

**Toward Understanding the Physiological Determinants of
Microbial Competitiveness in Methanogenic Processes**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

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DEDICATION

This dissertation is dedicated in loving memory to my maternal grandfather.

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I would like to express the highest level of gratitude to my advisor, Dr. Qiang He, for all the knowledge and wisdom he imparted on me over the last six years.

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ABSTRACT

Methanogenesis is of great significance in both natural and engineered processes. Anaerobic digestion technology represents the engineering-scale implementation of methanogenesis in waste treatment processes. Despite the broad application of anaerobic digestion as a common waste treatment option, much remains to be learned on the anaerobic food web underlying methanogenesis for more effective process modeling and control.

Following an initial screening of various substrates, six continuous lab-scale anaerobic digesters were developed with animal waste as the substrate. The linkage between microbial community composition and process performance was studied by initiating process imbalance with organic overloading. As a result, accumulation of short chain fatty acids, particularly acetate, occurred in response to the onset of process imbalance. Populations related to *Methanosaeta* vastly outnumbered those of *Methanosarcina* as the dominant acetoclastic methanogen throughout the study period, even at elevated acetate concentrations during process imbalance, which is unexpected as it is considered that *Methanosaeta* can out-compete *Methanosarcina* only at low acetate concentrations, typical for balanced anaerobic food webs. Subsequent methanogenic batch cultures enriched with high concentrations of acetate further confirmed the dominance of *Methanosaeta* over *Methanosarcina*. Given the significance of acetoclastic methanogenesis in the anaerobic food web and global carbon cycling, it is evident that the interactions between *Methanosaeta* and *Methanosarcina* need to be better defined. Other novel microbial populations were also identified as abundant constituents of the microbial communities in the anaerobic digesters, including populations related to *Crenarchaeota*. It is critical to understand the roles these abundant but poorly-described populations play in the anaerobic food web.

TABLE OF CONTENTS

Chapter I Introduction.....	1
Introduction.....	2
Research Outline.....	8
References.....	10
Chapter II Optimizing Methane Production from Organic Wastes.....	14
Improving Biogas Production from Dilute Dairy Waste by Anaerobic Co-digestion with Food Waste.....	15
Abstract.....	15
Introduction.....	15
Materials and methods.....	16
Results and discussion.....	18
Conclusion.....	27
References.....	28
Chapter III Linkage between Microbial Populations and Process Conditions in Anaerobic Digestion.....	30
Part I: Impact of Substrate Overloading on Archaeal Populations in Anaerobic Digestion of Animal Waste.....	31
Abstract.....	31
Introduction.....	31
Materials and Methods.....	33
Anaerobic digester set-up.....	33
Chemical analysis.....	33
Clone library analysis of archaeal microbial populations.....	34
Statistical Data Analysis.....	34
Results.....	35
Anaerobic co-digester operation and performance.....	35
Archaeal populations in pre and post-overloading co-digesters by clone library analysis.....	37
Discussion.....	40
References.....	43
Part II: Dynamics of Two Dominant Bacterial Phyla, <i>Bacteroidetes</i> and <i>Firmicutes</i> in Anaerobic Digestion Process with Organic Loading Perturbations.....	46
Abstract.....	46
Introduction.....	46
Materials and Methods.....	48
Anaerobic co-digester setting and performance monitoring.....	48
Sample collection and DNA extraction.....	48
454 pyrosequencing and sequence analysis.....	48
Community diversity analysis.....	49
Statistical analyses.....	49
Canonical Corresponding Analysis (CCA).....	50
Results.....	51
Anaerobic co-digester performance.....	51

Sequence taxonomy classification with RDP	51
Dynamics of <i>Bacteroidetes</i> and <i>Firmicutes</i> populations	55
Bacterial community diversity analysis within sample - α diversity	58
Comparison of bacterial structures between samples - β diversity	58
Correlation analysis between digester performance and bacterial communities	62
Discussion	62
Conclusions	67
References	68
Appendix I	72
Chapter IV Development of Optimized qPCR Assays for Specific Archaeal Populations in Anaerobic Digestion	74
Part I: Development of <i>Methanoculleus</i> -specific Real-time Quantitative PCR Assay for Assessing Methanogen Communities in Anaerobic Digestion	75
Abstract	75
Introduction	75
Materials and methods	76
Primer and probe design	76
Experimental evaluation of <i>Methanoculleus</i> -specific primer/probe set	77
Field testing of <i>Methanoculleus</i> -specific primer/probe set	77
TaqMan qPCR procedure	77
Results	79
Design of <i>Methanoculleus</i> -specific primer/probe set	79
In silico evaluation of <i>Methanoculleus</i> -specific primer/probe set	80
Experimental evaluation of <i>Methanoculleus</i> -specific primer/probe set	80
Field testing of <i>Methanoculleus</i> -specific qPCR assay	82
Discussion	84
References	85
Part II: Enrichment of <i>Methanocalculus</i> and Development of <i>Methanocalculus</i> - Specific qPCR Assay	88
Abstract	88
Introduction	88
Materials and methods	89
Anaerobic digester set-up	89
Formate enrichment for methanogens	89
Clone library analysis of formate enrichment cultures	90
Primer/probe design for <i>Methanocalculus</i> -specific qPCR assay	91
Experimental evaluation and field application of <i>Methanocalculus</i> -specific qPCR assay	91
TaqMan qPCR protocol	91
Results	92
Enrichment of Methanogen populations with formate	92
<i>Methanocalculus</i> populations enriched by formate	92
Design of <i>Methanocalculus</i> -specific primer/probe set	94
<i>In Silico</i> evaluation of <i>Methanocalculus</i> -specific primer/probe set	94
Experimental evaluation of <i>Methanocalculus</i> -specific qPCR assay	98
Field application of <i>Methanocalculus</i> -specific qPCR assay	99

Discussion	100
References	101
Part III: <i>Crenarchaeota</i> in Anaerobic Digestion of Animal Waste	104
Abstract	104
Introduction	104
Materials and methods	105
Anaerobic digester setup and operation	105
Enrichment setup and monitoring	106
<i>Crenarchaeota</i> sequence analysis	107
Primer/probe design for CrenAD-specific qPCR	107
TaqMan qPCR protocol	107
Results	108
Phylogeny of <i>Crenarchaeota</i> identified in anaerobic digestion	108
Development of CrenAD-specific qPCR	116
Linkage of <i>Crenarchaeota</i> abundance to anaerobic digestion performance	119
Enrichment of <i>Crenarchaeota</i> as a minor population	119
Correlation between <i>Crenarchaeota</i> and acetoclastic methanogens	119
Discussion	122
References	124
Appendix II	127
Chapter V Dominance of <i>Methanosaeta</i> at High Acetate Concentrations in Anaerobic Digestion	133
Part I: Dynamics of Major Methanogenic Populations in Anaerobic Digesters with Organic Loading Fluctuation	134
Abstract	134
Introduction	134
Materials and Methods	136
Anaerobic digesters operation and biomass sampling	136
Chemical analysis	136
TaqMan qPCR analysis	139
Results	139
Digester performance	139
qPCR assays	140
<i>Methanosaeta</i> in anaerobic digestion	140
<i>Methanosarcina</i> in anaerobic digestion	142
<i>Methanoculleus</i> in anaerobic digestion	145
Discussion	145
Conclusion	151
References	152
Part II: Dominance of <i>Methanosaeta</i> Populations at High Acetate Concentrations in Anaerobic Digestion	155
Abstract	155
Introduction	155
Materials and Methods	157
Results	168
Discussion	179

Conclusion	183
References.....	184
Chapter VI Conclusion	189
Conclusion	190
VITA	192

LIST OF TABLES

Table II1 Various substrate loading rates in anaerobic digestion.....	17
Table II2 Chemical characteristics of substrates.....	17
Table III1 Archaeal community structure of clone libraries in anaerobic digestion of animal wastes pre- and post-overloading.....	39
Table III2 Summary of digester performance with perturbations of organic loading rates	52
Table III3 Pairwise correlations between <i>Bacteroidetes</i> and <i>Firmicutes</i> populations.....	57
Table III4 Biodiversity estimation of 16S rRNA sequences from the pyrosequencing analysis.....	57
Table IIIS1 List of primers and barcodes used in 454 pyrosequencing.....	72
Table IIIS2 List of major bacterial OTUs and their relative abundances.....	73
Table IV1 Characteristics of primer/probe sets for <i>Methanoculleus</i> -specific TaqMan qRT-PCR assays.....	79
Table IV2 Comparison of the coverage and specificity of <i>Methanoculleus</i> -specific TaqMan qRT-PCR assays.....	83
Table IV3 Quantification of <i>Methanoculleus</i> abundance with qRT-PCR in anaerobic digesters treating animal waste.....	83
Table IV4 Characteristics of primer/probe sets for <i>Methanocalculus</i> -specific TaqMan qPCR assays.....	95
Table IV5 Coverage and specificity of <i>Methanocalculus</i> -specific TaqMan qPCR primer/probe set.....	95
Table IV6 <i>Methanocalculus</i> abundance determined with qPCR in anaerobic digesters and formate enrichment cultures	100
Table IV7 Characteristics of TaqMan qPCR primer/probe set targeting <i>Crenarchaeota</i> populations in anaerobic digestion.....	113
Table IV8 Coverage and specificity of the CrenAD TaqMan qPCR primer/probe set in <i>Archaea</i> and <i>Bacteria</i>	114
Table IV9 Coverage and specificity of the CrenAD-specific qPCR primer/probe set in Miscellaneous Crenarchaeotal Groups (MCG).....	115
Table IV10 Quantification of <i>Crenarchaeota</i> abundance in methanogenic enrichments.....	121
Table IVS3 List of <i>Crenarchaeota</i> 16S rRNA gene sequences from anaerobic digestion processes	128
Table V1 Time points for both of the control and co-digester samplings.....	137
Table V2 List of the primer and probe sets for TaqMan real time PCR assays in this study	138
Table V3 Summary of TaqMan real time PCR standard curves with primer and probe sets applied in this study	138
Table V4 Quantification of 16SrRNA gene abundances of major methanogens with qPCR in acetate and methanol enrichment cultures from anaerobic digesters	159
Table V5 Quantification of 16SrRNA gene abundances of major methanogens.....	173
Table V6 Summary of growth kinetics parameters of enrichment cultures of <i>Methanosaeta</i> and <i>Methanosarcina</i>	182

LIST OF FIGURES

Fig. I1. Methanogenic food web	5
Fig. I2. Growth kinetics of <i>Methanosaeta</i> and <i>Methanosarcina</i> of acetate utilization.....	6
Fig. I3. Compositions of major arhaeal populations after enrichment with various chemicals.....	7
Fig. I4. Research outline of this study	8
Fig. II1. Methane potential of dairy waste (DW), food waste (FW) and inoculum.....	19
Fig. II2. Methane production from anaerobic mono-digestion.....	21
Fig. II3. Temporal Concentration profiles of volatile fatty acids during anaerobic mono-digestion of food waste	22
Fig. II4. Methane productions of food wastes (FW) in anaerobic co-digestion	23
Fig. II5. Volatile fatty acids concentrations of anaerobic co-digestion of food waste.....	25
Fig. II6. Alkalinity (ALK) and pH values at the end of anaerobic mono and co-digestion of food wastes	26
Fig. III1. Biogas production in anaerobic co-digesters.....	36
Fig. III2. Concentration of volatile fatty acids in anaerobic co-digesters.....	38
Fig. III3. Relative percentages of archaeal populations in anaerobic digesters.....	40
Fig. III4. Neighbor-joining phylogenetic tree showing the relationships of partial archaeal 16S rRNA gene sequences cloned from anaerobic codigesters	41
Fig. III5. Flow chart demonstrating procedures applied in 454 pyrosequencing data analysis.....	50
Fig. III6. Unit volume of biogas production from anaerobic co-digesters	53
Fig. III7. Classification of bacterial 16srRNA sequences from reactors with RDP II database.....	54
Fig. III8. Ratios of <i>Firmicutes</i> to <i>Bacteroidetes</i>	56
Fig. III9. Hierarchical cluster dendrogram based on OTU composition of bacterial populations.....	60
Fig. III10. Hierarchical clustering of major OTUs with significant changes	61
Fig. III11. Venn diagram of the bacterial community structures	63
Fig. III12. Principal Coordinates Analysis (PCoA) of the bacterial community structures	64
Fig. III13. Canonical Corresponding Analysis (CCA) for the correlations between major bacteria populations	65
Fig. IIIS1. Rarefaction curve of bacterial 16SrRNA gene sequences.....	72
Fig. IV1. Neighbour joining phylogenetic tree showing the grouping of reference <i>Methanoculleus</i> cultures and uncultured <i>Methanoculleus</i> clones from anaerobic digesters	78
Fig. IV2. Alignment of representative <i>Methanoculleus</i> 16S rRNA gene sequences	81
Fig. IV3. Amplification efficiency of <i>Methanoculleus</i> - specific qRT - PCR primer/probe set.....	81
Fig. IV4. Specific detection of <i>Methanoculleus</i> as increases in fluorescence using 16S rRNA gene templates	82
Fig. IV5. Methane production and substrate utilization in secondary enrichment cultures	93

Fig. IV6. Neighbor-joining tree showing the phylogenetic grouping of partial <i>Methanocalculus</i> 16S rRNA gene sequences cloned from formate enrichment cultures.	96
Fig. IV7. Alignment of representative <i>Methanocalculus</i> 16S rRNA gene sequences	97
Fig. IV8. Specific amplification of <i>Methanocalculus</i> 16S rRNA gene	98
Fig. IV9. Amplification efficiency of the <i>Methanocalculus</i> -specific qPCR assay	99
Fig. IV10. Organic loading scheme of the anaerobic digesters treating animal waste ...	109
Fig. IV11. Neighbor-joining tree showing the phylogenetic grouping of <i>Crenarchaeota</i> 16S rRNA gene sequences	110
Fig. IV12. Neighbor-joining phylogenetic tree showing the relationships between <i>Crenarchaeota</i> identified in anaerobic digestion (AD) processes	111
Fig. IV13. Neighbor-joining phylogenetic tree showing the relationships between Miscellaneous <i>Crenarchaeota</i> Group 6 (MCG-6)	112
Fig. IV14. Alignment of representative <i>Crenarchaeota</i> and <i>Thaumarchaeota</i> 16S rRNA gene sequences	117
Fig. IV15. Changes in <i>Crenarchaeota</i> relative abundance in anaerobic digesters	118
Fig. IV16. Abundance of acetoclastic methanogens in methanogenic enrichment cultures	120
Fig. IVS2. Amplification efficiency (E) and specificity of the <i>CrenAD</i> -specific qPCR	127
Fig. V1. Biogas production of anaerobic control and co-digesters	141
Fig. V2. Changes in <i>Methanosaeta</i> relative abundance in anaerobic co-digesters	143
Fig. V3. Changes in <i>Methanosaeta</i> relative abundance in anaerobic co-digesters	144
Fig. V4. Changes in <i>Methanosarcina</i> relative abundance in anaerobic co-digesters	146
Fig. V5. Changes in <i>Methanosarcina</i> relative abundance in anaerobic co-digesters	147
Fig. V6. Changes in <i>Methanoculleus</i> relative abundance in anaerobic co-digesters	148
Fig. V7. Changes in <i>Methanoculleus</i> relative abundance in anaerobic co-digesters	149
Fig. V8. Neighbour joining phylogenetic tree showing the grouping of reference <i>Methanosaeta</i> and <i>Methanosarcina</i> cultures	161
Fig. V9. Changes in the acetate loading rate of the treatment anaerobic digesters	162
Fig. V10. Methane production of control and treatment digesters with acetate loading	162
Fig. V11. Concentrations of acetate in both of control and treatment anaerobic digesters	164
Fig. V12. Volatile solid concentrations of both of the control and treatment anaerobic digesters	164
Fig. V13. 16SrRNA gen copy numbers of <i>Methanosaeta</i> and <i>Methanosarcina</i> in both of control and treatment digesters with acetate spiking	166
Fig. V14. Changes in the methanol loading rate of the treatment anaerobic digesters...	170
Fig. V15. Methane production of control and treatment digesters with methanol spiking	170
Fig. V16. Concentrations of methanol in both of control and treatment anaerobic digesters	171
Fig. V17. Concentrations of acetate in both of the control and treatment anaerobic digesters	171
Fig. V18. 16SrRNA gen copy numbers of <i>Methanosaeta</i> and <i>Methanosarcina</i> in both of control and treatment digesters with methanol spiking	172
Fig. V19. Epifluorescence micrographs of acetate or methanol enrichment cultures from anaerobic digester	174
Fig. V20. Concentration changes of acetate and methane in anaerobic batch reactors with	

inocula of mixed cultures.....	176
Fig. V21. Epifluorescence micrographs of mixed cultures of <i>Methansaeta</i> sp. and <i>Methanosarcina</i> sp.	181
Fig. V22. Specific growth curves of enrichment cultures of <i>Methansaeta</i> and <i>Methanosarcina</i>	183

Chapter I

Introduction

Introduction

Anaerobic digestion is the microbial conversion of organic matter to 'biogas' (methane) under anaerobic conditions. Various organic wastes have been treated by this technology, e.g., food waste (Tsukahara et al., 1999), household waste, agriculture waste and municipal waste (Amani et al., 2010). Anaerobic digestion technology has attracted increasingly interest in recent years, because of its advantages over conventional aerobic biological treatment technologies: (1) cost-effectiveness due to its ability to treat waste without aeration; (2) reduced production of excess sludge due to low biomass yield from anaerobic growth; and (3) production of methane as a renewable energy resource.

Anaerobic digestion of dairy farm waste

Animal waste generated from large-scale livestock operations has been a source of environmental pollution and public health risk, because the natural decomposition of animal waste releases large quantities of pathogens, excess nutrients, organic matter, solids, methane, ammonia and odorants into the environment (Jongbloed & Lenis, 1998). Anaerobic digestion has been shown to be an effective technology to overcome these challenges (Chen & Cheng, 2005). More recently, anaerobic co-digestion of animal waste has been explored to enhance the economic feasibility of animal waste treatment (Mata-Alvarez et al., 2011).

Anaerobic co-digestion

Despite the advantages of anaerobic digestion processes for waste stabilization and biogas production as an alternative treatment technology for animal waste management (Bekkering et al., 2010; Chen & Cheng, 2005; Sakar et al., 2009), the application of anaerobic digestion remains economically unattractive for the treatment of dilute dairy waste, which is characterized by low solids content (typically less than 10%) and subsequently low volumetric biogas yield (Garrison & Richard, 2005).

In contrast, organic-rich waste materials, e.g., food waste and poultry waste, have much higher solids content (>50%) than dilute dairy waste (Kelleher et al., 2002; Zhang et al., 2013). Thus, the addition of organic-rich waste as a co-substrate in the anaerobic digestion of dairy waste could potentially overcome challenges presented by the low solids content and low volumetric biogas yield of dilute dairy waste.

Challenges of anaerobic digestion application

Despite the advantages of anaerobic co-digestion, the broader adoption of this technology for waste treatment has been hindered by concerns of potential process instability resulting from the susceptibility of methanogenic microbial populations to changes in process conditions (Chen et al., 2008), such as overloading in organic loading rates (OLRs) and changes in waste composition, which are frequently encountered in the anaerobic digestion of animal wastes. Previous studies of anaerobic digestion processes have shown that substrate overloading frequently leads to process imbalance, characterized by the accumulation of organic acids and inhibition of biogas production (Leitao et al., 2006).

As anaerobic biotransformation of organic wastes involves a complex anaerobic food web, efficient anaerobic digestion requires the balanced activities of diverse anaerobic microbial populations including primary fermenters, secondary fermenters, and methanogens (Ahring, 2003; Schink, 1997). The methanogens, a group of microorganisms associated exclusively with *Archaea*, are responsible for methanogenesis, the terminal step of methane formation from CO₂/H₂ or acetate in the anaerobic decomposition of organic wastes (Ferry, 1999; Ferry, 1992) (Fig. 11). Methanogenesis is often considered as the limiting step in anaerobic digestion processes under organic overloading, due to both the slow growth rates and the susceptibility to overloading process conditions characteristic of methanogens (Chen et al., 2008; Kotzé et al., 1969). Thus, the dynamics of bacterial and archaeal populations in anaerobic digestion may provide insight into the linkage between process performance and microbial activities in anaerobic conversion under overloading. It has been noted that anaerobic digestion processes could adapt to perturbations resulting from organic overloading, likely through long-term changes in microbial community structures (Hashsham et al., 2000; Xing et al., 1997). While it is suggested that increased population diversity may be linked to the adaptation to process perturbations, much remains to be learned on the changes in specific microbial populations before and after organic overloading. Therefore, to gain insight into the responses of microbial populations to process disturbances in anaerobic digestion, one specific objective of this study is to investigate the impact of substrate overloading on the microbial populations.

qPCR assay development for specific archaeal populations in anaerobic digestion

To enhance the process efficiency and stability of anaerobic digestion, efforts have been made to understand the microbial communities underlying the anaerobic decomposition of organic materials. As a result, significant progress has been made in the quantification and monitoring of specific microbial populations frequently identified in anaerobic digestion processes, such as methanogenic archaea, using molecular techniques with high sensitivity and specificity (Narihiro & Sekiguchi, 2011). Real-time quantitative polymerase chain reaction (qPCR) has emerged as a rapid technique to target specific microbial populations with high sensitivity (Smith & Osborn, 2009). Of the two main chemistries used in qPCR, that is, TaqMan and SYBR Green, the former is considered to be capable of providing greater target specificity in microbial ecology studies with the use of an extra oligonucleotide probe in addition to the set of primers used by SYBR Green (Wang & Brown, 1999), leading to the development of a series of TaqMan qPCR assays for methanogen populations by targeting the 16S rRNA genes (Shigematsu et al., 2003; Tang et al., 2005; Yu et al., 2005).

qPCR for *Methanoculleus*

Given the potential metabolic diversity of methanogens, it is often desirable to differentiate and quantify methanogen populations at fine taxonomic resolutions (Garcia et al., 2000; Jetten et al., 1992). *Methanoculleus*-like organisms have been frequently identified as the dominant hydrogenotrophic methanogen populations in anaerobic digesters and other methanogenic environments, resulting in considerable interest in monitoring the population dynamics of these organisms (Barret et al., 2012; Cardinali-Rezende et al., 2012; Nettmann et al., 2010). As reported by a recent study, the only

Methanoculleus-specific TaqMan qPCR primer/probe set available in the literature targets bases 934–1221 (*E. coli* numbering) of the 16S rRNA gene (Shigematsu et al., 2003), making it difficult to assess the specificity and coverage of the qPCR assay. Moreover, this qPCR primer/probe set could not be applied with confidence for the specific quantification of *Methanoculleus* populations in these anaerobic digesters. Therefore, one of the objectives of this study is to design alternative *Methanoculleus*-specific TaqMan qPCR primer/probe set with high sequence coverage and specificity.

qPCR for *Methanocalculus*

Methanocalculus represents another group of hydrogenotrophic mesophilic methanogen populations found in diverse natural habitats in marine, estuarine, terrestrial environments as well as solid waste disposal sites and anaerobic digestion processes (Calderón et al., 2011; Lai et al., 2002; Lai et al., 2004; Mori et al., 2000; Ollivier et al., 1998; Shin et al., 2010; Zhilina et al., 2013), suggesting its potential significance in anaerobic environments. Understanding of the ecological functions of these methanogens, however, is incomplete, hindered by the lack of quantitative tools specifically targeting *Methanocalculus*. Therefore, one of the objectives of this study is to develop a *Methanocalculus*-specific qPCR assay with high sensitivity for the monitoring of these archaeal populations in the environment

qPCR for *Crenarchaeota*

In addition to methanogens, which belong to *Euryarchaeota*, recent molecular surveys of microbial communities in anaerobic digestion processes have unexpectedly detected the presence of 16S rRNA gene sequences related to uncharacterized *Crenarchaeota* populations (Chen et al., 2012; Chouari et al., 2005; Gomez et al., 2011; Leven et al., 2007; Riviere et al., 2009; Zhang et al., 2011). Moreover, *Crenarchaeota* have been reported as the predominant archaeal population in several studies of anaerobic digestion, representing 20-50% of the archaeal community (Acharya et al., 2011; Collins et al., 2005; Enright et al., 2009; Schauer-Gimenez et al., 2010; Siggins et al., 2011), suggesting the potential significance of these microorganisms in anaerobic decomposition of organic materials. In this study, therefore, a real-time quantitative PCR (qPCR) assay specifically targeting these *Crenarchaeota* populations was designed to monitor the population dynamics of *Crenarchaeota* during process perturbations in anaerobic digesters treating animal waste, revealing potential linkages between *Crenarchaeota* abundance and process condition

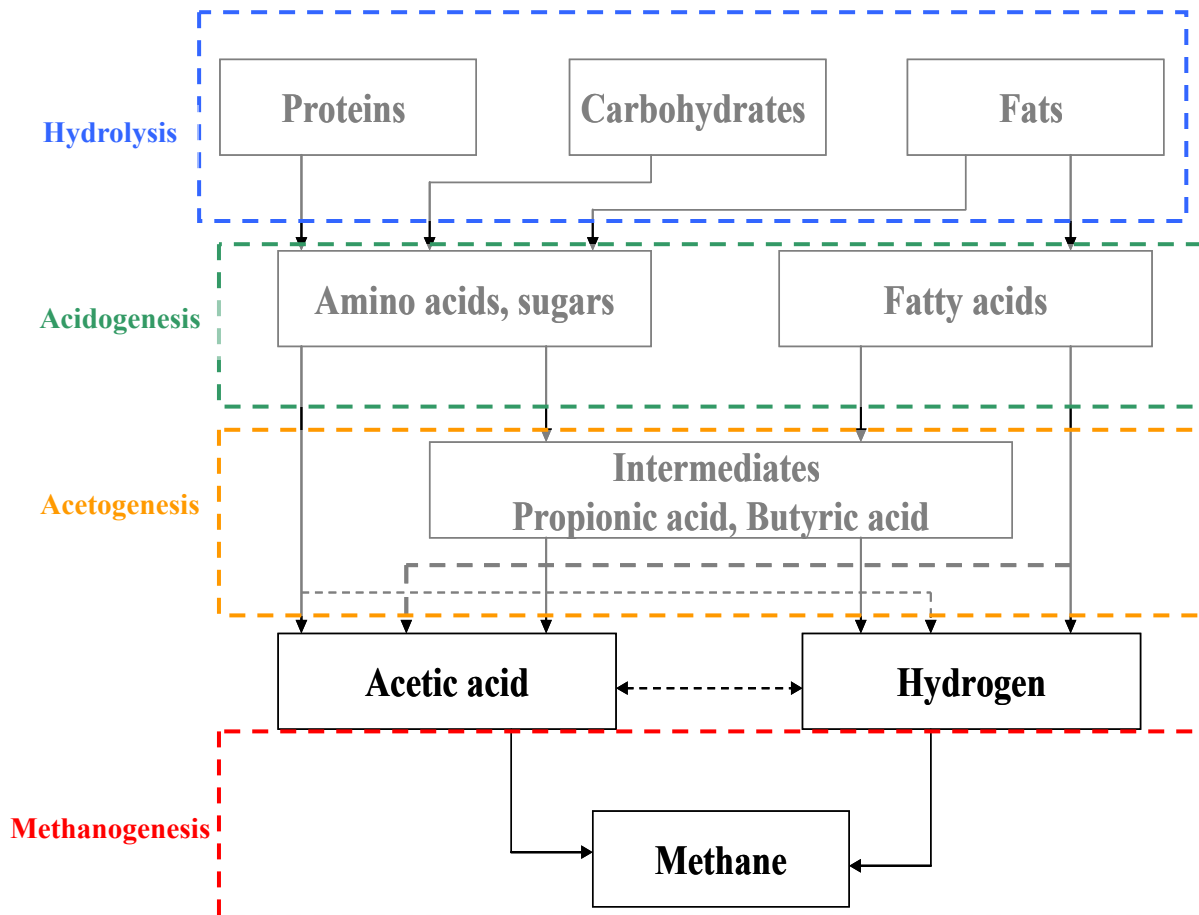


Fig. II. Methanogenic food web.

