the methylreductase system. The methylreductase system

involves two coenzymes each with a sulfhydryl active group, Coenzyme-M (CoM-HS) and Coenzyme-B (CoB-HS), as well as methyltransferases and electron shuttles (79). Liberation of methane begins with a methyl group being donated to CoM-SH to form CoM-S-CH_3. Using CoB-SH as an electron donor, the heterodisulfide bond CoM-S-SCoB is formed, releasing methane (79). The disulfide bond of CoM-S-S-CoB is then reduced by heterodisulfide reductase to yield the active sulfhydryl forms of the coenzymes (Co-M-SH and Co-M-SH) so that the cycle may begin again. Reduction of CoM-S-S-CoB is coupled to formation of a proton gradient which drives ATP synthesis (79).

As the general currency of the cell, the generation of one ATP molecule requires +32 kJ per mol under standard conditions, and +50 kJ per mol in an actively growing cell where some energy is used for growth and cell maintenance (233). An additional + 20 kJ per mol is lost as heat, although this value varies from organism to organism. Nonetheless, generally the energy requirement for ATP synthesis is at least + 60 kJ per mol (233). Studies of a number of bacterial and mitochondrial systems show that in general three protons cross the cell membrane per molecule of ATP hydrolyzed so that the smallest unit of metabolic energy can be considered one-third of an ATP unit. This requires a minimum of about -20 kJ per mol be released from any reaction for an organism to exploit the free energy change (233).

i. Description of methanogenesis pathways

Hydrogenotrophic methanogenesis involves seven steps with electrons derived from either hydrogen via hydrogenase, or formate via formate dehydrogenase (285). The first step is activation of carbon dioxide to formyl-methanofuran. The formyl group is next transferred to H_4MPT, where it is subsequently reduced to a methenyl, then methylene, and finally a methyl group before being transferred to CoM. The methyl group is then reduced to methane by the methylreductase system (285). Among hydrogenotrophic methanogens, those methanogens that possess cytochromes have a much higher growth yield than methanogens without cytochromes (272). All hydrogenotrophic methanogens that use formate as an electron donor lack cytochromes, restricting cytochromes to methanogens that primarily use hydrogen as an electron donor (272). Methanogens with cytochromes also have a hydrogen threshold concentration requirement for growth that is 2 to 3 orders of magnitude greater than hydrogenotrophic methanogens without cytochromes. Methanogens with cytochromes are all found within the order *Methanosarcinales* and are restricted to the genera *Methanosarcina*, *Methanosaeta*, and *Methanolobus*. Among these genera, only members of the *Methanosarcina* are able to grow on hydrogen and carbon dioxide (272).

During aceticlastic methanogenesis, the methyl and carboxyl groups of acetate are converted to methane and carbon dioxide, respectively (285). Acetate is first activated to acetyl-CoA, followed by cleavage of the carbon-carbon bond by carbon monoxide dehydrogenase which produces CoA-SH, enzyme-bound carbon monoxide, and a methyl group (285). The methyl group is transferred to CoM-SH via H_4MPT, and the enzyme-bound carbon monoxide is oxidized to hydrogen and carbon dioxide which provides the electrons for reduction of CoM-S-CH_3 to methane.

Methanogenesis from methylamines and methanol involves a disproportionation reaction where some molecules of substrate are oxidized and other molecules of substrate are reduced to produce methane (285). The methyl group from the substrate is first transferred to CoM-SH via methyltransferases to form CoM-S-CH_3. Electrons for reduction are obtained by oxidizing the methyl group of CoM-S-CH_3 to carbon dioxide by reversal of the carbon dioxide reduction pathway. Oxidation of one methyl group yields six electrons which is used to reduce three methyl groups to methane (79). This pathway is always used for conversion of methylamines, and is also the most common pathway for conversion of methanol. The one exception is members of the genus *Methanosphaera*, which reduce methanol with electrons from hydrogen. In this pathway, the methyl group of methanol is first transferred to CoM-SH to form CoM-S-CH_3 which is then reduced by hydrogen to produce methane (285).

There are a number of chemicals that are able to block some, or all, methanogenesis and have been used extensively in methanogen culture studies to deduce methanogenic metabolism. Chloroform and 2-bromoethanesulfonate (BES) inhibit all methanogenesis regardless of pathway (92, 182, 211, 236), and molybdate has been reported as a general methanogenesis inhibitor (211). Fluoroacetate and methyl-fluoride are used specifically to block aceticlastic methanogenesis (182, 208, 211, 236). The culture buffer may also affect the pathway for methanogenesis as Chin ***et al.*** (2003) found that phosphate buffer specifically inhibited aceticlastic methanogenesis, and the use of carbonate buffer stimulated hydrogenotrophic methanogenesis (45).

ii. Syntrophic associations of methanogens

Methanogens have limited substrates and so depend on fermentative microorganisms to convert complex organic material into acetate, formate, methylated compounds, hydrogen and carbon dioxide. Fermentative microorganisms associated with methanogens may be categorized as obligate or non-obligate syntrophic partners (285). Formation of acetate during fermentation requires NADH to be reoxidized. Many fermentative bacteria depend on NADH-linked

hydrogenases and formate dehydrogenases which require very low concentrations of hydrogen (10^{-5} kPa) or formate (<100 μM) for the reoxidation of NADH to be thermodynamically feasible (285). The presence of hydrogen- and formate-consuming methanogens helps to keep these concentrations low, and although many fermentative bacteria are able to grow without methanogens present (non-obligate syntrophs), more energy is generated during fermentation for these bacteria than would be without the presence of methanogens (285). Obligate syntrophs oxidize acetate, propionate, butyrate, and longer-chain volatile organic acids as well as aromatic compounds while in association with methanogens, and are unable to grow unless they are closely associated with hydrogen- or formate-consuming species (285).

Non-obligate interspecies electron transfer has been observed with members of *Clostridium*, *Cellulomonas*, *Desulfovibrio*, *Ruminococcus*, *Selenomonas*, *Sporomusa*, *Coprothermobacter*, and even anaerobic fungus (89). Syntrophic fermenters are often specific to one or a few substrates (89, 233). Species of *Thermoanaerobium* and *Pelobacter* are syntrophic ethanol-oxidizers, and in the absence of sulfate *Desulfovibrio vulgaris* acts as a syntrophic ethanol-oxidizer with methanogens. Syntrophic oxidation of butyrate and larger chain fatty acids is catalyzed by species of *Syntrophomonas*, syntrophic oxidation of propionate is carried out by members of the genus *Syntrophobacter*, and syntrophic acetate oxidation is performed by members of *Clostridium* and *Thermoacetogenium* (233). Other syntrophic fatty acid oxidizers include species of *Syntrophospora*, *Smithella*, *Thermosyntropha*, and *Eubacterium* (89). In general, degradation of fatty acids to acetate, hydrogen, and carbon dioxide is much more endergonic under standard conditions than is ethanol oxidation. This fact, coupled with the reality that fatty acids are often present in higher quantities than alcohols, makes syntrophic degradation of butyrate, propionate, and acetate the most important syntrophic associations in methanogenic environments (233).

The energy yield from syntrophic fatty-acid oxidation may involve more than two organisms, and the total energy yield must be divided among all participants (233, Table 2-1). Butyrate is oxidized to two moles of acetate, hydrogen and carbon dioxide which must be consumed by a methanogen partner to yield methane (233). The complete reaction is catalyzed by at least three bacteria; one to produce acetate and hydrogen from butyrate, one to consume acetate, and one to consume hydrogen. For the total reaction to yield about -20 kJ for each of the three organisms involved, hydrogen must be around 2 Pa and acetate around 50 μM. Syntrophic propionate oxidation is similar in that there must be a propionate metabolizer, an acetate consumer, and a hydrogen consumer present. The overall reaction yields approximately -22 to -23 kJ per mol for each of the three partners. While butyrate-oxidizers and propionate-oxidizers

are members of the domain *Bacteria*, acetate consumers may be either *Bacteria* or *Archaea* if they are aceticlastic methanogens (233). Syntrophic acetate oxidizers appear to be homoacetogens which may produce acetate from hydrogen and carbon dioxide, or alternatively ferment acetate to hydrogen and carbon dioxide in syntrophic association with a hydrogen consumer. Both metabolisms may be present in the same organism, and the metabolism is reversible and dependent upon acetate concentration and hydrogen partial pressure (1, 233). At high hydrogen concentrations, acetate is produced, and at low hydrogen concentrations (10-50 Pa), such as when in close proximity to a hydrogen-scavenging syntrophic partner, acetate is oxidized to produce hydrogen and carbon dioxide (54, 80, 153, 233). Although members of the *Methanosarcina* may produce methane from hydrogen and carbon dioxide, they do not appear to form syntrophic partnerships with VFA-degrading microorganisms (1), which may be due to their higher K_m for hydrogen which is beyond the lower threshold for syntrophic associations (271, 272). The acetate concentration also affects the pathway for conversion, with syntrophic acetate oxidation as the dominant pathway for at low acetate concentrations and methanogenesis as the dominant pathway at concentrations greater than the K_m for aceticlastic methanogens (1).

If methanogen activity is suppressed, or methanogens are absent, hydrogen-consuming reactions may be carried out by sulfur-, sulfate-, glycine-, and fumarate-reducing bacteria as well as homoacetogens (233). In anaerobic environments with no electron acceptors other than carbon dioxide, the only microorganisms able to consume hydrogen are the hydrogenotrophic methanogens and the homoacetogenic bacteria (54). In the absence or suppression of methanogenesis, homoacetogenesis becomes the sink for hydrogen (250). If examining the production of methane from glucose, at maximum two moles of acetate and four moles of hydrogen may be produced from one mole of glucose (54). Four moles of hydrogen are required to reduce one mole of carbon dioxide to methane, whereas one mole of acetate may produce one mole of methane. It then appears that approximately two-thirds of methane should be produced from acetate and only one-third from hydrogen, but this is not always the case in part because of the action of homoacetogens (54).

Although much research has been conducted on syntrophic associations between hydrogen- producing and consuming partners, most syntrophic fermenters require co-culture with a methanogen able to use both hydrogen and formate (233). This fact suggests that formate transfer is also important in interspecies electron transfer. The standard redox potential of the carbon dioxide/formate couple (-420 mV) is close to that of the H^+/H_2 couple (-414 mV) so both couples could be used interchangeably. Despite this fact, hydrogenase activities are typically much higher in syntrophic partners than are formate dehydrogenase activities suggesting

hydrogen-transfer is the dominant electron transfer mechanism in methanogenesis. It has been proposed that formate transfer may be the preferred mode of electron transfer for dispersed cells in an aqueous environment whereas electron transfer via hydrogen would be the preferred mode of electron transfer for densely packed cells such as those that occur in anaerobic digesters and sediments (233). Formate may also be syntrophically-oxidized to hydrogen and bicarbonate in the presence of a hydrogen-scavenging methanogen, or formate and hydrogen may be used to produce acetate (68). In addition, both of these metabolisms may reside in the same organisms, as a genomic analysis of species of *Moorella* have suggested (68).

C. Methanogenic habitats

Methanogenesis only proceeds under strict anaerobic conditions, and most commonly where electron acceptors other than carbon dioxide, such as nitrate, ferric, and sulfate, are limiting (285). For this reason, methanogens are widely found in water-saturated areas where the underlying soil and organic matter is at a negative redox potential. Common methanogen habitats include sediments of oceans, lakes, ponds, bogs, fens, marshes, swamps, estuaries, and rice paddies. Methanogens are also members of the internal flora of a wide variety of animals, and the activity of methanogens is also utilized in constructed reactors for digestion of organic waste (285). Approximately 30% of yearly methane emissions are from natural sources, primarily wetland soils (149). Of the 70% of methane emissions attributed to humans, agriculture, mainly domesticated ruminants and rice paddies, is the largest contributor (149).

i. Acidic peatlands and lake sediments

Acidic wetlands cover less than 3% of the terrestrial surface of the Earth but sequester approximately 30% of global carbon and nitrogen through the accumulation of layers of decaying organic matter referred to as peat (114, 149). Most organic matter in wetlands originates from plant matter consisting primarily of lignin, cellulose, and hemicellulose (54). Under anaerobic conditions, lignin is rather recalcitrant and the bulk of methane derives from degradation of the polysaccharides (54, 149). The water inputs to the wetland determine buffering capacity and thus acidity of the soil and water, so that wetlands range from neutral or even slightly alkaline (296) to pH values less than 4 (26). The source of water into the wetland affects the concentration of mineral nutrients, and wetlands range from minerotrophic swamps and marshes to oligotrophic fens and bogs (127). Acidic peatlands themselves differ widely in the water and soil pH values, seasonal temperatures, and vegetation (17, 26, 112) even within the same wetland (86). Acidic peatlands may be oligotrophic or minerotrophic, and may receive water inputs only from

precipitation (bogs), or from a combination of precipitation, surface- and ground-water flow (fens, swamps). Temperatures change seasonally, as may redox conditions, depending the flow of water into and out of the area (94, 149). Common foliage in these peatlands include acid-tolerant *Sphagnum* mosses, *Carex* sedges, as well as shrubs and conifers.

Typically maximum methane production occurs 10-20 cm below the water table (73, 87, 88, 127, 146), but methane production may occur throughout the soil depth (86, 141). Many researchers have reported the dominance of hydrogenotrophic methanogenesis and accumulation of acetate in these environments suggesting that acetate is not an important precursor for methanogenesis (69, 87, 110, 181, 251) Duddleston *et al.* (2002) observed acetate slowly oxidized to carbon dioxide through aerobic oxidation or anaerobic respiration instead of metabolized by methanogens (69).

The dominance of hydrogenotrophic methanogenesis in acidic wetlands is reinforced by community analysis with clone libraries dominated by members of the Fen Cluster, a deeply branching clade within the *Methanomicrobiales*, and Rice-Cluster I, a clade related to the *Methanobacteriales* and commonly found in rice fields (30, 31, 73, 86-88, 103, 127). The Fen cluster has also been referred to as the R10 group (103), or the E1/E2 clade (30). Brauer *et al.* (2006) isolated a member of the Fen cluster, *Candidatus* Methanoregula boonei, which was found to be hydrogenotrophic and acidophilic with maximal growth near a pH of 5 (25). Another member of the Fen Cluster, isolate E1-9c, is closely related to *Candidatus* Methanoregula boonei and was isolated from a minerotrophic fen (31). Cells of E1-9c were non-motile, hydrogenotrophic cocci with optimal growth near 30°C and a pH of 5.3-5.5. A member of Rice Cluster I, referred to as strain SANAE, was isolated by Sakai *et al.* (2007). SANAE was also found to be hydrogenotrophic utilizing either hydrogen and carbon dioxide or formate as the substrate for methanogenesis, but this organism could not be isolated with hydrogen added directly to the headspace. Instead, SANAE was isolated from a co-culture with a syntrophic hydrogen-producing partner (31). Their isolation method was based in part on earlier studies which showed that incubation of rice roots under high hydrogen partial pressure suppressed the methanogenic activity of members of Rice Cluster I (163). The authors hypothesized that members of Rice Cluster I are outcompeted for hydrogen by faster-growing hydrogenotrophic methanogens, and can only compete when closely associated with syntrophic partners.

Research has suggested that the pathway for methanogenesis depends on both the *in situ* temperature and pH. Although incubation studies of acidic peat have shown methanogenesis increasing with increasing pH, maximal methanogenesis usually occurs at a pH of 5-6 (26, 87, 94, 251, 286). The more acidic the peat (especially below pH 5) the more methane is produced from

hydrogen and carbon dioxide, and addition of these substrates into the headspace of acidic peat cultures stimulates methane production, but the addition of acetate inhibits methanogenesis (26, 114, 142). The more acidic the environment, the more hydrogenotrophic methanogenesis dominates as temperature drops (142). At slightly more neutral pH values (above 5), aceticlastic methanogenesis has instead been reported to dominate (142).

Incubations of peat from acidic Northern peatlands typically show methanogenesis at temperatures near freezing and as great as 45°C (26, 181). Despite the fact that the temperatures in Northern acidic wetlands rarely reach temperatures above 25°C, the optimum temperature for methanogenesis in incubation studies of peat is usually mesophilic (25-35°C) (26, 94, 141, 181, 251). There is also evidence for psychrophilic communities from these environments. Williams & Crawford (1984) obtained methanogenic enrichments from 120 cm below the water table in an acidic peat bog which showed maximal methanogenesis at 12°C (286).

Other commonly detected methanogen sequences in acidic peatlands are related to *Methanosarcina*, *Methanosaeta*, Rice Cluster II, and *Methanobacterium,* with sequences related to *Methanoculleus* and *Methanospirillum* occasionally detected (17, 30, 31, 40, 86, 112). Kotsyurbenko *et al.* (2007) isolated an acidophilic strain of *Methanobacterium* capable of growth at pH values as low as 3.8 (142). Hydrolytic and fermentative bacteria detected in acidic peatlands include members of the *Clostridia, Acidobacteria,* the alpha-, beta-, delta-, and gamma-*Proteobacteria, Verrucomicrobia*, *Actinobacteria*, *Planctomycetes*, *Bacteriodetes*, *Sphingobacteria*, *Lactobacillus*, and *Chlorobia* (62, 94, 105). Acidophilic aerobic methanotrophs within the *Methylosinus-Methylocystis* clade as well as methanotrophs distantly related to both this clade and *Methylococcus* have been detected in these environments (61, 174). Enrichment cultures of acidophilic aerobic methanotrophs have exhibited optimal growth at pH from 4.5-5.5. In addition, two new genera of aerobic methanotrophs, *Methylocella* and *Methylocapsa*, have been isolated from acidic peat bogs which exhibit maximal growth at pH values of 4-5 (251).

ii. Tundra and other permanently cold sediments

Methanogenesis has been detected in a number of permanently cold sediments, including tundra, or permafrost, soils and deep lake sediments. The distinction between acidic peatlands and tundra is not a sharp one, as many tundra soils are acidic water-saturated peatlands with seasonal periods of thawing (36). What is notable in these environments is that methanogenesis occurs year-round, with methane produced up to complete freezing of the soil and a release of the stored methane upon thawing in the spring (282). Arctic permafrost soils, which have a mean annual temperature of less than 0°C, contain greater than 14% of the global organic soil carbon

and are responsible for up 20% of global methane emission (140, 182). These sediments are very important in the sequestration of organic carbon and the regulation of carbon in the atmosphere. Microbial decomposition is slow in Arctic and deep lake sediments due to the high moisture content and low temperature, but as global temperatures increase, microbial mineralization of these carbon reserves also increases (140). The greatest number of active microorganisms, both methanogens and other bacteria, is in the first 10 cm of soil, but active microorganisms have been obtained from depths up to 45 cm (140).

Enrichment cultures and incubation studies from permanently cold sediments have demonstrated methanogenesis at temperatures from 1-70°C (133, 193, 194, 248) although optimal temperatures for methanogenesis were typically 25-30°C (133, 182, 193). The pathway for methanogenesis changes as the incubation temperature changes, as was noted for other acidic peatland environments, but the results are quite different. In contrast to other acidic peatland environments, in tundra soils at temperatures less than 30°C methanogenesis is dominated by the aceticlastic pathway, and a shift to the hydrogenotrophic pathway occurs at higher temperatures (182, 236, 237). Aceticlastic methanogenesis at low temperatures relies on acetate produced by homoacetogens which outcompete hydrogenotrophic methanogens for hydrogen at low concentrations (193, 194, 236, 237). When hydrogen is added to the headspace of enrichment cultures, hydrogenotrophic methanogenesis dominates (237).

Clone libraries and bacterial isolates have supported this pathway for methanogenesis at low temperatures. Clone libraries from freshwater and marine sediments as well as tundra soil have contained sequences predominantly related to the genera *Methanosarcina* and *Methanosaeta*, which are the only aceticlastic methanogens, but also related to the methylotrophic methanogens *Methanolobus* and *Methanococcoides*, and the hydrogenotrophic methanogen *Methanogenium* (133, 182, 193, 211, 296). Other researchers have also obtained clones related to the genera *Methanobacterium*, *Methanospirillum*, Rice Cluster I, and the Fen Cluster (182, 193, 211, 296). Although enrichment and incubation studies of permanently cold sediments have suggested a mesophilic optimum for methanogenesis, some researchers have found evidence for psychrophilic organisms and communities. Kendall *et al.* (2007) isolated a methanogen from sediments of Skan Bay, Alaska, related to the genus *Methanogenium* which exhibited optimal growth at 20°C (133). Nozhevnikova *et al.* (2001) isolated acetogens related to *Acetobacterium* from tundra soil that demonstrated optimal growth at 20°C (193). Nozhevnikova *et al.* (2003) incubated deep lake sediments at temperatures from 2-70°C for 8 months before changing the incubation temperature and noting the methanogenic pathway (194). The researchers found sediments which were incubated at temperatures of 50°C or above could no longer produce

methane at temperatures below 15°C. Enrichments which were maintained at temperatures from 2-15°C over the eight month period were dominated by aceticlastic methanogens and acetogens whereas enrichments kept at mesophilic or thermophilic temperatures were dominated by hydrogenotrophic methanogens (194).

In addition to methanogens, a number of diverse *Bacterial* communities have been identified in tundra soil. Sequences for aerobic methane oxidizers related to *Micrococcaceae* and *Methylocystaceae* have been identified in Arctic tundra soil (283). Common acetogens found in permanently cold sediments belong to the genus *Acetobacterium*, whose members have been found in a variety of habitats including lake and pond sediments, wetland soil, and cattle manure digesters (193). Bacterial clone libraries from Arctic tundra soil have included high G+C gram positive bacteria, alpha and gamma *Proteobacteria*, and the *Cytophaga-Flavobacterium-Bacteriodes* cluster (140).

iii. Rice paddy soils

Rice fields experience seasonal flooding and draining events, which cause seasonal fluctuations in the redox potential of the soil (165). Flooding corresponds to the growing season of the rice plants, and flooding reduces the redox potential of the soil. The aerenchyma of rice plants is a hollow stem area which translocates air to the roots of the plant while simultaneously releasing methane produced in the soil to the atmosphere (99). The translocation of gases through the aerenchyma means that even during the flooded season methanogens at the rice roots may be exposed to oxygen. Theoretically, methanogenesis is only thermodynamically feasible at low redox potential (<200 mV) and when other electron acceptors are not present (165). In truth, methanogenesis in rice paddy soils has been observed at redox potentials above this threshold and when there are other electron acceptors. Despite the potential oxygen exposure, methanogens are prominent members of the community on rice roots. Rice roots that are incubated anoxically immediately show methane production even if they were exposed to oxygen during collection suggesting some oxygen tolerance of these methanogenic communities (99).

A number of novel methanogen lineages have been identified in rice field soil. Rice Cluster I and Rice Cluster II are two clades deeply branching from the *Methanosarcinales* and *Methanomicrobiales* (99). Members of Rice Cluster I appear to be ubiquitous and often the dominant methanogen in rice paddies, regardless of location, temperature, or time of year (45, 46, 97, 154, 163-165, 226). Members of Rice Cluster II are less often identified, but have still been found in a variety of rice paddy soils and as members of the community on rice plant roots (46, 99, 154, 165, 207). Members of Rice Cluster I appear to be widespread in wetland environments

and have been identified in Arctic tundra soil (112) and a number of acidic peatlands (30, 87, 126). Members of Rice Cluster II are rarely identified outside of rice paddy environments, but members of this clade have occasionally been detected in tundra soil (112) and peatlands (31).

Besides members of Rice Cluster I and II, other methanogens identified in both bulk soil and associated with rice roots include members of the *Methanosarcina*, *Methanosaeta*, *Methanogenium*, *Methanoculleus*, *Methanomicrobiaceae*, and *Methanobacteriaceae* (45, 97, 163-165). Flooding of the rice field signals the start of the growing season and initiates methanogenesis. The pathway for methanogenesis and the methanogen community itself often changes throughout the growing season (145). Early in the growing season when rice fields are first flooded, hydrogenotrophic methanogenesis dominates and acetate accumulates (145, 154, 165). As the growing season progresses, more acetate is used for methanogenesis until at the end of the season the ratio of methane produced from acetate compared to that produced from hydrogen and carbon dioxide reaches the theoretical ratio of 2:1 (145). Incubations of rice roots have also showed this progression from hydrogenotrophic to aceticlastic methanogenesis after flooding with members of Rice Cluster I initially dominating the community and later being replaced by members of the *Methanosarcina* (45, 163).

As was found in the isolation of Rice Cluster I species SANAE, the hydrogen partial pressure affects the activity, and thus the methanogenic pathway, in rice field soil. Lu ***et al.*** (2005) examined methanogen activity and community structure of rice root incubations after the initiation of flooding. Methanogens on the rice roots included *Methanosarcina*, *Methanobacteriaceae*, and Rice Cluster I, although Rice Cluster I showed much greater activity than members of the other two clades (163). When the hydrogen partial pressure was increased in the incubation, the activity of Rice Cluster I methanogens was completely suppressed but the activity of *Methanosarcina* increased suggesting members of the metabolically-diverse *Methanosarcina* may act as both hydrogenotrophs and aceticlasts in rice paddy communities (163).

Temperature also plays a large role in the pathway of methanogenesis and carbon cycling in rice field soil. Rice fields are typically mesophilic environments with yearly temperatures ranging from 15-30°C, although enrichment cultures have been obtained with growth at temperatures as high as 50°C (164). Rice paddy bulk soil incubations maintained at 30°C were dominated by hydrogenotrophic methanogenesis. When these incubations were shifted to 15°C, homoacetogenesis outcompeted methanogenesis for available hydrogen, similar to that which has been reported for tundra and other permanently cold sediments (44). To deduce the shift in methanogen community, enrichment cultures of rice soil were grown at 30°C and then shifted to

15°C (47). The shift from 30 to 15°C showed a rapid drop in methanogenesis and an initial accumulation of acetate, which was later consumed. The enrichment culture grown at 30°C was dominated by members of Rice Cluster I, but the shift to 15°C showed the appearance of sequences related to *Methanomicrobiaceae*, *Methanobacteriaceae*, and *Methanosaeta*. An enrichment culture maintained at 30°C showed the appearance of *Methanosarcina* and Rice Cluster II, but prolonged incubation at 30°C resulted in the dominance of *Methanosarcina* with fewer numbers of *Methanobacteriaceae*. Prolonged incubation of an rice paddy soil enrichment culture maintained at 15°C instead showed dominance by *Methanosaeta* with fewer numbers of *Methanobacteriaceae* suggesting that temperature not only affects the methanogenic pathway, but also the dominant aceticlastic methanogens present (47).

iv. Temperate freshwater river and lake sediments

Methanogenesis may occur in any water-saturated, anaerobic environment including pond, lake, and river sediments, but these methanogenic communities have been less studied than others. These environments may be subject to seasonal changes in temperature, source of organic matter, and oxidation due to changes in temperature and seasonal water turnover in lakes (92). The impact of nutrients has been of interest to some researchers due to the potential intensification of methane production in these areas. Portions of the Florida Everglades receiving nutrients or that had been recently farmed showed higher rates of methane production than non-impacted areas (255). This effect was also seen in a hypereutrophic lake receiving agricultural runoff which showed seasonal increases in methane production in response to increases in both temperature and nutrient load (72). Methanogen sequences obtained in freshwater, temperate sediments are similar to those seen in other wetland environments, and most often include members of the *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, and *Methanomicrobiaceae* (35, 72, 92, 255).

v. Marine and estuarine sediments

Methanogens have been found in estuarine, near-coastal, and deep marine sediments (189). Increases in temperature have been found to increase hydrogen concentrations in marine sediments (80), but deep marine sediments are permanently cold. Some researchers have reported the dominant pathway of methanogenesis to be hydrogenotrophic in both deep sea (189) and estuarine sediments (14), but this is unusual due to typically high concentrations of sulfate compared to other methanogenic environments. In the presence of sulfate, sulfate-reducing bacteria (SRB) often out-compete methanogens for hydrogen due to their lower K_m, so most

methane is produced from noncompetitive substrates such as methanol and methylamine (80, 211, 285). In systems with both sulfate-reducers and methanogens, the fate of the methyl group of acetate may be used to determine the dominate pathway for oxidation of organic carbon (285). SRB oxidize the methyl group of acetate to carbon dioxide whereas methanogens reduce the methyl group of acetate to methane. This assumption is only valid when acetate-oxidizing bacteria are not active because otherwise the methyl group of acetate is oxidized to carbon dioxide prior to reduction to methane by methanogens (285).

Although sulfate concentrations are the primary determinant of the methanogenic pathway in marine sediments, salinity may also influence the methanogenic pathway and the methanogens that are present. Typically, sulfate and hydrogen concentrations are inversely related with increasing sulfate concentrations reducing hydrogen concentrations, and decreasing sulfate concentrations resulting in higher hydrogen concentrations (80). In hypersaline microbial mats near salterns in the Pacific ocean, sulfate concentrations were found to be six times greater than typical seawater concentrations, and sulfate-reducing bacteria (SRB) were the primary mediators of carbon mineralization (254). When the sulfate concentration was lowered or sulfate reduction was inhibited in incubation studies, methane production increased, and the addition of acetate did not stimulate methanogenesis . Methanogen sequences were all related to the hydrogenotrophic *Methanogenium* and the methylotrophic *Methanolobus*. When both the salinity and sulfate were high, all sequences were related to *Methanolobus*. Only when either the salinity, the sulfate concentration, or both were lowered did sequences related to *Methanogenium* appear (254). The dominance of methylotrophic methanogenesis usually results in the dominance of the methylotrophic members of the *Methanosarcinaceae* in clone libraries, especially the genera *Methanolobus* and *Methanococcoides* (211), but this is not always the case. Newberry *et al.* (2004) found hydrogenotrophic methanogenesis to be the dominant in a deep marine sediment, and clone libraries contained mainly sequences related to *Methanobrevibacter* and *Methanosarcina* (189). Banning *et al.* (2005) found sequences of aceticlastic, methylotrophic, and hydrogenotrophic methanogens in a brackish lake sediment including representatives of *Methanosarcina*, *Methanosaeta*, *Methanolobus*, *Methanospirillum*, and *Methanoculleus* (14).

Some of the more interesting marine environments are methane seeps or hydrothermal vents. In these locations, organic rich sediments are pyrollyzed by hydrothermal processes to release aliphatic and aromatic hydrocarbons, volatile fatty acids, methane, ammonia, and other gases into the subsurface through vents or fractures in the seabed (66). These are challenging environments for life with both high temperatures (300-400°C) as well as high hydrostatic pressures (89). Cold methane seeps may also occur where reservoirs of gas hydrates, or

clathrates, exist (20). Clathrates depend upon low temperatures and high pressures to remain crystalline, and for this reason, they tend to be restricted to polar and deep-sea locations (20). Cold methane seeps often have abundant clam colonies of genus *Calyptogena* and mats of filamentous sulfur-oxidizing bacteria of the genus *Beggiatoa* which cover the sediments overlying the hydrates (119, 139). A diverse array of metabolisms occur in both hot and cold methane seeps, including methanogenesis and both aerobic and anaerobic methane oxidation (66, 119, 187). Methanogens identified in these sediments include methylotrophic *Methanococcoides* and *Methanohalophilus*, and hydrogenotrophic *Methanocorpusculaceae*, *Methanomicrobiaceae*, *Methanococcus*, *Methanocaldococcus*, *Methanothermococcus*, and *Methanopyrus* (66, 187). Methanotroph sequences retrieved from these sediments include aerobic methane-oxidizers of genera *Methylobacter*, *Methylomonas, Methylophaga, Methylothermus,* and *Methylococcus* and anaerobic methane oxidizers of clade ANME-1 and 2 (66, 119, 133).

vi. Animal intestinal tracts

Ruminants lack cellulolytic enzymes and thus depend on anaerobic microorganisms to degrade cellulose to compounds which can be absorbed by their intestines for nutrition (89). Methanogens have been identified in the guts of a diverse range of animals including sheep (82), cows (287), pigs (273), deer (273) chickens (223), termites (197, 247), humans (261), terrestrial arthropods, and marine animals such as plankton, fish, and baleen whales (89). Methanogens found in animals are often quite different from those found in other habitats as the animal gut is typically of neutral pH and moderate temperature (247). In the rumen, acetate is produced by fermentation and often accumulates to concentrations of 50 to 100 mM where it is absorbed by the host organism. Therefore, the majority of methane derives from hydrogen and carbon dioxide as opposed to formate (89). In addition, there may be associations of methanogens with rumen and termite protozoa.

Although *Methanosarcina* is often present in the rumen environment, growth on acetate is too slow to maintain their presence and *Methanosarcina* grow by either hydrogenotrophic or methylotrophic methanogenesis (285). In the large intestines of animals, methanol may be formed from metabolism of the methoxy groups of pectin, providing a substrate for *Methanosarcina* whose K_m for hydrogen is much higher than the other hydrogenotrophic methanogens present (285). A diverse group of hydrogeneotrophic methanogens have been identified in the rumen, most commonly belonging to the orders *Methanobacteriales*, especially in the genera *Methanobrevibacter* and *Methanosphaera*, and in the order *Methanomicrobiales* within the genus *Methanocorpusculum* (247, 261, 82, 197, 210, 273). Many animal methanogens

are host-specific or even geographically limited due to differences in the gastrointestinal environment and feed of different animals (287). For example, Ufnar *et al.* (2007) showed that functional gene fragments from pigs could be used as swine-specific markers of fecal contamination, distinct from those recovered from cow, deer, sheep, horse, and chicken (273, 274). (102)

vii. Landfills

Landfills have essentially infinite retention times for microbiota but the temperature, pH, and available carbon source change over time (148). For this reason, the methanogens that are identified in landfills depends heavily upon the location and age of the sampling point. Methanogenesis in landfills has been observed at pH values down to 5.5 and as great as 8.6 suggesting both acido- and alkalitolerant methanogenic consortia exist in these environments (116, 148). In addition, methanogens in landfills are able to survive very high VFA concentrations, as evidenced in a study by Huang *et al.* (2002) who identified methanogens in a treatment pond degrading landfill leachate that contained greater than 14,000 mg/L of VFAs. The methanogen community was dominated by *Methanoculleus* and *Methanosarcina*, although sequences belonging to *Methanocorpusculum*, *Methanospirillum*, and *Methanogenium* were also identified (116). These researchers also found isolates of an uncultured clade that has been identified in several other landfills as well as hydrocarbon- and chlorinated solvent-contaminated aquifers (115, 116, 275). Enrichment cultures suggest methanogenesis in these environments is mainly hydrogenotrophic and members of the *Methanomicrobiales* are dominant, especially the genus *Methanoculleus* but also *Methanospirillum*, *Methanocorpusculum*, *Methanogenium*, and *Methanofollis* (115, 116, 275,168). Other methanogens that have been identified include *Methanobacterium, Methanobrevibacter*, *Methanosarcina*, and *Methanosaeta*, although in most cases only *Methanosarcina* or *Methanosaeta* is identified (115, 116, 168). In only one landfill environment were both *Methanosaeta* and *Methanosarcina* identified (275).

viii. Miscellaneous habitats

In some environments, such as hot springs and volcanic fissures, geothermal hydrogen is released and used as a substrate for methanogenesis rather than decaying organic matter (285). Generally thermophilic and hyperthermophilic methanogens have been isolated from these environments (285). Methanogens have also been isolated from deep oil-bearing rocks which range from slightly saline to saline, and mesophilic to thermophilic in temperature (89). Hydrogenotrophs isolated from oil reservoirs include *Methanobacterium*, *Methanococcus*,

Methanoplanus, and *Methanocalculus*, and methylotrophic methanogens belonging to *Methanohalophilus* and *Methanosarcina* (89).

Environments containing fossil fuels such as petroleum or coal are often home to methanogens. Water from a methane-producing coal bed showed the presence of methanogens, and enrichment cultures yielded exclusively members of *Methanocorpusculum* (262). Methanogen sequences obtained from petroleum-contaminated aquifers include *Methanosaeta*, *Methanospirillum*, and *Methanobacterium* (138, 264). A high-temperature, high-sulfur petroleum reservoir yielded methanogen sequences belonging to *Methanobacteriaceae*, *Methanococcales*, and *Methanoculleus* (200).

A rarely studied, but interesting methanogenic location is mud volcanoes, produced from the emission of semi-liquid, gas-enriched mud from deeper sediment layers (4). Researchers found sequences related to *Methanosarcina* and ANME-2, and a relatively heavy δ^{13} of -30‰ suggesting methane was not completely biogenic in origin. The researchers concluded that methane in these environments is both of biogenic and non-biogenic in origin, and methane production and oxidation was occurring within the same environment (4).

Although methanogens have generally been considered oxygen-intolerant, active methanogenic enrichments have been recovered from oxic environments. Sheppard ***et al.*** (2005) obtained methanogen sequences related to *Methanosarcina*, *Methanomicrobiaceae,* and *Methanobacteriaceae* from the oxic layer of grassland soils receiving digested sewage sludge in land application (244). Peters and Conrad (1995) obtained active methanogenic cultures from five oxic soils, including forest soil, termite mound, savanna soil, desert soil, and the A horizon of farmed soil (209). In addition to methanogens, these researchers also obtained active sulfur-reducing bacteria and acetogens from all five soils. Although some species of sulfur-reducing bacteria and acetogens are known spore-formers which provides oxygen and dessication tolerance, no known methanogen species is a spore-former and the mechanism of survival in oxic environments is unknown (209). Methanogens are active members of the microbial communities of rice roots, where the aerenchyma of the rice plants translocates oxygen to the subsurface and releases the methane produced on the roots to the atmosphere (99, 165). Methanogens have also been obtained from dental caries and subgingival plaque in the human mouth, both of which are aerobic environments (285). These observations have raised the question of just how oxygen-sensitive methanogens are. When grown in culture alone, methanogens are extremely sensitive to oxygen, but in natural habitats methanogens are found in aerobic and microaerophilic habitats, and even under anoxic conditions methanogenesis occurs when alternate electron acceptors are still available (165, 285). Researchers have theorized that this is due in large part to the action of

facultative anaerobes which rapidly consume oxygen and create anoxic microenvironments in which the methanogens exist (285).

D. Methanotrophy

In most wetland environments, aerobic methanotrophs cultivate the oxic layers of the soil, sediment, or lake and oxidize methane to carbon dioxide and water (149). Two forms of methanotrophy occur in soils and sediments: high affinity oxidation, and low affinity oxidation. High-affinity oxidation occurs at methane concentrations less than 12 ppm, and appears to be ubiquitous in the natural environment, whereas low-affinity oxidation occurs at methane concentrations higher than 40 ppm. In anaerobic environments oxygen availability is the main limitation for methanotrophy which is negatively correlated with both increasing water content and decreasing redox potential of the soil. Some wetland plants possess aerenchyma that translocate oxygen to methanotrophic communities at their roots and facilitate the oxidation of methane (149). Aerobic methanotrophs are classified as Type I or Type II depending on phylogeny and the enzymes used for methane oxidation. Type I methanotrophs belong to the gamma-*Proteobacteria* whereas Type II methanotrophs belong to the alpha-*Proteobacteria* (119). All known aerobic methanotrophs contain the particulate monooxygenase (*pmoA*) but type II methanotrophs and the type I methanotroph *Methylococcus* also contain a soluble methane monooxygenase (*mmoX*).

Anaerobic methane oxidation was recently observed in deep sea hydrothermal vents in association with sulfur-reducing bacteria. Several researchers have observed *mcrA*-like sequences belonging to the Anaerobic Methane-Oxidizing Archaea (referred to as ANME or MOA) and have resolved the phylogenetic relationships into three deeply branching clades (66, 104, 183). The ANME-1 are related to the *Methanomicrobiales* and *Methanosarcinales*, and may be further divided into subgroups a and b (183). The ANME-2 with subgroups a, b, and c are related to the *Methanosarcinaceae* and *Methanosaetaceae*, and the ANME-3 are related the *Methanococcoides* within the *Methanosarcinaceae* (183). The three groups of ANME seem to occupy different niches in the environment. Although ANME-1 and ANME-2 may co-occur, typically a habitat is dominated by one group or the other (139). ANME-2 form aggregates with members of the *Desulfosarcina-Desulfococcus* branch of SRB, with the SRB on the outside of the aggregates and the methanogens on the inside (22, 91) (139). Members of the *Desulfosarcina-Desulfococcus* are also observed to associate with ANME-1 in *Beggiatoa* mats, but not in aggregates (139). Instead, ANME-1 are more often observed as single cells or filaments without a sulfate-reducing partner (199). In the water column, as compared to

sediments, both ANME-1 and ANME-2 have been observed as single cells (71). The ANME-3 are observed to form aggregates with *Desulfobulbus*, and in some locations have been shown to be the dominant methane oxidizer in *Beggiatoa* mats (162).

It is currently not known the mechanism by which MOA oxidize methane, but three hypotheses exist. The first hypothesis is that methane is oxidized to carbon dioxide and hydrogen by "reverse methanogenesis" according to the equation $CH_4 + 2H_2 \rightarrow CO_2 + 4H_2$ (33, 276). Methanol or acetate may also be produced depending on the *Archaeal* strain (33) with these products used by SRB to convert sulfate to hydrogen sulfide. This theory is based in part on the observation that methyl coenzyme-M reductase is produced in high quantities during anaerobic methane oxidation, suggesting this enzyme may be used in either direction (246, 272). In addition, some species of methanogens have been shown to oxidize methane in trace quantities whereas members of the sulfate-reducing genus *Archaeoglobus*, which lack the Mcr gene, were not (185). A complication to this theory is that under typical *in-situ* conditions, methane oxidation by this mechanism with concomitant sulfate reduction utilizing the produced hydrogen only yields approximately -25 kJ/mol^{-1} methane oxidized for the net reaction (276). This energy must be shared between the methane-oxidizing and sulfate-reducing partners, an energy yield for each that is below the -20 kJ/mol^{-1} considered the minimum energy currency of a reaction (233). This fact, coupled with the inability of researchers to obtain significant methane oxidation in methanogen cultures, has led to the development of two other hypotheses. The second hypothesis suggests a reversal of aceticlastic methanogenesis in which two moles of methane are oxidized to produce acetate and hydrogen according to the equation $2CH_4 + 2H_2O \rightarrow CH_3COOH + 4H_2$, or one molecule of methane and one molecule of carbon dioxide are used to produce acetate according to the equation $CH_4 + HCO_3^- \rightarrow CH_3COO^- + H_2O$. The acetate and hydrogen that are produced are then used by SRB (33, 276). Although both equations are feasible given typical methane seep conditions, these theories have yet to be proven in culture studies. The most recent hypothesis is that a methane-oxidizing and carbon dioxide-reducing *Archaea* produces methyl sulfides which are then used by SRB to reduce sulfate (184). In sediment incubations from methane seeps the researchers found that high H_2 concentrations did not inhibit methane oxidization suggesting it is not an intermediate between methane oxidizers and SRB. In addition, cultivation of an ANME-2 related methanogen resulted in the production of large quantities of methyl sulfides (184). A limited number of SRB are known to use methyl sulfides, and this pathway may explain the limited diversity of SRB in these consortia (33).

III. Techniques for studying methanogen communities

A. Culture-dependent techniques

Methanogens may be autotrophic or heterotrophic, yet nearly all cultured methanogens require some acetate in the growth media (89). In addition, most cultured methanogens require the addition of complex organic substrates such as yeast extract, rumen fluid, digests of casein, amino acids, and vitamins such as riboflavin, pantothenic acid, thiamin, and biotin (89). Complex nutritional requirements mean that it is difficult to grow many methanogens in culture alone. Enrichments for methanogens are most successful if alternate electron acceptors are kept from the media and stored in the dark both to prevent the growth of phototrophic organisms and also because methanogens are sensitive to light (198, 285). Antibiotics are often employed to prevent the growth of members of the domain *Bacteria* as the difference in cell wall structure makes methanogens generally insensitive to most antibiotics (285). As with the culturing of any microorganism, considerations must be made to pH, salinity, temperature, and the presence of minerals necessary for growth (285). In addition, the extreme sensitivity of methanogens to oxygen when grown as isolates makes culturing and long-term storage much more difficult, so a reducing agent is often used in both liquid and solid media to ensure anaerobic conditions. Although enrichments are generally performed in liquid culture, the isolation of single colonies requires solid media. The development of the Hungate roll tube method was instrumental in isolating methanogens and other strictly anaerobic bacteria (49). This technique combined liquid anaerobic enrichments with molten agar in a tube which was capped and rolled in an ice bath to create an agar film around the inside of the tube. The atmosphere of the tube was filled with oxygen-free carbon dioxide to create an anaerobic environment and allow the growth of individual colonies that could later be transferred to liquid growth media. The Hungate roll-tube method allowed the isolation of dozens of anaerobic bacteria, including many fermentative and cellulolytic organisms as well as methanogens (49).

B. Culture-independent techniques: molecular studies

Methanogens are difficult to isolate and culture, and so many researchers have chosen to examine microbial communities through molecular techniques. The most common target for molecular studies in the 16S rRNA gene (16S rDNA), but many researchers have also begun to use functional genes in molecular studies. For methanogens, the functional gene that is most often targeted is the alpha subunit of the methyl coenzyme-M reductase (*mcrA*) and its isoenzyme *mrtA* which is only found in members of the *Methanobacteriales* and *Methanococcales*. The Mcr

is exclusive to methanogens which allows distinction from non-methanogen sequences. The Mcr is composed of a five gene cluster organized as mcrBDCGA, which includes sequences for subunits α, β, γ, and two other components arranged in the complete enzyme as a 2α2β2γ complex with two active sites (285). The alpha subunit was chosen for molecular studies as it is the largest subunit at approximately 1500 bp, and thus contains the greatest amount of phylogenetic information. Several researchers have compared phylogenetic relationships of methanogens obtained with 16S rDNA and *mcrA* sequences and found them to be nearly identical (168, 257). Springer *et al.* (1995) observed that *mcrA* sequences were approximately three times more distant than comparable 16S sequences within the *Methanosarcinaceae* (257). Bapteste *et al.* (2005) performed phylogenetic analysis for 53 ribosomal proteins, including 20 proteins involved in hydrogenotrophic methanogenesis and 15 proteins involved in the synthesis of coenzymes for this pathway. No evidence of lateral gene transfer was found for enzymes involved in the last two steps of methanogenesis, which includes the final step catalyzed by the Mcr. This finding lends support for the use of the Mcr as a phylogenetic marker in conjunction with, or independently of, the 16S rRNA gene (15).

i. Non-quantitative molecular techniques

The most popular non-quantitative molecular technique used to analyze methanogen communities is the construction of clone libraries. The clones obtained in a library are heavily dependent on a number of factors including, but not limited to, the type of DNA extraction and purification method used, the target gene, and the PCR cycling conditions. Some researchers have found a cloning bias may be due to differences in the lysis susceptibility of different cells, which is especially apparent in clone libraries targeting members of the *Bacteria* as well as methanogens (243). Due to differences in cell-wall and membrane structure, methanogens may not be effectively lysed using methods developed for the *Bacteria*, and methanogens themselves show different lysis susceptibility based on phylogeny (243).

There are currently three primer sets used to amplify the *mcrA*; the MCR set developed by Springer *et al.* (1995), the ME set developed by Hales *et al.* (1996), and the ML set developed by Luton *et al.* (1996) (103, 168, 257, Table 2-1). The reverse primer for all three sets is at nearly the same location on the gene, but the forward primers target different conserved regions. All three primer sets have been used extensively, but coverage of the methanogen community differs depending on the primer set used. In general, the MCR set has been able to recover a greater range of sequences, most likely due to its greater degeneracy, but this degeneracy also means that non-*mcrA* sequences are obtained (127, 164). The ME primer set has been reported to not

amplify sequences of the *Methanosaeta* or the *mrtA* of the *Methanobacteriaceae* (164). In addition to coverage differences, the choice of primer set affects the apparent dominance of operational taxonomic units (OTUs) in a library and the calculated diversity indices for the library (127). The choice of cycling parameters, specifically the annealing temperature and the number of cycles, also affects the diversity and dominance of clones in a library. Lueders and Friedrich (2003) examined the effect of annealing temperature and cycle number on libraries obtained for the 16S rDNA and *mcrA*. The researchers found that neither library was affected by the number of PCR cycles, but while the 16S rDNA libraries were also not affected by annealing temperature, the *mcrA* libraries were. The researchers hypothesized these differences to be due to differences in degeneracy, as the 16S rDNA primers were much less degenerate than the *mcrA* primers (166).

Primers used for the 16S rDNA are typically either general *Archaeal* or *Euryarchaeal* primers. As methanogens are not monophyletic, there has been little to no success in creating methanogen-specific 16S rDNA primers. Most members of the *Euryarchaeota* outside of methanogens are extremophiles and not expected to be found in most methanogenic environments, so *Euryarcheota*-specific 16S rDNA primers may be considered methanogen-specific primers in these environments (29). An advantage to creating libraries for both the 16S rDNA and *mcrA* is that one library may reveal diversity that the other does not. Banning ***et al.*** (2005) was able to show greater diversity with libraries created with order-specific 16S rDNA methanogen primers versus *mcrA* libraries (14). In contrast, while studying oligotrophic and nutrient-impacted areas of the Florida Everglades, Castro ***et al.*** (2004) found similar 16S rDNA sequences in both areas, but was able to differentiate the methanogen communities in the *mcrA* libraries (35).

ii. Quantitative molecular techniques

Due to biases in amplification, clone abundances in a library cannot be presumed to reflect actual abundances of an organism in an environment. For this reason, researchers have developed techniques that reveal abundances of a given methanogen or OTU. Some of these techniques are based on PCR and therefore subject to the same biases as clone libraries, such as single-stranded conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP). With both SSCP and T-RFLP, nucleic acids must be extracted from the environment and amplified by PCR. With SSCP, the PCR products are heated to separate the DNA strands and then rapidly cooled resulting in reannealing of some strands, formation of heteroduplexes in others, and a portion remaining as single-stranded DNA (152). Under non-denaturing conditions single-stranded DNA forms a 3-dimensional folded structure determined by

intramolecular interactions in the strand. These single-stranded products may be separated from each other and from duplex DNA by gel electrophoresis. Electrophoretic mobility is dependent on length, molecular weight, and the shape of the molecule allowing fragments of the same size but different sequences to be separated from one another (152). T-RFLP is based upon the digestion of DNA from a single clone sequence with endonucleases and then separation of the resulting fragments based on size with gel electrophoresis. At least one primer is labeled with a fluorophore so that each strand of the resultant PCR products contains a fluorescent molecule (172). The use of a fluorescently-labeled primer allows several different sequences to be analyzed simultaneously so that an entire microbial community may be examined at one time. With both SSCP and T-RFLP, the use of capillary electrophoresis and sensitive detection equipment generates a succession of peaks with peak area directly correlated to fragment abundance (172, 210). Both SSCP and T-RFLP may be used to obtain both qualitative (sequence diversity) and quantitative (sequence abundance) results with the understanding that the same biases that affect the generation of clone libraries also affect these techniques.

Two commonly-used PCR-independent techniques involve probing of extracted RNA and the labeling of whole cells using Fluorescent In-situ Hybridization (FISH). Raskin *et al.* (1994) developed probes for the 16S rRNA to differentiate members of the *Methanobacteriaceae, Methanococcales, Methanomicrobiales, Methanosarcinaceae, Methanosarcina*, and *Methanosaetaceae* (215). The researchers extracted RNA from the environment and bound them to a nylon membrane. The probes were radioactively labeled with ^{32}P, and hybridized to the bound nucleic acids. Unbound probe was removed with subsequent washing steps, and quantitation was achieved by determining radioactivity using either exposure of X-ray film or scintillation counting. Probe specificity was determined not only by sequence, but also by the ionic strength and solvent concentration of the hybridization buffer, and the temperature of the washing conditions. The researchers also experimented with dual-probing of a single strand of RNA to enhance the signal strength which depends upon the probes having similar hybridization and wash conditions (215). Membrane variability was the major source of experimental error in quantitative membrane hybridizations, so the researchers used RNA standards as a reference, as well as the statistical analysis of several membrane hybridizations and several locations on each membrane to evaluate the precision of values for relative abundances (213, 214).

