Quantitative Analysis of Methangenic Community in Anaerobic Digesters and its Response to Freeze Drying and Exposure to Oxygen

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QUANTITATIVE ANALYSIS OF METHANOGENIC COMMUNITY IN
ANAEROBIC DIGESTERS AND ITS RESPONSE TO FREEZE
DRYING AND EXPOSURE TO OXYGEN

by

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ABSTRACT

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Methanogens are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases. Methane is produced in a wide variety of highly reduced anaerobic environments, as well as by degradation of organic compounds in industrial and municipal wastewater. This process is carried out by the concerted activity of an interdependent microbial community, composed of Bacteria and Archaea, the later including methanogens which complete the final step and produce methane and carbon dioxide. Methanogenesis is often the rate limiting step and is sensitive to processing imbalances. Therefore, an understanding of the microbial community structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion for increased renewable energy production. To examine the relationship between methane production and methanogen community structure, quantitative polymerase chain reaction (qPCR) was used to quantify the total methanogen community (*mcrA* gene) as well as specific genera (16S rRNA gene) in biomass from industrial scale digesters. Results from this study revealed that there was a positive correlation between methane production and *mcrA* and *Methanospirillum* transcripts. It was also found that reactors not dominated by any particular genus, but those that had a balanced community of hydrogenotrophic and aceticlastic methanogens had a higher capacity to resist organic overload and produce methane. One of the major problems faced in anaerobic digestion process is its inherent instability and sensitivity to frequent exposure to oxygen. qPCR analyses of 16S rRNA revealed that *Methanoculleus* had significantly lower activity, while *Methanospirillum* and *Methanosaeta* had significantly higher activity at higher oxygen concentrations. Finally, this study also presents the use of freeze drying as a viable method for preserving anaerobic methanogenic biomass. qPCR with 16S rRNA genus specific primers revealed that methanogens varied in their ability to tolerate the process of freeze drying. *Methanospirillum* had the highest 16S rRNA transcripts before and after drying, followed by *Methanosaeta* and *Methanoculleus*. Therefore, the data obtained from this study helps to determine the identity of desirable organisms and community architecture in relation to digester performance, exposure to oxygen and low temperature desiccation encountered during preservation by freeze drying.

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Background and Significance

1.1 Anaerobic Wastewater Treatment. According to the United Nations World Water Assessment Programme report (2003), approximately 1.5×10^{15} L of wastewater is produced annually. Inadequate handling of waste water has serious consequences for the environment and human health. It may contaminate the water supply, thereby increasing the risk of infectious diseases and deteriorating groundwater ecosystems. However, the cost of treating wastewater is high and therefore it is very important to choose the right method of treatment. Wastewater treatment is divided into two types: aerobic and anaerobic. Aerobic treatment requires aeration and has several advantages including fast start up, and rapid growth and adaptability of microbial community in the biomass (Speece, 2008). However, aerobic treatment can also be very expensive as it requires aeration.

On the other hand, anaerobic wastewater treatment is a biological process that converts organic matter using naturally occurring microorganisms under anaerobic conditions. Anaerobic digestion has been used commercially for the treatment and stabilization of organic wastes for many years, particularly by municipalities to dispose most of its sewage sludge and many industries to treat solid and liquid wastes in anaerobic treatment facilities (Braber, 1995; Speece, 1996; Ward, 2008). Anaerobic digestion has certain advantages over other alternative methods, such as high organic loading rates, low energy consumption and low sludge production (Eckenfelder, 2009;

Speece, 1996). Another important advantage that makes this method suitable for the treatment of wastewater is that, biogas is produced by the anaerobic biomass, as microorganisms degrade the waste. The biogas is composed of 55 to 70% methane (CH_4) and 30 to 45% carbon dioxide (CO_2). The methane can be used to generate electricity and heat (Speece, 2008). Therefore, anaerobic digestion is a favorable process, both for renewable energy generation and as a waste stabilization method.

1.2 Microbial community in anaerobic digestion. While most anaerobic treatment research has been focused on understanding and improving the engineering aspect of the technology, there has been less focus on the microorganisms that enable this process. Successful anaerobic treatment of organic wastes is a concert of biochemical and physicochemical reactions that requires the stable function of a complex, interdependent microbial community (Liu and Whitman, 2008). Anaerobic digestion is carried out by different microorganisms and is divided into five steps - disintegration, hydrolysis, acidogenesis (fermentation), acetogenesis (generation of acetate) and methanogenesis (generation of CH_4) (Liu and Whitman, 2008). Disintegration involves the breakdown of large particulate matter. This is followed by hydrolysis, involving the enzyme mediated biochemical transformation of lipids, proteins and polysaccharides into soluble and smaller organic molecules (Schluter, 2008). Hydrolysis, is catalyzed by the extracellular enzymes secreted by microorganisms responsible for fermentation (Schluter, 2008). In the third stage of digestion, acidogenesis, the hydrolysis products are degraded into volatile fatty acids such as acetic, propionic, butyric or valeric acids, CO_2, H_2 and ethanol (Schluter, 2008). During acetogenesis, a syntrophic group of strict anaerobes called acetogens catalyze the conversion of all the volatile fatty acids and short chain organic

products to acetate, hydrogen and carbon dioxide (Schink, 1997; White, 2000). Efficient

flow of electrons through this metabolic intermediate necessitates efficient methanogenic

metabolism of hydrogen and acetate and efficient metabolism of propionic acid

(C_2H_5COOH) (Speece, 2008). The hydrogen required for methanogenesis is often

provided via interspecies hydrogen transfer and if methanogens fail to draw off hydrogen

produced by syntrophic fatty acid-oxidizing bacteria, then fatty acid oxidation becomes

energetically unfavorable for the syntrophs (Stams, 1994). During methanogenesis,

acetate is converted to methane by a group of organisms called aceticlastic methanogens

(70% of methane is generated through the acetic acid pathway (Liu, 2008)), while a part

of the generated CO_2 and H_2 are utilized by another group of methanogens called

hydrogenotrophic methanogens, which also produce methane as the end product (Gujer,

1983).

1.3 Methanogens in Anaerobic Digestion. As already mentioned, several different

groups of microorganisms are required for the complete degradation of waste.

Methanogens are especially important as they are required for the production of methane

and methanogenesis is often regarded as the rate limiting step in anaerobic digestion (Liu,

2008). Methanogens belong to the strictly anaerobic group of Archaea, within the

kingdom and phylum *Euryarchaeota* (Liu et al., 2008). They are phylogenetically divided

into five orders, *Methanobacteriales, Methanococcales, Methanosarcinales*

Methanopyrales and Methanomicrobiales (Lange and Ahring, 2001; Liu et al., 2008).

From these orders, the genera *Methanobacterium, Methanobrevibacter, Methanoculleus,*

Methanospirillum, Methanolinea, Methanothermobacter, Methanogenium, Methanosaeta

and *Methanosarcina* have been found in anaerobic digesters (Hori, 2006; Imachi, 2008; Rastogi, 2008).
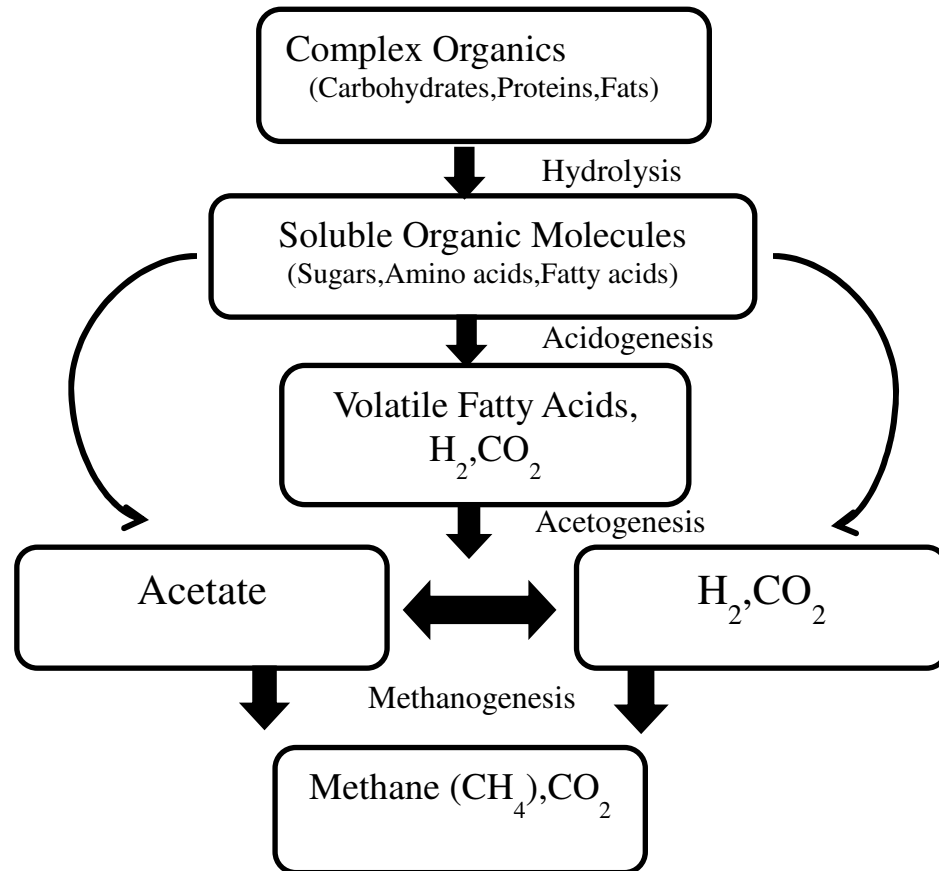
Figure 1.1. Path of anaerobic digestion. A schematic representing the step-wise degradation of organic waste in anaerobic digesters. Adapted from Speece, (1996) and Liu and Whitman, (2008).

These are further categorized based on their substrate requirements, as aceticlastic and hydrogenotrophic methanogens. Members of *Methanosaeta* and *Methanosarcina* are acetate-utilizing methanogens; the other genera found in digesters require hydrogen and carbon dioxide, although some are capable of using formate and alcohols (Liu, 2008; Stams, 1994).

Methanogenesis is catalyzed by a unique set of enzymes. Methyl coenzyme M reductase (MCR) is the enzyme which catalyzes the final reaction in both types of methanogenesis, the reduction of CH_3-CoM to CH_4 and is specific to methanogens and the anaerobic methane oxidizing archaea (Hallam et al., 2003). Several studies have established that the gene which encodes the alpha subunit (*mcrA*) can be used to detect the presence of methanogens in the environment (Luton et al., 2002; Springer et al., 1995). A PCR primer set developed by Luton et al., (2002), which amplifies an approximately 460 base pair segment of the *mcrA* sequence, has been shown to consistently amplify a wide range of methanogenic groups (Hallam et al., 2003; Juottonen et al., 2006). Unlike the 16S rRNA which has 1-4 copies per genome, there are only one or two copies of *mcrA* in the genome of methanogens.

1.4 Instability due to propionate. Anaerobic treatment suffers from a drawback in that methanogens are highly sensitive to environmental perturbations (Connaughton et al., 2006). A sudden change in temperature, pH or an increase in organic loading or toxic compounds could lead to an entire system failure. In contrast, acid forming bacteria are more robust and can tolerate and operate in a wider range of the above mentioned conditions than methanogens (Connaughton et al., 2006). An increase in fatty acids (propionate, butyrate) in an anaerobic digester can lead to a decrease in pH (Liu et al., 2008). This decrease in pH can be detrimental to methanogenesis, as most methanogens can tolerate a pH range of 6-9 (Hori et al., 2006; Liu et al., 2008). Hori et al. (2006) also demonstrated that the composition of the methanogen community in a thermophilic anaerobic digester changed from dominantly *Methanoculleus* species to *Methanothermobacter,* as the concentration of volatile fatty acids (VFAs) changed.

Propionic acid is an important intermediate in the production of methane from complex feedstocks, and its degradation often controls the rate of methane production (Speece, 2008). Metabolism of propionate to acetate and hydrogen is energetically unfavorable (ΔG^o,+71.67 kJ/mole), but the conversion of acetate to methane and carbon dioxide (ΔG^o,-35.83 kJ/mole) and conversion of hydrogen and carbon dioxide to methane (ΔG^o,-98.06 kJ/mole) moves the overall reaction in a forward direction (McCarty and Smith, 1986). The bioconversion of fatty acids to acetate and H_2 is not thermodynamically favorable under standard conditions. H_2 consuming reactions, such as CH_4 production, are required to reduce H_2 concentration and drive the bioconversion of propionate in the forward direction. It has also been reported by McCarty and Smith (1986), that degradation of propionate is favorable only when hydrogen concentration in an anaerobic digester is between 10^{-4} to 10^{-6} atm, and at higher concentration of hydrogen, this usually results in accumulation of propionic and butyric acids, leading to decrease in pH, and ultimately terminating methane production (McCarty and Smith, 1986). Therefore, propionate accumulation is usually indicative of an imbalance in anaerobic digester process. de Garcia et al. (2006) evaluated the chemical oxygen demand (COD) of a simulated organic compound, utilizing 100g COD and found that 20g COD was utilized by carbohydrates, 20g by proteins and 30g by lipids. The remaining 30g was non-biodegradable material present in the substrate. Out of the 70%, although only 8.7% passed through propionate, they reported that this amount of propionate can lower pH and affect process performance and lead to digester upset. Therefore, propionate accumulation is an indicator of organic overload or process imbalance in anaerobic digesters.

Propionic acid degradation is not only energetically unfavorable, but its accumulation can also be inhibitory to methanogens at high concentrations (Ahring and Angelidaki, 1995; Barredo, 1991; Savant, 2004; Smith, 1990). Smith and McCarty (1990) studied the effect of substrate overloading and found that propionate concentrations can remain chronically elevated for a significant time after a process overload. Barredo and Evison (1991) studied the effect of propionate toxicity on methanogen enriched sewage sludge and pure cultures of hydrogen utilizing methanogens, and found that propionate accumulation was characterized by a decrease in the amount of methane produced as well as the microbial count.

1.5 Instability due to oxygen. Another major problem faced by anaerobic digesters is their inherent instability and sensitivity to frequent exposure to oxygen. Oxygen is regarded as a potentially toxic compound during anaerobic treatment, especially for methanogens, which are known to be strict anaerobes (Liu et al., 2008). It has been well established that the sensitivity and intolerance of strict anaerobes to oxygen is due to the accumulation of oxygen radicals (OH$^.$ and O$_2^-$) and hydrogen peroxide (White, 2000). Other microorganisms that can tolerate oxygen are protected by superoxide dismutase (SOD) and catalase, enzymes which convert oxygen radicals and hydrogen peroxide to oxygen and water (White, 2000). Zehnder et al., (1977), also demonstrated that *Methanobacterium ruminantium, Methanobacterium* strain AZ and *M. mobile* were highly sensitive to oxygen, since their growth and methane production were prevented at 0.01 ppm dissolved oxygen. Kiener et al., (1983) showed that pure cultures of the methanogenic species *Methanococcus voltae* and *M. vannelii* are considered to be highly

sensitive to oxygen and were killed without any lag after exposure to oxygen, possibly due to their lack of Superoxide Dismutase (SOD).

Further investigations have demonstrated that the sensitivity of methanogens to oxygen may not necessarily mean that the effect is bactericidal, but maybe bacteriostatic, and methanogens can tolerate varying degrees of oxygen exposure (Conklin, 2007; Kato, 1993; Zitomer and Shrout, 2000). *Methanobacterium thermoautotrophicum*, *Methanobrevibacter arboriphilus* and *Methanosarcina barkeri* showed an ability to survive for hours in the presence of oxygen without a decrease in the number of colony forming units (Kato, 1997). *Methanobacterium* and *Methanosarcina* are also commonly isolated from dry and oxic paddy soil demonstrating that they can survive under aerobic conditions between flooding periods (Kato, 1997). It has also been confirmed that some methanogens have the ability to produce SOD enzyme (Brioukhanov et al., 2006; Brioukhanov and Netrusov, 2007; Kirby et al., 1981). Besides, SOD, some methanogenic species, for example *Methanosarcina barkeri* strain Fusaro, was shown to have a number of redox carriers which decreased the redox potential when chemical oxidant agents were used (Kato, 1997). This capacity to adjust the redox potential in its environment may explain its survival in dry and oxic soil, to a certain extent (Kato, 1997). Another mechanism for the methanogens to cope with exposure to air could be the presence of cells as aggregates, in the case of *Methanosarcina* which showed higher oxygen tolerance in cell aggregates than in dispersed cells (Kiener and Leisinger, 1983). Another mechanism for survival of methanogens maybe because of its dependence upon facultative microorganisms also present in the biomass community which scavenge the oxygen and create an anaerobic environment (Kato, 1997). Thus, several studies have

demonstrated that methanogenic species can tolerate oxygen exposure to a certain extent, and that adding some oxygen in the anaerobic digestion process may even be beneficial (Jarrell, 1985; Kato, 1997; Zitomer and Shrout, 1998). Therefore, obligate anaerobes differ in their sensitivity to oxygen, varying from strict intolerance to possessing some intrinsic tolerance.

1.6 Understanding the relationship between methanogens and methane production. As mentioned above, a variety of microorganisms coexist in anaerobic digesters and their concerted activity is necessary for the complete conversion of organic materials to methane. Several studies have focused on uncovering the diversity of the methanogen community in anaerobic digesters. Raskin et al., (1994) used family or genus specific fluorescent oligonucleotide probes to follow methanogen community dynamics in a mesophilic and themophilic digester over time, and found that the community shifted from *Methanosaeta* to *Methanosarcina* with an increase in acetate levels. Steinberg and Regan (2008) studied the methanogen communities in acidic fen and an anaerobic digester using *mcrA* and 16S rRNA genes and found that the sequences from the fen and the digester were very different. Several other studies have also shown that the methanogen community dynamics change in different digesters depending on its composition. For example, Goberna et al., (2010) reported that *Methanosarcina* was the dominant methanogen in a digester treating cattle manure, and it increased six-fold when olive mill waste was added to it. They also showed that in a digester operated at $55^{o}C$, hydrogenotrophic methanogens such as *Methanobacterium, Methanoculleus* and *Methanothermobacter* were detected. Rastogi et al. (2008) used *mcrA* and reported that seasonal shifts occurred in the methanogen community in a digester degrading cattle

manure. They found that there was an increase in *Methanocorpusculum* related sequences in winter samples, indicating that this genus had higher tolerance for cold.

Therefore, performance and community dynamics are not the same in all digesters and vary considerably. Lusk et al. (1995) reported that of the 74 digesters in the US producing methane from animal manure, 40% of them operated poorly. Therefore, an understanding of the microbial community structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion for increased renewable energy production. At present, new anaerobic digesters are started by adding biomass from an existing digester, and the exact identity of the microbes in these digesters has not been determined and the community structure has not been defined. So far, very little work has been done to understand the link between digester performance and microbial community structure. Tale et al. (2011) showed that the rates at which different biomass samples from different full scale digesters produced methane vary widely (e.g., from <0.1 to >10 mL CH_4/gVS-hr), and the microbial community structure significantly affected the rate and extent of methane production. Microbial community structure also influences the stability of the digester during process upsets that occur due to organic overloads, high concentration of volatile fatty acids (VFAs), and low pH, and as a result, affect the performance of a digester. Therefore, the microbial community structure in a digester should be optimized to produce more methane. While several studies, mentioned above, have examined the microbial communities in various anaerobic digesters, they used qualitative and semi-quantitative techniques, such as clone libraries, nucleic acid hybridization and molecular finger printing (Lee, 2009). It is important to pay more attention to quantitative as well as qualitative approaches, as function of anaerobic

digesters may be related to relative abundance of microbial populations as well as the composition of the community (Akarsubasi, 2005). Therefore, the scope of this work is to use culture independent molecular techniques, particularly based on 16S rRNA gene sequences and methanogen specific functional gene, *mcrA* to link microbial community structure and dynamics to process performance.

1.7 Preservation and storage of anaerobic biomass. Methanogens are slow growing organisms and require significant time to reproduce. Therefore, it would be beneficial if various defined methanogenic cultures could be preserved for application in anaerobic processes such as seeding or reseeding and bioaugmentation of full scale digesters to increase biogas production and process stability. The use of wet cultures for this process maybe problematic, due to difficulty in handling high volumes, distribution cost and culture aging during storage. Therefore, there is a need to develop methods to economically transport and store biomass without significant loss of viability. Preservation methods such as refrigeration, freezing and freeze-drying are routinely used with anaerobic pure cultures (Castro et al., 2002), but data is scarce for the storage of anaerobic sludge. Drying, is one such method that reduces mass and facilitates less expensive shipping and handling and can provide a relatively stable product (Aguilera and Karel, 1997). Common technologies to preserve microorganisms in laboratory as well as bio-industries are freeze-drying, liquid drying and spray drying. It has been reported that different drying methods have different effects on different species (Castro et al., 2002). For example, freeze drying of different strains of *Campylobacter pylori* resulted in varying cell viability in different strains (Owen, 1989). All drying technologies are associated with their own set of advantages and disadvantages and

varying loss in cell viability. Freeze drying is particularly advantageous for preserving heat sensitive microorganisms due to the use of ultra low temperature drying as compared to other drying techniques. The preservation of methanogenic cultures in the presence of air would make it more convenient, practical and economical, but there is very limited information regarding the effect of exposure to air during preservation on methanogenic cultures. Investigating the effect of drying on methanogenic populations will enhance our knowledge to understand the sensitivity of different groups of methanogens to drying and this information can be useful to improve techniques for their preservation.

1.8 Introduction to Specific Aims. An understanding of the microbial community structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion for increased renewable energy production. At present, it is still unknown whether the number of methanogens in a digester plays a role in the ability of biomass to make methane or if composition of the waste water contributes to methane producing ability of methanogens. The scope of this study attempts to answer these questions and provide important information regarding the function and structure of methanogens in anaerobic wastewater treatment which can be used to optimize this technology.