Competition between *Methanosaeta* and *Methanosarcina* in anaerobic digestion

In anaerobic digestion process, the acetoclastic pathway is often observed to be the dominant mechanism of methanogenesis (Ferry, 1992; Jeris & McCarty, 1965; Smith & Mah, 1966). Notably, more than 60% of the CH₄ generated in natural environments also follows this mechanism (Bridgham et al., 2013; Ferry, 1992; Penning et al., 2006). There are two well known genera of acetoclastic methanogens, i.e. *Methanosaeta* and *Methanosarcina*, converting acetate to methane and carbon dioxide. It has been considered that *Methanosarcina* has higher maximum growth rate, $\mu_{\max}$ and half-saturation coefficient, K_S than *Methanosaeta* (Fig. I2) (Conklin et al., 2006; De Vrieze et al., 2012). Thus, *Methanosarcina* is expected to be predominant in anaerobic digestion processes with relatively high acetate concentration and short hydraulic retention time (HRT). Accordingly, *Methanosaeta* would become the predominant acetoclastic methanogen population due to its high affinity to acetate under lower acetate concentrations or longer HRT conditions. (De Vrieze et al., 2012; Hulshoff Pol et al., 2004; Liu & Whitman, 2008; Smith & Ingram-Smith, 2007).

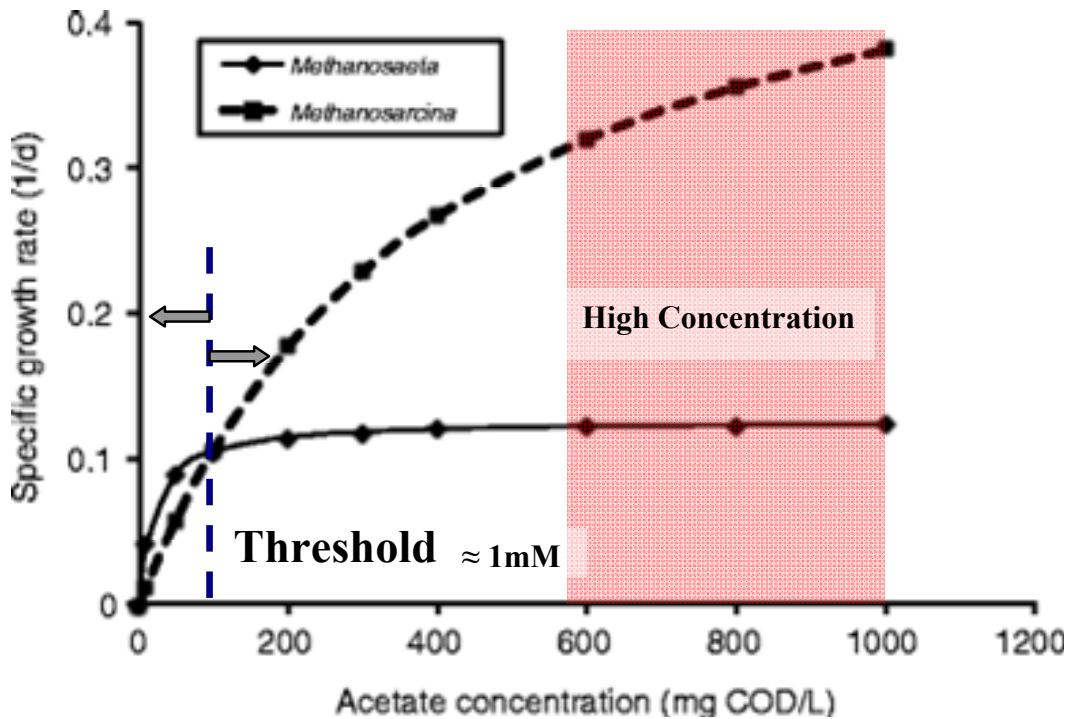


Fig. I2. Growth kinetics of *Methanosaeta* and *Methanosarcina* of acetate utilization (Conklin et al., 2006; De Vrieze et al., 2012).

However, inconsistent with this kinetic model, *Methanosaeta* populations were found to dominate the acetoclastic methanogen communities at acetate concentration exceeding 10mM in anaerobic digestion processes (Angenent et al., 2002). Similarly, dominance of *Methanosarcina* could not be observed in a CSTR during startup period with relatively high acetate concentrations (10 ~ 20mM) (Moertelmaier et al., 2013) or in an upflow anaerobic sludge bed reactor (UASB) despite acetate concentrations favorable for the competitiveness of *Methanosarcina* growth (van Haandel et al., 2013). Additionally, *Methanosaeta* was reported to be the dominant methanogens after enrichment with high acetate concentration (up to 10mM) (Ito et al., 2011).

In this study, preliminary results have shown the predominance and persistence of *Methanosaeta* in lab-scale semi-CSTR digesters during organic over loading with acetate accumulations (Chen et al., 2012) or in enrichment cultures fed with high acetate concentration (20mM) (Fig. I3). In contrast, *Methanosarcina* seemed to be enriched by methanol (Fig. I3). Further investigations, therefore, were conducted in this study to understand the microbial physiology underlying the unexpected dominance of *Methanosaeta* over *Methanosarcina* in anaerobic digesters established in this study.

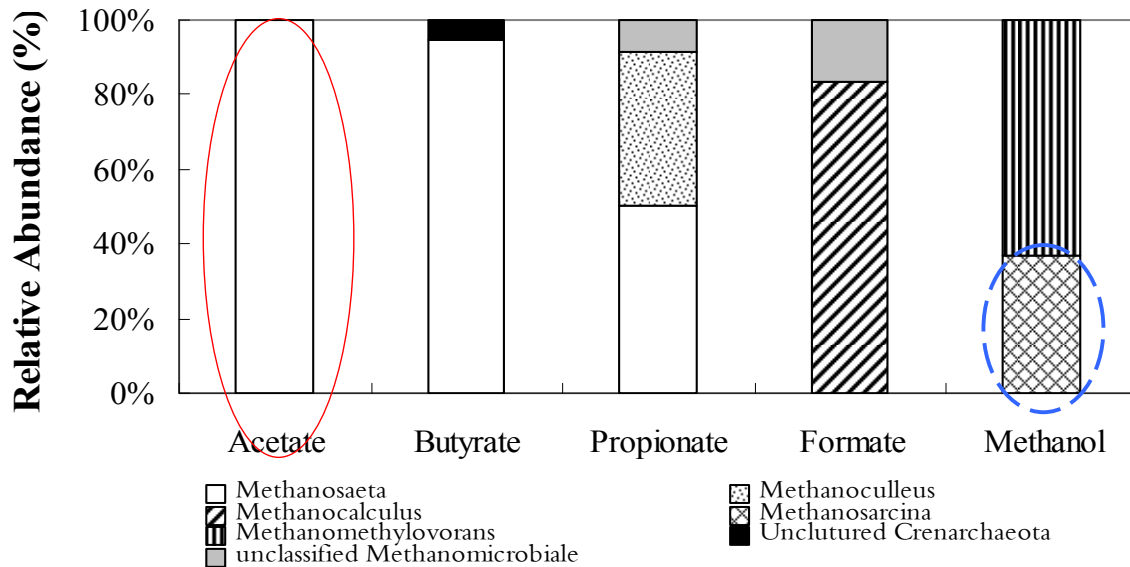


Fig. I3. Compositions of major archaeal populations after enrichment with various chemicals. Dominances of *Methanosaeta* and *Methanosarcina* are labeled by red and blue circles respectively.

Research Outline

To close the knowledge gaps discussed above with regard to the microbial dynamics in response to the performance of anaerobic digestion processes, research tasks carried out in this dissertation are summarized in Fig. I4. Results from all tasks were organized into chapters as listed below.

- Optimizing methane production from organic wastes (Chapter II)
 - ✓ *Food waste in anaerobic co-digestion with dairy farm waste.*
- Microbial populations in response to anaerobic digestion performance (Chapter III)
 - ✓ *Archaeal populations (16S rRNA gene clone library).*
 - ✓ *Bacterial populations (454 pyrosequencing).*
- qPCR development for specific archaeal populations in anaerobic digestion (Chapter IV)
 - ✓ *Methanoculleus*
 - ✓ *Methanocalculus*
 - ✓ *Crenarchaeota*
- Dominance of *Methanosaeta* at high acetate concentrations (Chapter V)
 - ✓ *Methanosaeta and Methanosarcina in digesters.*
 - ✓ *Methanosaeta and Methanosarcina in enrichments.*
 - ✓ *Growth kinetics of Methanosaeta and Methanosarcina.*

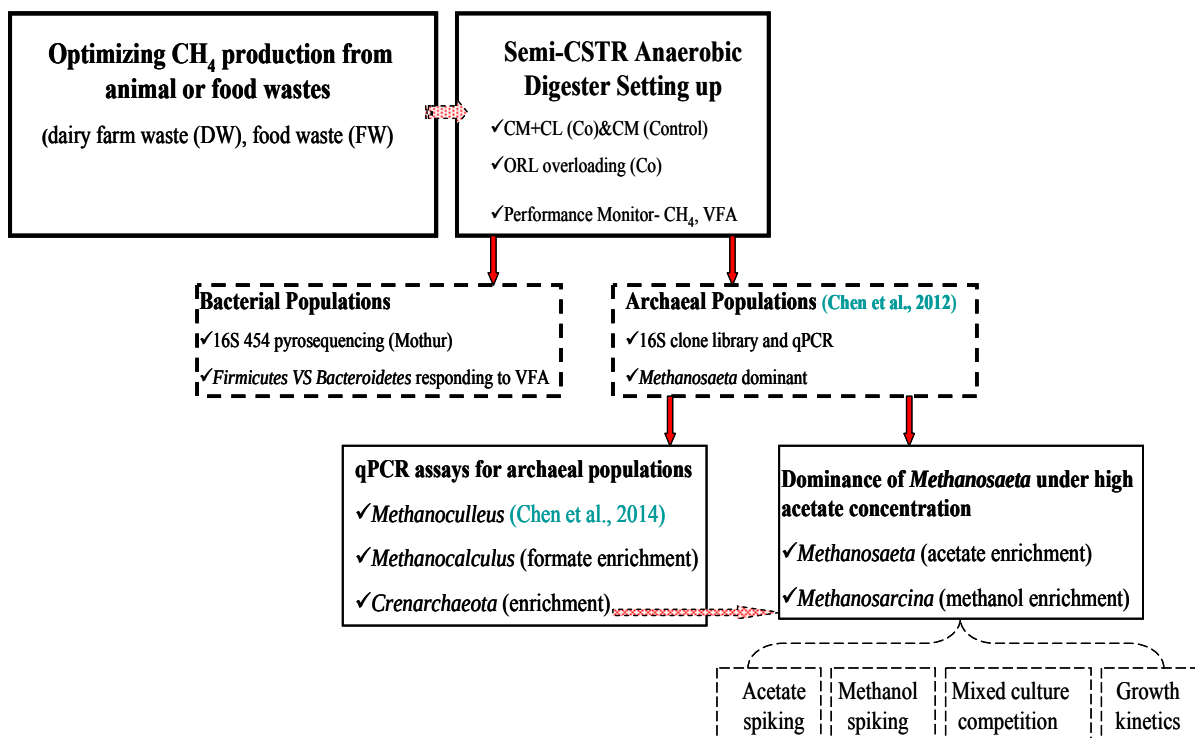


Fig. I4. Research outline of this study.

First, food waste (FW) was evaluated as an organic-rich co-substrate to enhance CH₄ production from the anaerobic co-digestion with diluted dairy farm waste (DW) (Chapter II). Supported by the performance data of CH₄ production and VFA concentrations under various organic loading rates of FW, it was found that process instability in fermentation and methanogenesis as a result of substrate overloading appeared to be major obstacles to the adoption of anaerobic digestion technology.

To reveal linkages between process performance and microbial community in anaerobic digestion process disturbed by overloading, triplicate continuous anaerobic co-digesters were developed as model anaerobic digestion processes, which were disrupted by substrate overloading and subsequently restored. Throughout the whole process, both bacterial and archaeal community structures were studied (Chapter III). Based on results of archaeal populations in anaerobic digestion, it was necessary to design qPCR assays for the quantitative monitoring of specific archaeal populations, e.g., *Methanoculleus*, *Methanocaldococcus* and *Crenarchaeota*, in order to study the dynamics of these populations in methanogenic processes (Chapter IV).

The unexpected dominance of acetoclastic methanogen, *Methanosaeta* rather than *Methanosarcina* in both CSTR digesters and enrichment cultures under high acetate concentrations in this study was inconsistent with current understanding of niche differentiation in acetate utilization between these two populations (Fig. I2). Meanwhile, the enrichment of *Methanosarcina* with methanol suggested that methylotrophy was likely preferred to acetotrophy for *Methanosarcina* in anaerobic digesters in this study. Thus, to gain insight into the discrepancy between prior knowledge and experimental observations on the competitiveness of *Methanosaeta* and *Methanosarcina* in anaerobic digestion, a systematic research approach was adopted integrating the assessment of microbial physiology, microbial ecology, and process performance in anaerobic digestion. Details on experimental designs and results were described in Chapter V.

References

- Acharya, B.K., Pathak, H., Mohana, S., Shouche, Y., Singh, V., Madamwar, D. 2011. Kinetic modelling and microbial community assessment of anaerobic biphasic fixed film bioreactor treating distillery spent wash. *Water Res*, **45**(14), 4248-4259.
- Ahring, B. 2003. Perspectives for Anaerobic Digestion. in: *Biomethanation I*, (Eds.) B. Ahring, I. Angelidaki, E.C. Macario, H.N. Gavala, J. Hofman-Bang, A.J.L. Macario, S.J.W.H.O. Elferink, L. Raskin, A.J.M. Stams, P. Westermann, D. Zheng, Vol. 81, Springer Berlin Heidelberg, pp. 1-30.
- Amani, T., Nosrati, M., Srekrishnan, T.R. 2010. Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects — a review. *Environ Rev*, **18**(NA), 255-278.
- Angenent, L.T., Zheng, D., Sung, S., Raskin, L. 2002. Microbial community structure and activity in a compartmentalized, anaerobic bioreactor. *Water Environ Res*, **74**(5), 450-61.
- Barret, M., Gagnon, N., Morissette, B., Topp, E., Kalmokoff, M., Brooks, S.P.J., Matias, F., Masse, D.I., Masse, L., Talbot, G. 2012. *Methanoculleus* spp. as a biomarker of methanogenic activity in swine manure storage tanks. *FEMS Microbiol Ecol*, **80**(2), 427-440.
- Bekkering, J., Broekhuis, A.A., van Gernert, W.J.T. 2010. Optimisation of a green gas supply chain - A review. *Bioresour Technol*, **101**(2), 450-456.
- Bridgman, S.D., Cadillo-Quiroz, H., Keller, J.K., Zhuang, Q. 2013. Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. *Global Change Biol*, **19**(5), 1325-1346.
- Calderón, K., Rodelas, B., Cabirol, N., González-López, J., Noyola, A. 2011. Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment. *Bioresour Technol*, **102**(7), 4618-4627.
- Cardinali-Rezende, J., Colturato, L., Colturato, T.D.B., Chartone-Souza, E., Nascimento, A.M.A., Sanz, J.L. 2012. Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions. *Bioresour Technol*, **119**, 373-383.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Chen, Y., Cheng, J.J. 2005. Anaerobic processes in waste treatment. *Water Environ Res*, **77**(6), 1347-1388.
- Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. *Bioresour Technol*, **99**(10), 4044-4064.
- Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., Sghir, A. 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. *Environ Microbiol*, **7**(8), 1104-1115.
- Collins, G., Mahony, T., McHugh, S., Gieseke, A., de Beer, D., O'Flaherty, V. 2005. Distribution, dynamics and in situ ecophysiology of Crenarchaeota in anaerobic wastewater treatment granular biofilms. *Water Science and Technology*, **52**(7), 233-239.
- Conklin, A., Stensel, H.D., Ferguson, J. 2006. Growth kinetics and competition between

- Methanosarcina and Methanosaeta in mesophilic anaerobic digestion. *Water Environ Res*, **78**(5), 486-496.
- De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W. 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. *Bioresour Technol*, **112**(0), 1-9.
- Enright, A.M., McGrath, V., Gill, D., Collins, G., O'Flaherty, V. 2009. Effect of seed sludge and operation conditions on performance and archaeal community structure of low-temperature anaerobic solvent-degrading bioreactors. *Syst Appl Microbiol*, **32**(1), 65-79.
- Ferry, J.G. 1999. Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbiol Rev*, **23**(1), 13-38.
- Ferry, J.G. 1992. Methane from acetate. *J Bacteriol*, **174**(17), 5489-5495.
- Franke-Whittle, I.H., Goberna, M., Insam, H. 2009. Design and testing of real-time PCR primers for the quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of uncultured methanogens. *Canadian Journal of Microbiology*, **55**(5), 611-616.
- Garcia, J.L., Patel, B.K.C., Ollivier, B. 2000. Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. *Anaerobe*, **6**(4), 205-226.
- Garrison, A.V., Richard, T.L. 2005. Methane and manure: Feasibility analysis of price and policy alternatives. *Trans ASAE*, **48**(3), 1287-1294.
- Gomez, E., Martin, J., Michel, F.C. 2011. Effects of organic loading rate on reactor performance and archaeal community structure in mesophilic anaerobic digesters treating municipal sewage sludge. *Waste Manage Res*, **29**(11), 1117-1123.
- Hashsham, S.A., Fernandez, A.S., Dollhopf, S.L., Dazzo, F.B., Hickey, R.F., Tiedje, J.M., Criddle, C.S. 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *Appl Environ Microbiol*, **66**(9), 4050-4057.
- Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., Lens, P.N.L. 2004. Anaerobic sludge granulation. *Water Res*, **38**(6), 1376-1389.
- Ito, T., Yoshiguchi, K., Ariesyady, H.D., Okabe, S. 2011. Identification of a novel acetate-utilizing bacterium belonging to Synergistes group 4 in anaerobic digester sludge. *ISME J*, **5**(12), 1844-56.
- Jeris, J.S., McCarty, P.L. 1965. The Biochemistry of Methane Fermentation Using [image] Tracers. *Journal (Water Pollution Control Federation)*, **37**(2), 178-192.
- Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B. 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothermobacter soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Lett*, **88**(3-4), 181-197.
- Jongbloed, A.W., Lenis, N.P. 1998. Environmental concerns about animal manure. *J Anim Sci*, **76**(10), 2641-2648.
- Kelleher, B.P., Leahy, J.J., Henihan, A.M., O'Dwyer, T.F., Sutton, D., Leahy, M.J. 2002. Advances in poultry litter disposal technology - a review. *Bioresour Technol*, **83**(1), 27-36.
- Kotzé, J.P., Thiel, P.G., Hattingh, W.H.J. 1969. Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Res*, **3**(7), 459-494.
- Lai, M.-C., Chen, S.-C., Shu, C.-M., Chiou, M.-S., Wang, C.-C., Chuang, M.-J., Hong, T.-Y., Liu, C.-C., Lai, L.-J., Hua, J.J. 2002. *Methanocalculus taiwanensis* sp. nov.,

- isolated from an estuarine environment. *Int J Syst Evol Microbiol*, **52**(5), 1799-806.
- Lai, M.-C., Lin, C.-C., Yu, P.-H., Huang, Y.-F., Chen, S.-C. 2004. *Methanocalculus chunghsingensis* sp. nov., isolated from an estuary and a marine fishpond in Taiwan. *Int J Syst Evol Microbiol*, **54**(1), 183-189.
- Leitao, R.C., van Haandel, A.C., Zeeman, G., Lettinga, G. 2006. The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review. *Bioresour Technol*, **97**(9), 1105-1118.
- Leven, L., Eriksson, A.R.B., Schnurer, A. 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *FEMS Microbiol Ecol*, **59**(3), 683-693.
- Liu, Y., Whitman, W.B. 2008. Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea. *Annals of the New York Academy of Sciences*, **1125**(1), 171-189.
- Mata-Alvarez, J., Dosta, J., Mace, S., Astals, S. 2011. Codigestion of solid wastes: A review of its uses and perspectives including modeling. *Crit Rev Biotechnol*, **31**(2), 99-111.
- Moertelmaier, C., Li, C., Winter, J., Gallert, C. 2013. Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. *Bioresour Technol*(0).
- Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M., Takamizawa, K. 2000. *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. *Int J Syst Evol Microbiol*, **50**(5), 1723-9.
- Narihiro, T., Sekiguchi, Y. 2011. Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea. *Microbial Biotechnology*, **4**(5), 585-602.
- Nettmann, E., Bergmann, I., Pramschufer, S., Mundt, K., Plogsties, V., Herrmann, C., Klocke, M. 2010. Polyphasic Analyses of Methanogenic Archaeal Communities in Agricultural Biogas Plants. *Appl Environ Microbiol*, **76**(8), 2540-2548.
- Ollivier, B., Fardeau, M.-L., Cayol, J.-L., Magot, M., Patel, B.K.C., Prensier, G., Garcia, J.-L. 1998. *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well. *Int J Syst Bacteriol*, **48**(3), 821-828.
- Penning, H., Claus, P., Casper, P., Conrad, R. 2006. Carbon Isotope Fractionation during Acetoclastic Methanogenesis by *Methanosaeta concilii* in Culture and a Lake Sediment. *Appl Environ Microbiol*, **72**(8), 5648-5652.
- Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *Isme Journal*, **3**(6), 700-714.
- Sakar, S., Yetilmezsoy, K., Kocak, E. 2009. Anaerobic digestion technology in poultry and livestock waste treatment - a literature review. *Waste Manage Res*, **27**(1), 3-18.
- Schauer-Gimenez, A.E., Zitomer, D.H., Maki, J.S., Struble, C.A. 2010. Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. *Water Res*, **44**(12), 3555-3564.
- Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev*, **61**(2), 262-&.

- Shigematsu, T., Tang, Y., Kawaguchi, H., Ninomiya, K., Kijima, J., Kobayashi, T., Morimura, S., Kida, K. 2003. Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. *J Biosci Bioeng*, **96**(6), 547-558.
- Shin, S.G., Lee, S., Lee, C., Hwang, K., Hwang, S. 2010. Qualitative and quantitative assessment of microbial community in batch anaerobic digestion of secondary sludge. *Bioresour Technol*, **101**(24), 9461-9470.
- Siggins, A., Enright, A.-M., O'Flaherty, V. 2011. Low-temperature (7 degrees C) anaerobic treatment of a trichloroethylene-contaminated wastewater: Microbial community development. *Water Res*, **45**(13), 4035-4046.
- Smith, C.J., Osborn, A.M. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol*, **67**(1), 6-20.
- Smith, K.S., Ingram-Smith, C. 2007. Methanosaeta, the forgotten methanogen? *Trends Microbiol*, **15**(4), 150-155.
- Smith, P.H., Mah, R.A. 1966. Kinetics of Acetate Metabolism during Sludge Digestion. *Applied Microbiology*, **14**(3), 368-&.
- Tang, Y.Q., Shigematsu, T., Morimura, S., Kida, K. 2005. Microbial community analysis of mesophilic anaerobic protein degradation process using bovine serum albumin (BSA)-fed continuous cultivation. *J Biosci Bioeng*, **99**(2), 150-164.
- Tsukahara, K., Yagishita, T., Ogi, T., Sawayama, S. 1999. Treatment of liquid fraction separated from liquidized food waste in an upflow anaerobic sludge blanket reactor. *J Biosci Bioeng*, **87**(4), 554-556.
- van Haandel, A., De Vrieze, J., Verstraete, W., dos Santos, V.S. 2013. Methanosaeta dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. *Journal of Chemical Technology & Biotechnology*, n/a-n/a.
- Wang, T., Brown, M.J. 1999. mRNA quantification by real time TaqMan polymerase chain reaction: Validation and comparison with RNase protection. *Anal Biochem*, **269**(1), 198-201.
- Xing, J., Criddle, C., Hickey, R. 1997. Effects of a long-term periodic substrate perturbation on an anaerobic community. *Water Res*, **31**(9), 2195-2204.
- Yilmaz, V., Ince-Yilmaz, E., Yilmazel, Y.D., Duran, M. 2014. Is acetoclastic methanogen composition in full-scale anaerobic processes related to acetate utilization capacity? *Appl Microbiol Biotechnol*, **98**(11), 5217-26.
- Yu, Y., Lee, C., Kim, J., Hwang, S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*, **89**(6), 670-679.
- Zhang, C.S., Xiao, G., Peng, L.Y., Su, H.J., Tan, T.W. 2013. The anaerobic co-digestion of food waste and cattle manure. *Bioresour Technol*, **129**, 170-176.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhilina, T.N., Zavarzina, D.G., Kevbrin, V.V., Kolganova, T.V. 2013. Methanocalculus natronophilus sp. nov., a new alkaliphilic hydrogenotrophic methanogenic archaeon from a soda lake, and proposal of the new family Methanocalculaceae. *Microbiology*, **82**(6), 698-706.