The probes developed by Raskin *et al.* (1994) have been popular in quantifying methanogen communities from a variety of environments (213, 214, 216), and have been adapted for use in FISH as well as quantitative PCR. FISH may be used to examine individual cell morphology as well as biofilm architecture and the proximity of different cell types to one

another in an environment (241, 256). FISH involves the use of fluorescently-labeled probes bound to three-dimensionally folded 16S rRNA within a cell, so cell permeability to the probe as well as accessibility of the 16S rRNA site to the probe must be considered. Cells are first fixed to prevent degradation of RNA, and are permeabilized to allow probe entry and hybridization. Specificity is determined by the probe sequence as well as the hybridization conditions, specifically the temperature, ionic strength, and organic solvent concentration (56, 256). Excitation of the fluorophores with light of a specific range of wavelengths causes emission of light of longer wavelengths from the bound probe and allows quantitation based on the strength of fluorescence (56). The increase in fluorescence is linear with an increase in cell number, but must be normalized to controls to account for background fluorescence as well as differences in cell size and shape (256). Many primers or probes originally designed for PCR or other quantitation methods, such as radiolabeled probes used in Northern blots, have been adapted for FISH with appropriate optimization performed (56).

Real-time quantitative PCR (qPCR) is not a PCR-independent technique, but attempts to correct for PCR bias with the use of internal controls. The amplification of target DNA is quantified at each cycle of PCR with the use of fluorescent dyes. A dilution series of known copy numbers of target DNA is run in the same assay with samples containing unknown quantities of target DNA. The standard curve is based on the relationship of the initial amount of template to the cycle number at which that reaction reaches a fluorescence threshold, denoted the C_T value (125). It is important that the threshold is crossed during the exponential phase of amplification for quantitation purposes (109). During PCR amplification is initially exponential, but eventually reaches a plateau in which few or no additional copies of template are created (125). This is due to depletion of reagents such as dNTPs and primers, reduced efficiency of the DNA polymerase, and the accumulation of inhibitory substances such as pyrophosphate. Even during the exponential phase of amplification, complete doubling of the target DNA in each cycle is not ensured. For this reason, the efficiency term e is incorporated into the equation describing target amplification as $X_n=X_o(1+e)^n$ where X_o is the starting template concentration, X_n is the target concentration when the fluorescence threshold is crossed, and n is the number of PCR cycles (125). If complete doubling occurs, e is equal to 1. A plot of the log of the starting quantity of DNA versus C_T for the standard dilution series yields a linear response with a negative slope. A slope of approximately -3.32 equates to 100% efficiency, with percent efficiency calculated from $(10^{(-1/m)})-1$ where m is the slope of the standard curve (109). The y-intercept of the standard curve is related to the sensitivity of the reaction with a lower y-intercept correlated with greater sensitivity of the assay (252).

Quantitative PCR uses fluorescence to track amplification of the target sequence in real-time with a camera mounted to a thermocycler. The chemistry for qPCR involves either a non-specific fluorophore or a fluorescently-labeled probe with sequence complementary to the target strand. The most common non-specific fluorophore used in qPCR is SYBR-Green I, an intercalating dye which exhibits fluorescence only when bound to double-stranded DNA. Additional specificity may be achieved with the use of a dual-labeled TaqMan probe used in 5' nuclease assays (109). TaqMan assays take advantage of the 5' nuclease activity of *Taq* polymerase which will cleave a non-extendible hybridization probe bound to the template DNA. A TaqMan probe is dual-labeled with two fluorescent dyes, a reporter dye and a quencher dye. The emission spectra from the reporter dye is quenched by the second fluorescent dye through the process of fluorescence resonance energy transfer when both dyes are in close proximity bound to the TaqMan probe. During PCR, the activity of the *Taq* polymerase releases both the reporter and quencher dye allowing emissions from the reporter dye to be detected (109). As SYBR-Green I is a non-specific fluorophore, it will bind to any double stranded DNA including non-target strands, so care must be taken to ensure the correct product is amplified. TaqMan probes allow additional specificity, but require the additional design of a probe as well as being more expensive than the SYBR-Green I technology. An additional advantage to TaqMan assays is the ability to multiplex, or follow the amplification of several targets in one reaction with the use of different reporter dyes, each with a unique absorption and emission spectra.

The absolute quantitation of target DNA from an environmental sample can be problematic and most qPCR results should be seen as relative rather than absolute. The efficiency of the DNA extraction method, variations from extraction to extraction, as well as variations in sample handling and preparation all affect qPCR results. That being said, accurate relative quantitations can be achieved. A no-template control should be included in every qPCR assay, and the C_T value for the lower limit of detection should always be greater than that for no-template controls (252). The template for the standard curve should be accurately quantified, and multiple standard curves should be run in each assay, and should be reproducible both within and between assays (252). Research has shown qPCR to be a reliable quantitation technique when compared with other methods. Malinen *et al.* (2003) found similar quantitation results with dot-blot hybridizations and qPCR assays for fecal coliform, but sensitivity was much greater with the qPCR method. In addition, both SYBR-Green I and TaqMan assays yielded similar results in quantifying the same target (170). Stewart *et al.* (2006) compared qPCR quantitation of methanogens in the guts of human and adult children and compared the results to a traditional breath analysis for methanogens. Although similar results were obtained with each method, the

qPCR assay was much more sensitive in identifying low numbers of methanogens in the human gut (261).

IV. Anaerobic digestion

A. Introduction to anaerobic digestion

The anaerobic digestion (AD) of organic wastes is used to reduce pathogens, conserve nutrients, and convert organic matter into a methane-containing biogas which may be used for energy recovery (83). AD can be used to treat any organic waste, but has been found to be most economical for manure treatment, and thus most AD has been used for the treatment of animal waste (58, 59). Although in the U.S. raw manure may be land-applied, AD of manure prior to land application reduces volatile organics, odors, and pathogens, as well as the total amount of waste that needs to be land applied (59). In addition, the retention of nutrients such as nitrogen, phosphorous, and sulfur in the organic fraction prevents water pollution and eutrophication due to runoff, as well as preventing air pollution from methane emissions (58).

In the U.S. aerobic processes are most commonly used for treating municipal wastewater because of the shorter retention times which allow reactor sizes to be smaller (155). Major disadvantages to aerobic treatment of wastewater are the higher energy requirements, especially the cost of aeration, and the higher sludge production compared to anaerobic treatment. Additional advantages to using AD for treating wastewater are lower solid waste handling and disposal costs, and the production of energy-yielding biogas which allows the recovery of some or all of the cost of waste treatment (155). Despite these advantages, AD is most commonly used as a secondary treatment to reduce pathogens and total solids from aerobic process prior to disposal or land application (83). A reluctance to accept AD for municipal wastewater treatment is based in part on the longer retention times, but also on the belief that AD treatment results in odor production and is more unstable than aerobic processes (155). The increasing cost of energy, incentives for use of renewable resources, and the development of high-rate reactor designs have increased the use of AD in primary wastewater treatment (83).

Waste may be treated prior to AD in order to improve COD removal and biogas generation. Generally pretreatment is used to enhance hydrolysis of particulate matter during the AD process (278). Methods may include mechanical ones such as grinding, shredding, or ultrasonic disintegration, chemical treatment with strong acids or bases, treatment with enzymes, thermal hydrolysis, or even the use of shock waves (1, 278). Pretreatment is especially helpful in

treating lignocellulosic wastes which are composed of particulate matter largely inaccessible to microbial enzymes (1).

i. Explanation of terms used and waste treatment goals

Waste entering an anaerobic digester is termed influent, and waste exiting the reactor is the effluent (220). If solids and liquids are separated prior to exiting the reactor, the liquid portion is commonly referred to as the effluent while the sludge removed from the reactor is referred to as wasted sludge. A primary purpose of anaerobic digestion is to reduce organic as well as inorganic material in the waste stream which may occur in soluble, suspended, or particulate form. The suspended and particulate matter which may be collected by filtering is referred to as the total solids and quantified in terms of dry mass per volume. A portion of the total solids are organic and referred to as volatile solids. Organic matter is also classified in terms of the amount of oxygen which would be needed to completely mineralize it to carbon dioxide and water. The chemical oxygen demand (COD) is a reference to the total amount of oxygen needed to react all the organic carbon, but not all organic material is able to be mineralized microbially. The portion of organic matter accessible to microbial degradation is classified instead as the biochemical oxygen demand (BOD) with the COD always equal to or greater than the BOD (220).

Products from AD include the treated liquid effluent, wasted sludge, and biogas. The liquid effluent may undergo further treatment steps, but is ultimately discharged to either surface or groundwater. The wasted sludge from the reactor still contains quite a bit of water, and so this material is dewatered and dried to produce biosolids. Biosolids may be land-applied or landfilled, depending on the quantities of regulated pollutants, such as heavy metals, that may be present. The biogas produced from the reactor is largely composed of carbon dioxide and methane with traces of other gases including water vapor. The use of the biogas depends on its quality and the current needs of the AD facility, but the biogas should never be vented directly to the atmosphere because of the presence of traces of toxic hydrogen sulfide, as well as the methane which has a global warming potential 20-30 times that of carbon dioxide. The biogas yield is the volume of methane obtained per mass of COD destruction during the AD process (220).

Reactor operation is classified, in part, in terms of the operating temperature and the time the waste remains in the reactor. AD reactors are most commonly operated at mesophilic temperatures of 30-40°C (average is 37°C), but may also be operated at thermophilic (50-65°) or, more rarely, at hyperthermophilic (65-80°C) temperatures. Psychrophilic operation refers to any reactor operation at a temperature less than 20°C. The retention time is the amount of time waste

as well as biomass spends in the reactor and is referred to in terms of hours or days. When there is solids-liquid separation of effluent, then retention time is referred to in terms of the hydraulic and solids retention times for the liquid and solid portions of the effluent, respectively. Solids retention times (SRTs) are always in terms of days, but in some high-rate reactors hydraulic retention times (HRTs) might be on the order of minutes or hours. The retention time is also dependent on the operating temperature with increasing operating temperature generally correlated with shorter retention times. The rate at which waste, also referred to as substrate for the microbial community, enters the reactor is termed the organic loading rate and is described as the mass of COD applied per reactor volume per day.

ii. Production and use of biosolids

Biosolids refer to the dewatered, dried solids from waste treatment which are high in organic matter as well as nutrients such as nitrogen, phosphorous, and sulfur (58). The easily degraded organic fraction and pathogen numbers have been substantially reduced during the AD process, so the biosolids are referred to as "stabilized". Regulations for the use and disposal of biosolids are found in Title 40 of the Code for Federal Regulations, Part 503. There are generally three routes of disposal for biosolids: land-application on croplands, land-application on land not used for growing crops or in a landfill, or incineration. Regulations were designed to allow biosolids to be used for a beneficial purpose while still protecting public health, and therefore apply to the levels of potential infectious agents in the biosolids. Biosolids may be classified as either "Class A" or "Class B" according to pathogen concentration. Class A biosolids have no detectable levels of pathogens, whereas Class B biosolids have low, but detectable levels of pathogens. Class B biosolids have restrictions on where and when they may be land-applied, but Class A biosolids have no such restrictions and may be sold as any other fertilizer or fill soil would be. The operation of the AD system is vital in determining the pathogen load in the resulting biosolids. Much research has suggested that thermophilic AD is able to meet Class A standards more easily than mesophilic or psychrophilic AD, but an increase in the retention time may be adequate for the production of Class A biosolids from psychrophilic and mesophilic reactors (1). During the AD process, greater pathogen destruction has been correlated to higher VFA, ammonia, and sulfide concentrations in the reactor, or when the operating pH is alkaline. In addition, further pathogen reduction may be achieved by composting or irradiating the biosolids (1).

iii. Production and use of biogas

With energy prices continuing to increase, one of the most valuable products of the AD process is the biogas. Typically about 50% of the organic matter introduced into an anaerobic digester is converted to biogas (58) with a theoretical yield of 0.35 m^3/kg COD removed at standard temperature and pressure (190). Biogas is mainly a mix of methane and carbon dioxide with traces of other gases including hydrogen sulfide, nitrogen, hydrogen, oxygen, carbon monoxide, ammonia, and volatile organics. Hydrogen sulfide is a concern not only because of its odor and toxicity, but also because it is corrosive to metals and may damage gas lines and generator equipment if not removed prior to use (190). In addition, there is quite a bit of water vapor in biogas which must also be removed. The quality of the biogas, which is based on the percent of the biogas composed of methane, affects the heating value, air-to-fuel ratio, and minimum auto-ignite temperature, all factors which affect the end-use of the biogas (190). Carbon dioxide and hydrogen sulfide may be removed from biogas by passing through columns packed with specifically absorbent materials or under alkaline conditions. Pure methane has a heating value of 50 MJ/kg, or 35.9 MJ/m^3 at STP, but biogas is typically 50-70% methane so the heating value is less. Flammable biogas is 5-12% methane in air, and a biogas of less than 25% methane will not burn (190).

The biogas from AD has several potential uses and can represent a significant economic offset to the cost of waste treatment. Biogas may be used directly in boilers or other heating devices, combusted in gas generators for the production of electricity, and used in combined heat and power (CHP) plants. Additional reformation of biogas to enrich for methane can produce a gas that may be used in municipal gas lines, used as a fuel for compressed natural gas vehicles, or reformed to produce hydrogen for fuel cells or SynGas, for “synthesis gas”, a mixture of hydrogen and carbon monoxide (190). In rural areas of the world, biogas is often used directly for electricity production, lighting, heating, and cooking with little clean-up performed (59). Dilute biogas is non-combustible and represents a loss of energy. For biogas with a low methane percentage, solid oxide fuel cells (SOFCs) may be a way to convert poor quality biogas to electricity (277). Under high temperatures, SOFCs convert the methane and carbon dioxide in biogas to SynGas which may then be used for electricity production. Typically biogas conversion to electricity is about 20% efficient, but SOFCs have efficiencies of 30-40% (277).

iv. Reactor design and operation

There are three basic reactor types; batch, plug-flow, and continuously stirred tank reactors (CSTRs) (220). A batch reactor involves sequential treatment steps in which waste enters the digester, treatment occurs during a reaction phase, and the treated waste is then removed from the digester. A plug-flow design introduces waste at one end of the reactor and allows it to flow the length of the reactor as treatment takes place such that reaction is completed and treated effluent exits the other end of the reactor. In a CSTR, the contents of the reactor are mixed such that the concentration of material in the reactor is equal to that in the effluent exiting the reactor. For all three of the above designs, a solids-liquid separation typically follows treatment with the treated liquid effluent eventually discharged to surface or groundwater systems and wasted sludge processed for use as biosolids. For the plug-flow and CSTR designs feeding and wasting, and even mixing may be continuous or occur periodically. Early AD designs showed poor efficiency in treating waste, and later designs sought to not only improve the rate and quality of waste treatment but also the capture and use of biogas (101). Improved waste treatment was met by increasing the retention time of the active microbial population as well as increasing the contact of the active biomass with the incoming waste through proper mixing. Both of these goals were largely attained though the separation of the hydraulic and solids retention times thereby allowing a relatively short hydraulic retention time and a much longer solids retention time (101). The type of reactor system will depend upon the nature of the waste being treated as well as the end-use for the products of AD.

Anaerobic digesters may be inoculated from already existing reactors, or may be started without the use of an exogenous inoculum from the waste material itself. The latter technique is common for manure digesters and municipal wastewater digesters as fermenters and methanogens are common microflora of a variety of animals including humans (82, 197, 223, 261, 273, 287). A new reactor inoculum taken from an existing reactor operating at the same temperature and treating the same waste reduces the start-up time (57, 243). Start-up time is also reduced when the number of active microorganisms added to a new reactor is high in comparison to the amount of substrate being digested (84, 178). The active biomass in the reactor may exist in a suspended, or flocculent, form consisting mainly of individual cells and small clumps of cells aggregated with waste particles, as larger aggregates of cells that form layered granules, or as a biofilm attached to an inert material in the reactor.

a. Continuously-stirred tank reactors

CSTRs are traditionally operated with continuous feeding, wasting, and mixing so that ideally concentrations of waste and active biomass are equal throughout the reactor, and the microorganisms produced during waste treatment equal those removed from the reactor in the effluent (220). Part of the wasted biomass may be recirculated back into the reactor to maintain a higher biomass concentration and increase the biomass retention time in the reactor. CSTRs are also referred to as chemostats, and are frequently used to determine the kinetics of microbial growth and metabolism (2, 220). Researchers have examined variations on these processes in lab-scale CSTRs in order to optimize AD waste treatment in these types of reactors. The start-up of CSTRs usually involves an initially long retention time to allow sufficient growth of active biomass, with retention time gradually lowered to the desired operational value (74, 135). Feeding or wasting may be conducted periodically instead of continuously, but Kim *et al.* (2002) found that continuously fed lab-scale CSTRs were more stable during variable organic loading rates than reactors which were instead fed once daily. Both continuously-fed and daily-fed CSTRs were more stable than batch-fed reactors which showed greater pH variations between feedings than did the CSTRs (137).

Mixing may also be continuous or periodic, although periodically mixed systems are referred to instead as semi-continuous reactors. Several researchers have compared continuous mixing to minimal or periodic mixing regimes in which reactors are only mixed after feeding and prior to wasting, or are mixed for short periods of time once to several times daily. Periodically mixed reactors have more stable operation, especially during sudden changes in substrate concentration in the reactor, as well as removing more COD and generating more methane than continuously stirred reactors (128, 137, 263). Periodic mixing as well as feeding and wasting is more common in full-scale operations due to the reduced cost and greater ease of operation over continuous systems (101).

b. Plug-flow reactors

The earliest anaerobic reactors were septic tanks and anaerobic ponds or lagoons (101). All of these designs are basically plug-flow reactors, with waste entering one end and treated effluent exiting the other. These reactors were designed to capture and retain solids only as early in waste treatment technology the removal of non-solid components of waste was not recognized as important in environmental and human health protection (101). Waste flows horizontally through the top part of a septic tank and solids settle to the bottom where they are further degraded microbially. Some plug-flow reactors incorporate areas of mixing to eliminate dead

zones in the reactor and to increase contact between the retained biomass and the influent waste. Effluent from the septic tank is infiltrated into the soil through a leach bed as part of further waste treatment (169). Imhoff tanks, named for their designer, are 2-chambered septic tanks with an upper chamber used to enhance solids retention and a lower chamber to allow digestion of the solids (101). Anaerobic ponds or lagoons are open-air shallow earthen dams with plastic or clay liners to prevent soil infiltration. Waste flow is also horizontal along the length, and typically several ponds or lagoons are placed in series to enhance waste treatment (101).

Septic tanks and anaerobic lagoons and ponds are open to the atmosphere, allowing both biogas escape and oxygen infiltration. Oxygen that enters these systems is quickly scrubbed by facultative anaerobes allowing deeper layers to remain anaerobic (169). Traditionally, these designs have low removal efficiencies and biogas is not captured for energy recovery, but current plug-flow AD designs have improved waste treatment efficiency and are covered to capture biogas (101). The major problems with plug-flow reactors are the formation of a frothy scum on the surface and the accumulation of inorganic solid material at the bottom that periodically needs to be removed (169).

c. Anaerobic sequencing batch reactors

The anaerobic sequencing batch reactor (ASBR) is based on a traditional batch reactor design but current technology allows automation of the waste treatment steps. Waste treatment is performed sequentially as feeding, reaction, settling, and liquid withdrawal (295). Several ASBRs can also be operated in parallel so that while one reactor is being fed, one is undergoing reaction, another is undergoing settling, and treated effluent is being removed from another (220). This allows flow into and out of the reactor system to be continuous even if the individual reactors are operated as batch reactors. The ASBR technology was developed to improve the retention of the active biomass in the reactor as well as improve process control, especially in terms of the feeding rate. Reactors may go through several reaction cycles a day or one reaction cycle over several days or even weeks (173). The length of the reaction cycle can be extended or shortened to accommodate changes in the type or concentration of substrate or the operating temperature of the reactor (173). During the reaction step, the mixing rate may also be adjusted to ensure a good substrate to biomass contact (295). This flexibility in operation make ASBRs suitable for treating a variety of wastes and even combining aerobic and anaerobic treatment in one cycle of the reactor (220).

d. Fixed-film reactors

Fixed-film reactors depend on the colonization of an inert support material with an anaerobic microbial biofilm. Waste is introduced into the bottom of the reactor and flows upward through the bed of colonized material where treatment takes place with treated effluent exiting the top of the reactor. This design allows separation of the hydraulic and solids retention times both by retaining active biomass in the reactor as biofilm and because particulate matter is filtered from the waste stream as it flows through the support material (101). The anaerobic filter was the first design of such a reactor in which a heavy support material composed of stones or gravel, bricks, or plastic pieces is placed in the bottom of the reactor and serves as a site for the anaerobic biofilm to grow (101). The heavy nature of the inert material allows it to remain in the reactor even under high loading rates which result in rapid waste flow rates through the reactor. Reactors with immobilized biomass have been reported to withstand the stress of changing substrate loading conditions better than reactors with a flocculent, or suspended, biomass (212). Several support materials have been tested for use in fixed-film reactors, and although gravel, bricks, and clay media have all been successfully used, material with high porosity and high-surface area, such as specially produced plastic media, produce faster waste treatment and greater methane yield (279). An additional concern in the choice of media for a fixed-film reactor is the flow of wastewater through the reactor. If the flow rate in a fixed-film reactor is too great, flow goes from laminar to turbulent, increasing the shear stress and reducing the thickness and compactness of the biofilm as it is washed off the support media (155).

A variation on the fixed-film reactor is a membrane reactor which utilizes a membrane with a pore size smaller than that of the anaerobic microorganisms to retain the active biomass in the reactor (155). Membrane reactors are subject to fouling and scaling in which the membrane becomes coated with a biofilm or inorganic material and prevents the waste from passing through the membrane. In addition to keeping the membrane clean, another challenge of operating these reactors is the high hydraulic pressures needed to force the waste through the membrane to allow for solids-liquid separation (155).

e. Granular sludge reactors

Wastewater treatment engineers discovered that under the right conditions, anaerobic microbes would form granules of 1-5 mm characterized by a high density, and thus a high settling velocity, as well as a high mechanical strength (101). The properties of these granules allow them to be retained in a bioreactor with high upward flow velocities of waste without the use of an inert support material for colonization. The first granulated sludge reactor used for waste

treatment was the Upflow Anaerobic Sludge Blanket (UASB) designed and developed by Dr. Gatze Lettinga (83). The UASB was popularized for direct treatment of municipal and industrial wastewater in the 1980's in the Netherlands. Granulated reactors, also referred to as fluidized bed reactors, are operated much in the same way as fixed bed or fixed film reactors that utilize a support material. In the UASB design, waste is introduced equally along the bottom of the reactor and pumped upward through the sludge granules where treatment occurs with treated effluent exiting the top (101). The upflow velocity fluidizes the granules into a suspended bed of active microbial biomass referred to as the sludge bed. A phase separator is often used in the upper portion of the reactor which increases the retention of the granules. A later variation on the UASB is the Expanded Granular Sludge Bed (EGSB) which was designed to circumvent some of the problems of the UASB such as the development of preferential flow paths, hydraulic short circuiting, and dead zones. The EGSB uses a higher upflow velocity to further expand the sludge bed and improve mixing to promote better contact of the granules with the influent substrate (101, 169). The success of the UASB in treating dilute waste streams such as municipal and industrial wastewaters is due to large separation in hydraulic and solids retention times, thereby allowing retention of the slow-growing anaerobic biomass but treating a large-volume of wastewater in a relatively short time and with a small reactor footprint (101). The operating challenge for fluidized bed reactors is retention of the granules when treating a low COD wastewater. A dilute or low COD wastewater requires a large upflow velocity to achieve acceptable treatment rates (157). As with the anaerobic filter, if the upflow velocity is too great, flow may change from laminar to turbulent causing damage to the granules and a greater loss of active biomass in the effluent of the reactor (157).

If a granular sludge is not used as an inoculum for the reactor, the proper start-up conditions are important to allow the development of an active granular biomass with the right characteristics. Researchers have found the most effective strategy for start-up with flocculent or suspended biomass is to gradually increase the organic loading rate, and thus the upflow velocity, in a step-wise fashion while recirculating a portion of the effluent to better retain biomass in the reactor (57, 299). This places a strong selection pressure on the anaerobic microorganisms to form granules and non-granulated biomass is eventually lost from the reactor. The solids concentration in the effluent waste needs to be kept low as a high solids concentration during start-up inhibits granulation (57).

Granular-sludge reactors are generally better at withstanding periodic increases in substrate loading, but are sensitive to the solids content in the influent waste even after the start-up period (101, 299). If the solids content of the influent waste changes suddenly, excess solids

become trapped in the sludge bed causing the compaction of the sludge blanket and trapping the gas produced in the sludge bed. Accumulation of gas in the sludge bed increases its buoyancy and eventually leads to sludge washout (299). As the reactor temperature decreases, the effect of the solids content of the waste is increased because hydrolysis of particulate matter slows and solids accumulation in the sludge bed increases (169). Most granular reactors are operated at mesophilic or psychrophilic temperatures because researchers have found it difficult to obtain and maintain good granule quality at thermophilic temperatures (299). Increasing the operating temperature of a UASB from the mesophilic to thermophilic range resulted in granule disintegration and eventual reactor failure in one study. These researchers found the greatest granule strength and resiliency to changes in operating conditions in reactors operated at temperatures ranging from 24-50°C (299).

f. Multi-stage reactors

Anaerobic digesters may exist as stand-alone treatments or may be used in series with other reactors for waste treatment. There are several possible schemes for multi-stage reactors in anaerobic digestion (294). The first and most common is acid-phase digestion which consists of at least two reactors differing in retention time and operational characteristics. Hydrolysis of particulate matter and fermentation to VFAs largely takes place in the first reactor, referred to as the acidification reactor, which is smaller with a shorter retention time, high VFA concentration, and acidic pH. Little methane is produced in the first reactor because the retention time is typically too short to retain methanogens, and the acidic pH inhibits methane production (169, 294). Methanogenesis mainly takes place in the second reactor which is larger and has a longer retention time and cirucumneutral pH (219). Acid-phase reactors may improve the rate of waste treatment by trapping and partially hydrolyzing particulate organic matter in the first stage, the step which is most often the bottleneck in methanogenesis (83). These type of reactors are particularly advantageous for treating wastewaters with high organic carbon content or rapidly fluctuating organic carbon loads, as the acidification reactor helps buffer the methanogenic reactor against changes in substrate concentration and pH variations (169). Thermophilic AD typically has higher concentrations of VFAs than mesophilic AD, and thermophilic acid-phase reactor systems help to reduce the concentration of VFAs in the methanogenic reactor (137). The largest disadvantage to acid-phase systems is the accumulation of solids in the first reactor which may eventually cause reactor upset (169).

Other types of multi-stage reactor designs include temperature-phased AD and series mesophilic AD (219, 294, 295). In temperature-phased AD a small thermophilic reactor with a

short retention time is followed by a mesophilic reactor with a long retention time. Although methane may be produced in either reactor, most of the methane is produced in the second mesophilic reactor. Operation of the first reactor at thermophilic temperatures enhances hydrolysis of particulate matter as well as enhancing pathogen destruction (1). Often reactors are used in series to achieve greater COD and solids removal for more stringent effluent quality standards. Mesophilic AD reactors operated in series are one example, but others include a septic tank followed by an anaerobic filter or a UASB followed by either an anaerobic filter, an EGSB, or a CSTR (65, 101, 169). In these examples, all reactors in the series serve as methanogenic reactors with each operated in order to maximize waste treatment (101). Multi-stage AD has been reported to be better than single-stage AD for removing greater amounts of solids and organic matter, especially at lower temperatures (167, 294), producing more methane, and improving the stability of reactors operating at thermophilic temperatures or under fluctuating organic loads (137, 294). The greatest disadvantage to multi-stage AD is greater complexity in running two reactors in series which leads to greater operation-and-maintenance costs, as well as a larger capital investment for installing the system (158).

Another potential use for acid-phase waste treatment is the production of hydrogen and methane together in one system. Biohydrogen for fuel cells and as a transportation fuel becomes more cost-efficient when coupled with a methanogenic reactor treating the effluent waste from the biohydrogen reactor (144). Under these circumstances, hydrogen is collected from the first stage, the acidification reactor, and methanogenesis proceeds mainly from VFA degradation in the second stage (55, 144, 158). For biohydrogen production, methanogenesis needs to be inhibited in the first stage. This may be accomplished by heat-treatment of the inoculum to select for spore-forming *Clostridium sp.*, control of pH in the acidic range, and addition of the general methanogen inhibitor BES (158). Despite these precautions, methane is typically detected in the first reactor and cannot be completely prevented, especially when using non-sterile waste streams as substrate (55, 144, 158).

v. Anaerobic co-digestion of wastes

In an effort to provide a better quality organic feed to an AD system, two or more types of waste may be blended together, referred to as co-digestion. Combining wastes of several different types is often used to balance the carbon-to-nitrogen (C/N) ratio in order to optimize waste treatment and biogas production (102). The optimal C/N ratio in terms of methane production is around 20-30, with a lower ratio resulting in the release of excess nitrogen as ammonia. Ammonia serves as a buffering agent in AD, but is also toxic to anaerobic

microorganisms at increased concentrations. At ratios greater than 20-30 there is not enough nitrogen to promote cell growth and maintenance, and a large portion of the COD in the waste goes untreated (102). Combining a high nitrogen waste such as animal manure with a low nitrogen waste, such as fruit and vegetable remains, was found to improve methane production and effluent quality from an anaerobic digester (102). Co-digestion of meat industry wastewater with fruit and vegetable waste enhanced the buffer capacity and reduced the effect of ammonia inhibition in another study (28). In another study, municipal solid waste, which is largely lignocellulosic, was successfully co-digested with sewage sludge, a nitrogen-rich waste, from a municipal wastewater treatment plant (202).

In Europe, wastes from agriculture, industry, and municipal solid waste as well as wastewater treatment plants are often combined in centralized AD plants (278). The use of AD in the United States is largely decentralized, so that individual farms, landfills, or wastewater treatment plants have their own reactors on-site. Decentralized AD systems may treat only one type of waste, typically manure or municipal wastewater sludge, leaving material from other areas of agriculture or industry largely unexploited for energy production (28, 284). Crop residues and other lignocellulosic wastes as well as the protein-rich wastewater of the meat processing industry are particularly difficult to process but co-digestion increases the rate of conversion to biogas (28, 284). Co-digestion of wastes from several industries improves the economics of centralized AD by providing a substrate that will maximize methane yield (58, 188). Additionally, transporting waste to a centralized AD reduces the cost of treatment and increases the potential profits from producing biosolids and methane (58, 188).

vi. Causes of anaerobic digester failure and lack of acceptance

Many wastes that could be treated through AD are treated aerobically, leaving potential energy sources untapped. The largest reason cited for not adopting AD for waste treatment is reduced process stability and the increased chance for process failure of anaerobic treatment when compared to aerobic treatment (1, 37, 101). Process failure is usually first seen as a cessation of biogas production, followed by an accumulation of VFAs and an eventual end to all biological activity. A reactor in this state is referred to as a “stuck” or “sour” digester. Reasons for failure include a sudden change in reactor pH or temperature, an influx of toxic or inhibitory substances, or a change in the rate waste enters or leaves the digester which may result in the loss of a large portion of the active biomass (1, 132). Fermentation can proceed much faster than methanogenesis and an imbalance in these two process leads to the accumulation of VFAs faster than their removal by secondary fermenters and methanogens, leading to a drop in pH and a

further inhibition of methanogenesis (137). Some of the substances inhibitory to AD processes include sulfide (135), ammonium (235), long-chain fatty acids (18), and heavy metals (202). High protein wastes increase the amount of toxic sulfide and ammonium in the reactor (1).

AD reactors can be adapted to treat waste under non-optimal conditions or wastes containing large quantities of toxics, but typically treatment is only possible if conditions remain stable and the concentration of toxic in the influent is constant (1). This has limited AD use of rapidly changing waste streams such as municipal or industrial wastewater, although reactors with granulated or immobilized biomass have shown greater process stability under these conditions (1). Variability in the quantity and type of waste affects not only process stability but the quality of the biogas and treated effluent (59). Research has suggested that the addition of inorganic nutrients may aid anaerobic digestion for difficult to treat wastes including wastes containing high concentrations of toxic compounds (13, 205). Some researchers have used mineral supplementation to prevent reactor failure, or even to recover reactor activity after sudden changes in temperature or organic loading rate (13, 18). For some wastes, inorganic nutrients may present in sufficient quantities but be largely unavailable, and addition of these nutrients increases the conversion of COD to biogas as well as the stability of the reactor (137).

If biogas yields are low or the quality of the biogas is poor, then there is less economic gain in using AD for waste treatment (59). For decentralized systems, biogas is often used on-site, but usage depends on a high-quality product. For this reason, energy prices are also a large determinant of how widely AD is used for waste treatment. When energy prices are low, AD is not as economically feasible and the energy cost of aerobic or other waste treatments is also lower, leading many industries to reject AD (238). The introduction of net metering, the use of a utility meter that can run in either direction, for many states has improved the economics of AD greatly. Net metering allows utility consumers to produce energy for, or consume energy from, the local utility grid and pay or be compensated only for the net energy consumed or produced (238).

Besides process stability and biogas and effluent quality, other factors have lead to AD failure and a general reluctance to accept AD technology. These include lack of digester monitoring and maintenance (59, 132), poor digester design (132, 238), and failure of technology transfer or lack of technical support for AD operators (59, 132). Decentralized AD reactors are often located on farms and require the farmer to be the main reactor operator, so technological support is needed in case problems arise or a transfer of farm ownership occurs (59). Also, traditional AD reactor designs were intended for a large amount of waste, such as in a centralized system, and these designs are not always suitable for on-site usage for small waste streams, such

as those that typically occur on farms (238). In addition to improving AD process stability and efficiency, these additional concerns need to be addressed in order to broaden AD.

B. The microbiology of anaerobic digestion

Descriptions of anaerobic digester communities in a variety of reactors have revealed that the type of waste treated in the reactor has little influence on the methanogens detected, but the reactor type, operational conditions, and the time the reactor has been operating do (151). Microbial diversity is typically greater in pilot- or full-scale digesters when compared to lab-scale digesters. The most commonly detected methanogens in all anaerobic reactors are the genera *Methanosaeta* and *Methanobacterium* (150, 151, 159). CSTR reactors often have greater mixing rates and VFA concentrations than other types of reactors, and *Methanosarcina* and *Methanomicrobiales* numbers are greater under these conditions (48, 151). The most commonly detected members of *Methanomicrobiales* are the genera *Methanospirillum* and *Methanocorpusculum* which form relationships with syntrophic *Bacteria* (176, 290). Reactors that are undergoing a start-up period or that are showing poor methane production typically contain higher concentratios of VFAs and this also selects for *Methanosarcina* (60). Fluidized-bed reactors that utilize a granular sludge contain large numbers of filamentous *Methanosaeta* that are important in the granule formation process, but also contain large numbers of *Methanobacterium* (151, 241, 242).

Diversity is generally greater for the *Bacterial* than the *Archaeal* community in a reactor, and the *Bacterial* community fluctuates more over time than the *Archaeal* community (60, 301). Dominant *Bacterial* clades identified in anaerobic digesters include the *Proteobacteria*, especially the delta-*Proteobacteria*, *Firmicutes*, especially *Clostridium*, and the *Cytophaga-Flavobacterium-Bacteriodetes* group (48, 216). The type of waste that is treated has a greater influence on the members of the *Bacterial* community present than it does for the *Archaeal* community, but *Bacteria* are responsible for primary processing of the waste and this places a selection pressure on the community.

The two operational parameters that have the greatest affect on the microbial community are the operating temperature and pH. In general, enzyme kinetics as well as microbial growth and substrate utilization rates increase with increasing temperatures within the typical environmental range of a given microorganism. The microbial consortia of anaerobic digesters are classified according to their optimal growth and metabolism temperatures as mesophilic (30-40°C), thermophilic (45-65°C), hyperthermophilic (65-80°C) and psychrophilic (<20°C) (1, 37). Most fermenters and methanogens isolated from AD reactors, regardless of operating

temperature, have been found to be mesophilic (278). The vast majority of anaerobic digesters are operated at mesophilic temperatures both because of the dominance of mesophilic organisms in methanogenic communities, but also because this operation usually achieves the best balance between the expense of heating a digester and the rate of processing the waste. The reactor pH is also crucial to effective methanogenesis and is the most oft-cited reason for reactor failure (13, 18, 137). Even though individual organisms in the reactor consortia may have different optimal pH values for metabolism, the optimal pH for anaerobic digestion has been found to be 6.3-7.8 (74). Volatile fatty acids are produced as part of the methanogenesis process so control of reactor pH typically involves preventing acidification. There are advantages to operation of anaerobic digesters outside of mesophilic and circumneutral pH ranges, and this has led researchers to examine how operating temperature and pH not only affect reactor operation, but the microbial community as well.

i. The effect of temperature

Although the vast majority of AD reactors are operated at mesophilic temperatures, but there are some advantages to operation at non-mesophilic temperatures. Anaerobic digestion at lower temperatures reduces the heating costs for the digester, as well as the temperature of the effluent waste stream. For on-site waste treatment of municipal or industrial wast water and release of treated effluent to natural water bodies, effluent streams must be near ambient temperatures which makes psychrophilic treatment more desirable (157). Lower temperatures require longer retention times which mean larger reactors and a larger footprint for the process which is not always possible or desirable (21). This is the largest advantage to AD at thermophilic or hyperthermophilic temperatures because of the much shorter retention times and thus smaller reactor footprints. The disadvantage to treatment at increased temperatures is the greater cost for digester heating and increased process instability (137).

a. Thermophilic operation

Thermophilic anaerobic digestion refers to reactor operation at temperatures of 45°C or greater. Some of the advantages to using thermophilic AD rather than mesophilic AD for waste treatment include shorter retention times and thus smaller reactor size, a greater reduction of solids and pathogens, as well as better solids-liquid separation which reduces costs for biosolids production (7, 137). Microbial growth yields are also typically lower at thermophilic temperatures which reduces the amount of solids produced in the process, and therefore increases the amount of organic material converted to biogas (137). Some of the disadvantages to

thermophilic AD include greater energy inputs for heating the digester, a longer start-up period, reduced process stability, and increased susceptibility to toxics in the influent waste stream or sudden changes in environmental conditions including organic loading rate and temperature (74).

Despite faster waste treatment kinetics, thermophilic reactors often suffer from increased concentrations of ammonia as well as VFAs (74, 137). Total ammonia as well as the portion that exists as free ammonia both increase with increased operating temperature, but toxicity of free ammonia to the microbial community is much greater than that of the ammonium ion (74). Changes in organic loading rate in thermophilic reactors cause an imbalance between fermentation and methanogenic processes, resulting in increased VFAs which can cause process instability. For these reasons, thermophilic reactors are more sensitive to both reactor configuration and operation than mesophilic reactors. Operation of thermophilic reactors is best under continuous organic loading rate and low mixing intensities (137). Multi-stage thermophilic AD systems are often more stable and efficient than one-stage systems, and this effect is greater than for mesophilic systems (137).

The long start-up period for thermophilic anaerobic digesters, as well as the accumulation of VFAs and process instability, has largely been attributed to the operation of these reactors without the use or adaptation of an appropriate thermophilic microbial community (1, 7, 137). Ideally, an inoculum for any AD system should be taken from a reactor that is operated at similar temperature and treating a similar waste stream. If a thermophilic AD reactor is inoculated with a mesophilic community, or is started with just the microbes associated with the influent waste material, then a sufficiently long time is needed to allow the reproduction of the relatively small percent of the microbial community that can grow and metabolize under thermophilic conditions (1, 7). Adaptation is typically accomplished through a step-wise increase in the operating temperature from mesophilic to thermophilic conditions. To prevent process failure from substrate overloading and VFA accumulation, it is important to limit substrate early in the adaptation process and gradually increase the organic loading rate as the active biomass increases (7).

Even if a microbial community from a mesophilic inoculum can be established for a thermophilic reactor, it is likely that the microbial consortia is thermotolerant and not thermophilic, resulting in the process instability associated with thermophilic AD (7). Nozhevnikova *et al*. (1999) examined the long-term microbial adaptation to anaerobic waste treatment at temperatures ranging from 55-82°C. The inoculum for all incubation temperatures was a mesophilic AD community, and serial batch incubations were maintained for up to 15 months. The researchers observed methanogenesis up to 73°C, but it was not sustained and

subsequent batch cultures showed no methane production. Stable methanogenesis was only achieved at temperatures up to 55°C (192).

Molecular analysis of anaerobic digester communities has generally shown microbial diversity, especially methanogen diversity, to be greater at mesophilic than thermophilic temperatures (129, 243). In general, methanogens identified in thermophilic anaerobic digesters are commonly identified in mesophilic reactors as well. Both *Methanosarcina* and *Methanosaeta* have been observed in thermophilic anaerobic digesters, but the methanogenic community is generally dominated by hydrogenotrophic methanogens belonging to the genera *Methanobacterium* and *Methanothermobacter* (1, 42, 129, 243, 269). *Bacterial* community members that have been found in both mesophilic and thermophilic reactors include *Clostridium*, *Propionibacterium*, *Thermodesulfovibrio*, *Desulfovibrio*, *Desulfolobus*, *Syntrophobacter*, and members of the delta-*Proteobacteria*, which have been reported to dominate mesophilic *Bacterial* reactor communities (242, 243, 269). Other genera identified in thermophilic reactors include *Coprothermobacter*, *Pelotomaculum*, *Desulfonispora*, *Symbiobacterium*, *Thermoacetogenium*, *Pseudomonas*, *Empedobacter*, *Sporomusa*, and *Arcobacter* (269). Thermophilic reactors have been reported to contain more members of the *Clostridium*, *Thermotagales*, green non-sulfur bacteria and *Thermodesulfovibrio* than mesophilic reactors (42, 243, 269). Clones related to green non-sulfur bacteria, *Thermodesulfovibrio*, and *Desulfotomaculum* were isolated from thermophilic UASB granules in co-cultures with *M. thermoautotrophicum* degrading carbohydrates and fatty acids, suggesting their roles as syntrophic partners of hydrogenotrophic methanogens (242).

b. Psychrophilic operation

Psychrophilic AD refers to any reactor operating at a temperature of 20°C or less (157). The main advantages to using psychrophilic AD is the reduced energy inputs for digester heating, and the production of effluents that do not need to be cooled to ambient temperature prior to discharge in natural water bodies (53, 157). The main disadvantage to psychrophilic AD is the longer retention times needed for waste to be degraded (132, 157). There are other challenges for psychrophilic AD, including the increased solubility of gases and the increased viscosity of liquids are reduced temperatures. As the temperature drops and the solubility of gases increases, it becomes more difficult to harvest methane from the wastewater and the increased concentrations of methane in the waste can reduce the rate of methanogenesis (53). The increased solubility of carbon dioxide might reduce the reactor pH to an unacceptable level (157). An increased liquid viscosity means more energy is needed for mixing and pumping waste, and

solids-liquid separation becomes more difficult as particles settle more slowly (157). A lower temperature also means diffusion is slower, and as hydrolysis of particulate matter is largely catalyzed by extracellular enzymes, the rate of hydrolysis is also slowed (5).

Despite these challenges, psychrophilic AD can produce the same quality effluent, biogas, and biosolids as mesophilic or thermophilic AD (5). Some researchers have even reported higher methane concentrations in biogas from psychrophilic reactors in comparison to mesophilic ones treating the same waste (53). Granulated or fixed-film reactors are popular for psychrophilic waste treatment because of their ability to operate with very different solids and liquid retention times. Granular reactors treating brewery wastewater at 37 and 15°C showed similar COD removal rates at the same upflow velocity (53). Other research has shown similar results, with psychrophilic granular reactors able to treat wastewater at the same rate as mesophilic ones (175). This statement holds true as long as the organic loading rate does not exceed the capacity of the reactor to remove organics from the waste stream. If this capacity is exceeded, VFAs accumulate and the reactor will eventually fail (52, 177). This is true for any AD reactor, but because of the reduced kinetics at low temperature the point where the reactor capacity is exceed is at a lower organic loading rate for psychrophilic AD than for mesophilic AD.

As with thermophilic AD, the development of a psychrophilic or psychrotolerant anaerobic consortia is vital to efficient and stable reactor operation. Inocula are often from a mesophilically-operated reactor and adapted for psychrophilic waste treatment (52, 53, 175, 177). Similar to the development of a thermophilic biomass from a mesophilic one, the development of a psychrophilic biomass requires gradual adjustment of both temperature and organic loading rate as the activity of the biomass increases (175, 177, 249). The biomass that develops is always what has been termed psychrotolerant instead of truly psychrophilic, with the active biomass showing optimal methanogenesis at 35-37°C (52, 175, 192). Adaptation to psychrophilic temperatures does increase the activity of the microbial consortia at psychrophilic temperatures (134, 177). With sufficiently long adaptation periods, several researchers have obtained AD microbial communities able to digest waste at psychrophilic temperatures at the same rates as the community was originally digesting waste at mesophilic temperatures (192, 249).

Recent research has suggested the introduction of two new terms describing cold-adapted microorganisms based on growth range and maximal growth temperature. Stenopsychrophiles, also referred to as “true” psychrophiles, are organisms with a narrow range of growth restricted to cold temperatures, whereas eurypsychrophiles, also referred to as psychrotolerant, have wider growth ranges and larger maximum temperatures for growth (36). Often eurypsychrophiles have

optimal and maximum growth temperatures well above the typical *in-situ* temperature of their habitats, but this fact does not suggest a lack of adaptation to cold temperatures. For example, the growth rate of the eurypsychrophile *Methanococcoides burtonii* (maximum growth temperature 28°C) at 4°C is greater than the growth rate of the stenopsychrophile *Methanogenium frigidum* (maximum growth temperature 18°C). Adaptation to a wide temperature range makes sense in places such as permafrost, which experience annual freeze-thaw cycles and temperatures ranging from -45 to 25°C, or deep-sea sediments where microorganisms may be carried by currents to warmer parts of the water column (36). Cold-adapted organisms survive and even thrive in changing temperatures by the production of antifreeze proteins or cryoprotectants, the production of more unsaturated lipids in cell membranes, and the production of cold-adapted proteins (77). Cold-adapted proteins are characterized by an increasing size of the active site to make it more accessible to ligands and easier for products to exit, and a reduction in the overall stability of the enzyme itself to prevent rigidity. These and other changes allow more rapid processivity at cold temperatures (77) (Feller 2003).

This research suggests that efficient psychrophilic AD is not dependent upon culturing of psychrophilic organisms, but rather psychro-adaptive organisms which can adjust metabolism as temperatures drop to maintain a similar level of activity. This research also partially explains why methanogen communities from psychrophilic reactors often contain the same organisms as mesophilic ones. Researchers have reported the dominance of members of the *Methanomicrobiales*, especially the *Methanocorpusculum*, as well as members of the *Methanosaeta* and *Methanobacterium* (50, 52, 175, 176, 290). *Crenarchaeota* and beta-*Proteobacteria* have also been reported as dominant members of psychrophilic reactor communities (50, 52).

c. Temperature changes

Occasionally reactors may be subjected to sudden changes in temperatures, such as when a heating element fails or when environmental temperatures change rapidly in unheated digesters. This is more likely to occur in small, decentralized AD systems such as those treating farm or industrial waste (173). A increase or decrease in temperature is characterized by reduced methane production, accumulation of VFAs resulting in a drop in the reactor pH, and reduction of the rate of COD removal (155, 173, 177). A drop in temperature of 5°C or greater in reactors operating within the range of approximately 18-35°C is accompanied by an initial cessation of methanogenic activity, followed by a gradual increase to original operation rates at the new temperature (37, 155, 173, 175). This is consistent with the theory of eurypsychrophilic

organisms which can adjust their cell machinery to deal with decreasing temperatures. Although operation of psychrophilic AD at temperatures down to near freezing is possible, rates do not mimic those of mesophilic AD when operated below about 18°C (173, 175, 177, 192).

An increase in temperature of greater than 5°C also causes a temporary cessation of microbial activity as the microorganisms adjust with the transitory accumulation of VFAs and COD. If the reactor is operating in a psychrophilic to mesophilic range, this increase in temperature results in fairly rapid recovery of activity to previous levels (37, 173, 278). A shock that increases the temperature above the maximum growth temperature of the microbial consortia increases the decay rate thereby decreasing the amount of active bacterial mass in the digester and delaying or preventing recovery of activity (74, 155). As the temperature of the shock increases, the number of microorganisms capable of survival at that temperature also decreases, which prolongs the recovery of digester activity until the adaptive microorganisms can reproduce to acceptable levels (74). An additional challenge in digester recovery under increasing temperature shocks is that levels of total ammonia as well as free ammonia increase as temperature increases, and both are toxic to the microbial consortia (74). Temperature shocks have been shown to impose stability on thermophilic AD reactors, which are considered more unstable than either psychrophilic or mesophilic AD reactors. El-Mashad *et al.* (2004) found that for reactors operated at 50°C, periodic upward or downward shocks of 10°C imposed stability. The researchers also found this trend for periodic downward, but not upward, shocks of 10°C imposed on reactors operated at 60°C (74).

ii. The influence of pH

Methanogenesis is a balance between VFA production and consumption by different trophic groups, and any change in reactor operation may disrupt this balance can cause a drop in pH. Changes in reactor temperature or mixing intensity, or sudden increases in the feed rate or substrate concentration, reduce methane production and cause the accumulation of VFAs and a resultant drop in pH (128, 173, 18, 137). A change in the feed rate affects reactor pH both because of the change in retention time of active biomass and because of the change in concentration of fermentable material in the reactor. A decrease in solids retention time, and thus biomass retention, is of greater impact to the slower-growing methanogens and syntrophs than the faster-growing primary fermenters (55, 144, 158). In addition, primary fermenters can produce VFAs faster than syntrophic fatty acid-oxidizers and methanogens can consume them. This imbalance is usually constrained by hydrolysis which is most often the rate-limiting factor in AD, but if hydrolysis is not rate-limiting, than an increase in substrate may overwhelm the VFA-

consumption processes causing VFA accumulation and a drop in pH. The nature of the substrate also impacts the reactor pH by influencing the buffer capacity in the reactor which is primarily due to VFAs, bicarbonate, and ammonia (74). Lignocellulosic wastes and other wastes with a high carbon-to-nitrogen ratio are difficult to treat in anaerobic digestion because they offer little buffering from the release of excess nitrogen as ammonium (13). Co-digestion with a nitrogen-rich waste, such as manure, or the use of a multi-stage reactor may be used to prevent reactor upset due to acidic pH (13).