1.9 Specific Aim I: Effect of methanogen community composition on methane production and response to organic overload conditions

1.9.a Introduction: A variety of microorganisms coexist in anaerobic digesters and their concerted activity is necessary for the complete conversion of organic materials to methane. The overall goal of this project was to analyze the methanogenic community structure (diversity and abundance) in anaerobic biomass from several industrial and lab-

scale digesters. This will help gain an understanding of the impact of methanogenic diversity and abundance on process stability to provide better knowledge for optimizing management practices in the future.

1.9.b Aims and Hypothesis: This work is based on the hypothesis that, the coexistence of a balanced community of hydrogenotrophic and aceticlastic methanogens, is necessary for stable process performance, both for higher methane production rates and resilience to organic loading shocks. This was tested by obtaining a fingerprint of the diversity of the methanogen community in different digesters by Denaturing Gradient Gel Electrophoresis (DGGE) and determining the abundance of methanogens in the different anaerobic samples, by qPCR using *mcrA* and 16S rRNA specific primers on DNA and RNA extracts.

The data supported the hypothesis and demonstrated that performance and community structure were not the same in all digesters and varied considerably. This is particularly important as the inoculum used for starting a new digester or for bioaugmentation of upset digesters may significantly influence the operating characteristics of a reactor.

1.10 Specific Aim II: Response of hydrogenotrophic and aceticlastic methanogen populations in mesophilic anaerobic digesters to increasing oxygen exposure

1.10.a Introduction: One of the major problems faced in anaerobic digestion process is its inherent instability and sensitivity to frequent exposure to oxygen. However, it is unknown at this point whether oxygen exposure affects only methane production capacity, survival of methanogens, or both. Therefore, the aim of this study was to

quantitatively investigate how increasing the concentration of oxygen can affect the methanogenic community structure and function.

1.10.b Aims and Hypothesis: This specific aim is based on the hypothesis that although methanogens are obligate anaerobes, they can tolerate a broad range of oxygen exposure and thus, these digesters have a significant capacity to maintain stable performance. This hypothesis was tested was estimating the diversity and abundance of methanogens in anaerobic reactors fed varying doses of oxygen.

The results from this study demonstrated that methanogens showed variable resistance to oxic conditions and that the structure of the methanogenic archaeal community changed with increasing oxygen exposure.

1.11 Specific Aim III: Changes in methanogen community structure and activity following freeze drying of anaerobic sludge

1.11.a Introduction: This study investigates the use of freeze drying as a viable method for preserving anaerobic methanogenic biomass. The aim of this study was to determine the response and recovery of diverse methanogens in anaerobic biomass to low temperature desiccation and oxygen stress faced during freeze drying. The results will lead to a better understanding of the sensitivity of different groups of methanogens to stress faced during drying and this information can be useful to improve their preservation.

1.11.b Aims and Hypothesis: This study is based on the hypothesis that the response and recovery of methanogenic archaea to freeze drying and subsequent rehydration varies

based on the characteristic properties of the genera and also growth conditions prior to freeze drying. Therefore, the overall objectives of this study were to **(i)** evaluate the ability of methanogens to endure freeze drying, as well as **(ii)** the role of acclimation to oxygen prior to freeze drying in improving recovery of methanogens from damage caused by oxic cryodesiccation.

The potential tolerance of three methanogenic genera, *Methanospirillum, Methanoculleus* and *Methanosaeta* to low temperature and oxygen stress was evaluated. The data supported the hypothesis and demonstrated that methanogens varied in their ability to tolerate the process of freeze drying and retaining their functional activity.

Chapter 2

Materials and Methods

2.1 Sample Sources: Anaerobic biomass was collected from anaerobic hydrogen enrichment cultures (HR1 and HR2), anaerobic propionate enrichment cultures (PR1-PR12), a lab-scale digester fed non-fat dry milk and municipal/industrial full scale digesters (Table 2.1). Lab scale digester and enrichment cultures were maintained at the Water Quality Center (WQC) in the Civil, Construction and Environmental Engineering Department at Marquette University, Milwaukee, WI. They were all maintained at 35°C and continuously mixed. The sources for industrial and municipal biomass are mentioned below (Table 2.1).

Table 2.1: Anaerobic Biomass Samples. Description of anaerobic cultures and digesters used in this study.

S.No	Source of sample	Name	Type of AD	Substrate
1	WQC[a]	HR1	CSTR[b]	H_2 and CO_2 (50:50) Basal Media
2	WQC	HR2	CSTR	H2 and CO2 (50:50) Basal Media Oxygen (75mg/day)
3	WQC	PR1	CSTR	Calcium Propionate (1g COD/L-day) Basal Medium
4	WQC	PR2	CSTR	Calcium Propionate (1g COD/L-day) Basal Medium Oxygen (1.3% COD = 8mL/L-d added O_2)
5	WQC	PR3	CSTR	Calcium Propionate (1g COD/L-day) Basal Medium Oxygen (6.7% COD = 40mL/L-d added O_2)

6	WQC	PR4	CSTR	Calcium Propionate (1g COD/L-day) Basal Medium Oxygen (12.5% COD = 75mL/L-d added O_2)
7	WQC	Lab Scale (LS)	CSTR	Non-Fat Dry Milk
8	Philadelphia	Municipal WWTP[c]-1	CSTR	Municipal Sludge
9	Des Moines	Municipal WWTP-2	CSTR	Municipal Sludge
10	Kerry	Sugar Industry WWTP	CSTR	Food Flavoring and Ingredients
11	Wis-Pak	Soft drink bottling WWTP	UASB[d]	Soft Drink Bottling Waste
12	F&A Dairy Products	Dairy WWTP	CSTR	Dairy Waste
13	City Brewery	Brewery WWTP-1	UASB	Brewery Waste
14	New Belgium	Brewery WWTP-2	UASB	Brewery Waste
15	Sierra Nevada	Brewery WWTP-3	UASB	Brewery Waste

[a]WQC = Water Quality Center, Marquette University, [b]CSTR = Continuous Stirred-Tank Reactor, [c]WWTP = Waste Water Treatment Plant, [d]UASB = Upflow Anaerobic Sludge Blanket.

All the anaerobic biomass samples were collected using sterile pipets in RNase and DNase free centrifuge bottles (treated with 0.1% Diethylpyrocarbonate, DEPC, and autoclaved) and placed on ice during transport. Chemical Oxygen Demand (COD) was calculated and basal medium was prepared by Ben Bocher (Bocher, 2012).

2.2 Nucleic Acid Extraction: DNA was extracted from each of the biomass samples using one of two methods. The first extraction method was performed in tandem with RNA extractions on

biomass samples using the RNA Powersoil[TM] Total RNA Isolation kit with DNA Elution

Accessory Kit (MO BIO, Carlsbad, CA), according to the manufacturer's standard protocol

[Chapter 4&5]. The second method [Chapter 3] involved combining the use of RNA

Powersoil[TM] Total RNA Isolation kit (Step 1-10 of experienced user protocol) followed by

Powersoil DNA Extraction kit (MO BIO, Carlsbad, CA). DNA was purified using the DNA

Ultra Clean Kit (MO BIO, Carlsbad, CA).

RNA from the biomass samples was extracted along with DNA using the RNA

Powersoil[TM] Total RNA Isolation kit (MO BIO, Carlsbad, CA) with the DNA Elution Accessory

Kit (MO BIO, Carlsbad, CA) according to manufacturer's protocol. RNA samples were then

treated with RNase free DNase (Qiagen, Valencia, CA) and purified using the RNeasy Mini Kit

(Qiagen, Valencia, CA).

Following purification, RNA and DNA were checked for integrity on agarose gels (1%

w/v) stained with ethidium bromide, and quantified using a spectrophotometer (Nanodrop ND-

1000, ThermoScientific, Waltham, MA, USA).

2.3. Polymerase chain reaction amplification:

(a) *mcrA*: The primer pair designed by Luton et al (2002) mcrF and mcrR [Table 2.2] was used

for PCR amplification. The final component concentrations per 50µl PCR reaction were: 100nM

each primer, 0.2 mM dNTPs, 1X Colorless Go Taq Reaction Buffer containing 1.5 mM $MgCl_2$

(Promega, Madison, WI), and 1.25U Go Taq Polymerase (Promega, Madison, WI). The template

concentration was approximately 100ng per reaction. The PCR conditions were as follows:

initial denaturation at 95^oC (5 min), 35 cycles of 95^oC (1 min), 49^oC (1min) and 72^oC (3 min),

and a final extension of 10 minutes at 72^oC. The program included a slow ramp in temperature

$(0.1\,^{o}Cs^{-1})$ between the annealing and extension steps of the first 5 cycles of the protocol to assist in the initial formation of product due to degenerate primers (Luton et al., 2002). The size of the expected PCR products was confirmed using a λ (Hind III digest) and φX174 (Hae III digest) DNA ladder in a 1% agarose gel stained with ethidium bromide.

**(b) *mcrA*-GC*:* The *mcrA* forward primer was modified to include a GC clamp (5`-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGTGGTGTMGGATTCA CACARTAYGCWACAGC-3') (Luton et al.2002, Muyzer et al. 1993). The final component concentration per 50 µl PCR reaction were: 100nM each primer, 0.2 mM dNTPs, 1X Colorless GoTaq Reaction Buffer containing 1.5 mM $MgCl_2$ (Promega, Madison, WI), and 1.25 U Go Taq Polymerase (Promega, Madison, WI). Template concentrations were approximately 100ng per reaction. The PCR conditions were as follows: initial denaturation at $95\,^{o}C$ (5 min), 35 cycles of $95\,^{o}C$ (1 min), $58\,^{o}C$ (1min) and $72\,^{o}C$ (3 min), and a final extension of 10 minutes at $72\,^{o}C$. The program included a slow ramp in temperature $(0.1\,^{o}Cs^{-1})$ as mentioned above. The size of the expected PCR products was confirmed as described above.

2.4. Reverse Transcription (RT-PCR). RT-PCR was performed using the iScript Select cDNA synthesis kit (Biorad, Hercules, CA) on 1200ng (16S rRNA) or 1400ng (*mcrA)* of each purified RNA extract. Controls included no-reverse transcriptase controls for each sample and no-template controls for each run. Each 20 µl reverse transcriptase reaction contained 1X iScript select reaction mix, 500nM mcr-R (Luton et al., 2002) or 16S rRNA 1000R (Gantner et al., 2011) primer, 2 µl GSP enhancer solution, 1 µl iScript reverse transcriptase (RNase H^{+} MMLV reverse transcriptase and RNase inhibitor protein) and RNA. The RT reaction conditions were: $42\,^{o}C$ for 90min followed by $85\,^{o}C$ for 5 min. The resulting cDNA samples were stored at $-20\,^{o}C$.

2.5. Quantitative PCR. qPCR was performed according to the guidelines suggested by Smith (Smith and Osborn, 2009).

(a) *mcrA:* qPCR was performed using the mcr primers (Luton et al.,2002). The product of these primers is 460 base pairs of *mcrA,* the gene encoding the α subunit of methyl coenzyme M reductase. The final qPCR mix (25 µl) included: 1X iQ SYBR Green Supermix reaction buffer containing dNTPs, iTaq DNA polymerase and 3mM $MgCl_2$ (Biorad, Hercules, CA), 750 nM mcrF and mcrR, and 1ng template DNA or cDNA (1 µl of RT-PCR reaction). Each qPCR included a no-template control and no-RT controls from the RT reactions.

Table 2.2: Primers. Description of primers used in this study.

Target organism	Size	Primer Sequence
Methanospirillum (Rowe et al., 2008)	122bp	F-5`-AGTAACACGTGGACAATCTGC CCT R5`ACTCATCCTGAAGCGACGGATCTT
Methanoculleus (Whittle et al., 2009)	262bp	F-5`- GGAGCAAGAGCCCGGAGT R-5`-CCAAGAGACTTAACAACCC
Methanosaeta (Rowe et al., 2008)	266bp	F-5`-GGGGTAGGGGTGAAATCTTGTAATCCT R-5`-CG-GCGTTGAATCCAATTAAACC GCA
Methanosarcina (Whittle et al., 2009)	325bp	F-5`-CCTATCAGGTAGTAGTGGGTGTAAT R-5`-CCCGGAGGACTGACCAAA
All Methanogens (Luton et al,2002)	460bp	F-5'- GGTGGTGTMGGATTCACACARTAYGC R-5'- TTCATTGCRTAGTTWGGRTAGTT

PUC		PUC-F-5´-GTAAAACGACGGCCAG-3´
		PUC-R-5´-CAGGAAACAGCTATGAC-3´

The qPCR reactions were performed with the Biorad MyIQ Single Color Real Time PCR Detection System using the following protocol: initial denaturation at 95°C (10 min), 45 cycles of $95\,^{o}$C (30 sec) and $58.5\,^{o}$C (1min) and a final extension of 7 min at $72\,^{o}$C. The amplification program was followed by a denaturation/melt curve program (80 cycles of 10 sec length starting at $55\,^{o}$C and increasing in increments of $0.5\,^{o}$C) to check for product specificity. Starting quantity amounts were calculated using the MyIQ optical system software version 1.0.

qPCR standards were used in all runs. They were created using pooled *mcrA* DNA clones from anaerobic biomass samples in a study by Morris (2011). A broad spectrum of *mcrA* sequences representing methanogen genera commonly found in anaerobic digesters (*Methanospirillum, Methanobacterium, Methanosaeta, Methanoculleus, Methanobrevibacter)* were selected. 50 ng of each was added to the standard mix, and the mix was diluted to 0.1 ng/µl. 3 µl aliquots of the mix were stored at -80°C.

(b) 16S rRNA genus specific primers: qPCR was performed with 16S rRNA genus specific primers for *Methanospirillum, Methanoculleus, Methanosaeta* and *Methanosarcina.* The protocol used to develop standard curve is similar to that mentioned above for *mcrA*. A clone library was developed using the PCR products from each of the 16S rRNA primer sets. 16S rDNA clones were pooled and diluted to 0.1ng/µl and stored at -80°C for later use.

The final qPCR mix (25 µl) is as follows: 1X iQ SYBR Green Supermix reaction buffer containing dNTPs, iTaq DNA polymerase and 3mM $MgCl_2$ (Biorad, Hercules, CA), 300 nM of 16S rRNA primers, and 1ng template DNA or cDNA (1 µl of RT-PCR reaction). Thermal cycling included an initial denaturation at $95^{o}C$ (10 min), 45 cycles of $95\,^{o}C$ (30 sec) and 60-65 ^{o}C (1min) and a final extension of 7 min at $72\,^{o}C$. Annealing temperature of $60\,^{o}C$ was used for *Methanospirillum* and *Methanosaeta,* $65\,^{o}C$ for *Methanoculleus* and *Methanosaeta.* The amplification program was followed by a denaturation/melt curve program (80 cycles of 10 sec length starting at $55\,^{o}C$ and increasing in increments of $0.5\,^{o}C$) to check for product specificity. Starting quantity amounts were calculated using the MyIQ optical system software version 1.0.

2.6 Denaturing Gradient Gel Electrophoresis (DGGE): DNA extracts were amplified with *mcrA* specific primers, followed by *mcrA*-GC primer as described in 2.3 a&b. The PCR products were analyzed on polyacrylamide gels. Equal PCR product concentrations from each PCR reaction were used for DGGE in a 1mm thick 8% polyacrylamide gel (37:5:1 acrylamide to bis-acrylamide) with 40-70% denaturing gradient (urea and formamide). Electrophoresis at 100V for 15 h was performed using the Universal DCode Mutation Detection System (Biorad). The DGGE gel was stained with 1% SYBR Gold Nucleic Acid Stain (Invitrogen) for 30 min and visualized using the GelDoc-It Imaging System (UVP). A marker was run on every gel to compare the densiometric data from different gel images. Unique bands from each gel were excised, cloned and sequenced. Jaccard similarity coefficients (r) were calculated with unweighted data (binary data representing migration position only) to compare community fingerprints.

The Jaccard similarity was calculated according to the formula (Paul, 2001): $S_{Jaccard} = N_{AB}/(N_A+N_B-N_{AB})$, where N_{AB} is the number of bands common to both samples and N_A and N_B represent the total number of bands in sample A and B, respectively.

2.7 Cloning: The excised bands were suspended in 50µL of sterile water and DNA was allowed to elute for 24 hrs. PCR was performed using *mcrA* primers and these *mcrA* PCR products were then ligated into the pCR 2.1-TOPO® vector followed by transformation into One Shot TOP10™ chemically competent *E. coli* using the TOPO TA® cloning kit, according to manufacturer's instructions (Invitrogen Carlsbad, CA). X-gal (40µL of 40mg/ml) and Ampicillin (25µL of 50mg/ml) amended Luria-Bertani agar was used for blue-white screening of the transformants. Randomly selected white colonies were used for direct PCR with the vector-specific primers PUCF (5´-GTAAAACGACGGCCAG-3´) and PUCR (5´-CAGGAAACAGCTATGAC-3´) (Invitrogen, Carlsbad, CA). The PCR reaction component concentrations were as described above. The PCR conditions for the PUC primers were as follows: denaturing temperature of 94°C (1 min), annealing temperature of 55°C (1 min), and elongation temperature of 72°C (1 min), and a final extension of 10 minutes at 72°C. The size of the PUC-amplified PCR products were confirmed as described above.

2.8 Sequence Analysis. The PCR products were purified using Qiaquick™ PCR Purification Kit (Qiagen, Valencia, CA) and sequenced with a capillary automated DNA sequencer (Applied Biosystems, Carlsbad, CA) at the University of Chicago Cancer Research Center DNA Sequencing Facility. The sequences were analyzed using FinchTV (Geospira Inc., Seattle, WA). Nucleotide-nucleotide BLAST searched were conducted with the *mcrA* sequences to determine their relationship to reference *mcrA* sequences in GenBank®.

2.9 Specific Methanogen Activity Assays. Specific Methanogen Activity Assays were performed by U. Bhattad (Chapter 3) and B. Bocher (Chapters 4&5) (Department of Civil, Construction, and Environmental Engineering, Marquette University) in triplicate. All assays were performed under anaerobic conditions in 160-ml serum bottles with 25 ml (< 3g VSS/L) of biomass. The VSS concentration was determined at the beginning and end of activity tests and the average of the two values was employed for specific activity calculations.

For H_2/CO_2 specific activity assays, 100 ml of the H_2:CO_2 gas blend at ambient pressure and temperature was injected through the stopper using a syringe and needle. For acetate and propionate specific activity tests (Zitomer et al. 2008b), 3g/L propionate in the form of calcium propionate or 10g/L calcium acetate were used, whereas the control assays were not supplied with any substrate. All the propionate and acetate assays were then sparged with gas (7:3 v/v N_2:CO_2) to establish anaerobic conditions. Immediately after the addition of substrate to the test assays, all bottles were incubated at 35°C and shaken at150 rpm using an incubator shaker (model C25KC, New Brunswick Scientific, Edison, NJ). Bottle head space volume was measured at ambient pressure (approximately 1 atm) for 30 days by inserting the needle of a glass syringe with wetted barrel. Syringe content was re-injected into the serum bottle after volume measurement. Headspace methane content was analyzed using gas chromatography.

2.10 Organic Overload Perturbation Assay (OOPA). OOPA test was developed to provide information about biomass' response to organic overload. A 25 mL sample of standard active biomass was placed in a 160 mL serum bottle and sparged with oxygen free gas (3:7 v/v mix of CO_2 and N_2). OOPA tests were incubated at 35°C in a gyratory shaker at 150 rpm. The sample was given a one time dose of 5.2 g glucose/L. Dosage was selected to be high enough to perturb the system, but not high enough to stop CH_4 production. The time taken to produce 66.7% of

theoretical maximum biogas as well cumulative amount of biogas production after 20 days was calculated.

2.11 Principal Component Analysis (PCA). PCA was done using MATLAB (v. R2010bSP1, Math Works, Natick, MA). Optical densities of DGGE bands were used as dimensional values for community structure. PCA was used to relate DGGE banding patterns to CH_4 production rates.

Chapter 3

**Effect of methanogen community composition on methane production and response
to organic overload conditions**

3.1 Introduction. Anaerobic digestion is an environmentally beneficial and economically viable

solution for the degradation of organic pollutants in industrial and municipal wastewater. This

process is carried out by the concerted activity of an interdependent microbial community,

composed of Bacteria and Archaea, the latter including methanogens, which complete the final

step and produce biogas, composed of 55 to 70% methane (CH_4) and 30 to 45% carbon dioxide

(CO_2) (Speece, 1996). Anaerobic digestion has been used commercially for the treatment and

stabilization of organic wastes for many years (Speece, 1996). Municipalities use it to dispose of

most of their sewage sludge and many industries use it to treat solid and liquid wastes in

treatment facilities (Braber, 1995; Speece, 1996; Ward et al., 2008). Anaerobic digestion has

certain advantages over other alternative methods, such as high organic loading rates, low energy

consumption, low sludge production and most importantly, the recovery of CH_4 as a source of

clean energy (Lee, et al., 2009). Therefore, anaerobic digestion is a favorable process, both for

renewable energy generation and as a waste stabilization method.

   As mentioned above, a variety of microorganisms coexist in anaerobic digesters and their

concerted activity is necessary for the complete conversion of organic materials to CH_4.

Methanogenesis is often the rate limiting step of anaerobic digestion of organic wastes and is

also the most sensitive to processing imbalances, that may occur, due to organic overloads, high

concentration of volatile fatty acids (VFAs), high concentrations of ammonia or low pH (Braun

et al., 2009; Curtis and Sloan, 2004). Therefore, an understanding of the microbial community

structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion, for increased renewable energy production.

Leclerc et al., (2004) reported that the functioning and stability of an anaerobic digester is directly related to the microbial community within the digester and the community structure may significantly vary from one anaerobic culture to another. Raskin et al., (1995) used family or genus specific fluorescent oligonucleotide probes to follow methanogen community dynamics in mesophilic and themophilic digesters. They found that methanogen community abundance shifted depending on acetate levels, in particular, *Methanosaeta* was dominant at low acetate levels and *Methanosarcina* at high acetate levels. Steinberg and Regan (2008) found that the methanogen community sequences were significantly different among acidic fen and an anaerobic digester, based on methyl coenzyme M reductase (*mcrA)* and 16S rRNA genes. Goberna et al., (2009) reported that the methanogen community dynamics changed in different digesters depending on the composition of the waste. They found that *Methanosarcina* was the dominant methanogen in a digester treating cattle manure, and its abundance increased six-fold when olive mill waste was added to the digester. Another study showed that in a digester operated at $55^{\circ}C$, hydrogenotrophic methanogens such as *Methanobacterium, Methanoculleus* and *Methanothermobacter* were also detected (Goberna et al., 2009).