Chapter II

Optimizing Methane Production from Organic Wastes

Improving Biogas Production from Dilute Dairy Waste by Anaerobic Co-digestion with Food Waste

Abstract

Dairy waste generated in regions of warm and humid climate represents a unique challenge due to significant dilution by large quantities of water used for sanitation and cooling which is important to maintain animal productivity under such climate conditions. The objective of this study was to evaluate the feasibility of food waste as an organic-rich co-substrate to improve biomethane production in the anaerobic co-digestion of dilute dairy waste. Characterization of both waste materials shows that the organic content of food waste was 418 gVS/L, which was more than 20 times higher than that of dairy waste (18 gVS/L). Accordingly, the volumetric methane potential of food waste ($102 \text{ L}_{\text{methane}}/\text{L}_{\text{FW}}$) was 21 times greater than that of dairy waste ($4.8 \text{ L}_{\text{methane}}/\text{L}_{\text{DW}}$), suggesting the potential of food waste in improving methane yield. The anaerobic mono-digestion of food waste as the sole substrate indicates that methane production was completely inhibited at food waste loadings above 27.2 gVS/L due to significant accumulation of volatile fatty acids and drop in pH. When food waste was added as the co-substrate into the anaerobic co-digestion of dairy waste, volumetric methane production was improved significantly by more than 200% as compared with the methane yield achieved in anaerobic mono-digestion of dilute dairy waste. Therefore, significant improvements of methane production from the anaerobic digestion of dilute dairy waste could be enhanced by insignificant loadings of food waste as the co-substrate.

Keywords: *Methane, Food waste, Dairy farm waste, Anaerobic digestion, Loading rate, Renewable energy*

Introduction

Anaerobic digestion has been implemented as a sustainable technology for the treatment of various types of organic wastes and the production of biogas as a renewable energy source. Given the large amount of animal manure generated from the livestock industry, major efforts have been made to broaden the application of anaerobic digestion technology in the treatment of animal waste. One challenge to the implementation of anaerobic digestion is the low organic content of certain animal waste, such as cattle manure, and the low biogas yield as a result, which could negatively impact the economic feasibility of anaerobic digestion for animal waste treatment.

One strategy to overcome this challenge is the development of anaerobic co-digestion process by using organic-rich waste materials as co-substrates to compensate for the low organic content of certain types of animal waste (Ward et al., 2008). Indeed, various waste streams have been used as co-substrates in the anaerobic digestion of cattle manure and other animal wastes with varying successes. Food wastes (FW), particularly post-consumer wastes generated at increasing rates due to population growth and rising living standards (Parfitt et al., 2010), have drawn recent interest as potential co-substrates

for the anaerobic digestion of animal wastes with low organic content (Li et al., 2010; Zhang et al., 2007).

Dairy waste generated in regions of warm and humid climate represents a unique challenge due to significant dilution by large quantities of water used for sanitation and cooling which is important to maintain animal productivity under such climate conditions (Morse Meyer et al., 1997; West, 2003). Previous studies have shown that such dilute dairy waste exhibited total solids content of less than 3% (Zhang et al., 2011b), which is considerably lower than the total solids content of greater than 15% in dairy wastes generated from dairy farms in other regions (El-Mashad & Zhang, 2010; Neves et al., 2009; Zhang et al., 2013). Given the substantial differences in substrate characteristics, the feasibility of dilute dairy waste for anaerobic digestion could not be evaluated readily with results from previous studies using more concentrated dairy waste. Therefore, the objective of this work was to assess the feasibility of anaerobic co-digestion of dilute dairy waste with organic-rich cafeteria food waste to enhance process efficiency.

Materials and methods

Materials

Dilute dairy waste (DW), a mixture of raw dairy manure and wastewater discharged during milking/cleaning operations, was obtained from the waste storage basin of a dairy farm located in Loudon County, Tennessee, USA. The DW was used as the substrate to establish a set of 3.6-L continuous-flow anaerobic digesters with a solids retention time of 20 days at a constant temperature of 35°C. The digestate was used as the inoculum for the subsequent development of batch anaerobic digesters (Chen et al., 2012; Zhang et al., 2011b). Food waste (FW) was collected from the student cafeteria on the campus of the University of Tennessee, Knoxville. To prevent clogging of tubing, the dairy waste slurry and the food waste were prepared by homogenization using a blender and subsequently passing through a 2-mm mesh sieve to remove large debris. All waste materials were preserved at 4°C until use.

Methane potential determination

Methane potentials of the DW, FW and inoculum used in anaerobic digestion were determined in batch digesters following previously described procedures (Hansen et al., 2004). Batch digesters were set up in sealed 160-mL serum bottles with an effective volume of 100 mL. Loading rates of DW and FW were 1.7 and 3.4g VS/L, respectively (Fig. II1). Subsequently, all batch digesters were flushed with a gas mixture of nitrogen and carbon dioxide (80:20 v/v) for 5 min in order to establish anaerobic condition (He & Sanford, 2002). The control digesters contained 100 mL of inoculums only. All digesters mentioned above were operated in triplicate and incubated at 35°C with constant agitation with a stirring bard at 80 rpm in an incubator (New Brunswick Scientific, Edison, New Jersey, USA). Biogas production and methane content were monitored periodically until gas production ceased. The removal efficiency of volatile solids (VS) was quantified following the termination of the methane potential test.

Anaerobic mono-digestion of FW

Methanogenic feasibility of food waste in anaerobic digestion was evaluated with different loading rates (Table II1) as the sole substrate (mono-digestion) in anaerobic batch digesters, using the same inoculum and FW material as those in methane potential measurements. For each batch digester, the inoculum was mixed with FW and the total volume was adjusted to 100 mL. The control batch digesters were set up with the inoculum as the only substrate. All digesters were established in triplicates and operated at 35°C with constant agitation. Biogas production and methane content were monitored as described previously (Zhu et al., 2011).

Table II1 Various substrate loading rates in anaerobic digestion applied in this study.^a

Batch reactors ^d	FW (gVS/L)	DW (gVS/L)
<i>Mono-digester^b</i>		
M-D1	3.4	0
M-D2	6.8	0
M-D3	13.6	0
M-D4	27.2	0
M-D5	54.4	0
<i>Co-digesters^c</i>		
C-D1	3.4	12.42
C-D2	6.8	12.42
C-D3	13.6	12.42

^a Two substrates were applied in this experiment: food waste (FW) and dairy farm waste slurry (DW).

^b Mono-digester: FW was added as the sole substrate with operational volume of 100mL.

^c Co-digester: both FW and DW were added with operational volume of 40mL.

^d 4.46 and 0.93 gVS/L of inoculum were initiated in mono and co-digesters respectively. Triplicate digesters were set up for each FW loading rate in both mono and co-digesters.

Table II2 Chemical characteristics of substrates used in this study.

Parameters	FW ^a	DW ^a	Inoculum ^a
pH	4.44 ± 0.02	7.37 ± 0.03	7.80 ± 0.01
Total solids (g/L)	438.76 ± 0.08	24.70 ± 0.04	12.35 ± 1.06
Volatile solids (g/L)	417.94 ± 27.55	17.70 ± 0.21	7.44 ± 0.17
Total alkalinity (mg CaCO ₃ /L)	NA ^b	6250 ± 150	7367 ± 161
Water content (%)	81.21 ± 0.05	98.66 ± 0.01	97.57 ± 0.01

^a Data are means of triplicate measurements ± standard deviations.

^b NA: not detectable since FW sample's pH was below the titration end point at pH 4.5 for total alkalinity according to standard method.

Anaerobic co-digestion of DW with FW

To further evaluate the methane production of FW as a co-substrate in anaerobic digestion, anaerobic co-digesters were set up with both FW and DW as the substrates. Similar to the experimental setting in anaerobic mono-digestion test, various loading rates of FW (Table II1) along with DW (12.42 gVS/L) and inoculum (9.36 gVS/L) were added into batch anaerobic co-digesters. Biogas production and methane content were monitored periodically as described in section 2.3. Volatile solids (VS) concentration and its removal were quantified when the digestion test was terminated.

Chemical analysis

Biogas production from the anaerobic digesters was determined with a previous described water-displacement method (Demirer & Speece, 1998). Methane content was analyzed by a Hewlett Packard (HP) 5890 Series II gas chromatograph (GC) with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen-1000). Argon was used as the carrier gas with a flow rate of 5mL/min and the following the temperature program: oven 125°C, injection port 150°C and detector 170°C (Zhu et al., 2011). According to standard methods (APHA/AWWA/WEF, 2005), total solids (TS) and volatile solids (VS) were determined according to Method 2540B and 2540E respectively; total alkalinity (ALK) was quantified according to Method 2320B. Volatile fatty acids (VFA), including acetate, propionate and butyrate, were determined with an Agilent 1200 HPLC system (Agilent Technologies, CA, US) equipped with a 1260 Infinity Multiple Wavelength detector (MWD) (set to 210nm) and a Aminex HPX- 87H ion exclusion organic acid analysis column, 300 × 7.8 mm (Bio-Rad, CA, US) (Karr et al., 1983). The eluent was 8 mM H₂SO₄, pumped at a flow rate of 0.6 ml min⁻¹ through a heated (30°C) column. Samples (0.5 ml) periodically withdrawn from the batch reactors were spun down by a microcentrifuge (14,000 rpm, 15 min at 4°C). The supernatants (475µL) were transferred to autosampler vials and acidified with 1M H₂SO₄ (25µL) before analysis.

Results and discussion

Waste characterization

To evaluate the methane production potential as a co-substrate in anaerobic digestion with DW, the characteristics of FW and DW were analyzed and compared as well as the inoculum material (Table II2). Similar to the characteristics of FW collected from university dining halls and cafeteria in other studies (Kim et al., 2004; Kwon & Lee, 2004; Shin et al., 2004), the total solids (TS) content was 43.9%, more than twenty times higher than that in DW, indicative of the much greater methane potential of FW than that of DW (Zhu et al., 2011). It should be noted that the TS content of DW in this study was less than 2.5%, which was significantly lower than the TS content of dairy wastes reported in other studies (El-Mashad & Zhang, 2010; Neves et al., 2009; Zhang et al., 2013), representing the potentially major differences in dairy waste characteristics due to differences in production practices in regions of distinct climates.

The low pH (pH =4.8) and lack of alkalinity in FW (Table II2), however, indicate its potential inhibitory effects on anaerobic digestion when used as the sole substrate or at high loading rates (Zhu et al., 2011). DW, however, had a much higher pH at 7.4 and a greater alkalinity of 6250mg CaCO₃/L. It suggests that DW would have much higher buffer capacity than FW in anaerobic digestion. Due to the high VS content of FW and high buffering capacity of DW, it could be hypothesized that biogas production from DW could be significantly enhanced by the anaerobic co-digestion with FW as a co-substrate with higher organic content while the acidity of FW could be balanced by the high buffering capacity of DW (Table II2). To evaluate the potential enhancement in methane production by anaerobic co-digestion, the methane potential of DW and FW as the sole substrate was first quantified.

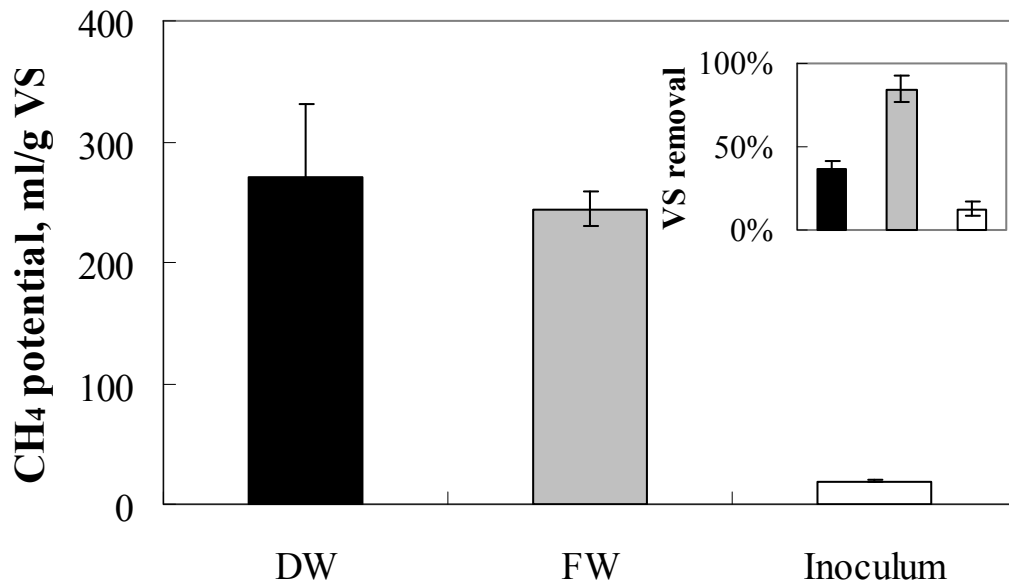


Fig. III1. Methane potential of dairy waste (DW), food waste (FW) and inoculum, with the inset indicating the corresponding removal of volatile solid (VS). The inoculum was the digestate discharged from anaerobic digesters treating dairy waste. Data points are means of triplicates and the error bars indicate the standard deviations.

Methane potential

In order to determine the methane potentials, batch anaerobic digesters were added with DW or FW as the sole substrate and inoculated with anaerobic digestate. The controls had only the inoculums. Methane potential was determined as the cumulative methane production when anaerobic digestion reached completion.

The methane potential of DW (270 ± 60 mL CH₄/gVS) was not significantly different from that of FW (244 ± 14 mL CH₄/gVS) (Fig.1). However, the utilization of organic matter, measured as VS removal, reached only 40% for DW, which was much lower than the VS removal of 80% for FW (Fig. II1), suggesting that the organic matter in DW was less readily biodegradable than that in FW, likely due to the presence of recalcitrant cellulosic materials (Kaparaju & Rintala, 2005). The minor degradable fraction was likely comprised with lipids and proteins, which may have much higher methane potential than carbohydrates, the main component for cellulosic materials (Kaparaju et al., 2008). Although VS removal of FW in anaerobic digestion was considerably higher than DW, the similar methane potentials of these two materials indicated the biodegradable fraction of DW had greater energy potential than that of FW. Consistent with results of methane potential and VS removal rate of FW in anaerobic digestion obtained from previous studies (El-Mashad & Zhang, 2010; Lee et al., 2009; Wang et al., 1997; Zhang et al., 2007), While the methane potentials for DW and FW were comparable, the volumetric methane potentials were considerably different given the much higher VS content of FW as compared with DW. Indeed, the volumetric methane potential was $102 \text{ L}_{\text{methane}}/\text{L}_{\text{FW}}$, approximately 21 times greater than that of DW ($4.8 \text{ L}_{\text{methane}}/\text{L}_{\text{DW}}$) (Table II2). Thus, biogas production from anaerobic digestion of DW could be improved by adding FW as the co-substrate even at relatively low volumetric loading rates.

The methane potential of the inoculum was determined as 18.9 ml CH₄/gVS, which was negligible as compared with those of DW and FW (Fig. II1). Correspondingly, VS removal rate was only $13 \pm 4\%$, which was expected as the inoculum material was essentially digested DW with most of the biodegradable materials depleted. Since both methane potential and VS removal rate of inoculum were substantially less than those of DW and FW, the contribution of the inoculum to biogas production would be insignificant in the anaerobic digestion of DW and FW.

Anaerobic mono-digestion

Although FW had much higher volumetric methane potential and was demonstrated to improve biogas production as co-substrate in anaerobic digestion with DW, it should be noted that FW could contain significant levels of long chain fatty acids (Carucci et al., 2005; Jiang et al., 2013), which may be hydrolyzed into volatile fatty acids (VFA) during fermentation process (Hanaki et al., 1981). The accumulation of VFA may become inhibitory to methanogenesis (Chen et al., 2008; Lin et al., 2011). The improper loading rates of FW could disturb the anaerobic digestion process and eliminate the benefits of the addition of FW as co-substrate in order to improve biogas production. Thus, optimum FW loading rates with minimum inhibitory effects were further investigated in anaerobic

batch reactors using FW as sole substrate.

Results from anaerobic mono-digestion indicate that there were no obvious inhibitory effects with FW loading rate at 13.6gVS/L or below (Fig. II2B). The methane production from FW ranged from 216 ± 40 to 244 ± 14 mLCH₄/gVS following 20 days of anaerobic digestion with FW loading rates increased from 3.4 to 13.6gVS/L, which were almost identical to the methane potential of FW tested before (Fig. II1). The completion of FW digestion with loading rates from 3.4 to 13.6gVS/L was also demonstrated by the high VS removal rates of FW, which were all above 85% (data not shown).

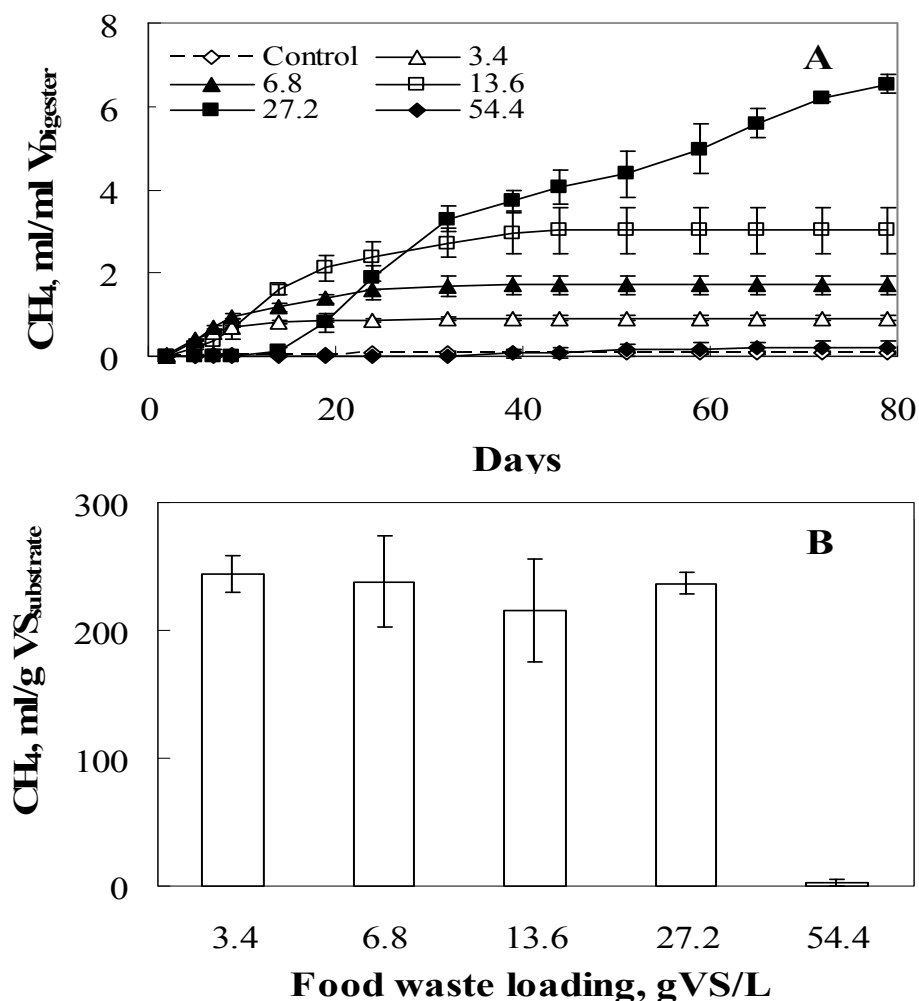


Fig. II2. Methane production from anaerobic mono-digestion of food waste as the only substrate at various loading rates. (A) Cumulative methane production over time; and (B) comparison of methane yields from food waste. Data points are means of triplicate batch reactors and the error bars indicate the standard deviations. The loading rates of food waste were shown as gVS/L of digestion volume.

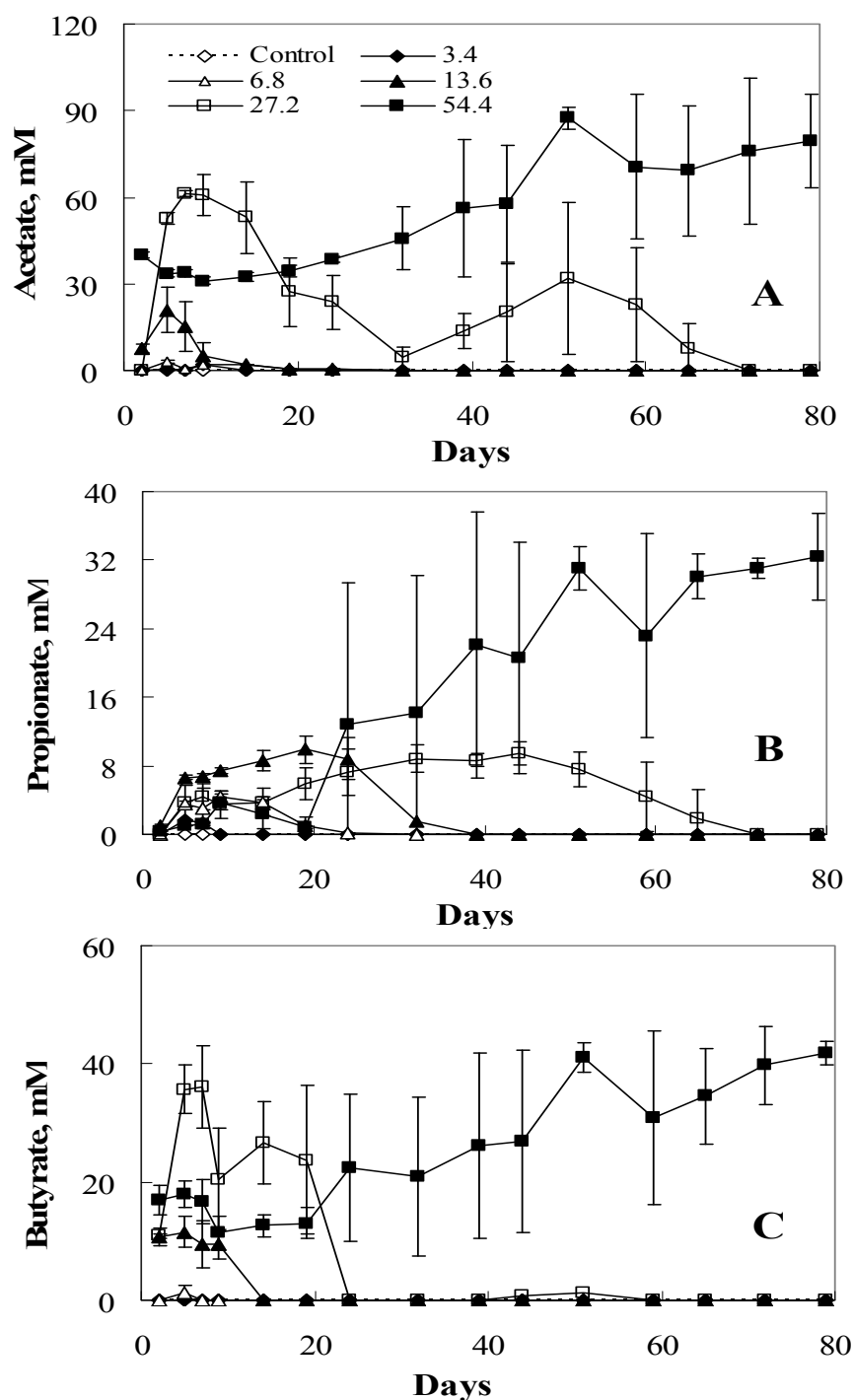


Fig. II.3. Temporal concentration profiles of volatile fatty acids during anaerobic mono-digestion of food waste at various loading rates. (A) acetate; (B) propionate; and (C) butyrate. Data points are means of triplicate batch reactors and the error bars indicate the standard deviations. The loading rates of food waste were shown as gVS/L of digestion volume.