Anaerobic digestion is an alkalizing process because VFAs are consumed and alkalinity increases through the production of CO_2 during fermentation (204). This explains why effluent pH is higher than influent pH in reactors with high COD removal rates. During these reactor upsets methane production drops as VFAs continue to accumulate which has led to the belief that methanogens are the most sensitive members of the reactor consortium to acidic pH (13, 18, 137). But methanogens capable of growth and metabolism at acidic pH have been isolated from both natural and constructed environments (25, 230). Some methanogens in the microbial consortia may be sensitive to reduced pH, but it is likely that other members of the consortium such as the syntrophic fatty acid-oxidizers are also sensitive. The toxicity of VFAs in anaerobic digestion is dependent both on their concentration and the reactor pH (18). Toxicity at acidic pH arises from undissociated VFAs which may pass the cell membrane where they dissociate at the circumneutral pH in the cell, and cause an uncoupling of the proton motive force. Anaerobic digestion can proceed well at acidic pH when there are little to no VFAs present (278). The toxicity of VFAs increases with a decrease in pH, and differs with the type of acid as propionate is much more toxic to anaerobic digestion than acetate or butyrate, regardless of pH (16, 120, 229).

Despite problems with toxicity, anaerobic digester consortia have been adapted to treat a variety of wastes at acidic pH, including high-VFA wastes. Acclimation is slow and typically involves step-wise reductions in operating pH either through control of the feed rate or artificial reduction with acid such as HCl (123, 204, 265, 266). Patel *et al.* (2000) adapted a fixed-film reactor to treat a petrochemical waste of pH 2.5 without prior neutralization by gradually increasing the feeding rate. COD removal efficiency was so great in the adapted reactor that effluent pH was 7.5-8.5 (204). Jain and Mattiasson (1998) adapted an anaerobic digester inoculum to produce methane in batch culture at pH values as low as 4. A non-acclimatized culture subjected to a sudden pH decrease from 7 to 4 ceased methane production for over a month. When the culture acclimatized to methanogenesis at pH 4 was subjected to an increase in pH to 7, methane production at first ceased for 2-3 days, but returned to normal within one week.

A return to an operational pH of 4-4.5 showed the same pattern, with initial suppression of methanogenesis and a subsequent return to normal methane production within one week. The authors found that although methane production was slower at acidic pH, methane yields were greater (123). Similar results were found by Taconi *et al.* (2007) who examined batch degradation of acetate by an anaerobic digester consortium at pH 4.5 and 7. Methanogenesis took twice as long to complete at pH 4.5, but methane yields were greater (265). The authors then went on to adapt another methanogenic consortium to treat an acetate waste at acidic pH by step-wise reduction in operating pH from neutral to 4.5 with the addition of HCl. Although COD degradation was comparable at all pH values, the decrease in pH decreased COD removal and methanogenesis rates so that treatment was slower (266).

a. Organic overloading and pH changes

One of the challenges of anaerobic digestion is dealing with an inconsistent waste stream to the reactor in terms of sudden changes in type of substrate or feed rate (52). An easily fermentable, soluble substrate is more quickly converted to VFAs than complex, particulate substrate (155). Increases in substrate concentration may cause an increase in hydrogen and carbon dioxide in the biogas as well as the accumulation of VFAs, which cause shifts in the metabolic pathways used for substrate processing (34, 155). Few researchers have examined the effects of substrate perturbations and subsequent pH changes in anaerobic reactors. Even when pH is controlled, addition of excess substrate may cause inhibition of methanogenesis or even reactor failure. Paulo *et al.* (2003) studied a thermophilic CSTR treating a methanol wastewater subjected to periodic increases in substrate concentration resulting in a temporary cessation of methanogenesis, accumulation of VFAs, and drop in pH. Restoring the pH to neutral did not immediately result in recovery, and a return to normal methanogenesis rates from the overfeeding was slow (205). Dupla *et al.* (2004) conducted a similar a experiment with a fixed bed reactor treating vinasses wastewater and subjected to periodic increases in organic loading both with and without pH control. When pH was controlled at 6, the reactor rapidly recovered to the increase in organic load. When the organic load was increased without pH control, it dropped to approximately 5.3 and the reactor was not able to recover without addition of alkalinity to raise the pH to circumneutral levels (70). A successful adaptation of anaerobic digester biomass to periodic substrate overloads was described by Xing *et al.* (1997) who operated a glucose mesophilic CSTR under periodic substrate perturbations for over 200 days. Initially after the perturbations were begun, COD removal and methane production rates were low and VFAs

accumulated, but after 160 days of operation the reactor returned to pre-perturbation rates and was able to handle the overloads with no decrease in performance (288).

The microbiology of a reactor subjected to organic loading rate shocks has an enormous effect on the ability of the reactor to handle periodic changes in substrate concentration. Regardless of substrate concentration, easily fermentable carbohydrates such as glucose are completely converted by primary fermenters during organic loading rate shocks suggesting these members of the reactor consortia are not strongly inhibited by high VFA concentrations and low pH (18, 106, 137). Products of primary fermentation include alcohols, such as ethanol, fatty acids such as lactate, propionate, butyrate, and acetate, carbon dioxide, and hydrogen (34). Hashsham *et al.* (2000) examined the response of two reactors, each inoculated with a morphologically different biomass, to additions of a high concentration of glucose. Prior to the perturbation both reactors showed similar performance, but after the perturbation, performance was quite different. One reactor, referred to as LS, showed the consumption of glucose followed by the sequential production and consumption of lactate, butyrate, acetate, then propionate. The other reactor, referred to as HS, consumed the glucose with simultaneous production of these fermentation products, and a faster recovery than the LS reactor. The authors attributed the superior performance of the HS reactor under glucose perturbations to the multiple pathways by which the additional substrate was processed, preventing bottlenecks in its degradation (106).

b. Acetate cycling

Acetate along with hydrogen are the two most important intermediates in methanogenesis. If methane is produced from glucose, at most two moles of acetate and four moles of hydrogen may be produced from one mole of glucose (54). Four moles of hydrogen are needed to produce one mole of methane from carbon dioxide, whereas one mole of acetate produces one mole each of methane and carbon dioxide. From this stoichiometry it appears that approximately 2/3 of methane should result from aceticlastic methanogenesis and only 1/3 from hydrogenotrophic methanogenesis (54). This does not account for the production of acetate from hydrogen and carbon dioxide by homoacetogens, nor does it consider syntrophic acetate oxidation to hydrogen and carbon dioxide with subsequent conversion of these products to methane (1). Homoacetogenesis and syntrophic acetate oxidation are often performed by the same microbe with acetate concentrations and hydrogen partial pressure determining which metabolism is used (1, 245). Isotope studies have been able to somewhat untangle the complex metabolism of acetate by selective labeling of the methyl or carboxyl carbons. Aceticlastic methanogenesis converts the methyl carbon of acetate to methane with electrons generated from

the oxidation of the carboxyl carbon to carbon dioxide. If acetate is metabolized through syntrophic acetate oxidation, then both carbons are oxidized to carbon dioxide before being reduced to methane (245).

Acetate and hydrogen levels determine the pathway for acetate consumption and methanogenesis. High acetate concentrations favor aceticlastic methanogenesis, with low acetate concentrations favoring syntrophic acetate oxidation in conjunction with hydrogenotrophic methanogenesis (1). If aceticlastic methanogenesis is inhibited, syntrophic acetate oxidation will proceed at high acetate concentrations. Aceticlastic methanogens consist of the fast-growing *Methanosarcina* with low affinity for acetate, and the slow-growing *Methanosaeta* with high affinity for acetate (285). If acetate concentrations are high, the fast-growing *Methanosarcina* outcompete both *Methanosaeta* and syntrophic acetate oxidizers for substrate, but at lower acetate concentrations, *Methanosaeta* and syntrophs compete for acetate (245). High hydrogen partial pressures and increasing pH favor homoacetogenesis to produce acetate as well as hydrogenotrophic methanogenesis (34, 136). Greater hydrogen partial pressures also result in greater production of products such as lactate, ethanol, and propionate over butyrate and acetate (34). Lower hydrogen partial pressures result in more substrate converted to first butyrate then to acetate, and more acetate converted to hydrogen and carbon dioxide by syntrophs (34, 120). Incubation studies of methanogenic soil from temperate acidic peatlands showed increased methane production from hydrogen and carbon dioxide as the pH of the soil became more acidic (26, 114). In tundra soils, aceticlastic methanogenesis using acetate produced by homoacetogens dominated at temperatures less than 30°C (182, 236, 237, 193, 194, 236, 237).

Both *Methanosarcina* and *Methanosaeta* are often found co-existing in anaerobic digesters, even when competing for acetate. At acetate concentrations below about 30 mg/L, *Methanosaeta* has a faster specific growth rate, and above this acetate concentration *Methanosarcina* has a faster specific growth rate (293). The *Methanosarcina* are quite metabolically diverse, and may use hydrogen and carbon dioxide as well as methylated substrates for methanogenesis. As hydrogenotrophic methanogens, the *Methanosarcina* do not form syntrophic relationships with acetate-oxidizers because their high K_m for hydrogen is beyond the lower threshold for this partnership (272). When producing methane from a methylated compound, *Methanosarcina* compete with acetate producers for substrate. Acetogens have a higher specific growth rate, but also a higher K_m and can only outcompete methanogens at high substrate concentrations (81). Although *Methanosarcina* may use other substrates for methanogenesis, many researchers have found fluctuations in *Methanosaeta* and *Methanosarcina* numbers controlled by acetate concentrations with high concentrations favoring *Methanosarcina*

and low concentrations favoring *Methanosaeta* and syntrophic acetate oxidizers which compete for substrate (130, 201, 245, 297).

iii. The influence of mixing

Regardless of the structure of a reactor, mixing is an important parameter to consider during design and operation. Mixing enhances contact of the substrate with the active biomass, improves the uniformity of pH and temperature within the reactor, enhances the rate of heat transfer to the digester, prevents stratification and the formation of a surface scum layer as well as preferential flow paths and dead zones, and facilitates biogas removal from the digester (111, 128). Digester mixing is characterized by the intensity, duration, and location of the mixing, and the strategy employed for mixing. Mixing may be accomplished through mechanical means, or may be achieved through recirculation of effluent or biogas through the reactor (111). Mixing strategies may be continuous or intermittent, or may involve periods of higher mixing intensity at certain times such as after feeding (128).

Mixing in lab-scale reactors is typically much more intense and thorough than what can be achieved in full-scale reactors, so it may be difficult to relate what is observed at the lab-scale to larger-scale applications. The start-up period for lab-scale AD reactors is inversely related to mixing intensity, and the shortest time to the onset of methane production is often seen in reactors with no mixing (111, 128, 137). For continuously mixed reactors, similar biogas production and COD removal rates have been observed over a wide range of mixing intensities (111) but when an intermittent mixing strategy is compared to continuous mixing, intermittently mixed reactors show increased biogas production, increased COD removal, and an increased retention of active biomass (128, 137, 178, 263). Reactors shifted from a continuous to an intermittently mixed strategy show improved performance, and performance decreases when reactors are returned to continuous mixing. Continuous mixing or an increase in mixing intensity improves the ability of a reactor to deal with a transient increase in the organic loading rate, perhaps by reducing variations in substrate concentration and pH throughout the reactor (111, 128).

The mixing strategy and intensity affect the methanogen community by influencing VFA concentrations and the formation of microbial flocs in the reactor. Continuous mixing reduces or eliminates flocculent biomass (111). In addition, reactors that are continuously mixed have larger concentrations of VFAs, especially acetate, which select for the faster-growing *Methanosarcina* over *Methanosaeta* (111, 137, 178, 201). In addition, increased mixing intensity may cause more damage to the filamentous *Methanosaeta* as compared to the rod-shaped cells of *Methanosarcina*. Hoffman *et al.* (2008) examined the methanogen populations in lab-scale CSTRs operated at

different mixing intensities. Although similar levels of *Methanobacteriaceae* and *Methanomicrobiales* were observed in all reactors, *Methanosarcina* increased and *Methanosaeta* decreased in proportion to mixing intensity (111). McMahon *et al.* (2001) observed the methanogen populations of lab-scale reactors operated with either continuous or minimal mixing. Operation under continuous mixing conditions led to higher VFA levels, decreased *Archaea* numbers, and showed a decrease in *Methanosaeta* with an increase in *Methanosarcina*. A change to minimal mixing resulted in the increase of *Archaea* and *Methanosaeta*, with a decrease in *Methanosarcina* (178).

iv. Granulated and fixed-film biomass

Reactor designs may contain an active microbial population in either a flocculent form, or as a fixed-film or granulated biomass. Flocculent biomass consists of small suspended colonies of microorganisms whereas the structure of microbial biofilms or granules is more complex. In all three conditions, a portion of cells will exist as planktonic, although the majority will be a part of a floc, biofilm, or granule structure. Sawayama *et al.* (2006) quantified the proportion of cells living as planktonic or as part of the biofilm in a fixed-film reactor community. The researchers found that while there were approximately three times more *Bacteria* in the biofilm community as were planktonic, there were nearly two orders of magnitude more methanogens in the biofilm than were planktonic. The most dramatic difference among the methanogens was for the genera *Methanosaeta* which were only detected in the biofilm community (231).

The development of fixed-film and granulated reactor designs allowed a greater separation of the hydraulic and solids retention times, thereby allowing slow-growing organisms to be retained in the reactor. This fact is illustrated well by the presence of the slow-growing *Methanosaeta* which are vital in biofilm and granule formation in these types of reactors (9, 221, 243, 299). Granule formation can be induced from flocculent material by slowly increasing the upflow velocity through the reactor and recirculating the effluent containing some of the flocculent biomass (299). *Methanosaeta* are the only filamentous methanogens, and the presence of these organisms as well as other filamentous microbes allows several points of contact for cells to adhere. In addition, extracellular polymeric substances are produced during granule formation, eventually resulting in a compact matrix of filaments and cells whose density allows it to settle in the reactor even as fluid is pumped upward through the sludge bed (299). Rochealeau *et al.* (1999) studied granules from a UASB enriched for either *Methanosarcina* (high acetate concentrations) or *Methanosaeta* (low acetate concentrations). The researchers found that while the *Methanosaeta*-enriched granules contained no *Methanosarcina*, the *Methanosarcina*-enriched

granules contained both *Methanosarcina* and *Methanosaeta*, underlining the importance of *Methanosaeta* in granule structure (221). Granular reactors subjected to transient increases in organic loading rate may accumulate VFAs, and this increase, especially for acetate, causes a decrease in the number of filamentous microorganisms and a general disintegration of the granules (137, 155).

Granulated sludge contains a number of granules of different sizes, and even colors, with all trophic groups involved in methanogenesis present in one granule (67, 243). FISH analysis with probes targeting different trophic groups have allowed the visualization of the architecture of reactor granules. Granules have a layered structure and spatial distribution of phylogenetically distinct microbes with more *Bacteria* in the outer layers and more *Archaea*, mainly methanogens, located in the interior (67, 243). Inner layers are typically more dense than outer layers, with a interior section mainly composed of dead cells. Despite the dense matrix of filaments, granules are still porous allowing the transport of metabolic waste out of the granule and the transport of substrate into the granule. Methanogens that have been identified in granular sludge include *Methanomicrobiales*, *Methanosarcina*, *Methanobacterium*, and the *Methanosaeta* (9, 39, 67, 93, 159, 301). The *Bacterial* community is often dominated by *Clostridium* and delta-*Proteobacteria*, especially *Desulfovibrio* (39, 67, 93, 159), but other *Bacteria* identified in these reactors include *Nitrospira*, *Deferribacteres*, *Lysobacter*, *Synergistes*, and members of the *Cytophaga-Flavobacterium-Bacteriodetes* cluster (39, 67, 93). Outer layers of the granules are typically composed of primary fermenters such as *Clostridium* with methanogens in the interior layers. *Methanosaeta* were the predominant methanogens in the core of the granule with a outer layer of *Methanobacteriaceae* cells, and delta-*Proteobacteria*, which were mainly *Desulfovibrio*, evenly distributed throughout the inner and outer layers of the granules (159). SRB may be syntrophic partners with hydrogenotrophic methanogens which explains their presence throughout the granules (216). Long-term monitoring of methanogen and *Bacterial* communities in granular reactors has suggested that the methanogen population is rather stable but the *Bacterial* community changes rapidly and never reaches a consistent profile (301).

Granulated sludge may be formed at nearly any temperature, but the microbial consortia of the granules differs from mesophilic to thermophilic conditions. Both mesophilic and thermophilic granules have a similar structure with outer layers of *Bacteria* cells and inner layers of methanogens with an innermost center containing few to no active cells (241, 242). The dominant methanogen in both mesophilic and thermophilic granules were members of the filamentous *Methanosaeta*. Mesophilic granules also contained methanogens belonging to the *Methanobacterium* and *Methanospirillum*, while thermophilic granules also contained members

of the *Methanobacterium* as well as *Methanosarcina*. Both mesophilic and thermophilic granules also contained large numbers of filamentous green non-sulfur bacteria which are important in maintaining granule structure. Mesophilic granules were dominated by the delta-*Proteobacteria*, including members of the *Syntrophobacter* and *Desulfobulbus* (242). The *Bacterial* community of thermophilic granules was dominated by green non-sulfur bacteria, but granules also contained members of *Thermodesulfovibrio, Desulfotomaculum*, and *Clostridium* (241, 242).

The authors were able to isolate clones of *Thermodesulfovibrio* and green non-sulfur bacteria from the granules in co-culture with the hydrogenotrophic methanogen ***M.*** ***thermoautotrophicus***. Isolation without co-culture proved difficult, and the excellent growth of these clones in the co-culture suggests their role as syntrophic partners of the hydrogenotrophic methanogens (242). A co-culture of *Pelotomaculum thermopropionicum* with *Methanothermobacter thermoautotrophicus* delta H showed aggregation of cells when grown on propionate, but not when cells were grown on ethanol or propanol (122). The degradation of propionate, but not ethanol or propanol, is thermodynamically unfavorable unless hydrogen partial pressure is kept low by the hydrogenotrophic methanogen, and aggregation of cells was catalyzed by the extracellular polymeric substances and filaments produced by ***P.*** ***thermopropionicum*** (122). These observations suggest that granulation by anaerobic microorganisms improves the thermodynamics and energy yield for both syntrophs and methanogens.

Several researchers have reported the *Methanosaeta* and *Methanobacterium* as the dominant methanogens in granular sludge (9, 39, 67, 75). While the presence of *Methanosaeta* is likely due to its filamentous nature, the frequent occurrence of *Methanobacterium* may be due to its ability to form syntrophic relationships with fatty-acid and carbohydrate degraders.

v. The effect of toxics

Contrary to what has been popularized about anaerobic digestion, methanogens do not appear to be more sensitive to toxics than other members of the methanogenic consortium, and AD has been used to successfully treat a wide range of toxic and xenobiotic substances (278). Some examples include halogenated organics including chlorinated aliphates and chlorinated aromatics, nitroaromatics, formaldehyde, methanol, phenol, benzoic acids, terephthalic and toluic acids (43, 205, 278). The potential concentration and type of toxic depends on the waste stream. Industrial wastes may contain a wide range of xenobiotic and potentially toxic compounds, and the waste stream may change frequently (1). Heavy metals, especially zinc, chromium, copper, cadmium, nickel, and lead, are toxic to the AD process (202). Heavy metals are typically a

concern in industrial wastes, but some municipal wastes may also contain these, and the presence of heavy metals in the resultant biosolids prevents land-application (1). More typical toxics found in municipal waste are pesticides, herbicides, detergents, and chlorinated solvents. Agricultural waste may also contain pesticides and herbicides as well as antibiotics and high ammonia levels, all of which may inhibit anaerobic digestion. High concentrations of sulfate or sulfide may be found in some industrial, municipal, or agricultural wastes. Competition with sulfate-reducers for substrate inhibits methanogenesis, but the presence of sulfide is toxic to many microorganisms (1). Long chain fatty acids which are present in oily food wastewaters appear to be toxic to all members of the AD community (18). Even oxygen can be considered a toxic as its presence in sufficient concentration will completely inhibit anaerobic digestion (268).

a. Ammonia and ammonium

Ammonium may be present in industrial effluents as part of the waste stream, but ammonium in municipal and agricultural wastes is typically due to the presence of excess nitrogen in the substrate which is released as ammonium (102). Ammonium (NH_4^+) dissociates to ammonia and hydrogen ion with approximately 50% of each form present at a pH of 9.2. Ammonia is more toxic than ammonium and the ratio of ammonia to ammonium increases with an increase in pH or temperature (102, 173). An anaerobic digester consortium can be adapted to digest high-ammonium waste streams, but the general limit seems to be approximately 4 g/L of the ammonia species (173, 278). In addition to toxicity, ammonium may cause a shift in the metabolic processing of wastes. Schnurer and Nordberg (2007) operated two mesophilic CSTRs treating the organic fraction of municipal solid waste. Egg albumin powder was added to one reactor to simulate an increase in protein content, and release of excess nitrogen as ammonium during degradation. In the control reactor ammonium was low and acetate was primarily degraded by aceticlastic methanogens. In the test reactor ammonium was increased from less than 1 g/L to nearly 7 g/L causing a shift in acetate degradation from aceticlastic methanogens to syntrophic acetate-oxidizing *Bacteria* associated with hydrogenotrophic methanogens (235). Angenent *et al.* (2002) studied shifts in the methanogen community during the start-up of an anaerobic sequencing batch reactor treating a high-ammonium swine waste. Early in the start-up period the organic loading rate, and thus the ammonium concentration, were low and methanogens were mainly members of *Methanomicrobiales* with fewer *Methanobacteriaceae*, *Methanosarcina*, and *Methanosaeta*. As the loading rate and ammonium concentrations increased, *Methanosarcina* and *Methanosaeta* declined, resulting in a primarily hydrogenotrophic community dominated by *Methanobacteriaceae* (8).

Quaternary ammonium compounds may also be a concern in treating domestic or industrial waste streams. These compounds contain four functional groups attached to a central nitrogen atom as R_4N^+, and are frequently found as part of surfactants, emulsifiers, fabric softeners, disinfectants, and corrosion inhibitors (270). Quaternary ammonium compounds have been found to inhibit methanogenesis but not fermentation in AD processes, even with pH controlled to circumneutral levels (270).

b. Sulfate and sulfide

Sulfate may occur at high concentrations in industrial wastewaters from oil production, molasses fermentation, and from tannery, pulp, and paper industries (206). Sulfate reduction in AD is undesirable both because of the inhibitory effect on methane production, and because sulfate reduction removes COD while producing hydrogen sulfide, a toxic and corrosive product with no fuel value (135). Inhibition of anaerobic digestion is due to competition of methanogens with sulfate-reducing bacteria for substrate and the toxicity of reduced sulfur compounds produced by SRB (135, 214). The inhibitory effects of sulfur compounds to methanogenesis occurs in decreasing order of effect as sulfide, then sulfite, thiosulfite, and finally sulfate. Sulfide is toxic to other microbes in AD as well as the SRB themselves (135, 206). SRB use hydrogen or organic acids as electron donors for sulfate reduction, but when little sulfate is present, SRB may grow syntrophically with hydrogen- or formate-consuming methanogens by fermenting carbohydrates, alcohols, and fatty acids (214).

High-sulfate papermill wastewater has been successfully treated at a number of sites in fluidized-bed reactors with granular sludge (75, 222, 228). Granulated sludge from these reactors have shown high rates of methanogenesis, and most-probable number determinations have even shown methanogens to outnumber SRB (75). The methanogenic and SRB populations are spatially separated in granular sludge with sulfate reduction occurring in outer layers and methanogenesis taking place in the interior (228). This spatial separation may be the reason that high rates of methanogenesis and sulfate reduction can occur simultaneously in these reactors. Most-probable number determinations of SRB and methanogens in granular sludge taken from a reactor treating sulfidic papermill wastewater showed greater numbers of both hydrogenotrophic and aceticlastic methanogens than SRB (75). Enrichment cultures of these granules were grown with sulfate and either acetate, hydrogen and carbon dioxide, propionate, or butyrate. Acetate cultures were dominated by *Methanosaeta*, formate cultures were dominated by *Methanobacterium*, and cultures grown with hydrogen and carbon dioxide yielded high numbers of both *Methanobacterium* and SRB. Enrichments on propionate showed the presence of SRB,

syntrophs, and methanogens related to *Methanomicrobiales*, *Methanobacterium*, and *Methanosaeta* whereas enrichments on butyrate only showed the presence of *Methanobacterium* and *Methanosaeta* (75). These results suggest that methanogens in this reactor were able to compete successfully with SRB for substrates, a result that has been confirmed in other studies. Santegoeds *et al.* (1999) examined methane production and sulfate reduction in a granular sludge taken from a reactor also treating papermill wastewater. The addition of up to 10 mM of sulfate showed no effect on methanogenesis rates even as sulfate was quickly reduced (228). The *Bacterial* community of these reactors is diverse, containing both SRB of *Desulfovibrio*, *Desulfobulbus*, and *Desulfuromonas*, syntrophic fatty-acid and carbohydrate degraders of *Syntrophobacter*, *Syntrophomonas*, and *Clostridium*, and members of other genera including *Actinobacterium*, *Propionibacterium*, *Eubacterium*, *Holophaga*, *Halobacteroides*, *Cellulomonas*, *Oerskovia*, as well as members of the *Crenarchaeota* (222, 228).

Because SRB may also grow syntrophically with methanogens, they are nearly always present as members of the AD microbial community regardless of sulfate concentrations in the reactor (27, 206, 214, 216)). SRB are able to respond rapidly to changes in sulfate concentration, and an influent pulse of sulfate is quickly reduced to sulfide. During sulfate reduction, VFAs are consumed and carbon dioxide is produced resulting in an increase in alkalinity and decrease in reactor pH (135). Increases in sulfate in the waste stream may not alter COD removal efficiency, but the percentage of methane in the biogas is reduced at mesophilic temperatures. Sulfate has been reported to be more inhibitory at thermophilic than at mesophilic temperatures, causing accumulation of acetate (206). Pender *et al.* (2004) examined the response of reactors operated at mesophilic temperatures with or without sulfate to an increase in temperature to thermophilic conditions. As would be expected, the increase in temperature reduced methanogenesis in both reactors, but the affect on hydrogenotrophic and aceticlastic methanogenesis was different in the sulfate-fed and non-sulfate reactors. Aceticlastic methanogenesis was reduced more in the sulfate-fed reactor than in the reactor operated without sulfate, and a decrease in the number of *Methanosaeta* occurred concomitantly with an increase in *Methanobacteriaceae* during the shift from mesophilic to thermophilic temperature (206).

Several researchers have examined changes in methanogen and SRB communities in reactors operated under fluctuating sulfate loads. Reactors operated with no influent sulfate showed the presence of SRB of the genera *Desulfovibrio*, *Desulfobulbus*, *Desulfobacterium*, and *Desulfococcus* (214, 216). Researchers have reported increases in sulfate correlated with increased numbers of *Desulfococcus* and decreased numbers of methanogens with members of *Methanomicrobiales* replacing members of *Methanobacteriales* as the dominant methanogens

(214). Numbers of *Desulfovibrio* and *Desulfobacterium* were constant in the reactors, independent of sulfate concentration (214). Even when operated under sulfidic conditions, methanogens were still present, although at low levels, but their abundance rose when sulfate concentrations decreased (214).

c. Other toxics

Anaerobic digestion can be used to treat industrial waste streams containing a wide range of toxic compounds, with few organic materials completely resistant to anaerobic digestion. Some examples of these resistant compounds include aromatic substrates composed of multiple rings in condensed formation, plant polymers such as lignin, and certain fractions of coal (232). For some organic materials, anaerobic digestion is difficult and requires adaptation of the active biomass and careful operation of the reactor to maintain optimal environmental conditions for degradation. Long-chain fatty acids, such as palmitate, stearate, oleate, and linoleate, are difficult to degrade anaerobically and are even considered toxic to the AD microbial consortia (107, 108). Toxicity appears to affect all members of the community, not just methanogens (1). Olive mill wastewater is a common industrial waste containing not only long-chain fatty acids, but also polyphenols which are inhibitory to the anaerobic digestion process (18, 107, 108). The degradation of long-chain fatty acids is endergonic unless the partial pressure of hydrogen is kept extremely low by the syntrophic association of fatty-acid degrading bacteria with hydrogen- or formate-consuming methanogens (107). Even under ideal syntrophic associations, degradation yields little energy and growth of these organisms is slow. Reactors must be operated with long retention times, and also with a pH that is neutral or slightly alkaline as lower pH further inhibits degradation (18). In addition, fatty acids appear to be degraded faster at thermophilic temperatures than at mesophilic temperatures, and by different *Bacterial* communities (107, 108). Hatamoto *et al.* (2007) examined *Bacteria* from mesophilic and thermophilic granular sludge degrading ^{13}C-labeled palmitate to identify the organisms active in the metabolism of this lipid. Members of the mesophilic community included *Clostridium*, *Spirochaetes*, *Syntrophaceae*, and *delta-Proteobacteria*, and members of the thermophilic community also included *Clostridium* and *Spirochaetes* as well as *Syntrophomonadaceae*, *Thermotoga*, *Anaerobaculum*, *Coprothermobacter* and *Bacteriodetes* (107, 108).

Another common toxin that the anaerobic digester process may be exposed to is oxygen. In many waste treatment schemes, waste material is concentrated, or thickened, prior to addition to the anaerobic digester. The waste material is exposed to oxygen during the thickening process as this is not conducted under anaerobic conditions (51). In addition, waste streams with high

sulfate may be micro-aerated to suppress SRB and prevent the formation of hydrogen sulfide (268). When anaerobic biomass is exposed to oxygen, oxygen consumption by facultative anaerobes is critical to protect methanogens from oxygen exposure and contribute to the oxygen tolerance of anaerobic digester biomass (51). When facultative anaerobes are active, anaerobic digester biomass is quite oxygen tolerant. Conklin *et al.* (2007) showed no effect on methane production or COD removal rates for AD biomass exposed to oxygen four hours a day for three months with dissolved oxygen levels reaching 3-4 mg/L during exposure. In addition, continuous oxygen exposure for 5 days only decreased methanogenesis to 77% of its original level (51). Microaeration in lab scale CSTRs used to prevent sulfate reduction showed a decrease in hydrogen sulfide production and an increase in methane production over a control reactor operated with no microaeration (268). Active methanogens have also been identified in the aerated activated sludge of municipal wastewater treatment plants (95). Researchers obtained clones related to *Methanosarcina* and *Methanobrevibacter* as well as other sequences within the *Methanosarcinales* and *Methanobacteriales* from activated sludge samples obtained from four treatment plants. Incubation of activated sludge from these treatment plants under anoxic conditions showed methane production within four hours. Samples which were aerated, autoclaved, or treated with the methanogen inhibitor BES showed no methane production suggesting that the observed methanogenesis was from active microorganisms (95).

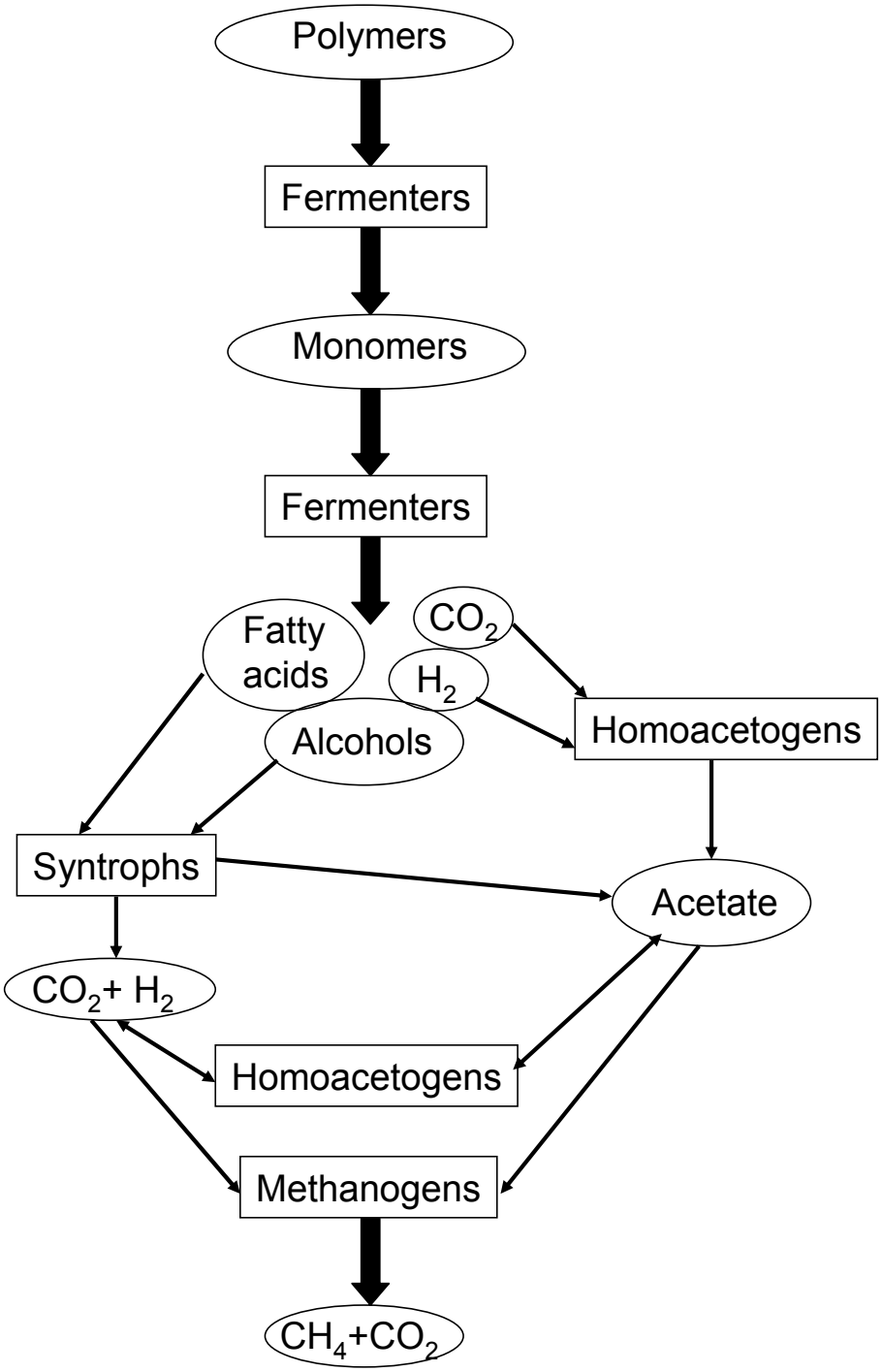


Figure 2-1. The steps of methanogenesis and the trophic groups catalyzing each.

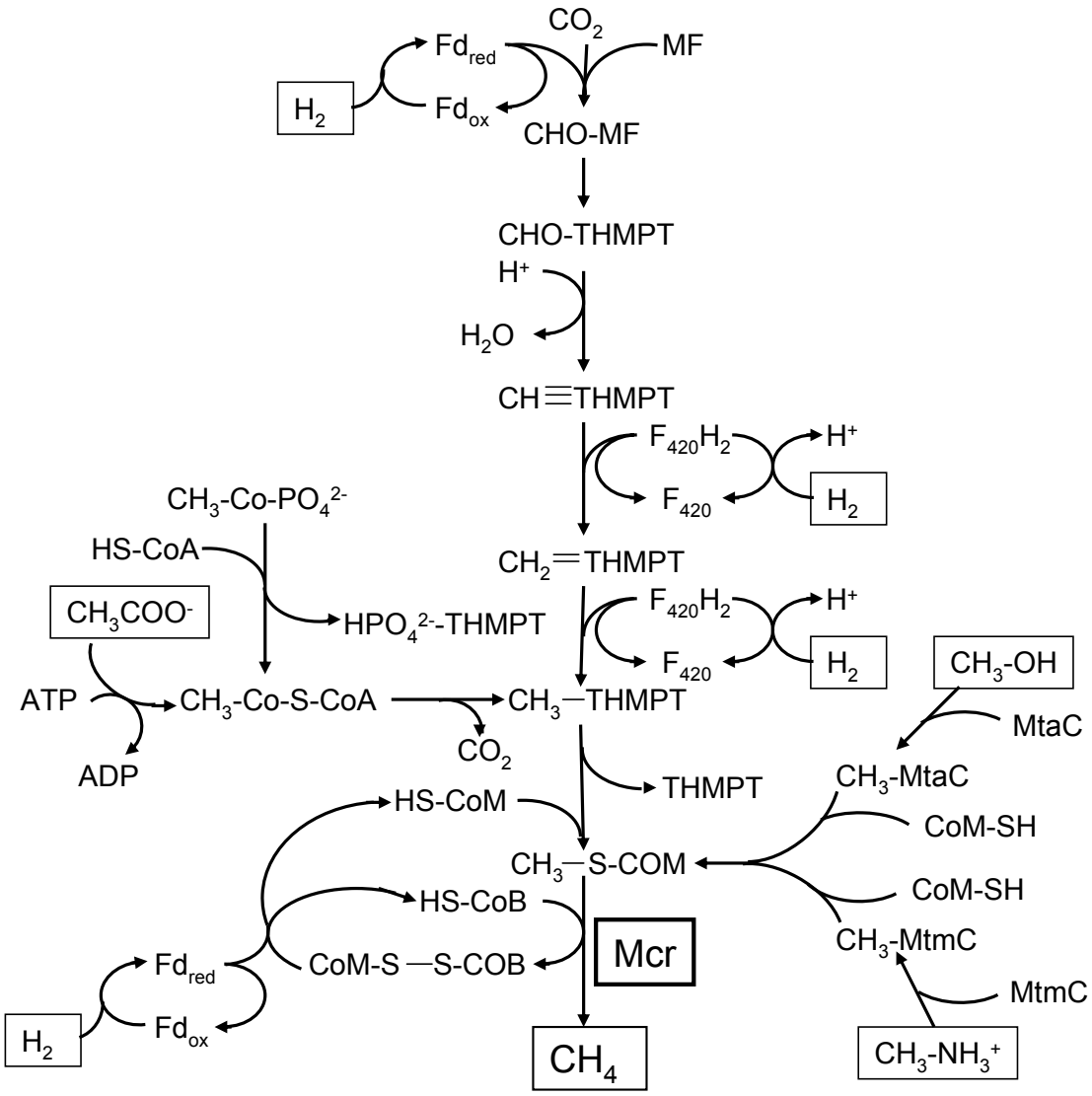


Figure 2-2. The biochemistry of methanogen pathways.

Figure adapted from Ferry (2007) (79); THMPT=tetrahydromethanopterin, MF=methanofuran, CoM=coenzyme M, CoB=coenzyme B, CoA=coenzyme A, F_{420}=coenzyme F_{420}, Fd=ferredoxin, Mta=corrinoid/methyltransferase 1 pair for methanol, MtmC=corrinoid/methyl transferase 1 pair for methylamine, Mcr=methyl coenzyme M reductase

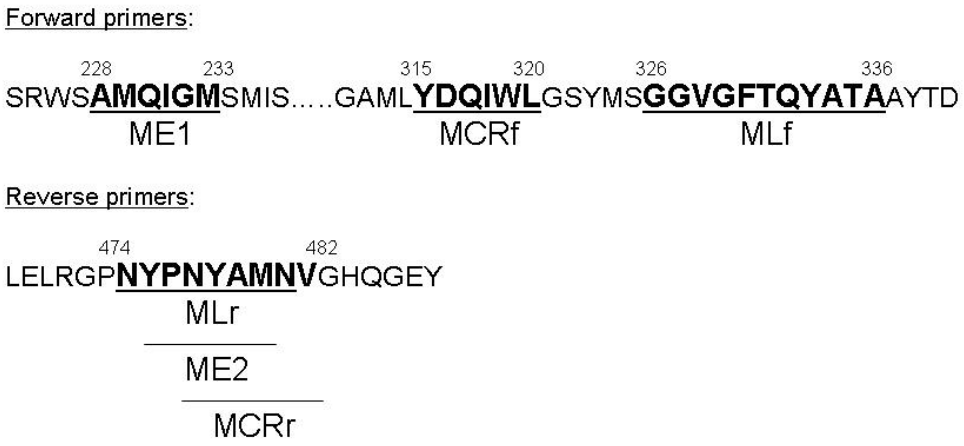


Figure 2-3. Current primers for the gene encoding the methyl-coenzyme M reductase, alpha subunit.

ME1 and ME2 were designed by Hales *et al.* (103), MCRf and MCRr were designed by Springer *et al.* (257), and MLf and MLr were designed by Luton *et al.* (168) Numbering is based on *M. thermoautotrophicum* delta H (U10036). Figure adapted from Juottonen *et al.* (127)

Table 2-1: Standard free energies for common reactions in methanogenesis.

Equations adapted from [a]Whitman et al. (285) and [b]Garcia et al. (89)

Fermentation/acetogenesis	**ΔG°' (kJ/mol)**
[a] $C_6H_{12}O_6 + 2H_2O \rightarrow 0.7CH_3(CH_2)2COOH + 0.6CH_3COOH + 1.3H^+ + 2CO_2 + 2.6H_2$	-233.0
[a] $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	9.6
[a] $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$	48.3
[a] $CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	76.0
Syntrophic acetate oxidation	
[a] $CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$	94.9
Homoacetogenesis	
[a] $4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	-94.9
Methanogenesis	
[a] $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-131.0
[a] $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36.0
[b] $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-130.1
[b] $CH_3(CH_2OH)CH_2 + CO_2 \rightarrow CH_4 + 4CH_3COCH_3 + 2H_2O$	-36.5
[b] $2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COO^- + H^+$	-116.3
[b] $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-112.5
[b] $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-104.9
[b] $4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	-75.0
[b] $2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$	-73.2
[b] $4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4\ NH_4^+$	-74.3
[b] $(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + H_2S$	-73.8

Chapter 3

Phylogenetic Comparison of the Methanogenic Communities from an Acidic Oligotrophic Fen and an Anaerobic Digester Treating Municipal Wastewater Sludge

This chapter is an adaptation of Steinberg and Regan.(2008)

ABSTRACT

Methanogens play a critical role in the decomposition of organics under anaerobic conditions. The methanogenic consortia in saturated wetland soils are often subjected to large temperature fluctuations and acidic conditions, imposing a selective pressure for psychro- and acidotolerant community members; however, methanogenic communities in engineered digesters are frequently maintained within a narrow range of mesophilic and circumneutral conditions to retain system stability. To investigate the hypothesis that these two disparate environments have distinct methanogenic communities, the methanogens in an oligotrophic, acidic fen and a mesophilic anaerobic digester treating municipal wastewater sludge were characterized by creating clone libraries for the 16S rDNA and methyl coenzyme M reductase alpha subunit (*mcrA*) genes. A quantitative framework was developed to assess the differences between these two communities by calculating the average sequence similarity for 16S rDNA and *mcrA* within a genus and family using sequences of isolated and characterized methanogens within the approved methanogen taxonomy. The average sequence similarities for 16S rDNA within a genus and family were 96.0 and 93.5%, respectively, and the average sequence similarities for *mcrA* within a genus and family were 88.9 and 79%, respectively. The clone libraries of the bog and digester environments showed no overlap at the species level, and almost no overlap at the family level. Both libraries were dominated by clones related to uncultured methanogen groups within the *Methanomicrobiales*, although members of the *Methanosarcinales* and *Methanobacteriales* were also found in both libraries. Diversity indices for the 16S rDNA library of the bog and both *mcrA* libraries were similar, but these indices indicated much lower diversity in the 16S digester library when compared with the other three libraries.

INTRODUCTION

Methanogens are an integral part of carbon cycling on the planet, anaerobically catalyzing the production of methane primarily through carbon dioxide reduction with hydrogen or the conversion of methylated compounds such as acetate (285). These *Archaea* are essential community members in anaerobic digesters, which may be used to treat a variety of domestic, agricultural, and industrial wastes with concomitant energy production from the combustion of the methane-containing biogas. Anaerobic digestion is typically operated at circumneutral pH and mesophilic (30-37°C) or thermophilic (55-65°C) temperatures, with process stability decreasing dramatically outside of this range of conditions (21, 132, 156, 157). In these systems, methanogens are considered to be the most sensitive members of the methanogenic consortium to non-optimal conditions (10, 89, 113), yet methanogenesis is known to proceed in both cold and acidic natural environments such as tundra (112, 283), *Sphagnum*-dominated peatlands (146, 203), and lake sediments (196, 236). Presumably this difference in psychro- and acidotolerance can be attributed, in part, to differences in the methanogen communities, and knowledge of these differences might lend insights into community-based strategies to increase digester stability with reduced chemical and energy inputs necessary to maintain narrow operating conditions.

Methanogens are difficult to isolate or culture under laboratory conditions, so communities are often examined through culture-independent techniques, such as the amplification and sequencing of target DNA from environmental samples. The most widely used target for molecular analysis is the 16S rRNA gene (16S rDNA), and a number of primers and probes have been developed specifically for methanogens or groups of methanogens (35, 171, 211, 215, 221, 251, 256, 297). To eliminate potential problems with nonspecific amplification, some researchers have developed primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) (103, 168, 257). Mcr catalyzes the last step of methanogenesis and is conserved among all methanogens. Phylogenetic inference with *mcrA* sequences is similar to that obtained with 16S rDNA sequences, suggesting no lateral transfer (15, 168, 257). Moreover, Mcr is absent in all non-methanogens, with the exception of the anaerobic methane-oxidizing *Archaea* (ANME), which are closely related to the methanogens (104). Due to the fact that methanogens may be examined exclusively from other bacteria present in an environment, *mcrA* has been increasingly used for phylogenetic analysis coupled with, or independent of, 16S rDNA.

The increasing use of molecular-based community analysis has led to the identification of phylogenetic clusters of methanogens that are quite divergent in sequence from isolated, phenotypically described methanogens. Ecological indices such as the Shannon-Wiener or

Simpson's indices and species accumulation curves, which are used to determine the extent of sampling of an environment, have been adapted for use in microbial gene surveys (117). The use of these ecological indices requires that genes or gene fragments be organized into operational taxonomic units (OTUs) representing a species or strain. Boone *et al*. (23) suggested a 16S rDNA sequence similarity of less than 98% as evidence for a separate methanogen species, which is slightly more restrictive than the minimum 97% sequence similarity suggested by Stackebrandt and Goebel (258) for sequences to be considered from the same species. Although these studies provide a basis for determining if gene sequences represent new species, little information is available about sequence similarities used to determine new taxonomic levels of methanogens above the species level. In addition, although many researchers use *mcrA* sequences alone or coupled with 16S rDNA sequences to examine methanogenic communities, little attention has been given to appropriate sequence similarity limits for *mcrA* in establishing a separate species or genus.

The hypothesis of this research is that the methanogen community of a mesophilic anaerobic digester is distinct from that of an acidic peat bog. The methanogen communities from these two environments were explored by creating 16S rDNA and *mcrA* clone libraries. To characterize the extent of community differences between these libraries, *in silico* analysis was performed on existing *mcrA* and 16S rDNA sequences for phenotypically characterized methanogens to describe the range of sequence similarity within currently accepted taxonomic levels.

MATERIALS AND METHODS

Site characteristics and sampling.

Bear Meadows Bog is an acidic, boreal peatland situated approximately 15 miles southeast of State College, Pennsylvania. It is classified as a transitional bog, and vegetations predominantly consists of *Sphagnum* mosses, sedge grasses, and highbush blueberry, with peat approximately 1 m thick. The bog pH varies from 3.8 to 5.0, and bog waters contain little alkalinity, sulfate, or iron (19). Porewater methane reaches a maximum of 5500 ppm at 30 cm below the water-sediment interface. Methane emitted from the bog has an isotopic signature of -60‰ suggesting a biogenic origin (19). Temperatures in the region range from approximately -15 to 35°C and precipitation is fairly evenly spaced throughout the year with an average of 98 cm of rain and 115 cm of snow (www.nws.noaa.gov).

Cores were taken from two locations at Bear Meadows Bog covered with both *Sphagnum* mosses and sedges. Each core was collected in a series of three six-inch long Teflon sleeves (Ben Meadows Company, Janesville, WI) for a total of 1.5 feet extracted at each location. Immediately upon collection, the cores were placed into a sealed container with a GasPak (BBL Inc, Franklin Labs, NJ) to create an anaerobic environment (H_2/CO_2) for transport.

Anaerobic digester sludge was collected from the State College Wastewater Treatment Plant. The plant operates two fixed-cover primary digesters, which receive primary sludge and thickened secondary sludge and are constantly mixed and heated to 37°C. The pH is kept near-neutral by adjusting the organic loading rate, and the solids retention time is 25 d. Collected sludge was transported anaerobically in a sealed container with a GasPak.

Construction of 16S rDNA and mcrA clone libraries.

Clone libraries were constructed for both methanogen-specific 16S rDNA and *mcrA* from the Bear Meadows Bog and anaerobic digester samples. DNA was extracted using a PowerSoil DNA Extraction Kit (MoBio Labs, Carlsbad, CA). For the bog peat cores, DNA extractions were performed on sediment taken from the central portions of the top and bottom of each of the three 6-inch core segments for a total of six DNA extractions from the bog peat. All of the DNA extractions from the bog peat were pooled before performing PCR for library construction.

A fragment of the 16S rDNA was amplified with A21f, an *Archaea*-specific forward primer (64) and Eury498 (29), a reverse primer developed from an *Euryarchaeota*-specific probe. PCR conditions were based on Girguis et al. (91). The *mcrA* sequences were amplified with three different forward primers: ME1 (103), ML-f (168), and mlas (5'GGTGGTGTMGGDTTCACMCARTA 3'), which is a truncated version of the forward primer ML-f designed for a better melting temperature match with the reverse primer, a lower 3' stability to improve specificity, and two additional degeneracies to improve coverage (121). The ML primer set is reported to have wide coverage of methanogen groups, detecting members of *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriales* (both *mcrA* and *mrtA*), *Methanococcales*, and the uncultured groups Fen Cluster, Rice Cluster I, and MCR-2 (72, 85, 126, 168, 255). The ME primer set also captures a wide range of methanogens (14, 87, 88), and has been reported to capture methanogen groups related to the Fen cluster not detected by the ML primer set, although the ME primers are reported to have difficulty amplifying members of the *Methanosarcinaceae* (127). We thought that by using both the ML and ME forward primers we would capture a greater representation of the methanogen diversities at these sites. All three forward primers were paired with the same reverse primer, mcrA-rev

(5'CGTTCATBGCGTAGTTVGGRTAGT 3'), which is a consensus sequence of three published reverse primers for *mcrA* (103, 168, 257). Reaction conditions were the same for all three *mcrA*-targeted primer sets and included an initial denaturation at 95°C for 3 min, followed by 5 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 30 s with a ramp rate of 0.1°C/s from the annealing to extension temperature. These initial five cycles were followed with 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s followed by a final extension at 72°C for 10 min.

PCR products were ligated into pCR 2.1 vector using a TA cloning kit, and the ligation products were used to transform *Escherichia coli* Top10 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Ampicillin- and X-Gal amended LB agar was used for blue-white screening of transformants, which were subject to whole-cell PCR directly to amplify the plasmid insert. PCR products were sent to the Nucleic Acid Facility at Penn State University for sequencing, and sequencing electropherograms were examined for accuracy using Sequence Scanner v1.0 (Applied Biosystems, Inc., Foster City, CA). Sequences derived from the analysis were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) under accession numbers DQ680352 to DQ680670, and accession numbers DQ781023 to DQ781063.

Determination of sequence similarity cutoffs for methanogen taxonomic levels.