Start-up is generally considered the most critical step in the operation of an anaerobic digester and its stable performance depends on the establishment of a suitable microbial community. At present, new anaerobic digesters are started by adding biomass from an existing digester, usually from which the exact identity of microbes has not been determined and the community structure has not been defined. While several studies, mentioned above, have examined the microbial communities in various anaerobic digesters at steady state, using

qualitative and semi-quantitative techniques, such as clone libraries, nucleic acid hybridization and molecular finger printing (Lee, 2009) at steady state, the methanogen composition of the inoculum used for starting new digesters or for bioaugmentation and its role in methane production and operational success during organic overload, is poorly understood. A balanced ratio of acetogens and methanogens is required to initiate gas production in reactors. Typically, during start up, the biomass in a reactor takes 1-3 weeks to acclimatize to the new environment (Pandey et al., 2011). This period maybe prolonged due to the slow growth rate of methanogens, which often results in an accumulation of volatile fatty acids (VFAs), and may lead to delayed startup or failure of the digester. Therefore, it is important to have sufficient levels of methanogens or the "right" methanogens during startup, to avoid these problems. While Fang and Lau, (1996) reported the relationship between successful startup and initial loading rate, solid and hydraulic retention times (SRT and HRT), relatively little work has been done to demonstrate how the methanogen community varies based on the substrate in the seed sludge source, and how this difference may effect the performance of the digester during steady state and its response to overload shock. Therefore, the scope of this work was to use culture independent molecular techniques, based on quantitative as well as qualitative approaches using methanogen specific 16S rRNA gene and the functional gene, *mcrA,* to link the effect of methanogenic community structure in the seed sludge to process performance. This is particularly important as function of anaerobic digesters may be related to relative abundance of specific methanogen populations and not just the composition of the community .

Rationale and Hypothesis. The overall goal of this project was to analyze the methanogenic community structure (diversity and abundance) of anaerobic biomass from several industrial and lab-scale digesters. This information is crucial during critical stages such as start up and bioaugmentation of upset digesters, as it will help determine the methanogenic community architecture of the anaerobic biomass, which results in either excellent or poor digester performance. This work is based on the hypothesis that, the coexistence of a balanced community of hydrogenotrophic and aceticlastic methanogens is necessary for stable process performance, both for higher methane production rates and resilience to organic loading shocks. This will help gain understanding of the impact of methanogenic diversity and abundance on process stability to provide better knowledge for optimizing management practices in the future.

This hypothesis was tested by:

a. DGGE using *mcrA* specific primers on various full scale digesters and lab scale cultures to fingerprint the diversity of the methanogen community in different digesters.

b. Determing the abundance of methanogens in the different anaerobic samples, by qPCR using *mcrA* and 16S rRNA genus specific primers on DNA and RNA extracts.

c. Relating microbial community structure and abundance to biomass activity – specific methanogenic activity assay (SMA) and organic overload perturbation assay (OOPA, measure of functional resilience during an organic overload event).

3.2 Results

3.2.a Methanogen Community Structure in anaerobic digesters. DGGE provides a genetic fingerprint of the microbial diversity based on physical separation of unique nucleic acid sequences (Muyzer, 1999). The analysis of the methanogen community structure in sludge samples from full scale and lab scale reactors for the *mcrA* gene (Fig 3.1) showed distinctive profiles, but only a minor diversity. The most dominant bands in all reactors were affiliated to *Methanobacterium.* (B1), *Methanospirillum* (B2), *Methanosaeta* (B3) and *Methanosarcina* (B4).
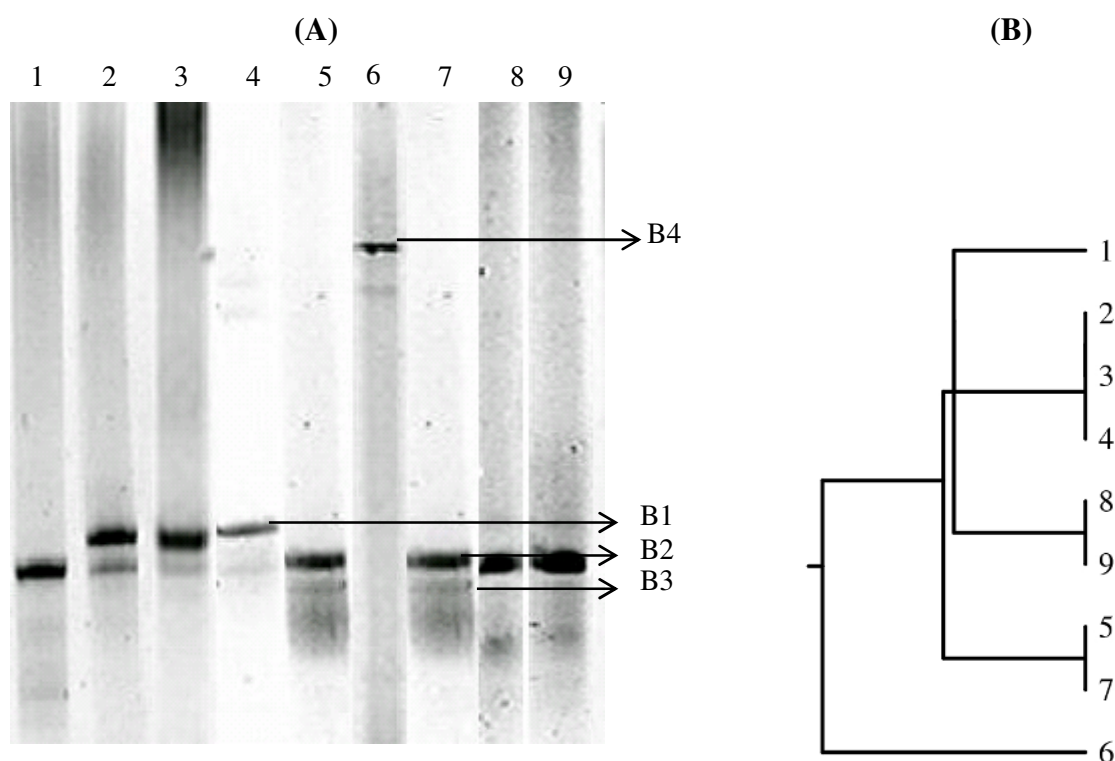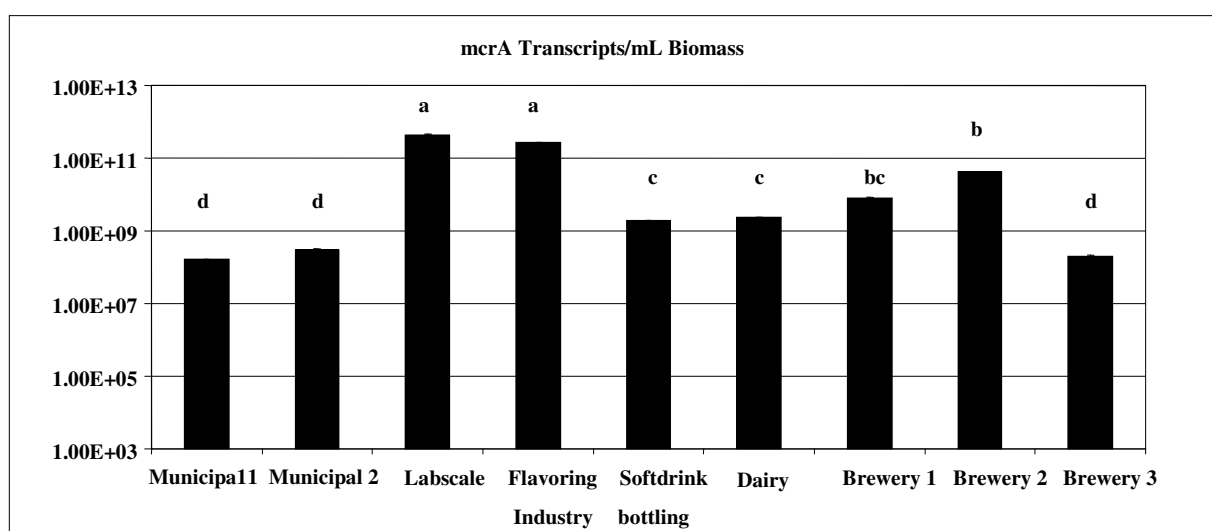

Fig.3.1. DGGE of *mcrA* from industrial digesters. DGGE fingerprint of *mcrA* genes present in industrial biomass samples. Lanes are as follows -1.Municipal 1, 2. Municipal 2, 3. Labscale, 4. Flavoring Industry, 5. Softdrink Bottling, 6. Dairy, 7. Brewery 1, 8. Brewery 2 and 9. Brewery 3. **(B)** Dendrogram showing relationship between methanogen communities in based on distance matrix using Jaccard coefficient.

Fig 3.2. Results of qPCR experiment for industrial biomass. Quantification of (**a**) *mcrA* gene copies and (**b**) *mcrA* transcripts and (**c**) *mcrA* transcript:gene ratio. Each bar represents results from two technical replicates and error bars show standard deviation from the mean. Statistical differences (ANOVA, P<*0.05*, Tukey test) among different biomass samples are indicated by different letters. Similar alphabets indicate no significant differences among the groups.
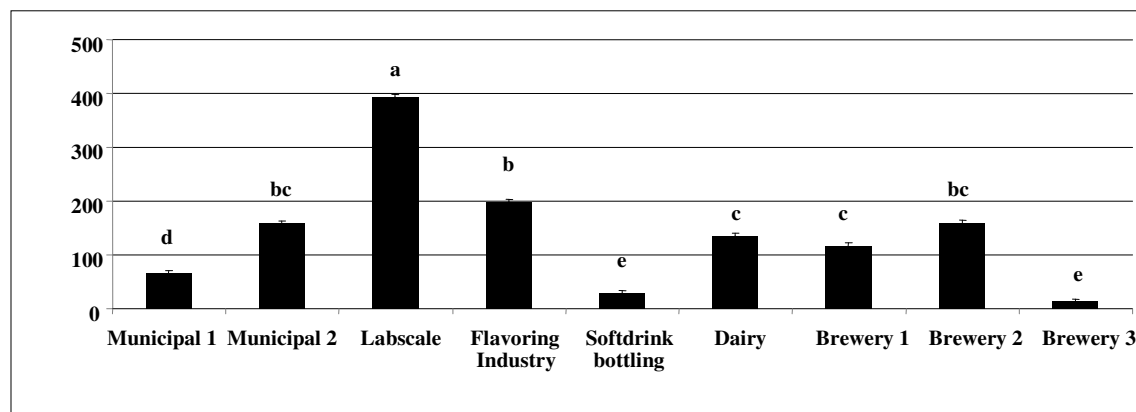
(a)

(b)

3.2.c Quantification of 16S rRNA gene copies and transcripts. Gene copy numbers and transcripts of the 16S rRNA gene were quantified using 16S rRNA genus specific primers for the genera *Methanospirillum, Methanosaeta (*Franke-Whittle et al., 2009) and *Methanoculleus* (Rowe et al., 2008). *Methanosarcina* levels were either very low or undetected in most samples, and therefore have not been used in this study.

Transcripts of the 16S rRNA gene for *Methanospirillum* were estimated to be between 4.71 x10^5 and 1.33 x10^{13} transcripts/mL biomass (Fig.3.3.b). They were significantly higher in the lab scale reactor, followed by flavoring and brewing industry 2 (p<0.05, ANOVA, Tukey test). *Methanoculleus* transcripts were estimated to be 1.18 x10^4 and 2.55 x10^{11} /mL biomass. *Methanosaeta* were estimated to be between 2.57 x10^6 and 1.63 x10^{11} transcripts/mL biomass and were significantly higher in biomass from MWWTP1 (p<0.05, ANOVA, Tukey test). A similar trend was also observed among 16S rRNA gene copies.

The 16SrRNA transcript:gene copy ratio (Fig 3.3.c) was significantly higher for *Methanoculleus,* with 60-80 16S rRNA transcripts/cell (p<0.05, ANOVA, Tukey test), while *Methanospirillum* and *Methanosaeta* were estimated to be 11-20 and 9-21 16S rRNA transcripts/gene, respectively.

Fig 3.3. qPCR results of industrial biomass. 16S rRNA **(a)** gene copies **(b)** transcripts and **(c)** transcript:gene ratio. Each bar represents results from two technical replicates and error bars show standard deviation from the mean. Statistical differences (*P*<0.05, ANOVA, Tukey) among different biomass samples are indicated by different letters [*Methanospirillum* (A-E), *Methanoculleus* (a-d) and *Methanosaeta* (*a-c*)]. Letters of similar case and style indicate no significant differences among the groups

(a)
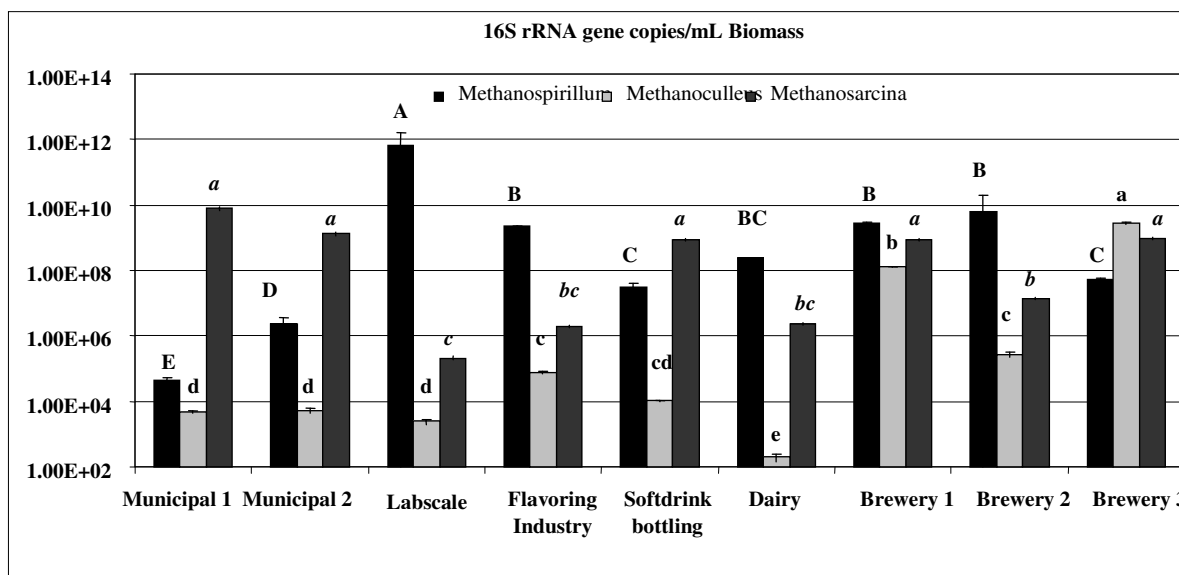
(b)
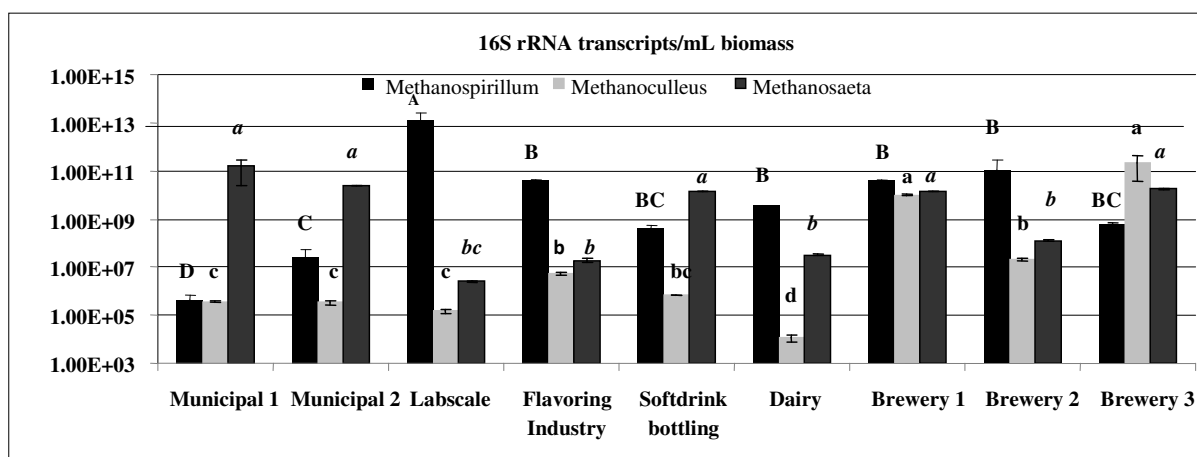
(c)
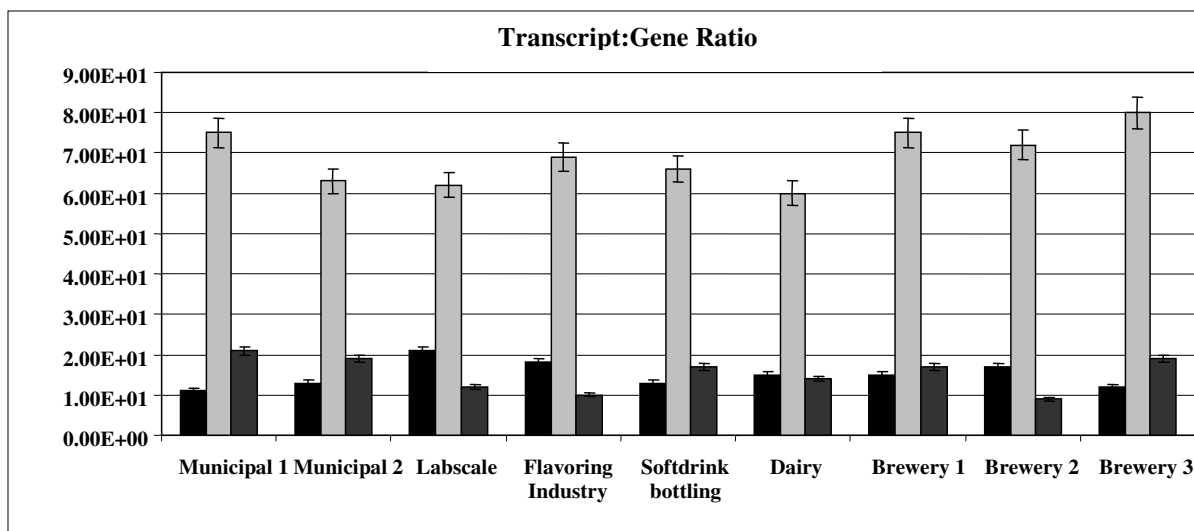
3.2.d Anaerobic digester performance

(i) Specific Methanogen Activity. SMA assays were used to compare the biomass samples ability to produce methane, given a particular substrate. The SMA assays for the lab scale and industrial biomass (Fig.3.4.a) showed that the biomass from the lab scale reactor had the highest methane production rate ($p<0.05$, ANOVA, Tukey test). This was followed by the flavoring industry and brewery 2 ($p<0.05$, ANOVA, Tukey test). The average cumulative methane production was estimated to be between 3.2 ± 0.3-27 ± 3.6 mL CH_4/h*gVSS).

(ii) Organic Overload Perturbation Assay (OOPA). OOPA was performed to demonstrate the reslience of the anaerobic systems to organic overload. This was measured based on the time taken by the system to produce 66.7% of the maximum biogas after an overload shock with glucose. As shown in Fig. 3.4.b, while most of the industrial samples took less than 150 hrs to produce 2/3rd the maximum biogas, MWWTP1 followed by labscale reactor, took significantly

longer time, (p<0.05, ANOVA, Tukey test). There was no significant difference between the

average biogas production in 20 days among the samples.

3.2.e Relationship between methanogenic activity and performance. Transcripts of *mcrA* and

Methanospirillum 16S rRNA genes correlated positively with SMA results against propionate

(R^2=0.86 and 0.78 respectively, p<0.05, ANOVA, Appendix II). However, there was no

correlation between *Methanoculleus* and *Methanosaeta* transcript number and SMA. A positive

correlation was established between *Methanosaeta* activity and resilience of the system to

organic overload (R^2=0.67, p<0.05, ANOVA).