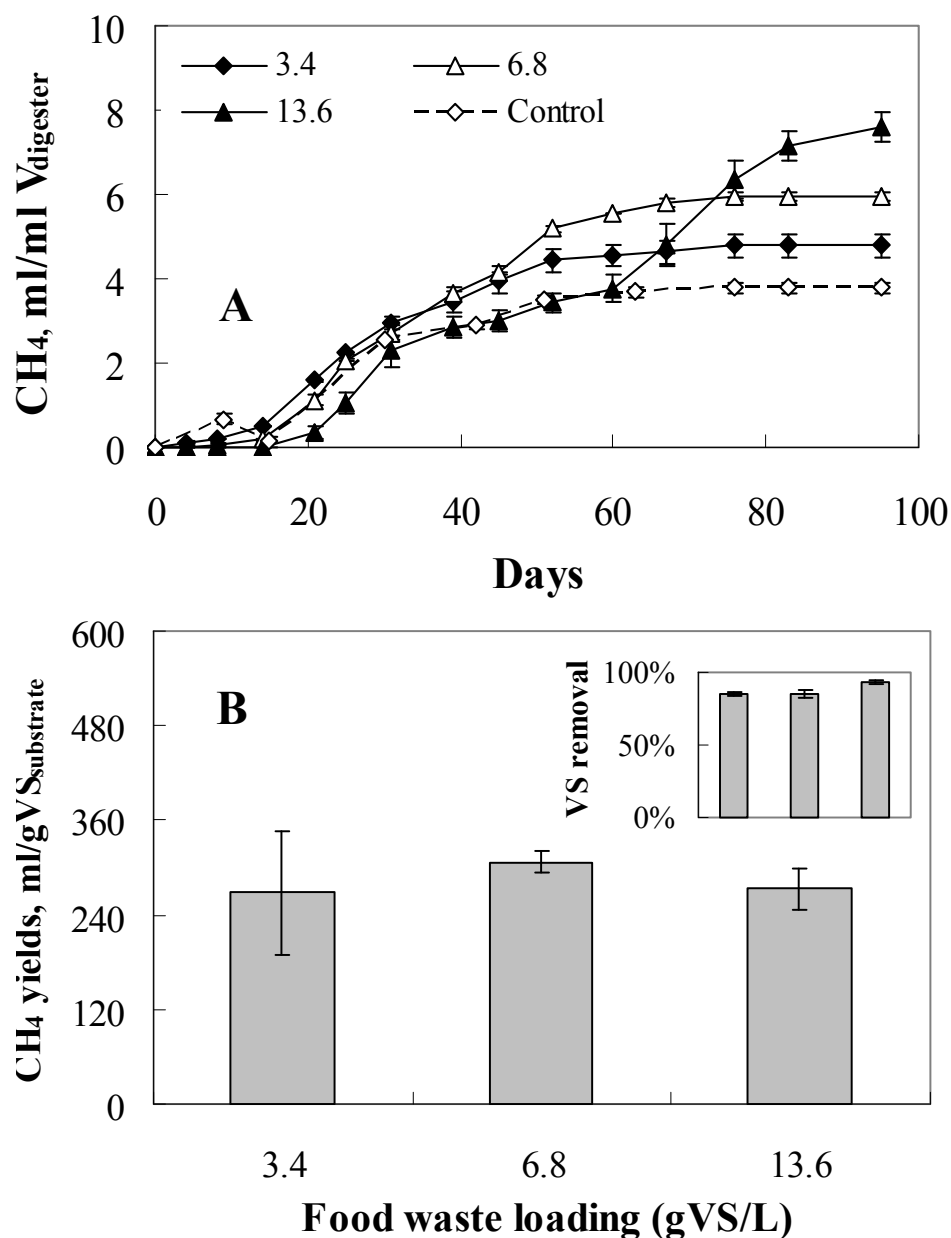


Fig. II.4. Methane productions of food wastes (FW) in anaerobic co-digestion at various loading rates. (A) Cumulative methane production over time; and (B) comparisons of methane yields only from food wastes at various loading rates, with the inset showing FW utilization efficiencies as VS removal at the completion of digestion. All methane yields from co-digestion indicated were only from FW by subtracting methane produced from cow manure. Data points are means of triplicate batch reactors and the error bars indicate the standard deviations.

When the FW loading rate was raised to 27.2 gVS/L, a much prolonged period (70 days) was needed for the completion of anaerobic digestion, when cumulative methane production reached 237 ± 8 mL CH₄/gVS and VS removal rate reached approximately 90%, both comparable to those achieved at lower FW loading rates of 3.4-13.6gVS/L (Fig. II2B), suggesting that the anaerobic digestion process was inhibited to some extent at the loading rate of 27.2 gVS/L. A closer examination of the anaerobic digestion process revealed the transient accumulation of VFAs, which gradually diminished as anaerobic digestion progressed (Fig. II3), providing evidence that the addition of FW at this loading rate could potentially cause process imbalance.

When the FW loading rate was further increased to 54.4gVS/L, however, the anaerobic digestion process was severely inhibited with no significant methane production (Fig. II2). Moreover, VFA levels kept increasing, reaching 79, 32 and 42 mM for acetate, propionate, and butyrate, respectively, with no signs of subsiding (Fig. II3). Accordingly, the accumulation of VFAs reduced the alkalinity from 4800 to 1700 mg CaCO₃/L and depressed pH from near neutral to the acidic level of 4.8 (Fig. II6A). These results suggest that FW loading rates greater than 27.2 gVS/L may cause process inhibition and only lower loading rates could be beneficial in anaerobic co-digestion using FW as a co-substrate.

Anaerobic co-digestion

The effectiveness of anaerobic co-digestion of DW with FW was tested with FW loading rates from 3.4 to 13.6 gVS/L, in order to prevent the potential inhibition from excess FW (Table II1). In the anaerobic co-digestion of DW, representing FW/DW loading ratios (VS_{FW}/VS_{DW}) between 0.27 and 1.1 (Table II1). As compared to CH₄ yields in mono-digestion with the same FW loading rates (Fig. II2B), methane productions under all FW dosages in anaerobic co-digestion were promoted ranging from 268 to 307mLCH₄/gVS (Fig. II4B). At FW dosage of 6.8gVS/L or the optimum ratio of FW/DW, 0.55, it reached the maximum value (Fig. II4B). Generally, CH₄ productions in anaerobic co-digestion had been promoted significantly by 10 ~ 29% comparing to those in anaerobic mono-digestion. The high VS removal rates of FW (above 85%) in anaerobic co-digestion with all dosages also indicated the complete utilization of biodegradable components of FW in biogas production. Although FW had low pH and almost no alkalinity, the methanogenesis process of FW was likely to be enhanced in anaerobic co-digestion with DW in this experiment as it was reported by previous studies that adding FW as co-substrate in anaerobic digestion could be beneficial due to the dilution of toxic chemicals (Azbar et al., 2008; Khalid et al., 2011), balance of nutrients such as C/N ratio (Zhang et al., 2013), trace element addition (Zhang et al., 2011a) and synergistic effect of microorganisms (Chen et al., 2008).

The VFAs including acetate, propionate and butyrate in co-digesters with FW loading rates from 3.4 to 13.6gVS/L were depleted at the end of reactions despite of initial accumulations (Fig. II5A-C), suggesting well balanced reactions between fermentation and methanogenesis in anaerobic co-digestion. For acetate concentration, however, the multiple peaks occurring in co-digesters with FW loading rate, 13.6gVS/L

over the whole process (Fig. II5A) may indicate the acetogenesis and methanogenesis is not synchronous. It could also explain the unique cumulative methane production curve at FW loading rate of 13.6gVS/L comparing with other curves at lower FW loading rates (Fig. II4A). During first 30 days, CH₄ accumulated gradually as other curves with lower FW loading rates following the deceleration of CH₄ accumulation until the 60th day. Finally, the CH₄ production rate boosted with fast consumption of acetate in co-digesters. Unlike acidification effects with high FW loading rate, i.e. 54.4gVS/L observed in anaerobic mono-digestion (Fig. II6A), constant neutral pH values and the increase of alkalinity in anaerobic co-digestion with FW loading rates from 3.4 to 13.6gVS/L after completion of reactions also indicated no inhibitory effects but enhancement for the addition of FW in anaerobic co-digestion with DW (Fig. II6B).

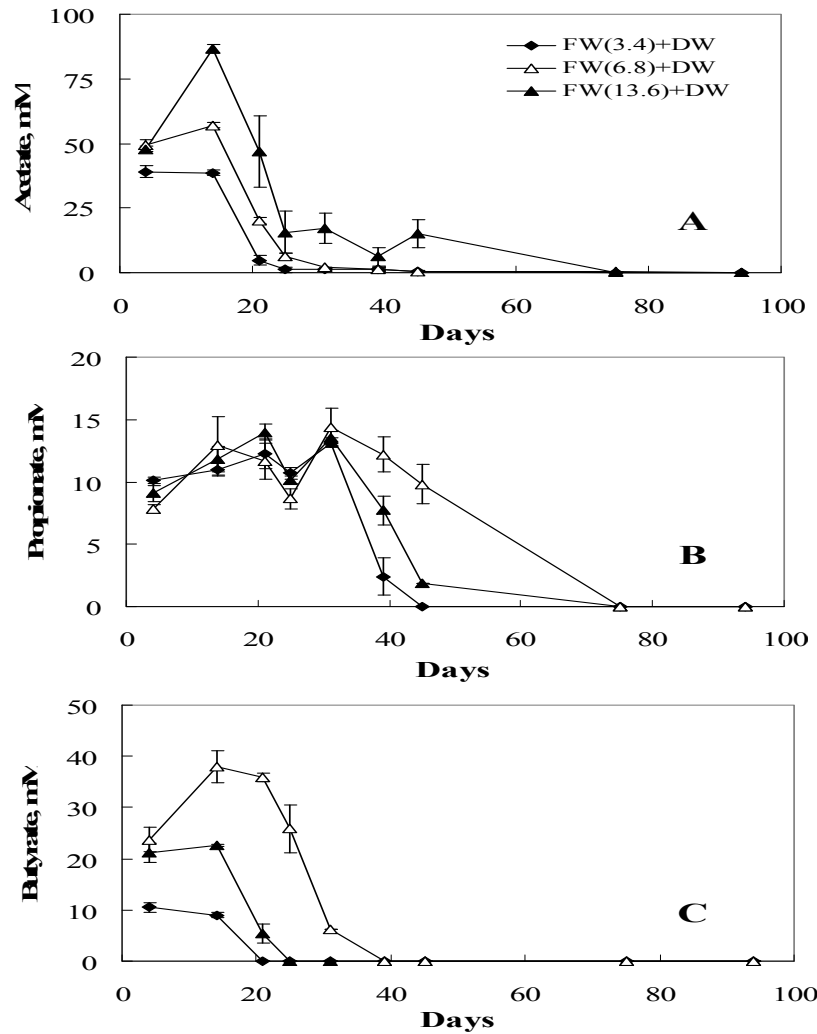


Fig. II5. Volatile fatty acids concentrations of anaerobic co-digestion of food waste (FW) with dairy waste (DW) at various loading rates over time. (A) acetate; (B) propionate; and (C) butyrate. Data points are means of triplicate batch reactors and the error bars indicate the standard deviations.

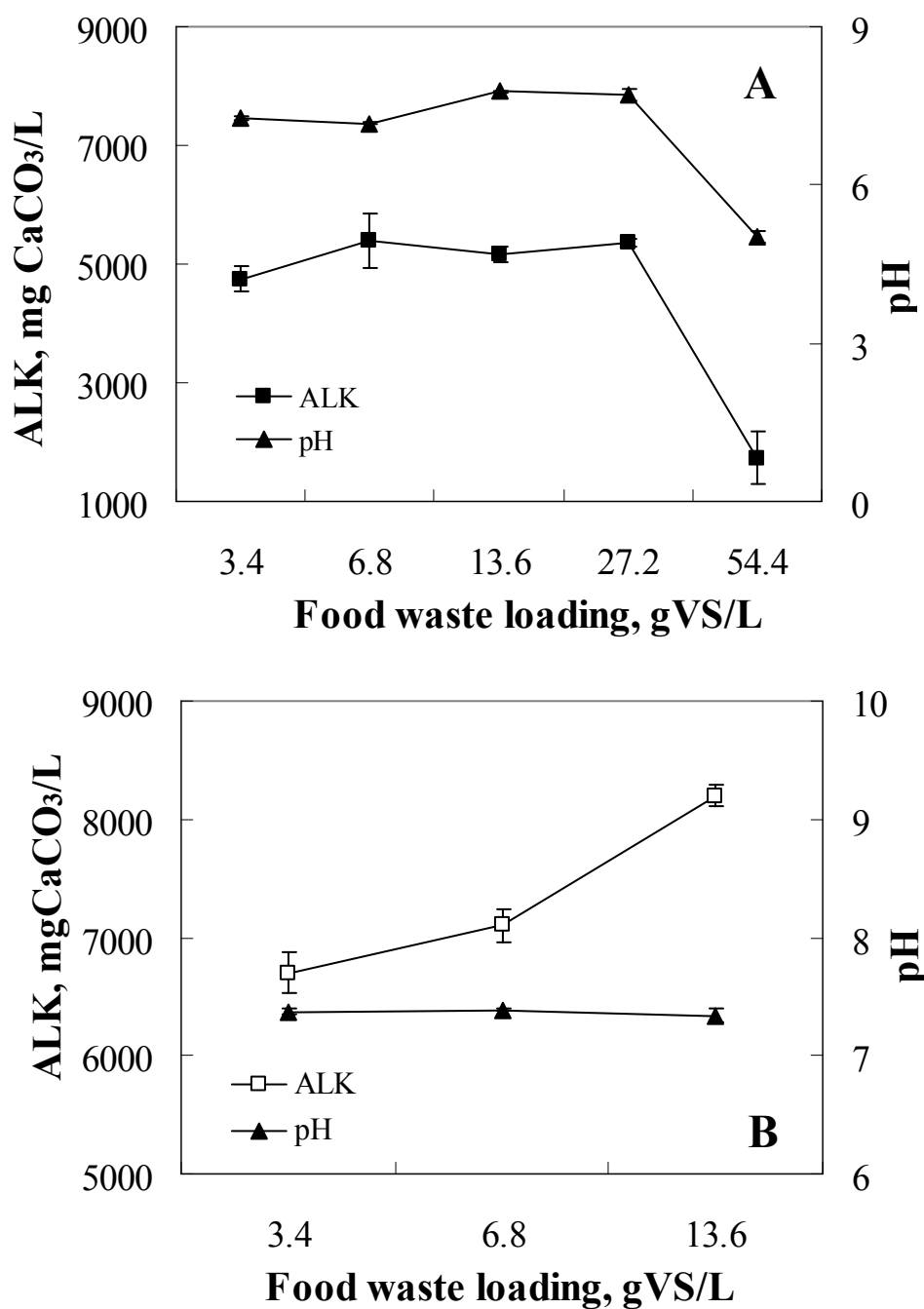


Fig. II6. Alkalinity (ALK) and pH values at the end of anaerobic mono (A) and co-digestion (B) of food wastes (FW) at various loading rates. Data points are means of triplicate batch reactors and the error bars indicate the standard deviations.

Conclusion

The methane potential of the organic rich material, FW was evaluated in this study. It was shown as a substrate with high VS content and volumetric methane potential as compared to DW. However, the anaerobic mono-digestion of FW with various loading rates indicated that the inhibition of anaerobic digestion could happen at high loadings with accumulation of VFAs and dramatic decreasing of pH and alkalinity in digesters, suggesting the importance of proper FW loading decided in practical operations. In contrast, the use of FW as co-substrate in anaerobic co-digestion at proper loading rate or VS ratio between FW and DW could significantly improve methane production by up to 29% at FW/DW ratio close to 0.5 approximately. The operational feasibility of anaerobic co-digestion using FW as co-substrate could be enhanced by insignificant volumetric loading rates (less than 3%) for considerable improvements of methane production. Therefore, significant increase of biomethane production could be achieved at relatively low FW loading rates without inhibitive effects.

References

- APHA/AWWA/WEF. 2005. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.
- Azbar, N., Keskin, T., Yuruyen, A. 2008. Enhancement of biogas production from olive mill effluent (OME) by co-digestion. *Biomass & Bioenergy*, **32**(12), 1195-1201.
- Carucci, G., Carrasco, F., Trifoni, K., Majone, M., Beccari, M. 2005. Anaerobic Digestion of Food Industry Wastes: Effect of Codigestion on Methane Yield. *Journal of Environmental Engineering*, **131**(7), 1037-1045.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *Journal of Applied Microbiology*, **113**(6), 1371-1379.
- Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, **99**(10), 4044-4064.
- Demirer, G.N., Speece, R.E. 1998. Anaerobic biotransformation of four 3-carbon compounds (acrolein, acrylic acid, allyl alcohol and n-propanol) in UASB reactors. *Water Research*, **32**(3), 747-759.
- El-Mashad, H.M., Zhang, R. 2010. Biogas production from co-digestion of dairy manure and food waste. *Bioresource Technology*, **101**(11), 4021-4028.
- Hanaki, K., Matsuo, T., Nagase, M. 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnology and Bioengineering*, **23**(7), 1591-1610.
- Hansen, T.L., Schmidt, J.E., Angelidaki, I., Marca, E., Jansen, J.I.C., Mosbæk, H., Christensen, T.H. 2004. Method for determination of methane potentials of solid organic waste. *Waste Management*, **24**(4), 393-400.
- He, Q., Sanford, R.A. 2002. Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. *Biodegradation*, **13**(5), 307-316.
- Jiang, J.G., Zhang, Y.J., Li, K.M., Wang, Q., Gong, C.X., Li, M.L. 2013. Volatile fatty acids production from food waste: Effects of pH, temperature, and organic loading rate. *Bioresource Technology*, **143**, 525-530.
- Kaparaju, P., Buendia, I., Ellegaard, L., Angelidakia, I. 2008. Effects of mixing on methane production during thermophilic anaerobic digestion of manure: Lab-scale and pilot-scale studies. *Bioresource Technology*, **99**(11), 4919-4928.
- Kaparaju, P.L.N., Rintala, J.A. 2005. The effects of post-treatments and temperature on recovering the methane potential of > 2 mm solid fraction of digested cow manure. *Environmental Technology*, **26**(6), 625-631.
- Karr, D.B., Waters, J.K., Emerich, D.W. 1983. Analysis of Poly- β -Hydroxybutyrate in *Rhizobium japonicum* Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV Detection. *Applied and Environmental Microbiology*, **46**(6), 1339-1344.
- Khalid, A., Arshad, M., Anjum, M., Mahmood, T., Dawson, L. 2011. The anaerobic digestion of solid organic waste. *Waste Management*, **31**(8), 1737-1744.
- Kim, S.H., Han, S.K., Shin, H.S. 2004. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *International Journal of Hydrogen Energy*, **29**(15), 1607-1616.
- Kwon, S.H., Lee, D.H. 2004. Evaluation of Korean food waste composting with fed-

- batch operations I: using water extractable total organic carbon contents (TOC_w). *Process Biochemistry*, **39**(10), 1183-1194.
- Lee, D.H., Behera, S.K., Kim, J.W., Park, H.-S. 2009. Methane production potential of leachate generated from Korean food waste recycling facilities: A lab-scale study. *Waste Management*, **29**(2), 876-882.
- Li, R.P., Chen, S.L., Li, X.J. 2010. Biogas Production from Anaerobic Co-digestion of Food Waste with Dairy Manure in a Two-Phase Digestion System. *Applied Biochemistry and Biotechnology*, **160**(2), 643-654.
- Lin, J., Zuo, J.E., Gan, L.L., Li, P., Liu, F.L., Wang, K.J., Chen, L., Gan, H.N. 2011. Effects of mixture ratio on anaerobic co-digestion with fruit and vegetable waste and food waste of China. *Journal of Environmental Sciences-China*, **23**(8), 1403-1408.
- Morse Meyer, D., Garnett, I., Guthrie, J.C. 1997. A Survey of Dairy Manure Management Practices in California. *Journal of Dairy Science*, **80**(8), 1841-1845.
- Neves, L., Oliveira, R., Alves, M.M. 2009. Fate of LCFA in the co-digestion of cow manure, food waste and discontinuous addition of oil. *Water Research*, **43**(20), 5142-5150.
- Parfitt, J., Barthel, M., Macnaughton, S. 2010. Food waste within food supply chains: quantification and potential for change to 2050. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**(1554), 3065-3081.
- Shin, H.S., Youn, J.H., Kim, S.H. 2004. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *International Journal of Hydrogen Energy*, **29**(13), 1355-1363.
- Wang, Y.S., Odle, W.S., Eleazer, W.E., Barlaz, M.A. 1997. Methane potential of food waste and anaerobic toxicity of leachate produced during food waste decomposition. *Waste Management & Research*, **15**(2), 149-167.
- Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L. 2008. Optimisation of the anaerobic digestion of agricultural resources. *Bioresource Technology*, **99**(17), 7928-7940.
- West, J.W. 2003. Effects of Heat-Stress on Production in Dairy Cattle. *Journal of Dairy Science*, **86**(6), 2131-2144.
- Zhang, C.S., Xiao, G., Peng, L.Y., Su, H.J., Tan, T.W. 2013. The anaerobic co-digestion of food waste and cattle manure. *Bioresource Technology*, **129**, 170-176.
- Zhang, L., Lee, Y.W., Jahng, D. 2011a. Anaerobic co-digestion of food waste and piggery wastewater: Focusing on the role of trace elements. *Bioresource Technology*, **102**(8), 5048-5059.
- Zhang, R., El-Mashad, H.M., Hartman, K., Wang, F., Liu, G., Choate, C., Gamble, P. 2007. Characterization of food waste as feedstock for anaerobic digestion. *Bioresource Technology*, **98**(4), 929-935.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011b. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresource Technology*, **102**(2), 779-785.
- Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.

Chapter III

**Linkage between Microbial Populations and Process Conditions in
Anaerobic Digestion**

Part I: Impact of Substrate Overloading on Archaeal Populations in Anaerobic Digestion of Animal Waste

Abstract

Aim: To characterize adaptive changes in archaeal microbial community in response to substrate overloading and identify potential linkages between process performance and microbial community composition.

Methods and Results: Triplicate continuous anaerobic digesters were developed as model anaerobic digestion processes, which were subsequently disrupted by substrate overloading. The clone library analysis of archaeal communities experiencing substrate overloading showed that populations related to *Methanosaeta* were the dominant methanogens before and after substrate overloading, suggesting the functional importance of these acetoclastic methanogens in balanced anaerobic digestion processes characterized with low organic acids concentrations. Population redundancy in *Methanosaeta* increased following substrate overloading with the emergence of additional populations of *Methanosaeta*. More importantly, the methanogenic community responded to process imbalance with greater functional diversity with increased abundance of functionally distinct hydrogenotrophic and acetoclastic methanogens, which likely enhanced the functional stability of anaerobic digestion during disruptions in the anaerobic food web under process perturbation. *Crenarchaeota* were identified as persistent constituents of the archaeal communities in anaerobic digestion, warranting further efforts to identifying the functions of these phylogenetically distinct populations in anaerobic digestion.

Conclusions: Substrate overloading in anaerobic digestion resulted in an increased functional diversity of the methanogenic community, which enhanced the capacity to overcome subsequent occurrences of process perturbations without performance disruption, providing a potential strategy to maintain process stability in anaerobic digestion.

Significance and Impact of the Study: Anaerobic digestion is a sustainable option for waste treatment and renewable energy production. However, process instability resulting from variations in substrate loading has been one of the obstacles to the broader adoption of anaerobic digestion technology. Insight into the linkages between process performance and microbial community gained in this study is valuable for developing strategies for the mitigation of the impact of substrate overloading on anaerobic digestion processes.

Introduction

Anaerobic digestion is an important biological process capable of simultaneous waste treatment and renewable energy recovery. Interest in anaerobic digestion has grown considerably in recent years, particularly with the need for increases in renewable energy production and reduction in greenhouse gas emission (McKendry, 2002). Despite the advantages of anaerobic digestion, the broader adoption of this technology for waste

treatment has been hindered by concerns of potential process instability resulting from the susceptibility of methanogenic microbial populations to changes in process conditions (Chen et al., 2008), such as fluctuations in organic loading rates (OLRs) and changes in waste composition, which are frequently encountered in the anaerobic digestion of animal wastes.

Previous studies of anaerobic digestion processes have shown that substrate overloading frequently leads to process imbalance, characterized by the accumulation of organic acids and inhibition of biogas production (Leitao et al., 2006). As anaerobic biotransformation of organic wastes involves a complex anaerobic food web, efficient anaerobic digestion requires the balanced activities of diverse anaerobic microbial populations including acidogenic and methanogenic micro-organisms (Ahring, 2003; Schink, 1997). The terminal step in anaerobic decomposition of organic wastes, methanogenesis, is carried out exclusively by methanogens, a group of micro-organisms associated with *Archaea* (Luo et al., 2009). Methanogenesis is often considered as the limiting step in anaerobic digestion processes under perturbation, due to both the slow growth rates and the susceptibility to fluctuating process conditions characteristic of methanogens (Chen et al., 2008; Kotzé et al., 1969). Thus, the dynamics of archaeal populations in anaerobic digestion may provide insight into the linkage between process performance and microbial activities in anaerobic conversion under perturbation.

Animal waste generated from large scale livestock operations has been a source of environmental pollution and public health risk, because the natural decomposition of animal waste releases large quantities of pathogens, excess nutrients, organic matter, solids, methane, ammonia and odorants into the environment (Jongbloed & Lenis, 1998). Anaerobic digestion has been shown to be an effective technology to overcome these challenges (Chen & Cheng, 2005). More recently, anaerobic co-digestion of animal waste has been explored to enhance the economic feasibility of animal waste treatment (Mata-Alvarez et al., 2011). The success of anaerobic co-digestion typically exploits the potentially significant improvement in biogas production by supplementing the primary substrate with an organic-rich co-substrate, thus overcoming challenges presented by the low biogas yield of the primary substrate. This practice, however, frequently leads to fluctuations in OLRs, which may subsequently result in undesirable disruptions in process performance. Interestingly, it has been noted that anaerobic digestion processes can adapt to perturbations resulting from organic overloading, likely through long-term changes in microbial community structures (Hashsham et al., 2000; Xing et al., 1997). While it is suggested that increased population diversity may be linked to the adaptation to process perturbations, much remains to be learned on the changes in specific microbial populations before and after perturbations. Therefore, to gain insight into the responses of microbial populations to process disturbances in anaerobic digestion, the specific objective of this study is to investigate the impact of substrate overloading on the methanogenic populations, which complete the terminal step of the anaerobic food web, by comparing the archaeal populations before and after performance disruption in continuous anaerobic co-digestion of dairy and poultry wastes as a model anaerobic digestion process.