16S rDNA and *mcrA* sequences from isolated methanogens were analyzed to determine how to group clone sequences from the libraries into appropriate OTUs. A current list of methanogens with recognized taxonomic standing is maintained by the Subcommittee on Taxonomy of Methanogens of the International Committee on Systematics of Prokaryotes (ICSP) (http://www.the-icsp.org/taxa/methanogenslist.htm). Using this accepted taxonomic structure, corresponding 16S rDNA and *mcrA* sequences for these methanogens were obtained from GenBank when available. Sequences were eliminated from analysis if they were too short or contained several undetermined bases (>2% of the sequence). Sequences belonging to *Methanocalculus* sp. were eliminated from analysis as the placement of this group is still under study by the ICSP. Sequences for *Methanospirillum hungatei* and *Methanopyrus kandleri* were also eliminated from analysis as they each represent the only sequence for their genus and family. Sequences were truncated from 114 to 1435 bp (*E. coli* numbering) for 16S rDNA, and 1014 to 1423 bp (*Methanobacterium thermautotrophicum* numbering) for *mcrA*. These truncations were necessary to ensure that all sequences were of the same length prior to analysis. The sequences were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/), and Mega3 (147) was used to create distance matrices based on the Jukes-Cantor algorithm. Sequences were grouped together

by taxonomic level, and Mega3 was used to calculate the mean sequence similarities within each taxonomic unit and between taxonomic units. For instance, within the genus *Methanobacterium*, the average sequence similarity was calculated based on comparisons of each species to every other species in that genus. To calculate the percent similarity within the family *Methanobacteriaceae*, the mean similarity was calculated based on the similarities between each species in this family and every other species in a separate genus of this family.

Phylogenetic and diversity analyses of bog and digester communities.

The program DOTUR (234) was used to group clone sequences into OTUs based upon decreasing levels of similarity using the furthest-neighbor algorithm, which states that any two sequences in a group defined by a percent similarity are at least that similar to one another. The input files for DOTUR were distance matrices generated by Mega3 using a Jukes-Cantor algorithm. EstimateS (version 7.5, R. K. Colwell [http://purl.oclc.org/estimates]) was used to calculate Coleman rarefaction curves, Shannon-Wiener and Simpson indices, and Chao1 and ACE values. For the 16S rDNA libraries, statistics were conducted for OTUs of 98 and 96% similarity, respectively representing similarity within a methanogen species suggested by Boone (23) and similarity within a methanogen genus as determined by this study. For the *mcrA* libraries, statistics were conducted for OTUs of 89% similarity, representing the average *mcrA* sequence similarity of methanogens within a genus as there is no standard *mcrA* divergence for determining a new methanogen species. For each run, 100 randomizations were made with sample replacement. To construct phylogenetic trees, clones were grouped with DOTUR by family-level associations as determined in this study. One representative sequence was chosen from each clone group, and these clone sequences were aligned with ClustalW, and phylogenetic trees were created in Mega3 using the maximum parsimony method with a clone-neighbor interchange search level 1 and bootstrapped with 100 trials. Trees for both the 16S rDNA and *mcrA* were rooted with *Methanopyrus kandleri* as the outgroup.

RESULTS

Sequence similarities for methanogen taxonomic levels.

The mean percent similarities of methanogen 16S rDNA sequences were calculated for 15 genera containing at least two sequences, and 8 families representing four orders of methanogens (Table 3-1). Results obtained using a Kimura 2-Parameter algorithm (data not shown) were nearly identical to those obtained with the Jukes-Cantor algorithm. The average

sequence similarity of species within a genus was 96.0% with a range of 88.2 to 100%. The average sequence similarity within a family was 94.3% when considering all methanogen families, and was 93.5% for families containing at least two genera. The mean percent similarities of *mcrA* sequences were calculated for 15 genera and 8 families of methanogens (Table 3-2). The average percent similarity of *mcrA* sequences within a genus was 88.9%, which ranged from 69.3 to 100%. The average similarity overall within a family was 83.5%, and was 79.0% for families containing at least two genera.

Collector's curves and diversity indices of bog and digester communities.

A total of 97 methanogen 16S rDNA sequences were obtained, 47 from the bog sediment and 50 from the digester sludge. A total of 259 *mcrA* sequences were obtained, 105 from the bog sediment and 154 from the digester sludge. For the 105 *mcrA* sequences derived from the bog, 35, 38, and 32 sequences were obtained with ME, ML, or mlas as the forward primer, respectively. For the 154 *mcrA* sequences obtained from the digester sludge, 21, 72, and 61 sequences were obtained with ME, ML, and mlas as the forward primer, respectively.

The 16S rDNA and *mcrA* libraries were analyzed by constructing species accumulation curves and calculating diversity indices. Minimum sequence similarities of 98 and 96% were used to group16S rDNA sequences into OTUs as these values represented the suggested sequence similarity within a methanogen species (23), and the calculated average sequence similarity of species within a genus from this study (Tables 3-1, 2). Sequences for *mcrA* generated from all three forward primers were analyzed together, but sequences identified as the isoenzyme *mrtA* were excluded from this analysis. Only members of the *Methanobacteriales* and *Methanococcales* possess the Mrt operon (89), so these sequences were excluded to avoid a biased overrepresentation of species from these orders in the library. At a sequence similarity of 98%, the 16S rDNA libraries were grouped into 26 and 16 clades for the bog and digester, respectively. At a sequence similarity of 96%, the 16S rDNA libraries were grouped into 19 and 7 clades for the bog and digester, respectively. At a sequence similarity of 89%, *mcrA* sequences were grouped into 24 clades each for the bog and digester libraries.

Based on these clone groupings, Coleman rarefaction curves, which represent how extensively the genetic diversity at a site has been sampled, were constructed for both environments and gene targets (Fig. 3-1). The diversity indices ChaoI, abundance-based coverage estimator (ACE), Shannon-Wiener index (H'), and Simpson's index (D) were calculated using EstimateS (Table 3-3). The values for Chao1 and ACE represent the expected number of OTUs present in an environment if sampling were complete (117). The Shannon-Wiener index (H') and

Simpson's index are measures of species richness and evenness of distribution of species within a community, and both increase with increasing genetic diversity at a site. Comparing the 16S rDNA libraries, diversity was lower and dominance was greater in the digester versus the bog library at both the 96 and 98% similarity cutoffs for OTU groupings (Table 3-3). As expected, for both 16S rDNA libraries the rarefaction curves show a flattening with a cutoff of 96 versus 98%, but the change in curve shape was much more dramatic for the 16S rDNA library (Fig. 3-1). Comparing the *mcrA* libraries from the bog and digester, there was no difference in diversity or dominance indices (Table 3-3). The diversity indices for the *mcrA* libraries were estimated for a genus-level grouping and were compared with the 16S rDNA libraries at a genus-level grouping of 96% similarity. There was no difference in diversity when comparing the 16S rDNA library to the *mcrA* libraries of the bog and digester, but the 16S rDNA library did show less diversity than the other three libraries. Ecological indicators often underestimate the true richness of a community at low sample sizes (117) and as the 16S rDNA libraries were much smaller than the *mcrA* libraries, this may have contributed to the lower richness values for the 16S rDNA digester library.

Phylogeny of bog and digester communities.

Sequences in the libraries were grouped at the family level using DOTUR with minimum sequence similarity values of 93.5 and 79% for 16S rDNA and *mcrA*, respectively. One representative sequence from each group was chosen for incorporation in the phylogenetic trees and was identified by BLAST analysis (Appendix 1 Figs. A1-1, 2). The 16S rDNA sequences grouped into 15 clades for the bog and 7 clades for the digester, with no incidence of clones from both environments being present in the same clade (Fig. 3-2). For the 16S rDNA libraries, nearly all of the clones from Bear Meadows Bog closely grouped with clones from acidic freshwater environments, including two central New York state peatlands (17, 30), and a freshwater lake located downstream of an acidic *Sphagnum* bog (40). The 16S rDNA digester sequences were related to clones obtained from a variety of environments, including full-scale anaerobic digesters (76, 186), lotus field soil (225), and part of the Florida Everglades impacted by agricultural runoff (41).

The *mcrA* sequences grouped into 12 clades for the bog and 14 clades for the digester, with two clades containing representative sequences from both the bog and digester libraries (Fig. 3-3). Similar trends are seen in the *mcrA* libraries as with the 16S rDNA libraries, with most of the bog sequences most closely related to clones from oligotrophic, freshwater lakes and

peatlands (72, 85, 87, 126) and the majority of the digester sequences most closely related to clones from anaerobic digesters (143, 218), and pasture and rice paddy soil (90, 244).

Both the bog and digester libraries were dominated by clones that were related to uncultured methanogen groups. The majority of clones from the bog environment were members of the Fen cluster, a deeply-branching clade of the *Methanomicrobiales* which was first identified by Galand *et al*.(88), but has also been labeled by other researchers as MCR-5 and E1 and E2 (17, 35). Three groups of uncultured methanogens were represented exclusively within the bog clone libraries. These include Rice Clusters I and II, first described in Groβkopf *et al*. (98), and two sequences that are related (89% similarity) to a branch within cluster MCR-5 (Figure 3-3). Members of this cluster are related to members of the *Methanomicrobiales* isolated from an oligotrophic area of the Florida Everglades (35). The majority of the digester 16S rDNA clones grouped with sequences from Cluster I, isolated from a variety of bioreactors and described by McHugh *et al*.(176), and with sequences from a nutrient-impacted area of the Florida Everglades that are grouped in *Methanomicrobiales* clusters E1 and E2, as named by Cadillo-Quiroz *et al*. (30). Most of the *mcrA* digester clones group with cluster MCR-7, which was identified by Castro *et al*. (35) during a molecular survey of oligotrophic and nutrient-impacted areas of the Florida Everglades. A number of digester clones grouped in cluster MCR-2, a cluster also defined in the study by Castro *et al.* (35). In this study all sequences belonging to MCR-2 and MCR-7 were only found in the digester environment.

Although the majority of clones obtained in this study were closely related to other uncultured clone sequences, a number of clone sequences from both libraries were closely related to cultured methanogen species. The bog environment included clones related to *Methanocorpusculum parvum* (GenBank accession no. AF414045) and the *mrtA* of *Methanothermobacter thermophilus* (AY289753), and several clones from the digester environment were closely related to *Methanospirillum hungatei* strain JF-1 (NC007796) and *Methanobrevibacter ruminantium mcrA* (AF414406).

DISCUSSION

The methanogens of the bog and digester environments were found to be very different from one another, and although this result was expected, the degree of difference in comparing sequences from both environments was surprising. There was nearly no overlap in either 16S rRNA or *mcrA* gene sequences even at a level of similarity representing the average similarity within a methanogen family. Few studies have compared methanogens in natural wetlands to those found in constructed environments, and the present study is the first to directly compare

methanogen communities in a naturally-occurring wetland and a constructed anaerobic reactor. In addition to containing very different methanogen sequences, both the bog and digester libraries were dominated by clones belonging to uncultured environmental groups. The majority of clones from both the 16S rDNA and *mcrA* libraries of the bog environment were related to the Fen Cluster, a deeply branching clade within the *Methanomicrobiales*. The Fen cluster may no longer be considered an uncultured group, as recently Braüer *et al.* (25) isolated and described a member of this group, *Candidatus* Methanoregula boonei, which was found to be acid tolerant and hydrogenotrophic. The Fen cluster has been identified in a number of other peatland environments, many of which are acidic, oligotrophic bogs. The majority of clones from the 16S rDNA library of the digester environment grouped with Cluster I, members of which have been found in a variety of anaerobic digesters (48, 176, 206). The majority of clones in the *mcrA* library from the digester grouped with clade MCR-7, which has been found in a wide range of environments including oligotrophic and nutrient-impacted areas of the Florida Everglades (35), biogas plants (218), and a brackish lake sediment (14). Cluster I is related to the *Methanobacteriales*, and MCR-7 is found within the *Methanomicrobiales*, so it is likely that members of Cluster I and MCR-7 are hydrogenotrophic. This suggests that both environments are dominated by hydrogenotrophic methanogens, although acetotrophic methanogen sequences closely related to *Methanosaeta* were also found in both environments. Members of the *Methanosaeta*, which are strictly acetotrophic, appear to be ubiquitous and have been isolated from a number of different environments including freshwater marshes and lakes (35, 38, 40), acidic peatlands (17, 85, 126), rice field soil (164), and from anaerobic digesters treating municipal solid waste and sewage sludge (96, 179), dairy wastewater (160), or industrial wastewater (50). Despite the abundance of clones representing hydrogenotrophic methanogens in all libraries, it should be noted that sequence abundance in a clone library does not necessarily relate to abundance of the organism in the environment.

The analyses of sequences from accepted methanogen taxonomy performed in this study illustrate the difficulty in gene fragment-based surveys of environmental microorganisms. Springer *et al.* (257) found that the average *mcrA* sequence distance between species was approximately three times greater than the distance of the 16S rDNA sequences. This correlates well to the difference between the average sequence distances found for the 16S rDNA and *mcrA* in this study. Despite this fact, the range of similarity within a methanogen genus varied from 88.2 to 99.9% for the 16S rDNA, and from 69.3-100.0% for the *mcrA*. Currently, the definition of species applied to methanogen 16S rDNA clone libraries varies from researcher to researcher, and there is no species definition applied to *mcrA* libraries. Ecological richness estimators can be

used for microbial communities only if strict OTU definitions are consistently applied (117). Consistent application of an OTU definition also makes it possible to compare diversity measurements among studies. The goal for determining average sequence similarity within genera and families of methanogens in this study was primarily to calculate richness estimators and characterize differences between the two methanogen communities we studied, but these data were also used to relate *mcrA* sequence disparity to phylogenetic distance.

The clone libraries for ME, ML, and mlas as the forward primer were compared to detect any difference in *mcrA* sequence recovery among the three forward primers. In general, a clone group sharing at least 80% similarity was detected by all three primers. Clone groups with only one or two clones were not detected by all three primers, but this is likely a limitation of the number of clones sampled in the different libraries. With both primers ML and mlas, clone groups with more than one clone were obtained solely with that primer. This was most dramatic for the forward primer mlas, which had four groups of unique clones. These results suggest that the forward primers ML and mlas may capture gene sequences not captured by the forward primer ME. The ME primer set has been reported to not detect sequences from *Methanosaeta* sp. or sequences of the isoenzyme *mrtA* (14), a finding mimicked in the current study.

A few authors have looked at primer bias in obtaining *mcrA* sequences from the natural environment. Lueders *et al*. (164) found that a cluster of *mcrA* sequences, which was named "unidentified rice field soil *mcrA*" by the authors, could be detected with the MCR, but not the ME, primer pair. Nercessian *et al*. (187) amplified *mcrA* sequences from deep-sea hydrothermal vents with ML and ME primer sets, and recovered different sequences in both libraries with only some overlap in clones (187). Juottonen *et al*. (127) examined potential bias in the three *mcrA* primer sets in obtaining clones from drained peatland sediment. Similar clone sequences were obtained with all sets, but four minor OTUs were not detected by all sets although the authors could not rule out the number of clones sequenced as responsible for these differences in coverage. The dominant OTUs obtained with each primer set differed, which in turn affected ecological diversity measurements of the clone libraries, namely the Shannon-Wiener and Simpson's dominance indices (127). Results from these studies seem to suggest more comprehensive libraries of *mcrA* diversity may be obtained by using two or more primer sets. A limitation in comparing the results of these studies is that different PCR parameters, notably the annealing temperature, were used in each study. Lueders and Friedrich demonstrated that the numbers and types of clones obtained in an *mcrA* library generated with the ML primer set was determined by the choice of annealing temperature (166), so both the choice of primer set and PCR conditions will affect the clones obtained in a library.

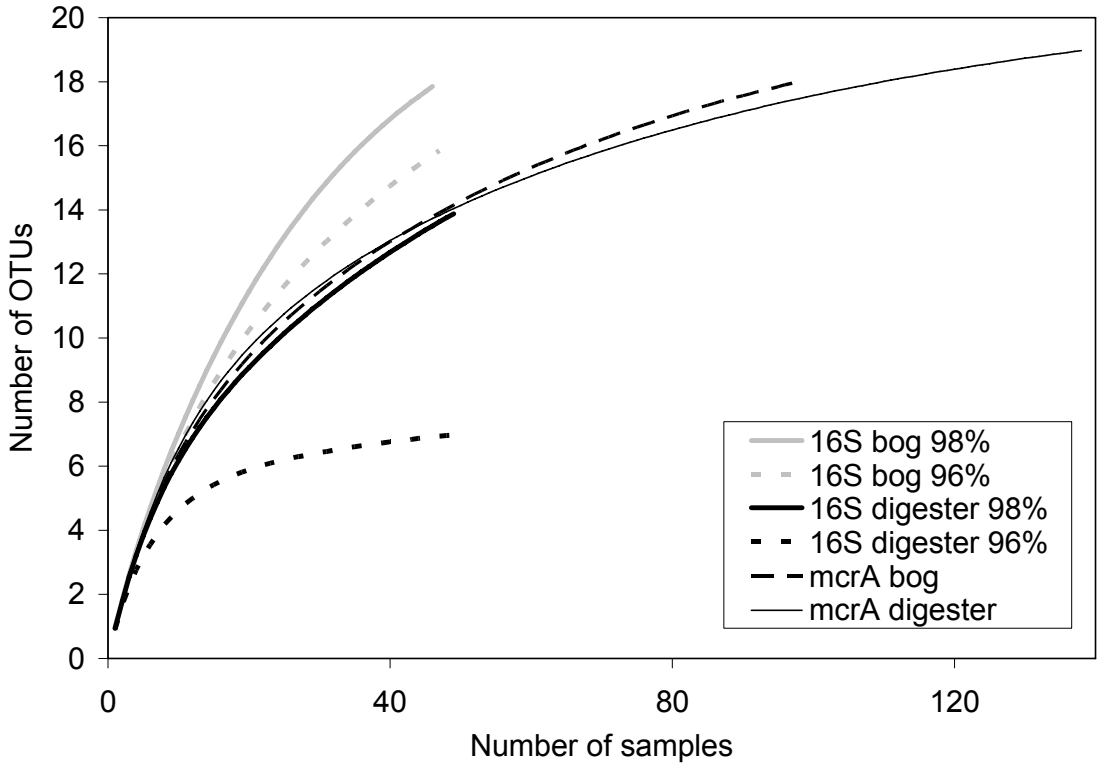


Figure 3-1. Coleman rarefaction curves of 16S rDNA and *mcrA* clone libraries obtained from an acidic peatland and an anaerobic digester.

The 16S rDNA sequences were grouped by 98 or 96% similarity and *mcrA* sequences were grouped by 89% similarity using the program DOTUR. Rarefaction curves were generated using the program EstimateS at 100 randomizations made with sample replacement.

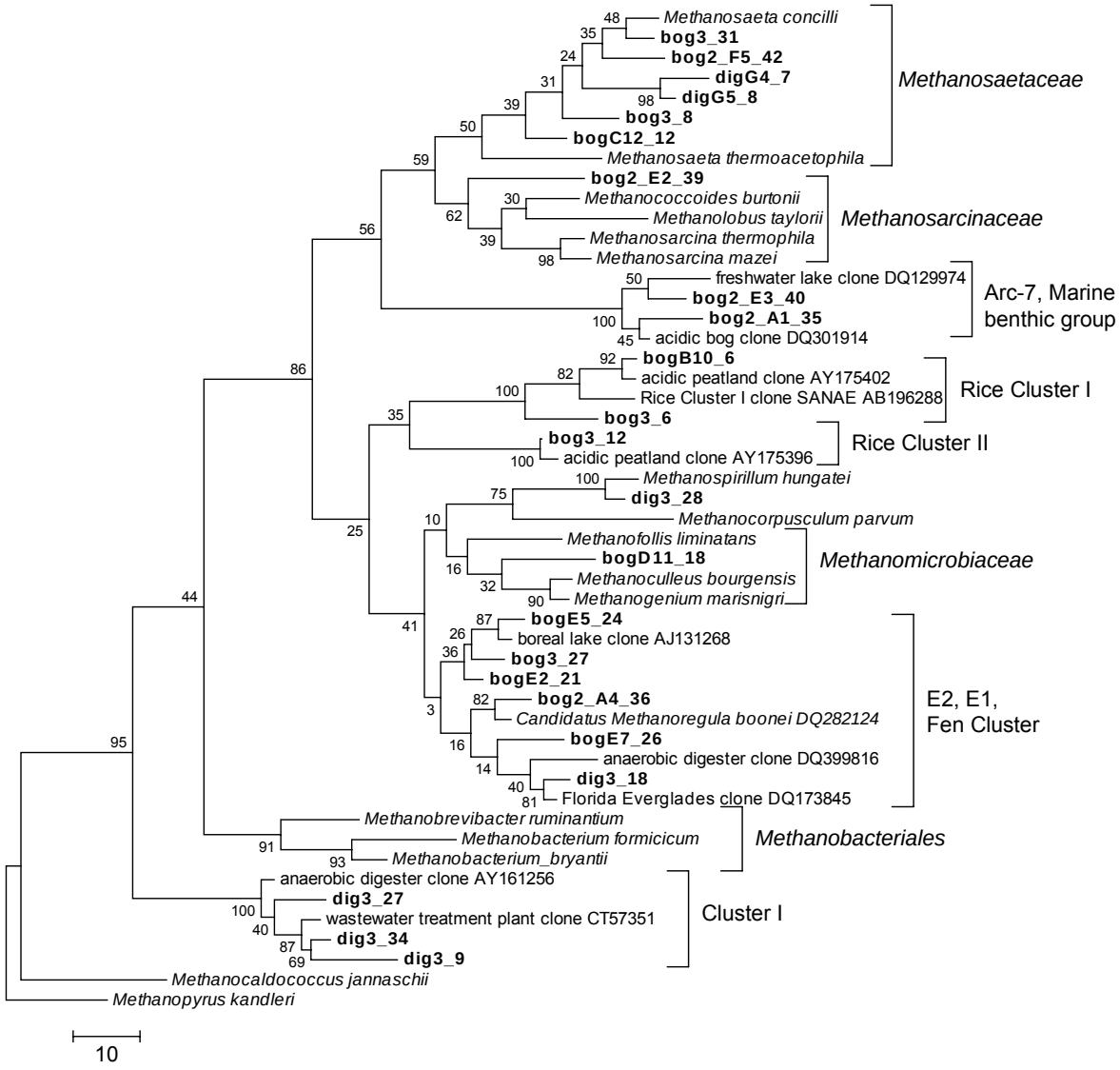


Figure 3-2. Phylogenetic tree for representative 16S rDNA sequences from an acidic peatland and an anaerobic digester.

Closest GenBank sequence matches are shown in Appendix 1, Table A1-1. Clones obtained from the acidic peatland are denoted "bog" and clones obtained from the anaerobic digester are denoted "dig". The tree was constructed as a maximum parsimony tree using a close-neighbor interchange level 1 and bootstrapped with 100 trials. All positions containing gaps and missing data were eliminated from the dataset. The scale bar represents the number of changes over the whole sequence. Classification of clusters is based on Vetriani *et al.* (280) for the marine benthic group, which was also named Arc-7 in Castro *et al.* (35), Groβkopf *et al.* (98) for Rice Clusters I and II, Cadillo-Quiroz *et al.* (30) for E1 and E2, also named Fen cluster in Juottonen *et al.* (126), and McHugh *et al.* for Cluster I (176).

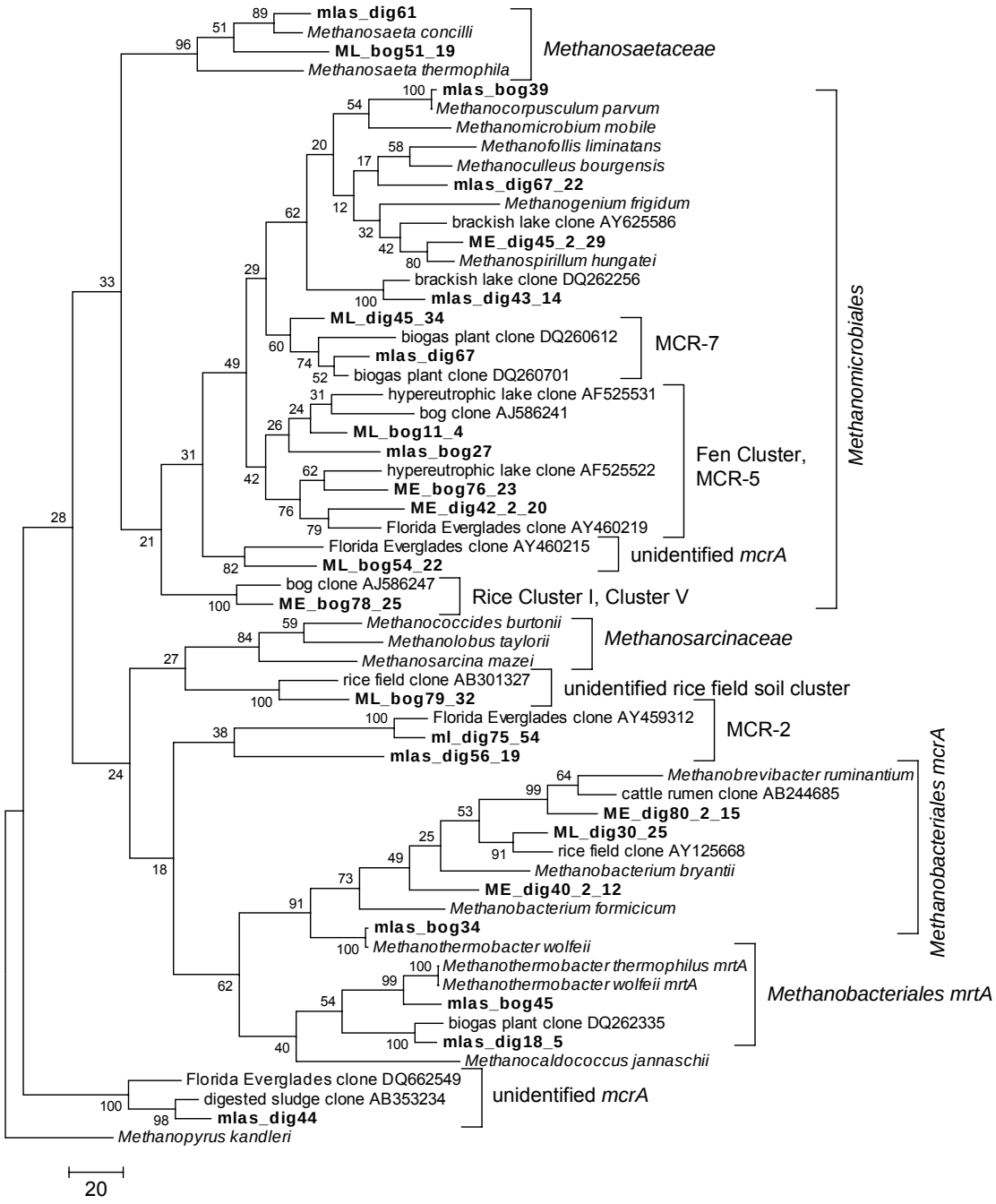


Figure 3-3. Phylogenetic tree for representative *mcrA* sequences obtained from an acidic peatland and an anaerobic digester.

Closest GenBank sequence matches are shown in Appendix 1, Table A1-2. Acidic peatland clones are denoted "bog", and anaerobic digester clones are denoted "dig". The tree was constructed as a maximum parsimony tree using a CN-1 and bootstrapped with 100 trials. All positions containing gaps and missing data were eliminated from the dataset. The scale bar represents the number of changes over the whole sequence. Classification of clusters is based on Castro *et al.* (35) for MCR-2, MCR-5, and MCR-7, Juottonen *et al.* (126) for the Fen cluster, Groβkopf *et al.* (98) for Rice Cluster I, also named Cluster V in Galand *et al.* (88), and Lueders *et al.* (164) for the unidentified rice field soil cluster.

Table 3-1. Sequence percent similarity values within the methanogens for the 16S rRNA gene. Ranges and means were calculated with distance matrices created with the Jukes-Cantor algorithm.

Family	mean % similarity	Genus	# in analysis	range of % similarity (mean)	GenBank accession numbers
Methanobacteriaceae	92.8	*Methanobacterium*	11	92.1-99.8 (95.4)	M36508, AY386124, DQ649335, AY350742, M59124, AF233586, AF095261, AF028690, AF093061, DQ649330, AF095264
		Methanobrevibacter	9	88.2-97.1 (94.0)	AY196666, AF242652, AB065294, U62533, U41095, U82322, U55239, U55237, U55240
		Methanosphaera	1	N/A	M59139
		Methanothermobacter	5	98.6-99.9 (99.3)	X68720, X99046, X99047, X99048, AB104858,
Methanococcaceae	93.4	*Methanococcus*	4	91.4-95.9 (93.5)	M36507, U39016, U38486, U38461,
		Methanothermococcus	2	(95.0)	M59128, AB057722,
Methanocaldococcaceae	95.6	*Methanocaldococcus*	5	95.7-98.8 (96.8)	M59126, AF056938, AF547621, AF025822, AF051404
		Methanotorris	1	N/A	AB100884
Methanomicrobiaceae	92.7	*Methanomicrobium*	1	N/A	M59142
		Methanoculleus	6	95.7-99.9 (97.3)	AB065298, AB038795, M59134, Y16382, AB065297, AF531178
		Methanofollis	3	95.7-99.7 (97.3)	AF095272, AY186542, Y16428,
		Methanogenium	3	90.0-98.4 (93.2)	M59130, AJ862839, DQ177345
		Methanolacinia	1	N/A	AY196678
Methanoplanaceae	94.4	*Methanoplanus*	2	(94.4)	M59143, U76631,
Methanocorpusculaceae	99.9	*Methanocorpusculum*	4	99.7-100.0 (99.9)	AY260435, AF095266, AY260436, AF095268
Methanosarcinaceae	92.9	*Methanosarcina*	8	93.7-99.1 (96.6)	AY196682, AE010299, AY663809, DQ058823, AB065296, AJ012742, U89773, M59140
		Methanococcoides	2	(97.6)	M59127, X65537
		Methanohalobium	1	N/A	U20149
		Methanohalophilus	1	N/A	M59133
		Methanolobus	4	96.6-98.2 (97.4)	U20148, U20152, U20154, U20155,
		Methanomethylovorans	1	N/A	AF120163
		Methanimicrococcus	1	N/A	AJ238002
Methanosaetaceae	92.5	*Methanosaeta*	2	(92.5)	M59146, AB071701
Mean % similarity	**94.3**			**96.0**	
with multiple genera	**93.5**				

Table 3-2. Sequence percent similarity values within the methanogens for *mcrA*.
Ranges and means were calculated using distance matrices created with the Jukes-Cantor algorithm.

Family	mean % similarity	Genus	# in analysis	range of % similarity (mean)	GenBank accession numbers
Methanobacteriaceae	71.7	*Methanobacterium*	4	69.3-75.8 (73.1)	AF414050, AY386125, AF313806, AY289750
		Methanobrevibacter	4	77.2-90.0 (82.6)	AF414046, AF414035, DQ251045, DQ251046
		Methanosphaera	1	N/A	AF414047
		Methanothermobacter	4	97.2-100.0 (98.6)	U10036, AY303950, AY289752, AY289748
Methanothermaceae	98.0	*Methanothermus*	2	(98.0)	J03375, AY289747
Methanococcaceae	83.2	*Methanococcus*	4	76.0-92.7 (83.3)	M16893, AY354034, BX957223, X07793
		Methanothermococcus	2	(85.9)	AF414048, AY354033
Methanocaldococcaceae	81.4	*Methanocaldococcus*	2	(82.5)	AF414040, AY354035
		Methanotorris	1	N/A	AF414039
Methanomicrobiaceae	79.5	*Methanomicrobium*	1	N/A	AF414044
		Methanoculleus	5	89.3-100.0 (92.1)	AF414036, AB288270, NZ AASI01000002, AF313804, DQ229156
		Methanofollis	1	N/A	AF414041
		Methanogenium	4	82.8-93.8 (86.9)	DQ229157, DQ229158, DQ229159, DQ229160
Methanocorpusculaceae	94.9	*Methanocorpusculum*	3	93.8-97.1 (94.9)	AY260445, AF414049, AY260441
Methanosarcinaceae	79.4	*Methanosarcina*	7	88.3-99.3 (91.8)	Y10058, AE010299, AY260443, AF414043, U22248, U22250, U22251
		Methanococcoides	2	(96.0)	U22235, U22234
		Methanohalobium	1	N/A	U22236
		Methanohalophilus	3	95.0-99.0 (96.7)	U22237, U22259, U22239
		Methanolobus	5	87.4-96.3 (91.6)	U22244, U22257, U22242, U22243, U22245
		Methanomethylovorans	1	N/A	AY260442
		Methanosalsum	1	N/A	U22252
Methanosaetaceae	79.6	*Methanosaeta*	2	(79.6)	AF313802, CP000477
Mean % similarity	**83.5**			**88.9**	
with multiple genera	**79.0**				

Table 3-3. Diversity indices for bog and digester 16S rDNA and *mcrA* libraries using species-level groupings.

Library	Chao1 (95% confidence intervals)	ACE	Shannon-Wiener Index (H')	Simpson's diversity index (1/D)
16S bog—98%	28.02 (20.16, 65.43)	30.2±8.96	2.51±0.16	10.99±3.15
16S bog—96%	20.66 (15.32, 54.5)	19.07±4.58	2.28±0.14	8.44±1.9
16S digester—98%	17.33 (12.72, 48.36)	17.19±4.78	2.05±0.15	6.85±1.18
16S digester—96%	10.73 (9.3, 24.78)	10.74±1.86	1.84±0.12	5.59±0.81
mcrA bog	25.83 (21.69, 52.1)	24.27±3.07	2.65±0.09	11.93±1.71
mcrA digester	26.06 (21.17, 57.1)	24.81±4.3	2.5±0.08	9.94±0.93

Chapter 4

An *mcrA*-Targeted Real-Time Quantitative PCR Method to Examine Methanogen Communities

This chapter is an adaptation of Steinberg and Regan (2009)

ABSTRACT

Methanogens are of great importance in carbon cycling and alternative energy production, but they are difficult to isolate and thus typically studied through culture-independent molecular techniques. Biases inherent in PCR prevent inference of an organism's environmental abundance based on end-point PCR product abundance, but real-time quantitative PCR (qPCR) overcomes some of these biases. We developed a SYBR-Green I qPCR assay to quantify total numbers of methyl-coenzyme M reductase alpha subunit (*mcrA*) genes. TaqMan probes were also designed to target several different phylogenetic groups of methanogens in qPCR assays. Total *mcrA* and *mcrA* of different methanogen phylogenetic groups were determined from six samples: four samples from anaerobic digesters treating either primarily cow or pig manure, and two aliquots from an acidic peat sample stored at 4°C or 20°C. Only members of the *Methanosaetaceae, Methanosarcina, Methanobacteriaceae, Methanocorpusculaceae*, and Fen cluster were detected in the environmental samples. The three samples obtained from cow manure digesters were dominated by members of the genus *Methanosarcina*, whereas the sample from the pig manure digester contained detectable levels of only members of the *Methanobacteriaceae*. The acidic peat samples were dominated by both *Methanosarcina* and members of the Fen cluster. In two of the manure digester samples only one methanogen group was detected, but in both of the acidic peat samples and two of the manure digester samples, multiple methanogen groups were detected.

INTRODUCTION

Methanogens are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases, during organic matter degradation in anaerobic soils and sediment (32). Methanogens are widespread in anaerobic environments including tundra (211), freshwater lake and wetland sediments (35, 72), estuarine and marine sediments (14), acidic peatlands (17, 88), rice field soil (45, 99), animal guts (224), landfills (168), and anaerobic digesters treating animal manure (8), food processing wastewater (159) and municipal

wastewater and solid waste (213, 297). Methane produced in anaerobic digesters may be captured and used for energy production thus offsetting some or all of the cost of operation, and reducing the global warming potential of methane release to the atmosphere.

Methanogens are difficult to study through culture-based methods, and therefore many researchers have instead used culture-independent techniques to study methanogen populations. The 16S rRNA gene (16S rDNA) is the most widely used target for gene surveys, and a number of primers and probes have been developed to target methanogen groups (35, 56, 171, 211, 215, 221, 251, 256, 297). To eliminate potential problems with nonspecific amplification, some researchers have developed primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) (103, 168, 257). The Mcr is exclusive to the methanogens with the exception of the methane-oxidizing *Archaea* (104), and shows similar phylogeny to the 16S rDNA, allowing *mcrA* analysis to be used in conjunction with, or independently of, the 16S rDNA (15, 168, 257). A number of researchers have examined methanogen communities with the *mcrA* and have found uncultured clades quite different in sequence from cultured methanogen representatives (35, 45, 72, 88, 103, 127, 164, 255).

Researchers have quantitatively described methanogen communities through the use of rRNA- or rDNA-targeted probes using techniques such as dot-blot hybridization (8, 159, 213, 215, 256) and fluorescent *in situ* hybridization (FISH) (56, 221, 241, 297). Real-time quantitative PCR (qPCR) is an alternate technique capable of determining the copy numbers of a particular gene present in the DNA extracted from an environmental sample. Only a few researchers have used qPCR to quantitatively examine methanogen communities, and most of these studies have exclusively targeted the 16S rDNA (113, 224, 231, 291-293). Far fewer researchers have used qPCR to quantify methanogens by targeting the *mcrA* (119, 195, 245), and these studies were limited to only a few phylogenetic groups.

In this paper we present a methodology for determining methanogen gene copy numbers through the use of qPCR targeting the *mcrA*. Methanogens were quantified in total using methanogen-specific primers in SYBR-Green assays and also as members of several different phylogenetic groups using TaqMan probes targeting specific subsets of methanogens.

MATERIALS AND METHODS

Design of methanogen-specific probes.

We previously constructed clone libraries for the *mcrA* and *mrtA* genes from two distinct methanogenic environments: sediment of an acidic transitional fen known as Bear Meadows Bog,

and the primary digester of a municipal wastewater treatment plant treating combined primary and secondary sludge (260). The alignment of these clone sequences along with sequences from cultured methanogens were used to design TaqMan probes for groups of methanogens dominant in both environments (Fig. 4-1, Table 4-1). Targeted groups that were observed in the municipal wastewater sludge digester include members of the *Methanosaetaceae*, *Methanobacteriaceae mcrA* and the isoenzyme *mrtA*, *Methanospirillaceae*, MCR-7, and two subgroups of MCR-2 which were labeled MCR-2a and MCR-2b. Uncultured clades MCR-7 and MCR-2 derive from a naming scheme used by Castro *et al.* (35). Targeted groups observed in the clone library from the acidic fen include *Methanosarcina*, *Methanocorpusculaceae*, and the Fen Cluster, which was named in a study by Galand *et al.* (88). Probes were designed for a melting temperature of 68-72°C, a G+C content of 40-60%, and a length of 23-30 base pairs. The melting temperatures of probes were calculated with Integrated DNA Technologies' Oligo Analyzer program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Probe specificity was checked with a BLAST search of GenBank sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Probes were ordered from Biosearch Technologies (Novato, CA) labeled with FAM, Cal Fluor Orange, or Cal Fluor Red as the reporter dye, and Black Hole 1 or Black Hole 2 as the quencher dye.

Collection of environmental DNA.

Methanogen communities were analyzed from four anaerobic digesters and two incubations of acidic peat. Case studies for the design and operation of the digesters examined in this study are available through the Pennsylvania State University Department of Agricultural and Biological Engineering website (www.biogas.psu.edu). Penn England dairy farm began operating a two-compartment reactor with a flexible cover in August of 2006. Each compartment is heated to 37°C and mixed approximately one hour twice daily. The design retention time in each compartment of the reactor is 20 d with 8-9% solids, and the system receives dairy manure, bedding, and milk parlor wash water. Bedding used in the dairy barns is composed entirely of the dried digested solids from the reactor. Grease trap waste from local restaurants is added to increase methane production. Brookside farms began operating a plug-flow reactor in April of 2006 with a retention time of 30-33 d and 8-10% solids. The reactor is heated to 37°C and receives manure, sawdust bedding, and milk parlor wash water. Brewery waste and cheese whey are also added to the influent of the reactor. Schrack dairy farm began operating a plug-flow reactor in August of 2006 with a retention time of 30 d and 10-12% solids. The reactor is heated to 37°C and receives manure, sawdust bedding, and milk parlor wash water. Crone farms

operates a plug-flow digester with a retention time of 30 d and 8-10% solids. The reactor is heated to 37°C and treats only raw pig manure with no bedding or other amendments.

In addition to the anaerobic digesters, samples were also taken from acidic peat that had been stored for nearly two years at either room temperature (about 20°C) or 4°C. The peat incubations were included because, based on the previous cloning results, we expected that some of the groups targeted by these TaqMan probes would not be present in manure digesters but would be present in incubations of peat. The peat was collected in September of 2006 from Bear Meadows Bog, an acidic transitional fen which has been previously described (19). Peat was transferred to sterile Pyrex media bottles under aerobic conditions, and bottles were capped and stored with no amendments for nearly 2 years before withdrawing samples for DNA analysis. DNA from all samples was extracted using a PowerSoil DNA Extraction Kit (MoBio, Carlsbad, CA) according to manufacturer's instructions. Contaminants carried over during DNA extraction inhibited PCR, but inhibition was eliminated by diluting extracts fivefold in PCR-grade water.

Preparation of templates for qPCR standard curves.

Cultures of *Methanobacterium thermoautotrophicum, Methanobacterium wolfeii, Methanosarcina acetivorans,* and *Methanosarcina barkeri* MS were generously provided by the laboratory of Christopher House (Associate Professor of Geosciences, Penn State University, University Park, PA). DNA from these methanogen cultures was extracted with a DNeasy DNA Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions for DNA extraction from gram-positive cells. The *mcrA* or *mrtA* gene was amplified with primers mlas and mcrA-rev using a previously described method (260). PCR products were ligated into a pCR 2.1 vector and used to transform *Escherichia coli* Top10 cells according to the manufacturer's instructions (TA Cloning Kit, Invitrogen, Carlsbad, CA). Blue-white screening was used to identify transformants, and positive clones were grown overnight at 37°C in LB broth containing ampicillin. Plasmids were purified with a PrepEase Plasmid Purification kit (USB, Cleveland, OH) and quantified by absorbance at 260 nm. To confirm the presence of the correct insert, plasmids were sequenced at the Nucleic Acid Facility at Penn State University. As we did not have methanogen cultures representing *Methanospirillaceae*, *Methanocorpusculaceae*, or the Fen cluster, we used environmental clones previously obtained from a municipal sludge anaerobic digester and Bear Meadows Bog (260). Plasmid DNA containing each of the 18 cloned fragments was used as templates for standard curves (Table 4-1). Plasmid DNA from each clone was diluted 10-fold in PCR-grade distilled water to create standard dilution series for qPCR

ranging from 2.1×10^7 to approximately 207 copies per µl, which translated to 4.15×10^7 to 415 copies per reaction.

SYBR-Green I assays for total mcrA gene copies.

Quantitation of total *mcrA* gene copies was performed with primers mlas and mcrA-rev (260) using the non-specific fluorophore SYBR-Green I (Molecular Probes, Invitrogen). The full genomes from 19 methanogens submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were examined for the presence of multiple copies of *mcrA*. All the genomes contained only one copy of *mcrA*, but members of the *Methanococcales* and *Methanobacteriales* also contained a copy of the gene for isoenzyme Mrt. The primers mlas and mcrA-rev also amplify *mrtA*, so methanogen numbers inferred by gene copy numbers may be overestimated by as much as a factor of 2 when these two orders are prevalent.

The SYBR-Green qPCR conditions included 1x *Taq* Polymerase buffer and 0.03 U/µl *Taq* (USB), 2.5 mM $MgCl_2$, 0.2 mM dNTPs containing uracil in place of thymidine, 0.01 U/µl of heat-labile uracil-DNA glycosylase, 0.25 µM each primer, 0.3 M betaine, 250 µg/mL of bovine serum albumin, 10 nM fluorescein (Bio-Rad, Hercules, CA) as a reference dye, and a 75,000x dilution of SYBR-Green I dye (Molecular Probes). Quantitative PCR was run on a Bio-Rad iCycler with the following protocol: 20 min at 37°C to degrade contaminating PCR products, 3.5 min at 95°C followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55° for 45 sec, extension at 72°C for 30 sec, and image capture at 83°C, followed by a final extension at 72°C for 7 min. Melt-curve analysis to detect the presence of primer-dimers was performed after the final extension by increasing the temperature from 50° to 95°C in 0.5°C increments every 10 sec. Image capture at 83°C was necessary to exclude fluorescence from the amplification of primer-dimers.

SYBR-Green qPCR was first performed in triplicate on a dilution series of each clone used as a standard for the group-specific methanogen TaqMan probes (Table 4-1). Three separate 96-well plates were necessary to quantify the dilution series for all clones. The mean, standard deviation, and coefficient of variance were calculated for the dilution series within each plate and among all three plates. After testing the assay with these 18 standards, total *mcrA* copies from environmental DNA were determined in triplicate, with dilution series of clones containing the *M. thermoautotrophicum mrtA* and *M. concilli mcrA* genes run in duplicate as controls.

TaqMan qPCR for mcrA copies of individual methanogen groups.

TaqMan assays for *mcrA* copies from methanogen groups were verified before quantitation of environmental DNA. Each probe was checked for specificity in TaqMan assays by running all of the non-target clone standards as negative controls. A dilution series of clones used as standards for each probe was run in three separate reactions to test the reproducibility of the TaqMan probe quantitation. The master mix for TaqMan qPCR was identical to that used for SYBR-Green I assays, including the primers, except that it contained 3.5 mM $MgCl_2$ instead of 2.5 mM $MgCl_2$, and 150 nM of TaqMan probe in place of SYBR-Green I and fluorescein dyes. Quantitative PCR was performed with the following protocol: 20 min at 37°C to degrade any contaminating product, 3.5 min at 95°C followed by 40 cycles of denaturation at 95°C for 30 sec, and annealing/extension with image capture at 55° for 1 min. For quantitation of environmental DNA, one or two clones were used as standards for each TaqMan assay. One dilution series was constructed for each standard, and standards were run in duplicate while environmental samples were run in triplicate. The standard deviation of *mcrA* concentration for each environmental sample was determined from the standard deviation of the three threshold cycle (C_T) values, which was then log transformed to gene copy number.

RESULTS

Quantitative PCR assays.

Standard curves for SYBR-Green I qPCR were run with dilution series of all clones used as standards for methanogen group-specific TaqMan probes. A total of 18 curves were collected from three separate qPCR 96-well plates, and the mean and standard deviation were calculated for assays within each plate and for all the data combined together (Table 4-2). Similar results were obtained for each standard curve within and among plates, with an average slope of -3.451, average efficiency of 94.9%, average y-intercept of 39.40, average R^2 of 0.9962, and a lower limit of detection of approximately 415 copies/reaction. A slope of -3.32 represents 100% efficiency, or a doubling of the DNA products in each cycle of PCR.

TaqMan probes were tested in triplicate for specificity and reproducible amplicon detection with dilution series of clones used as standards for each probe (Table 4-1). Amplification and detection varied from probe to probe, with efficiencies ranging from 91-99% (Table 4-3). The lower limit of detection for each TaqMan probe was approximately 415 copies/reaction, and similar y-intercepts were obtained with each probe suggesting all probes had about the same sensitivity for their respective targets. Quantitative PCR assays with the same

probe were also similar for all three assays, which demonstrated the reproducibility of these methods. No negative controls or controls lacking template were amplified in any of the TaqMan qPCR assays, which confirmed the specificity of the probes to their targets. Several probes had two clones that were used as standards, and amplification of both clones was nearly identical for these probes (data not shown).

Quantitation of environmental DNA.

DNA was extracted from six environmental samples, including four anaerobic digesters treating primarily animal waste and two incubations of an acidic peat maintained at either 20°C or 4°C for nearly two years. Total *mcrA* was measured using a SYBR-Green I method, and *mcrA* from different methanogen groups was measured using the TaqMan probe method. Total *mcrA* was also calculated as the addition of the methanogen numbers obtained with each TaqMan probe. Total *mcrA* numbers obtained with both the SYBR-Green I and TaqMan probe methods were similar, although not identical (Table 4-4). The structure of the methanogen communities differed in the six environments both in terms of total *mcrA* numbers and in the numbers of different methanogen groups that were represented. All three cattle manure digesters were dominated by members of the *Methanosarcina*, whereas the two incubations of peat were dominated by both members of *Methanosarcina* and the Fen cluster. The Brookside digester sample showed detectable levels of only *Methanosarcina*, and the Crone digester sample showed only *Methanobacteriaceae*. The Penn England digester sample contained *Methanosaetacae*, *Methanosarcina*, *Methanobacteriaceae*, and *Methanocorpusculaceae*, and the Schrack digester sample contained *Methanosarcina* and *Methanocorpusculaceae*. The two peat incubations were the only environmental samples to contain members of the Fen cluster, but although both incubations were inoculated from the same sample, the methanogen communities were different in the two incubations. Besides members of the *Methanosarcina* and Fen cluster, the 20°C incubation also contained *Methanosaetaceae*, whereas the 4°C incubation contained members of *Methanobacteriaceae*.

DISCUSSION

A number of studies have quantified methanogens belonging to different phylogenetic groups with techniques such as dot-blot hybridization and fluorescent *in-situ* hybridization (159, 215, 297), as well as qPCR (113, 224, 231, 291-293). Quantitation in these studies has targeted 16S rRNA or rDNA, but studies by Springer *et al.* (257) and Bapteste *et al.* (15) suggest that the *mcrA* gene demonstrates the same phylogenetic relationships as the 16S rDNA gene between

organisms. A major advantage to the use of *mcrA* as a target is that this gene is exclusive to methanogens, with the exception of the closely-related anaerobic methane-oxidizing *Archaea*. Despite this fact, very few researchers have used the *mcrA* as a target for quantitation studies (119, 195, 245).

To the best of our knowledge, the only other study besides ours that quantified phylogenetically different groups of methanogens by targeting *mcrA* is by Shigematsu *et al.*, who designed TaqMan probes to target *Methanosaeta*, *Methanosarcina*, and *Methanoculleus* in acetate-fed anaerobic reactors (245). Our study is the first to quantify methanogens of certain phylogenetic groups using *mcrA* as the target, including methanogens of the uncultured group MCR-2, and also members of the previously uncultured groups Fen cluster and MCR-7. Bräuer *et al.* recently isolated and characterized a member of the Fen cluster, *Candidatus* Methanoregula boonei, from an acidic peatland (25). This isolate was found to be hydrogenotrophic and acidophilic, with optimum growth at pH 5. The Fen cluster, which has also been referred to as MCR-5 (35) or E1 and E2 (17), has been detected in a number of diverse environments including neutral freshwater sediments (253), acidic peatland sediments (126), and rice field soil (45). Imachi *et al.* recently isolated a member of MCR-7 from a propionate-degrading enrichment originally cultured from an anaerobic digester stabilizing municipal sewage sludge (118). *Candidatus* Methanolinea tarda is related to the *Methanomicrobiales*, and most closely related to *Candidatus* Methanoregula boonei (93.7% 16S rRNA sequence similarity). The isolate was neutrophilic and thermophilic with an optimum growth temperature of 50°C, and formed long multicellular filaments. Members of MCR-7 have been detected in oligotrophic and nutrient-impacted areas of the Florida Everglades (35), anaerobic digesters (217), and estuarine sediments (14).