Fig.3.4. (a) SMA. Average cumulative CH4 production rate for calcium propionate assays. Error
bars represent the standard deviation of 3 replicates. **(b) OOPA.** Average time taken to produce
66.7% max biogas and average biogas produced in 20 days after organic overload. Error bars
represent the standard deviation of 3 replicates

(a)
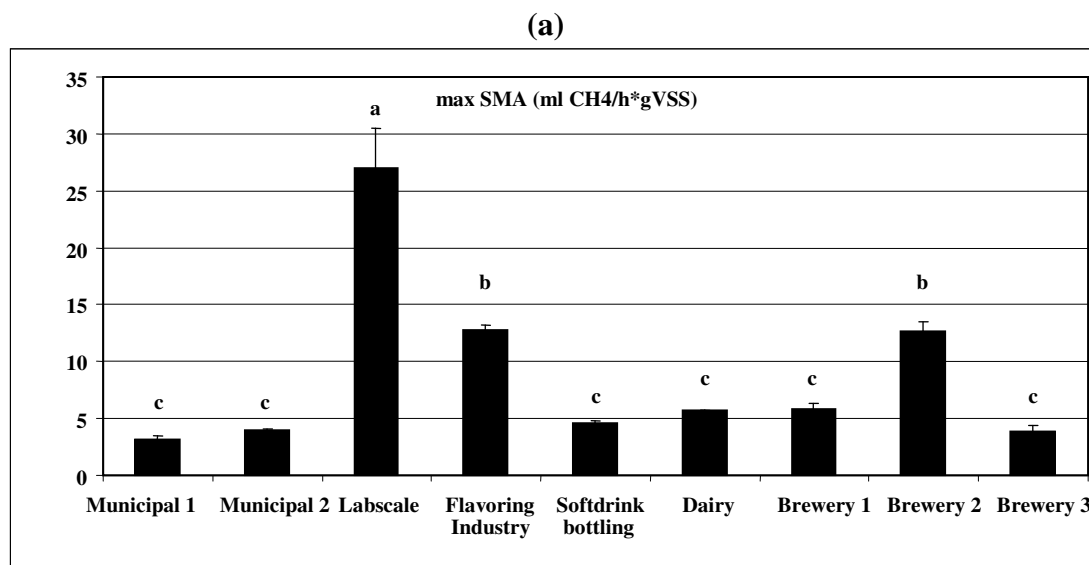
(b)
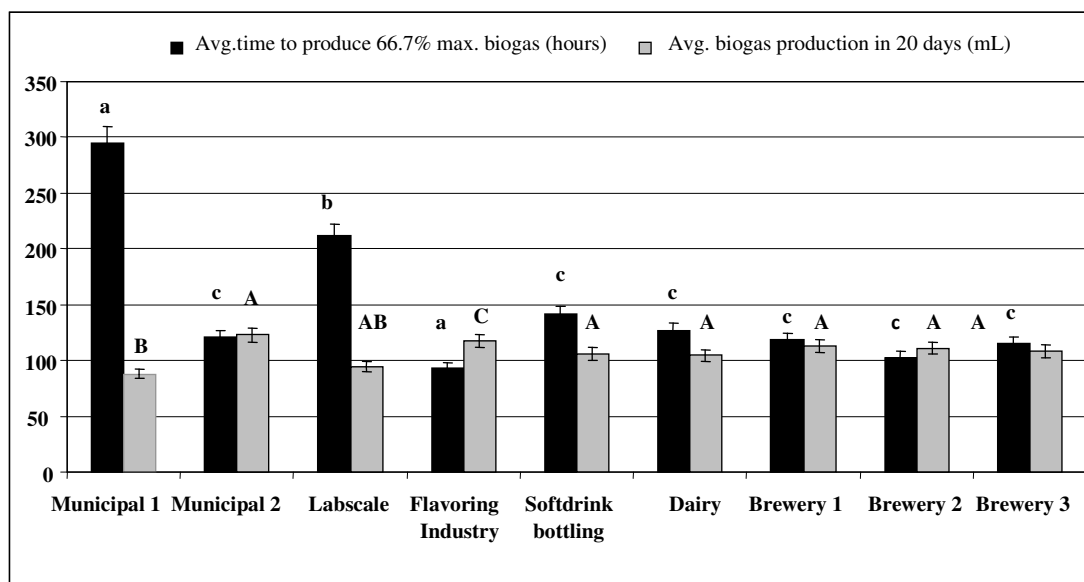
3.2.f Principal Component Analysis

PCA was used in this study to analyze the *mcrA* DGGE patterns of 10 samples (Fig.3.5). First two principal components explained considerable variance (PC1= 66.12% and PC2=24.28%). PCA indicated that there was a relationship between DGGE banding pattern and SMA. Based on principal component coordinates, biomass samples clustered in to two primary groups. In the PCA score plot (PC1 vs PC2), samples 1,5,7,8 and 9 (Cluster 1) were grouped negatively, while samples 2,3 and 4 (Cluster 2) grouped positively, along the PC1 axis indicating that samples in Cluster 1 and 2 were clearly distinct from each other. All the brewery samples were in Cluster 1, whereas the lab scale and flavoring industry sample were in cluster 2. The average SMA of biomass samples within Cluster 1 and 2 were 5.7 ±0.28 and 14.6 ± 0.73 mL mL CH4/h*gVSS, respectively (p>0.05).
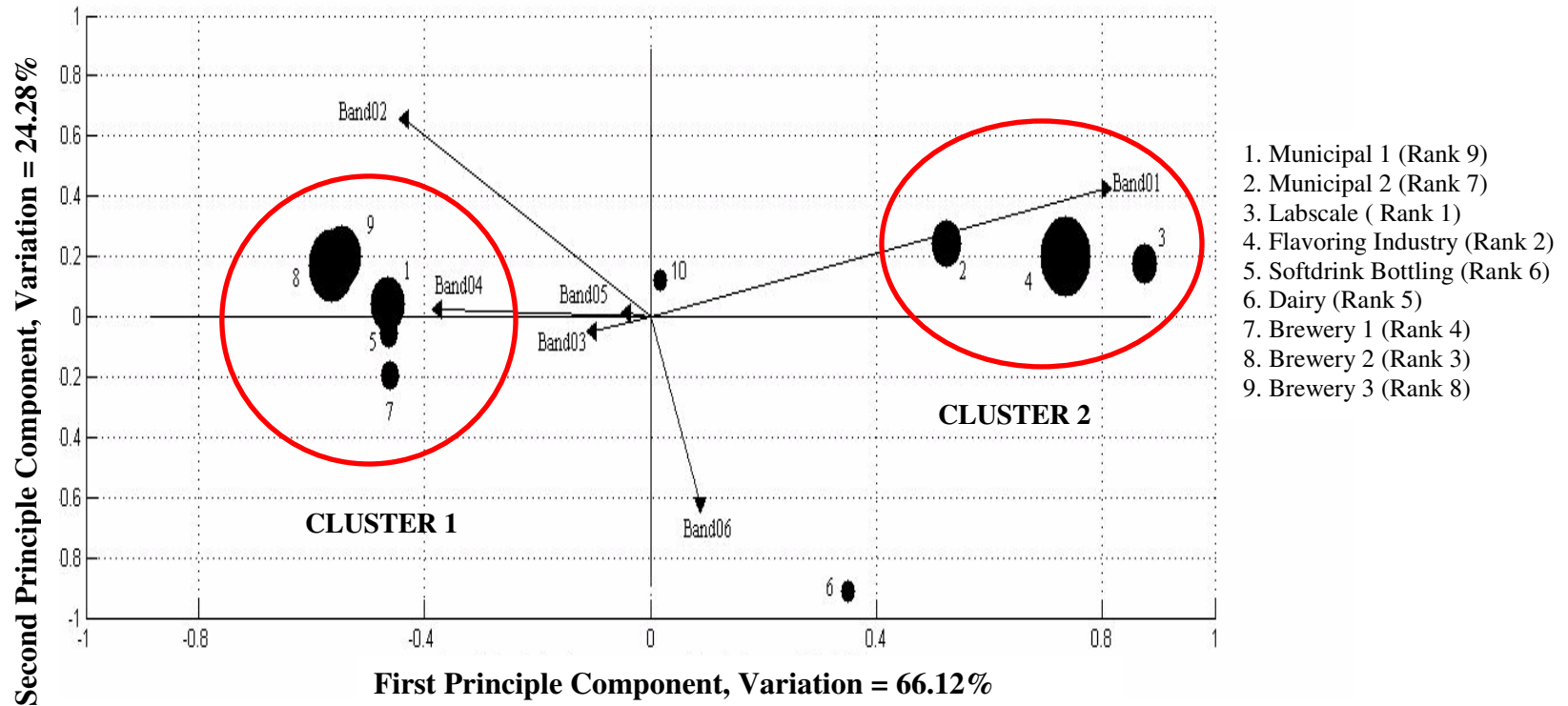
Fig.3.5. Principal component analysis using DGGE band intensities. Points represent individual biomass samples that are clustered according to their methanogenic community structure (i.e., DGGE band intensities). The diameter of each point is proportional to the measured methanogenic activity (i.e., SMA values). Vectors Band 1, Band 2, Band 3, Band 4 and Band 6 represent DGGE bands that have the greatest influence on differences in community structure. Clones from Band 1, Band 4 and Band 6 were most similar to *Methanobacterium*, *Methanospirillum* and *Methanosarcina*, respectively.

3.3 Discussion. Anaerobic digestion is an under-utilized technology to treat organic wastes, primarily due to the reason that anaerobic treatment processes may be less stable and more prone to failure than aerobic processes. Therefore, an understanding of the microbial community structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion for increased renewable energy production. To the best of our knowledge, this is the first study to report methanogenic community composition and abundance from a wide variety of anaerobic digesters treating a wide variety of waste substrates. The key to this project was the analysis of methanogenic community structure (diversity and evenness) as well as methane production rate for a large number of methanogenic cultures to possibly determine the identity of desirable organisms and the community architecture that results in either excellent or poor digester performance.

The anaerobic biomass samples demonstrated SMA values that varied over an order of a magnitude and were statistically categorized in to 3 different groups (p<0.05). The samples with the highest SMA also had the greatest number of *mcrA* and *Methanospirillum* transcripts (R^2=0.86 and 0.78, respectively, P<0.05). While a significant correlation could not be established, it was observed that samples that initially had lower SMA had higher *Methanosaeta* activity. Our results are in agreement with Tale et al., (2011), who reported that the rates at which different biomass samples from different full scale digesters produce methane varied widely (e.g., from <0.1 to >10 mL CH_4/gVS-hr), and the methanogen community structure significantly affected the rate and extent of methane production. Therefore, performance and community structure are not the same in all digesters and vary considerably. This is particularly important as the inoculum used for starting a new digester or for bioaugmentation of upset digesters may significantly influence the operating characteristics of a reactor. Raskin et al.,

(2000) have shown that *Methanosaeta* was the dominant aceticlastic methanogen in a variety of anaerobic reactors at low acetate concentrations , whereas, *Methanosarcina* was dominant at high acetate levels (McMahon et al., 2001). McMahon et al. (2004), investigated microbial population dynamics in anaerobic digesters treating municipal solid waste and sewage sludge during startup and overload conditions. They reported that digesters which contained high levels of *Archaea,* particularly dominated by *Methanosaeta concilii,* started successfully, whereas, digesters dominated by *Methanosarcina* had poor performance during startup.

Analysis of methanogenic activity from the biomass samples used in this study revealed that municipal sludge and softdrink bottling samples were dominated by *Methanosaeta,* whereas the brewery, lab scale, flavoring and dairy samples are dominated by hydrogenotrophic methanogens, particularly *Methanospirillum.* These results also support the findings of Karakashev et al., (2005), who reported that *Methanosaeta* was the dominant aceticlastic methanogen in digesters fed with sludge, but were never found to be dominant in digesters treating manure. Yu et al., (2005) performed quantitative real time PCR (16S rRNA gene copies) on waste activated sludge and also found that *Methanosaetaceae* was found to be dominant. They reported that operating conditions such as hydraulic retention time (HRT) and type of substrate used were important parameters for the development of a methanogenic community.

A comparison of the methanogenic community activity with the ability of the system to tolerate organic overload shows that OOPA values correlated positively with *Methansaeta* activity (R^2=0.67, p<0.05). It was also found that reactors not dominated by any particular genus, but those that had a balanced community of hydrogenotrophic and aceticlastic methanogens had a higher capacity to resist organic overload and produce methane. This was evident in MWWTP1 and lab scale reactors, which were dominated by *Methanosaeta* and

Methanospirillum, respectively, and took significantly longer time to recover from the overload shock. Previous work by McMahon et al., (2001) showed that during periods of rapid consumption of volatile fatty acids (VFAs), typically found after start up, syntrophic fatty acid oxidizing bacteria (SFAOB) were dependent on both aceticlastic and hydrogenotrophic methanogens to consume their metabolic products. The long lag periods before SFAOB began to metabolize large quantities of fatty acids may have occurred because adequate syntrophic interactions between SFAOB and methanogens have not been established (McMahon et al., 2001). Other researchers have reported that levels of propionate degrading bacterial populations are probably low in stable digesters which do not produce propionate (Xing et al., 1997a,b). The reason for the poor performance of the lab scale reactor to overload, in spite of having high methane production rate was supported by McMahon et al., (2004), who found that those digesters with a history of very stable operation may be particularly susceptible to failure during a sudden influx of organic material. This may also be due to the imbalance in the methanogen community. Fermentative bacteria can acclimate more quickly to new conditions because of their relatively high growth rates while the metabolic capacity of the methanogens, due to their population imbalance, may not be sufficient to balance the increasing activity of the fermenters (Griffin et al., 1998). Therefore, acetate and hydrogen are not consumed at the same rate they were produced and may lead to longer recovery periods.

In summary, it is critical to analyze the performance history and community composition of biomass that may be used as inoculum for start up or bioaugmentation. Our results suggest that biomass with a balance of aceticlastic to hydrogenotrophic methanogenic populations are more successful at withstanding VFA accumulation, typically encountered during start up and organic overload conditions. To further investigate our hypothesis, studies on methanogenic

population dynamics in digester systems need to be complemented with studies on population

dynamics of propionate degrading syntrophs and other syntrophic fatty acid oxidizing bacteria.

Chapter 4

Determine the response of hydrogenotrophic and aceticlastic methanogen populations in mesophilic anaerobic digesters to increasing oxygen exposure

4.1 Introduction

Methanogens are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases. They are found in a variety of anaerobic environments including tundra(Morozova and Wagner, 2007), freshwater lake and wetland sediments (Biderre-Petit et al.,2011, Earl et al., 2003), acidic peatlands (Basiliko et al., 2003; Galand et al., 2002), estuarine and marine sediments (Banning et al., 2005), paddy field soils (Chin et al., 2004; Großkopf et al., 1998), animal guts (Saengkerdsub et al., 2007), landfills (Luton, 2002) and anaerobic digesters treating animal manure, and municipal wastewater and solid waste (Angenent et al., 2002; Raskin, 1994).

During anaerobic digestion, organic matter is sequentially degraded by a complex microbial consortia to simple precursor compounds such as acetate, H_2/CO_2, formate and methanol, from which methanogenic archaea produce biogas, composed of methane and carbon dioxide (McKeown et al., 2009). Methanogens are strictly anaerobic, chemolithotrophic microorganisms (Morozova and Wagner, 2007). that can be highly sensitive to environmental perturbations (Connaughton, 2006). A sudden change in temperature, pH or an increase in organic loading rate or toxic compounds could lead to an entire system failure. An increase in fatty acids such as propionate or butyrate in an anaerobic digester can lead to a decrease in pH (Liu, 2008), which can be detrimental to methanogenesis, as most methanogens can only tolerate a pH range of 6-9 (Hori, 2006; Liu, 2008). Hori et al. (2006) also demonstrated that the composition of the methanogen community in a thermophilic anaerobic digester changed from

dominantly *Methanoculleus* species to *Methanothermobacter,* with the accumulation of volatile fatty acids (VFAs).

One of the major problems faced in anaerobic digestion process is its inherent instability and sensitivity to frequent exposure to oxygen. Most anaerobic digesters are subjected to stress by varying oxygen loading conditions as the reactors are operated in an open environment, and oxygen can enter during feeding or mixing. Because anaerobic digestion involves strictly anaerobic acetogens and methanogens, it is commonly perceived that oxygen is a toxic inhibitor and can cause slow start-ups, reactor instability, low methane yield and even total reactor failure. It has been reported that these strict anaerobes are very sensitive to oxygen as they cannot synthesize the enzymes superoxide dismutase (SOD) or catalase, used by aerobes to neutralize toxic oxygen radicals (Kato et al., 1997). Zehnder et al., (1977) demonstrated that *Methanobacterium ruminantium, M. mobile* and *Methanobacterium* strain AZ were highly sensitive to oxygen, because their growth and methane production were prevented at 0.01 ppm dissolved oxygen. Kiener et al. (1983) showed that pure cultures of the methanogenic species *Methanococcus voltae* and *M. vannielii* were highly sensitive to oxygen and were killed without any lag after exposure to oxygen, possibly due to their lack of SOD. Whereas, some methanogens like *Methanobacterium thermoautotrophicum*, *Methanobrevibacter arboriphilus* and *Methanosarcina barkeri* have the ability to produce the SOD enzyme and showed an ability to survive for hours in the presence of oxygen without a decrease in the number of colony forming units (Kato, 1997). Besides SOD, some methanogenic species, for example *Methanosarcina barkeri* strain Fusaro, was shown to have a number of redox carriers which decreased the redox potential when chemical oxidant agents were used (Kato, 1997). Therefore,

methanogens differ in their sensitivity to oxygen, varying from strict intolerance to possessing some intrinsic tolerance.

Rationale and hypothesis. The scope of this work was to provide quantitative insight into the response of methanogenic communities to exposure to oxygen. Zitomer et al., (1998) reported that although high concentrations of O_2 are toxic to methanogenic species, low doses (1 to 0.1 O_2/L-day) may even be beneficial and have been to show to increase methanogenic activity of mixed methanogenic cultures by up to 20% under some conditions. However, the influence of O_2 stress on the changes in microbial community structure due to different O_2 doses are unknown. This specific aim is based on the hypothesis that although methanogens are obligate anaerobes, they can tolerate a broad range of oxygen exposure and thus, these digesters have a significant capacity to maintain stable performance. However, it is unknown at this point whether oxygen exposure affects only methane production capacity, or survival of methanogens or both. Therefore, the aim of this study was to quantitatively investigate how increasing concentration of oxygen can affect the methanogenic community structure and function. This hypothesis was tested by:

a. Extracting DNA and RNA from digesters fed varying doses of oxygen and performing quantitative PCR (qPCR) and quantitative reverse transcriptase PCR (qRT-PCR) using *mcrA* and 16S rRNA genus specific primers on DNA and RNA, respectively.

b. Estimating the diversity of methanogen community structure using denaturing gradient gel electrophoresis (DGGE) of *mcrA* genes.

c. Comparing qPCR results to methane production rates for biomass from each digester.

4.2 Results

4.2.a Quantification *mcrA* gene copies and transcripts. The total *mcrA* gene copies and transcripts (Fig.4.1.a&b) were calculated and expressed per volume of biomass. The metabolically active fraction of the methanogenic community was analyzed by quantifying the *mcrA* transcripts in reactors PR1-PR4, each receiving different doses of oxygen (0, 1.3, 6.7 and 12.5%. of COD, respectively). The *mcrA* transcript numbers ranged between $6.50 \times 10^6 \pm 1.70 \times 10^6$ and $1.07 \times 10^8 \pm 4.79 \times 10^6$. PR1 had significantly lower transcripts compared to all the other reactors that received oxygen ($p < 0.05$, ANOVA, Tukey test). Amongst the reactors exposed to varying concentrations of oxygen, PR4 had significantly greater *mcrA* transcripts/mL biomass ($p < 0.05$, ANOVA, Tukey test). A similar trend was also observed among *mcrA* gene copies, where PR1 had significantly lower gene copy numbers when compared to the reactors that received oxygen. Transcript:gene abundance ratios (Fig 4.1.c) were between 15 and 143 transcripts of *mcrA* per gene and were significantly greater in biomass from PR1 ($p < 0.05$, ANOVA, Tukey test).
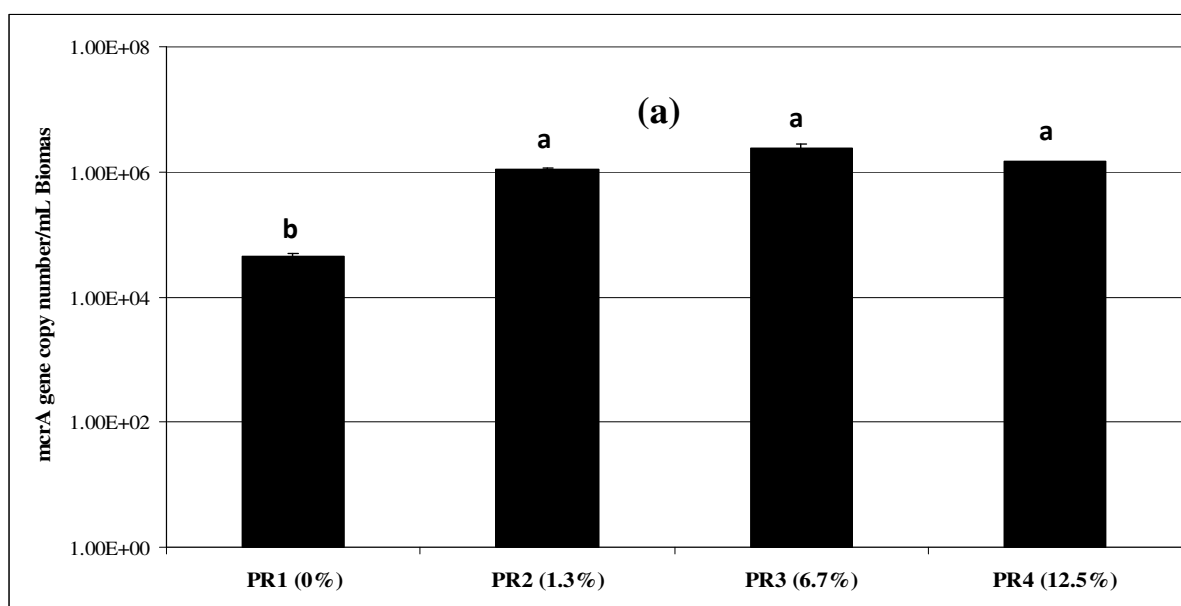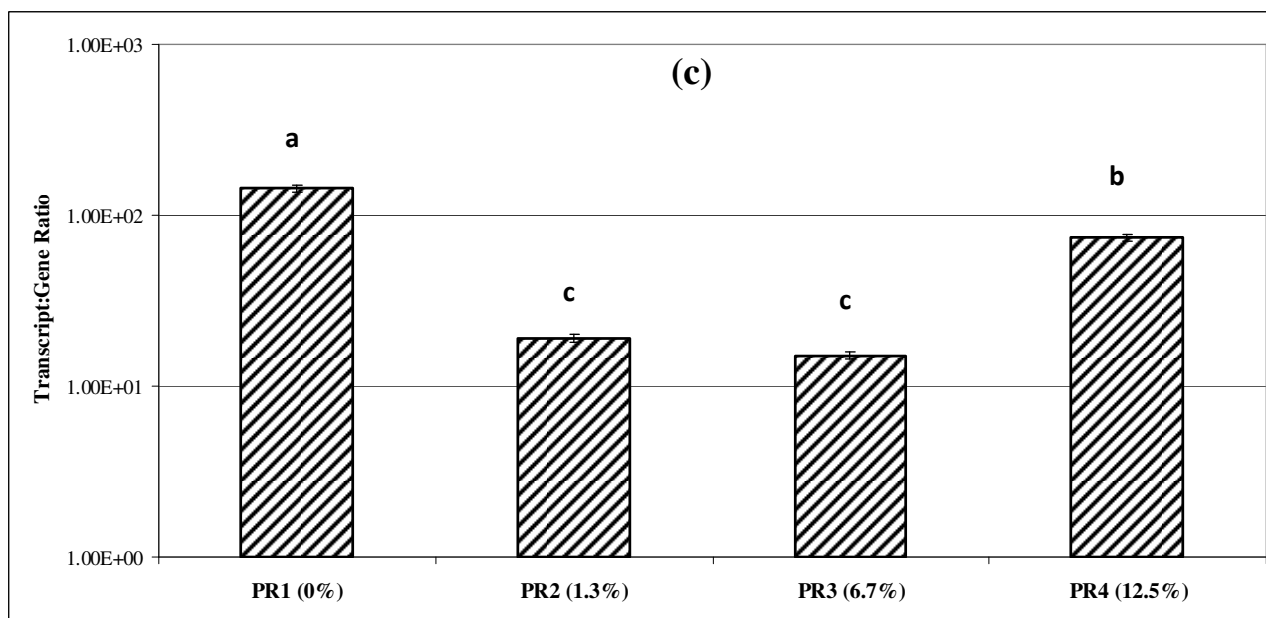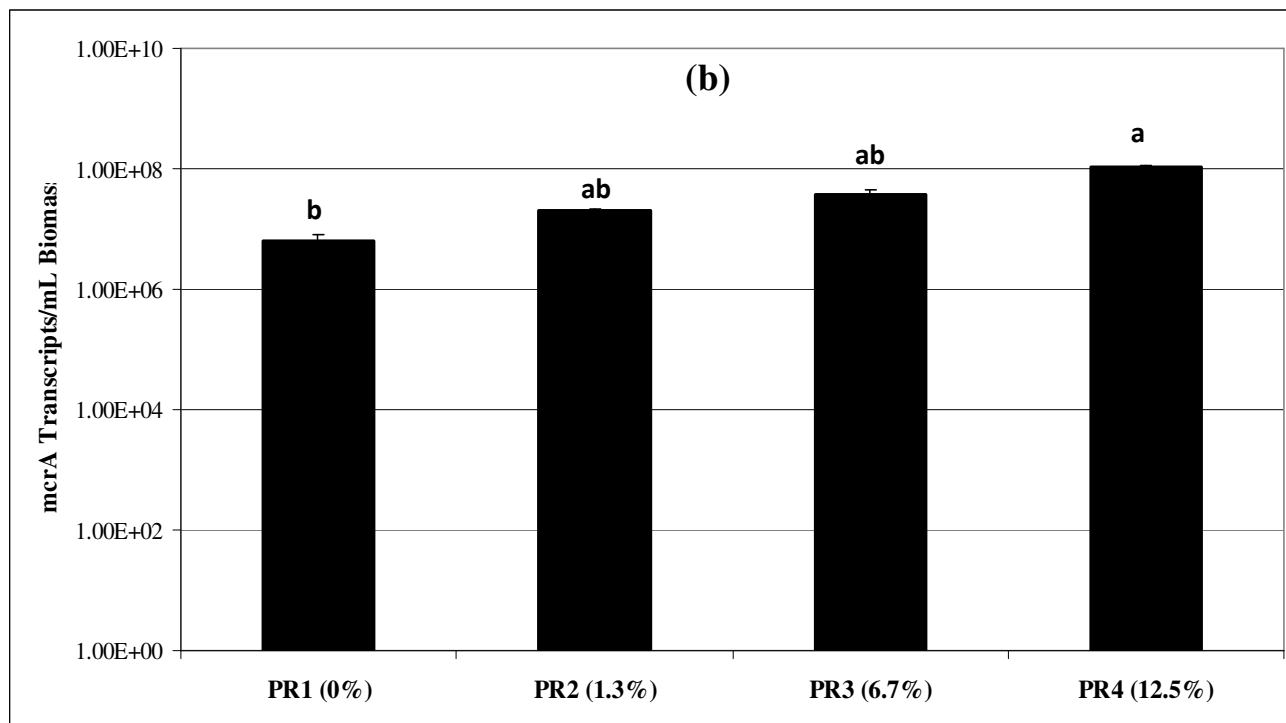
Fig 4.1. Results of qPCR experiment. Quantification of **(a)** *mcrA* gene copy number **(b)** *mcrA* transcripts, **(c)** transcript:gene ratio. Each bar represents results from two technical replicates. Error bars show standard deviation from the mean. Statistical differences ($P<0.05$) tested by Tukey test among different biomass samples are indicated by different letters. Similar letters indicate no significant differences among the groups.