Materials and Methods

Anaerobic digester set-up

Triplicate mesophilic continuous anaerobic co-digesters were established with dairy waste as the primary substrate and poultry waste as the co-substrate following the configuration described previously (Fernandez et al., 2000). The co-digestion process exploited the higher solids content of poultry waste as compared with the primary substrate dairy waste (Table II) to enhance biogas production. All three completely mixed digesters had a working volume of 3.6 L and were operated in a constant temperature room at 35°C. During normal operation, the digesters were fed at 4h intervals and the hydraulic retention time was maintained at 20 days. These digesters were initiated with inoculum from an operating laboratory scale dairy manure anaerobic digester and established using dilute dairy waste as the only feed. Following the establishment of stable anaerobic digestion performance, poultry waste was added as an organic-rich co-substrate to achieve enhanced methane production. Poultry waste was also used to cause organic overloading when added at excessive levels. All digesters exhibited stable operation with consistent pH, methane yield and volatile fatty acids (VFAs) level prior to stepwise increases in the loading rate of poultry waste that eventually resulted in organic overloading and inhibition of biogas production. During this study, the initial OLR in the triplicate digesters was maintained at 1.3 g volatile solids (VS) L⁻¹day⁻¹, consisting of 1.0 g VS L⁻¹day⁻¹ from dairy waste and 0.3 g VS L⁻¹day⁻¹ from poultry waste. With the addition of more poultry waste to the feed, the OLR in the anaerobic digesters were raised stepwise to 1.5 g VS L⁻¹day⁻¹ and subsequently to 1.8 g VS L⁻¹day⁻¹ when normal process performance was disrupted by substrate overloading (Fig. III1). To recover the digesters from disturbance, feeding was stopped and later restored stepwise to 0.5, 1.0 and eventually 1.5 g VS L⁻¹day⁻¹, when stable operation was again achieved. Dairy waste was the only substrate in the feed, when OLR was 0.5 or 1.0 g VS L⁻¹day⁻¹; subsequently, poultry waste was added to the feed to raise the OLR from 1.0 to 1.5 g VS L⁻¹day⁻¹, resulting in a substrate mixture consisting of dairy waste and poultry waste with a ratio of 2 : 1 (gVS/gVS).

Chemical analysis

Biogas production from the anaerobic digesters was used as the primary parameter to monitor digestion performance (Michaud et al., 2002) and was determined using a water-displacement method described previously (Zhu et al., 2011). VFAs in the digestate were quantified using a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and a Restek Stabilwax ®-DA column as previously described (He et al., 2009). Methane content in biogas was analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen®-1000; Sigma-Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 ml min⁻¹ and the following temperature scheme: oven 125°C, injection port 150°C and detector 170°C. Chemical oxygen demand (COD), total alkalinity (TA), total solids (TS), VS and ammonia-nitrogen (NH₄⁺-N) were all determined according to standard methods (APHA 2005): COD was measured with the '5220C' Close Reflux-Titrimetric Method; TA was quantified using the '2320B' titration method; TS and VS were measured using the '2540 B and E'

method; and NH₃-N was quantified using the '4500- NH₃ D' method with an Orion 9512 ammonia ion selective electrode (Orion Research Inc., Beverly, MA, USA).

Clone library analysis of archaeal microbial populations

For archaeal microbial community analysis, pre-overloading sludge samples were taken from the anaerobic codigesters at day 0 when the performance of the digesters were stable; post-overloading samples were taken at day 340 following the complete recovery from performance disruption due to substrate overloading. Samples were stored at -80°C until analysis. Triplicate samples taken at the same time point were pooled before whole community DNA was extracted and purified as previously described (Zhang et al., 2009). Archaeal 16S rRNA genes were subsequently amplified by polymerase chain reaction (PCR) using the Archaea-specific primers Arch21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch958R (5'-YCCGGCGTTGAMTCCAATT-3') following PCR conditions described previously (Delong, 1992).

The amplified products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions. For each clone library, approximately 100 cloned plasmid inserts were randomly selected for sequencing with the ABI Prism BigDye chemistry (Applied Biosystems, Foster City, CA, USA) using M13 forward and reverse primers. The obtained sequences were screened for chimeric artefacts by the programme Chimera Check at the Ribosomal Database Project II (Cole et al., 2003). Subsequently, the 16S rRNA gene sequences were searched in the NCBI GenBank database using the BLAST program to locate the most similar sequences as the closest relatives to the 16S rRNA gene clones retrieved from the anaerobic digesters. Sequences were assigned preliminary phylogenetic associations based on the RDP CLASSIFIER programs at [http:// rdp.cme.msu.edu/classifier/classifier.jsp](http://rdp.cme.msu.edu/classifier/classifier.jsp) (Cole et al., 2003). These 16S rRNA gene sequences were further aligned with homologous sequences using CLUSTALX (Thompson et al., 1997) and used for the construction of phylogenetic trees by the neighbor-joining algorithm (1000 bootstrap resamplings) with MEGA 4.0 (Tamura et al., 2007). A sequence or cluster of sequences with <3% dissimilarity to the adjacent sequence(s) was defined as an operational taxonomic unit (OTU) as previously described (Zhang et al., 2011). Partial 16S rRNA gene sequences recovered in this study were deposited at GenBank under the following accession numbers: JN052741–JN052771 and JN083826–JN083830.

Statistical Data Analysis

To compare the archaeal community structures of the anaerobic co-digesters pre-disturbance and post-disturbance, the Shannon diversity index (H') was calculated for the pre-overloading and post-overloading clone libraries as previously described (Hill et al., 2003):

$$p_i = \frac{n_i}{N}, \quad H' = - \sum (p_i \ln p_i),$$

n_i - the number of clones in the i th OTU

N- the total number of clones in the library.

The similarity between the methanogen communities was further characterized with the Morisita–Horn similarity index (C_{M-H}), which ranges between 0 and 1. A C_{M-H} value of 1 indicates that two identical communities are identical, and a C_{M-H} value of 0 indicates that two communities share no common members. The Morisita–Horn similarity index (C_{M-H}) was calculated using the equation below for the pre-overloading and post-overloading clone libraries (i.e. community A and B) as previously described (Magurran, 2004):

$$C_{M-H} = \frac{2 \sum \frac{S_{A,i} S_{B,i}}{n m}}{\sum (\frac{S_{A,i}}{n})^2 + \sum (\frac{S_{B,i}}{m})^2}$$

where $S_{A,i}$ = the number of clones from community A in the i th OTU

$S_{B,i}$ = the number of clones from community B in the i th OTU

n = the number of clones in community A

m = the number of clones in community B

Results

Anaerobic co-digester operation and performance

Following the establishment of the triplicate continuous bench-scale anaerobic co-digesters with dairy waste as the primary substrate and poultry waste as the co-substrate, stepwise increases in OLR were achieved by increasing the proportion of poultry waste in the feed to the digesters (Fig. III1). Given that the volatile fraction of the solids content of poultry waste was slightly higher than that of dairy waste, poultry waste offered much higher total VS content than that of dairy waste. Thus, the addition of poultry waste raised the OLR of anaerobic digestion without altering the hydraulic loading rate. As expected, stepwise increases in biogas production were observed in response to the addition of poultry waste before the process was ultimately overwhelmed when OLR reached $1.77 \text{ g VS L}^{-1} \text{ day}^{-1}$ (Fig. III1), confirming the effectiveness of poultry waste as an organic-rich co-substrate to enhance biogas production in anaerobic digestion.

Before the onset of substrate overloading, balanced anaerobic digestion performance was evident with stable biogas production at $1583 \pm 41 \text{ ml day}^{-1}$ when the OLR was maintained at $1.28 \text{ g VS L}^{-1} \text{ day}^{-1}$ (Fig. III1). Upon the increase in OLR by 15% to $1.5 \text{ g VS L}^{-1} \text{ day}^{-1}$, biogas production reached $1912 \pm 20 \text{ ml day}^{-1}$, an increase proportional to that of the OLR, suggesting that anaerobic digestion performance remained stable at this OLR level. Accordingly, the concentration of VFAs remained constantly below 0.5 mM at OLR levels between 1.28 and $1.5 \text{ g VS L}^{-1} \text{ day}^{-1}$ (Fig. III-2), despite increases in OLR and biogas production, further confirming process stability at these OLR levels (Ahiring et al., 1995).

A further increase in OLR, however, led to substrate overloading and disruptions in anaerobic digestion performance. Following the sampling of pre-overloading biomass at Day 100, the OLR was raised from 1.5 to 1.77 g VS L⁻¹ day⁻¹ (Fig. III1). The digesters responded with an initial spike of biogas production to 2431 ± 38 ml day⁻¹; but a rapid decline in biogas production ensued, indicative of the deterioration of process performance (Fig. III1). Accompanying the rapid decline in biogas production, VFA concentration rose quickly (Fig. III2), providing further evidence for process imbalance as a result of substrate overloading. To prevent further deterioration of process performance, feeding of substrate was ceased at Day 135. However, VFA concentration continued to climb until reached ~40mM, while biogas production continued its precipitous drop (Fig. III1).

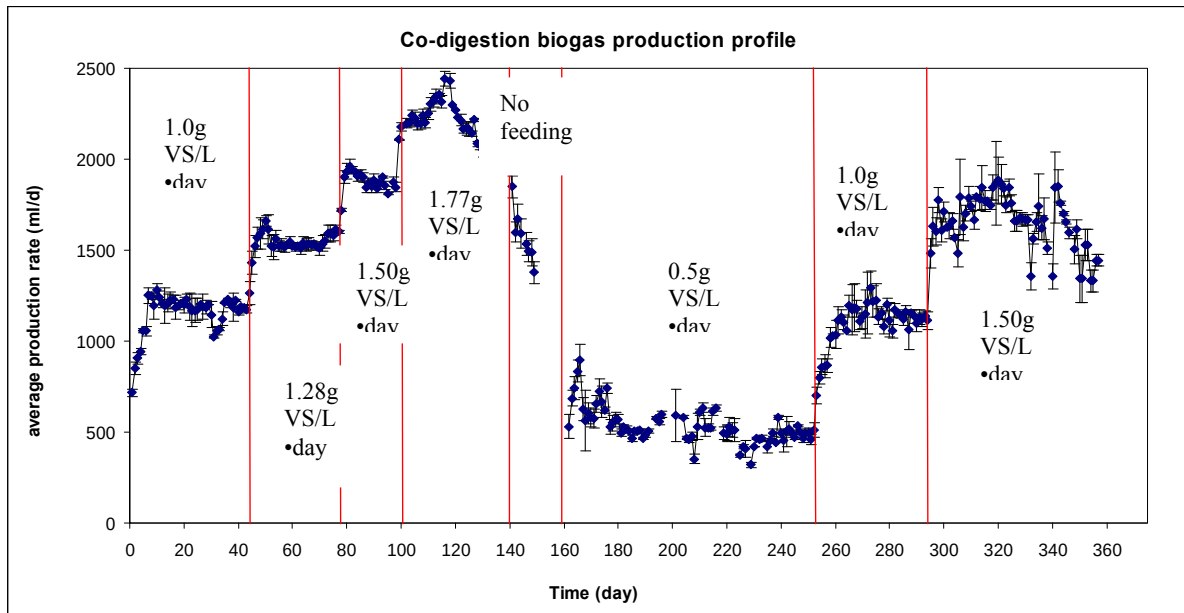


Fig. III1. Biogas production in anaerobic co-digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation.

To recover the anaerobic digestion process from substrate overloading, feeding of animal waste was suspended for a period of 30 days (Fig. III1). Feeding was resumed with a low OLR of 0.5 g VS L⁻¹ day⁻¹. Immediately following the resumption of substrate feeding, the VFA level rose quickly from 14.5 to 44mM (Fig. III2). However, the VFA level fell below 1 mmol/L on Day 240 and stable anaerobic digestion performance was re-established at this feeding rate with biogas production stabilized at 522 ± 20 ml day⁻¹ in a 90-day period (Fig. III1).

With the objective to restore the digestion process to the stable performance achieved at the original OLR of 1.5 g VS L⁻¹ day⁻¹ prior to overloading, stepwise increases in OLR were implemented to raise OLR from 0.5 g VS L⁻¹ day⁻¹, to 1.0 g VS L⁻¹ day⁻¹ and then to 1.5 g VS L⁻¹ day⁻¹ (Fig. III1). Consequently, biogas production increased to 1086 ± 102 ml day⁻¹ and 1620 ± 149 ml day⁻¹ with OLR at 1.0 and 1.5 g VS L⁻¹ day⁻¹, respectively (Fig. III1). Similar to the spike in VFA concentration observed immediately following the resumption of substrate feeding to 0.5 g VS L⁻¹ day⁻¹, the digesters experienced spikes in VFA concentration when the OLR was raised from 0.5 to 1.0 g VS L⁻¹ day⁻¹ and again from 1.0 to 1.5 g VS L⁻¹ day⁻¹ (Fig. III2). In all three occurrences of VFA accumulation following increases in OLR, spikes in VFA level followed by rapid declines and subsequently remained below 1mM as indications of the establishment of stable performance. Notably, the spike in VFA level in response to increases in OLR became less and less pronounced, with the maximum VFA accumulation decreasing from a high of 44mM at OLR of 0.5 g VS L⁻¹ day⁻¹, to 8.0mM at OLR of 1.0 g VS L⁻¹ day⁻¹ and to a very moderate 2.5mM at OLR of 1.5 g VS L⁻¹ day⁻¹ (Fig. III2). While fluctuations in biogas production post-overloading were still visible with the relatively greater standard deviations in biogas production as compared to those under pre-overloading conditions (Fig. III1), it could be concluded that the anaerobic digesters had recovered from the performance collapse induced by substrate overloading.

Archaeal populations in pre and post-overloading co-digesters by clone library analysis

As methanogens as members of the *Archaea* are among the microbial populations most sensitive to process disturbances in anaerobic digestion (Chen et al., 2008), the responses of archaeal microbial populations before and after substrate overloading were further studied by clone library analysis to understand the responses of these populations to process disturbance. Clone library analysis identified 11 archaeal OTUs involved in the anaerobic digestion process (Table III1). OTUs 2, 3, 4, 7, 8 and 11 persisted throughout the disturbance and recovery process, representing 98% of the archaeal abundance in the pre-overloading archaeal community. The most abundant methanogen population in pre-overloading closely related to *Methanosaeta concilii* (OTU2) and *Methanocorpusculum*-like organisms (OTU8) (Fig. III3). *Methanosaeta*-related organisms are acetoclastic methanogens suggested to be competitive in established methanogenic communities with low acetate concentration (Jetten et al., 1990). Indeed, 16S rRNA gene sequences related to *Methanosaeta* represented 52% of the methanogen sequences in the pre-overloading clone library (Fig. III3). The dominance of *Methanosaeta* related methanogens is consistent with the pre-overloading condition in the anaerobic codigesters with balanced performance and low VFA concentration. The roles of *Methanocorpusculum*-like

populations, however, are less clear. These hydrogenotrophic methanogens have been found to be the predominant methanogen populations in psychrophilic anaerobic digestion processes and are suggested to be specifically adapted to psychrophilic conditions (McKeown et al., 2009; O'Reilly et al., 2010). The persistence of these methanogens in the mesophilic anaerobic digestion processes of this study raises the possibility that *Methanocorpusculum*-like organisms may function in a broader temperature range.

To characterize the microbial community in response to substrate overloading, the Morisita–Horn similarity index (C_{M-H}) was calculated as a measure of the similarity in the population structure between the pre- and post-overloading methanogenic communities. The C_{M-H} value of 0.71 indicates that the two microbial communities shared considerable similarities. The differences between the two communities could be attributed to the observation that the post-overloading methanogenic community became more diverse (Fig. III3), as the Shannon diversity index (H') increased from the pre-overloading value of 1.45 to the post-overloading value of 2.07. Similar to the pre-overloading methanogenic community, methanogens related to *M. concilii* (OTU2) remained prevalent; however, the relative abundance of these micro-organisms in the methanogen populations decreased from 52% pre-overloading to 33% post-overloading (Fig. III3). Contributing to the increased diversity was the emergence of additional methanogen OTUs in response to substrate overloading, which included those closely related to *Methanosaeta harundinacea* (OTU10), *Methanosarcina barkeri* (OTU5) and *Methanobacterium beijingense* (OTU9).

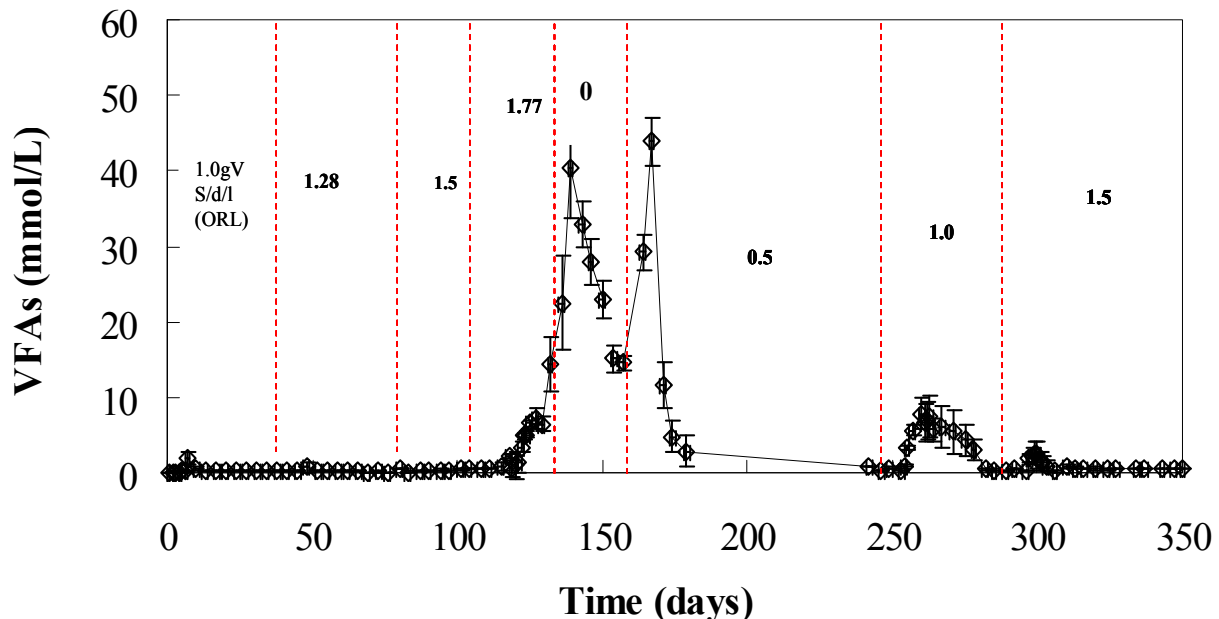


Fig. III2. Concentration of volatile fatty acids in anaerobic co-digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation.

Notably, the sequence abundance of OTU10 reached approximately the same level as that of OTU2, which was the most abundant methanogen population both pre- and post-overloading (Fig. III4). As both OTU10 and OTU2 are closely related to *Methanosaeta* (Ma et al., 2005), it is likely that both would share the same physiology of adaptation to methanogenic processes with low acetate levels (Jetten et al., 1990). Thus, the presence of OTU10 post-overloading resulted in a significant level of functional redundancy for methanogenesis at low acetate conditions. In contrast, *Methanosarcina*-related methanogens (OTU5) are known to be more competitive than *Methanosaeta*-related methanogens (OTUs 2 and 10) at high acetate conditions (Jetten et al., 1990). Therefore, the presence of *Methanosarcina*-related methanogens in post-overloading microbial community could be a response to the accumulation of organic acids accompanying process imbalance resulting from organic overloading.

While methanogens were the most prominent members of the archaeal community in the anaerobic digesters analysed in this study, a number of clones phylogenetically unrelated to methanogens formed a cluster within the archaeal phylum *Crenarchaeota* (Fig. III4), which was unexpected because it is typically assumed that all archaeal populations involved in anaerobic digestion are methanogens, yet no known methanogens belong to *Crenarchaeota* (Luo et al., 2009). In particular, of the 11 archaeal OTUs identified in the anaerobic co-digesters, 4 represented the archaeal phylum *Crenarchaeota* and 7 represented the *Euryarchaeota* (Table III1). Thus, a considerable portion of the archaeal community in the anaerobic digesters was likely nonmethanogen populations.

Table III1 Archaeal community structure of clone libraries in anaerobic digestion of animal wastes pre- and post-overloading.

OTU	Taxonomic identification	Closest relative	GenBank accession no.	Representative clones*	Relative abundance, %†	
					Pre-O	Post-O
1	<i>Crenarchaeota</i>	Uncultured <i>Crenarchaeote</i>	CU916928	12B (99%)	0	4
2	<i>Euryarchaeota</i>	<i>Methanosaeta concilii</i>	NR_028242	10A (99%); 16B (99%)	22	23
3	<i>Crenarchaeota</i>	Uncultured <i>Crenarchaeote</i>	AY464784	27A (97%); 60B (97%)	6	3
4	<i>Crenarchaeota</i>	Uncultured <i>Crenarchaeote</i>	GU196174	7A (99%); 9B (99%)	50	13
5	<i>Euryarchaeota</i>	<i>Methanosarcina barkeri</i>	AF028692	2B (99%)	0	9
6	<i>Euryarchaeota</i>	<i>Methanomethylovorans</i> sp.	EF174501	65A (99%)	2	0
7	<i>Crenarchaeota</i>	Uncultured <i>Crenarchaeote</i>	EF552166	1A (100%); 38B (99%)	2	12
8	<i>Euryarchaeota</i>	<i>Methanocorpusculum</i> sp.	AY260434	2A (99%); 81B (96%)	10	9
9	<i>Euryarchaeota</i>	<i>Methanobacterium beijingense</i>	AY552778	4B (98%)	0	3
10	<i>Euryarchaeota</i>	<i>Methanosaeta harundinacea</i>	AY970347	41B (98%)	0	21
11	<i>Euryarchaeota</i>	<i>Methanoculleus palmolei</i>	NR_028253	60A (98%); 6B (97%)	8	5

OTU, operational taxonomic units; Pre-O, pre-overloading; Post-O, post-overloading.

*Clone designation: A-Clones retrieved from pre-overloading samples; B-Clones retrieved from post-overloading samples.

The value in the parenthesis indicates % sequence similarity of each representative clone to the closest relative.

†Relative abundance of OTUs is the number of clones in a particular OTU as per cent of the total number of clones in the entire clone library.

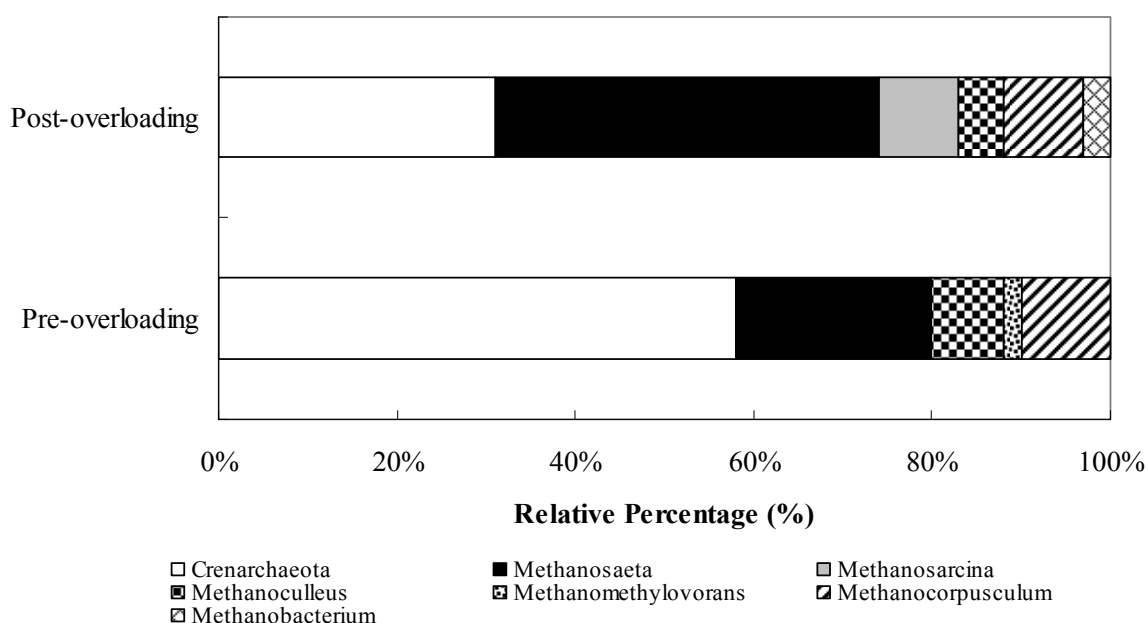


Fig. III3. Relative percentages of archaeal populations in anaerobic digesters.

Discussion

Anaerobic digestion is a sustainable option for waste treatment and renewable energy producing. However, process instability resulting from variations in substrate loading has been one of the obstacles to the broader adoption of anaerobic digestion technology. It is thus important to identify mechanisms that can mitigate the impact of substrate overloading on anaerobic digestion processes. Using anaerobic co-digesters treating dairy and poultry wastes as the model system, results from this study show that the archaeal community recovered from substrate overloading acquired greater population diversity, suggesting the development of enhanced functional diversity potentially beneficial to overcoming subsequent occurrences of higher substrate loading.