Both *Methanosarcina* and *Methanosaetaceae* were detected in the Penn England digester sample and the acidic peat incubation at 20°C, and in both samples there were approximately four times more *Methanosarcina* than *Methanosaetaceae* detected. Members of the genera *Methanosarcina* and *Methanosaetaceae* are the only aceticlastic methanogens, but whereas *Methanosarcina* are quite metabolically diverse, *Methanosaetaceae* are obligately aceticlastic (285). Slow-growing *Methanosaetaceae* have a lower K_m for acetate than the faster-growing *Methanosarcina,* and will outcompete *Methanosarcina* for acetate at low concentrations (300). *Methanosarcina* are able to produce methane from H_2/CO_2 and methylated compounds as well as acetate. Of all three pathways, cleavage of acetate yields the least energy for *Methanosarcina*, so other substrates may be preferentially used (285). As *Methanosarcina* and *Methanosaetaceae* may not be in direct competition for acetate in an environment, it is not unusual to find both

genera present in a wide range of environments, including natural wetlands and lakes (35, 72, 126) as well as constructed anaerobic reactors (78, 96, 245). What is interesting is that *Methanosaetaceae* were found in the 20°C acidic peat incubation but not the 4°C incubation (Table 4). Under standard conditions, hydrogenotrophic methanogenesis yields more energy than aceticlastic methanogenesis, but as the temperature drops, more methane is produced from acetate than H_2/CO_2 (211). In addition, at colder temperatures homoacetogenic bacteria, which produce acetate from H_2 and CO_2, outcompete hydrogenotrophic methanogens for H_2 (191, 236). Thus, in tundra, deep lake sediments, and other permanently cold environments methanogenesis proceeds predominantly from cleavage of acetate (182, 191, 236). It is possible that in the 20°C incubation, acetate was sufficiently low to allow *Methanosaetaceae* to outcompete *Methanosarcina*, but at 4°C homoacetogenesis may have led to the accumulation of acetate and dominance of the faster-growing *Methanosarcina*. Similar to our findings, researchers studying permanently cold environments have typically found *Methanosarcina* to be the only aceticlastic methanogen present (131, 182) although a study by Purdy *et al.* found *Methanosaeta* but not *Methanosarcina* in both freshwater and marine Antarctic sediments (211).

It is also interesting that *Methanobacteriaceae* were the dominant methanogens at 4°C but were not detected at 20°C. The *Methanobacteriaceae* are quite widespread and have been detected in freshwater sediments (17, 253), the guts of animals (224), anaerobic reactors (176, 217), and rice fields (45). Although *Methanobacteriaceae* have been detected in Northern wetlands and freshwater lake sediments, which experience yearly cold seasons (114, 141, 182), they are rarely detected in permanently cold sediments such as tundra and polar lakes. This observation, coupled with the fact that no psychrophilic members of the *Methanobacteriaceae* have been isolated, suggests that methanogens of this family do not compete well at cold temperatures. So while it is not unusual that *Methanobacteriaceae* would be present in the original peat prior to incubation, it is unusual that they would become the predominant methanogen at 4°C but not at 20°C.

Although *mcrA* of *Methanobacteriaceae* was detected, the isoenzyme *mrtA* of *Methanobacteriaceae* was not. The primers used in this study previously amplified both *mcrA* and *mrtA* from *Methanobacteriaceae* (260), so the failure to detect the *Methanobacteriaceae* *mrtA* is most likely due to a limitation in the coverage of the probe designed for this study. Probes were designed both from previously obtained clone sequences (260) and from sequences of cultured methanogens available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). There are far fewer *mrtA* sequences than *mcrA* sequences available, so although this probe was able to detect *mrtA* from isolated and

sequenced members of the *Methanobacteriaceae*, it may not have been able to detect *mrtA* from uncultured members whose *mrtA* sequences may be significantly different. For quantitative analysis, a probe for the *mcrA* of *Methanobacteriaceae* alone is sufficient. Originally probes for both the *mcrA* and *mrtA* of *Methanobacteriaceae* were designed to allow greater coverage of this diverse family.

The Fen cluster was detected in both acidic peat incubations but not in any of the digester samples. Members of this cluster are widespread in natural wetland environments. *Candidatus* Methanoregula boonei, an isolate of the Fen cluster, demonstrated a doubling time of about two days even under optimal growth conditions (25). If other members of the Fen cluster are also slow growers, this may explain why members of the Fen cluster have rarely been detected in anaerobic reactors, which typically have retention times of 20-40 days. Members of the Fen cluster are often found in acidic environments, and as *Candidatus* Methanoregula boonei was found to be acidophilic, members of this cluster may have a competitive advantage in acidic environments such as the incubated peat samples (25).

Researchers studying methanogen communities in swine and cattle have primarily obtained sequences of *Methanobacteriaceae*, especially of the genera *Methanobacterium* and *Methanobrevibacter*, *Methanomicrobiaceae*, *Methanocorpusculaceae,* and less frequently, *Methanosarcina* (124, 273, 287). In the rumen, acetate is produced by fermentation and absorbed by the host organism so that *Methanosarcina* present in the rumen environment usually grow by hydrogenotrophic or methylotrophic methanogenesis (285). Although the Penn England, Brookside, and Schrack dairy manure digesters treat similar waste, only *Methanosarcina* could be detected in the Brookside sample (Table 4-4). The Schrack sample was similarly dominated by *Methanosarcina*, although low numbers of *Methanocorpusculaceae* were also detected. In the Crone reactor, which treats only pig manure, *Methanobacteriaceae* was the sole methanogen group detected. Of all the reactor samples, Penn England had the most diverse methanogen community. The Brookside, Schrack, and Crone digesters are all plug-flow designs whereas the Penn England reactor is a periodically-mixed tank reactor. In addition, bedding used in the Penn England barns is composed of the dried, digested solids from the reactor which return to the reactor when the barns are cleaned. If some methanogens present in the digested solids survive dessication and oxygen exposure, they are eventually returned to a favorable environment in the reactor. Active methanogens have been obtained from oxic environments including aerated activated sludge (95) and the A horizon of forest, savanna, and desert soils (209). This may essentially increase the retention time for both methanogens and other bacteria in this reactor and may allow a more diverse methanogen population to emerge.

As the detection limit for the TaqMan qPCR method was approximately 415 copies per reaction, minor methanogen groups may have existed in numbers less than this in the environmental samples but could not be detected by this qPCR method. In fact, most of the TaqMan probes were unable to detect any amplification from the environmental DNA extracts, and results of quantitative PCR suggest that only one to a few methanogen groups dominate the methanogen population in all environments (Table 4-4). The inability to detect minor community members may partially explain the difference between total *mcrA* numbers as detected by SYBR-Green I assays and the total *mcrA* calculated by adding the numbers obtained in TaqMan assays, although it is more likely that differences in these numbers is due to the error of the methods themselves. Even though the two calculations of total *mcrA* do not perfectly agree, they are close (Table 4-4). Sufficiently large clone libraries may be able to detect minor community members that are present in numbers too low to be detected by qPCR, but PCR bias makes abundance of a sequence in a clone library an unreliable predictor for abundance of that organism in the actual sample. Quantitative PCR overcomes these biases to allow the determination of relative abundance of a sequence in an environment (127, 166), though potentially missing minor target populations.

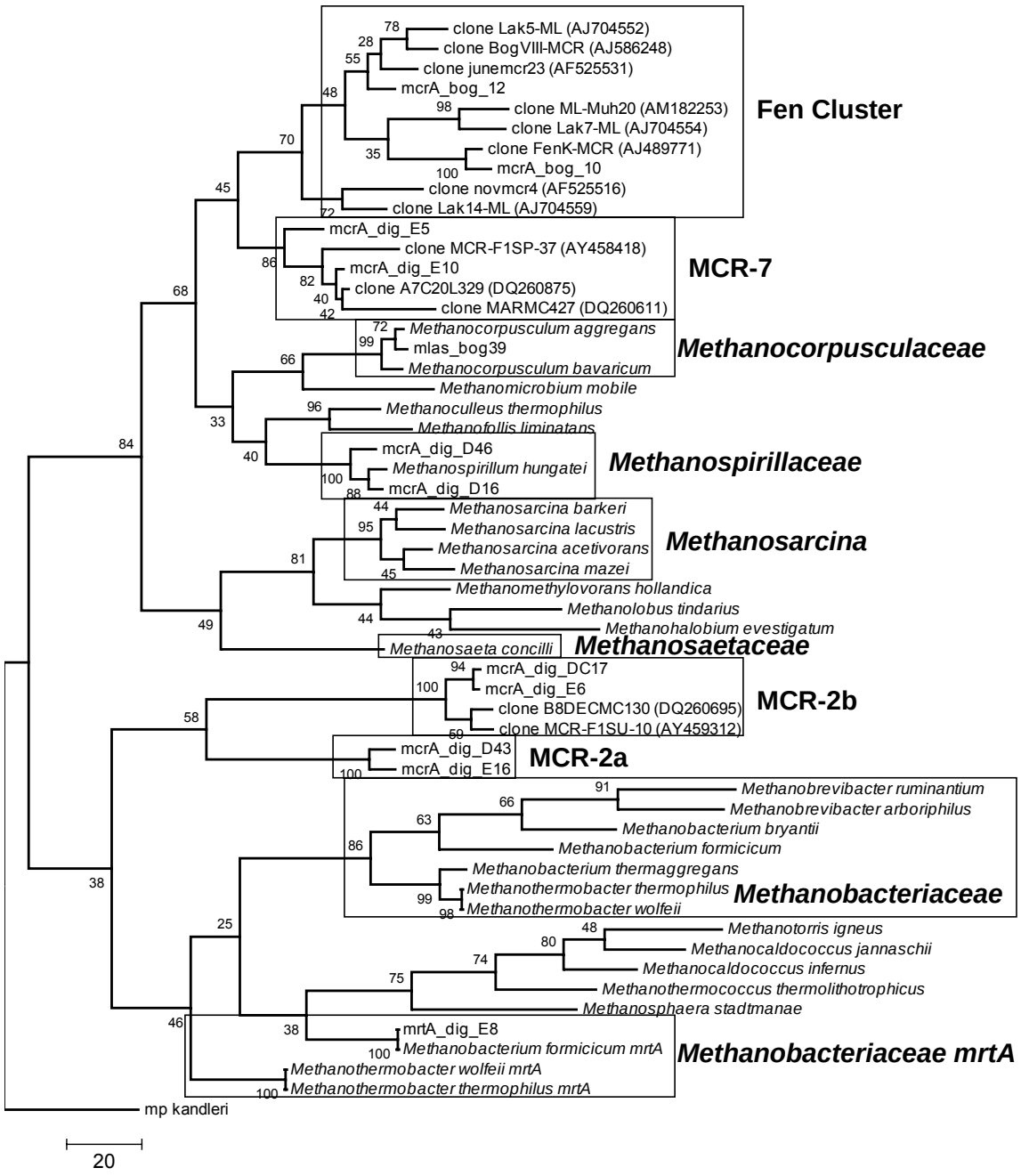


Figure 4-1. Phylogenetic tree of representative *mcrA* sequences targeted by TaqMan probes designed for real-time quantitative PCR.

Boxes denote sequences targeted by the respective TaqMan probe listed in Table 1. The tree was constructed as a maximum parsimony tree using a close-neighbor interchange level 3 and bootstrapped with 1000 trials. All positions containing gaps and missing data were eliminated from the dataset. The scale bar represents the number of changes over the whole sequence. Classification of clusters is based on Castro *et al.* (8) for MCR-2, MCR-5, and MCR-7, and Juottonen *et al.* (24) for the Fen cluster.

Table 4-1. TaqMan probes designed for this study and used to quantify different methanogen groups from environmental DNA.

Probe name	Target group	Probe sequence (5'-3')[a]	Position[b]	Clones used as standards (GenBank accession number)
mbac-mcrA	*Methanobacteriaceae mcrA*	ARGCACCKAACAMCATGGACACWGT	1154	*M. wolfeii* (AB300780) *M. arboriphilus* (AF 414035)
mrtA	*Methanobacteriaceae mrtA*	CCAACTCYCTCTCMATCAGRAGCG	1433	*M. thermoauto-trophicum* (NC 000916) mrtA_dig_E8 (EU980398)
mcp	*Methanocorpusculaceae*	AGCCGAAGAAACCAAGTCTGGACC	1319	mlas_bog39 (DQ680603)
msp	*Methanospirillaceae*	TGGTWCMACCAACTCACTCTCTGTC	1419	mcrA_dig_D16 (EU980421) mcrA_dig_D46 (EU980419)
MCR-7	Uncultured MCR-7 group	TGSCTTGACCTTRTCCWTCTCGYTS	1134	mcrA_dig_E5 (EU980422) mcrA_dig_E10 (EU980423)
MCR-2a	uncultured MCR-2 group	CCACTCTACTGCCGGTATCAACG	1317	mcrA_dig_D43 (EU980402) mcrA_dig_E16 (EU980418)
MCR-2b	uncultured MCR-2 group	ATGTATCTCTGCAGCAGCCGGTACA	1269	mcrA_dig_DC17 (EU980412) mcrA_dig_E6 (EU980407)
Fen	uncultured Fen Cluster group	AAVCACGGYGGYMTCGGMAAG	1071	mcrA_bog_10 (EU980434) mcrA_bog_12 (EU980424)
msar	*Methanosarcina*	TCTCTCWGGCTGGTAYCTCTCCATGTAC	1272	*M. barkeri* (Y00158) *M. acetivorans* (NC 003552)
msa	*Methanosaetaceae*	CCTTGGCRAATCCKCCGWACTTG	1107	*M. concilli* (AF414037)

[a] R=A/G, K=G/T, M=A,C, W=A,T, Y=C,T, S=G,C, V=A,C,G

[b] 5' end of the sense strand, based on *M. thermoautotrophicum mcrA* delta H (U10036) numbering

Table 4-2. Reproducibility of the SYBR-Green I assay for total *mcrA* copies conducted for 17 standard curves in three separate qPCR assays.

Plate	Mean±Standard deviation			
	Slope	Efficiency (%)	Y-intercept	R^2
1	-3.416±0.054	96.2±2.1	38.78±0.72	0.9954±0.0037
2	-3.470±0.097	94.2±3.7	39.48±0.49	0.9964±0.0030
3	-3.464±0.099	94.4±3.9	40.24±0.36	0.9974±0.0069
Combined	-3.451±0.079	94.9±3.1	39.40±0.69	0.9962±0.0030

Table 4-3. Reproducibility of TaqMan assays targeting different methanogen groups from three standard curves conducted in three separate qPCR assays.

Probe	Mean±Standard deviation			
	Slope	Efficiency (%)	Y-intercept	R^2
mbac-mcrA	-3.518±0.141	92.4±5.3	42.20±0.89	0.9949±0.0004
mrtA	-3.538±0.064	91.7±2.3	41.06±0.32	0.9937±0.0035
mcp	-3.487±0.040	93.6±1.5	42.75±1.44	0.9912±0.0041
msp	-3.570±0.104	90.6±3.7	41.66±0.43	0.9930±0.0040
MCR-7	-3.505±0.040	92.9±1.5	41.41±0.26	0.9964±0.0014
MCR-2a	-3.513±0.077	92.6±2.8	40.67±0.97	0.9953±0.0057
MCR-2b	-3.427±0.355	95.8±15.8	42.73±1.47	0.9864±0.0130
Fen	-3.345±0.121	99.4±5.2	41.24±1.46	0.9930±0.0043
msar	-3.477±0.094	93.9±3.6	43.64±1.24	0.9953±0.0034
msa	-3.481±0.074	93.8±2.8	42.45±1.45	0.9977±0.0016

Table 4-4. Quantitation results for total methanogen *mcrA* copies and *mcrA* from different methanogen groups from six different environmental samples.

Numbers represent copies mL^{-1} of original sample, which was digester effluent for the Penn England, Brookside, Schrack, and Crone environments, and peat slurry for the 20°C and 4°C incubations. Only those methanogen groups detected in at least one environment are shown.

Targeted group	Penn England	Brookside	Schrack	Crone	20°C incubation	4°C incubation
Methanosaetaceae	$7.07\times10^5\pm 1.91\times10^5$	0	0	0	$3.45\times10^4\pm 1.88\times10^4$	0
Methanosarcina	$3.20\times10^6\pm 9.18\times10^5$	$1.54\times10^6\pm 2.42\times10^5$	$4.49\times10^6\pm 1.54\times10^6$	0	$1.24\times10^5\pm 1.19\times10^5$	$1.27\times10^6\pm 1.12\times10^5$
Methanobacteriaceae	$6.81\times10^5\pm 3.96\times10^5$	0	0	$1.40\times10^6\pm 2.29\times10^6$	0	$2.98\times10^6\pm 1.50\times10^6$
Methanocorpusculaceae	$5.95\times10^5\pm 9.78\times10^4$	0	$3.90\times10^5\pm 5.23\times10^4$	0	0	0
Fen Cluster	0	0	0	0	$6.36\times10^5\pm 1.62\times10^5$	$6.94\times10^5\pm 1.25\times10^5$
Total of TaqMan assays	$5.18\times10^6\pm 1.60\times10^6$	$1.54\times10^6\pm 2.42\times10^5$	$4.88\times10^6\pm 1.59\times10^6$	$1.40\times10^6\pm 2.29\times10^6$	$7.95\times10^5\pm 3.00\times10^5$	$4.94\times10^6\pm 1.74\times10^6$
SYBR-Green I	$2.53\times10^6\pm 3.99\times10^5$	$1.04\times10^6\pm 4.36\times10^5$	$3.13\times10^6\pm 6.68\times10^5$	$3.63\times10^6\pm 2.37\times10^5$	$3.55\times10^6\pm 3.61\times10^5$	$3.95\times10^6\pm 5.57\times10^5$

Chapter 5

Response of lab-scale methanogenic reactors inoculated from different sources to organic loading rate shocks

This chapter represents a manuscript in preparation

ABSTRACT

Anaerobic digestion has the potential to couple waste treatment with energy production, but is under-utilized mainly because it is perceived as less stable than other waste treatment processes. The most often-cited reason for digester failure is a drop in pH, and reactor failure has mainly been attributed to the sensitivity of methanogens despite the fact that,methanogenesis in acidic wetlands proceeds at pH values less than 4. A reactor inoculated from an acidic wetland may be more resistant to low pH than a traditional inoculum. To investigate this hypothesis, lab-scale semi-batch reactors were inoculated with either acidic bog sediments (Bog reactors), sludge from a municipal wastewater sludge anaerobic digester (Digester reactors), or a combination of these two inocula (Hybrid reactors), and challenged on three occasions with periodic pulses of glucose without pH control to simulate organic loading rate shocks. After the first glucose pulse, the Digester- and Hybrid-test reactors failed and had to be re-inoculated from control reactors. After the second glucose pulse, all reactors failed but activity was recovered by raising the pH to neutral conditions. After the third glucose pulse, all reactors failed, but activity was recovered by raising pH to only 4.8-5. Control reactors showed less methanogen diversity than test reactors, with *Methanosarcina* and *Methanobacteriaceae* dominating all reactors. The acidophilic Fen Cluster was originally observed in the Bog- and Hybrid-test reactors after the first two sets of glucose pulses, but could not be detected later in reactor operation. Eventually *Methanosarcina* and *Methanobacteriaceae* came to dominate test reactors as well, and the presence of *Methanosarcina* correlated with reactor recovery after glucose pulses.

INTRODUCTION

Anaerobic digestion (AD) is often used in domestic wastewater processing to treat settled materials and waste-activated sludge from aerobic treatment processes. AD of wastewater sludge reduces the volume of sludge that needs be handled, reduces potential pathogens, and produces a methane-rich biogas that may be used for energy recovery (1). AD involves the coordination of several metabolic groups of prokaryotes to convert organic material to primarily carbon dioxide

and methane. Members of the *Bacteria* generally catalyze hydrolysis of particulate matter to small organic molecules followed by fermentation to mainly volatile fatty acids (VFAs), hydrogen, and carbon dioxide. Subsequent conversion of these fermentation products to methane is catalyzed by members of the *Archaea* known as methanogens (79, 233). Because of the dynamic nature of waste treatment, digester conditions may change on a daily basis in terms of waste flow and composition (155). An increase in the amount of fermentable substrate can result in fermentation exceeding methanogenesis, and lead to the accumulation of VFAs and a decrease in pH leading to process instability and even failure (24, 281). Reduced methane production is often concomitant with the accumulation of VFAs and reduction in pH (24, 70, 227), which has been suggested as evidence for the sensitivity of methanogens to low pH and the cause of digester failure during overloading events (1, 233). Although methanogens appear to be the most sensitive members of the anaerobic digester consortium to low pH, methanogenesis is common in acidic natural wetlands (25, 30, 94, 142). Improving anaerobic digester process stability is important for situations where influent substrate composition or amount may rapidly change.

Functioning and stability of an anaerobic digester is directly related to the microbial community within the digester. With the development of culture-independent molecular techniques, researchers have been able to follow changes in the methanogen community of anaerobic digesters exposed to shock-loading conditions. Delbes *et al.* (2001) followed 16S rDNA and rRNA levels of methanogens in a continuously mixed lab-scale digester during a period of high acetate accumulation. The researchers found no change in total methanogen rDNA levels during the high acetate period, but did note that rRNA levels of the aceticlastic methanogen *Methanosaeta concilli* increased while rRNA levels of hydrogenotrophic methanogens decreased (63). Scully *et al.* (2005) subjected two identical lab-scale expanded granular sludge reactors, R1 and R2, to a 50% reduction in hydraulic retention time, initially resulting in VFA accumulation in both reactors. Reactor R2 showed VFA consumption and a return to original COD removal efficiency at the reduced retention time, but R1 continued to accumulate VFAs and eventually failed. Examination of the methanogen communities revealed differences in dominant methanogens and an increase in R2 *Archaeal* diversity but a decrease in R1 *Archaeal* diversity after the reduction in hydraulic retention time (239). McMahon *et al.* (2004) examined the microbial community structure of two lab-scale semi-continuous reactors before and after a 5-fold increase in organic loading rate. The researchers found that one digester, which was dominated by *Methanosarcina*, survived the organic loading rate increase while a second digester dominated by *Methanosaeta* failed (179). Similar results were obtained by Hori *et al.* (2006), who saw a

shift in the predominant hydrogenotrophic methanogens and an increase in *Methanosarcina* concurrent with increases in VFA concentration in a lab-scale reactor with no pH control.

Relatively few studies have examined the role of the inoculum source of an anaerobic digester in determining both the operational success and the resultant methanogen composition. Forster-Carneiro *et al.* (2007) ran lab-scale anaerobic digesters treating solid food waste that were inoculated from five sources including corn silage, digested restaurant waste, cattle and swine manure, and digested municipal wastewater sludge. With all reactors inoculated at the same volatile solids concentration, the researchers found the best methanation rates were obtained with swine manure and wastewater sludge as inocula (84). Some researchers have found bioaugmentation with either bovine rumen fluid (161) or hydrogen-producing bacteria (12) has increased methane production. Often the inoculum for an anaerobic digester derives either from animal or human waste, such as municipal wastewater, or from an already established reactor that was inoculated from one of these sources. Very few researchers have explored the idea of using a naturally-derived methanogenic inoculum in an anaerobic digester to better degrade waste under difficult conditions. Aspé *et al.* (1997) studied the methanation of fish processing effluents using either marine sediment or pig manure as the inoculum. The researchers found that only the marine sediment inoculum was successful in digesting these high-sulfate, high-salinity wastes to methane (11). Taconi *et al.* (2008) demonstrated successful acetate degradation at pH values as low as 4 in a reactor inoculated with a mixture from three sources: an anaerobic digester treating municipal wastewater sludge, an anaerobic lagoon treating animal processing wastes, and sediments from a nearby peatland. In this study, the sediments from the peatland composed less than 7% of the initial inoculum and were not enriched for microbial growth prior to inoculation of the reactor (266).

The hypothesis of this research was that the methanogenic community of an acidic peatland would be a better inoculum for an anaerobic digester operating under low pH conditions. To date, no research has utilized acidic peatland soils as the sole inoculum for an anaerobic digester. To study this hypothesis, we inoculated lab-scale digesters with samples from either an acidic bog or a digester treating wastewater sludge, and subjected the digesters to organic shock-loading without pH control. Digester response was monitored chemically and microbiologically by following methanogen population dynamics with real-time quantitative PCR and methanogen group-specific primers and probes.

MATERIALS AND METHODS

Reactor configuration

Lab-scale anaerobic digesters were constructed from 1L Pyrex aspirator bottles (Corning Inc., Corning, NY) with a serrated bottom outlet to accommodate tubing. The top of the reactor vessel was closed with a size 6 neoprene stopper which was bored for $^3/_8$" and ¼" holes to accommodate tubing. These tubing penetrations in the stopper were sealed with silicone adhesive. Headspace gas from the reactor was collected through $^3/_8$" PVDF Flex tubing (Cole-Parmer, Vernon Hills, IL) to a 1L Cali-5-Bond gas sampling bag equipped with a 2/3 way valve stopcock (Calibrated Instruments, Inc., Hawthorne, NY). Reactors were fed via ¼" PVDF Flex tubing through the stopper and connected to a 3" piece of silicone tubing (¼" outer diameter) that could be sealed between feedings with a hose clamp. The same silicone tubing was also placed over the serrated bottom outlet of the reactor for sludge wasting and sealed with a hose clamp. PVDF Flex tubing and Cali-5-Bond gas sampling bags were chosen for their low permeability for H_2 and CH_4 gases. The reactors were sterilized by autoclaving prior to inoculation, and each 1-L reactor contained 500 mL of sludge and 500 mL of headspace.

Inoculum source

Two control reactors were inoculated from different sources. One reactor (Digester-control reactor) was inoculated with 500 mL of effluent sludge from the primary digester treating municipal wastewater sludge at the Pennsylvania State Wastewater Treatment Plant. The primary anaerobic digester at the State College plant is a fixed-cover, continuously stirred tank reactor operated at approximately 35°C and a hydraulic retention time of 25 days with no solids-liquid separation. The digester processes a combination of primary settled material and waste-activated sludge, and the reactor is maintained at neutral pH via adjustment of the feeding rate. The second reactor (Bog-control reactor) was inoculated with 500 mL of sediment from a local acidic peatland known as Bear Meadows Bog (19). This bog is described as a transitional bog, receiving most of its inflow from rainwater, but also receiving some groundwater flow. Vegetation in the bog is primarily *Sphagnum* mosses, sedge grasses, and highbush blueberry, with peat approximately 1 m thick. The bog pH varies from 3.8 to 5.0, and bog waters contain little alkalinity, sulfate, or iron (19). The inoculum for the Bog-control reactor was derived from two separate cores taken from areas of the bog covered with *Sphagnum* moss and *Carex* sedge grass. Cores were taken from the first 12" of peat beneath the root layer of the moss and sedges. The cores were transported anaerobically in an air-tight chamber containing a GasPak (BBL, Inc.)

from the sampling site to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Within the anaerobic chamber, sediment from the two cores were mixed in equal proportions and added to the Bog-control reactor. Sampling was conducted in early September of 2006 and water temperature was measured as 15°C. A third reactor (Hybrid-control reactor) was inoculated with equal volumes of wasted sludge from the other two control reactors after they had been operating for several weeks.

Start-up conditions

Reactors were always incubated in the dark. After inoculation, the Bog-control reactor was incubated at 15°C for approximately one month, and then moved to room temperature (approximately 25°C) for another month before being moved to a 30°C temperature-controlled room. Methane could not be detected in the headspace gas of the Bog-control reactor for the first 60 days of incubation. During this start-up period new material was only fed approximately once a week until 117 days, at which point feeding and wasting were conducted every four days. Initially, the reactors were mixed using a magnetic stir bar and stir plate, but it was observed that methane concentrations in the headspace increased when stirring was discontinued. Due to this finding, stirring was discontinued for both the Bog-control and Digester-control reactors at 112 days. During the start-up period, pH in the Bog-control reactor increased from an initial value of 4.5 to approximately 5.3.

The Digester-control reactor was maintained at 35°C for approximately two months before being moved to a 30°C temperature-controlled room. Originally the reactor was continuously mixed with a magnetic stir bar and stir plate, and operated at a hydraulic retention time of 30 days to mimic that of the anaerobic digester from which it was inoculated. At 112 days into the start-up period, the retention time was also increased to 40 days and stirring was discontinued so that operational conditions for both control reactors would be the same. During the start-up period, pH in the Digester-control reactor was steady at 7.2-7.5.

The Hybrid-control reactor was inoculated on day 85 of operation of both the Digester-control and Bog-control reactors. The Hybrid-control reactor was inoculated with approximately 250 mL of sludge each from the Bog-control and Digester-control reactors. The Hybrid-control reactor was stored in a 30°C temperature-controlled room and was initially continuously mixed with a magnetic stir bar on a stir plate, but mixing was discontinued on day 112. Day 112 for the Hybrid-control reactor in actuality corresponds to 26 days of operation, but the numbering system was not changed to avoid confusion. Here and elsewhere throughout the document the day of operation corresponds to the number of days the original seed reactors, Digester-control and Bog-

control, were operated. During the start-up period, the pH in the Hybrid-control reactor was steady at 6.1-6.4.

Routine operation and data collection

After the start-up period, reactors were operated in semi-batch mode with feeding and wasting of 10% of the sludge volume conducted every 4 days. Reactors were mixed gently by hand once daily, and thoroughly mixed before feeding and wasting for a combined hydraulic and solids retention time of 40 days. Feed material was composed of primary settled materials and waste-activated sludge from the Pennsylvania State University Wastewater Treatment Plant amended with a mixture of amorphous and microcrystalline cellulose (MN301, Fisher) at a ratio of 0.25 g cellulose per 50 mL feed sludge. Feed material was sterilized and stored at 4°C in separate 50 mL aliquots prior to feeding the reactors. Biogas was continuously collected passively in the attached gas sampling bags to maintain atmospheric pressure in the headspace of all reactors.

Reactor performance was monitored by gas production and composition, total solids and volatile solids destruction, pH, alkalinity, COD removal, volatile fatty acid concentration, and methanogen numbers. Reactor headspace gas was withdrawn directly from gas sampling bags through a silicone septum, and gas composition was monitored by a gas chromatogram (SRI Instruments, Torrance, CA). One gas chromatogram, used to detect H_2, N_2, O_2, and CH_4, contained a 3' molecular sieve 5 A° column with argon at 20 psi as the carrier gas. The second gas chromatogram, which was used to detect CO_2, contained a 3' silica gel column with helium at 20 psi as the carrier gas. For both gas chromatograms, the injector and column were kept at 70°C, and detection was via a thermal conductivity detector operated at low gain at 250°C. Gas volume was measured directly from the gas sampling bag using a 60 mL gas-tight syringe with a Luer-lock fitting.

Total solids (TS) and volatile solids (VS) of the feed material and waste sludge were measured by drying at 105°C (method 2540B) and combustion at 550°C (method 2540E), respectively, for duplicate samples (180). COD was measured using high-range vials (0-1500 mg/L, Hach method 8000). Total VFAs and bicarbonate alkalinity were measured by a 2-point titration with sulfuric acid to pH 5.1 and 3.5 (6). Individual VFAs were measured by high-performance liquid chromatography (HPLC) on a Waters, Inc. model 2695 machine equipped with a BioRad organic acid column (Aminex HPX-87H). The injector and column temperature were 60°C, with 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min. Samples were filtered through 0.2 uM syringe filters and acidified with sulfuric acid (final concentration of

0.1 M) before 100 uL of each sample was injected for separation on the HPLC. A standard mix of 10 mM each of formic, acetic, propionic, 2-methyl-propionic, butyric, 3-methyl-butyric, valeric, hexanoic, and heptanoic acids (Supelco, Sigma-Aldrich Co.) was used for standard dilutions ranging from 10 mM to 100 uM.

Total methanogen numbers were inferred with real-time quantitative PCR (qPCR) targeting the functional gene methyl coenzyme M reductase using a previously described SYBR Green-based method (259). Total methanogen numbers corresponding to different phylogenetic groups were determined with TaqMan probes in qPCR (259).

Organic loading rate shocks

To test the responses of the enrichment cultures to periodic substrate overloading, new lab-scale reactors were inoculated from the seed reactors on day 117. For each seed reactor (Digester-control, Bog-control, and Hybrid-control), a sterile test reactor with identical configuration as the seed reactors was inoculated by transferring one-half (250 mL) of the sludge from the seed reactor to the test reactor (Digester-test, Bog-test, and Hybrid-test reactors, respectively). After inoculating the test reactors, both the seed and test reactors were operated with feeding but without wasting until the sludge volume in the reactors had again reached 500 mL (approximately 20 d).

The first organic loading rate (OLR) shock was delivered to the test reactors on day 146. This shock consisted of 10 g of glucose dissolved in 50 mL distilled water and sterilized. The feed sludge amended with cellulose that was routinely used as substrate for the reactors contained about 2% VS, so 10 g of glucose in one 50 mL feeding was equal to approximately 10 times the typical amount of VS. The volume of the OLR shock was kept the same as the regular feedings so that the retention time would remain at 40 days, and pH in the reactors was not controlled during OLR shocks. After the first OLR shock, feeding and wasting was continued every four days, but the Digester-test and Hybrid-test reactors did not recover activity and were disposed of on day 210. New test reactors were inoculated on day 214 from the Digester-control and Hybrid-control reactors as previously described for further OLR shock tests.

In the second set of OLR tests, the test reactors were shocked with progressively larger amounts of substrate. In the first of these OLR shocks, the test reactors were fed a sterile, anoxic solution of 1 g glucose in 50 mL distilled water with 1x trace mineral solution on day 262. The trace mineral solution was prepared according to a recipe provided by the Oregon Collection of Methanogens (Portland State University, http://methanogens.pdx.edu). Trace minerals were added with the OLR shock so that any inhibition of methane production after the OLR shocks

would not be caused by mineral limitations. A second OLR shock was delivered at the next feeding four days later, when the test reactors were fed a sterile 50 mL solution containing 5 g glucose and 5x trace minerals solution. After this second OLR shock, the test reactors were fed and wasted every four days as usual. Twenty-one days after the second OLR shock on day 287, the test reactors were again shocked by feeding a sterile 50 mL solution of 10 g glucose and 10x trace minerals solution. After this shock loading event, the test reactors were not fed again for two weeks, and then were only fed and wasted every eight days instead of every four for one month. One week after this third OLR shock, 5 mL of 10 M NaOH was added to the test reactors to raise the pH to approximately 7. Ten days later, another 1 mL of 10 M NaOH was added to the Hybrid-test reactor, as the pH had again dropped. All three test reactors eventually recovered activity, so none of the test reactors needed to be restarted from control reactors after this series of OLR shocks.

A third set of OLR shocks were delivered to test the effect of adding trace minerals to the reactors with the glucose feedings. First, a second set of test reactors were inoculated from the original test reactors on day 399. For each test reactor (Digester-test, Bog-test, and Hybrid-test), a second sterile reactor was inoculated by transferring one-half (250 mL) of the sludge from each test reactor to the second test reactor. After inoculating the second test reactors, both sets of test reactors were operated with feeding but without wasting until the sludge volume in the reactors had again reached 500 mL. Approximately 5 weeks after inoculating the second set of test reactors, on day 436, all the test reactors were fed a glucose solution to simulate an OLR shock. The first set of test reactors were fed with a sterile solution of 10 g glucose in 50 mL distilled water, and the second set of test reactors were fed a sterile solution of 10 g glucose and 10x trace minerals in 50 mL distilled water. Two weeks after delivering the OLR shock, 1 mL of 1 M NaOH was added to each test reactor. Four days later another 2 mL of 1 M NaOH was added to the test reactors. Four days after the second NaOH addition, on day 457, feeding and wasting for all reactors was resumed on the same four day schedule.

RESULTS

Start-up and performance of control reactors

Initially the Digester-control reactor showed more methane production, higher pH, greater solids and COD removal, greater bicarbonate alkalinity, and lower VFAs than the Bog-control reactor (Appendix 2 Figs. A2-1,2,3,4). The pH in the Digester-control and Bog-control reactors during this time was about 7.3 and and 5.2, respectively, and the headspace methane

concentrations varied from 40-60% in the Digester-control reactor, and less than 2% in the Bog-control reactor (Appendix 2 Figs. A2-1, 2). The average methane production and pH for the Hybrid-control reactor were between the values for these two reactors. There were two major problems encountered during the start-up period. The first was that microbial density was much lower in the bog sediments than in the anaerobic digester sludge, and it was approximately 100 days before methanogen numbers were comparable in both reactors (Appendix 2 Fig. A2-5). The second problem was that the original gas sampling bags leaked out biogas and allowed the intrusion of atmospheric oxygen into reactors. These gas sampling bags were replaced with a superior product on day 235, after which all reactors showed an immediate increase in biogas production, and pH rose to neutral values in the Bog- and Hybrid- control reactors. Gas production and pH remained stable in the control reactors from this point through the duration of operation (508 days) with methane production averaging 70-80 mL per day, and pH of 6.8-7.0 (Appendix 2 Figs. A2-1, 4).

On the day of inoculation the Digester-control reactor was dominated by MCR-7 with fewer numbers of MCR-2a and *Methanosaeta* (Appendix 2 Fig. A2-5). By day 25 the methanogen community was dominated by *Methanosarcina*, and this dominance persisted throughout operation. *Methanobacteriaceae* and *Methanospirillum* were also periodically detected. The only methanogen detected in the Bog-control reactor during the first month was the Fen Cluster. By day 90 the Fen Cluster was replaced by *Methanosarcina* and *Methanobacteriaceae*. For most of the operation of this reactor, the methanogen population was dominated by *Methanosarcina* with fewer numbers of MCR-7. The Hybrid-control reactor was dominated by *Methanosarcina* and *Methanobacteriaceae* on the day of inoculation, and the population was relatively consistent throughout operation. Occasionally *Methanospirillum* or MCR-7 were detected, but by the end of operation only *Methanosarcina* was detected.

Response of test reactors to first OLR shock

When test reactors were inoculated from the control reactors on day 117 of operation, performance of the test reactors in terms of gas production and pH was nearly identical to that of the control reactor from which each was inoculated (Fig. 5-1). The Digester-test reactor contained 50% methane in the headspace with a pH of 7.4, the Bog-test reactor contained about 5% methane in the headspace with a pH of 5.5, and the Hybrid-test reactor contained about 35% methane in the headspace with a pH of 6.1. An OLR shock of 10 g glucose was delivered on day 146, and immediately following this OLR shock, the pH in all three reactors dropped to approximately 4.4 and methane production ceased. Over the next 70 days of operation, the

Digester- and Hybrid-test reactors did not produce methane, and while the pH remained at 4.4 in the Digester-test reactor, the pH continued to drop to 3.5 in the Hybrid-test reactor. Both the Digester- and Hybrid-test reactors periodically contained hydrogen in the headspace of up to 25%. In contrast, the Bog-test reactor showed almost no hydrogen in the headspace, and methane production began again on day 178 with methane concentrations and pH steadily increasing. Due to the failure of the Digester-test and Hybrid-test reactors to recover methanogenic activity, the contents of these reactors were disposed of, and new sterile test reactors were inoculated from these respective control reactors on day 214 using the previously described inoculation method.

The OLR shock caused an immediate accumulation of VFAs in all three test reactors (Appendix 2 Fig. A2-7). The Digester-test reactor accumulated only acetate, but the Bog- and Hybrid-test reactors accumulated both acetate and propionate. Although the Digester-test reactor showed the least accumulation of VFAs at about 9 mM of acetate, the acetate concentration remained stable until 214 when a new reactors was inoculated. In the Bog-test and Hybrid-test reactors, VFAs reached a maximum of about 35 and 40 mM, respectively, at around 160 days before steadily declining. The decline in VFAs corresponded to an increase in pH in the Bog-test reactor, but not in the Hybrid-test reactor, which showed a gradual decline in pH until day 214 when a new reactors was inoculated.

Total *mcrA* numbers and *mcrA* of methanogen groups were assayed on days 146, 172, and 203 of the first OLR shocks (Fig. 5-1). The lower limit of detection for this study was approximately 400-4000 gene copies which translates to 10^5-10^6 copies per mL of effluent reactor sludge (259). The non-detection of a methanogen group does not necessarily mean that it is not present in the community. Following the first OLR shock on day 146, total *mcrA* numbers dropped in the Digester- and Hybrid-test reactors, but total *mcrA* numbers stayed approximately the same in the Bog-test reactors. *Methanobacteriaceae* dropped to non-detectable levels in the Digester-test reactor after the first OLR shock and the reactor became dominated by *Methanosarcina*. The opposite happened to the Hybrid-test reactor, where *Methanosarcina* dropped to non-detectable levels and the reactor became dominated by *Methanobacteriaceae*. The Bog-test reactor was dominated by members of the Fen cluster, but after the first OLR shock, *Methansarcina* was also detected.

Response of test reactors to second OLR shocks

During the second OLR shocks, reactors were given 1, 5, and 10 g of glucose on days 262, 266, and 287, respectively. The 1 g shock resulted in no change in pH or gas production, but

the 5 g shock caused a rapid drop in both pH and methane production in the Digester-test reactor (Fig. 5-2). The Bog-test reactor showed little response to the 5 g shock, but the Hybrid-test reactor produced nearly three times as much methane as the usual daily volume. Prior to the 10 g OLR shock, pH in all reactors was neutral and gas production was approximately the same. After the 10 g OLR shock, the pH in all three reactors dropped to about 4.5 and methane production ceased. Concomitantly with the drop in pH and methane production, solids and COD removal and bicarbonate alkalinity all decreased (Appendix 2 Figs. A2-7, 8). Transitory accumulation of hydrogen was observed in the Digester- and Hybrid-test reactors, but not in the Bog-test reactor (Fig. 5-2). During this period of operation, less than 10 mM of VFAs were detected in the Digester-test reactor, but the Bog-test reactor accumulated up to 26 mM acetate and 11 mM propionate, and the Hybrid-test reactor accumulated up to 48 mM acetate and 23 mM of butyrate (Appendix 2 Fig. A2-9).

On day 295, NaOH was used to adjust the pH in each reactor to approximately 6.5-6.8 and immediately following this pH adjustment, methane production increased in the Digester- and Bog-test reactors as accumulated VFAs were consumed (Fig. 5-2, Appendix 2 Fig. A2-9). Following this rapid increase, methane production abruptly decreased and remained low until about day 374, when methane production increased to pre-OLR shock levels. The decline in methane production was not as profound for the Digester-test reactor as the Bog-test reactor. The Hybrid-test reactor showed no rapid increase in methane production after pH was increased, but methane production did increase to pre-OLR shock levels by day 374 as it did in the other two reactors. When the reactors were again producing methane, TS, VS, and COD removals all returned to pre-OLR shock levels of 40, 60, and 65%, respectively, and bicarbonate alkalinity increased in all reactors (Appendix 2 Figs. A2-7, 8).

The second OLR shocks were delivered on days 262, 266, and 287, and methanogen communities were assayed prior to the shocks on days 238 and 262, and afterwards on day 299, 346, and 379 (Fig. 5-2). By day 379, all reactors showed recovery of methane production. The Digester-test reactor methanogen community was dominated by *Methanosarcina* before the OLR shocks, with fewer numbers of *Methanobacteriaceae* and *Methanospirillum* (Fig. 5-2a). By day 299 the total *mcrA* had dropped by about one order of magnitude, but the community was still dominated by *Methanosarcina* with lesser numbers of *Methanobacteriaceae*. At day 346, and later at day 379, *mcrA* numbers had returned to original levels, but the community profile on both days looked similar to that seen on day 299. Prior to the shocks in the Bog-test reactor, the community contained mainly *Methanosarcina* and Fen Cluster methanogens with fewer *Methanobacteriaceae* (Fig. 5-2b). On day 299, *Methanosarcina* could not be detected and

Methanobacteriaeae became the dominant member of the community with fewer numbers of the Fen Cluster and clade MCR-2b. By day 346, *Methanosarcina* again became the dominant methanogen, members of the Fen Cluster increased, and MCR-7 also appeared as part of the community, but by day 379, only *Methanosarcina* could be detected. In the Hybrid-test reactor, the methanogen community was dominated by *Methanosarcina* with fewer numbers of *Methanobacteriaceae* and the Fen Cluster on day 238 (Fig.5-2c). By day 262 only *Methanosarcina* and *Methanobacteriaceae* were detected. After the last OLR shock, *Methanobacteriaceae* became the dominant methanogen with fewer numbers of *Methanosarcina*, but by day 346 *Methanosarcina* again dominated the community. The Fen Cluster was also detected again on day 346 along with *Methanobacteriaceae*.

Response of test reactors to third OLR shock

A second set of test reactors was inoculated from the first test reactors on day 399. Performance in terms of pH and gas production was nearly identical in all six reactors prior to the third OLR shock. On day 436, an OLR shock of 10 g glucose was delivered to all test reactors, but the second set of test reactors also received mineral nutrients with the shock. Immediately following the OLR shock, pH dropped to 4.5 in all reactors and methane and hydrogen production first increased and then ceased completely (Fig. 5-3). Following the OLR shock, solids and COD removal as well as alkalinity dropped in all test reactors (Appendix 2 Figs. A2-7, 8). VFAs also increased following the OLR shock, especially acetate and butyrate with smaller amounts of propionate (Appendix 2 Fig. A2-9))

On days 449 and 453, NaOH was added to reactors to adjust pH to approximately 4.8-5 (Fig. 5-3). For approximately one month after the addition of NaOH, neither gas production nor an increase in pH was noted in any of the reactors with the exception of Bog-test reactor 2, which showed a steady increase in pH. Gas production began again in the Bog-test reactors 1 and 2 on days 479 and 475, respectively, in the Digester-test reactors on day 483, and in the Hybrid-test reactors on day 500. An increase in pH corresponded with a large amount of methane being produced concomitant with the destruction of accumulated VFAs, followed by a return of methane production to pre-shock levels (Fig.5-4, Appendix 2 Fig. A2-9). The increase in methane production corresponded to an increase in solids and COD removal as well as bicarbonate alkalinity (Appendix 2 Figs. A2-7, 8).

Methanogen communities were assayed on days 411, 440, 457, 485, and 504 (Fig. 5-2). The Digester- and Bog-test reactors had recovered activity by day 485, but the Hybrid-test reactor never fully recovered activity during the testing period. For all six reactors, *mcrA* numbers

declined after the shock and began to increase as reactors showed increased pH and methane production (Fig. 5-4). Only the first set of reactors, which did not receive mineral nutrients, are shown in Figure 2, but communities in the second set of reactors mimic what was seen in the first set with a few minor differences (Appendix 2 Fig. A2-10). In the Digester-test reactor prior to the OLR shock, the community looked similar to the profile on day 379 with the community dominated by *Methanosarcina* with fewer numbers of *Methanobacteriaceae* and *Methanospirillum* (Fig. 5-2a). By day 440, four days after the shock, only *Methanosarcina* was detected. On day 457 the community became dominated by *Methanobacteriaceae* with fewer *Methanosarcina*. On days 485 and 504 the community returned to what it had been prior to the shock, with mostly *Methanosarcina* and *Methanobacteriaceae* and fewer numbers of *Methanospirillum*. For the Digester-test 2 reactor, *Methanospirillum* was never detected, and only *Methanosarcina* was detected on day 432, and only *Methanobacteriaceae* were detected on day 457 (Appendix 2 Fig. A2-10). In the Bog-test reactor, the community was dominated by *Methanosarcina* and fewer MCR-7 prior to the shock on day 411, and also directly afterwards on day 440 (Fig. 5-2b). By day 457 only *Methanobacteriaceae* were detected and by day 485 only *Methanosarcina* was detected. By the end of sampling on day 504 the community was again dominated by *Methanosarcina* with fewer *Methanobacteriaceae*. In the Bog-test 2 reactor, MCR-7 was also detected on day 457 and Methanobacteriaceae was also detected on day 485 (Appendix 2 Fig. A2-10). Only *Methanosarcina* could be detected in the Hybrid-test reactor on days 411 and 440, and only *Methanobacteriacae* could be detected on days 457 and 485 (Fig. 5-2c). By day 504 both *Methanobacteriacae* and *Methanosarcina* were detected. In Hybrid-test 2 reactor, MCR-7 was also detected on days 411, 440, and 457 (Appendix 2 Fig. A2-10). Although the response of test reactor to the OLR shocks varied with the inoculum, the operation of test reactors 1 and 2 for each inoculum mimicked each other well in response to the third OLR shock . Both chemical and microbiological data suggest consistency and reproducibility in the response of the reactors to the third OLR shock, regardless of nutrient amendment (Fig. 5-2, 4, Appendix 2 Figs. A2-7, 8, 10).

DISCUSSION

In this study, I explored the hypothesis that a methanogenic inoculum taken from an acidic environment would allow an anaerobic digester operating with periodic increases in fermentable substrate to better maintain or recover activity than a traditional inoculum. To the best of my knowledge, this is the first time acidic peat sediment was used as the sole inoculum for an anaerobic reactor. Taconi *et al.* (2008) used 200 mL of wetland sediments in a 2950 mL

inoculum to inoculate a lab-scale reactor. The remainder of the inoculum was taken from an anaerobic digester treating municipal wastewater sludge and an anaerobic lagoon treating animal processing wastes. Based on the findings in our study, it is likely that microbial density was much lower in the sediments than in the anaerobic digester and lagoon, and that the wetland microorganisms would be a small proportion of that initial inoculum (266).

The methanogen group that we were most interested in conferring acidotolerance to a lab-scale reactor was the Fen Cluster, a deeply-branching clade within the *Methanomicrobiales* often found to dominate clone libraries in wetlands (72, 87, 126), but rarely, if ever, detected in constructed reactors. Two members of the Fen Cluster have been isolated, and both isolates are hydrogenotrophic and acidophilic with optimal methanogenesis at pH 5-5.5 (25, 31). The Fen Cluster was the dominant sequence in clone libraries prepared from the acidic bog sediments used to inoculate the reactor in this study (260). A clone library was also previously described for the anaerobic digester used as the other inoculum in this study, and the dominant sequence in this library was of cluster MCR-7, also a deeply branching clade of the *Methanomicrobiales* (260). Sequences belonging to MCR-7 have been identified in parts of the Florida Everglades (35), anaerobic digesters (217), and estuarine sediments (14). Imachi ***et al.*** (2008) recently isolated a member of this clade, and found it to be hydrogenotrophic and neutrophilic with optimal growth near pH 7. Based on the previously obtained clone libraries and the descriptions of the dominant members in each, it appeared that the acidic bog sediment contained predominantly acidotolerant methanogens whereas the anaerobic digester sludge did not.

During the first OLR shocks, only the Bog-test reactor survived, and this reactor contained large numbers of the Fen cluster (Fig. 5-1, 2). The Fen Cluster persisted in the Bog-test reactor throughout the second OLR shocks, and was detected in the Hybrid-test reactor prior to and after the second OLR shocks, but by Day 379 the Fen Cluster was no longer detected in either reactor (Fig. 5-2). In the Bog-control reactor, the Fen Cluster was the only methanogen group detected until day 90 when it was no longer seen (Appendix 2 Fig. A2-5). It is likely that an acidic reactor pH confers a competitive advantage to the Fen Cluster, but that under neutral conditions, members of this clade may not be able to persist. That would explain the disappearance of this clade from the Bog-control reactor, as well as from the Bog- and Hybrid-test reactors during the period between the second and third OLR shocks. Although the Digester-control reactor was dominated by MCR-7 on the day of inoculation, by day 25 this clade was no longer detected and was never detected in the Digester-test reactor. Instead MCR-7 was periodically detected in the Bog- and Hybrid-control reactors as well as the Bog- and Hybrid-test reactors (Fig. 5-2, Appendix 2 Fig. A2-5).