4.2.b Quantification of 16S rRNA gene copies and transcripts. Gene copy numbers and transcripts of the 16S rRNA gene were quantified using 16S rRNA genus specific primers for the genera *Methanospirillum, Methanosaeta (*Franke-Whittle et al., 2009) and *Methanoculleus* (Rowe et al., 2008). *Methanosarcina* levels were either very low or undetected in most samples, and therefore have not been used in this study.

The 16S rRNA transcripts (Fig 4.2.b) for *Methanospirillum* were estimated to be between 2.91×10^{11} and 1.92×10^{12} .They were significantly higher in PR2 and PR3 compared to PR1 and PR4 ($p<0.05$, ANOVA, Tukey test). *Methanoculleus* transcripts were estimated to be between 1.76×10^{8} and 9.04×10^{9} transcripts/mL biomass and there was a significant decrease in its activity at higher oxygen concentrations (PR3 and PR4). *Methanosaeta* were estimated to be between 1.62×10^{10} and 9.5×10^{10} transcripts/mL biomass and were significantly higher in biomass from PR4 ($p<0.05$, ANOVA, Tukey test). A similar trend was observed among 16S rRNA gene copy numbers.

The transcript:gene ratio (Fig 4.2.c) was significantly higher for *Methanoculleus,* with 69-79 16S rRNA transcripts/gene ($p<0.05$, ANOVA, Tukey test), while *Methanospirillum* and *Methanosaeta* were estimated to be 16-21 and 11-16 16S rRNA transcripts/gene, respectively

Fig 4.2. qPCR results. Quantification of **(a)**16S rRNA gene copy number **(b)** 16S rRNA transcripts, **(c)** transcript:gene ratio Each bar represents results from two technical replicates. Error bars show standard deviation from the mean. Statistical differences (P<0.05, ANOVA, Tukey) among different biomass samples are indicated by different letters [*Methanospirillum* (A-B), *Methanoculleus* (a-b) and *Methanosaeta* (*a-b*)]. Letters of similar case and style indicate no significant differences among the groups

(a)

(b)

(c)
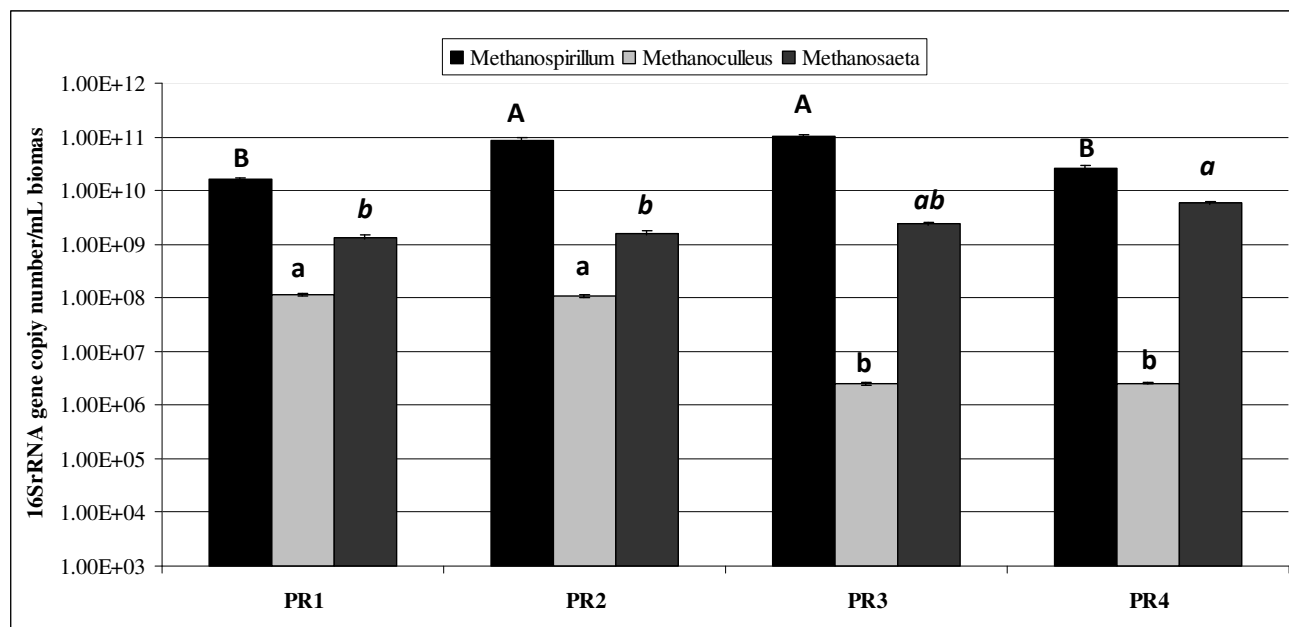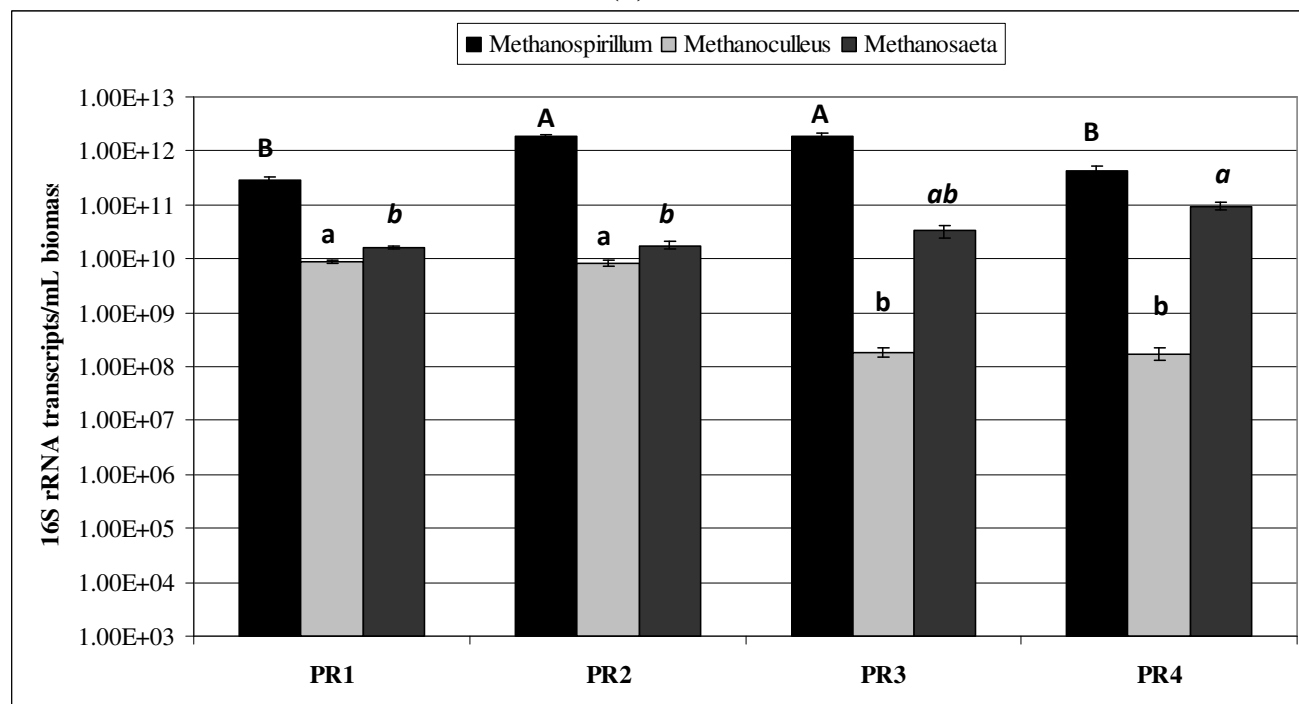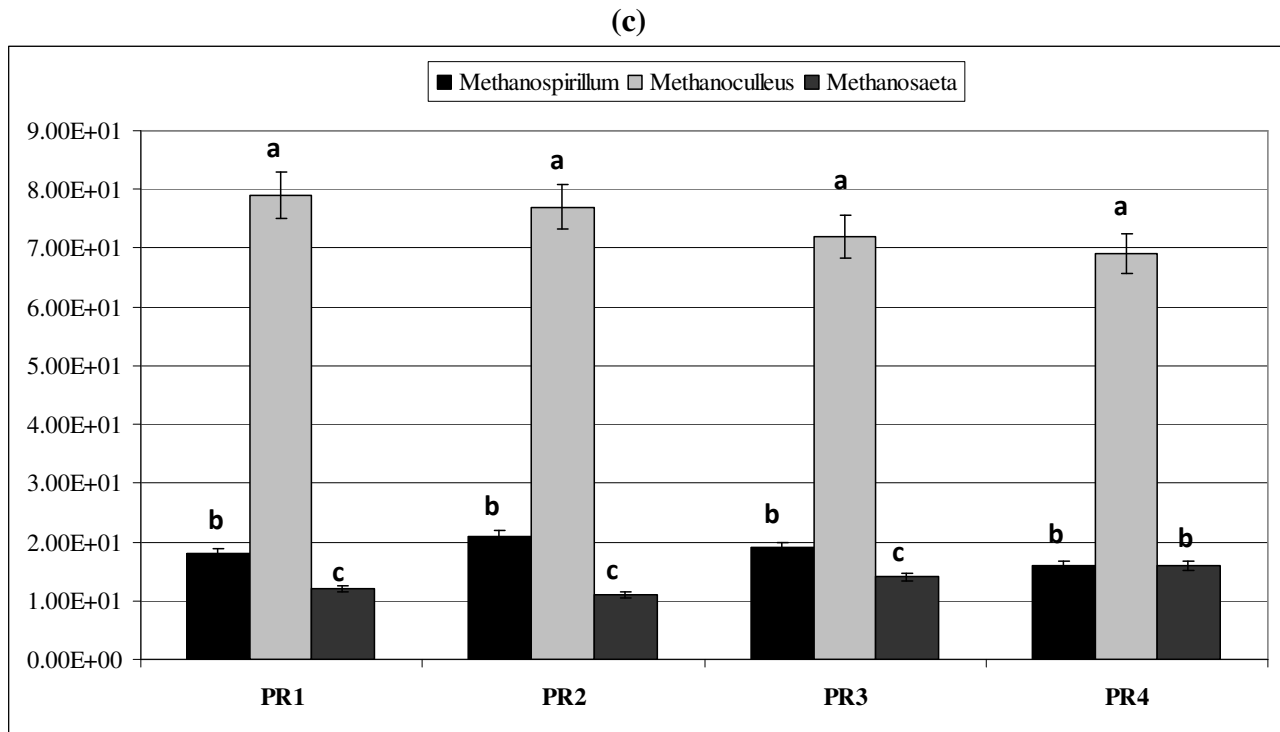
4.2.c Anaerobic digester performance

(i) Specific Methanogen Activity. SMA assays were used to compare the biomass samples ability to produce methane, given a particular substrate. The SMA assays for the reactors receiving increasing oxygen doses (Fig.4.3.a) showed a linear relationship between methane production rate and oxygen dosage. PR1 and PR2 had statistically similar SMA values, whereas PR4 had the highest methane production rate. The average cumulative methane production was estimated to be between 7 ± 0.3-15.3 mL CH_4/h*gVSS).

(ii) Organic Overload Perturbation Assay (OOPA). OOPA was performed to demonstrate the reslience of the anaerobic system to organic overload. This was measured based on the time taken by the system to produce 66.7% of the maximum biogas after an overload shock with

glucose. As shown in Fig. 4.3.b, the samples that received oxygen took significantly longer time to produce 2/3rd maximum biogas, ($p<0.05$, ANOVA, Tukey test). There was no significant difference between the average biogas production after 20 days among the samples.

4.2.d Relationship between methanogenic activity and performance. *mcrA* and *Methanosaeta* transcripts (Fig 4.4. a&b) correlated positively with SMA results against propionate ($R^2 =0.74$ and 0.76 respectively, $p<0.05$, F ratio test of ANOVA). However, there was no correlation between *Methanospirillum* and *Methanoculleus* transcript number and SMA. A positive correlation was also established between *mcrA* and *Methanosaeta* activity (Fig 4.4. c&d) and resilience of the system to organic overload ($R^2 =0.86$ and 0.63, respectively, $p<0.05$, F ratio test of ANOVA).

Fig.4.3. (a) SMA. Average cumulative CH4 production rate for calcium propionate assays. **(b) OOPA.** Average time taken to produce 66.7% max biogas and average biogas produced in 20days after organic overload. Error bars represent the standard deviation of 3 replicates. Statistical differences (*P<0.05*) tested by Tukey test among different biomass samples are indicated by different letters. Letters of similar case and style indicate no significant differences among the groups.
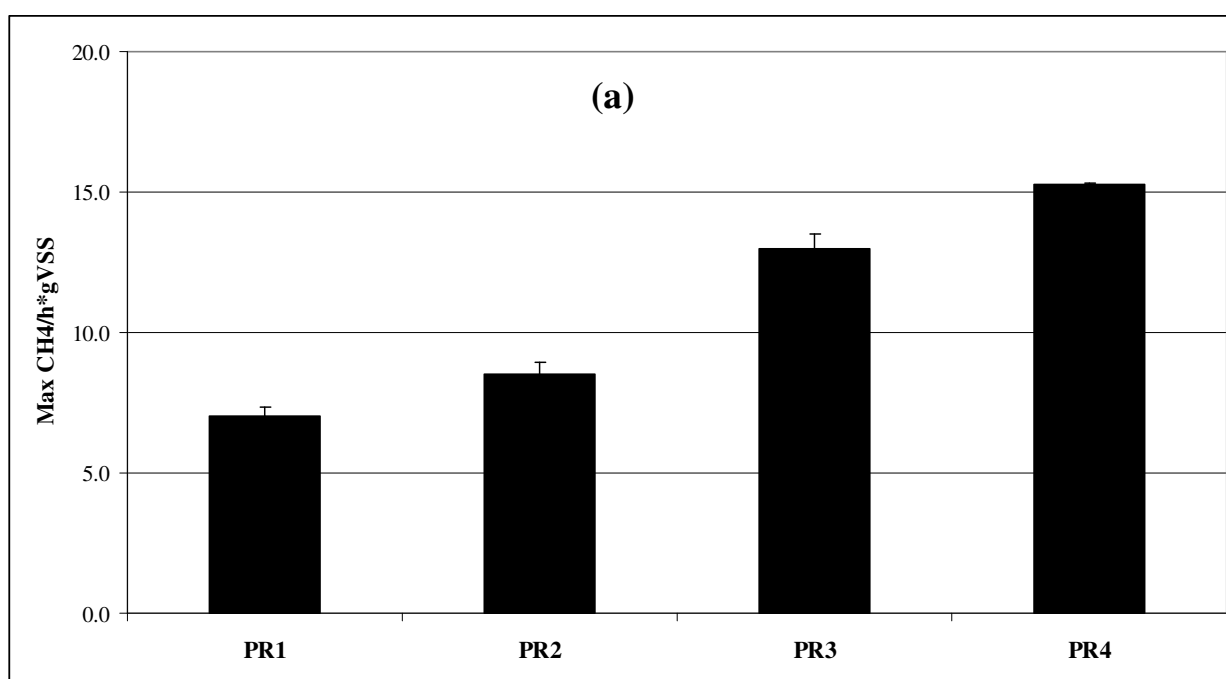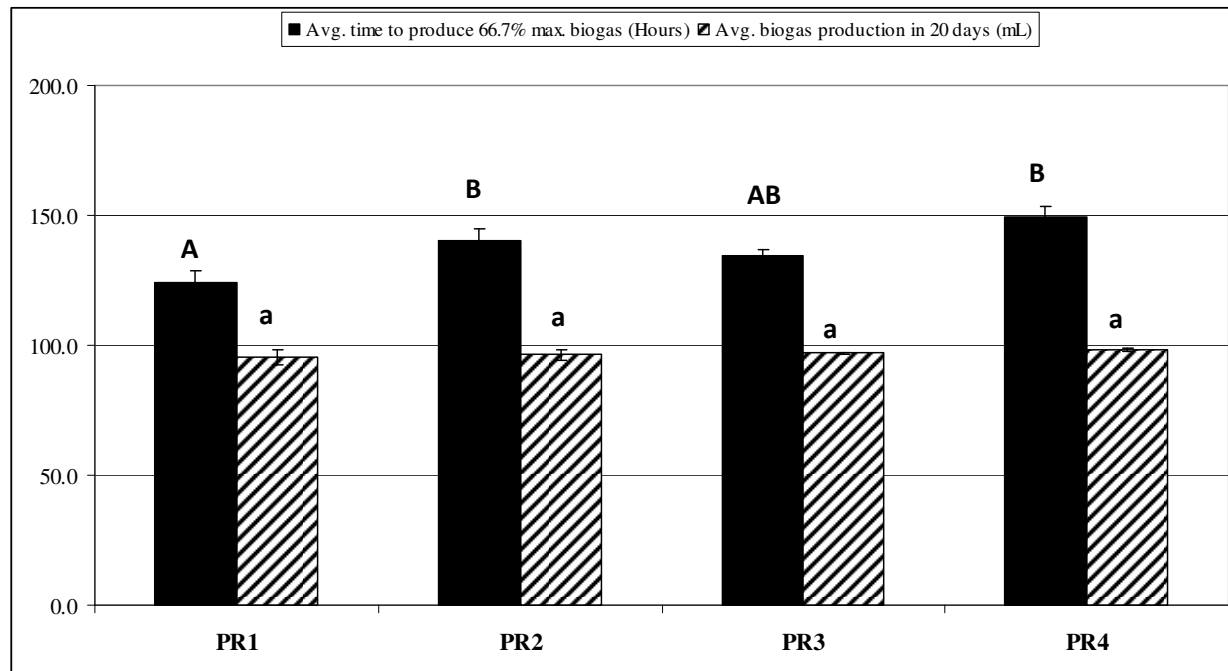
(b)