The linkage between greater archaeal diversity and adaptation to substrate overloading is reflected in the enhanced functional diversity in the post-overloading methanogenic community. *Methanosaeta*-related populations known to be competitive for acetate at low concentrations were found to be the dominant methanogens in both pre- and post-overloading conditions (Fig. III3), suggesting the importance of these methanogens in balanced anaerobic digestion processes characterized by low VFA concentrations (Ahring et al., 1995; Jetten et al., 1990). However, the identification of *Methanosarcina*-related acetoclastic methanogens recovered from overloading (Table III1) represented a major enhancement in the functional diversity of the post-overloading community, because the *Methanosarcina* related methanogens are known to be more competitive than *Methanosaeta* at high acetate conditions, which are frequently encountered in unstable anaerobic digestion processes (Jetten et al., 1990). Thus, the

greater functional diversity as a result of increased *Methanosarcina* abundance likely improved the capacity of the post-overloading methanogenic community to overcome the detrimental impact of fluctuating substrate loading rates characterized by the transient accumulation of high concentrations of VFAs (Briones & Raskin, 2003; Hashsham et al., 2000). Indeed, the post-overloading anaerobic digestion process was increasingly adaptable to higher OLR as the accumulation of VFAs diminished further following each stepwise OLR increase (Fig. III2).

The increase in methanogenic functional diversity post-overloading was also demonstrated by the detection of *Methanobacterium*-related populations (Table III1), which are hydrogenotrophic methanogens physiologically distinct from *Methanosarcina* and *Methanosaeta* (Ma et al., 2005), such as *Methanoculleus*, adding another element in functional diversity and parallel processing for improved process stability. Moreover, substrate overloading also increased the population diversity of *Methanosaeta*-related methanogens in the post-overloading methanogenic community, likely providing additional functional redundancy important for maintaining process stability under disturbance as suggested in other studies (Girvan et al., 2005).

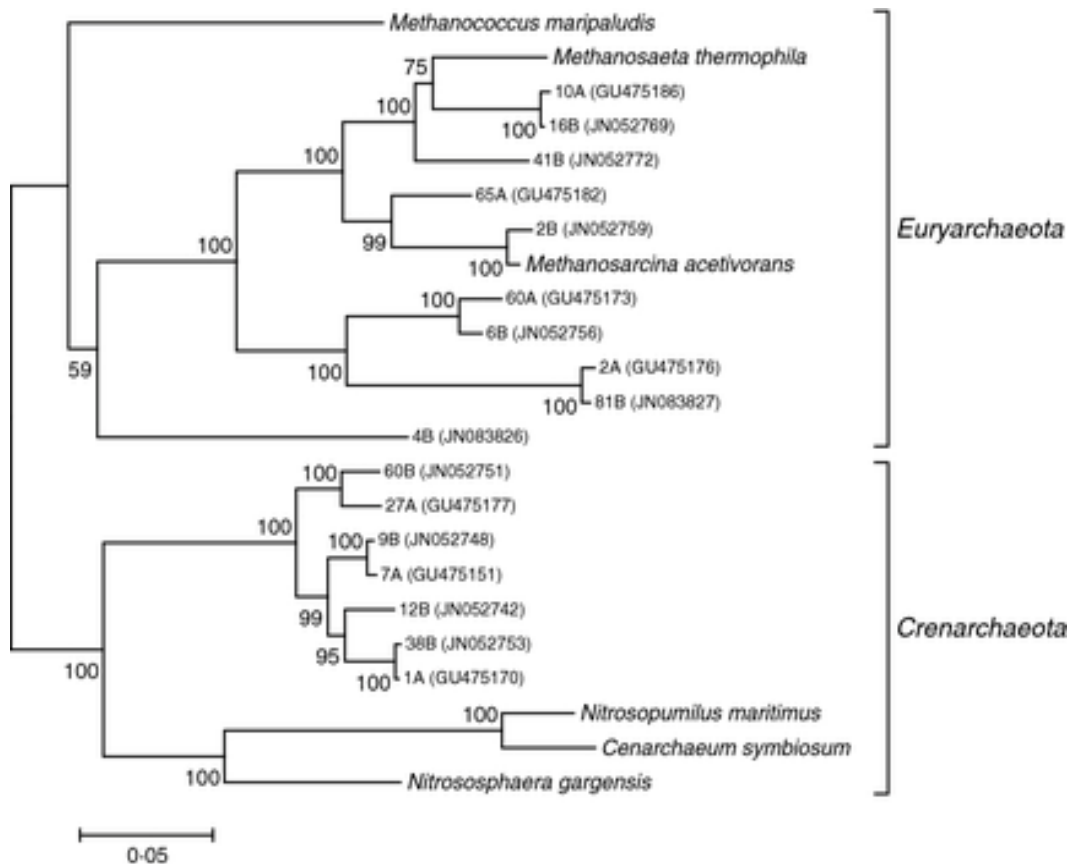


Fig. III4. Neighbor-joining phylogenetic tree showing the relationships of partial archaeal 16S rRNA gene sequences cloned from anaerobic codigesters. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. Sequence designation: (A) pre-overloading samples and (B) post-overloading

The *Crenarchaeota* OTUs, however, were more phylogenetically diverse and only had close relatives of uncultured clones lacking detailed physiological characterization (Table III1). More importantly, these *Crenarchaeota* populations were persistent, present archaeal communities during the whole operation process (Table III1). The closest known relative to OTU4 (Table III1), is *Candidatus Nitrososphaera gargensis*, a crenarchaeote shown to be an ammonia oxidizer in an enrichment culture (Hatzenpichler et al., 2008). Indeed, recent findings suggest the broad distribution of mesophilic *Crenarchaeota* and their roles in ammonia oxidation in the environment (Gubry-Rangin et al., 2010; Nicol & Schleper, 2006). However, ammonia oxidation is unlikely the function of the *Crenarchaeota* identified in the anaerobic digesters of this study due to the lack of relevant electron acceptors. Moreover, lineage of crenarchaeal 16S sequences from anaerobic digesters was different those of mesophilic *Crenarchaeota* by phylogenetic studies (Fig. III4) Given that *Crenarchaeota* were also found in other studies as abundant populations in anaerobic digestion (Collins et al., 2005; Godon et al., 1997; Zhang et al., 2011), more efforts are needed to identify their functions in anaerobic processes.

References

- Ahring, B. 2003. Perspectives for Anaerobic Digestion. in: *Biomethanation I*, (Eds.) B. Ahring, I. Angelidaki, E.C. Macario, H.N. Gavala, J. Hofman-Bang, A.J.L. Macario, S.J.W.H.O. Elferink, L. Raskin, A.J.M. Stams, P. Westermann, D. Zheng, Vol. 81, Springer Berlin Heidelberg, pp. 1-30.
- Ahring, B.K., Sandberg, M., Angelidaki, I. 1995. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. *Appl Microbiol Biotechnol*, **43**(3), 559-565.
- Briones, A., Raskin, L. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr Opin Biotechnol*, **14**(3), 270-276.
- Chen, Y., Cheng, J.J. 2005. Anaerobic processes in waste treatment. *Water Environ Res*, **77**(6), 1347-1388.
- Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. *Bioresour Technol*, **99**(10), 4044-4064.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., Tiedje, J.M. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res*, **31**(1), 442-443.
- Collins, G., Mahony, T., McHugh, S., Gieseke, A., de Beer, D., O'Flaherty, V. 2005. Distribution, dynamics and in situ ecophysiology of Crenarchaeota in anaerobic wastewater treatment granular biofilms. *Water Science and Technology*, **52**(7), 233-239.
- Delong, E.F. 1992. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America*, **89**(12), 5685-5689.
- Fernandez, A.S., Hashsham, S.A., Dollhopf, S.L., Raskin, L., Glagoleva, O., Dazzo, F.B., Hickey, R.F., Criddle, C.S., Tiedje, J.M. 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *Appl Environ Microbiol*, **66**(9), 4058-4067.
- Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A. 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environ Microbiol*, **7**(3), 301-313.
- Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F., Moletta, R. 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol*, **63**(7), 2802-2813.
- Gubry-Rangin, C., Nicol, G.W., Prosser, J.I. 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. *FEMS Microbiol Ecol*, **74**(3), 566-574.
- Hashsham, S.A., Fernandez, A.S., Dollhopf, S.L., Dazzo, F.B., Hickey, R.F., Tiedje, J.M., Criddle, C.S. 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *Appl Environ Microbiol*, **66**(9), 4050-4057.
- Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H., Wagner, M. 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(6), 2134-2139.
- He, Q., Lokken, P.M., Chen, S., Zhou, J.Z. 2009. Characterization of the impact of

- acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. *Bioresour Technol*, **100**(23), 5955-5965.
- Hill, T.C.J., Walsh, K.A., Harris, J.A., Moffett, B.F. 2003. Using ecological diversity measures with bacterial communities. *FEMS Microbiol Ecol*, **43**(1), 1-11.
- Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B. 1990. Acetate threshold values and acetate activating enzymes in methanogenic bacteria. *FEMS Microbiol Ecol*, **73**(4), 339-344.
- Jongbloed, A.W., Lenis, N.P. 1998. Environmental concerns about animal manure. *J Anim Sci*, **76**(10), 2641-2648.
- Kotzé, J.P., Thiel, P.G., Hattingh, W.H.J. 1969. Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Res*, **3**(7), 459-494.
- Leitao, R.C., van Haandel, A.C., Zeeman, G., Lettinga, G. 2006. The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review. *Bioresour Technol*, **97**(9), 1105-1118.
- Luo, H.W., Sun, Z.Y., Arndt, W., Shi, J., Friedman, R., Tang, J.J. 2009. Gene Order Phylogeny and the Evolution of Methanogens. *Plos One*, **4**(6).
- Ma, K., Liu, X.L., Dong, X.Z. 2005. *Methanobacterium beijingense* sp nov., a novel methanogen isolated from anaerobic digesters. *Int J Syst Evol Microbiol*, **55**, 325-329.
- Magurran, A.E. 2004. *Measuring Biological Diversity*. Blackwell Publishing, Oxford, UK.
- Mata-Alvarez, J., Dosta, J., Mace, S., Astals, S. 2011. Codigestion of solid wastes: A review of its uses and perspectives including modeling. *Crit Rev Biotechnol*, **31**(2), 99-111.
- McKendry, P. 2002. Energy production from biomass (part 2): conversion technologies. *Bioresour Technol*, **83**(1), 47-54.
- McKeown, R.M., Scully, C., Enright, A.M., Chinalia, F.A., Lee, C., Mahony, T., Collins, G., O'Flaherty, V. 2009. Psychrophilic methanogenic community development during long-term cultivation of anaerobic granular biofilms. *Isme Journal*, **3**(11), 1231-1242.
- Michaud, S., Bernet, N., Buffiere, P., Roustan, M., Moletta, R. 2002. Methane yield as a monitoring parameter for the start-up of anaerobic fixed film reactors. *Water Res*, **36**(5), 1385-1391.
- Nicol, G.W., Schleper, C. 2006. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol*, **14**(5), 207-212.
- O'Reilly, J., Lee, C., Chinalia, F., Collins, G., Mahony, T., O'Flaherty, V. 2010. Microbial community dynamics associated with biomass granulation in low-temperature (15 degrees C) anaerobic wastewater treatment bioreactors. *Bioresour Technol*, **101**(16), 6336-6344.
- Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev*, **61**(2), 262-&.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*, **24**(8), 1596-1599.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, **25**(24), 4876-4882.

- Xing, J., Criddle, C., Hickey, R. 1997. Effects of a long-term periodic substrate perturbation on an anaerobic community. *Water Res*, **31**(9), 2195-2204.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhang, Y., Zhang, X.L., Zhang, H.W., He, Q., Zhou, Q.X., Su, Z.C., Zhang, C.G. 2009. Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis. *Bull Environ Contam Toxicol*, **82**(3), 367-372.
- Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.

Part II: Dynamics of Two Dominant Bacterial Phyla, Bacteroidetes and Firmicutes in Anaerobic Digestion Process with Organic Loading Perturbations

Abstract

Triplicate mesophilic anaerobic digesters fed with dairy farm and poultry wastes were perturbed by organic loading rate (OLR) increase. OLR was ceased when biogas production begun to collapse. After digester methanogenic activity resumed, OLR was increased to the same level before perturbation under stable performance. Bacterial populations during different digester operational periods revealed by 454 pyrosequencing showed diverse transitions corresponding to the process performance. It was also validated by typical biodiversity indexes, Shannon (H) and Chao1 and β diversity analyses including Hierarchical Cluster Analysis, Principle Coordinate Analysis (PCoA) and Venn diagram. Ratios of *Firmicutes* to *Bacteroidetes*, two dominant phyla classified by RDP database declined during digester performance deterioration and increased when the performance was resumed. Supported by Canonical Corresponding Analysis (CCA) between bacterial community structure and reactor performance variables, phylum *Firmicutes*, particularly including the genera *Clostridium* and *Turicibacter*, was correlated with methane production, while phylum *Bacteroidetes* including genus *Petrimonas* was likely correlated with volatile fatty acids accumulation in respond to organic loading rate increase.

Keywords: 454 pyrosequencing, Bacterial Community, Anaerobic digestion, Organic loading rate, Firmicutes, Bacteroidetes

Introduction

Anaerobic digestion of organic material to methane is a multi-step process mediated and balanced between *Bacteria* and methanogenic *Archaea* (Wang et al., 2010). Firstly, polymers are hydrolyzed into soluble compounds under fermentative conditions. Then, acidogenic bacteria convert these intermediates (e.g., sugars, amino acids and fatty acids) into acetic acids, simple or low carbon volatile fatty acids (e.g., propionic and butyric acids), CO₂ and H₂ (Angenent et al., 2004). In the acetogenesis process, syntrophic bacteria convert volatile fatty acids into acetic acids, CO₂ and H₂ (Angenent et al., 2004; Stams & Plugge, 2009), which in turn can be reduced directly by acetotrophic and hydrogenotrophic methangens into methane (Wang et al., 2010). In anaerobic digestion, however, bacterial and archaeal populations for acids and methane generations are different in terms of physiologies, nutritional needs, growth kinetics, and sensitivities to environmental conditions (Chen et al., 2008). An imbalanced reaction between these two populations is the primary cause of reactor instability and inhibition of methane production (Demirel & Yenigun, 2002). Since all reactions from polymer hydrolysis to methanogenesis usually happen in one phase reactor, all reaction rates or kinetics for each step should be well balanced in order to generate methane. Otherwise, the performance of anaerobic digestion is susceptible to be perturbed by rapid accumulation of volatile fatty acids and the decrease in the pH of the bulk phase, subsequently inhibiting the

methanogenesis and leading to process failure (Liu et al., 2002). Because accumulated organic acids were mainly responsible to bacterial populations in anaerobic digestion (Pohland & Ghosh, 1971), it was meaningful to investigate bacterial community structures and their dynamics in response to methane production inhibition.

Among all bacterial groups in anaerobic digesters, the phyla, *Firmicutes* and *Bacteroidetes* are the two important and predominant groups revealed by many previous studies. Moreover, their relative abundances in anaerobic digesters were likely to be enriched under stable performance and high COD removal efficiency conditions (Chen et al., 2014). *Firmicutes* including many well known fermenters and syntrophic bacteria exhibits diverse metabolic functions, such as volatile fatty acid degradation (Garcia-Pena et al., 2011; Krakat et al., 2011), while another dominant phylum *Bacteroidetes*, may be proteolytic and involved in the degradation of the proteins (Kindaichi et al., 2004). Generally, *Bacteroidetes* was considered as an ideal indicator of the active role of protein degradation in anaerobic digestion process, which could be proved by the evidence of the increased concentration of ammonia nitrogen in reactors. As the core populations in anaerobic digesters, species belonging to *Firmicutes* or *Bacteroidetes* were known for being extremely resistant (Wan et al., 2013). With versatile capacities to degrade a wide range of complex organic compounds including carbohydrates, proteins, these two populations become more metabolic flexible upon fermentation disturbances (Krakat et al., 2011).

The discrepancies, however, of dynamics of *Firmicutes* and *Bacteroidetes* in anaerobic digestion upon different operational conditions were also reported from other studies. Some studies reported the shift of *Bacteroidetes* community with reactor condition changes but not for *Firmicutes* (Kampmann et al., 2012) or no clear linkages between either *Firmicutes* or *Bacteroidetes* and process parameters (Krakat et al., 2011). Meanwhile, the negative correlation between these two communities was revealed by surveying many types of anaerobic digesters with different substrate characteristics (Briones et al., 2014). The high ratios of *Firmicutes* or *Bacteroidetes* (F/B) (close to or more than 1) indicated the more liable digester performance than low F/B ratios combined with fibrous substrate feeding. In this study, therefore, dynamics of the relative abundances of two major populations, *Firmicutes* and *Bacteroidetes* were investigated throughout the whole digester operation period with OLR perturbations.

Although operational condition influences on microbial ecology in anaerobic digestion process were intensively studied in full and bench scale systems with high throughput sequencing (Briones et al., 2014; Jang et al., 2014; Luo et al., 2013; Sundberg et al., 2013; Yang et al., 2014), dynamics of a microbial community in one system over a relatively long period of operation were rarely studied. For anaerobic digestion, one of the biggest challenges is performance instability caused by fluctuation of organic loading rate (OLR) (Chen et al., 2008). In this study, therefore, the linkage between bacterial community dynamics and the performance and operation conditions of anaerobic digester was assessed. As described in previous study (Chen et al., 2012), OLRs of triplicate mesophilic anaerobic co-digesters fed with dairy farm and poultry wastes were increased stepwise until their performance collapsed. Then substrate feeding was ceased until

digester performance of methane production recovered. Finally, OLR was increased back to the original level gradually with stable performance without any inhibition. Bacterial communities at four time points representing different operational stages were analyzed by 454 pyrosequencing in order to investigate: (1) Are bacterial communities in anaerobic digestion determined or linked by fluctuation of OLR and digester performance parameters? (2) Did bacterial structures between pre-overloading and post-overloading differ even when digester performances recovered back to normal level after the organic overloading?

Materials and Methods

Anaerobic co-digester setting and performance monitoring

Triplicate mesophilic (35°C) semi-continuous anaerobic co-digesters with a 3.6L operation volume were fed daily with dairy farm waste as the primary substrate and poultry waste as the co-substrate following the configuration described previously (Chen et al., 2012; Zhang et al., 2011). As an organic-rich co-substrate, poultry waste was added into diluted dairy waste proportionally to accelerate reactor OLR stepwise and enhance methane generation until this resulted in organic overloading and biogas production inhibition at optimum OLR, 1.77gVS/day/L. Then organic loading was ceased to reduce fatty acid accumulation and increased back to 1.5gVS/day/L gradually when reactions in digester recovered from overloading. Digester performance parameters were monitored during whole process (Table III2), i.e. methane production, pH, volatile fatty acids (VFA), ammonia (NH_4^+) and alkalinity (ALK) following protocols described previously (Chen et al., 2012).

Sample collection and DNA extraction

Four time points during co-digester operation period, 45th day (pre-overloading), 73rd day (first OLR increase at 1.28gVS/g/day), 132nd day (digester performance collapse) and 289th day (performance recovered from overloading) were chosen for bacterial community structure analysis (Fig. III5 and Table III2). For each time point, samples from triplicate digesters were preserved individually and their DNA was extracted and purified following procedures used by previous study (Zhang et al., 2009).

454 pyrosequencing and sequence analysis

Extracted DNA samples were amplified by the universal bacterial primer set: Eub-p8F, 5'-AGAGTTTGATCMTGGCTCAG-3' and Uni-p907R, 5'CCGTCAATTCMTTTRAGTTTCCGTCAATTCMTTTRAGTTT-3' targeting the hyper-variable V4 region of 16SrRNA gene (Brosius et al., 1978). The PCR reaction system (25µL) contained 2.5µL Ex Taq 10× reaction buffer (Takara Inc, WI, USA), 1.5 mmol/L Mg^{2+} , 1.6 mmol/L dNTP, 1.5U Ex Taq DNA polymerase (Takara Inc, WI, USA), 0.2µmol/L primers and 5 ng genomic DNA. The PCR was carried out by a C1000 thermal cycler (Bio-Rad Inc, CA, USA) according to the program of 15 cycles at 94 °C for 40 s, 56 °C for 40 s and 72 °C for 50 s, and an extension at 72 °C for 10 min and

cooling to 4 °C. 10 base barcodes designed to allow sample multiplexing during pyrosequencing were incorporated between the 454 adapters and forward primer (Table S1). Compositions of a total of 12 PCR samples were analyzed by pyrosequencing using 454 FLX titanium sequencer (Roche, Nutley, NJ). Samples with unique barcodes were recognized individually. After pyrosequencing, sequences were analyzed by Mothur (version 1.30.2) following procedures outlined in Fig. III5. As procedures recommended previously (Schloss et al., 2011), sequence files were firstly denoised to improve highest data quality. After barcodes and primers were trimmed, sequences containing ambiguous (N) bases and shorter than 150bp were removed. In order to reduce sequence redundancy and save processing time by computer, unique sequences for each sample were screened and aligned. Then, chimeras from all these sequences aligned were removed to further reduce sequence errors (Edgar et al., 2011). Finally, 'contaminant' sequences belonging to non-bacterial lineages were removed. RDP Classifier was used to assign all effective sequences of each sample after processing to taxonomic ranks with confidence threshold of 80%.

Community diversity analysis

Sequences with high quality after pre-processing were used for sample diversity and community analyses based on operational taxonomic units (OTUs, with 97% similarity threshold). Because of the various numbers of sequences obtained from each sample, it is difficult to be perfectly even across all samples. Moreover, a variety of community diversity estimators (e.g., Chao1, Shannon and inverse Simpson, 1/D) is susceptible to be influenced by variable pyrosequencing library sizes (Gihring et al., 2012). Thus, an equal number of sequences were randomly sub-sampled based on the lowest among all these 12 samples prior to future OUT based analyses. Subsequently, alpha (α) and beta (β) diversity analyses were processed. Rarefaction curves (Fig. IIIS1), and diversity estimators including Shannon (H), Chao1, ACE and inverse Simpson (1/D) index were calculated based on 3% difference cutoff in sequences (Table IIIS3). Based on the commonly shared OUTs between samples, comparisons of bacterial community structure (β diversity) were analyzed by Principle Coordinates Analysis (PCoA), Cluster Analysis and Venn Diagram, in which PCoA and cluster dendrogram were performed by both un-weighted and weighted ways based on Jaccard and Theta (θ) index respectively (Fulthorpe et al., 2008).

Additionally, in order to visualize the transformations of the core OTUs during the whole period of the digester operation, an average linkage hierarchical clustering analysis of the major OTUs (top 20), comprising most of the sequences (>85%) for samples collected at all time points (Table IIIS2) was performed by Hierarchical Clustering Explorer, version 3.5 with the Euclidean distance method (Seo et al., 2004).

Statistical analyses

To demonstrate the correlation of any two related groups classified by RDP in phylum, family or genus level, the pairwise correlation analysis was implemented with statistical software JMP, version 11Pro (SAS, Cary, NC, USA). By controlling the level of

significance at $\alpha=0.05$ and 95% confidence interval, p value less than 0.05 means the correlations between the variables are statistically significant. Ratios of *Fimicutes* to *Bacteroidetes* were determined by RDP classification results. Subsequently, Oneway ANOVA test were implemented. Different categories with significant differences ($p<0.05^*$) for each sampling time point were determined by each pair student t test with the statistical software JMP (11Pro version) also.

Canonical Corresponding Analysis (CCA)

The corresponding relationships between anaerobic digester performance variables including pH, organic loading rate (ORL), CH₄ yield, alkalinity (ALK), NH₄⁺ and volatile fatty acids (VFA), and bacterial communities at four time points (45th, 73rd, 132nd and 289th day) representing different operational stages were assessed by Canonical Corresponding Analysis (CCA) using XLSTAT, version 2013 (Addinsoft, New York City, NY). Relative abundances of major bacterial populations, classified by three taxonomical levels, i.e. phylum, family and genus, were applied to investigate the corresponding relationship between the population distribution and some explanatory variables of the co-digester performance.

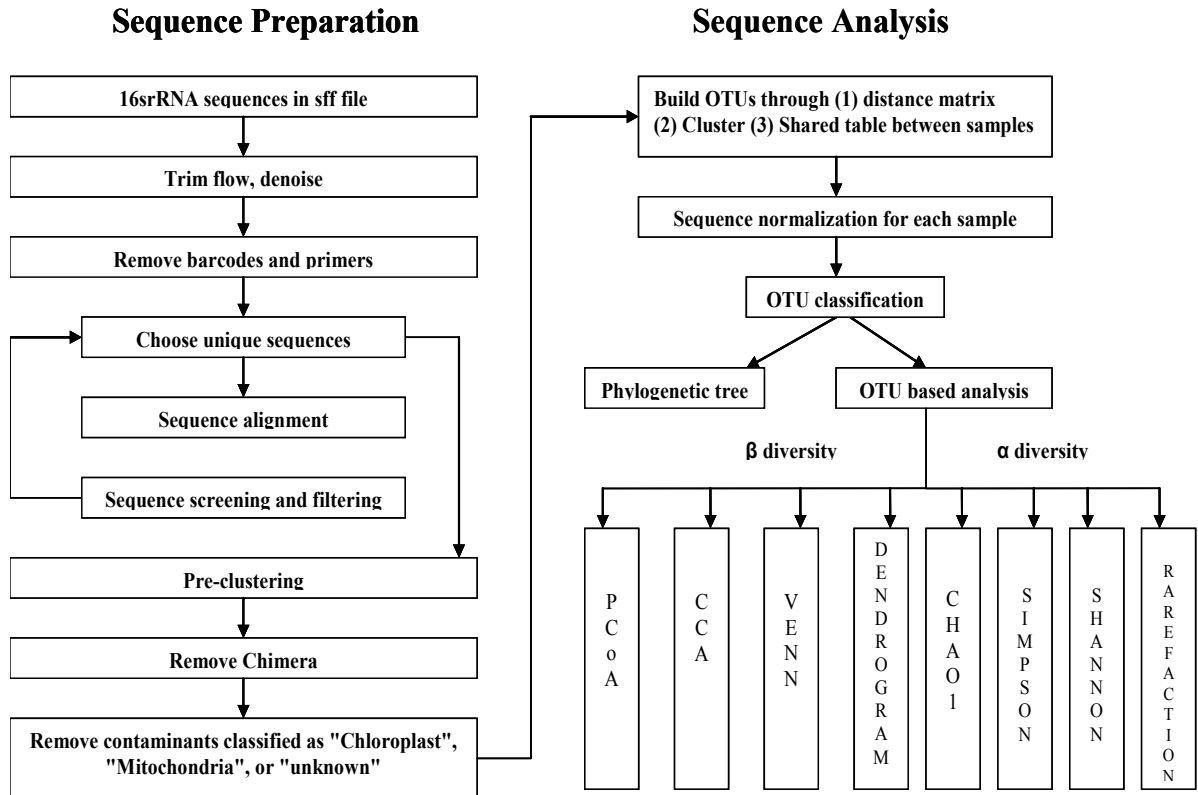


Fig. III5. Flow chart demonstrating procedures applied in 454 pyrosequencing data analysis.