Recovery of methane activity in reactors after OLR shocks appeared to be correlated with the presence of *Methanosarcina* and also the numbers of methanogens present. After each set of OLR shocks, the number of methanogens decreased in reactors, and recovery in methane production occurred with an increase in methanogen numbers (Fig. 5-2). After the first OLR shock, the Fen cluster was the only methanogen detected in the Bog-test reactor until day 203 when *Methanosarcina* appeared (Fig. 5-2). The appearance of *Methanosarcina* corresponds to a rapid increase in methane production as well as a decrease in VFAs and increase in pH (Fig. 5-1). After the second OLR shocks, the Digester-test reactor never completely stopped producing methane and *Methanosarcina* was always the dominant methanogen (Figs. 5-2, 3). In contrast, the Bog-test and Hybrid-test reactors were dominated by *Methanobacteriaceae*, but recovery of methane production in these reactors correlated to the return to dominance of *Methanosarcina*. After the third OLR shock on day 436, the Digester-test and Bog-test reactors showed similar performance. The reactors were dominated by *Methanobacteriaceae* on day 457 but showed no methane production, but when *Methanosarcina* again was the dominant methanogen on day 485, reactors again produced methane and pH had increased (Figs. 5-2, 4). In contrast, the Hybrid-test reactor did not recover from the third OLR shock by the end of the study period, and during this time this reactor was dominated by *Methanobacteriaceae*.

Several researchers have reported an increase in the numbers of *Methanosarcina* and *Methanobacteriaceae* at periods in reactor operation characterized by high acetate concentrations (63, 96, 113). Aceticlastic methanogens belong to the genera *Methanosarcina* and *Methanosaeta*. *Methanosarcina* are characterized by a faster growth rate but also a higher K_m for acetate compared to *Methanosaeta*, and *Methanosarcina* are typically the dominant aceticlastic methanogens in reactors with high acetate concentrations (201, 245, 297). A study by Yu ***et al.*** (2005) suggests that at acetate concentrations above about 30 mg/L (0.5 mM) the specific growth rate of *Methanosarcina* is greater than that of *Methanosaeta* allowing *Methanosarcina* to dominate aceticlastic methanogenesis. This could also explain the predominance of *Methanosarcina* in control reactors because although VFA concentrations were usually less than 10 mM, acetate concentrations were greater than 0.5 mM for the first 250 days of operation (data not shown).

Although the *Methanosarcina* can also use hydrogen and carbon dioxide for growth, their K_m for hydrogen is greater than that of other hydrogenotrophic methanogens (272). This prevents the *Methanosarcina* from forming syntrophic relationships with VFA oxidizers as this K_m is greater than the maximum hydrogen threshold for the syntrophic partner (272). Syntrophic fatty acid-oxidizers are usually specific to one or a few substrates (233). At high acetate

concentrations, aceticlastic methanogenesis catalyzed by *Methanosarcina* dominates, but at low acetate concentrations syntrophic acetate oxidizers and *Methansaeta* compete for substrate (245). Some syntrophic acetate oxidizers have been found to be homoacetogens which produce acetate under conditions of high hydrogen partial pressure and low acetate, or oxidize acetate when concentrations of acetate are high and hydrogen partial pressure is kept low by association with a hydrogen-consuming methanogen (1). Although *Methanosarcina* do not form syntrophic relationships, members of the *Methanobacteriales* and *Methanomicrobiales* do (233). Hydrogenotrophic methanogens in the test reactors were predominantly *Methanobacteriaceae* but also Fen Cluster and MCR-7, and the numbers of these groups increased during periods of high acetate concentrations which followed OLR shocks (Fig. 5-2, Appendix 2 Figs. A2-6, 9). Acidotolerant and acidophilic members of the *Methanobacteriaceae* have been isolated from anaerobic digesters (230) as well as peatlands (142, 251), and as stated previously, other researchers have seen an increase in *Methanobacteriaceae* during periods of high acetate and low pH in reactors (63, 96, 113). The control reactors eventually became dominated by *Methanosarcina*, and it is possible that the homoacetogens successfully outcompeted hydrogenotrophic methanogens for hydrogen, thereby providing a constant supply of acetate to the *Methanosarcina*. In the test reactors, periodic drops in pH may have provided a competitive advantage for hydrogenotrophic methanogens. Several studies of both acidic peatlands and anaerobic reactors have shown that as the pH decreases, an increasing proportion of methane is produced from hydrogen and carbon dioxide (26, 69, 85, 114, 136, 142).

A few researchers have successfully adapted methanogenic communities to process waste at low pH or under periodic high substrate loadings. Taconi *et al.* (2008) adapted a methanogenic community to digest batch additions of acetate at pH values as low as 4 with additions of hydrochloric acid. As each batch addition of acetate was consumed, pH would rise approximately 1-1.5 units before the next addition of acetate. Although acetate was consumed even at a pH of 4, each addition of acetate to the reactor caused a cessation of methane production for several days (266). Xing *et al.* (1997) subjected a lab-scale CSTR to consistent OLR shocks by feeding glucose for two days followed by no feeding for two days over a period of operation of more than 200 days. Initially the reactor accumulated high concentrations of VFAs, then showed a period of large fluctuations in COD and VFAs, followed by consumption of the accumulated VFAs and a return to pre-perturbation COD removal rates and methane production (289). In this study, the acidophilic Fen Cluster was detected during the first and second OLR shocks, but not during the third. There was approximately 120 d between the first OLR shock and the 1 g of glucose delivered during the second OLR shocks, and approximately 150 days

between the last of the second OLR shocks and the third OLR shocks. It is possible that without the selective pressure of low pH, the Fen Cluster could not be retained in sufficient numbers in the reactor. Although it is not clear how much of an influence the Fen Cluster had in providing pH tolerance to the reactor, the presence of this group during the first OLR shock in the Bog-test reactor was also the only example of a reactor that recovered activity without the addition of NaOH to raise the pH (Fig. 5-1, 2). After the third OLR shock, reactor pH only needed to be raised to approximately 4.8-5 to allow reactors to recover activity (Fig. 5-4). It is possible that members of the Fen Cluster are too slow growing to be retained in a reactor with a 40 d retention time, but this explanation seems unlikely in light of the fact that they were able to persist for 346 days in two of the test reactors. It is more likely that the operation of these lab-scale reactors selected for members of the *Methanosarcina* and other methanogens were effectively outcompeted for substrate. It is difficult to mimic full-scale operations in lab-scale reactors, and for this reason microbial diversity is often much less in lab-scale reactors than in pilot- or full-scale systems (151). Although methanogens of the Fen Cluster were not retained throughout the operational period, their presence during the early operation of these reactors lends further insight into the metabolic capabilities of this unique phylogenetic group.

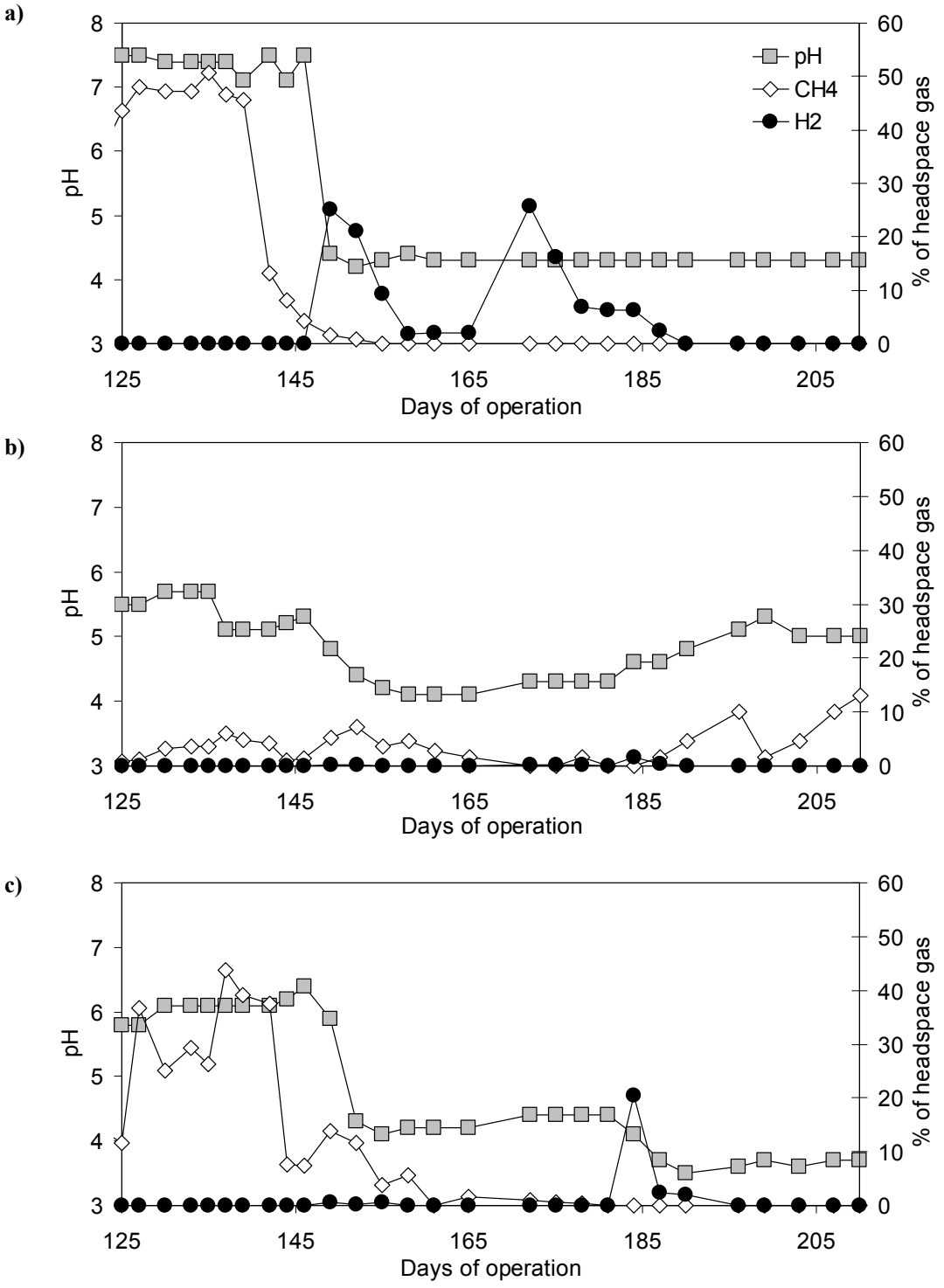


Figure 5-1. Performance of reactors during first organic loading rate shock.

Results are shown for a) Digester-test b) Bog-test, and c) Hybrid-test reactors. Reactors were opened on day 142 in an anaerobic glove chamber (headspace N_2:H_2 98%:2%) which replaced the headspace gas. Reactors received one shock of 10 g glucose on day 146.

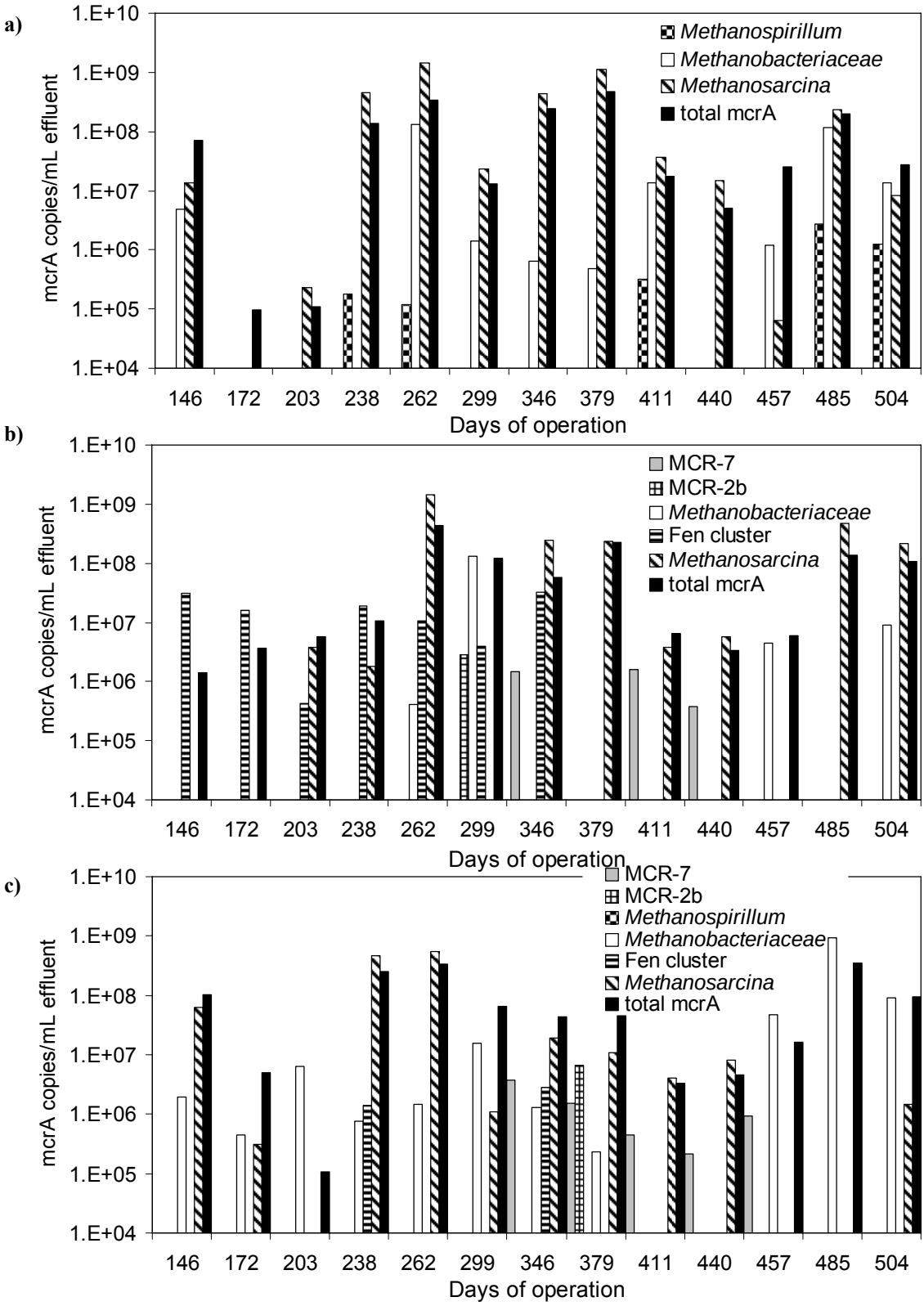


Figure 5-2. Methanogen community dynamics in test reactors during all three OLR shock tests.

Results are shown for a) Digester-test reactor 1, b) Bog-test reactor 1, c) Hybrid-test reactor 1. Digester- and Hybrid-test reactors were restarted from control reactors on day 210.

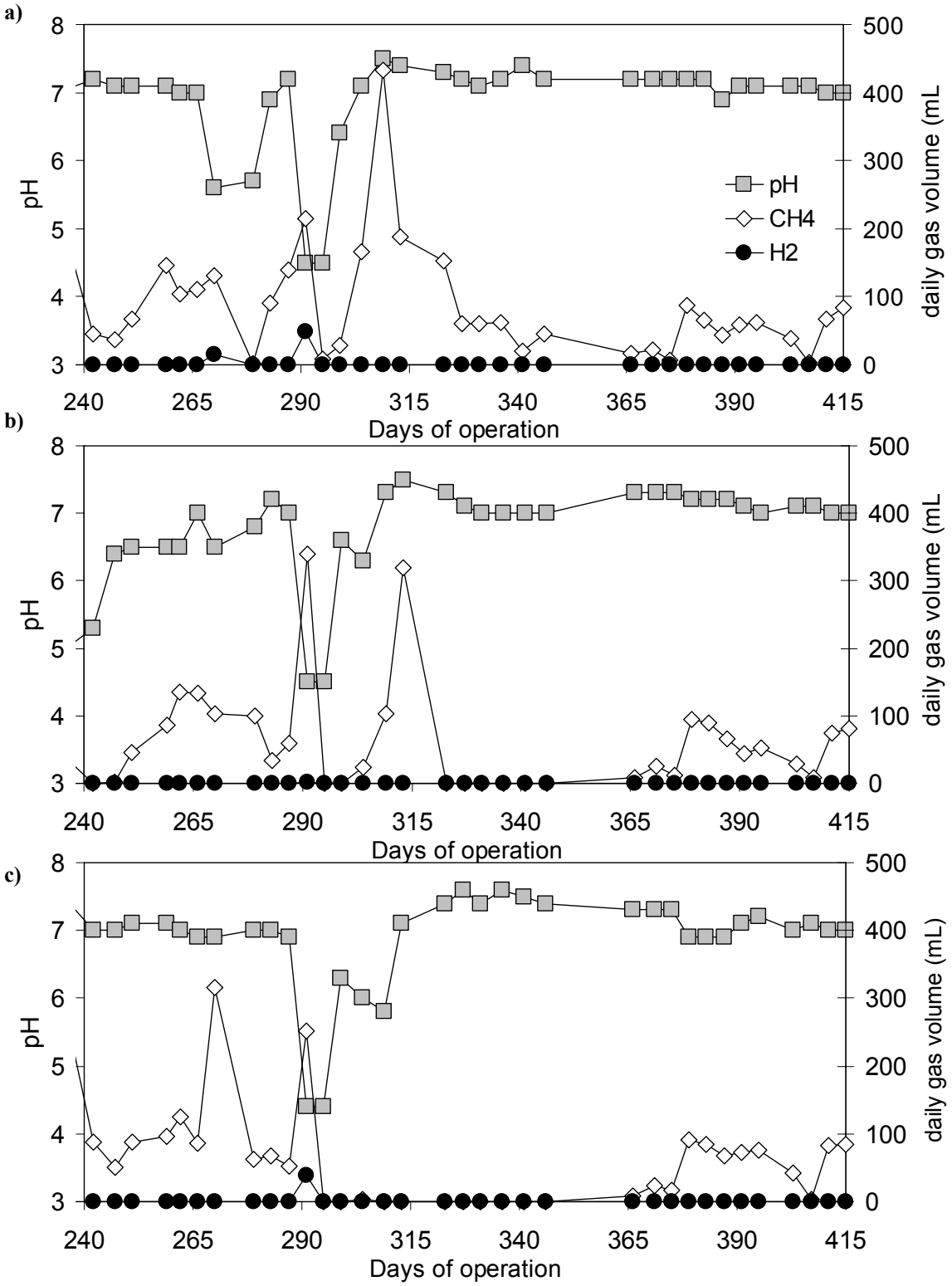


Figure 5-3. Performance of test reactors during the second OLR shocks.

Results are shown for a) Digester-test reactor, b) Bog-test reactor, c) Hybrid-test reactor. Reactors received 1 g, 5 g, and 10 g of glucose on days 262, 266, and 287 respectively.

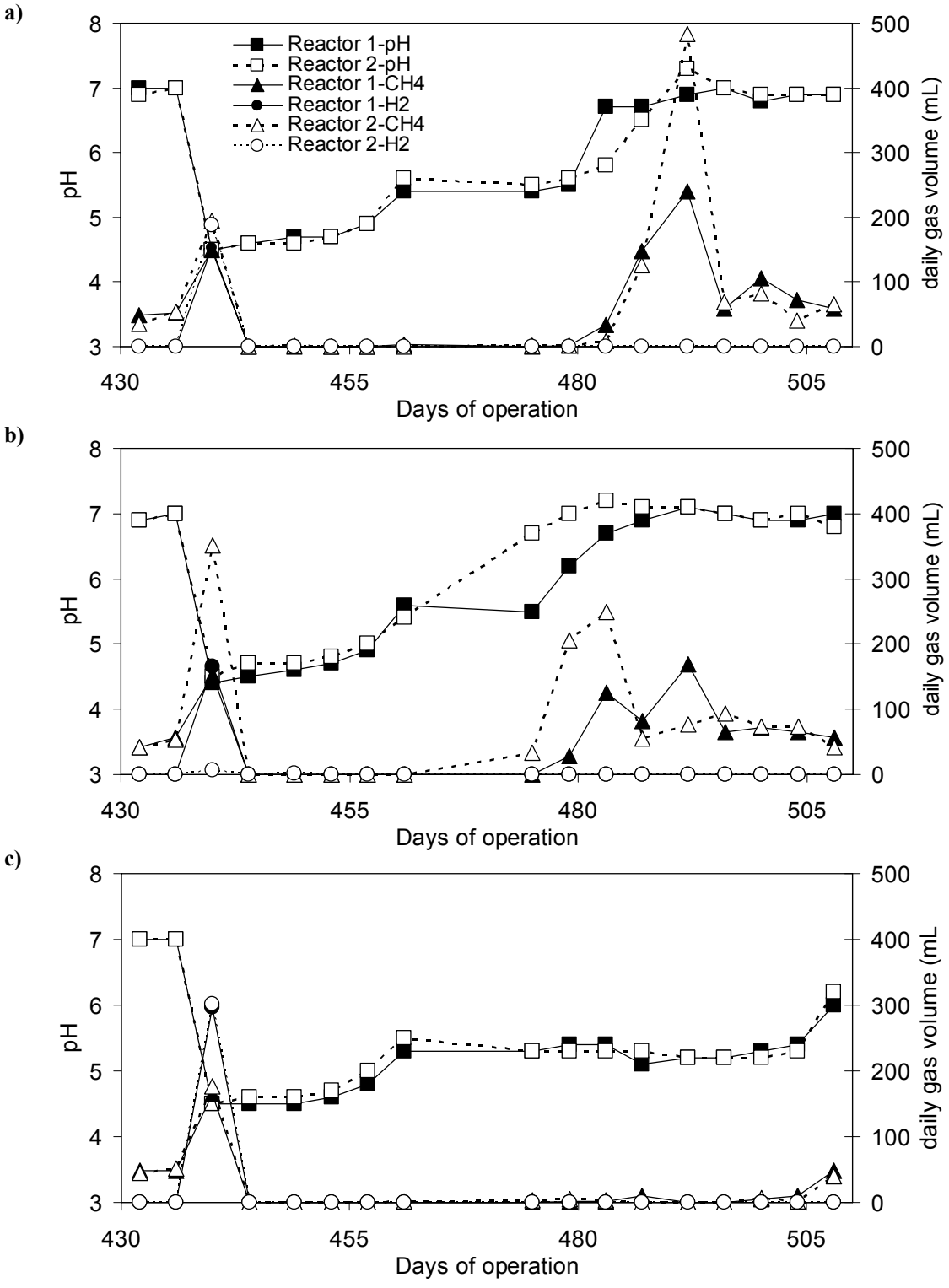


Figure 5-4. Performance of test reactors during the third OLR shocks.

Results are shown for a) Digester-test reactor, b) Bog-test reactor, c) Hybrid-test reactor. Reactors received 10 g of glucose on day 436. Test reactors labeled "2" received mineral nutrients with the OLR shock.

Chapter 6

FINAL CONCLUSIONS

Anaerobic digestion has been widely under-utilized to treat organic waste streams primarily because of the belief that anaerobic treatment processes are less stable and more prone to failure than aerobic ones. The use of aerobic over anaerobic treatment increases the expense of waste treatment as well as neglecting a source of alternative energy in the form of methane-rich biogas. The inocula for anaerobic digesters comes from a rather narrow range of sources typically originating from animal or human waste. A large percentage of natural methane emissions arise from cold to temperate acid peatlands where microbial consortia produce methane under conditions that would not be considered feasible in constructed reactors. To date, few if any research has been conducted on the use of an environmentally-derived inoculum for an anaerobic reactor operating under atypical conditions, for example, at acidic pH induced by shock loads of organic material.

The first hypothesis of this work was that the methanogen communities present in an acidic peatland and a constructed anaerobic digester would be different from each other. To address this hypothesis, clone libraries were prepared for the 16S rDNA and *mcrA* genes. Sequence data from cultivated and described methanogens were analyzed in order to quantify differences in the two clone libraries in terms of taxonomic levels. The second hypothesis was that a lab-scale reactor inoculated from an acidic peatland would be more resistant to periods of low pH induced by organic loading rate (OLR) shocks than a reactor inoculated from a municipal sludge digester. To assess differences in performance, lab-scale reactors were operated with periodic OLR shocks while following responses in the methanogen communities. In order to follow these changes, primers and probes were designed for the *mcrA* to distinguish different methanogenic clades in quantitative PCR. In this manner, total methanogen numbers as well as numbers of different methanogen groups could be followed throughout reactor operation.

Final conclusions of this project include:

- The methanogens present in an acidic peatland and an anaerobic digester are quite different from each other with almost no overlap at even the family level.
- The hydrogenotrophic *Methanomicrobiales* dominate both communities, with the Fen Cluster the most frequent sequence detected in the acidic peatland, and the MCR-7 clade the most frequent sequence detected in the anaerobic digester.

- Members of the Fen Cluster are able to survive lab-scale reactor conditions of ionic strength and VFA concentrations that are much higher than those found in natural wetlands.
- The Fen Cluster are selected for by acidic pH conditions in a reactor.
- Consumption of VFAs and a return to methane production in reactors subject to OLR shocks is concomitant with an increase in *Methanosarcina*.
- Lab-scale anaerobic reactors can recover activity and neutral pH after OLR shocks if reactor pH is raised to at least 4.8-5.

Chapter 7

FUTURE RESEARCH

The goal of this research was to improve anaerobic digester stability under organic shock loads using an acidotolerant methanogenic consortium as a reactor inoculum. Even though the operational differences that were seen in reactors after the first OLR shock did not persist in later OLR shocks, valuable information was still obtained. Isolates of the Fen Cluster are known to be acidophilic with optimal growth at a pH of 5-5.5, but it was not known until this study if they would be able to survive the conditions of an anaerobic reactor. This clade could be of great importance for anaerobic digesters treating acidic waste, but two questions would need to be addressed. The first question is whether the presence of the Fen Cluster would improve reactor operation under acidic conditions. If the answer to this first question is yes, then the second question would be how to maintain the presence of the Fen Cluster in an anaerobic reactor.

To address the first question, in this study the Fen Cluster appeared to be competitive when the operating pH of the Bog- and Hybrid-test reactors were low. Since the two known isolates of this clade show optimal growth at a pH of 5-5.5 (25, 31), the results seen in this study are consistent with what is known of this clade. During this study the pH became acidic because of VFA accumulation during overloading conditions, but a future study should look at maintaining acidic pH with HCl addition instead. Maintenance of an acidophilic methanogenic consortium in an anaerobic reactor would be useful for treating wastes where it is difficult to maintain neutral conditions in a reactor. Examples of these types of wastes include lignocellulosic wastes such as crop residues and by-products of wood and paper pulp industries (13), wastes from food and vegetable processing, including dairy and edible oil industries (18, 28, 102), brewery wastewater (53, 159), and petrochemical wastes (204). The high carbon-to-nitrogen ratios and low buffering capacity of most lignocellulosic and food wastes leads to rapid acidification of an anaerobic digester. Petrochemical wastes and other industrial wastes contain large quantities of phenols, aldehydes, cyclohexanes, and acids which result in a very acidic waste stream (204). Biohydrogen reactors produce an effluent waste rich in VFAs, especially acetate, with a pH of 4.5-5.5 (158, 298). For anaerobic treatment of any of these wastes, typically either alkalinity or a second nitrogen-rich waste needs to be added to maintain a neutral reactor pH and preven failure. It would be useful to see if a anaerobic reactor operating at acidic pH, possibly dominated by methanogens of the Fen Cluster, would be able to successfully treat these wastes.

There is a need for methanogenic consortia resistant to both OLR shocks as well as those that are acidophilic. Currently, most anaerobic digestion is used as a way to reduce the microbial biomass produced during aerobic waste treatment instead of direct anaerobic treatment of waste. For example, the flow and composition of municipal wastewater may change rapidly, and as anaerobic digesters are considered less stable under rapidly changing waste conditions, aerobic treatment is first used (155). A changing waste stream may cause reactor upset due to a sudden increase in the amount of fermentable material, and the glucose pulses delivered to lab-scale reactors in this study was meant to simulate this type of occurrence. Adaptation of an anaerobic methanogenic consortia to acidic pH may also confer stability of this consortia to rapid changes in pH. Jain and Mattiasson (1998) adapted an anaerobic consortium for methane production in batch culture by slowly lowering the pH down to 4 with addition of HCl. A non-acclimatized culture, which had always been maintained at neutral pH, was compared to a culture acclimatized to pH 4 during rapid changes in pH. The non-acclimatized culture ceased methane production for over a month when pH was shifted from 7 to 5, but the acclimatized culture was able to continue producing methane with little delay as pH was shifted from 5 to 7, and then back to 5 (123). In this study, maximal methanogenesis was always at neutral pH, but different findings should be obtained with an acidophilic consortia.

Cavicchioli (2006) suggested it may be more appropriate to classify microbes by their temperature range for growth rather than just the optimal temperature for growth. Part of the argument was based on the fact that *Methanococcoides burtonii*, which has a maximum growth temperature of 28°C, grows faster at 4°C than *Methanogenium frigidum*, which has a maximum growth temperature of 18°C (36). Perhaps a similar comparison based on growth pH could be made for methanogens, with frequent OLR shocks selecting for methanogens with a wide range of growth pH. Xing *et al.* (1997) adapted a glucose-fed mesophilic CSTR to survive constant OLR perturbations by maintaining a four day cycle of feeding two days followed by starving two days for over 200 days of operation. Initially after the perturbations were begun, COD removal and methane production rates dropped and VFAs accumulated, but after 160 days of perturbation the reactor returned to original rates of methane production and COD removal. Reactor pH never fell below about 6.3 and eventually returned to around 7 after the reactor recovered full methanogenic activity (288). In this study, more frequent glucose pulses may have eventually led to faster recovery from each pulse and retention of greater numbers of acidophilic methanogens.

Although this study focused on methanogens with the belief that these microbes are the most sensitive members of an anaerobic consortia to acidic or changing pH, this may not

necessarily be the case. Sensitivity to high VFA concentrations or changes in pH may instead lie with the syntrophic partners of the methanogens. Few studies have explored *Bacterial* populations in acidic peatlands (62, 94, 105) and it is likely that differences between the *Bacterial* populations of environmental and constructed methanogenic environments will be as great as the differences in methanogen populations. Many researchers have found high hydrogen partial pressures inhibited methanogenesis, and isolation of some hydrogenotrophic methanogens requires co-culture with a hydrogen- or formate-producing syntroph instead of just addition of these substrates to the culture medium (163, 226). Future research should explore the effect of changes in pH as well as VFA concentrations on co-cultures of syntrophic fatty-acid oxidizers with methanogen partners. Selection of methanogens resistant to acidic or rapidly changing pH will require co-selection of syntrophs able to withstand these environmental pressures as well.

A final area of exploration based on the findings of this study is the role of *Methanosarcina* in anaerobic digestion and possible ways to control their presence in a reactor. *Methanosarcina* were not seen in the clone libraries from either environment, but they quickly came to dominate methanogen numbers in both control and test reactors. Although the presence of *Methanosarcina* seemed to correlate with the consumption of VFAs and increasing methane production in the test reactors, it is difficult to know if *Methanosarcina* was beneficial or harmful to reactor recovery. It would be essential to compare a *Methanosarcina*-dominated reactor to a reactor containing few or no detectable *Methanosarcina* under OLR shocks. The *Methanosarcina* possess cytochromes, unlike most other methanogens, and this gives the *Methanosarcina* a larger growth yield (272). A large growth yield is undesirable in AD as the goal is to mineralize as much of the organic matter as possible to methane and carbon dioxide. In addition, the cytochromes of the *Methanosarcina* mean that their K_m for both acetate and hydrogen are higher than other methanogens, and the *Methanosarcina* are not able to form syntrophic relationships with *Bacteria* (272). It may be desirable for *Methanosarcina* to be present in large numbers after OLR shocks when acetate and hydrogen concentrations are high so that they may consume these products. But once levels of hydrogen and acetate have dropped below the K_m of the *Methanosarcina*, a reactor dominated with these methanogens may exhibit a bottleneck in methanogenesis as their growth rate slows.

Anaerobic digestion could be an important process for generating carbon-neutral energy from waste in the future, and optimization of the system must never neglect examination of microbial relationships. A greater understanding of the ecological relationships present in natural methanogenic environments will allow better use of methanogenesis to achieve human goals of waste treatment and renewable energy production.

REFERENCES

1. **Ahring, B. K.** 2003. Perspectives for anaerobic digestion. Advances in Biochemical Engineering/Biotechnology **81:**1-27.
2. **Ako, O. Y., Y. Kitamura, K. Intabon, and T. Satake.** 2008. Steady state characteristics of acclimated hydrogenotrophic methanogens on inorganic substrate in continuous chemostat reactors. Bioresource Technol **99:**6305-6310.
3. **Al-Masri, M. R.** 2001. Changes in biogas production due to different ratios of some animal and agricultural wastes. Bioresource Technol **77:**97-100.
4. **Alain, K., T. Holler, F. Musat, M. Elvert, T. Treude, and M. Kruger.** 2006. Microbiological investigation of methane- and hydrocarbon-discharging mud volcanoes in the carpathian mountains, romania. Environ Microbiol **8:**574-590.
5. **Alvarez, J. A., E. Armstrong, M. Gomez, and M. Soto.** 2008. Anaerobic treatment of low-strength municipal wastewater by a two-stage pilot plant under psychrophilic conditions. Bioresource Technol **99:**7051-7062.
6. **Anderson, G. K., and G. Yang.** 1992. Determination of bicarbonate and total volatile acid concentration in anaerobic digesters using a simple titration. Water Environ Res **64:**53-59.
7. **Angelidaki, I., X. Chen, J. Cui, P. Kaparaju, and L. Ellegaard.** 2006. Thermophilic anaerobic digestion of source-sorted organic fraction of household municipal solid waste: Start-up procedure for continuously stirred tank reactor. Water Res **40:**2621-2628.
8. **Angenent, L. T., S. W. Sung, and L. Raskin.** 2002. Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Water Res **36:**4648-4654.
9. **Angenent, L. T., D. D. Zheng, S. H. Sung, and L. Raskin.** 2002. Microbial community structure and activity in a compartmentalized, anaerobic bioreactor. Water Environ Res **74:**450-461.
10. **Arbeli, Z., A. Brenner, and A. Abeliovich.** 2006. Treatment of high-strength dairy wastewater in an anaerobic deep reservoir: Analysis of the methanogenic fermentation pathway and the rate-limiting step. Water Res **40:**3653-3659.
11. **Aspe, E., M. C. Marti, and M. Roeckel.** 1997. Anaerobic treatment of fishery wastewater using a marine sediment inoculum. Water Res **31:**2147-2160.

12. **Bagi, Z., N. Acs, B. Balint, L. Horvath, K. Dobo, K. R. Perei, G. Rakhely, and K. L. Kovacs.** 2007. Biotechnological intensification of biogas production. Appl Microbiol Biot **76:**473-482.

13. **Banks, C. J., and P. N. Humphreys.** 1998. The anaerobic treatment of a ligno-cellulosic substrate offering little natural ph buffering capacity. Water Science and Technology **38:**29-35.

14. **Banning, N., F. Brock, J. C. Fry, R. J. Parkes, E. R. C. Hornibrook, and A. J. Weightman.** 2005. Investigation of the methanogen population structure and activity in a brackish lake sediment. Environ Microbiol **7:**947-960.

15. **Bapteste, E., C. Brochier, and Y. Boucher.** 2005. Higher-level classification of the archaea: Evolution of methanogenesis and methanogens. Archaea **I:**353-363.

16. **Barredo, M. S., and L. M. Evison.** 1991. Effect of propionate toxicity on methanogen-enriched sludge, methanobrevibacter-smithii, and methanospirillum-hungatii at different ph values. Appl Environ Microb **57:**1764-1769.

17. **Basiliko, N., J. B. Yavitt, P. M. Dees, and S. M. Merkel.** 2003. Methane biogeochemistry and methanogen communities in two northern peatland ecosystems, new york state. Geomicrobiol J **20:**563-577.

18. **Beccari, M., F. Bonemazzi, M. Majone, and C. Riccardi.** 1996. Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. Water Res **30:**183-189.

19. **Biddle, J. F., C. Turich, S. Brantley, and M. A. Bruns.** 2002. Presented at the American Geophysical Union, San Francisco, CA, DEC 6-10.

20. **Bidle, K. A., M. Kastner, and D. H. Bartlett.** 1999. A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the cascadia margin (odp site 892b). Fems Microbiol Lett **177:**101-108.

21. **Bodik, I., B. Herdova, and M. Drtil.** 2000. Anaerobic treatment of the municipal wastewater under psychrophilic conditions. Bioprocess Eng **22:**385-390.

22. **Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jorgensen, U. Witte, and O. Pfannkuche.** 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407:**623-626.

23. **Boone, D. R., W. B. Whitman, and P. Rouviere.** 1993. Diversity and taxonomy of methanogens, p. 35-80. *In* J. G. Ferry (ed.), Methanogens. Chapman and Hall, Inc., New York.

24. **Borja, R., and C. J. Banks.** 1995. Response of an anaerobic fluidized-bed reactor treating ice-cream waste-water to organic, hydraulic, temperature and ph shocks. Journal of Biotechnology **39:**251-259.

25. **Brauer, S. L., H. Cadillo-Quiroz, E. Yashiro, J. B. Yavitt, and S. H. Zinder.** 2006. Isolation of a novel acidiphilic methanogen from an acidic peat bog. Nature **442:**192-194.

26. **Brauer, S. L., J. B. Yavitt, and S. H. Zinder.** 2004. Methanogenesis in mclean bog, an acidic peat bog in upstate new york: Stimulation by h-2/co2 in the presence of rifampicin, or by low concentrations of acetate. Geomicrobiol J **21:**433-443.

27. **Briones, A. M., B. J. Daugherty, L. T. Angenent, K. D. Rausch, M. E. Tumbleson, and L. Raskin.** 2007. Microbial diversity and dynamics in multi- and single-compartment anaerobic bioreactors processing sulfate-rich waste streams. Environ Microbiol **9:**93-106.

28. **Buendia, I. M., F. J. Fernandez, J. Villasenor, and L. Rodriguez.** 2009. Feasibility of anaerobic co-digestion as a treatment option of meat industry wastes. Bioresource Technol **100:**1903-1909.

29. **Burggraf, S., T. Mayer, R. Amann, S. Schadhauser, C. R. Woese, and K. O. Stetter.** 1994. Identifying members of the domain archaea with ribosomal-rna-targeted oligonucleotide probes. Appl Environ Microb **60:**3112-3119.

30. **Cadillo-Quiroz, H., S. Brauer, E. Yashiro, C. Sun, J. Yavitt, and S. Zinder.** 2006. Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central new york state, USA. Environ Microbiol **8:**1428-1440.

31. **Cadillo-Quiroz, H., E. Yashiro, J. B. Yavitt, and S. H. Zinder.** 2008. Characterization of the archaeal community in a minerotrophic fen and terminal restriction fragment length polymorphism-directed isolation of a novel hydrogenotrophic methanogen. Appl Environ Microb **74:**2059-2068.

32. **Cakir, F. Y., and M. K. Stenstrom.** 2005. Greenhouse gas production: A comparison between aerobic and anaerobic wastewater treatment technology. Water Res **39:**4197-4203.

33. **Caldwell, S. L., J. R. Laidler, E. A. Brewer, J. O. Eberly, S. C. Sandborgh, and F. S. Colwell.** 2008. Anaerobic oxidation of methane: Mechanisms, bioenergetics, and the ecology of associated microorganisms. Environ Sci Technol **42:**6791-6799.

34. **Calli, B., J. Zhao, E. Nijssen, and K. Vanbroekhoven.** 2007. Presented at the 11th IWA World Congress on Anaerobic Digestion, Brisbane, Australia, 23rd-27th September.

35. **Castro, H., A. Ogram, and K. R. Reddy.** 2004. Phylogenetic characterization of methanogenic assemblages in eutrophic and oligotrophic areas of the florida everglades. Appl Environ Microb **70:**6559-6568.
36. **Cavicchioli, R.** 2006. Cold-adapted archaea. Nat Rev Microbiol **4:**331-343.
37. **Chae, K. J., A. Jang, S. K. Yim, and I. S. Kim.** 2008. The effects of digestion temperature and temperature shock on the biogas yields from the mesophilic anaerobic digestion of swine manure. Bioresource Technol **99:**1-6.
38. **Chan, O. C., P. Claus, P. Casper, A. Ulrich, T. Lueders, and R. Conrad.** 2005. Vertical distribution of structure and function of the methanogenic archaeal community in lake dagow sediment. Environ Microbiol **7:**1139-1149.
39. **Chan, O. C., W. T. Liu, and H. H. P. Fang.** 2001. Study of microbial community of brewery-treating granular sludge by denaturing gradient gel electrophoresis of 16s rrna gene. Water Science and Technology **43:**77-82.
40. **Chan, O. C., M. Wolf, D. Hepperle, and P. Casper.** 2002. Methanogenic archaeal community in the sediment of an artificially partitioned acidic bog lake. Fems Microbiol Ecol **42:**119-129.
41. **Chauhan, A., and A. Ogram.** 2006. Fatty acid-oxidizing consortia along a nutrient gradient in the florida everglades. Appl Environ Microb **72:**2400-2406.
42. **Chen, C. L., J. H. Wu, and W. T. Liu.** 2008. Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. Water Res **42:**1963-1976.
43. **Chen, Y., J. J. Cheng, and K. S. Creamer.** 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technol **99:**4044-4064.
44. **Chin, K. J., and R. Conrad.** 1995. Intermediary metabolism in methanogenic paddy soil and the influence of temperature. Fems Microbiol Ecol **18:**85-102.
45. **Chin, K. J., T. Lueders, M. W. Friedrich, M. Klose, and R. Conrad.** 2004. Archaeal community structure and pathway of methane formation on rice roots. Microbial Ecol **47:**59-67.
46. **Chin, K. J., T. Lukow, and R. Conrad.** 1999. Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl Environ Microb **65:**2341-2349.
47. **Chin, K. J., T. Lukow, S. Stubner, and R. Conrad.** 1999. Structure and function of the methanogenic archaeal community in stable cellulose-degrading enrichment cultures at two different temperatures (15 and 30 degrees c). Fems Microbiol Ecol **30:**313-326.

48. **Chouari, R., D. Le Paslier, P. Daegelen, P. Ginestet, J. Weissenbach, and A. Sghir.** 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ Microbiol **7:**1104-1115.

49. **Chung, K. T., B. Hungate, and J. B. Russell.** 2005. Robert e. Hungate deceased members. Asm News **71:**42-42.

50. **Collins, G., A. Woods, S. McHugh, M. W. Carton, and V. O'Flaherty.** 2003. Microbial community structure and methanogenic activity during start-up of psychrophilic anaerobic digesters treating synthetic industrial wastewaters. Fems Microbiol Ecol **46:**159-170.

51. **Conklin, A., R. Bucher, H. D. Stensel, and J. Ferguson.** 2007. Effects of oxygen exposure on anaerobic digester sludge. Water Environ Res **79:**396-405.

52. **Connaughton, S., G. Collins, and V. O'Flaherty.** 2006. Development of microbial community structure and actvity in a high-rate anaerobic bioreactor at 18 degrees c. Water Res **40:**1009-1017.

53. **Connaughton, S., G. Collins, and V. O'Flaherty.** 2006. Psychrophilic and mesophilic anaerobic digestion of brewery effluent: A comparative study. Water Res **40:**2503-2510.

54. **Conrad, R.** 1999. Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. Fems Microbiol Ecol **28:**193-202.

55. **Cooney, M., N. Maynard, C. Cannizzaro, and J. Benemann.** 2007. Two-phase anaerobic digestion for production of hydrogen-methane mixtures. Bioresource Technol **98:**2641-2651.

56. **Crocetti, G., M. Murto, and L. Bjornsson.** 2006. An update and optimisation of oligonucleotide probes targeting methanogenic archaea for use in fluorescence in situ hybridisation (fish). J Microbiol Meth **65:**194-201.

57. **Cronin, C., and K. V. Lo.** 1998. Anaerobic treatment of brewery wastewater using uasb reactors seeded with activated sludge. Bioresource Technol **64:**33-38.

58. **Dagnall, S.** 1995. Uk strategy for centralized anaerobic-digestion. Bioresource Technol **52:**275-280.

59. **de Alwis, A.** 2002. Biogas-a review of sri lanka's performance with a renewable energy technology. Energy for Sustainable Development **4:**30-37.

60. **Dearman, B., P. Marschner, and R. H. Bentham.** 2006. Methane production and microbial community structure in single-stage batch and sequential batch systems anaerobically co-digesting food waste and biosolids. Appl Microbiol Biot **69:**589-596.

61. **Dedysh, S. N., N. S. Panikov, and J. M. Tiedje.** 1998. Acidophilic methanotrophic communities from sphagnum peat bogs. Appl Environ Microb **64:**922-929.

62. **Dedysh, S. N., T. A. Pankratov, S. E. Belova, I. S. Kulichevskaya, and W. Liesack.** 2006. Phylogenetic analysis and in situ identification of bacteria community composition in an acidic sphagnum peat bog. Appl Environ Microb **72:**2110-2117.

63. **Delbes, C., R. Moletta, and J. J. Godon.** 2001. Bacterial and archaeal 16s rdna and 16s rrna dynamics during an acetate crisis in an anaerobic digestor ecosystem. Fems Microbiol Ecol **35:**19-26.

64. **Delong, E. F.** 1992. Archaea in coastal marine environments. P Natl Acad Sci USA **89:**5685-5689.

65. **Demirer, G. N., and S. L. Chen.** 2005. Anaerobic digestion of dairy manure in a hybrid reactor with biogas recirculation. World J Microb Biot **21:**1509-1514.

66. **Dhillon, A., M. Lever, K. G. Lloyd, D. B. Albert, M. L. Sogin, and A. Teske.** 2005. Methanogen diversity evidenced by molecular characterization of methyl coenzyme m reductase a (mcra) genes in hydrothermal sediments of the guaymas basin. Appl Environ Microb **71:**4592-4601.

67. **Diaz, E. E., A. J. A. Stams, R. Amils, and J. L. Sanz.** 2006. Phenotypic properties and microbial diversity of methanogenic granules from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater. Appl Environ Microb **72:**4942-4949.

68. **Dolfing, J., B. Jiang, A. M. Henstra, A. J. M. Stams, and C. M. Plugge.** 2008. Syntrophic growth on formate: A new microbial niche in anoxic environments. Appl Environ Microb **74:**6126-6131.

69. **Duddleston, K. N., M. A. Kinney, R. P. Kiene, and M. E. Hines.** 2002. Anaerobic microbial biogeochemistry in a northern bog: Acetate as a dominant metabolic end product. Global Biogeochem Cy **16:**-.

70. **Dupla, M., T. Conte, J. C. Bouvier, N. Bernet, and J. P. Steyer.** 2004. Dynamic evaluation of a fixed bed anaerobic digestion process in response to organic overloads and toxicant shockloads. Water Science and Technology **49:**61-68.

71. **Durisch-Kaiser, E., L. Klauser, B. Wehrli, and C. Schubert.** 2005. Evidence of intense archaeal and bacterial methanotrophic activity in the black sea water column. Appl Environ Microb **71:**8099-8106.

72. **Earl, J., G. Hall, R. W. Pickup, D. A. Ritchie, and C. Edwards.** 2003. Analysis of methanogen diversity in a hypereutrophic lake using pcr-rflp analysis of mcr sequences. Microbial Ecol **46:**270-278.

73. **Edwards, C., B. A. Hales, G. H. Hall, I. R. McDonald, J. C. Murrell, R. Pickup, D. A. Ritchie, J. R. Saunders, B. M. Simon, and M. Upton.** 1998. Microbiological processes in the terrestrial carbon cycle: Methane cycling in peat. Atmos Environ **32:**3247-3255.

74. **El-Mashad, H. M., G. Zeeman, W. K. P. van Loon, G. P. A. Bot, and G. Lettinga.** 2004. Effect of temperature and temperature fluctuation on thermophilic anaerobic digestion of cattle manure. Bioresource Technol **95:**191-201.

75. **Elferink, S. J. W. H. O., W. J. C. Vorstman, A. Sopjes, and A. J. M. Stams.** 1998. Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater. Fems Microbiol Ecol **27:**185-194.

76. **Enright, A. M., G. Collins, and V. O'Flaherty.** 2006. Microbial community structure of anaerobic sludges, from dairy and chemical production full scale anaerobic digesters in ireland. GenBank, direct submission.

77. **Feller, G., and C. Gerday.** 2003. Psychrophilic enzymes: Hot topics in cold adaptation. Nat Rev Microbiol **1:**200-208.

78. **Fernandez, A. S., S. A. Hashsham, S. L. Dollhopf, L. Raskin, O. Glagoleva, F. B. Dazzo, R. F. Hickey, C. S. Criddle, and J. M. Tiedje.** 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microb **66:**4058-4067.

79. **Ferry, J. G., and K. A. Kastead.** 2007. Methanogenesis, p. 288-314. *In* R. Cavicchioli (ed.), Archaea: Molecular and cellular biology. ASM Press, Washington, D.C.

80. **Finke, N., T. M. Hoehler, and B. B. Jorgensen.** 2007. Hydrogen 'leakage' during methanogenesis from methanol and methylamine: Implications for anaerobic carbon degradation pathways in aquatic sediments. Environ Microbiol **9:**1060-1071.

81. **Florencio, L., J. A. Field, and G. Lettinga.** 1995. Substrate competition between methanogens and acetogens during the degradation of methanol in uasb reactors. Water Res **29:**915-922.

82. **Fonty, G., K. Joblin, M. Chavarot, R. Roux, G. Naylor, and F. Michallon.** 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. Appl Environ Microb **73:**6391-6403.

83. **Foresti, E., M. Zaiat, and M. Vallero.** 2006. Anaerobic processes as the core technology for sustainable domestic wastewater treatment: Consolidated applications,

new trends, perspectives, and challenges. Reviews in Environmental Science and Bio/Technology **5:**3-19.

84. **Forster-Carneiro, T., M. Perez, L. I. Romero, and D. Sales.** 2007. Dry-thermophilic anaerobic digestion of organic fraction of the municipal solid waste: Focusing on the inoculum sources. Bioresource Technol **98:**3195-3203.

85. **Galand, P. E., H. Fritze, R. Conrad, and K. Yrjala.** 2005. Pathways for methanogenesis and diversity of methanogenic archaea in three boreal peatland ecosystems. Appl Environ Microb **71:**2195-2198.

86. **Galand, P. E., H. Fritze, and K. Yrjala.** 2003. Microsite-dependent changes in methanogenic populations in a boreal oligotrophic fen. Environ Microbiol **5:**1133-1143.

87. **Galand, P. E., H. Juottonen, H. Fritze, and K. Yrjala.** 2005. Methanogen communities in a drained bog: Effect of ash fertilization. Microbial Ecol **49:**209-217.

88. **Galand, P. E., S. Saarnio, H. Fritze, and K. Yrjala.** 2002. Depth related diversity of methanogen archaea in finnish oligotrophic fen. Fems Microbiol Ecol **42:**441-449.

89. **Garcia, J. L., B. K. C. Patel, and B. Ollivier.** 2000. Taxonomic phylogenetic and ecological diversity of methanogenic archaea. Anaerobe **6:**205-226.

90. **Gattinger, A., M. G. Hoefle, M. Schloter, A. Embacher, F. Bohme, J. C. Munch, and M. Labrenz.** 2007. Traditional cattle manure application determines abundance, diversity and activity of methanogenic archaea in arable european soil. Environ Microbiol **9:**612-624.

91. **Girguis, P. R., V. J. Orphan, S. J. Hallam, and E. F. DeLong.** 2003. Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. Appl Environ Microb **69:**5472-5482.

92. **Glissmann, K., K. J. Chin, P. Casper, and R. Conrad.** 2004. Methanogenic pathway and archaeal community structure in the sediment of eutrophic lake dagow: Effect of temperature. Microbial Ecol **48:**389-399.

93. **Godon, J. J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta.** 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rdna sequence analysis. Appl Environ Microb **63:**2802-2813.

94. **Goodwin, S., and J. G. Zeikus.** 1987. Ecophysiological adaptations of anaerobic-bacteria to low ph - analysis of anaerobic-digestion in acidic bog sediments. Appl Environ Microb **53:**57-64.