4.2.e Methanogen Community Structure in anaerobic reactors. DGGE provides a genetic fingerprint of the microbial diversity based on physical separation of unique nucleic acid sequences. The analysis of the methanogen community structure in biomass samples for the *mcrA* gene (Fig 4.5) showed distinctive profiles. A matrix was constructed using the presence (1) or absence (0) of a band in each sample. Each significant band was assumed to represent one phylotype of methanogen. The most dominant bands in all reactors were affiliated to *Methanospirillum* (B1), *Methanobacterium* (B2), *Methanoculleus* (B3), *Methanosaeta* (B). Bands B5, B6, B7 and B8 were detected only in the samples that received oxygen. B7 was identified to belong to *Methanosarcina.* The other bands could not be identified due to difficulties in excising the bands.
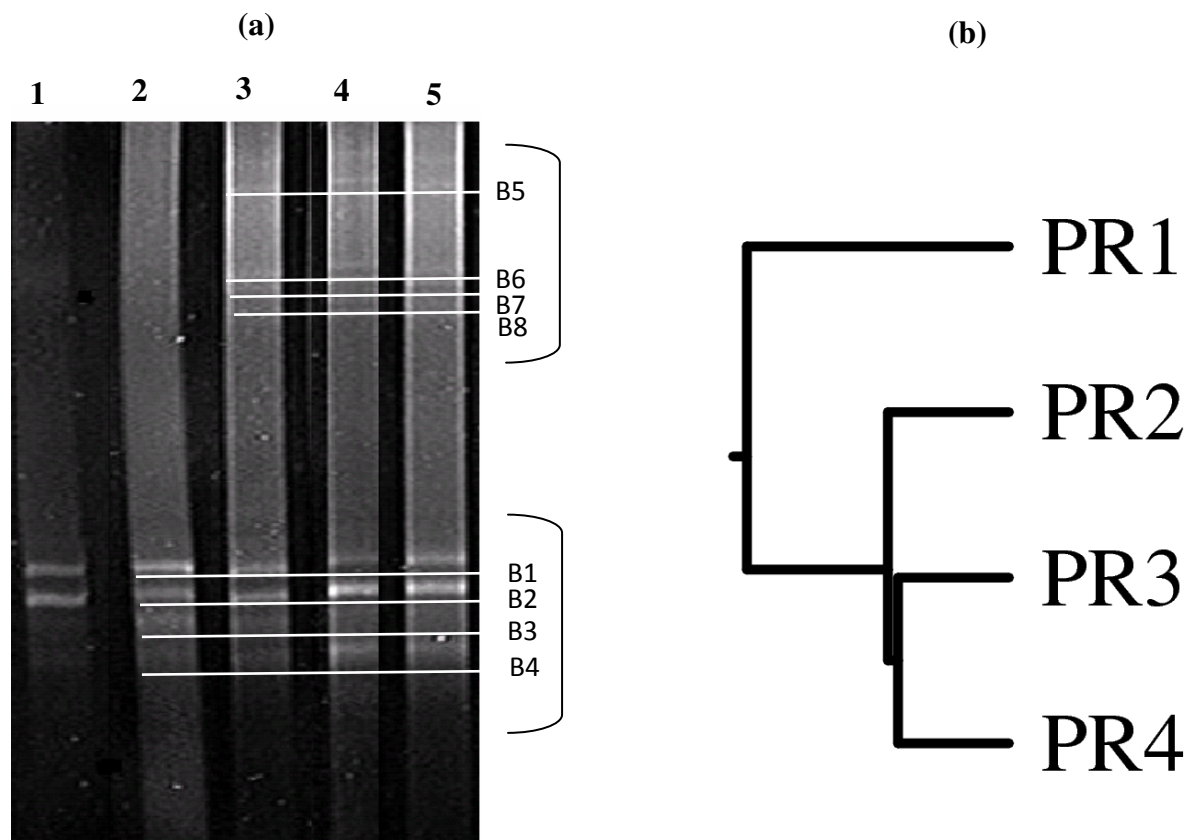
Fig.4.5.DGGE of *mcrA*: (a) DGGE fingerprint of *mcrA* genes present in biomass samples. Lanes represent **(1)** Ladder, **(2)** PR1 (0% Oxygen), **(3)** PR2 (1.3% Oxygen), **(4)** PR3 (6.7% Oxygen), **(5)** PR4 (12.5% Oxygen) **(b)** Dendrogram showing relationship between methanogen communities in based on distance matrix using Jaccard coefficient.

4.3 Discussion. As mentioned before, exposure of methanogens to oxygen during anaerobic digestion is problematic, given that they have been physiologically categorized as obligate anaerobes (Kato, 1997). Exposure of anaerobic digesters to minute and varying aerobic loading rates of oxygen may be unintentional and unavoidable as the reactors are operated in an open environment, and oxygen may enter during feeding or mixing. While comprehensive mechanistic models have been developed to understand inherent instability of digesters, these models do not include all necessary aspects to accurately predict the behavior of digesters when exposed to an inhibitor, such as oxygen. Therefore, an understanding of the effects of such aeration on

microbial community structure and dynamics in anaerobic process is a basic requirement to optimize anaerobic digestion for increased renewable energy production. To the best of our knowledge, this is the first study to provide quantitative insight into the behavior of methanogenic communities upon exposure to oxygen.

The results from our study show that methane production occurred in the presence of up to 12.5% added O_2. qPCR analyses of 16S rRNA revealed that *Methanoculleus* had significantly lower activity, while *Methanospirillum* (PR2 and PR3) and *Methanosaeta* (PR4) had significantly higher activity at higher oxygen concentrations. These results demonstrate that methanogens have variable resistance to oxic conditions, which maybe due to the activity of detoxification enzymes (Liu et al., 2008). Though, the presence of detoxification enzymes in the methanogens used in this study has not been reported in published works, the existence of these enzymes has been inferred from homology.

DGGE of *mcrA* rDNA showed that the structure of the methanogenic archaeal community also changed with increasing oxygen exposure. With increasing oxygen concentration, it was noticed that a second cluster including *Methanosarcina* emerged. This finding is in agreement with Angel et al., (2011), who reported the discovery of *Methanosarcina* in biological soil crusts. They showed that *Methanosarcina* was not only able to tolerate long periods of desiccation in arid soil, but also became metabolically active after wetting even in the presence of oxygen. This maybe due to the presence of 6 different putative genes whose function is associated with detoxification of ROS (Angel et al., 2011). Brioukhanov et al., also reported that the enzyme activity and transcriptional levels of SOD and catalase are up-regulated, and play a significant role in the protection of *Methanosarcina* against the toxic effects of ROS. However, these results contradict the findings of Yuan et al., (2008), who reported that the

structure of the methanogenic archaeal community remained stable and that oxygen stress did not differentially affect the various methanogenic populations, but inhibited CH_4 production.

Therefore, oxygen can have multifaceted effects in anaerobic digestion and the overall impact of aeration on methane yield thus depends on the resultant of these diverse biochemical and physiochemical reactions involving oxygen. Unfortunately, it is still not clear how methanogens survive the presence of oxygen, whether it is due to (i) intrinsic tolerance due to production of enzymes that neutralize toxic free radicals, (ii) aerobic oxidation, where oxygen is consumed or (iii) due to the presence of oxygen free micro niches, where methanogens survive and produce CH_4. However, through this study it is clearly understood that the sensitivity of methanogens to oxygen may not necessarily mean that the effect is bactericidal, but maybe bacteriostatic and digesters have a significant capacity to maintain stable performance under a broad range of oxygen input.

Chapter 5

Changes in methanogen community structure and activity following freeze drying of anaerobic sludge

5.1 Introduction. Methane is produced in a wide variety of highly reduced anaerobic

environments, from peat bogs, rice field soils, deep sea hydrothermal vents and permafrost to the

digestive tracts of animals (Florin et al., 2000; Horikoshi, 1999; McDonald et al., 1999;

Morozova and Wagner, 2007). Methane is the third most important greenhouse gas on Earth and

has 26-41 times the global warming potential of CO_2 (Angel et al., 2011). During anaerobic

digestion, organic matter is sequentially degraded by a complex microbial consortia to simple

precursor compounds such as acetate, H_2/CO_2, formate and methanol, from which methanogenic

archaea produce biogas, composed of methane (CH_4) and carbon dioxide (CO_2) (McKeown et

al., 2009). Methanogens are strictly anaerobic, chemolithotrophic microorganisms and are highly

abundant in extreme environments tolerating low/high temperatures, extreme salinity and

low/high pH (Morozova and Wagner, 2007).

Methanogens are slow growing organisms and require significant time to reproduce.

Therefore, it would be beneficial if various defined methanogenic cultures could be preserved for

application in anaerobic processes such as seeding or reseeding and bioaugmentation of full scale

digesters, to increase biogas production and process stability. The use of wet cultures for this

process is typically associated with difficulty in handling high volumes, distribution cost and

culture aging during storage. Therefore, there is a need to develop methods to economically

transport and store biomass without significant loss of viability. Preservation methods such as

refrigeration, freezing and freeze-drying/cryodesiccation are routinely used with anaerobic pure

cultures (Castro et al., 2002). While all drying techniques are associated with their own set of

advantages and disadvantages and varying loss in cell viability, freeze drying is particularly beneficial for preserving heat sensitive microorganisms due to the use of ultra low temperature drying.

However, during freeze drying, methanogens would be exposed to storage of viable cells in a desiccated state, which may impose physiological constraints that many species cannot tolerate. Some known desiccation tolerant microorganisms such as spore forming bacteria, heterocyst forming cyanobacteria, heteropolysaccharide forming *Deinococcus and Beijerinckia* synthesize an outer cell layer composed of extracellular polysaccharide (EPS) which enables the cells to retain the minimal intracellular water activity required for survival (Potts, 1994; Tamaru et al., 2005). So far, there is very little information available on the effect of subzero temperatures coupled with exposure to air, low water and nutrient availability on methanogens.

Bioaugmentation is defined as the practice of adding specific microorganisms to a system to enhance a desired activity (Rittmann and Whiteman, 1994). Bioaugmentation has been explored to improve start up of new digesters, odor reduction and recovery after organic overload (Duran, 2006; Saravanane, 2001). It was previously reported that acclimation of the microbial community to substrates they are used to treat or that may be toxic to them, showed potential for better degradation in an anaerobic digester (Hobson and Wheatley, 1993; McMahon et al., 2004). Schauer-Gimenez et al., (2010) investigated bioaugmentation as a method to decrease the recovery period of anaerobic digesters exposed to a transient toxic event. They bioaugmented two digesters inoculated with municipal wastewater solids and industrial wastewater with an H_2-utilizing culture and found that bioaugmentation is a useful tool to decrease recovery time and propionate concentration, while increasing biogas production after a toxic event.

The aim of this study was to investigate the response and recovery of diverse methanogens in anaerobic biomass to low temperature desiccation and oxygen stress faced during freeze drying. This investigation will lead to better understanding of the sensitivity of different groups of methanogens to stress faced during drying and this information can be useful to improve their preservation.

Hypothesis. This study is based on the hypothesis that the response and recovery of methanogenic archaea to freeze drying and subsequent rehydration varies based on the characteristic properties of the genus and also growth conditions prior to freeze drying. Therefore, the overall objectives of this study were to evaluate **(I)** the ability of methanogens to endure freeze drying, as well as **(II)** the role of acclimation to oxygen prior to freeze drying in improving recovery of methanogens from damage caused by oxic cryodesiccation.

5.2 Results

(I) Ability of methanogens to endure freeze drying

5.2.a Quantification of *mcrA* genes and transcripts. The methanogenic community in the biomass was analyzed before and after freeze drying. The total *mcrA* gene copies and transcripts were calculated and expressed per volume biomass. After reconstitution of the freeze dried samples, *mcrA* gene copy number was estimated to be 1.1×10^6 copies/mL biomass, which accounted to approximately $61 \pm 10\%$ of the fresh biomass (Fig.5.1.a). Similarly, the metabolically active fraction of the community analyzed by estimating the number of *mcrA* transcripts and was found to be 2.8×10^8 transcripts/mL biomass, which accounted to approximately $68 \pm 18\%$ of the fresh biomass (Fig.5.1.b). There was 99% correlation between *mcrA* gene copies and transcripts ($p<0.05$).

Transcript:gene abundance ratios were greater in freeze dried biomass, with 2.51×10^2 transcripts per gene for *mcrA,* whereas fresh biomass had 2.22×10^2 *mcrA* transcripts per gene (Fig.5.1.c).
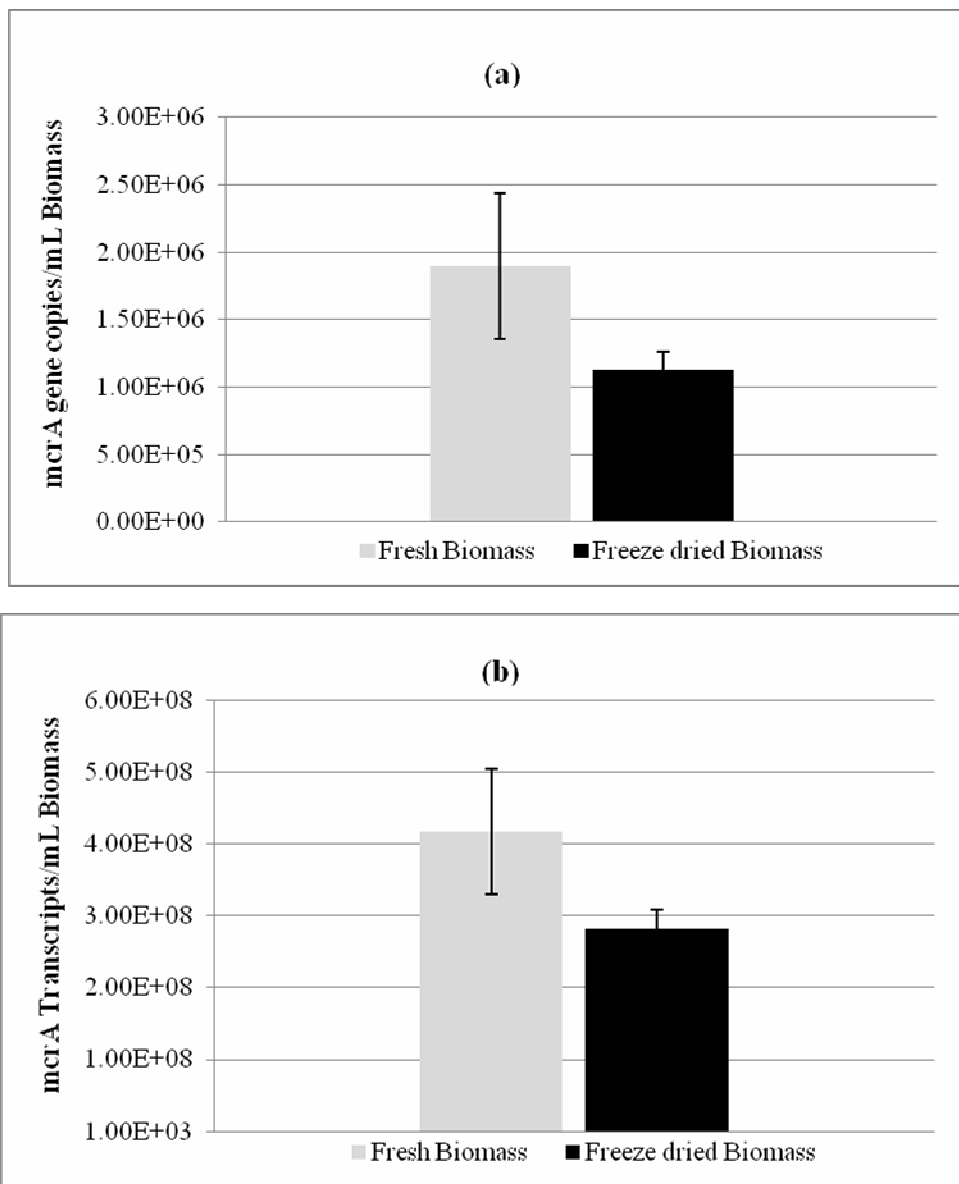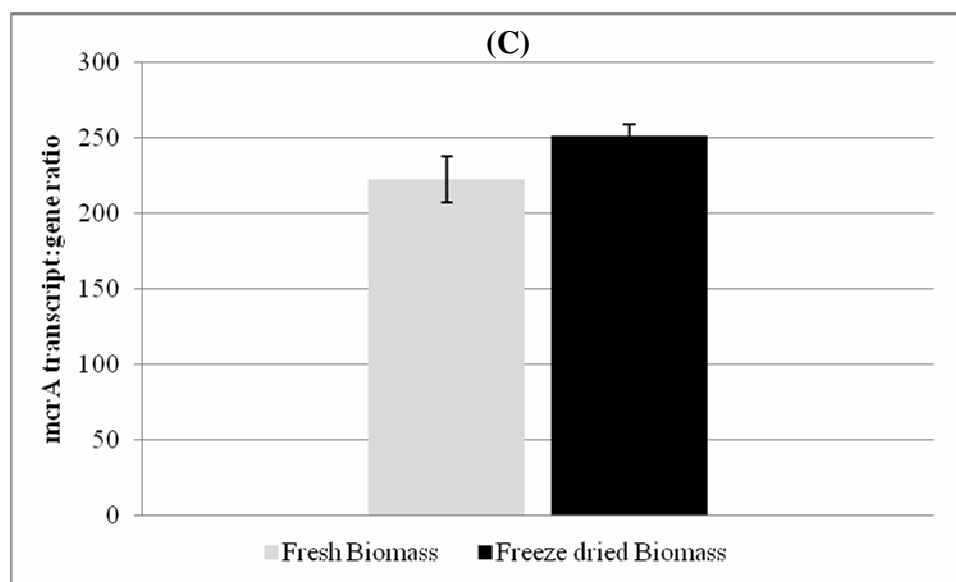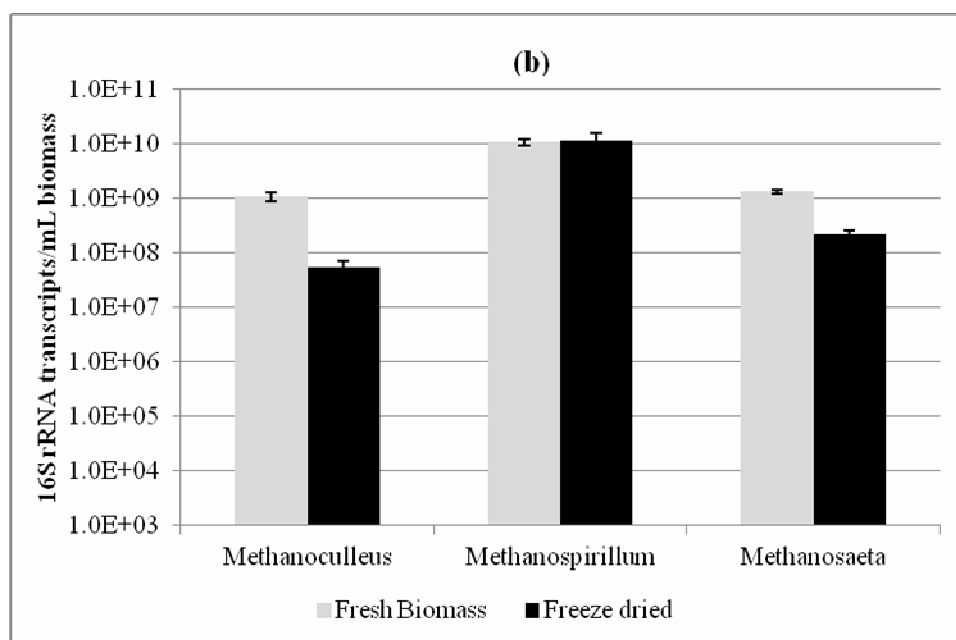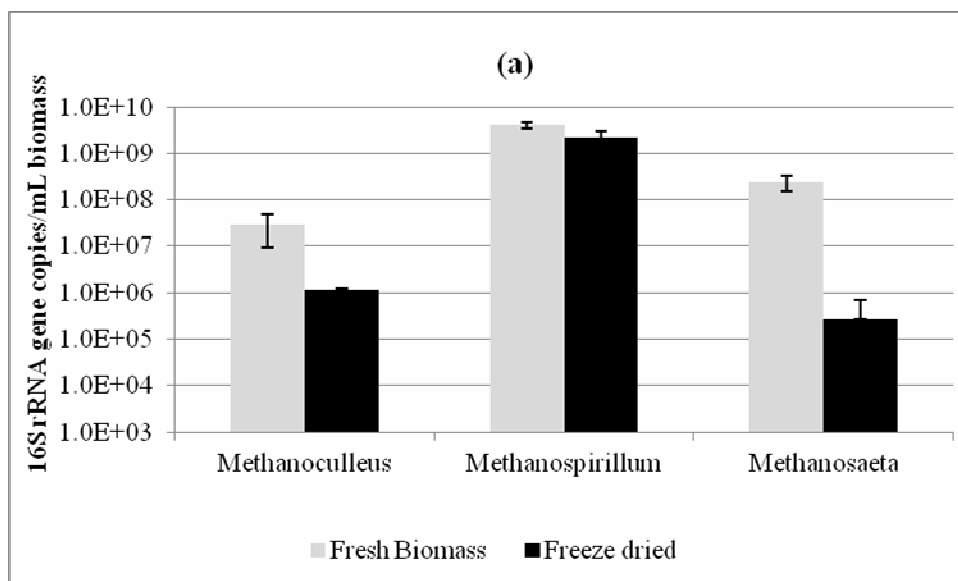
Fig.5.1. qPCR results. Quantification of **(a)** *mcrA* gene copies and **(b)** *mcrA* transcripts and **(c)** mean *mcrA* transcript:gene ratio, before and after freeze drying based on biomass volume.