Results

Anaerobic co-digester performance

As summarized (Table III2), without altering the hydraulic retention time (20days), increase of OLR was achieved by adding poultry waste into diluted dairy farm waste as co-substrate, due to its higher volatile solid content (Chen et al., 2012). Before OLR reached the highest level, 1.77gVS/L/day, gradual increase of biogas or methane production was observed in response to the addition of poultry waste (Fig. III5). Balanced anaerobic digestion performance was evident with proportional increase of methane production, from 230 to 353mL/Ldigester/day in average, with gradual OLR increase from 1.0 to 1.5gVS/L/day. Additionally, VFAs remaining constantly below 0.5 mmol/L (Table III2) with OLR less than 1.77gVS/L/day indicated anaerobic digestion stability (Ahring, 2003). With continuous increase of OLR, however, substrate overloading disrupted biochemical reaction balance and led to rapid declining of biogas generation although there was a sharp pulse of biogas production at maximum OLR level, 1.77gVS/L/day initially (Fig. III6). Accompanying the rapid decline of biogas production, VFA concentration accumulated rapidly, nearing 100 fold, from 0.4mmol/L to 40mmol/L approximately (Table III2), providing further evidence of process imbalance as a result of substrate overloading. To prevent further deterioration of process performance, feeding of substrate was ceased and diluted sodium hydroxide solution was added to neutralize the pH inside the digesters since low pH caused by high concentration of VFA could inhibit methanogenesis.

To recover anaerobic digestion performance from overloading, OLR was resumed to 0.5gVS/L/day after 30 days. Proportionally, methane production was half the amount of that at 1.0gVS/L/day when digesters were set up initially. Meanwhile, accumulation of VFA diminished rapidly from 40 to 3~7 mmol/L approximately. To recover digester performance back its initial status before OLR perturbation, OLR was increased from 0.5 to 1.0gVS/L/day the same as its initial OLR level. Similarly, methane production was obtained as its initial setting up status and low VFA concentration was achieved (0.4mmol/L in average) during this period. It could be concluded that the anaerobic digesters recovered completely from the performance collapse induced by substrate overloading.

Sequence taxonomy classification with RDP

The taxonomies of sequences of each sample were determined by RDP Classifier and summarized into three taxonomic levels: phylum, family and genus, which were represented as relative abundances of each sample (Fig. III7A-C, respectively). For these three taxonomic levels, major groups composing above 1% of total sequence of each sample were demonstrated. Others composing less than 1% or sequences not be classified by RDP were not shown. With small variations of the relative fractions of the bacterial populations in triplicate samples at three taxonomic levels (data not shown), relative abundances of bacterial populations at each time point were represented in average (Fig. III7A-C).

Table III2 Summary of digester performance with perturbations of organic loading rates^a

Phase	Operational period (day)	OLR ^c (gVS/L/day)	Biogas (mL/L/day)	CH ₄ content (%)	pH	VFA ^d (mmol/L)	NH ₄ ⁺ (mg/L as N)	ALK ^e (mg/L as CaCO ₃)
A	1-43	1.0	328 ± 26	70.63 ± 1.21	7.5 ± 0.1	0.25 ± 0.10	1032 ± 37	9515 ± 274
B	44-76	1.28	430 ± 19	68.52 ± 0.70	7.6 ± 0.1	0.19 ± 0.10	1401 ± 47	10682 ± 299
C	77-99	1.5	520 ± 19	67.66 ± 1.32	7.6 ± 0.1	0.29 ± 0.12	1554 ± 57	11719 ± 804
D ^b	100-160	1.77 — 0	678 — 12	65.06 ± 2.42	7.8 ± 0.1	0.44 — 40.43	1802 ± 117	14061 ± 864
E	161-253	0.5	145 ± 25	65.96 ± 3.54	7.7 ± 0.1	3.07 ± 4.15	2078 ± 127	13286 ± 1077
F	254-294	1.0	320 ± 22	66.56 ± 1.38	7.7 ± 0.1	0.39 ± 0.11	1802 ± 155	11678 ± 914

^aPerformance data of triplicate digesters were indicated as average ± standard deviation.

^bWhen ORL reached to maximum level, 1.77gVS/L/day, biogas was proportionally increased initially. With rapid accumulation of VFA, however, biogas production reduced gradually. In order to prevent methanogenic microbes from deteriorating, organic loading was ceased and sodium hydroxide was added into digester to make the pH neutral.

^cOrganic loading rates were adjusted by adding solid poultry waste with higher volatile solid content than diluted dairy farm manure.

^dVolatile fatty acids.

^eTotal alkalinity of effluents from digesters.

At the phylum level, all samples were mainly composed of major phyla, *Firmicutes*, *Bacteroidetes*, *Synergistetes*, *Proteobacteria*, and *Actinobacteria*. *Firmicutes* and *Bacteroidetes* were the two dominant phyla comprising 50% ~ 70% of total sequences approximately. The relative percentage of *Firmicutes* decreased from 50% to 28%, averagely, in response to OLR increase until performance collapsed. When methane production was recovered, its composition restored back to 35 ~ 40%. With opposite dynamics of *Firmicutes*, relative fractions of *Bacteroidetes* increased from 20% to 40 ~ 45% with OLR increase. Then it decreased to approximately 22 ~ 30% during stable performance period after recovery from overloading (Fig. III7A). For the other phyla, none comprised more than 10%. *Proteobacteria* and *Actinobacteria* varied between 3% ~ 4% and 4% ~ 10% respectively during the whole period of digester performance. The relative amount of *Synergistetes* increased gradually from 1% to 6%, when digester performance recovered.

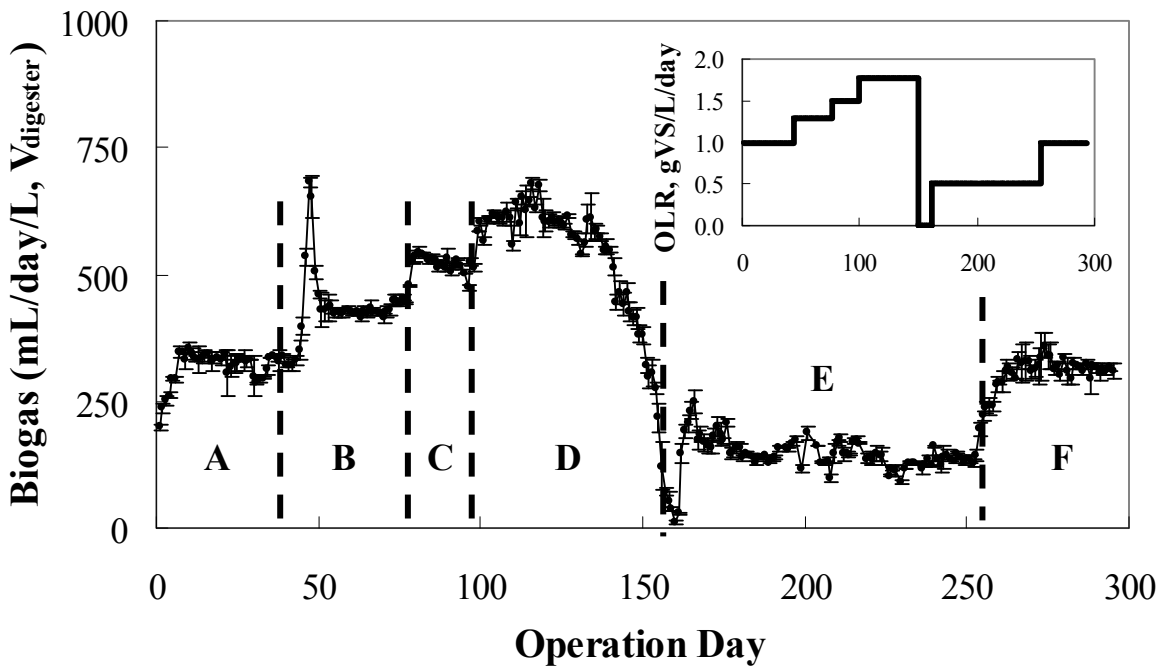


Fig. III6. Unit volume of biogas production from anaerobic co-digesters. Letters divided by solid dash lines indicate different organic loading rates between pre-and post-overloading of co-digesters, the same as indicated in Table 1. Inset indicates the organic loading rates (OLR). Data are means of triplicate digesters, with the error bars indicating the standard deviation.

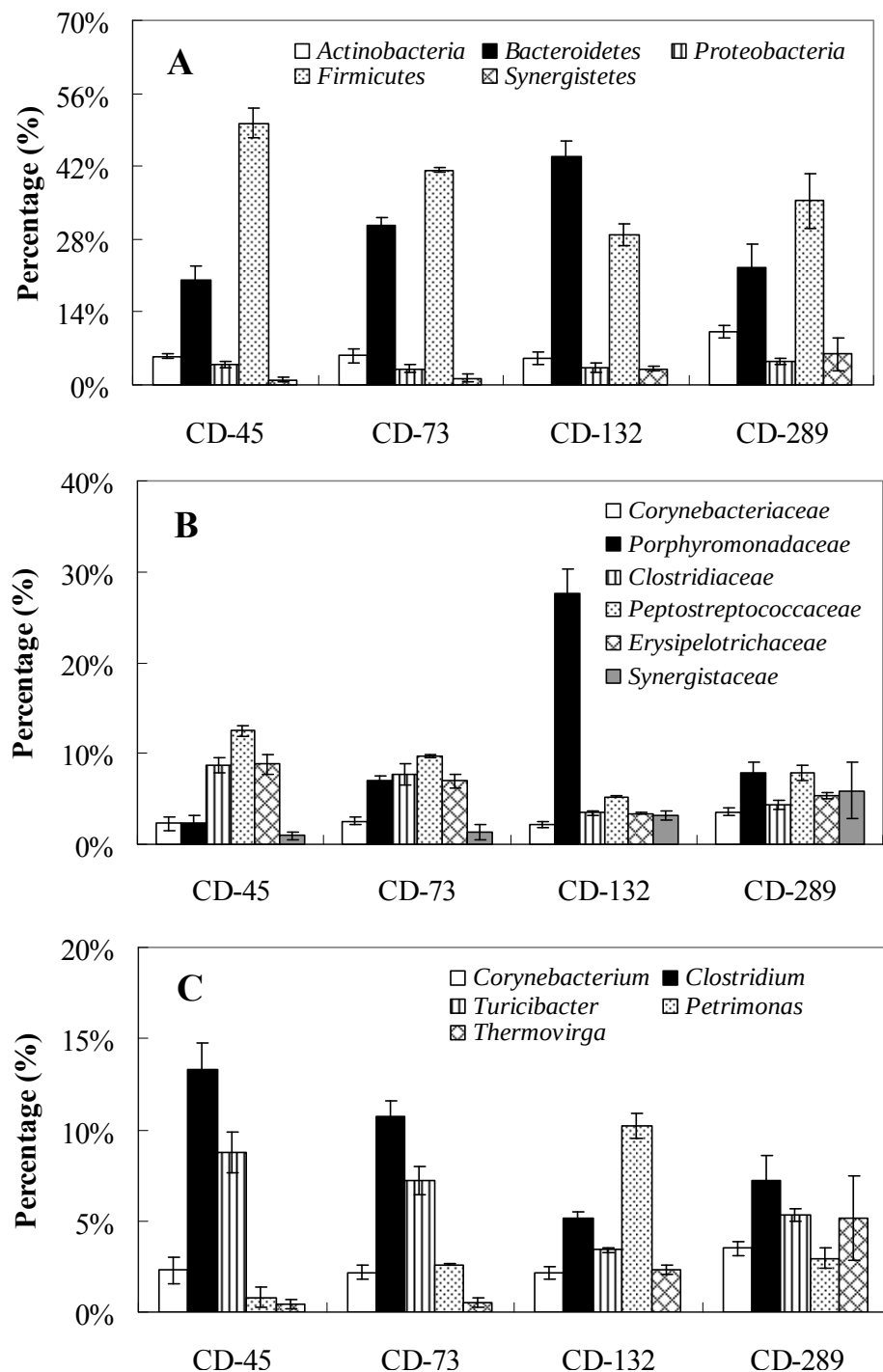


Fig. III7. Classification of bacterial 16sRNA sequences from reactors with RDP II database. The relative amounts (%) of major phyla (A), classified families (B) and genera (C) in total sequences for each sample were summarized respectively. The unclassified sequences were not included. Data are means of triplicate digesters, with the error bars indicating the standard deviation.

Excluding unclassified sequences comprising approximately 40% ~ 45%, the other sequences can be sorted into taxonomies in family level (Fig. III7B). Consistent with patterns of *Firmicutes* in response to OLR, the major families, *Clostridiaceae*, *Peptostreptococcaceae* and *Erysipelotrichaceae*, belonging to *Firmicutes* demonstrated declining pattern from initial setting up to performance collapse due to OLR increase. Their relative compositions recovered back to initial levels when methane production restored and performance became stable (Fig. III6). A typical family, *Porphyromonadaceae* affiliated to *Bacteroidetes* comprising only 2~3% of total sequences increased significantly to 25~30% when digester performance collapsed at maximum OLR, 1.77gVS/L/day. Therefore, increase of *Bacteroidetes* (Fig. III7A) should be mostly attributed to an increase of *Porphyromonadaceae*. Relative fractions of two major families, *Intrasporangiaceae* (not shown) and *Corynebacteriaceae* belonging to the phylum *Actinobacteria* remained constant during whole operational period, ranging from 2% to 4% of total sequences, while relative percentage of *Synergistaceae*, representing 100% of phylum *Synergistetes*, increased gradually after the digester performance was recovered from overloading (Fig. III7B).

When it comes to the genus level, less than 50% of total sequences can be classified by RDP Classifier. Genera, *Clostridium* and *Turicibacter* affiliated to families, *Clostridiaceae*, and *Erysipelotrichaceae*, comprising above 90% of sequences of their families respectively, decreased with OLR increase and then increased when performance of digester recovered (Fig. III7C). In contrast, representative genus, *Petrimonas* affiliated to the family, *Porphyromonadaceae* increased from less than 1% during initial setting to approximately 10% of the total sequences when methane production collapsed, in which *Petrimonas* comprised nearly 40% of sequences affiliated to *Porphyromonadaceae*. Correspondingly, 40% ~ 50% of the increase of *Petrimonas* should be attributed to *Porphyromonadaceae*. Similarly, relative percentages of *Thermovirga*, representing more than 90% of the sequences affiliated to *Synergistetes* or *Synergistaceae*, increased after digester performance recovered from organic overloading. Relative percentages of Genera, *Janibacter* (not shown in Fig. III7C) and *Corynebacterium* representing most compositions of families, *Intrasporangiaceae* and *Corynebacteriaceae* respectively, remained constant between pre-overloading or post-overloading and overloading stages (Fig. III7C), which were no more than 4% of total sequences together.

Dynamics of *Bacteroidetes* and *Firmicutes* populations

Supported by pairwise correlation analysis of *Bacteroidetes* and *Firmicutes* populations (Table III3), the significant negative correlations between these two populations in three taxonomic levels, i.e. phylum, family and genus also agreed the opposite dynamics of *Bacteroidetes* and *Firmicutes* populations (Fig. III7A-C) in response to OLR or VFA accumulation. Dissimilar with observations from previous studies about bacterial communities in mesophilic anaerobic digesters fed with various substrates that the most abundant *Bacteroidetes* and *Firmicutes* groups were unexpectedly steady and not clearly linked to process parameters (Kampmann et al., 2012; Krakat et al., 2011), the apparently opposite dynamics of these two major populations in response to reactor parameters, VFA

or CH₄ production, in this study, indicated their fundamental metabolic activities in maintaining bioreactor stability and biomethane genesis.

As ratio of *Firmicutes* to *Bacteroidetes* used as a critical indicator to study gut microbiota metabolisms or gastrointestinal microbial ecology (Callaway et al., 2010; Nicholson et al., 2012), it was also applied in the study of anaerobic digestion process (Briones et al., 2014). Similar with the observation reported previously (Briones et al., 2014), ratios of *Firmicutes* to *Bacteroidetes* (F/B) in this study were above 1 in both pre and post-overloading periods (Fig. III8). With the gradual OLR increase, F/B ratios decreased dramatically from 2.5 to 0.5 ~ 0.6 during digester performance collapse due to VFA accumulation. Sequentially, F/B ratio restored above 1 (~ 1.4) with stable performance recovered (Fig. III8). Based on results obtained in this study, thus, the shift of relative abundances between two major populations, *Firmicutes* and *Bacteroidetes* may indicate the feasibilities of biomethane production. Moreover, ratio of *Firmicutes* to *Bacteroidetes* can be applied as an important indicator in future studies.

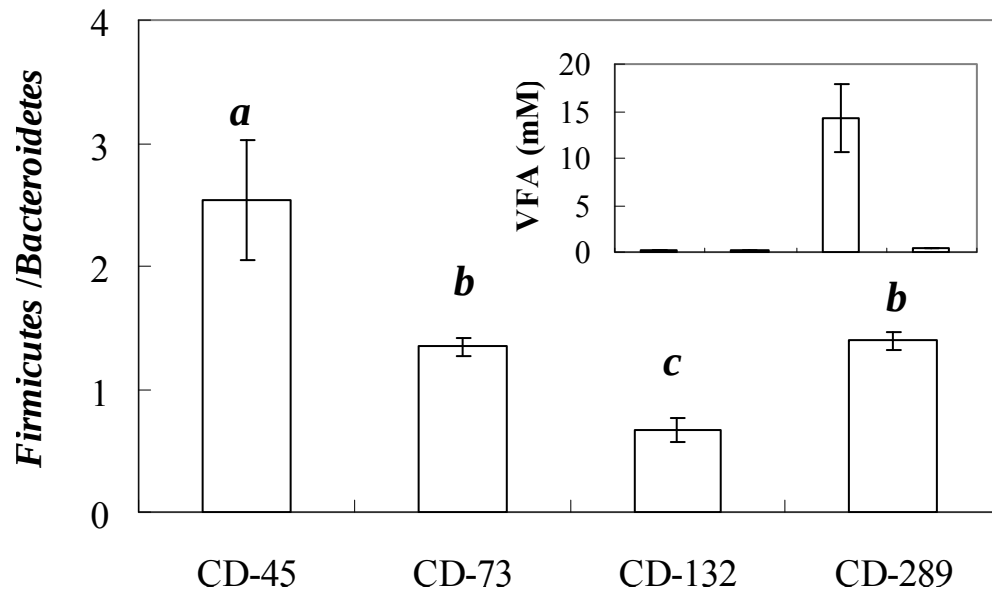


Fig. III8. Ratios of *Firmicutes* to *Bacteroidetes* at different sampling points during the whole period of anaerobic co-digestion. Inset indicates VFA concentrations. Different letters determined by oneway ANOVA analysis represents ratios between two categories with significant differences ($p=0.0001^*$). Data are means of triplicate digesters, with the error bars indicating the standard deviation.

Table III3 Pairwise correlations between *Bacteroidetes* and *Firmicutes* populations.

<i>Bacteroidetes</i> populations	<i>Firmicutes</i> populations	Correlation	p value
Phylum level			
<i>Bacteroidetes</i>	<i>Firmicutes</i>	-0.99	<0.0001 [*]
Family level			
	<i>Clostridiaceae</i>	-0.91	0.0002 [*]
<i>Porphyromonadaceae</i>	<i>Peptostreptococcaceae</i>	-0.96	<0.0001 [*]
	<i>Erysipelotrichaceae</i>	-0.92	0.0002 [*]
Genus level			
<i>Petrimonas</i>	<i>Turicibacter</i>	-0.92	0.0002 [*]
	<i>Clostridium</i>	-0.94	<0.0001 [*]

^{*} indicates the correlation between two variables is significant, p<0.05.

Table III4 Biodiversity estimation of 16S rRNA sequences from the pyrosequencing analysis.

Samples ^a	OUTs ^b	Coverage (%)	1/D ^c	Chao1 ^d	H _{shannon} ^e	ACE ^f
CD1.45.Bac	191	91.79	12.34	422.09	3.36	639.68
CD2.45.Bac	239	89.48	14.65	533.06	3.66	885.83
CD3.45.Bac	201	91.14	10.75	440.53	3.31	729.69
CD1.73.Bac	251	88.08	15.89	690.41	3.76	1320.68
CD2.73.Bac	226	89.88	13.19	588.12	3.59	850.05
CD3.73.Bac	225	89.88	11.27	497.34	3.48	956.28
CD1.132.Bac	276	87.50	15.87	719.25	4.00	1095.40
CD2.132.Bac	258	88.11	13.54	663.23	3.82	1300.39
CD3.132.Bac	274	87.05	13.66	739.87	3.86	1367.88
CD1.289.Bac	286	87.19	27.78	673.86	4.20	1004.76
CD2.289.Bac	310	84.83	28.73	874.99	4.23	1655.87
CD3.289.Bac	297	85.94	28.22	803.01	4.22	1453.98

^a Bacterial 16SrRNA sequences from co-digesters at operation day: 45, 73, 132 and 289. At each time point, triplicate samples were taken (CD1-CD3). For each sample, the number of sequences was normalized as the same.

^b Operational Taxonomy Unit- cutoff value=3%. It also represents observed richness.

^c Biodiversity index: inverse Simpson (Peet, 1974).

^d Chao1: richness estimator (Chao, 1984).

^e H_{shannon}: Shannon–Wiener index indicating population diversity index (Chao & Shen, 2003).

^f ACE: abundance coverage-based estimator (Colwell & Coddington, 1994).

Bacterial community diversity analysis within sample - α diversity

In order to eliminate influence from unequal sequence number on biological diversity estimator calculations for sample comparison (Gihring et al., 2012), an equal number of sequences for all samples were sub-sampled randomly based on samples with least sequences (1413 sequences). Then, biological diversity estimators (Table III4) and rarefaction curves (Fig. IIIS1) for each sample were analyzed. As shown in Table III4, sample community richness estimators, i.e. observed number of OTUs, Chao1 and ACE, with relatively small variations among triplicate samples at each time point, had an apparently increasing trend from pre-overloading to post-overloading periods with digester performance collapse. Similarly, community diversity estimators, i.e. inverse Simpson index, 1/D and Shannon index, H_{Shannon} , also had an increasing trend after digesters went through overloading perturbation, although there were some variations among triplicate samples for these two indices at each sampling point (Table III4).

Comparison of bacterial structures between samples - β diversity

A shared OTUs file or table between any two samples was summarized based on the OTUs determined (3% difference cutoff) from equally sub-sampled sequences of each sample. To visualize classifications of these 12 individual samples (triplicate samples at 4 time points) based on OTU, weighted and un-weighted cluster analyses were applied based on Theta, θ , and Jaccard calculations respectively. For both un-weighted and weighted clusters (Fig. III9A-B), triplicate samples from the same time point clustered in one lineage indicating considerable similarities or small composition variances among triplicate samples. Samples collected at different times, however, separated distinctly into different lineages suggesting the dynamics of bacterial communities during the whole digester operation period, especially under organic overloading condition.

To figure out bacterial community structures during different stages of digester performance, the top 20 major OTUs of all samples were selected, since sequences represented by these 20 OTUs for each sample comprised approximately 85 ~ 95% of sequences in each sample (Table IIIS2). In order to visualize dynamics of representative OTUs over time, two dimensional hierarchical cluster analyses of these 20 selected OTUs was performed (Fig. III10). Due to relatively small variations of these OTUs distributed in triplicate digesters at each sampling time (Table IIIS2), relative abundances of OTUs at each time point were applied in average. In consistent with results of taxonomic classification obtained from RDP (Fig. III7A-C), relative abundances of sequences represented by major OTUs affiliated to *Porphyromonadaceae*, unclassified *Bacteroidetes* and *Petrimonas* increased significantly in response to ORL increase from 45th to 132nd day and resumed the original status when digester performance was recovered under OLR level in the beginning, 1.0gVS/L/day (289th day). On the contrary, percentages of sequences represented by OTUs affiliated to *Firmicutes*, e.g., unclassified *Clostridiales*, *Clostridium_XI*, *Turicibacter* decreased when digester performance collapsed due to organic overloading at OLR, 1.77gVS/L/day. Similar to dynamics of *Synergistaceae* (Fig. III7B), relative abundance of sequences represented by OTUs affiliated to *Synergistaceae* did not show significant correspondence to OLRs and accumulated gradually during the whole digester operation process. As the new

established phylum, *Synergistetes* (Jumas-Bilak et al., 2009), one of the genera, *Thermovirga* grouped into this taxon represented its most sequences (more than 80%) in this study (Fig. III7C). Its metabolic roles in anaerobic digestion process appeared to be critical for future investigations. Other minor OTUs representing small percentages of sequences remaining constant or with no apparent correlations with digester performance parameters may have minor impacts on community structures.