95. **Gray, N. D., I. P. Miskin, O. Kornilova, T. P. Curtis, and I. M. Head.** 2002. Occurrence and activity of archaea in aerated activated sludge wastewater treatment plants. Environ Microbiol **4:**158-168.

96. **Griffin, M. E., K. D. McMahon, R. I. Mackie, and L. Raskin.** 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol Bioeng **57:**342-355.

97. **Grosskopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16s rrna gene sequence retrieval. Appl Environ Microb **64:**960-969.

98. **Grosskopf, R., S. Stubner, and W. Liesack.** 1998. Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl Environ Microb **64:**4983-4989.

99. **Groβkopf, R., S. Stubner, and W. Liesack.** 1998. Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl Environ Microb **64:**4983-4989.

100. **Guo, Y. Q., J. X. Liu, Y. Lu, W. Y. Zhu, S. E. Denman, and C. S. McSweeney.** 2008. Effect of tea saponin on methanogenesis, microbial community structure and expression of mcra gene, in cultures of rumen micro-organisms. Lett Appl Microbiol **47:**421-426.

101. **Haandel, A. v., M. Kato, P. Cavalcanti, and L. Florencio.** 2006. Anaerobic reactor design concepts for the treatment of domestic wastewater. Reviews in Environmental Science and Bio/Technology **5:**21-38.

102. **Habiba, L., B. Hassib, and H. Moktar.** 2009. Improvement of activated sludge stabilization and filterability during anaerobic digestion by fruit and vegetable waste addition. Bioresource Technol **100:**1555-1560.

103. **Hales, B. A., C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders.** 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by pcr amplification and sequence analysis. Appl Environ Microb **62:**668-675.

104. **Hallam, S. J., P. R. Girguis, C. M. Preston, P. M. Richardson, and E. F. DeLong.** 2003. Identification of methyl coenzyme m reductase a (mcra) genes associated with methane-oxidizing archaea. Appl Environ Microb **69:**5483-5491.

105. **Hamberger, A., M. A. Horn, M. G. Dumont, J. C. Murrell, and H. L. Drake.** 2008. Anaerobic consumers of monosaccharides in a moderately acidic fen. Appl Environ Microb **74:**3112-3120.

106. **Hashsham, S. A., A. S. Fernandez, S. L. Dollhopf, F. B. Dazzo, R. F. Hickey, J. M. Tiedje, and C. S. Criddle.** 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microb **66:**4050-4057.

107. **Hatamoto, M., H. Imachi, A. Ohashi, and H. Harada.** 2007. Identification and cultivation of anaerobic, syntrophic long-chain fatty acid-degrading microbes from mesophilic and thermophilic methanogenic sludges. Appl Environ Microb **73:**1332-1340.

108. **Hatamoto, M., H. Imachi, Y. Yashiro, A. Ohashi, and H. Harada.** 2007. Diversity of anaerobic microorganisms involved in long-chain fatty acid degradation in methanogenic sludges as revealed by rna-based stable isotope probing. Appl Environ Microb **73:**4119-4127.

109. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real time quantitative pcr. Genome Res **6:**986-994.

110. **Hines, M. E., K. N. Duddleston, and R. P. Kiene.** 2001. Carbon flow to acetate and c-1 compounds in northern wetlands. Geophysical Research Letters **28:**4251-4254.

111. **Hoffmann, R. A., M. L. Garcia, M. Veskivar, K. Karim, M. H. Al-Dahhan, and L. T. Angenent.** 2008. Effect of shear on performance and microbial ecology of continuously stirred anaerobic digesters treating animal manure. Biotechnol Bioeng **100:**38-48.

112. **Hoj, L., R. A. Olsen, and V. L. Torsvik.** 2005. Archaeal communities in high arctic wetlands at spitsbergen, norway (78 degrees n) as characterized by 16s rrna gene fingerprinting. Fems Microbiol Ecol **53:**89-101.

113. **Hori, T., S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi.** 2006. Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. Appl Environ Microb **72:**1623-1630.

114. **Horn, M. A., C. Matthies, K. Kusel, A. Schramm, and H. L. Drake.** 2003. Hydrogenotrophic methanogenesis by moderately acid-tolerant methanogens of a methane-emitting acidic peat. Appl Environ Microb **69:**74-83.

115. **Huang, L. N., Y. Q. Chen, H. Zhou, S. Luo, C. Y. Lan, and L. H. Qu.** 2003. Characterization of methanogenic archaea in the leachate of a closed municipal solid waste landfill. Fems Microbiol Ecol **46:**171-177.

116. **Huang, L. N., H. Zhou, Y. Q. Chen, S. Luo, C. Y. Lan, and L. H. Qu.** 2002. Diversity and structure of the archaeal community in the leachate of a full-scale recirculating landfill as examined by direct 16s rrna gene sequence retrieval. Fems Microbiol Lett **214:**235-240.

117. **Hughes, J. B., J. J. Hellmann, T. H. Ricketts, and B. J. M. Bohannan.** 2001. Counting the uncountable: Statistical approaches to estimating microbial diversity. Appl Environ Microb **67:**4399-4406.

118. **Imachi, H., S. Sakai, Y. Sekiguchi, S. Hanada, Y. Kamagata, A. Ohashi, and H. Harada.** 2008. Methanolinea tarda gen. Nov., sp nov., a methane-producing archaeon isolated from a methanogenic digester sludge. Int J Syst Evol Micr **58:**294-301.

119. **Inagaki, F., U. Tsunogai, M. Suzuki, A. Kosaka, H. Machiyama, K. Takai, T. Nunoura, K. H. Nealson, and K. Horikoshi.** 2004. Characterization of c-1-metabolizing prokaryotic communities in methane seep habitats at the kuroshima knoll, southern ryukyu arc, by analyzing pmoa, mmox, mxaf, mcra, and 16s rrna genes. Appl Environ Microb **70:**7445-7455.

120. **Inanc, B., S. Matsui, and S. Ide.** 1999. Propionic acid accumulation in anaerobic digestion of carbohydrates: An investigation on the role of hydrogen gas. Water Science and Technology **40:**93-100.

121. **Innis, M., D. Gelfand, and J. J. Sninsky.** 1999. Pcr applications: Protocols for functional genomics. Academic Press, San Diego.

122. **Ishii, S., T. Kosaka, K. Hori, Y. Hotta, and K. Watanabe.** 2005. Coaggregation facilitates interspecies hydrogen transfer between pelotomaculum thermopropionicum and methanothermobacter thermautotrophicus. Appl Environ Microb **71:**7838-7845.

123. **Jain, S. R., and B. Mattiasson.** 1998. Acclimatization of methanogenic consortia for low ph biomethanation process. Biotechnol Lett **20:**771-775.

124. **Janssen, P. H., and M. Kirs.** 2008. Structure of the archaeal community of the rumen. Appl Environ Microb **74:**3619-3625.

125. **Jansson, J. K., and T. Leser.** 1996. Quantitative pcr of environmental samples, p. 1-19, Molecular microbial ecology manual. Kluwer Academic Publishers.

126. **Juottonen, H., P. E. Galand, E. S. Tuittila, J. Laine, H. Fritze, and K. Yrjala.** 2005. Methanogen communities and bacteria along an ecohydrological gradient in a northern raised bog complex. Environ Microbiol **7:**1547-1557.

127. **Juottonen, H., P. E. Galand, and K. Yrjala.** 2006. Detection of methanogenic archaea in peat: Comparison of pcr primers targeting the mcra gene. Res Microbiol **157:**914-921.

128. **Kaparaju, P., I. Buendia, L. Ellegaard, and I. Angelidakia.** 2008. Effects of mixing on methane production during thermophilic anaerobic digestion of manure: Lab-scale and pilot-scale studies. Bioresource Technol **99:**4919-4928.

129. **Karakashev, D., D. J. Batstone, and I. Angelidaki.** 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl Environ Microb **71:**331-338.

130. **Karakashev, D., D. J. Batstone, E. Trably, and I. Angelidaki.** 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of methanosaetaceae. Appl Environ Microb **72:**5138-5141.

131. **Karr, E. A., J. M. Ng, S. M. Belchik, W. M. Sattley, M. T. Madigan, and L. A. Achenbach.** 2006. Biodiversity of methanogenic and other archaea in the permanently frozen lake fryxell, antarctica. Appl Environ Microb **72:**1663-1666.

132. **Kashyap, D. R., K. S. Dadhich, and S. K. Sharma.** 2003. Biomethanation under psychrophilic conditions: A review. Bioresource Technol **87:**147-153.

133. **Kendall, M. M., G. D. Wardlaw, C. F. Tang, A. S. Bonin, Y. T. Liu, and D. L. Valentine.** 2007. Diversity of archaea in marine sediments from skan bay, alaska, including cultivated methanogens, and description of methanogenium boonei sp nov. Appl Environ Microb **73:**407-414.

134. **Kettunen, R. H., and J. A. Rintala.** 1997. The effect of low temperature (5-29 degrees c) and adaptation on the methanogenic activity of biomass. Appl Microbiol Biot **48:**570-576.

135. **Khanal, S. K., and J. C. Huang.** 2005. Effect of high influent sulfate on anaerobic wastewater treatment. Water Environ Res **77:**3037-3046.

136. **Kim, I. S., M. H. Hwang, N. J. Jang, S. H. Hyun, and S. T. Lee.** 2004. Effect of low ph on the activity of hydrogen utilizing methanogen in bio-hydrogen process. Int J Hydrogen Energ **29:**1133-1140.

137. **Kim, M., Y. H. Ahn, and R. E. Speece.** 2002. Comparative process stability and efficiency of anaerobic digestion; mesophilic vs. Thermophilic. Water Res **36:**4369-4385.

138. **Kleikemper, J., S. A. Pombo, M. H. Schroth, W. V. Sigler, M. Pesaro, and J. Zeyer.** 2005. Activity and diversity of methanogens in a petroleum hydrocarbon-contaminated aquifer. Appl Environ Microb **71:**149-158.

139. **Knittel, K., T. Losekann, A. Boetius, R. Kort, and R. Amann.** 2005. Diversity and distribution of methanotrophic archaea at cold seeps. Appl Environ Microb **71:**467-479.

140. **Kobabe, S., D. Wagner, and E. M. Pfeiffer.** 2004. Characterisation of microbial community composition of a siberian tundra soil by fluorescence in situ hybridisation. Fems Microbiol Ecol **50:**13-23.

141. **Kotsyurbenko, O. R., K. J. Chin, M. V. Glagolev, S. Stubner, M. V. Simankova, A. N. Nozhevnikova, and R. Conrad.** 2004. Acetoclastic and hydrogenotrophic methane production and methanogenic populations in an acidic west-siberian peat bog. Environ Microbiol **6:**1159-1173.

142. **Kotsyurbenko, O. R., M. W. Friedrich, M. V. Simankova, A. N. Nozhevnikova, P. N. Golyshin, K. N. Timmis, and R. Conrad.** 2007. Shift from acetoclastic to h-2-dependent methanogenes is in a west siberian peat bog at low ph values and isolation of an acidophilic methanobactetium strain. Appl Environ Microb **73:**2344-2348.

143. **Kovacik, W. P., J. C. M. Scholten, W. Zhang, D. Culley, R. Hickey, and F. J. Brockman.** 2005. Changes in 16s rrna, mcra, and dsrab gene diversity in uasb bioreactor granules in response to short-term changes in substrate feed. GenBank, direct submission.

144. **Kraemer, J. T., and D. M. Bagley.** 2005. Continuous fermentative hydrogen production using a two-phase reactor system with recycle. Environ Sci Technol **39:**3819-3825.

145. **Kruger, M., G. Eller, R. Conrad, and P. Frenzel.** 2002. Seasonal variation in pathways of ch4 production and in ch4 oxidation in rice fields determined by stable carbon isotopes and specific inhibitors. Global Change Biol **8:**265-280.

146. **Krumholz, L. R., J. L. Hollenback, S. J. Roskes, and D. B. Ringelberg.** 1995. Methanogenesis and methanotrophy within a sphagnum peatland. Fems Microbiol Ecol **18:**215-224.

147. **Kumar, S., K. Tamura, and M. Nei.** 2004. Mega3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform **5:**150-163.

148. **Ladapo, J. A., and M. A. Barlaz.** 1997. Isolation and characterization of refuse methanogens. J Appl Microbiol **82:**751-758.

149. **Le Mer, J., and P. Roger.** 2001. Production, oxidation, emission and consumption of methane by soils: A review. Eur J Soil Biol **37:**25-50.

150. **Leclerc, M., C. Delbes, R. Moletta, and J. J. Godon.** 2001. Single strand conformation polymorphism monitoring of 16s rdna archaea during start-up of an anaerobic digester. Fems Microbiol Ecol **34:**213-220.

151. **Leclerc, M., J. P. Delgenes, and J. J. Godon.** 2004. Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16s rdna sequencing. Environ Microbiol **6:**809-819.

152. **Lee, D. H., Y. G. Zo, and S. J. Kim.** 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by pcr-single-strand-conformation polymorphism. Appl Environ Microb **62:**3112-3120.

153. **Lee, M. J., and S. H. Zinder.** 1988. Hydrogen partial pressures in a thermophilic acetate-oxidizing methanogenic coculture. Appl Environ Microb **54:**1457-1461.

154. **Lehmann-Richter, S., R. Grosskopf, W. Liesack, P. Frenzel, and R. Conrad.** 1999. Methanogenic archaea and co2-dependent methanogenesis on washed rice roots. Environ Microbiol **1:**159-166.

155. **Leitao, R. C., A. C. van Haandel, G. Zeeman, and G. Lettinga.** 2006. The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review. Bioresource Technol **97:**1105-1118.

156. **Lettinga, G., S. Rebac, S. Parshina, A. Nozhevnikova, J. B. van Lier, and A. J. M. Stams.** 1999. High-rate anaerobic treatment of wastewater at low temperatures. Appl Environ Microb **65:**1696-1702.

157. **Lettinga, G., S. Rebac, and G. Zeeman.** 2001. Challenge of psychrophilic anaerobic wastewater treatment. Trends Biotechnol **19:**363-70.

158. **Liu, D. W., D. P. Liu, R. J. Zeng, and I. Angelidaki.** 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Res **40:**2230-2236.

159. **Liu, W. T., O. C. Chan, and H. H. P. Fang.** 2002. Characterization of microbial community in granular sludge treating brewery wastewater. Water Res **36:**1767-1775.

160. **Liu, W. T., O. C. Chan, and H. H. P. Fang.** 2002. Microbial community dynamics during start-up of acidogenic anaerobic reactors. Water Res **36:**3203-3210.

161. **Lopes, W. S., V. D. Leite, and S. Prasad.** 2004. Influence of inoculum on performance of anaerobic reactors for treating municipal solid waste. Bioresource Technol **94:**261-266.

162. **Losekann, T., K. Knittel, T. Nadalig, B. Fuchs, H. Niemann, A. Boetius, and R. Amann.** 2007. Diversity and abundance of aerobic and anaerobic methane oxidizers at the haakon mosby mud volcano, barents sea. Appl Environ Microb **73:**3348-3362.

163. **Lu, Y. H., T. Lueders, M. W. Friedrich, and R. Conrad.** 2005. Detecting active methanogenic populations on rice roots using stable isotope probing. Environ Microbiol **7:**326-336.

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

165. **Lueders, T., and M. Friedrich.** 2000. Archaeal population dynamics during sequential reduction processes in rice field soil. Appl Environ Microb **66:**2732-2742.

166. **Lueders, T., and M. W. Friedrich.** 2003. Evaluation of pcr amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rrna and mcra genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl Environ Microb **69:**320-326.

167. **Luostarinen, S. A., and J. A. Rintala.** 2005. Anaerobic on-site treatment of black water and dairy parlour wastewater in uasb-septic tanks at low temperatures. Water Res **39:**436-448.

168. **Luton, P. E., J. M. Wayne, R. J. Sharp, and P. W. Riley.** 2002. The mcra gene as an alternative to 16s rrna in the phylogenetic analysis of methanogen populations in landfill. Microbiol-Sgm **148:**3521-3530.

169. **Mahmoud, N.** 2008. High strength sewage treatment in a uasb reactor and an integrated uasb-digester system. Bioresource Technol **99:**7531-7538.

170. **Malinen, E., A. Kassinen, T. Rinttila, and A. Palva.** 2003. Comparison of real-time pcr with sybr green i or 5 '-nuclease assays and dot-blot hybridization with rdna-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiol-Sgm **149:**269-277.

171. **Marchesi, J. R., A. J. Weightman, B. A. Cragg, R. J. Parkes, and J. C. Fry.** 2001. Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the cascadia margin as revealed by 16s rrna molecular analysis. Fems Microbiol Ecol **34:**221-228.

172. **Marsh, T. L.** 1999. Terminal restriction fragment length polymorphism (t-rflp): An emerging method for characterizing diversity among homologous populations of amplification products. Current Opinion in Microbiology **2:**323-327.

173. **Masse, D. I., L. Masse, and F. Croteau.** 2003. The effect of temperature fluctuations on psychrophilic anaerobic sequencing batch reactors treating swine manure. Bioresource Technol **89:**57-62.

174. **McDonald, I. R., M. Upton, G. Hall, R. W. Pickup, C. Edwards, J. R. Saunders, D. A. Ritchie, and J. C. Murrell.** 1999. Molecular ecological analysis of methanogens and methanotrophs in blanket bog peat. Microbial Ecol **38:**225-233.

175. **McHugh, S., M. Carton, G. Collins, and V. O'Flaherty.** 2004. Reactor performance and microbial community dynamics during anaerobic biological treatment of wastewaters at 16-37 degrees c. Fems Microbiol Ecol **48:**369-378.

176. **McHugh, S., M. Carton, T. Mahony, and V. O'Flaherty.** 2003. Methanogenic population structure in a variety of anaerobic bioreactors. Fems Microbiol Lett **219:**297-304.

177. **McHugh, S., G. Collins, and V. O'Flaherty.** 2006. Long-term, high-rate anaerobic biological treatment of whey wastewaters at psychrophilic temperatures. Bioresource Technol **97:**1669-1678.

178. **McMahon, K. D., P. G. Stroot, R. I. Mackie, and L. Raskin.** 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions - ii: Microbial population dynamics. Water Res **35:**1817-1827.

179. **McMahon, K. D., D. D. Zheng, A. J. M. Stams, R. I. Mackie, and L. Raskin.** 2004. Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. Biotechnol Bioeng **87:**823-834.

180. **Methods, S.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.

181. **Metje, M., and P. Frenzel.** 2005. Effect of temperature on anaerobic ethanol oxidation and methanogenesis in acidic peat from a northern wetland. Appl Environ Microb **71:**8191-8200.

182. **Metje, M., and P. Frenzel.** 2007. Methanogenesis and methanogenic pathways in a peat from subarctic permafrost. Environ Microbiol **9:**954-964.

183. **Meyerdierks, A., M. Kube, T. Lombardot, K. Knittel, M. Bauer, F. O. Glockner, R. Reinhardt, and R. Amann.** 2005. Insights into the genomes of archaea mediating the anaerobic oxidation of methane. Environ Microbiol **7:**1937-1951.

184. **Moran, J. J., E. J. Beal, J. M. Vrentas, V. J. Orphan, K. H. Freeman, and C. H. House.** 2008. Methyl sulfides as intermediates in the anaerobic oxidation of methane. Environ Microbiol **10:**162-173.

185. **Moran, J. J., C. H. House, K. H. Freeman, and J. G. Ferry.** 2005. Trace methane oxidation studied in several euryarchaeota under diverse conditions. Archaea **1:**303-309.

186. **Narihiro, T., Y. Sekiguchi, T. Terada, K. Kikuchi, M. Ikeda, T. Yamauchi, K. Shiraishi, Y. Kamagata, and K. Nakamura.** 2006. Comparative analysis of microbial communities in various upflow anaerobic sludge blanket granular sludges. GenBank, direct submission.

187. **Nercessian, O., N. Bienvenu, D. Moreira, D. Prieur, and C. Jeanthon.** 2005. Diversity of functional genes of methanogens, methanotrophs and sulfate reducers in deep-sea hydrothermal environments. Environ Microbiol **7:**118-132.

188. **Neves, L., R. Oliveira, and M. M. Alves.** 2009. Codigestion of cow manure, food waste and intermittent input of fat. Bioresource Technol **100:**1957-1962.

189. **Newberry, C. J., G. Webster, B. A. Cragg, R. J. Parkes, A. J. Weightman, and J. C. Fry.** 2004. Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the nankai trough, ocean drilling program leg 190. Environ Microbiol **6:**274-287.

190. **Noyola, A., J. Morgan-Sagastume, and J. Lopez-Hernandez.** 2006. Treatment of biogas produced in anaerobic reactors for domestic wastewater: Odor control and energy/resource recovery. Reviews in Environmental Science and Bio/Technology **5:**93-114.

191. **Nozhevnikova, A. N., C. Holliger, A. Ammann, and A. J. B. Zehnder.** 1997. Methanogenesis in sediments from deep lakes at different temperatures (12-70 degrees c). Water Science and Technology **36:**57-64.

192. **Nozhevnikova, A. N., O. R. Kotsyurbenko, and S. N. Parshina.** 1999. Anaerobic manure treatment under extreme temperature conditions. Water Science and Technology **40:**215-221.

193. **Nozhevnikova, A. N., M. V. Simankova, S. N. Parshina, and O. R. Kotsyurbenko.** 2001. Temperature characteristics of methanogenic archaea and acetogenic bacteria isolated from cold environments. Water Science and Technology **44:**41-48.

194. **Nozhevnikova, A. N., K. Zepp, F. Vazquez, A. J. B. Zehnder, and C. Holliger.** 2003. Evidence for the existence of psychrophilic methanogenic communities in anoxic sediments of deep lakes. Appl Environ Microb **69:**1832-1835.

195. **Nunoura, T., H. Oida, T. Toki, J. Ashi, K. Takai, and K. Horikoshi.** 2006. Quantification of *mcra* by quantitative fluorescent pcr in sediments from methane seep of the nankai trough. Fems Microbiol Ecol.

196. **Nusslein, B., K. J. Chin, W. Eckert, and R. Conrad.** 2001. Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical lake kinneret (israel). Environ Microbiol **3:**460-470.

197. **Ohkuma, M., S. Noda, K. Horikoshi, and T. Kudo.** 1995. Phylogeny of symbiotic methanogens in the gut of the termite reticulitermes speratus. Fems Microbiol Lett **134:**45-50.

198. **Olson, K. D., C. W. Mcmahon, and R. S. Wolfe.** 1991. Light sensitivity of methanogenic archaebacteria. Appl Environ Microb **57:**2683-2686.

199. **Orphan, V. J., C. H. House, K. U. Hinrichs, K. D. McKeegan, and E. F. DeLong.** 2002. Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. P Natl Acad Sci USA **99:**7663-7668.

200. **Orphan, V. J., L. T. Taylor, D. Hafenbradl, and E. F. Delong.** 2000. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. Appl Environ Microb **66:**700-711.

201. **Padmasiri, S. I., J. Z. Zhang, M. Fitch, B. Norddahl, E. Morgenroth, and L. Raskin.** 2007. Methanogenic population dynamics and performance of an anaerobic membrane bioreactor (anmbr) treating swine manure under high shear conditions. Water Res **41:**134-144.

202. **Pahl, O., A. Firth, L. MacLeod, and J. Baird.** 2008. Anaerobic co-digestion of mechanically biologically treated municipal waste with primary sewage sludge - a feasibility study. Bioresource Technol **99:**3354-3364.

203. **Panikov, N. S., and S. N. Dedysh.** 2000. Cold season ch4 and co2 emission from boreal peat bogs (west siberia): Winter fluxes and thaw activation dynamics. Global Biogeochem Cy **14:**1071-1080.

204. **Patel, H., and D. Madamwar.** 2000. Biomethanation of low ph petrochemical wastewater using up-flow fixed-film anaerobic bioreactors. World J Microb Biot **16:**69-75.

205. **Paulo, P. L., J. B. van Lier, and G. Lettinga.** 2003. Bicarbonate dosing: A tool to performance recovery of a thermophilic methanol-fed uasb reactor. Water Science and Technology **48:**95-101.

206. **Pender, S., M. Toomey, M. Carton, D. Eardly, J. W. Patching, E. Colleran, and V. O'Flaherty.** 2004. Long-term effects of operating temperature and sulphate addition on the methanogenic community structure of anaerobic hybrid reactors. Water Res **38:**619-630.

207. **Peng, J. J., Z. Lu, J. Rui, and Y. H. Lu.** 2008. Dynamics of the methanogenic archaeal community during plant residue decomposition in an anoxic rice field soil. Appl Environ Microb **74:**2894-2901.

208. **Penning, H., and R. Conrad.** 2006. Effect of inhibition of acetoclastic methanogenesis on growth of archaeal populations in an anoxic model environment. Appl Environ Microb **72:**178-184.

209. **Peters, V., and R. Conrad.** 1995. Methanogenic and other strictly anaerobic-bacteria in desert soil and other oxic soils. Appl Environ Microb **61:**1673-1676.

210. **Peu, P., H. Brugere, A. M. Pourcher, M. Kerouredan, J. J. Godon, J. P. Delgenes, and P. Dabert.** 2006. Dynamics of a pig slurry microbial community during anaerobic storage and management. Appl Environ Microb **72:**3578-3585.

211. **Purdy, K. J., D. B. Nedwell, and T. M. Embley.** 2003. Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting antarctic sediments. Appl Environ Microb **69:**3181-3191.

212. **Raposo, F., R. Borja, E. Sanchez, M. A. Martin, and A. Martin.** 2004. Performance and kinetic evaluation of the anaerobic digestion of two-phase olive mill effluents in reactors with suspended and immobilized biomass. Water Res **38:**2017-2026.

213. **Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl.** 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Appl Environ Microb **60:**1241-1248.

214. **Raskin, L., B. E. Rittmann, and D. A. Stahl.** 1996. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. Appl Environ Microb **62:**3847-3857.

215. **Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl.** 1994. Group-specific 16s ribosomal-rna hybridization probes to describe natural communities of methanogens. Appl Environ Microb **60:**1232-1240.

216. **Raskin, L., D. D. Zheng, M. E. Griffin, P. G. Stroot, and P. Misra.** 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes. Anton Leeuw Int J G **68:**297-308.

217. **Rastogi, G., D. R. Ranade, T. Y. Yeole, M. S. Patole, and Y. S. Shouche.** 2008. Investigation of methanogen population structure in biogas reactor by molecular characterization of methyl-coenzyme m reductase a (mcra) genes. Bioresource Technol **99:**5317-5326.

218. **Rastogi, G., and Y. S. Shouche.** 2005. Molcular phylogenetic analysis of methanogens from biogas plants. GenBank, direct submission.

219. **Reusser, S., and G. Zelinka.** 2004. Laboratory-scale comparison of anaerobic-digestion alternatives. Water Environ Res **76:**360-379.

220. **Rittmann, B. E., and P. L. McCarty.** 2001. Environmental biotechnology: Principles and applications. McGraw Hill, New York.

221. **Rocheleau, S., C. W. Greer, J. R. Lawrence, C. Cantin, L. Laramee, and S. R. Guiot.** 1999. Differentiation of methanosaeta concilii and methanosarcina barkeri in anaerobic mesophilic granular sludge by fluorescent in situ hybridization and confocal scanning laser microscopy. Appl Environ Microb **65:**2222-2229.

222. **Roest, K., H. G. H. J. Heilig, H. Smidt, W. M. de Vos, A. J. M. Stams, and A. D. L. Akkermans.** 2005. Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. Syst Appl Microbiol **28:**175-185.

223. **Saengkerdsub, S., R. C. Anderson, H. H. Wilkinson, W. K. Kim, D. J. Nisbet, and S. C. Ricke.** 2007. Identification and quantification of methanogenic archaea in adult chicken ceca. Appl Environ Microb **73:**353-356.

224. **Saengkerdsub, S., P. Herrera, L. Woodward, C. Anderson, D. J. Nisbet, and S. C. Ricke.** 2007. Detection of methane and quantification of methanogenic archaea in faeces from young broiler chickens using real-time pcr. Lett Appl Microbiol **45:**629-634.

225. **Sakai, S., H. Imachi, Y. Kamagata, A. Ohashi, and H. Harada.** 2005. Archaeal diversity in anaerobic environments. GenBank, direct submission **AB236107**.

226. **Sakai, S., H. Imachi, Y. Sekiguchi, A. Ohashi, H. Harada, and Y. Kamagata.** 2007. Isolation of key methanogens for global methane emission from rice paddy fields: A novel isolate affiliated with the clone cluster rice cluster i. Appl Environ Microb **73:**4326-4331.

227. **Sanchez, E., R. Borja, L. Travieso, A. Martin, and M. F. Colmenarejo.** 2005. Effect of organic loading rate on the stability, operational parameters and performance of a secondary upflow anaerobic sludge bed reactor treating piggery waste. Bioresource Technol **96:**335-344.

228. **Santegoeds, C. M., L. R. Damgaard, C. Hesselink, J. Zopfi, P. Lens, G. Muyzer, and D. De Beer.** 1999. Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. Appl Environ Microb **65:**4618-4629.

229. **Savant, D. V., and D. R. Ranade.** 2004. Application of methanobrevibacter acididurans in anaerobic digestion. Water Science and Technology **50:**109-114.

230. **Savant, D. V., Y. S. Shouche, S. Prakash, and D. R. Ranade.** 2002. Methanobrevibacter acididurans sp nov., a novel methanogen from a sour anaerobic digester. Int J Syst Evol Micr **52:**1081-1087.

231. **Sawayama, S., K. Tsukahara, and T. Yagishita.** 2006. Phylogenetic description of immobilized methanogenic community using real-time pcr in a fixed-bed anaerobic digester. Bioresource Technol **97:**69-76.

232. **Schink, B.** 2002. Anaerobic digestion: Concepts, limits and perspectives. Water Science and Technology **45:**1-8.

233. **Schink, B.** 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol R **61:**262-&.

234. **Schloss, P. D., and J. Handelsman.** 2005. Introducing dotur, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microb **71:**1501-1506.

235. **Schnürer, A., and A. Nordberg.** 2007. Presented at the 11th IWA World Congress on Anaerobic Digestion, Brisbane, Australia, 23rd-27th September.

236. **Schulz, S., and R. Conrad.** 1996. Influence of temperature on pathways to methane production in the permanently cold profundal sediment of lake constance. Fems Microbiol Ecol **20:**1-14.

237. **Schulz, S., H. Matsuyama, and R. Conrad.** 1997. Temperature dependence of methane production from different precursors in a profundal sediment (lake constance). Fems Microbiol Ecol **22:**207-213.

238. **Scruton, D., R. Achilles, and S. Weeks.** 2004. Presented at the American Society of Agricultural and Biological Engineers Annual International Meeting, Ottowa, Ontario, Canada, Aug 1-4.

239. **Scully, C., G. Collins, and V. O'Flaherty.** 2005. Assessment of anaerobic wastewater treatment failure using terminal restriction fragment length polymorphism analysis. J Appl Microbiol **99:**1463-1471.

240. **Sekiguchi, Y.** 2006. Yet-to-be cultured microorganisms relevant to methane fermentation processes. Microbes and Environments **21:**1-15.

241. **Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada.** 1999. Fluorescence in situ hybridization using 16s rrna-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl Environ Microb **65:**1280-1288.

242. **Sekiguchi, Y., Y. Kamagata, A. Ohashi, and H. Harada.** 2002. Molecular and conventional analyses of microbial diversity in mesophilic and thermophilic upflow anaerobic sludge blanket granular sludges. Water Science and Technology **45:**19-25.

243. **Sekiguchi, Y., Y. Kamagata, K. Syutsubo, A. Ohashi, H. Harada, and K. Nakamura.** 1998. Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16s rrna gene analysis. Microbiol-Sgm **144:**2655-2665.

244. **Sheppard, S. K., A. J. McCarthy, J. P. Loughnane, N. D. Gray, I. M. Head, and D. Lloyd.** 2005. The impact of sludge amendment on methanogen community structure in an upland soil. Appl Soil Ecol **28:**147-162.

245. **Shigematsu, T., Y. Q. Tang, T. Kobayashi, H. Kawaguchi, S. Morimura, and K. Kida.** 2004. Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. Appl Environ Microb **70:**4048-4052.

246. **Shima, S., E. Warkentin, R. K. Thauer, and U. Ermler.** 2002. Structure and function of enzymes involved in the methanogenic pathway utilizing carbon dioxide and molecular hydrogen. J Biosci Bioeng **93:**519-530.

247. **Shinzato, N., T. Matsumoto, I. Yamaoka, T. Oshima, and A. Yamagishi.** 1999. Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite reticulitermes speratus analyzed by pcr and in situ hybridization. Appl Environ Microb **65:**837-840.

248. **Simankova, M. V., O. R. Kotsyurbenko, T. Lueders, A. N. Nozhevnikova, B. Wagner, R. Conrad, and M. W. Friedrich.** 2003. Isolation and characterization of new strains of methanogens from cold terrestrial habitats. Syst Appl Microbiol **26:**312-318.

249. **Singh, L., M. S. Maurya, K. V. Ramana, and S. I. Alam.** 1995. Production of biogas from night soil at psychrophilic temperature. Bioresource Technol **53:**147-149.

250. **Siriwongrungson, V., R. J. Zeny, and I. Angelidaki.** 2007. Homoacetogenesis as the alternative pathway for h-2 sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis. Water Res **41:**4204-4210.

251. **Sizova, M. V., N. S. Panikov, T. P. Tourova, and P. W. Flanagan.** 2003. Isolation and characterization of oligotrophic acido-tolerant methanogenic consortia from a sphagnum peat bog. Fems Microbiol Ecol **45:**301-315.

252. **Smith, C. J., D. B. Nedwell, L. F. Dong, and A. M. Osborn.** 2006. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. Environ Microbiol **8:**804-815.

253. **Smith, J. M., H. Castro, and A. Ogram.** 2007. Structure and function of methanogens along a short-term restoration chronosequence in the florida everglades. Appl Environ Microb **73:**4135-4141.

254. **Smith, J. M., S. J. Green, C. A. Kelley, L. Prufert-Bebout, and B. M. Bebout.** 2008. Shifts in methanogen community structure and function associated with long-term manipulation of sulfate and salinity in a hypersaline microbial mat. Environ Microbiol **10:**386-394.

255. **Smith, K. S., and C. Ingram-Smith.** 2007. Methanosaeta, the forgotten methanogen? Trends Microbiol **15:**150-155.

256. **Sorensen, A. H., V. L. Torsvik, T. Torsvik, L. K. Poulsen, and B. K. Ahring.** 1997. Whole-cell hybridization of methanosarcina cells with two new oligonucleotide probes. Appl Environ Microb **63:**3043-3050.

257. **Springer, E., M. S. Sachs, C. R. Woese, and D. R. Boone.** 1995. Partial gene-sequences for the a-subunit of methyl-coenzyme-m reductase (mcri) as a phylogenetic tool for the family methanosarcinaceae. Int J Syst Bacteriol **45:**554-559.

258. **Stackebrandt, E., and B. M. Goebel.** 1994. A place for DNA-DNA reassociation and 16s ribosomal-rna sequence-analysis in the present species definition in bacteriology. Int J Syst Bacteriol **44:**846-849.

259. **Steinberg, L. M., and J. M. Regan.** 2009. An *mcra*-targeted real-time quantitative pcr method to examine methanogen communities. Applied and Environmental Microbiology, submitted.

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

261. **Stewart, J. A., V. S. Chadwick, and A. Murray.** 2006. Carriage, quantification, and predominance of methanogens and sulfate-reducing bacteria in faecal samples. Lett Appl Microbiol **43:**58-63.

262. **Strapoc, D., F. W. Picardal, C. Turich, I. Schaperdoth, J. L. Macalady, J. S. Lipp, Y. S. Lin, T. F. Ertefai, F. Schubotz, K. U. Hinrichs, M. Mastalerz, and A. Schimmelmann.** 2008. Methane-producing microbial community in a coal bed of the illinois basin. Appl Environ Microb **74:**2424-2432.

263. **Stroot, P. G., K. D. McMahon, R. I. Mackie, and L. Raskin.** 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions - i. Digester performance. Water Res **35:**1804-1816.

264. **Struchtemeyer, C. G., M. S. Elshahed, K. E. Duncan, and M. J. McInerney.** 2005. Evidence for aceticlastic methanogenesis in the presence of sulfate in a gas condensate-contaminated aquifer. Appl Environ Microb **71:**5348-5353.

265. **Taconi, K. A., M. E. Zappi, W. T. French, and L. R. Brown.** 2007. Feasibility of methanogenic digestion applied to a low ph acetic acid solution. Bioresource Technol **98:**1579-1585.

266. **Taconi, K. A., M. E. Zappi, W. T. French, and L. R. Brown.** 2008. Methanogenesis under acidic ph conditions in a semi-continuous reactor system. Bioresource Technol **99:**8075-8081.

267. **Talbot, G., E. Topp, M. F. Palin, and D. I. Masse.** 2008. Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. Water Res **42:**513-537.

268. **Tang, Y. Q., T. Shigematsu, Ikbal, S. Morimura, and K. Kida.** 2004. The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester. Water Res **38:**2537-2550.

269. **Tatara, M., T. Makiuchi, Y. Ueno, M. Goto, and K. Sode.** 2008. Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. Bioresource Technol **99:**4786-4795.

270. **Tezel, U., J. A. Pierson, and S. G. Paulostathis.** 2006. Fate and effect of quaternary ammonium compounds on a mixed methanogenic culture. Water Res **40:**3660-3668.

271. **Thauer, R.** 1997. Biodiversity and unity in biochemistry. Anton Leeuw Int J G **71:**21-32.

272. **Thauer, R. K., A. K. Kaster, H. Seedorf, W. Buckel, and R. Hedderich.** 2008. Methanogenic archaea: Ecologically relevant differences in energy conservation. Nat Rev Microbiol **6:**579-591.

273. **Ufnar, J. A., D. F. Ufnar, S. Y. Wang, and R. D. Ellender.** 2007. Development of a swine-specific fecal pollution marker based on host differences in methanogen mcra genes. Appl Environ Microb **73:**5209-5217.

274. **Ufnar, J. A., S. Y. Wang, D. F. Ufnar, and R. D. Ellender.** 2007. Methanobrevibacter ruminantium as an indicator of domesticated-ruminant fecal pollution in surface waters. Appl Environ Microb **73:**7118-7121.

275. **Uz, I., M. E. Rasche, T. Townsend, A. V. Ogram, and A. S. Lindner.** 2003. Characterization of methanogenic and methanotrophic assemblages in landfill samples. P Roy Soc Lond B Bio **270:**S202-S205.

276. **Valentine, D. L., and W. S. Reeburgh.** 2000. New perspectives on anaerobic methane oxidation. Environ Microbiol **2:**477-484.

277. **Van herle, J., Y. Membrez, and O. Bucheli.** 2004. Biogas as a fuel source for sofc co-generators. J Power Sources **127:**300-312.

278. **van Lier, J. B., A. Tilche, B. K. Ahring, H. Macarie, R. Moletta, M. Dohanyos, L. W. H. Pol, P. Lens, and W. Verstraete.** 2001. New perspectives in anaerobic digestion. Water Science and Technology **43:**1-18.

279. **Vartak, D. R., C. R. Engler, M. J. McFarland, and S. C. Ricke.** 1997. Attached-film media performance in psychrophilic anaerobic treatment of dairy cattle wastewater. Bioresource Technol **62:**79-84.

280. **Vetriani, C., H. W. Jannasch, B. J. MacGregor, D. A. Stahl, and A. L. Reysenbach.** 1999. Population structure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. Appl Environ Microb **65:**4375-4384.

281. **Voolapalli, R. K., and D. C. Stuckey.** 1998. Stability enhancement of anaerobic digestion through membrane gas extraction under organic shock loads. J Chem Technol Biot **73:**153-161.

282. **Wagner, D., S. Kobabe, E. M. Pfeiffer, and H. W. Hubberten.** 2003. Microbial controls on methane fluxes from a polygonal tundra of the lena delta, siberia. Permafrost Periglac **14:**173-185.

283. **Wagner, D., A. Lipski, A. Embacher, and A. Gattinger.** 2005. Methane fluxes in permafrost habitats of the lena delta: Effects of microbial community structure and organic matter quality. Environ Microbiol **7:**1582-1592.

284. **Wang, H., A. Lehtomaki, K. Tolvanen, J. Puhakka, and J. A. Rintala.** 2009. Impact of crop species on bacterial community structure during anaerobic co-digestion of crops and cow manure. Bioresource Technol **100:**2311-2315.

285. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 2005. The methanogenic bacteria, The prokaryotes, vol. 3.2. Springer-Verlag, New York, NY.

286. **Williams, R. T., and R. L. Crawford.** 1984. Methane production in minnesota peatlands. Appl Environ Microb **47:**1266-1271.

287. **Wright, A. D. G., C. H. Auckland, and D. H. Lynn.** 2007. Molecular diversity of methanogens in feedlot cattle from ontario and prince edward island, canada. Appl Environ Microb **73:**4206-4210.

288. **Xing, J., C. Criddle, and R. Hickey.** 1997. Effects of a long-term periodic substrate perturbation on an anaerobic community. Water Res **31:**2195-2204.

289. **Xing, J., C. Criddle, and R. Hickey.** 1997. Long-term adaptive shifts in anaerobic community structure in response to a sustained cyclic substrate perturbation. Microbial Ecol **33:**50-58.

290. **Xing, W., J. E. Zuo, J. Li, and Y. Sun.** 2007. Presented at the 11th IWA World Congress on Anaerobic Digestion, Brisbane, Australia, 23rd-27th September.

291. **Yu, Y., J. Kim, and S. Hwang.** 2006. Use of real-time pcr for group-specific quantification of aceticlastic methanogens in anaerobic processes: Population dynamics and community structures. Biotechnol Bioeng **93:**424-433.

292. **Yu, Y., C. Lee, and S. Hwang.** 2005. Analysis of community structures in anaerobic processes using a quantitative real-time pcr method. Water Science and Technology **52:**85-91.

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

294. **Zahller, J. D., R. H. Bucher, J. F. Ferguson, and H. D. Stensel.** 2007. Performance and stability of two-stage anaerobic digestion. Water Environ Res **79:**488-497.

295. **Zaiat, M., J. A. D. Rodrigues, S. M. Ratusznei, E. F. M. de Camargo, and W. Borzani.** 2001. Anaerobic sequencing batch reactors for wastewater treatment: A developing technology. Appl Microbiol Biot **55:**29-35.

296. **Zhang, G. S., J. Q. Tian, N. Jiang, X. P. Guo, Y. F. Wang, and X. Z. Dong.** 2008. Methanogen community in zoige wetland of tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster zc-i. Environ Microbiol **10:**1850-1860.

297. **Zheng, D., and L. Raskin.** 2000. Quantification of methanosaeta species in anaerobic bioreactors using genus- and species-specific hybridization probes. Microbial Ecol **39:**246-262.

298. **Zhu, H., A. Stadnyk, M. Beland, and P. Seto.** 2008. Co-production of hydrogen and methane from potato waste using a two-stage anaerobic digestion process. Bioresource Technol **99:**5078-5084.

299. **Zinatizadeh, A. A. L., A. R. Mohamed, M. D. Mashitah, A. Z. Abdullah, and M. H. Isa.** 2007. Characteristics of granular sludge developed in an upflow anaerobic sludge fixed-film bioreactor treating palm oil mill effluent. Water Environ Res **79:**833-844.

300. **Zinder, S. H.** 1994. Physiological ecology of methanogens, p. 128-206. *In* J. G. Ferry (ed.), Methanogenesis - ecology, physiology, biochemistry and genetics. vol. 367. Chapman & Hall, New York, NY.

301. **Zumstein, E., R. Moletta, and J. J. Godon.** 2000. Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain

reaction (pcr) single-strand conformation polymorphism analysis. Environ Microbiol **2:**69-78.

APPENDIX A

Supplemental Data for Chapter 3

Table A1-1. Representative 16S rDNA sequences from bog and digester clone libraries used to construct phylogenetic trees.

Representative clones presented here were based on a sequence similarity of 93.5% to group like sequences. Clones designated as "bog" or "dig" are for the bog and digester libraries, respectively. Clone numbers in bold indicate sequences in this group were retrieved from both environments.

Representative Clone (GenBank accession #)	Total clones (% range of sequence similarity)	BLAST search results (% identity)	Isolation source for BLAST clone	Bog clones	Digester clones
bogD11_18 (DQ781025)	12 (97.1-100)	AY175382 uncultured clone LH-03 (98)	acidic peatland	12	0
bogc12_12 (DQ781024)	8 (97.1-100)	AY175381 uncultured clone LH-02 (97)	acidic peatland	8	0
bog2_E2_39 (DQ680371)	6 (94.1-100)	DQ301884 uncultured clone CBd-466G (96)	acidic peatland	6	0
bog3_27 (DQ680354)	4 (94.8-96.7)	AF481343 uncultured clone Fuku08 (98)	acidic bog lake	4	0
bog2_A4_36 (DQ680358)	3 (96-98.9)	AJ459899 uncultured clone AMC 1 M21 (98)	acidic peatland	3	0
bog3_12 (DQ680370)	3 (97.1-99.3)	DQ301895 uncultured clone CBs-b2C (98)	acidic peatland	3	0
bog2_E3_40 (DQ680385)	2 (96.4)	EU155985 uncultured clone MH1100_C3E (97)	minerotrophic fen	2	0
bog3_8 (DQ680376)	2 (99.6)	DQ301883 uncultured clone CBs-a2H (98)	acidic peatland	2	0
bog3_6 (DQ680366)	1	DQ301893 uncultured clone CBs-b3B (99)	acidic peatland	1	0
bogB10_6 (DQ680367)	1	DQ301888 uncultured clone CBs-c1F (95)	acidic peatland	1	0
bog2_F5_42 (DQ680377)	1	AY652478 uncultured clone 353/B-3 (94)	nutrient-impacted freshwater marsh	1	0
bog2_A1_35 (DQ680383)	1	DQ301914 uncultured clone CBd-305F (94)	acidic peatland	1	0
bogE5_24 (DQ680356)	1	EU155921 uncultured clone MHLsu47_12F (96)	minerotrophic fen	1	0
bogE7_26 (DQ680360)	1	AY456732 uncultured clone ARC-U3SP-1 (97)	Florida Everglades	1	0
bog3_31 (DQ680382)	1	EU155946 uncultured clone MH1492_B12H (99)	minerotrophic fen	1	0
dig3_34 (DQ680406)	19 (92.9-100)	DQ386720 uncultured clone Mix Arch 7 (97)	dairy and chemical digester anaerobic sludge	0	19
dig3_18 (DQ781052)	12 (99.3-100)	AB236107 uncultured clone LF-ProM-C (97)	lotus field soil	0	12
dig3_27 (DQ781054)	8 (97.8-100)	DQ399803 uncultured clone R5A4 (97)	solvent wastewater digester anaerobic sludge	0	8
digG5_8 (DQ781042)	6 (95.6-99.6)	DQ173836 uncultured clone F4PM47 (99)	nutrient-impacted Florida Everglades	0	6
dig3_28 (DQ781055)	3 (98.9-99.6)	AB266913 uncultured clone HsA10f1 (97)	UASB sludge granules	0	3
dig3_9 (DQ680394)	1	AJ556500 uncultured clone OuI-15 (94)	oil-polluted saline soil	0	1
digG4_7 (DQ680411)	1	DQ201632 uncultured clone F4AM40 (97)	nutrient-impacted Florida Everglades	0	1

Table A1-2. Representative *mcrA* sequences from bog and digester clone libraries used to construct phylogenetic trees.

Representative clones presented here were based on a sequence similarity of 79.0% to group like sequences. Clones designated as "bog" or "dig" are for the bog and digester libraries, respectively. Clone numbers in bold indicate sequences in this group were retrieved from both environments.

Representative Clone (GenBank accession #)	Total clones (range of sequence similarity)	BLAST search results (% identity)	Isolation source for BLAST clone	Bog clones (ME/ML/ mlas)	Digester clones (ME/ML/ mlas)
ML_bog11_4 (DQ680490)	66 (80.9-100.0)	AJ853822 uncultured clone Lak15.1-ML (96)	acidic boreal fen	**23/29/12**	**0/2/0**
ME_bog76_23 (DQ680445)	8 (85.7-100.0)	AF525522 uncultured clone novmcr55 (89)	hypereutrophic lake	7/0/1	0/0/0
mlas_bog39 (DQ680603)	7 (98.5-100.0)	AY260444 *Methanocorpusculum parvum* (99)	*Methanocorpusculum parvum*	0/0/7	0/0/0
mlas_bog45 (DQ680587)	6 (99.5-100.0)	AY289753 *Methanothermobacter thermophilus mrtA* (91)	*Methanothermobacter thermophilus*	0/0/6	0/0/0
ML_bog79_32 (DQ680472)	5 (86.3-100.0)	AB301313 uncultured clone R-FL-14 (87)	rice field soil	0/5/0	0/0/0
ME _bog78_25 (DQ680413)	5 (80.9-98.5)	AJ704553 uncultured clone Lak6-ML (93)	acidic boreal fen	4/1/0	0/0/0
mlas_bog34 (DQ680579)	4 (99.5-100.0)	AB353232 uncultured clone TDS-J-M-D05 (97)	thermophilic digested sludge	0/0/4	0/0/0
ML_bog54_22 (DQ680476)	2 (89.7)	AY460217 uncultured clone MCR-U3SU-27 (89)	Florida Everglades	1/1/0	0/0/0
ML_bog51_19 (DQ680469)	2 (100.0)	AJ853818 uncultured clone Lak2.1-ML (94)	acidic boreal fen	0/2/0	0/0/0
mlas_bog27 (DQ680600)	1	AJ704558 uncultured clone Lak13-ML (90)	acidic boreal fen	0/0/1	0/0/0
mlas_dig67 (DQ680655)	66 (80.9-100.0)	DQ260701 uncultured clone E4DECMC311 (96)	biogas plant	0/0/0	10/32/24
ML_dig30_25 (DQ680524)	21 (83.4-100.0)	DQ260681 uncultured clone H7DECMC183 (98)	biogas plant	0/0/0	1/15/5
mlas_dig61 (DQ680640)	20 (82.8-100.0)	AF536522 uncultured clone (93)	soil amended with sewage sludge	0/0/0	0/9/11
mlas_dig18_5 (DQ680618)	15 (88.6-100.0)	DQ994860 uncultured clone SI-8 (95)	pasture soil	**0/0/1**	**0/7/7**
ML_dig45_34 (DQ680510)	10 (80.3-100.0)	AY937277 uncultured clone GranMCR7M9 (99)	UASB treating brewery waste water	**0/0/1**	**0/5/4**
mlas_dig56_19 (DQ680629)	9 (91.9-100.0)	AY460219 uncultured clone MCR-U3SU-35 (73)	Florida Everglades	0/0/0	0/2/7
ME_dig45_2_29 (DQ680449)	7 (89.7-100.0)	AF313805 *Methanospirillum hungatei* strain JF-1 (90)	rice field soil	0/0/0	4/0/3
ME_dig80_2_15 (DQ680456)	6 (84.0-100.0)	AF414035 *Methanobrevibacter arboriphilus* (93)	*Methanobrevibacter arboriphilus*	0/0/0	3/3/0
mlas_dig67_22 (DQ680649)	2 (96.6)	AY937276 uncultured clone GranMCR7M6 (94)	UASB treating brewery waste water	0/0/0	0/0/2
ME_dig40_2_12 (DQ680453)	2 (100.0)	DQ260578 uncultured clone MARMC26 (95)	biogas plant	0/0/0	2/0/0
ML_dig75_54 (DQ680553)	2 (96.6)	EF379243 uncultured clone CLI23 (97)	cattle rumen	0/0/0	0/1/1
ME_dig42_2_20 (DQ680447)	1	AB301396 uncultured clone R-UP-55 (91)	rice field soil	0/0/0	1/0/0
mlas_dig43_14 (DQ680648)	1	DQ262263 uncultured clone MARMCR366 (94)	biogas plant	0/0/0	0/0/1
mlas_dig44 (DQ680632)	1	AY937285 uncultured clone GranMCR4M17 (96)	UASB treating brewery waste water	0/0/0	0/0/1

APPENDIX B

Supplemental data for Chapter 5

Start-up and performance of reactors

Gas production was inconsistent for all reactors during the first 230 days of operation (Fig. A2-1a). Gas production in the Digester-control reactor dropped during the first 190 days of operation. Gas production in the Bog-control reactor was not detected until 80 days of operation, and remained low. Gas production in the Hybrid-control reactor dropped from inoculation to 190 days of operation. After mixing was discontinued on day 117, gas production began to increase in the Bog-control reactor, but gas production in the Digester-control and Hybrid-control reactors continued to drop. On day 190, reactors were shifted from a 30 d to a 40 d retention time, after which gas production increased in all reactors. The original gas sampling bags leaked, and were replaced on day 235, after which gas production stabilized and was similar in all three control reactors (Fig. A2-1b). Biogas contained 60-65% methane, 30-35% carbon dioxide, and 2-5% nitrogen, and approximately 70-80 mL of methane was produced per day.