5.2.b Quantification of 16S rRNA gene copies and transcripts. The 16S rRNA gene copies and transcripts were quantified using 16S rRNA genus specific primers for the genera *Methanospirillum, Methanosaeta (*Franke-Whittle et al., 2009) and *Methanoculleus* (Rowe et al., 2008). These genera were selected based on *mcrA* clone library analysis by Morris (2011) (data not shown). The *mcrA* clones were related to *Methanospirillum* (85%)*, Methanoculleus* (5%), *Methanosaeta* (3%), and unknown (7%).

The 16S rRNA gene copies for *Methanospirillum, Methanoculleus* and *Methanosaeta* were estimated to be 2.19×10^9, 1.13×10^6 and 2.72×10^5 gene copies/mL freeze dried biomass, respectively (Fig.5.2.a), whereas the transcripts were estimated to be 1.15×10^{10}, 5.38×10^7, and 2.17×10^8 /mL freeze dried biomass, respectively (Fig.5.2.b). This accounted for 53.5±13.5%, 39.6±7% and 11.3±4% based on gene copies and 98±4.3%, 4.92±2% and 16.4±2.7% based on transcript copy value of dried biomass to wet biomass, respectively.

Transcript:gene abundance ratio of *Methanosaeta* was significantly greater in freeze dried biomass (ANOVA, *P*<0.05). There were 6 16S rRNA transcripts of *Methanospirillum*, 41 of *Methanoculleus* and 1080 transcripts of *Methanosaeta* per cell, respectively (Fig.5.2.c).
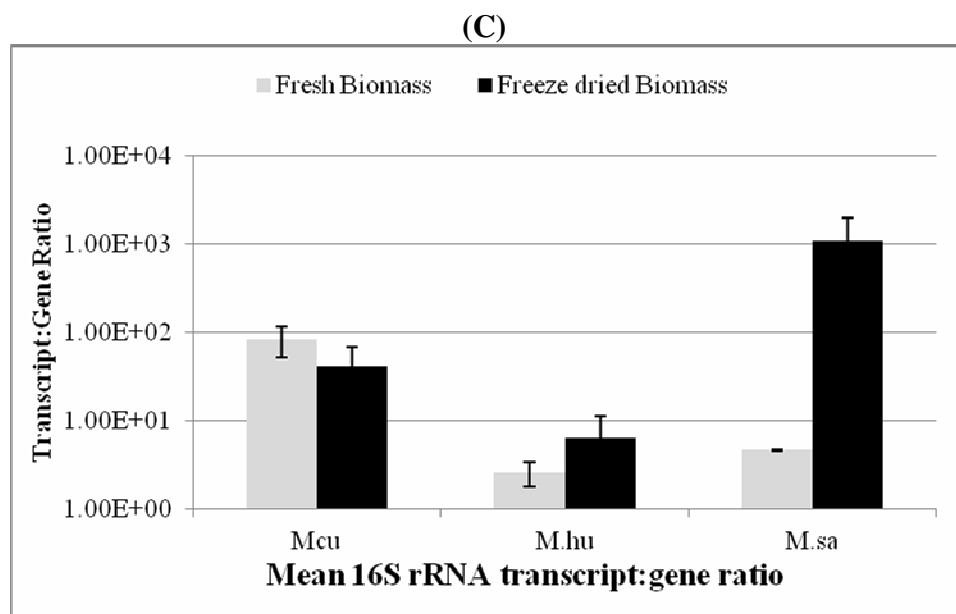
(C)

Fig.5.2. qPCR Results. Quantification of **(a)** 16S rRNA gene copies and **(b)** transcripts and **(c)** mean 16S rRNA transcript:gene ratio, before and after freeze drying based on biomass volume.

5.2.c *mcrA*-DGGE Analysis. Fig. 5.3 shows the DGGE image for the methanogen community composition in fresh and freeze dried biomass. A matrix was constructed using the presence (1) or absence (0) of a band in each sample. Each significant band was assumed to represent one phylotype of methanogen. The presence of the same band in different sample lanes indicated the presence of that particular phylotype in both samples. The methanogenic communities detected by DGGE were highly conserved throughout replicates and treatments. The most dominant bands were closely affiliated to *Methanobacterium beijingense* (B1), *Methanospirillum hungatei* (B2), *Methanosaeta concilli* (B3) and *Methanoculleus spp.* (B4).
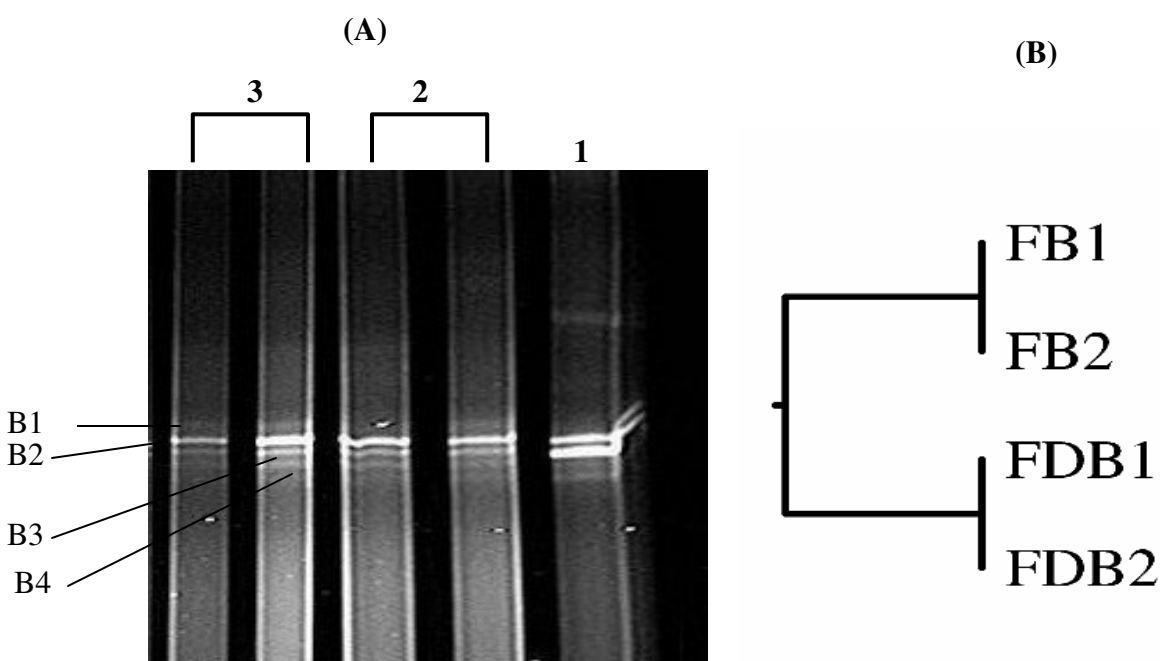
Fig.5.3. DGGE of *mcrA*: (A) DGGE fingerprint of *mcrA* genes present in fresh and freeze dried biomass samples. Lanes represent **(1)** Ladder, **(2)** Fresh biomass (FB1&2), and **(3)** Freeze dried biomass (FDB1&2). **(B)** Dendrogram showing relationship between methanogen communities in based on distance matrix using Jaccard coefficient.

5.2.d Specific Methanogenic Activity Assay. The activity lag, which is a measure of time required for obtaining maximum methane production, was significantly longer for dried biomass compared to fresh biomass. After freeze drying, the reconstituted biomass produced significant amount of methane from $H_2:CO_2$ and acetate. The SMA for $H_2:CO_2$ was estimated to be 7 ± 0.5 mL CH_4/gVSS-h for freeze dried biomass, which accounted for $65\pm5\%$ of the SMA from fresh biomass. The average activity lag measured was 152 hr for freeze dried biomass, compared to 21 hr for fresh biomass (Fig.5.4.a).

SMA values of freeze dried biomass for calcium acetate assays were 1 ± 0.3 mL CH_4/gVSS-h, which accounted for $41\pm10\%$ of the SMA from fresh biomass. The average activity lag (450 hr) for freeze dried biomass with calcium acetate as the substrate was significantly longer (ANOVA, $P<0.05$), compared to a lag of 37 hr for the fresh biomass (Fig.5.4.b).

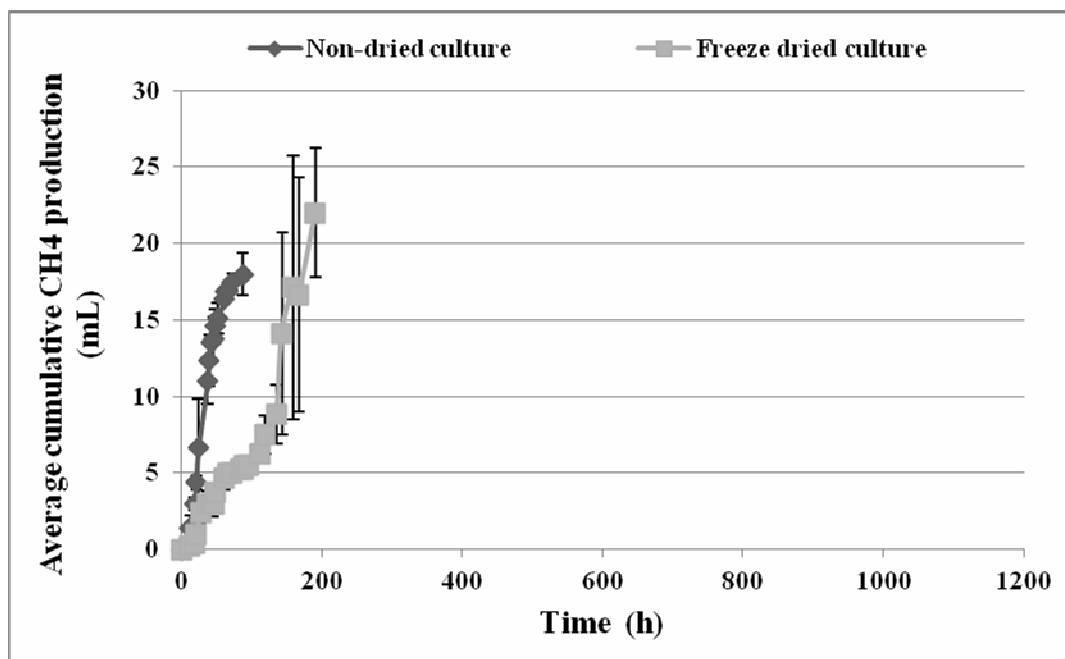**Fig.5.4.a. Specific Methanogen Activity Assay.** Average cumulative CH$_4$ production rate for H$_2$:CO$_2$ before and after drying in air. Error bars represent the standard deviation of 4 replicates.
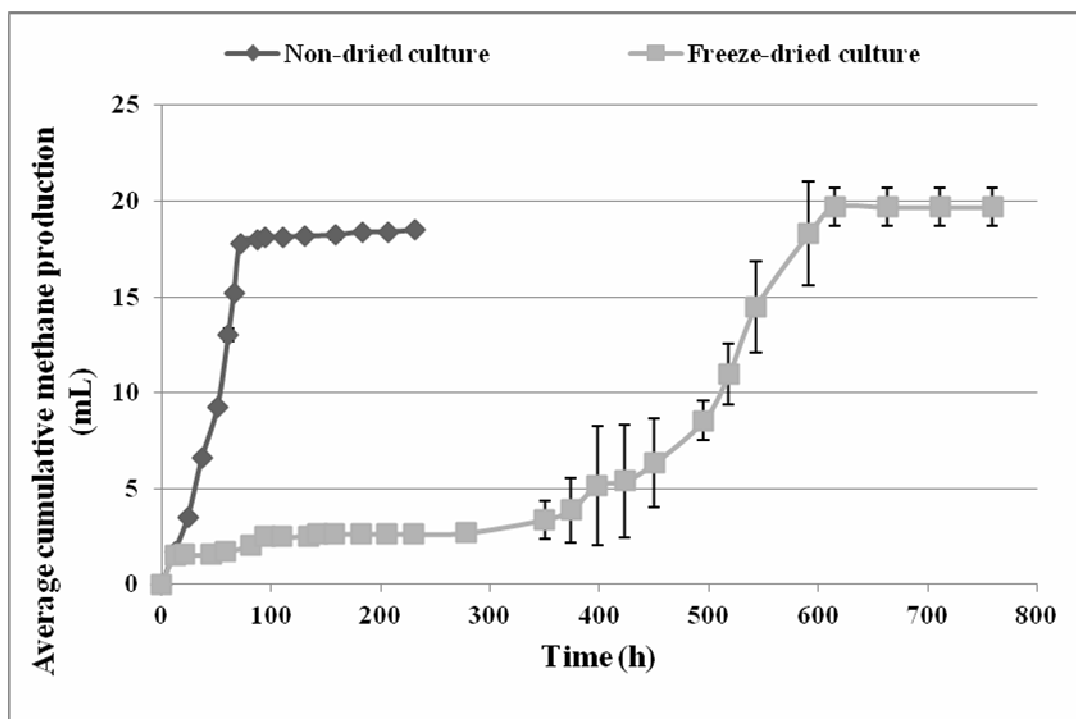

Fig.5.4.b. Specific Methanogen Activity Assay. Average cumulative CH4 production rate for calcium acetate assays before and after drying. Error bars represent the standard deviation of 4 replicates.

(II) Preservation of Hydrogen-utilizing culture by freeze drying

5.3.a Quantification of *mcrA* genes and transcripts. The effect of freeze drying was studied on H_2-utilizing cultures acclimated to (1) hydrogen and carbon dioxide [HR1] and (2) hydrogen, carbon dioxide and oxygen [HR2]. The total *mcrA* gene copies and transcripts were calculated and expressed per volume biomass (Fig.5.5 a&b). The biomass from culture HR2 had significantly greater abundance of *mcrA* gene copy and transcript numbers than biomass from culture HR1, both before and after freeze drying (ANOVA, $P<0.05$). However, there was no significant difference in the transcript:gene abundance ratios (ANOVA, $P<0.05$) (Fig.5.5.c).
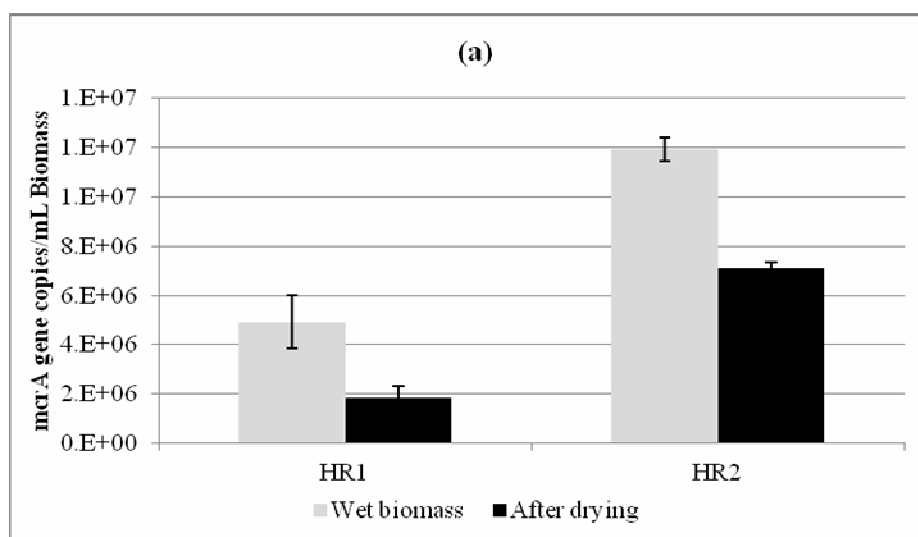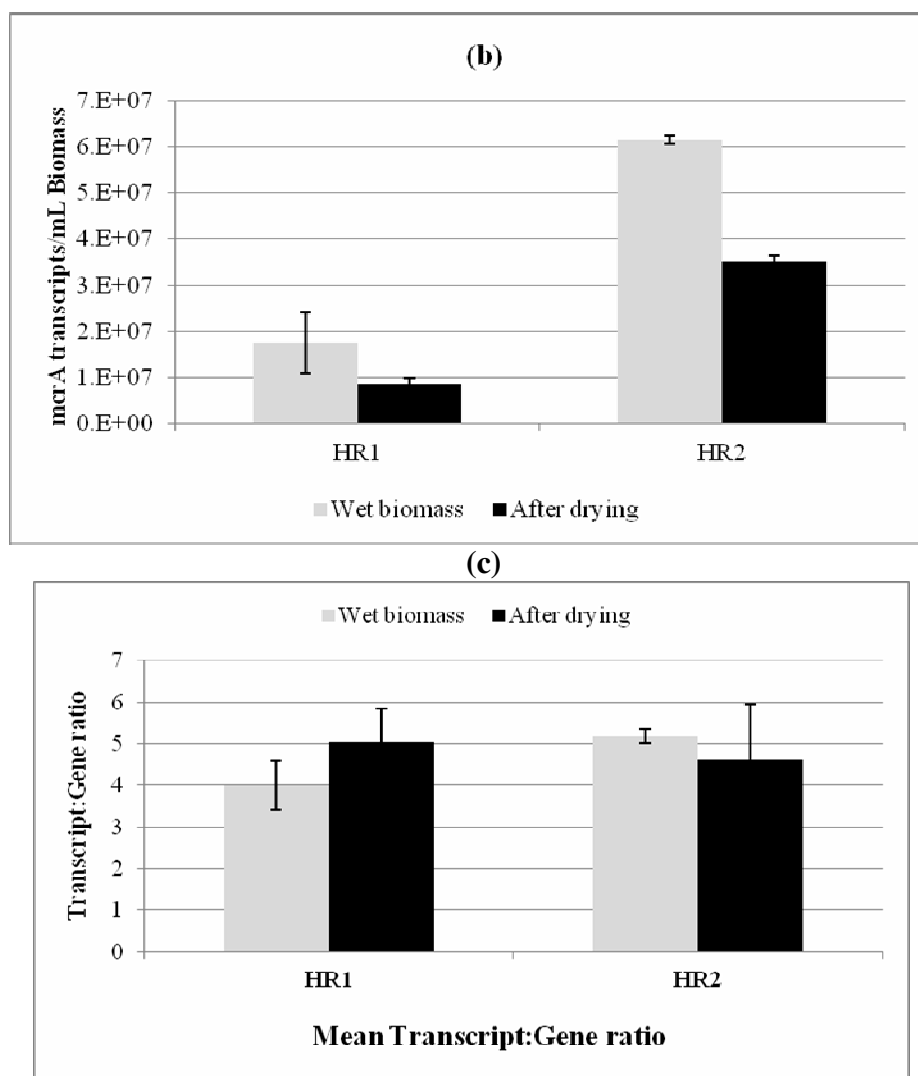
Fig.5.5. qPCR results. Quantification of **(a)** *mcrA* gene copies and **(b)** *mcrA* transcripts, **(c)** mean *mcrA* transcript:gene ratio, before and after freeze drying based on biomass volume.

5.3.b Specific Methanogen Activity Assay. SMA assays were used to compare the biomass samples' ability to produce methane given H_2:CO_2. The SMA values for enrichment cultures (HR1 and HR2), showed that HR2 had the highest overall methane production rate against H_2:CO_2, both before and after freeze drying. qPCR results (*mcrA* gene and transcript copies) correlated with SMA results, both for fresh ($R^2 = 0.57$ and 0.74, respectively) and freeze dried biomass ($R^2 = 0.63$ and 0.77, respectively).

Fig.5.6. Specific Methanogen Activity Assay Results. SMA values for enrichment cultures before and after freeze drying for $H_2:CO_2$. Error bars represent the standard deviation of 4 replicates.

5.4 Discussion. This study presents the use of freeze drying as a viable method for preserving anaerobic methanogenic biomass. So far, there is very little information ava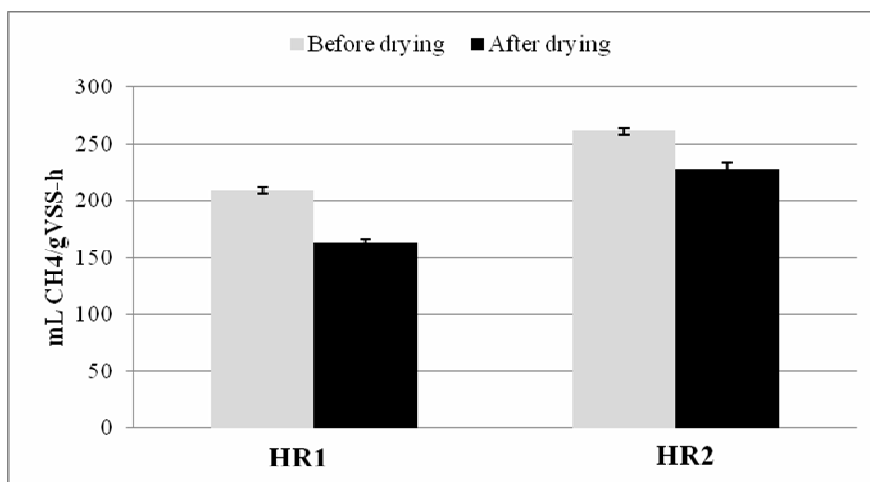ilable on the response and recovery of methanogens to stress faced during the process of cryodesiccation (freezing at -196^oC in liquid nitrogen followed by sublimation at -45^oC) and exposure to atmospheric air. The qPCR results and methanogenic activity assays in this study demonstrated that some methanogenic strains (*Methanospirillum* and *Methanosaeta*) retained their functional activity and have an ability to tolerate these stressful conditions. These data contradict the findings of Liu et al., (2008) who found that methanogens were vulnerable to the combination of desiccation and air coupled with low temperature, and reported that five out of the seven methanogenic strains they tested (*Methanospirillum hungatei,Methanosarcina mazei, Methanobacterium formicicum, Methanobrevibacter arborphilus* and *Methanococcus volatae)* lost their viability when desiccated at ambient temperature in atmospheric air. However, Morozova and Wagner (2007) reported the presence of biogenic methane in permafrost, which included methane formation at temperatures above 0^oC and subzero temperatures. Therefore, the

discovery of viable methanogens in permafrost sediments provides significant evidence of the stability of these microbial populations through extremely long existence at subzero temperature (Morozova and Wagner, 2007).