Measurements of reactor pH were begun on day 130 of operation (Fig. A2-2). The increase in retention time and the replacement of gas sampling bags increased pH values which eventually stabilized at day 240 and remained similar for all three control reactors for the remainder of operation. Beginning on day 145, TS and VS removal, COD removal, and bicarbonate alkalinity were assessed for effluent samples from all control reactors (Fig. A2-3). For the first 180 d of operation, solids and COD accumulated in the Bog-control reactor, but by day 245 all reactors showed similar total and volatile solids as well as COD removal rates. All control reactors had detectable levels of short-chain VFAs during the start-up period, primarily acetate, propionate, and butyrate (Fig. A2-4). The Bog-control and Hybrid-control reactors had high concentrations of acetate and propionate early in operation which likely caused the low pH of these reactors. Both the increase in retention time and replacement of the gas sampling bag reduced VFAs in all reactors which remained below 0.2 mM for the duration of operation.

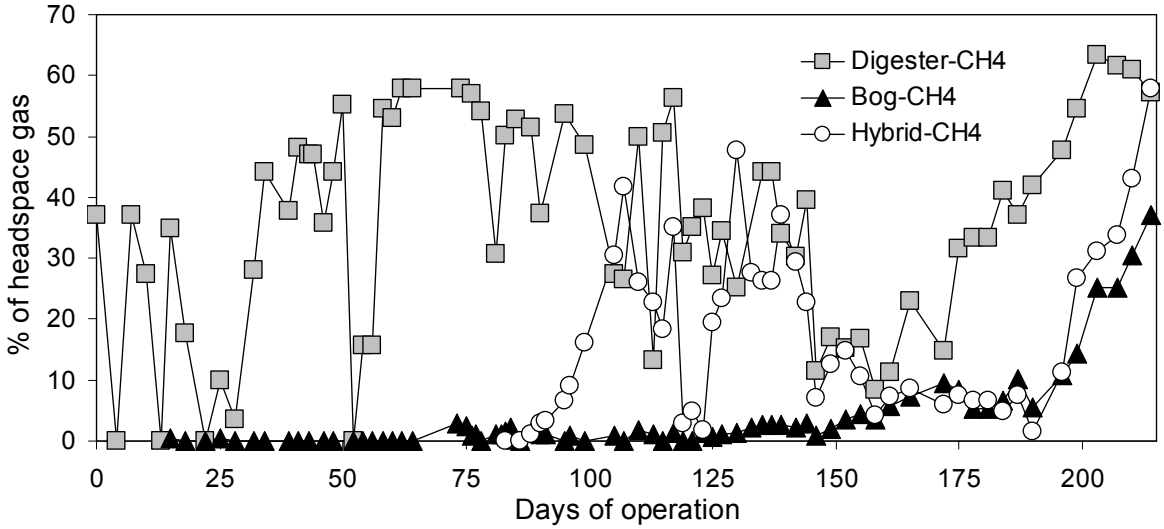
Initially, total *mcrA* copies were higher in the Digester-control reactor than in the Bog-control reactor (Fig. A2-5). The Hybrid-control reactor was inoculated on day 85 of operation of the Digester- and Bog-control reactors. The Digester-control reactor was initially dominated by MCR-7 with lesser numbers of MCR-2a and *Methanosaeta*, but became dominated by *Methanosarcina* by day 25. For the rest of operation this reactor was dominated by *Methanosarcina* with occasional detection of *Methanobacteriaceae*, *Methanosaeta*, and *Methanospirillum*. The Bog-control reactor was initially dominated by the Fen cluster, but by day 172 this reactor was also dominated by *Methanosarcina* with MCR-7, *Methanobacteriaceae*, and *Methanosaeta* also detected at some sampling points. The Hybrid-control reactor was always

dominated by *Methanosarcina*, and while early in operation *Methanobacteriaceae* was detected, by the end of operation only *Methanosarcina* was detected.

After the first OLR shock of 10 g glucose, VFAs mainly composed of acetate, propionate, and butyrate accumulated in all reactors (Fig. A2-6). The Digester-test reactor accumulated about 9 mM acetate, and very little propionate or butyrate, but no reduction in acetate was seen until this reactor was disposed of on day 210. In the Bog- and Hybrid-test reactors, mainly acetate and propionate accumulated, reaching maximum values around day 160 and then slowly decreasing. Although VFAs decreased in the Hybrid-test reactor, methane production was never detected again and this reactor was also disposed of on day 210.

A second set of test reactors for each inoculum was started on day 403. During the following OLR shocks test reactors 2 were supplemented with mineral nutrients, but test reactors 1 were not. The second set of OLR shocks was delivered to the test reactors on days 262 (1 g glucose), 266 (5 g glucose), and 287 (10 g glucose), and a third OLR shock was delivered on day 436 (10 g glucose). After each OLR shock, total and volatile solids removal was reduced in all reactors (Fig. A2-7), as well as bicarbonate alkalinity and COD removal (Fig. A2-8). During the second OLR shocks, only the 10 g glucose feeding resulted in accumulation of VFAs (Fig. A2-9). Although only test reactors 1 are shown for clarity, but both test reactors 1 and 2 showed similar results. After the 10 g glucose feeding on day 287, little VFas accumulated in the Digester-test reactors, but the Bog-test reactor accumulated acetate and propionate and the Hybrid-test reactor accumulated acetate and butyrate. Concomitant with VFA destruction in all test reactors, solids and COD removal efficiency as well as bicarbonate alkalinity rose in all three reactors (Fig. A2-7,8). After the third OLR shock, all reactors showed an accumulation of butyrate and acetate with little propionate (Fig. A2-9). VFAs were consumed within about 50 days in the Digester-test and Bog-test reactors as methane production increased, and COD removal and bicarbonate alkalinity increased (Fig. A2-8). Recovery was much slower in the Hybrid-test reactors and VFAs persisted until the end of operation. Test 2 reactors were dominated by *Methanosarcina* and *Methanobacteriaceae* with some MCR-7 also present in Bog- and Hybrid-test 2 reactors (Fig. A2-10).

a)



b)

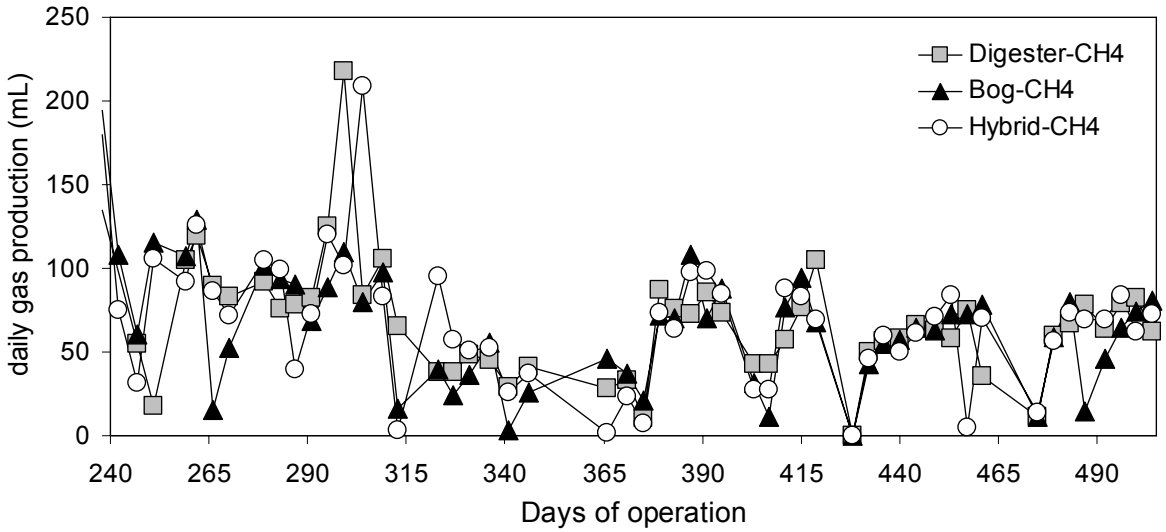


Figure A2-1. Methane production in control reactors during a) the first 215 days of operation, and b) the remainder of operation.

"Digester", "Bog", and "Hybrid" refer to the Digester-, Bog-, and Hybrid-control reactors. On day 117, new reactors were seeded from these three reactors for use in the organic-loading rate shock experiments.

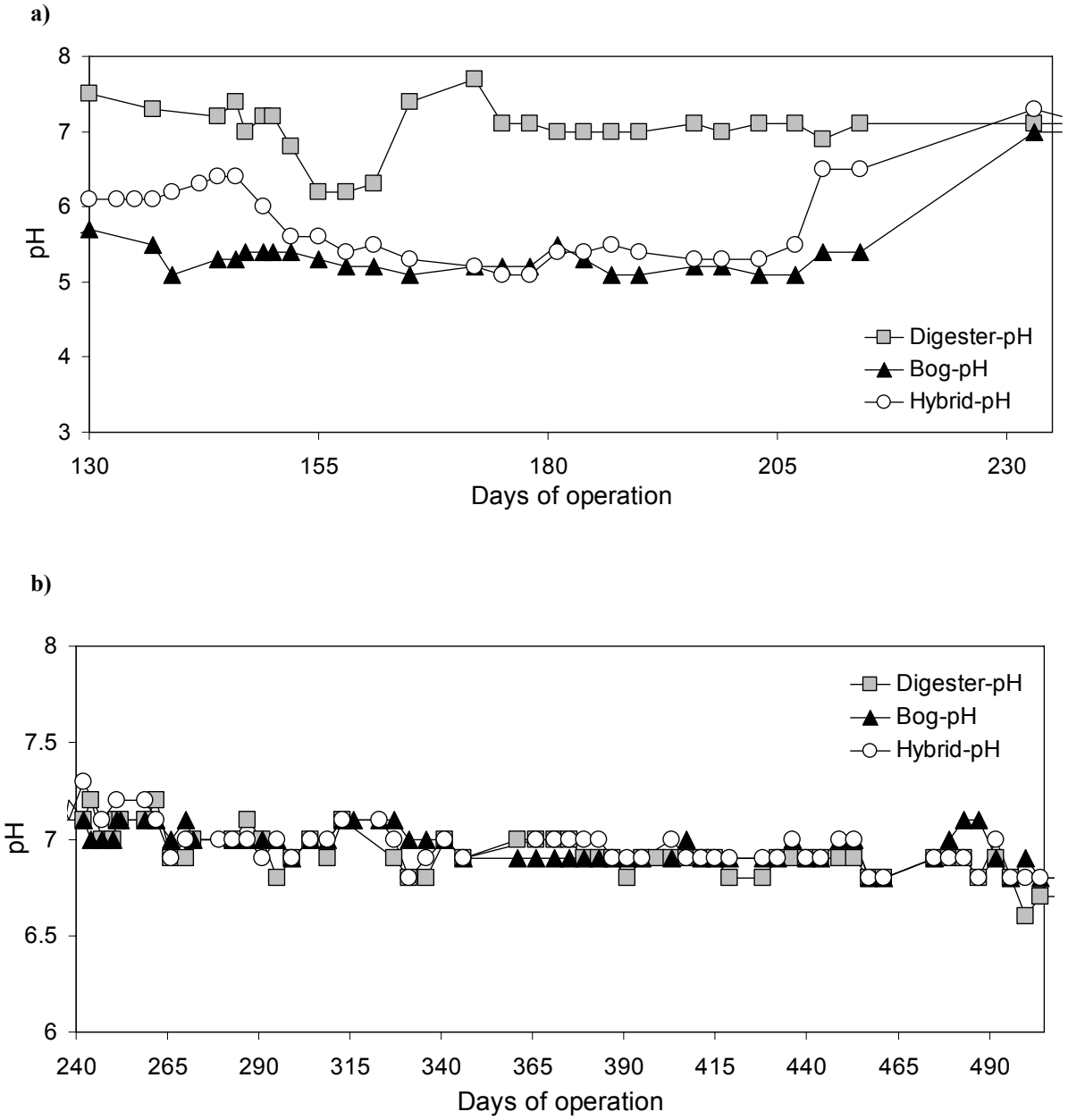


Figure A2-2. Measurement of pH in control reactors during a) the first 215 days of operation, and b) the remainder of operation.

Feedings were skipped on days 259, 279, 419, 423, 461, 465, and 469. From day 242-251 the temperature controls failed in the room where the reactors were stored, and room temperature was approximately 22°C. New feed material (settled material and waste-activated sludge) was obtained on days 235, 283, 375, and 415.

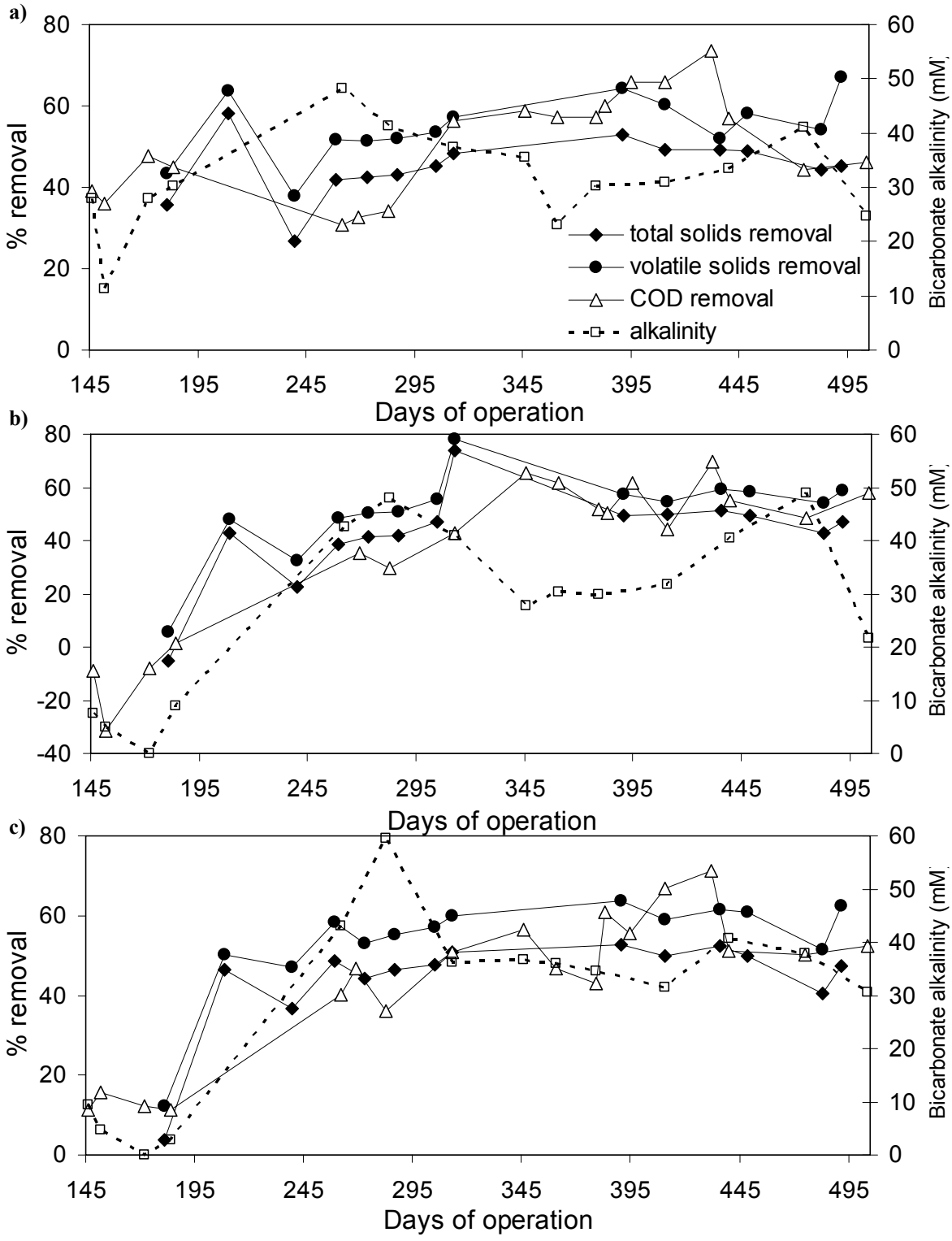


Figure A2-3. Bicarbonate alkalinity, total and volatile solids removal, and COD removal in control reactors during the course of operation.

Results are shown for a) Digester-, b) Bog-, and c) Hybrid- control reactors.

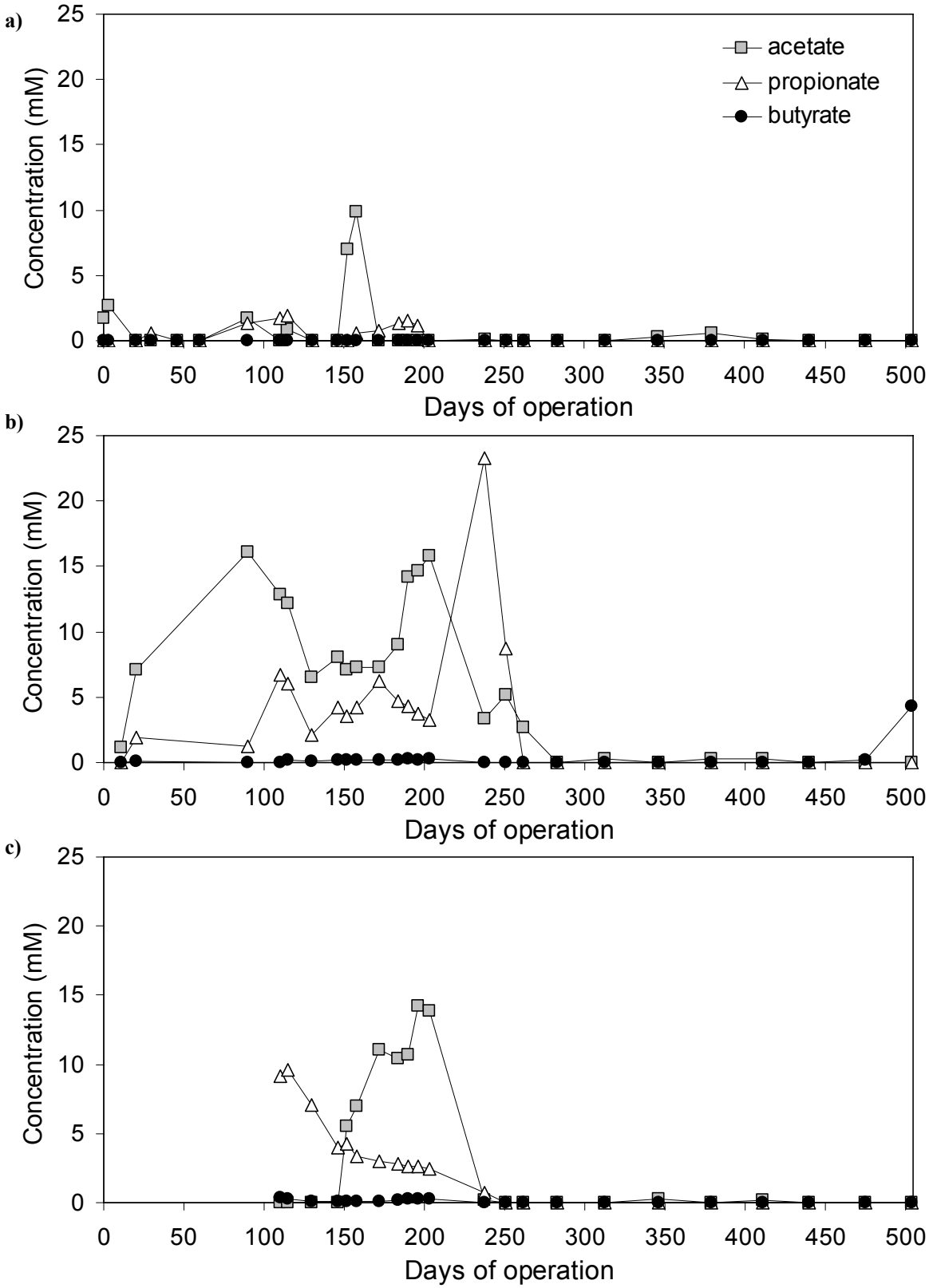
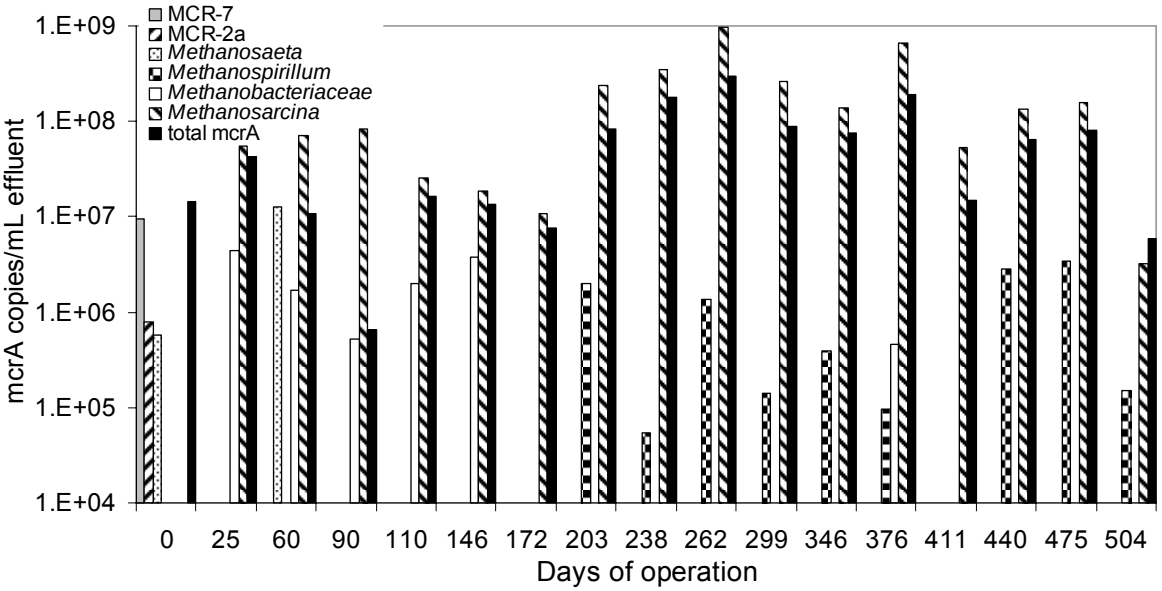
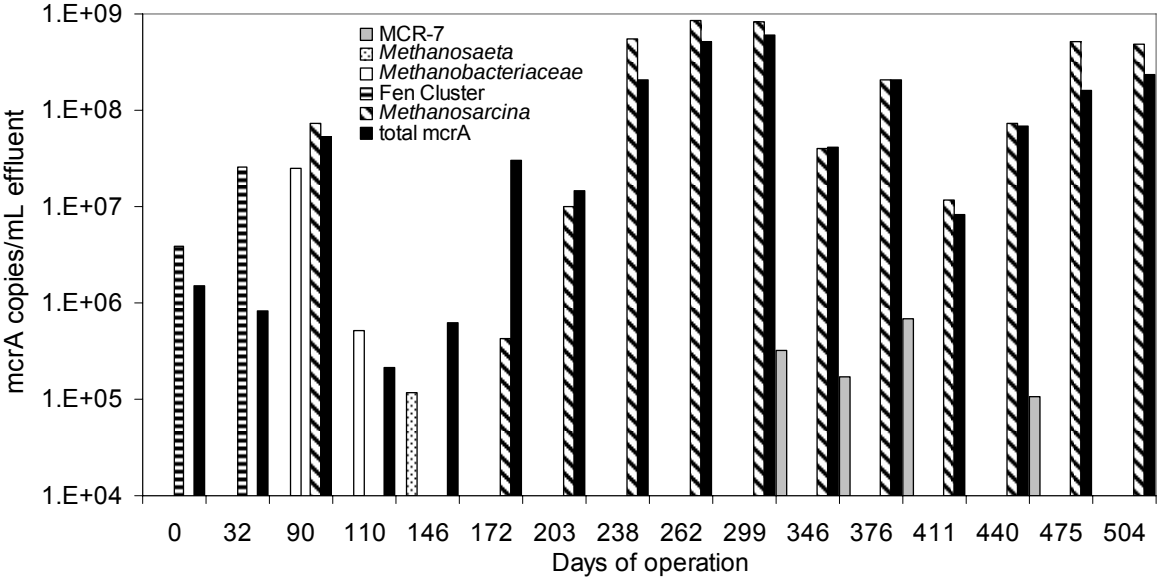


Figure A2-4. Volatile fatty acid production in control reactors during the course of operation. Results are shown for a) Digester-, b) Bog-, and c) Hybrid- control reactors .

a)


MCR-7
MCR-2a
Methanosaeta
Methanospirillum
Methanobacteriaceae
Methanosarcina
total mcrA
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
mcrA copies/mL effluent
0 25 60 90 110 146 172 203 238 262 299 346 376 411 440 475 504
Days of operation

b)


MCR-7
Methanosaeta
Methanobacteriaceae
Fen Cluster
Methanosarcina
total mcrA
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
mcrA copies/mL effluent
0 32 90 110 146 172 203 238 262 299 346 376 411 440 475 504
Days of operation

c)

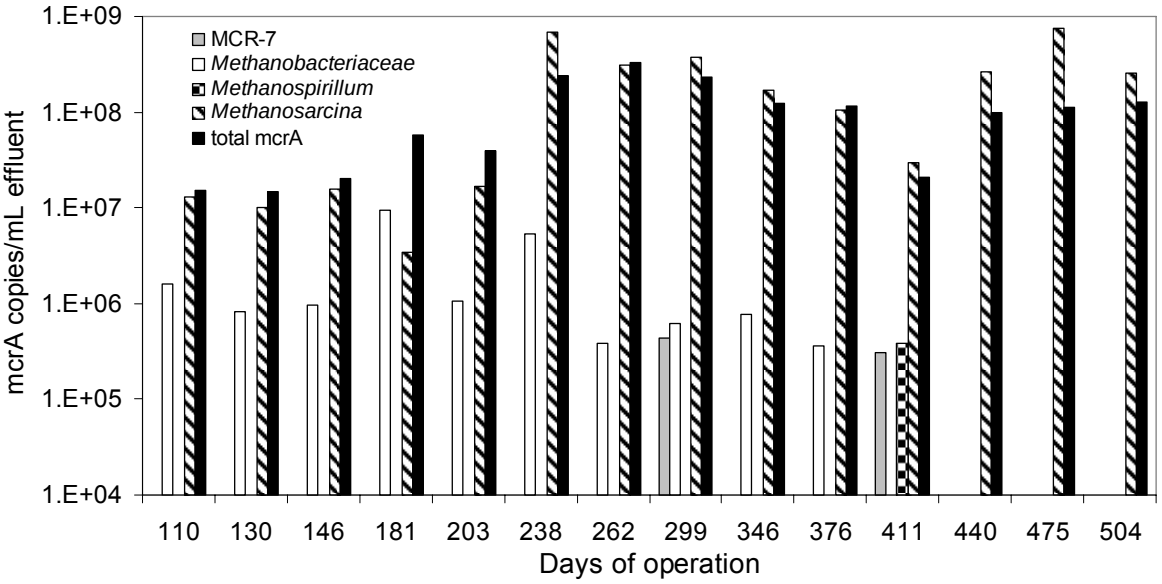


Figure A2-5. Methanogen community dynamics in control reactors over the course of operation. Results are shown for a) Digester-, b) Bog-, and c) Hybrid-control reactors.

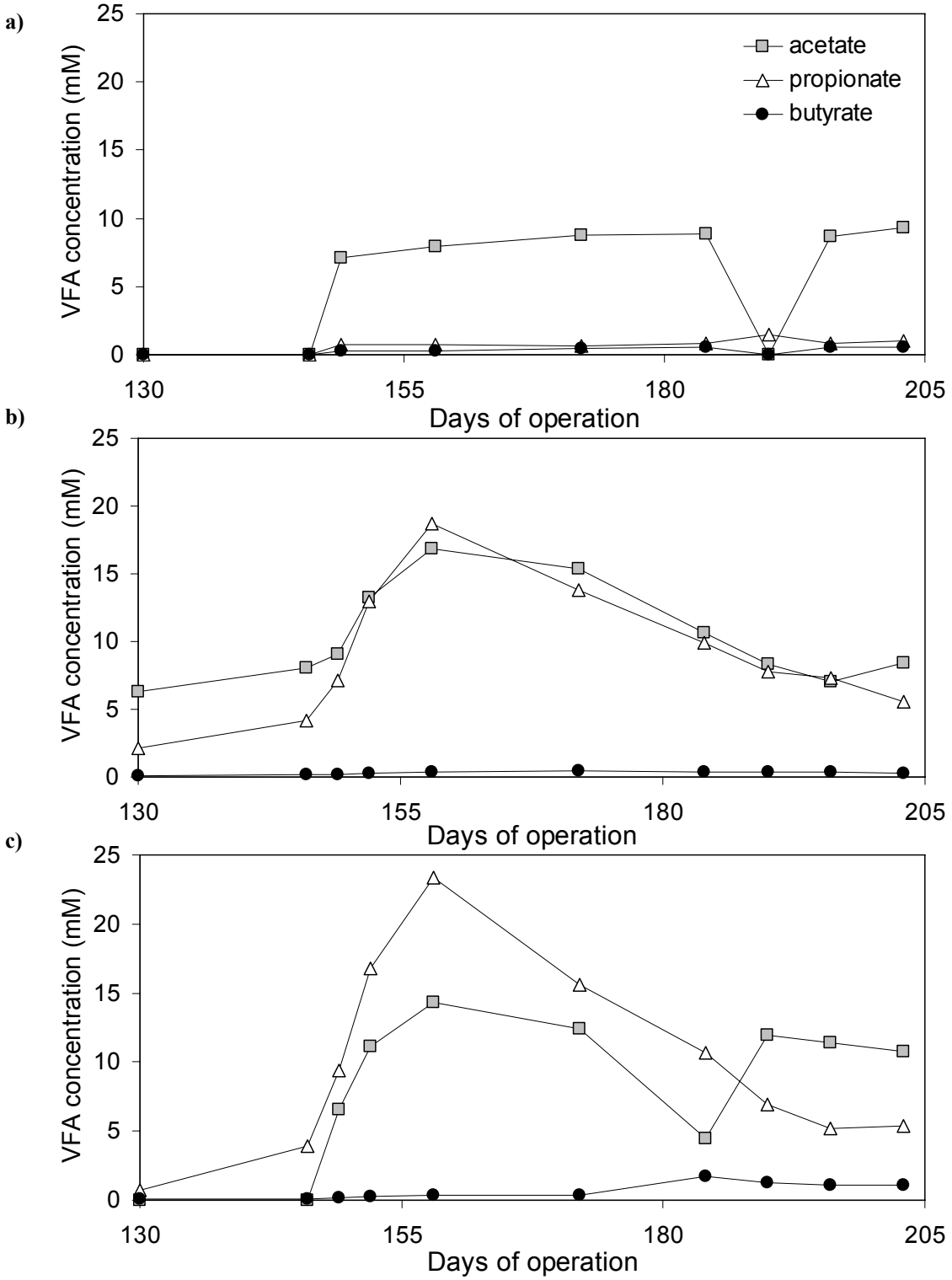


Figure A2-6. Volatile fatty acid production in test reactors during the first organic loading rate shock.

Results are shown for a) Digester-, b) Bog-, and c) Hybrid-test reactors. A shock of 10 g glucose was delivered on day 146. The Digester- and Hybrid-test reactors were restarted from control reactors on day 238.

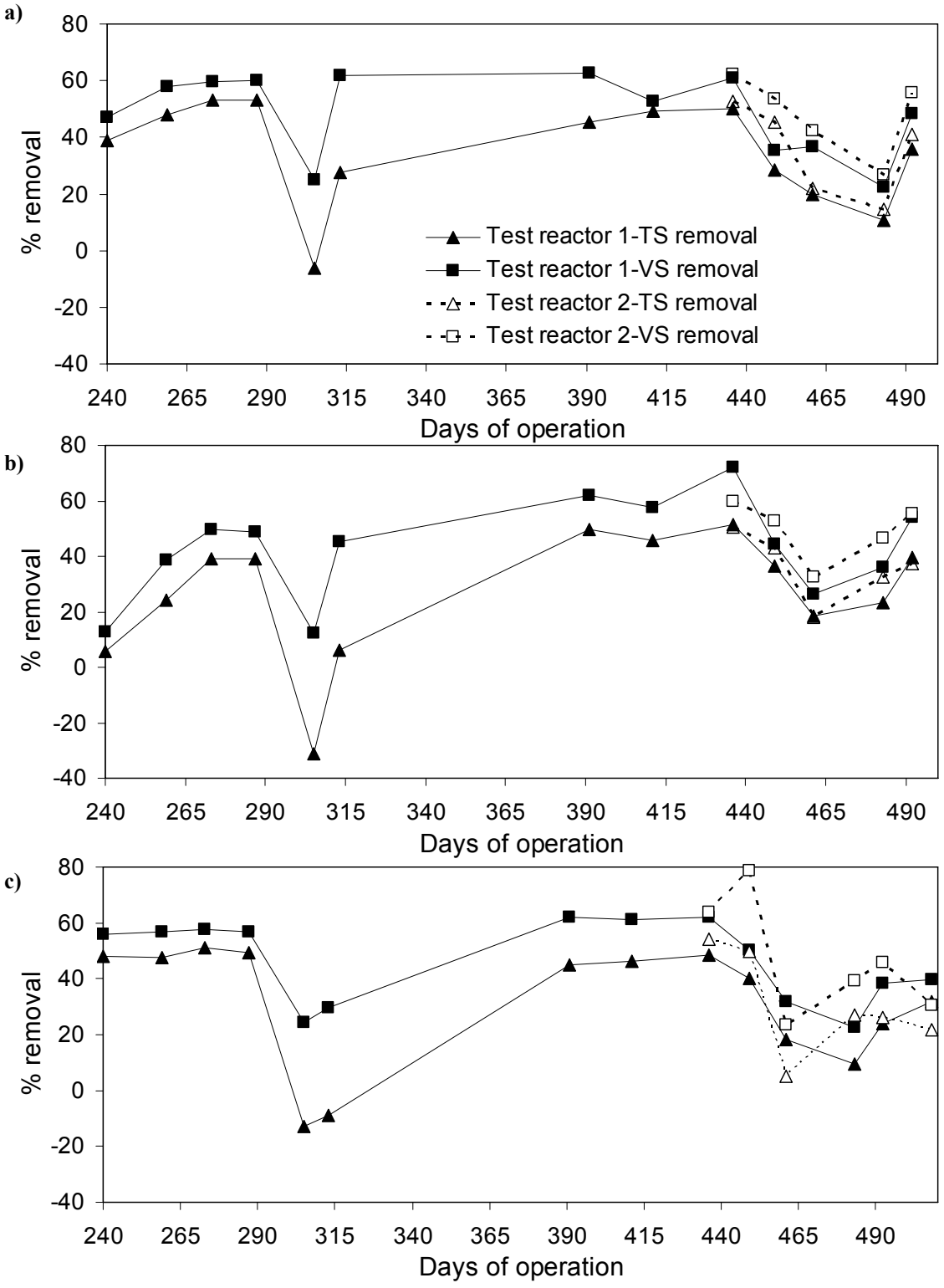


Figure A2-7. Solids removal in test reactors subjected to successive organic loading rate shocks.

Results are shown for a) Digester-, b) Bog-, c) Hybrid-test reactors. Number 2 test reactors received supplemental mineral nutrients during organic loading rate shocks, but number 1 test reactors did not. Reactors received shocks of 1g, 5g, 10g, and 10g glucose on days 262, 266, 287, and 436.

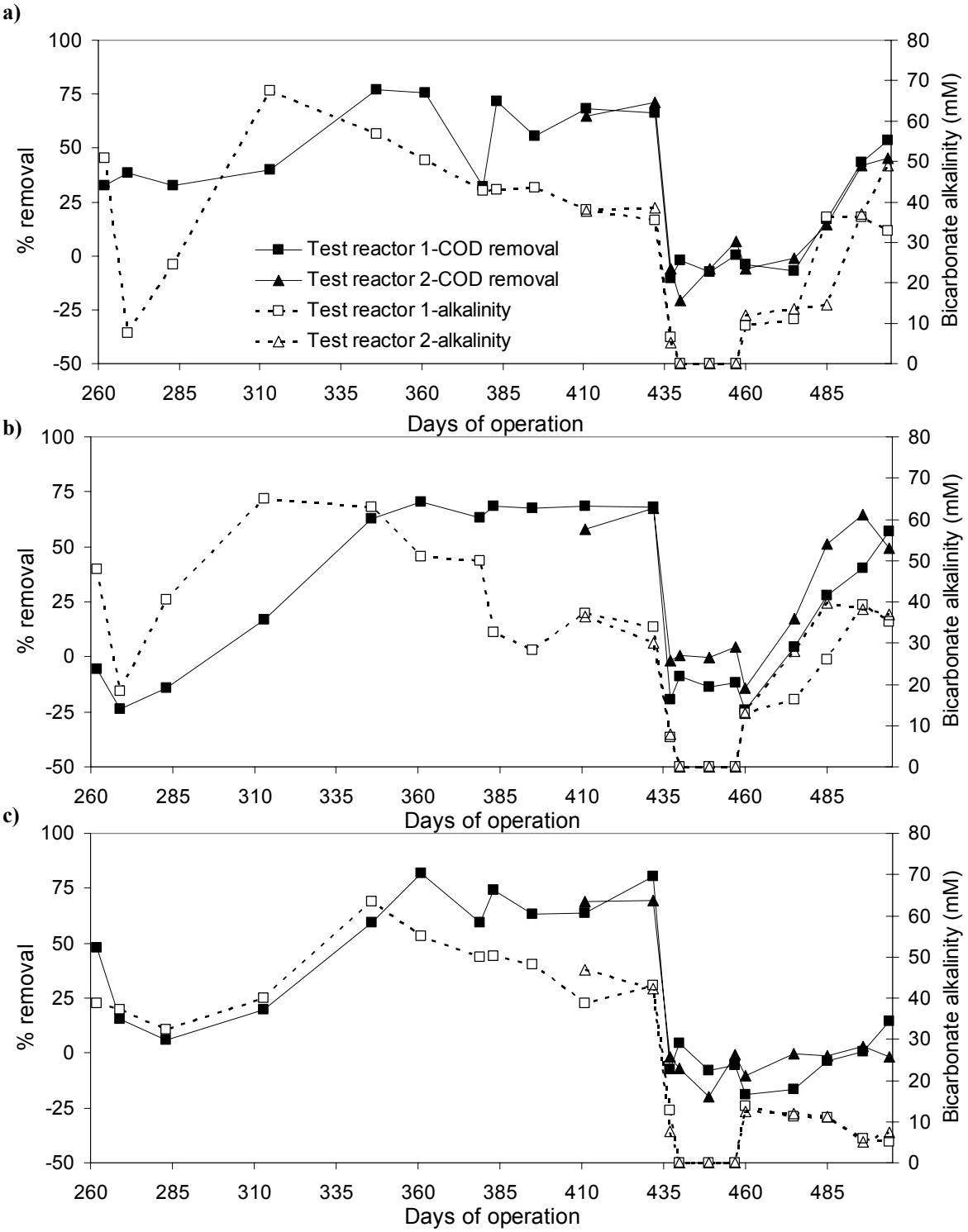


Figure A2-8. Bicarbonate alkalinity and COD removal in test reactors subjected to successive organic loading rate shocks.

Results are shown for a) Digester-, b) Bog-, and c) Hybrid-control reactors. Number 2 test reactors received supplemental mineral nutrients during organic loading rate shocks, but number 1 test reactors did not. Reactors received shocks of 1g, 5g, 10g, and 10g glucose on days 262, 266, 287, and 436.

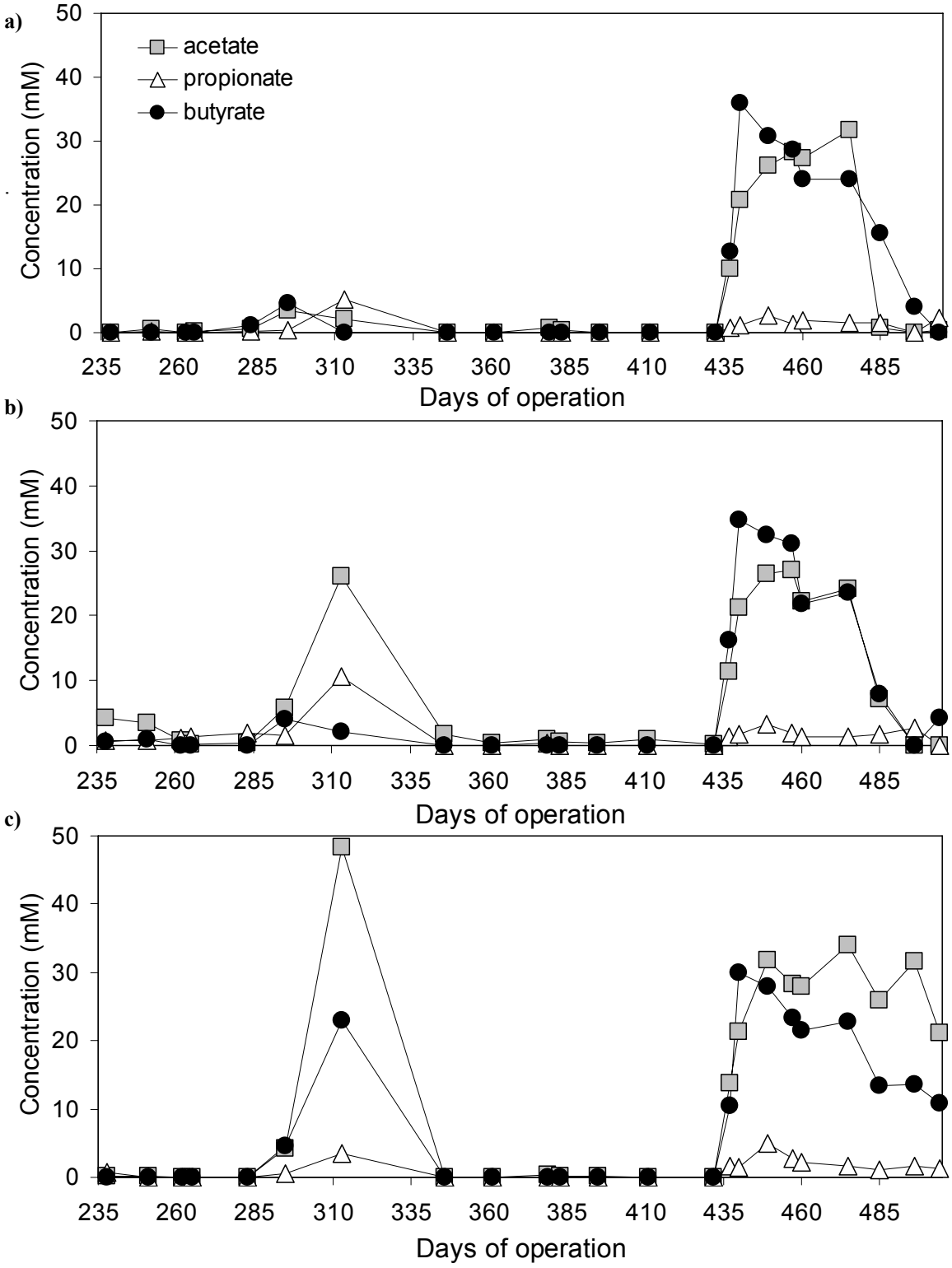
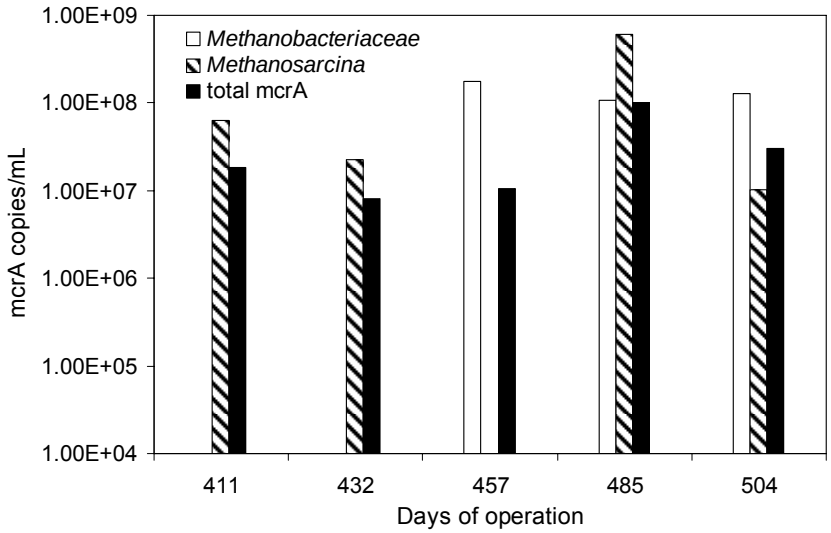


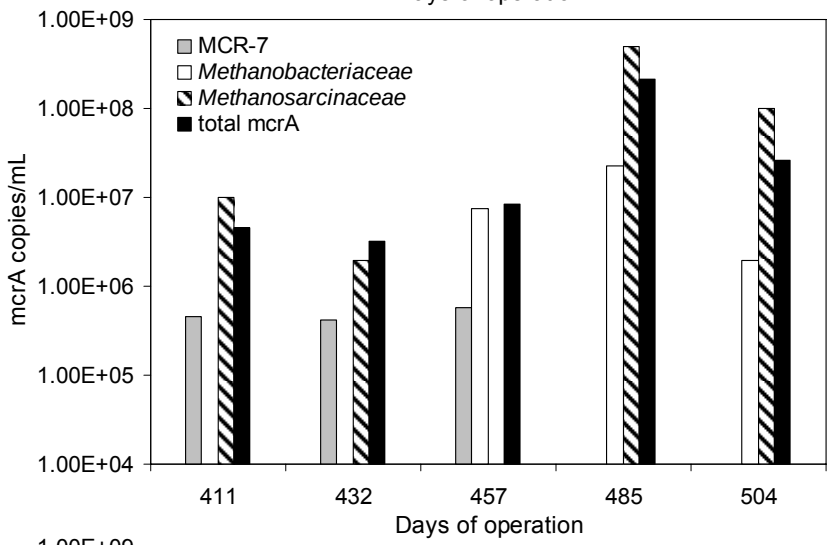
Figure A2-9. Volatile fatty acid production in test reactors subjected to successive organic loading rate shocks.

Results are shown for a) Digester-, b) Bog-, and c) Hybrid-test reactors. Reactors received shocks of 1g, 5g, 10g, and 10g glucose on days 262, 266, 287, and 436. Test reactors 2 received supplemental mineral nutrients during organic loading rate shocks, but test reactors 1 did not.

a)



b)



c)

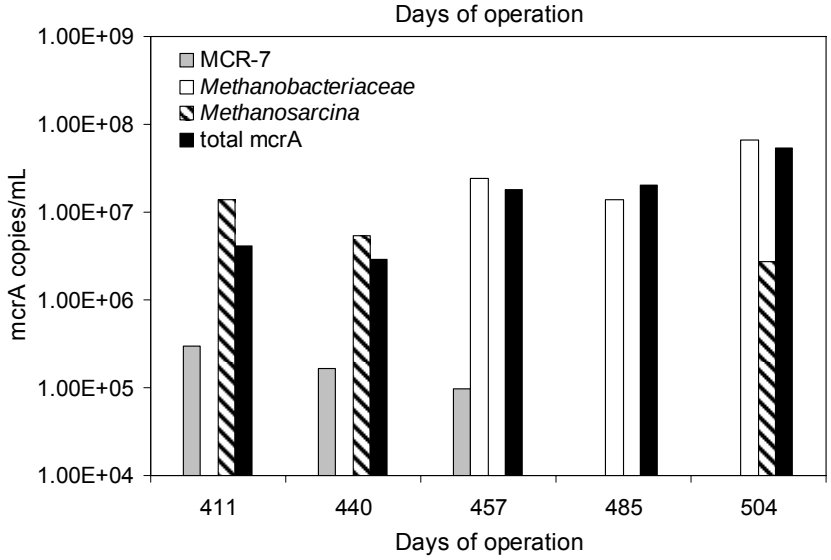


Figure A2-10. Methanogen community dynamics in test reactors which received mineral nutrients during the third OLR shocks.

Results are shown for a) Digester-, b) Bog-, and c) Hybrid-test reactors.

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PUBLICATIONS

LM Steinberg, JM Regan. (2008) Response of lab-scale reactors with different inocula to organic loading rate shocks. ***Water Research***. (*in preparation*)

LM Steinberg, JM Regan. (2008) Methods for the molecular survey of methanogen communities. ***Applied and Environmental Microbiology***. (*submitted*)

Z Ren, **LM Steinberg**, JM Regan. (2008) Electricity production and microbial biofilm characterization in cellulose-fed microbial fuel cells. ***Water Science and Technology***, 58(3): 617-622.

LM Steinberg, JM Regan. (2008) Phylogenetic characterization of the methanogenic communities from an acidic oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. ***Applied and Environmental Microbiology,*** 74(21): 6663-6671.

LM Steinberg, JJ Trimble, BE Logan. (2005) Enzymes responsible for chlorate reduction by Pseudomonas sp. are different from those used for perchlorate reduction by *Azospira* sp. ***FEMS Microbiology Letter****s*, 247: 153-159.

J Xu, JJ Trimble, **L Steinberg**, BE Logan. (2004) Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA. ***Water Research***, 38: 673-680.

LD Eirich, DL Craft, **L Steinberg**, A Asif, WH Eschenfeldt, L Stols, MI Donnelly, CR Wilson. (2004) Cloning and characterization of three fatty alcohol oxidase genes from *Candida tropicalis* strain ATCC 20336. ***Applied and Environmental Microbiology***, 70(8): 4872-4879.

J Xu, Y Song, B Min, **L Steinberg**, BE Logan. (2003) Microbial Degradation of Perchlorate: Principles and Applications. ***Environmental Engineering Science***, 20(5): 405-422.

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