5.4.a Recovery of methanogens: link between process rate and methanogenic activity after cryodesiccation. Anderson et al., (2010) reported that rehydration of dried cells can influence the activities and distribution of bacterial species on local and global scales. qPCR on freeze dried cultures revealed that methanogens retained 68±18% activity after freeze drying. Although transcript abundance did not correlate well with methane production rate with acetate as the substrate, there was evidence for linear correlation (regression coefficient, r^2 =0.99) between methane production rate and transcripts with H_2+CO_2 as the substrate. The transcript:gene abundance ratio reflects the transcript abundance per cell and has been suggested as a more direct measure of physiological activity than absolute abundance (Freitag and Prosser, 2009). The results obtained in this study are in accordance with pure culture studies on the thermophilic methanogen, *Methanococcus vannielli,* which demonstrated that there were 180 (lag phase), 200 to 400 (exponential phase) and 50 (stationary phase) *mcrA* mRNA transcripts per cell (Hennigan and Reeve, 1994).

We evaluated the potential tolerance of three methanogenic genera, *Methanospirillum, Methanoculleus* and *Methanosaeta* to low temperature and oxygen stress. qPCR with 16S rRNA genus specific primers revealed that methanogens varied in their ability to tolerate the process of freeze drying. *Methanospirillum* had the highest 16S rRNA transcripts before and after drying, followed by *Methanosaeta* and *Methanoculleus*. This suggests that there was less damage to *Methanospirillum* populations, which also corresponded with the SMA assay, where there was less relative activity lag of the freeze dried biomass in H_2:CO_2 when compared to assay with

calcium acetate. Dose et al., (1991) demonstrated that inactivation of *Bacillus subtilis* and *Deinococcus radiodurans* during long-term desiccation, resulted in increased DNA strand breaks and other DNA lesions. They found that these lesions continued to accumulate if the organism was not given intermittent periods of activity, and survival of these species appeared to depend on their ability to repair DNA damage. This variation in survival among *Methanospirillum, Methanosaeta* and *Methanoculleus* may therefore be due to differences in intrinsic and extrinsic defense systems. Liu et al., (2008) conducted a study to evaluate the ability of diverse methanogenic strains to endure aeration and water stress during periods of drainage in paddy soils. They reported that a *Methanospirillum spp.* showed viability for 3 days following desiccation under both oxic and anoxic atmospheres, whereas *Methanoculleus olentangyi* failed to survive at all (Liu et al., 2008). Some methanogenic strains, *Methanobacterium bryantii, Methanobrevibater arboriphilicus, Methanosarcina barkeri* etc., are equipped with detoxification enzymes, such as, superoxide dismutase (SOD) and catalase, and can survive in the presence of oxygen (Kato et al., 1997). Therefore, it seems reasonable to believe that the ability to survive oxic-desiccation for a long period of time is partially associated with the activity of detoxification enzymes, although, the presence of these enzymes has not yet been established in the methanogens present in this study.

The transcript:gene ratio also revealed that *Methanospirillum* and *Methanosaeta spp.* were more tolerant to low temperature and oxygen exposure, compared to *Methanoculleus.* Both of them had higher transcript:gene ratios in the freeze dried biomass, relative to fresh biomass, which suggests that the cells that survived freeze drying were more poised for transcription. The differences in survival reflect the ability of the cells to resist the effects of rapid freeze drying which may be due to structural differences in the cell wall and cell membranes of organisms

(Miyamoto-Shinohara et al., 2008). *Methanospirillum* and *Methanosaeta* are filamentous and their external surface is composed of a unique proteinaceous sheath (Southam and Beveridge, 1992). They contain an S-layer, similar to other Methanomicrobiales species, but individual cells are further enclosed by a sheath composed of an external paracrystalline layer. The sheath is a hollow tube which surrounds cells and separates them with multilayered cell spacers and end plugs (Firtel et al., 1993; Southam and Beveridge, 1992). 20% of the sheath is composed of phenol soluble proteins that confer rigidity (Southam and Beveridge, 1992) and the tight packing of the sheath particles produces a barrier of low porosity that limits the movement of even small molecules (Southam and Beveridge, 1992). The sheath is also extremely resistant to denaturants, salts, proteases and enzymes (Southam and Beveridge, 1992). A study on freeze fracture planes of methanogen membranes revealed that *Methanospirillum hungatei* GP1 was more resilient to freeze etching, a traditional electron microscopical technique to visualize bacterial membranes, when compared to *Methanosaeta concilii* (Beveridge et al., 1993) . The lack of membrane fractures in *M. hungatei* correlated with higher amounts of tetraether (50%) lipids in its plasmamembrane, whereas *Methanosaeta* did not contain tetraether lipids and easily fractured during freeze etching to reveal intramembranous particles. On the other hand, the cell envelope of *Methanoculleus* is composed of a regularly structured S layer complex that forms a tight but non-covalent association that is deformable and not rigid (Beveridge et al., 1993).

5.4.b Improving the survival of freeze dried biomass. While the ability of methanogens to survive the process of cryodesiccation has not been demonstrated so far, methanogens have been shown to survive periods of desiccation in nature (Angel et al., 2011; Boon et al., 1997; Mitchell and Baldwin, 1999). They reported that sediments in methanogenic reservoirs showed that methanogens consistently recovered upon rewetting of the sediments. Angel et al., (2011)

reported that methanogens in rice fields also survived in dry and oxic paddy soil. Kendrick and

Kral (2006) reported that *Methanosarcina barkeri* can survive desiccation in a Mars soil

simulant for at least 10 days, while *M. formicicum and M. wolfeii* survived for at least 25 days.

They also reported that there was a decrease in amount of methane produced by surviving

cultures with longer desiccation times, which indicated that not all cells survived or some cells

required longer periods of time to repair damage.

Several studies on anaerobic digestion systems have reported that addition of oxygen led

to significantly higher SMA (Morris, 2011; Tale, 2011; Zitomer and Shrout, 1998). The qPCR

data in this study suggests that acclimation of biomass to oxygen has an effect on the

methanogen abundance and activity, both before and after freeze drying. This trend is in

accordance to the findings of Morris (2011), who reported that addition of oxygen to a H_2-

utilizing culture resulted in greater *mcrA* gene and transcript copies, based on fresh biomass.

There was also a strong correlation between mean *mcrA* gene copy numbers from the hydrogen

enrichment cultures with SMA against $H_2:CO_2$. Similar to the SMA activity of the non-fat dry

milk biomass with acetate, a correlation could not be established between *mcrA* transcripts and

methane production (Morris,2011). This lack of correlation was possibly due to the fact that

several groups of microorganisms were able to utilize acetate, and acetate oxidation to H_2 and

CO_2 may also have occurred, reducing the available acetate for the methanogens for methane

production (Karakashev et al., 2006; Morris, 2011). Overall, the results from qPCR and SMA

assay suggest that the activity of an H_2-utilizing culture can be preserved by freeze drying.

Acclimation of the culture to oxygen prior to freeze drying also had a positive effect on the

survival and recovery of the freeze dried biomass following rehydration.

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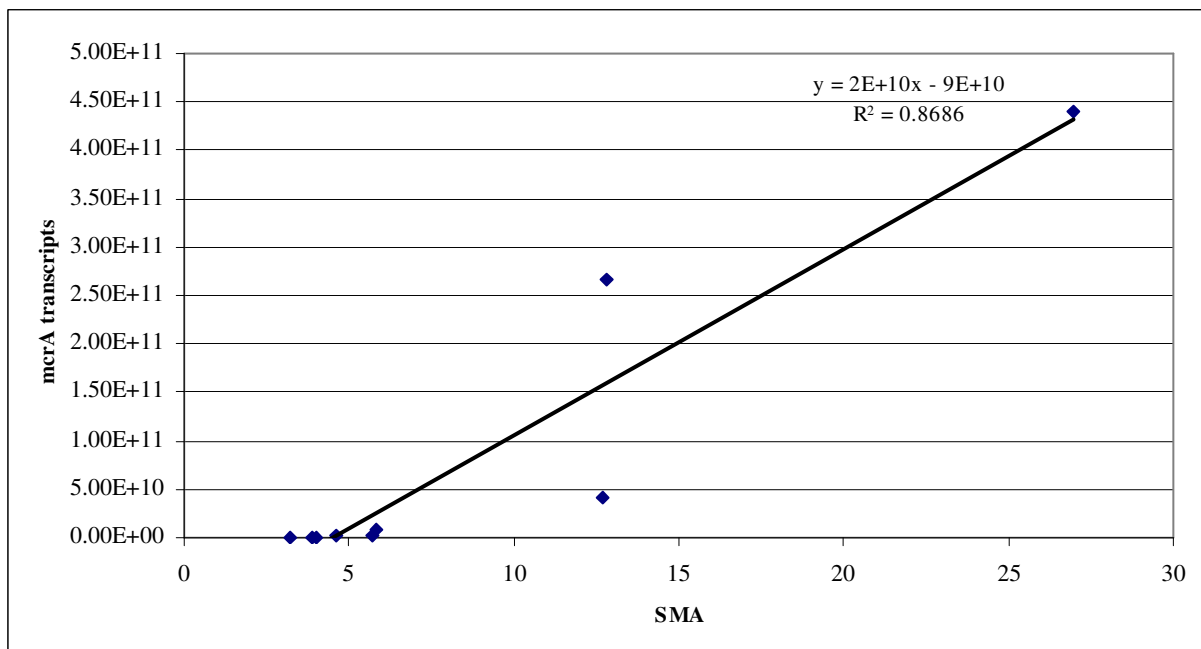
Appendix I

This appendix includes correlation graphs discussed in Chapter 4 but not included in the text.
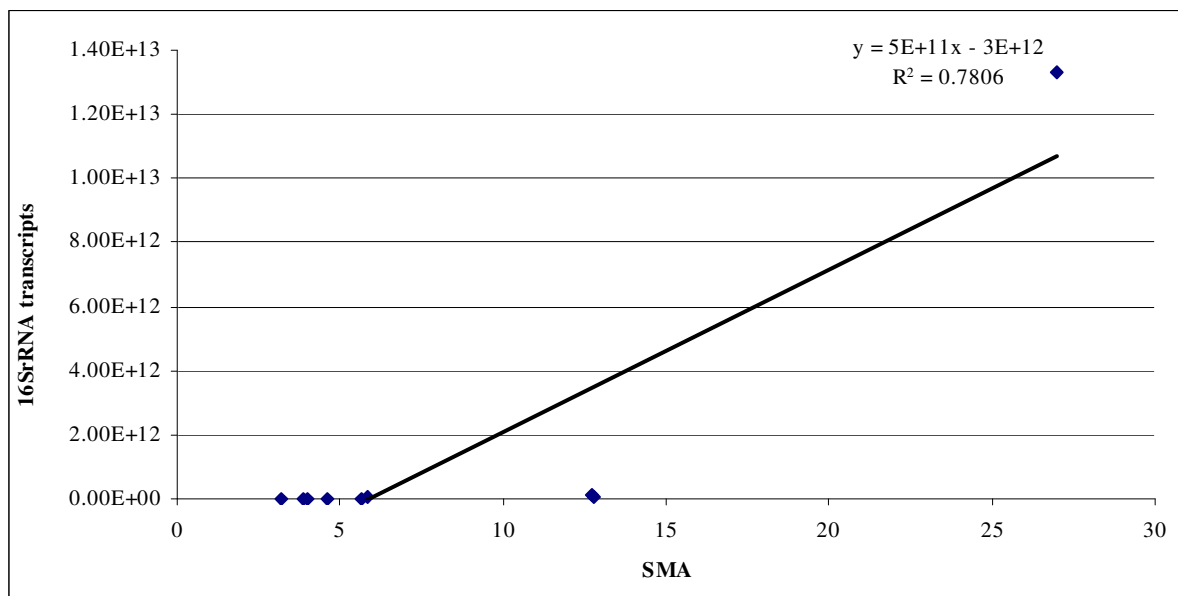
A. Correlation between *mcrA* transcripts and gene copies (4.2.b).
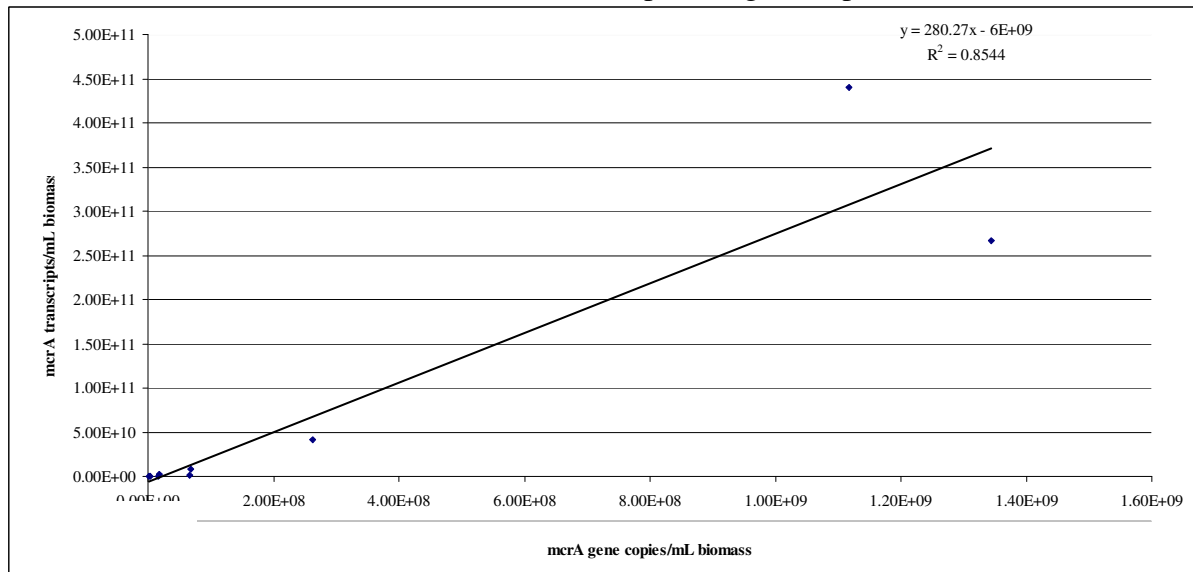
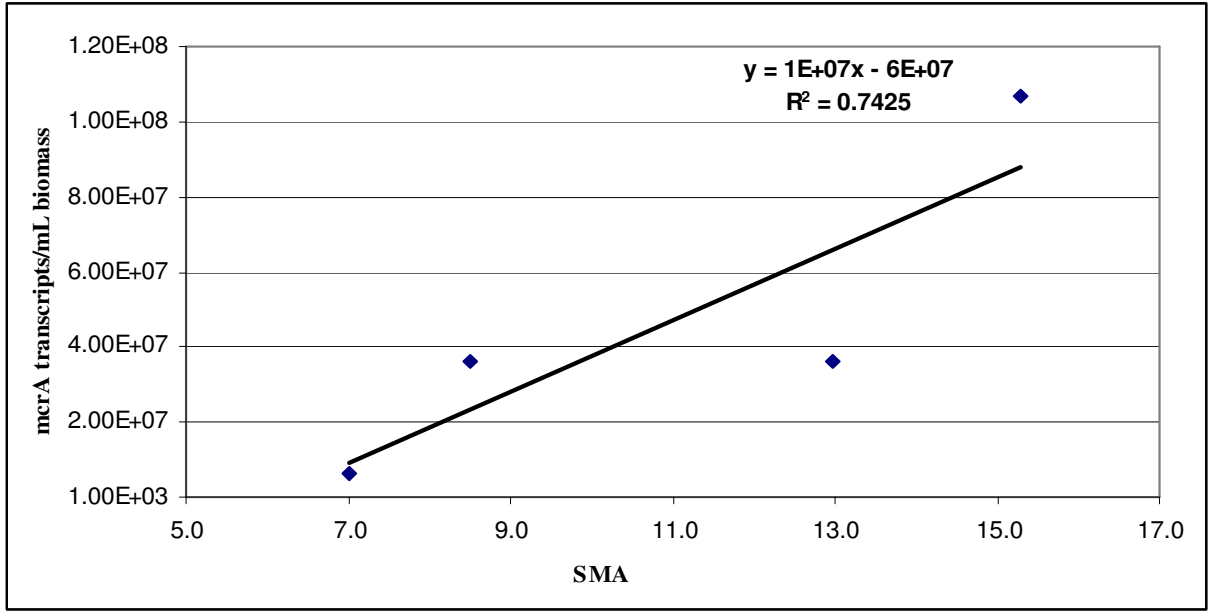B. Correlation between *mcrA* Transcripts and SMA (4.2.e)

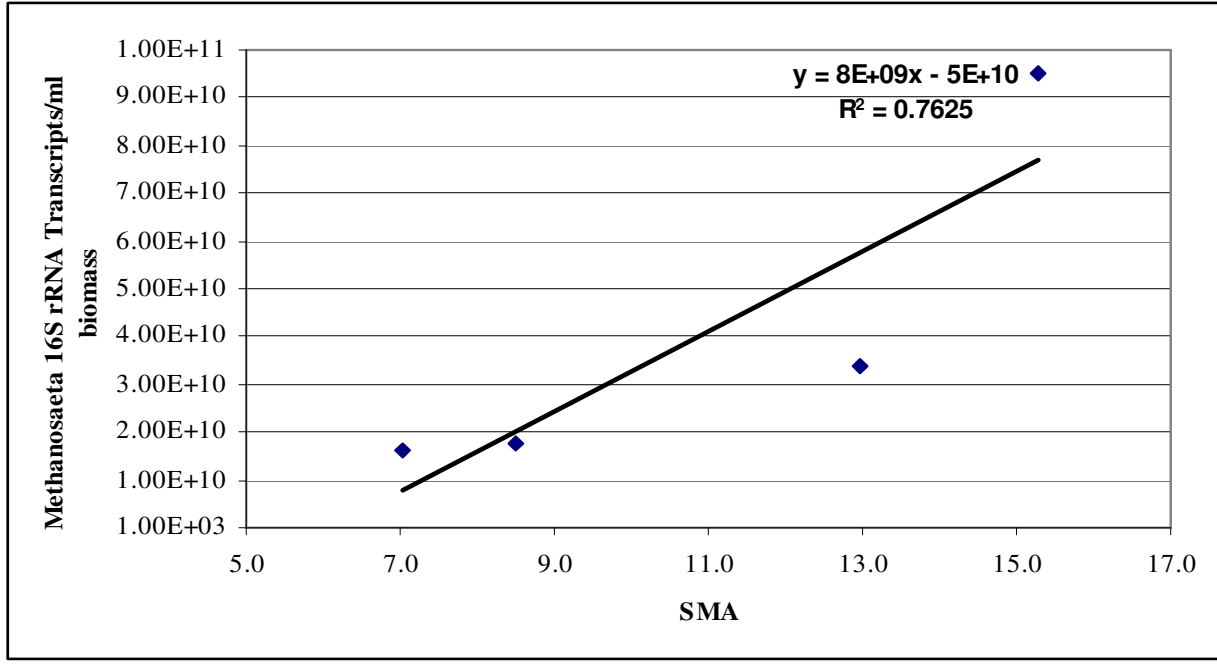C. Correlation between 16S rRNA Transcripts and SMA (4.2.e)

APPENDIX II

Fig.5.4. Correlation graphs. Graphs showing correlation between **(a)** *mcrA* transcripts and SMA, **(b)** Transcripts of 16S rRNA gene and SMA, **(c)** *mcrA* transcripts and OOPA, **(d)** Transcripts of 16S rRNA gene and OOPA.
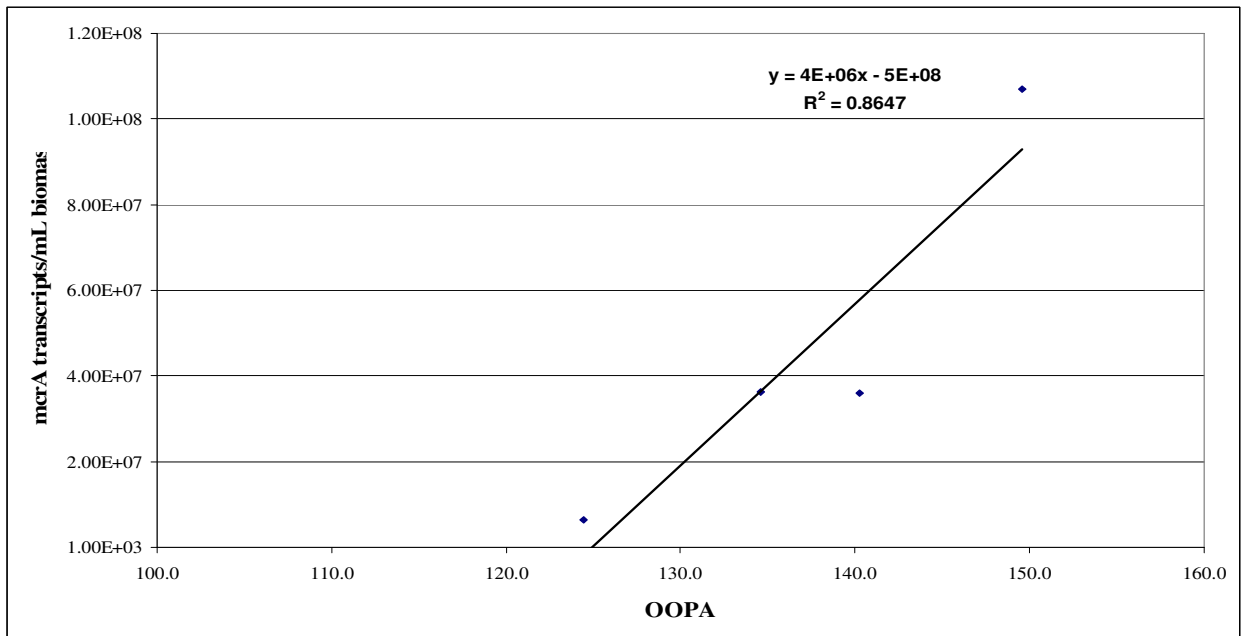
(a)

$y = 1E+07x - 6E+07$
$R^2 = 0.7425$

(b)

$y = 8E+09x - 5E+10$
$R^2 = 0.7625$

(c)

(d)