To compare similarities and show common shared or unique OTUs between samples collected from different operational stages, a Venn diagram was used to demonstrate bacterial community structures (Fig. III11A-B). Due to community similarities among triplicate samples at each point demonstrated by cluster analysis (Fig. III11A-B), sequences at the same time point were combined. As shown in Venn diagram (Fig. III11A), there were 153 OTUs commonly shared by all these four time points, which can be considered as core or persistent populations in digesters even they went through organic overloading. Numbers of unique OTUs for digester operation days, 45th, 73rd, 132nd and 289th were 214, 395, 706 and 675 respectively. The increase of unique OTUs from pre-overloading (45th day) to post-overloading (289th day) after digester performance recovery from OLR perturbation also suggested an increase of biodiversity of bacterial communities (Table III4). Other OTUs shared between samples from different time points were also indicated (Fig. III11A).

Consistent with RDP classification results of bacterial communities of samples collected at different time points, the 153 core OTUs shared by all samples were affiliated to several major phyla, *Bacteroidetes* (25%), *Firmicutes* (36%), *Proteobacteria* (10%), *Actinobacteria* (6%) and *Synergistetes* (5%) (Fig. III11B). Two dominant groups, *Bacteroidetes* and *Firmicutes* sustained in digesters through the whole operation with OLR perturbations indicated their importance for the metabolisms of fermentations in anaerobic biogas digesters (Cirne et al., 2007; Krause et al., 2008; Wang et al., 2009).

In addition, principle coordinates analysis (PCoA) plotted by two dimensional graphs was used to describe bacterial community profiles of samples collected from different time points. Both un-weighted and weighted bacterial communities differentiated by abundances between pairs of samples were analyzed (Fig. III12A-B). Comparing with un-weighted calculation, total variances explained by two axes were improved a lot by weighted calculation (Fig. III12B). For both un-weighted and weighted calculations, triplicate samples at the same time point had the smallest distance as compared to other time point samples. Samples collected from the 132nd and 289th days, however, were separated with relatively long distances, which was far away from samples of 45th and 73rd days (Fig. III12A-B). From two plots based on un-weighted and weighted calculations, bacterial community structures changed dramatically between pre-overloading and post-overloading. Moreover, bacterial communities during digester performance collapse (132nd day) was also distinct from others during other operation stages.

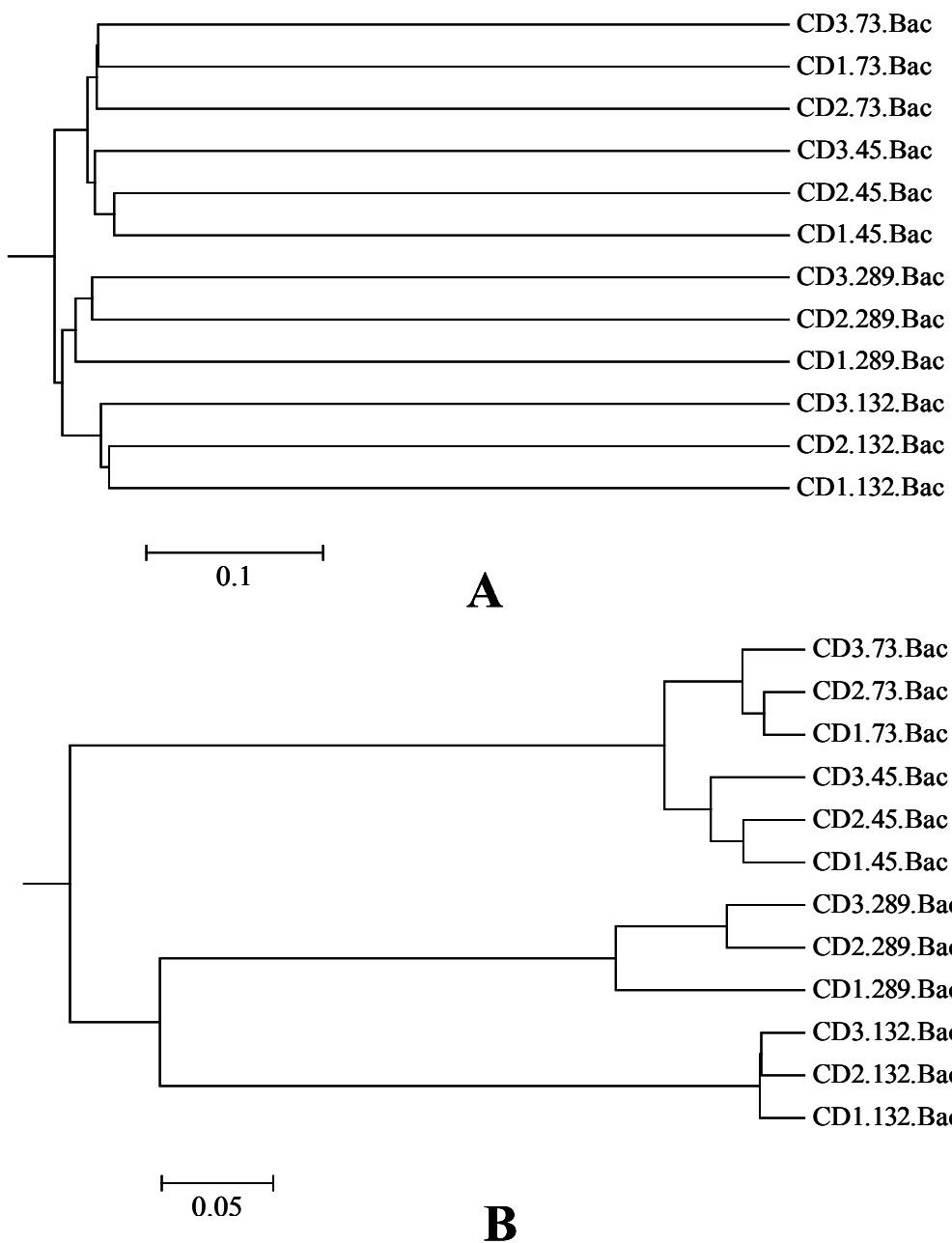


Fig. III9. Hierarchical cluster dendrogram based on OTU composition of bacterial populations following Jaccard (A) and Theta, θ (B) calculation methods. CD-co-digester; numbers indicate reactor operation date. For each time point, triplicate samples were collected.

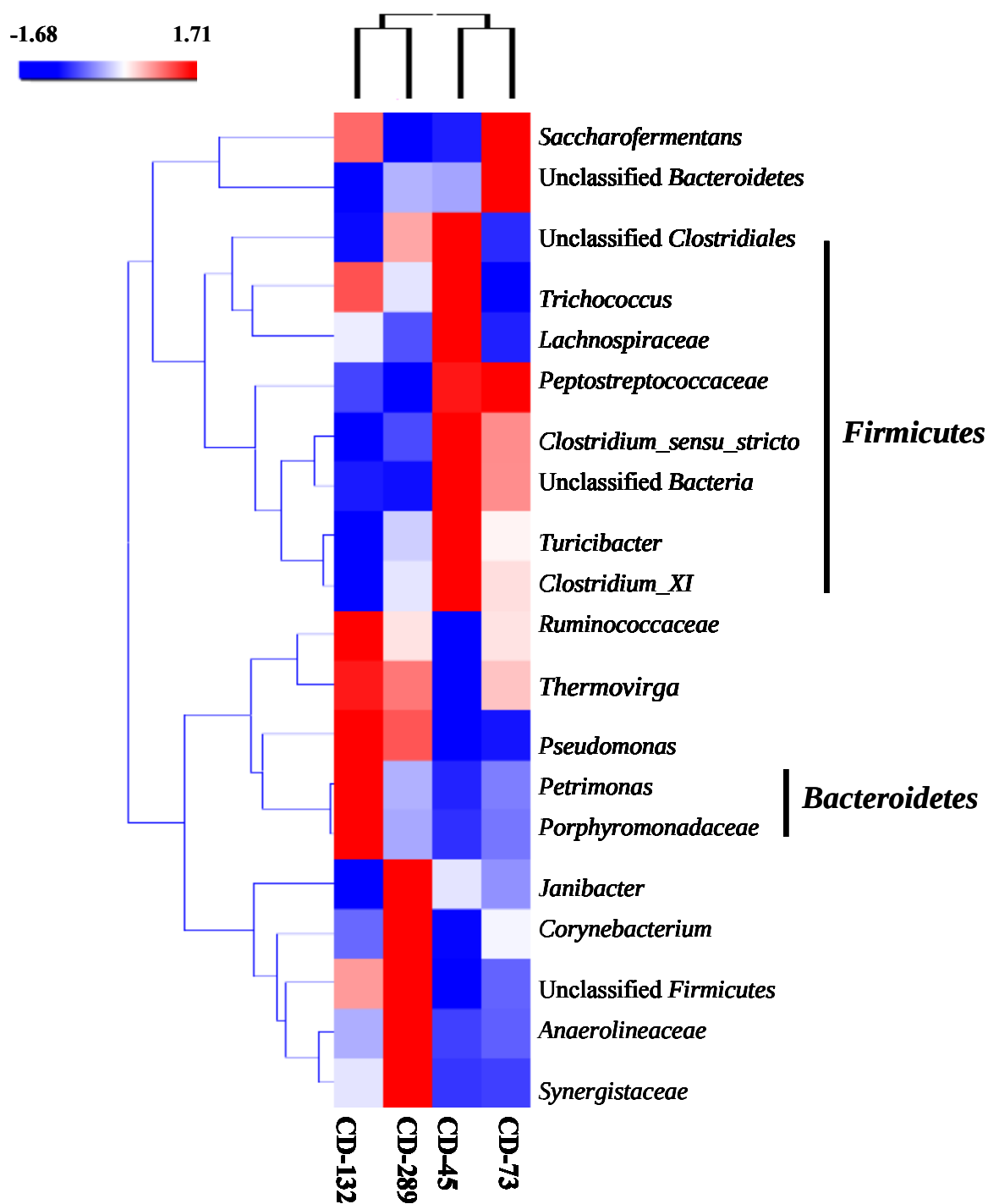


Fig. III10. Hierarchical clustering of major OTUs with significant changes (p<0.05) in response to digester performance disturbance. Each row represents major OTU affiliated to specific group (in family or genus levels). Each column represents individual time point of sampling. For each time point, triplicate digesters were sampled for sequencing analysis and relative percentages of OTUs were averaged.

Correlation analysis between digester performance and bacterial communities

Canonical Corresponding Analysis (CCA) was applied to investigate correlations between bacterial communities and digester performance parameters in three taxonomic levels, phylum, family and genus (Fig. III13A-C). For bacterial communities in three taxonomical levels, two factors representing the F1 and F2 axes can explain most of variations, over 90%. Among all chemical variables monitored, pH and VFA concentration had relatively high correlations with NH_4^+ concentration and OLR respectively. At the phylum level, *Firmicutes* positively corresponded to methane yield (Fig. III13A), in which it mainly included families, e.g., *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, *Clostridiaceae* and *Lachnospiraceae* (Fig. III13B). *Porphyromonadaceae* classified as a major family affiliated with phylum, *Bacteroidetes* positively corresponded to VFA accumulation with OLR increase (Fig. III13B). When it came to the genus level, *Turicibacter* and *Clostridium* belonging to families *Erysipelotrichaceae* and *Clostridiaceae* respectively were correlated with methane yield, while one genus identified as *Petrimonas* belonging to family, *Porphyromonadaceae* was in response to VFA accumulation (Fig. III13C).

Discussion

Consistent with previous studies about bacterial communities in anaerobic reactors, results obtained from the 454 pyrosequencing analysis in this study indicated that *Firmicutes*, *Bacteroidetes*, *Synergistetes*, *Proteobacteria* and *Actinobacteria* were commonly distributed (Krakat et al., 2011; Mnif et al., 2012). As described by previous research that *Firmicutes* and *Bacteroidetes* predominated in mesophilic continuous anaerobic reactors fed with cattle manure (Bertin et al., 2012; Briones et al., 2014), two dominant phyla, *Firmicutes* and *Bacteroidetes* compositing of more than 50% of the total sequences of digester samples, were also observed in this study. Moreover, these two populations in anaerobic digestion processes were negative correlated (Table III3) indicating opposite dynamics in response to operational parameters, i.e. OLR or VFA. However, this may not congruent with some previous observations of bacterial communities in long term operating anaerobic digesters that distribution patterns for *Firmicutes* and *Bacteroidetes* were not clearly linked to process parameters, e.g., hydraulic retention time (HRT), pH, OLR or acetate concentration (Kampmann et al., 2012; Krakat et al., 2011).

Firmicutes, the vast majority of fermentative microbiota in the acetogenic process may be an indicator of stable performance for methane generation, (Diamantis et al., 2012). Under stable performance of anaerobic digestion, the ratio of *Firmicutes* to *Bacteroidetes* generally ranged from 1 to 10 (Briones et al., 2014). Results obtained in this study also confirmed that dominant *Firmicutes* populations over *Bacteroidetes* populations was necessary to help maintain the stable metabolic functions in anaerobic digestion process, although this phenomena still needed more evidence provided by further studies.

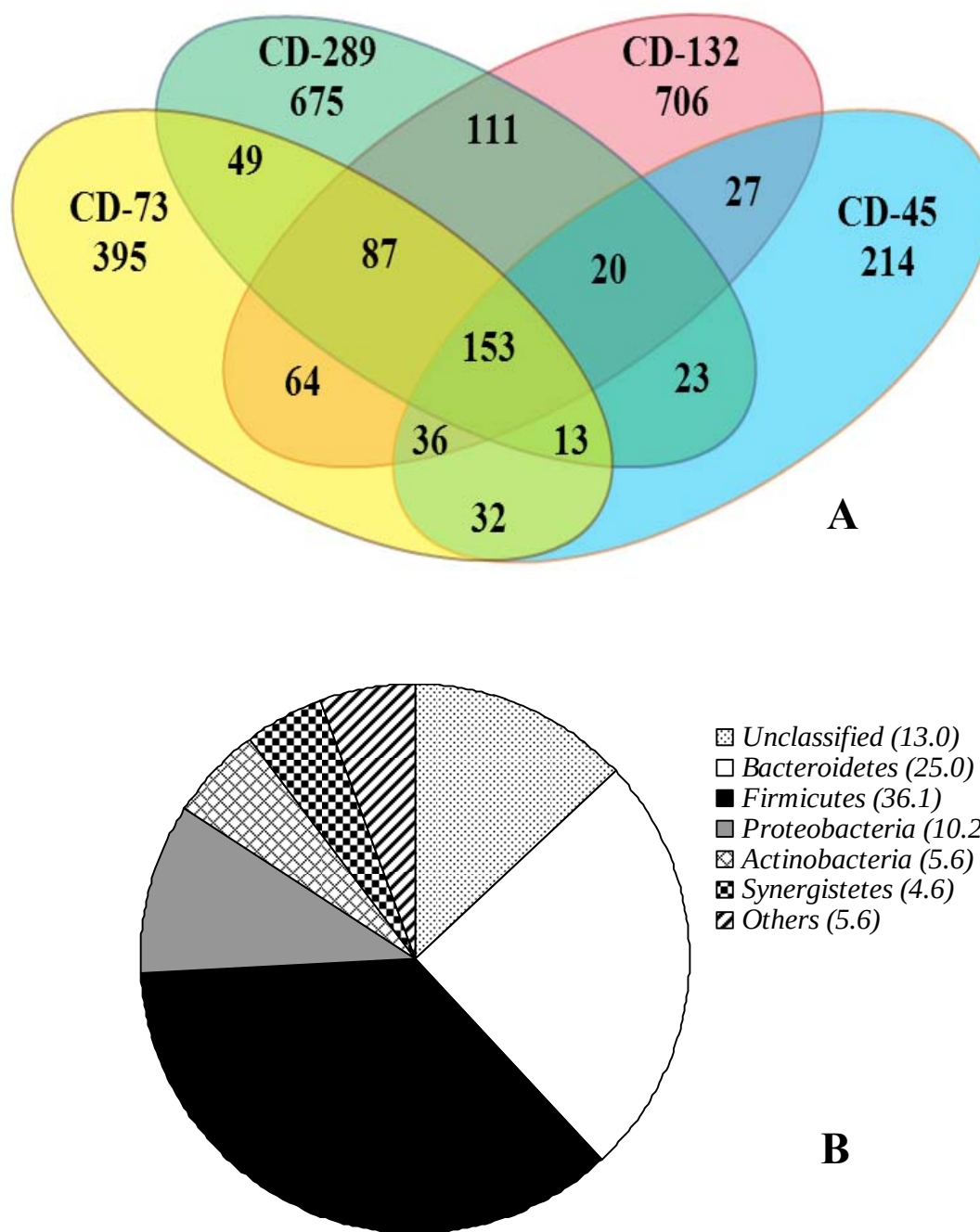


Fig. III11. (A) Venn diagram of the bacterial community structures during different reactor operation periods based on OTUs (cutoff = 0.03). The numbers indicated in this diagram is numbers of OTUs. (B) Taxonomic identification of core OTUs shared by all time points (153) in phylum level. Relative percentages (%) of the OTUs in each phylum were listed in parentheses.

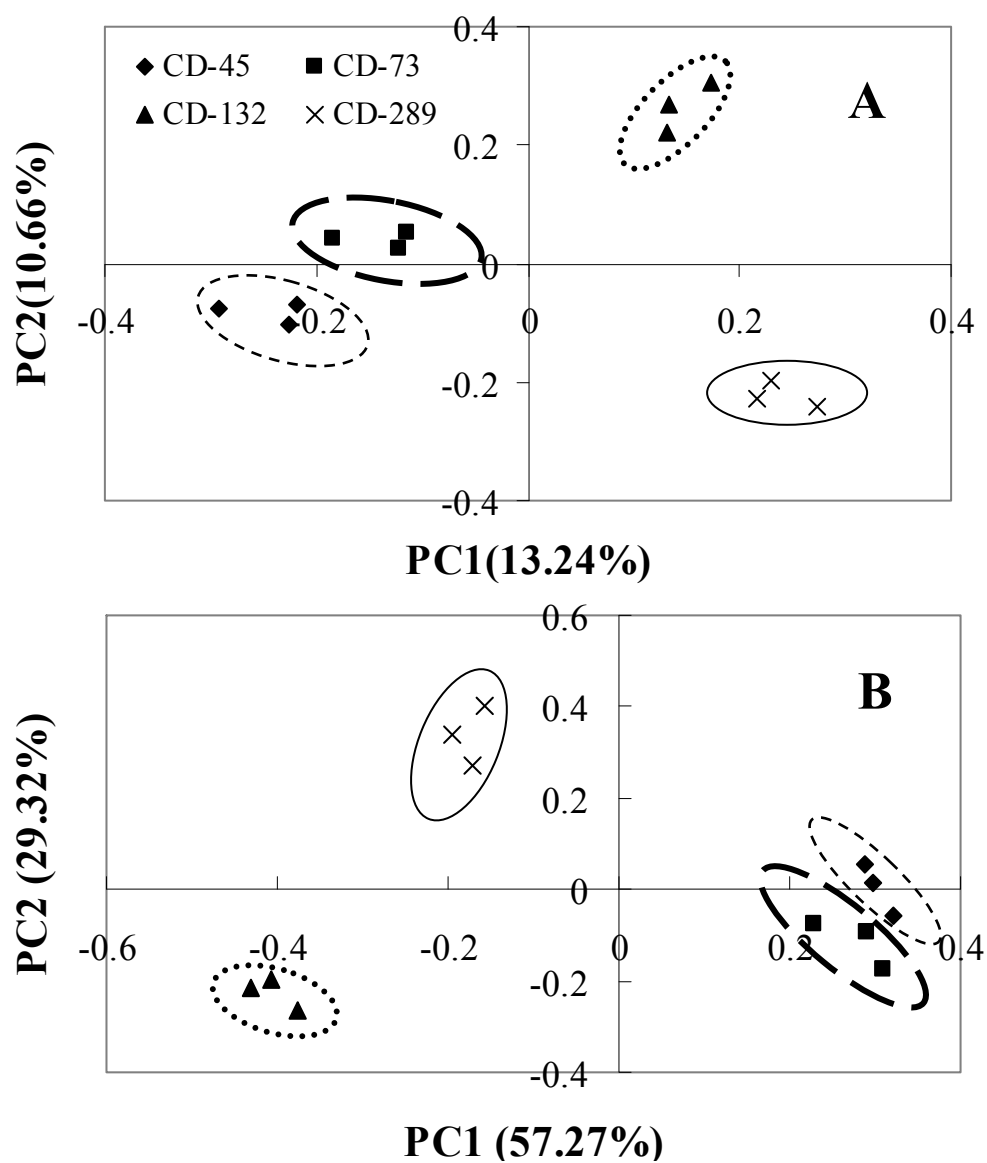


Fig. III12. Principal Coordinates Analysis (PCoA) of the bacterial community structures during different reactor operation periods based on OTUs (cutoff=0.03). (A) unweighted and (B) weighted.

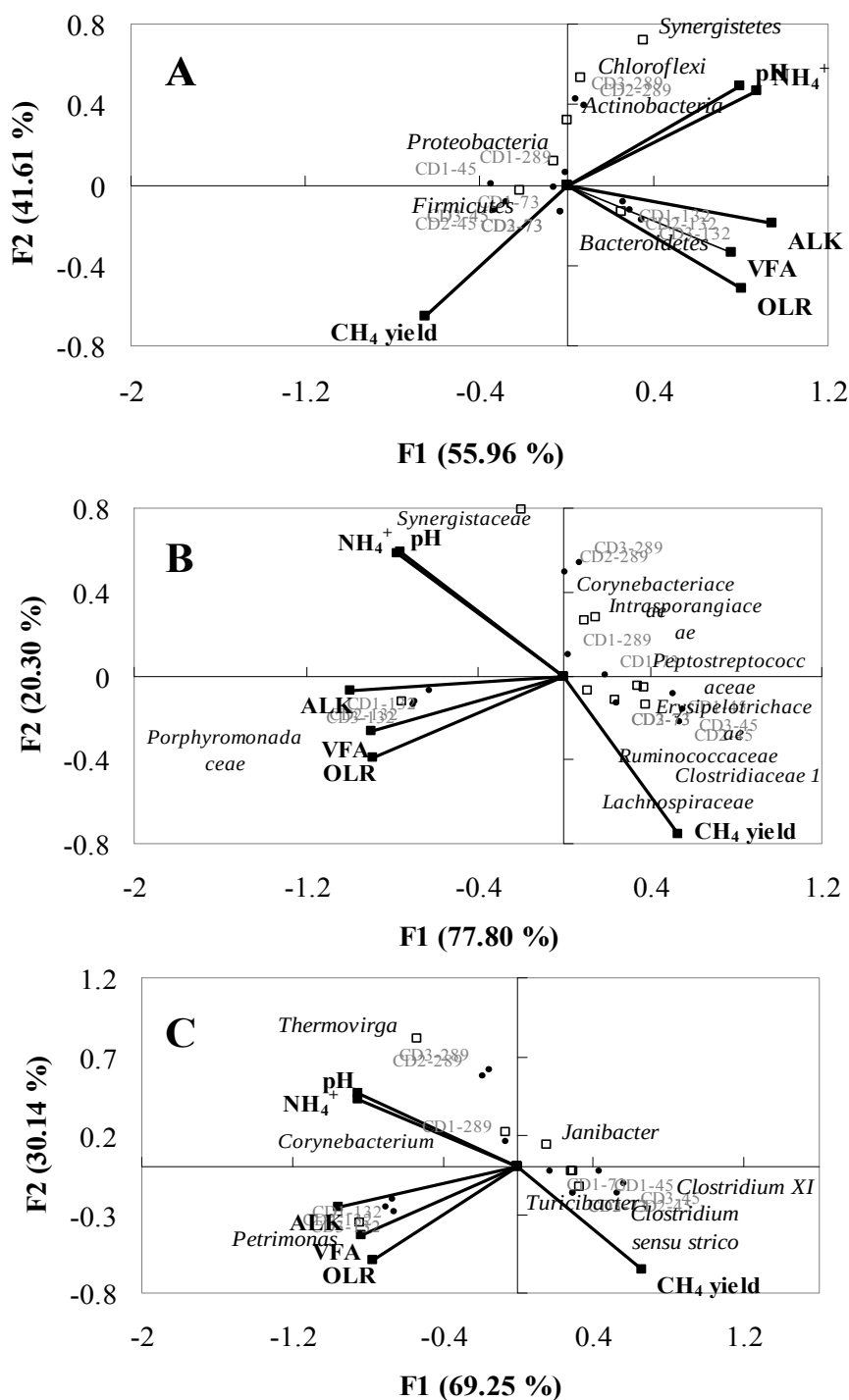


Fig. III13. Canonical Corresponding Analysis (CCA) for the correlations between major bacteria populations in phylum (A), family (B) and genus (C) level and reactor performance variables (pH, methane yield, alkalinity, ammonium, volatile fatty acids and organic loading rate) during different operation conditions respectively. Numbers represent reactor operation day and triplicate samples taken from each time point.

This phylum includes a highly diverse number of bacteria most of which show acetogenic metabolisms (Rincon et al., 2008). Most numbers in this phylum, particularly the class, *Clostridia* performs diverse metabolisms including homoacetogenesis syntrophic acetate oxidation, VFA and alcohol production from fermentations of cellulosic materials (Krakat et al., 2011). In the anaerobic digestion process, generally, bacteria generating acetate directly utilized by methanogenic *Archaea* were members of *Firmicutes*, mostly represented by the genus *Clostridium* and others less abundant but closely related to the genera within *Firmicutes*, especially under low OLR (Rincon et al., 2008; Rincon et al., 2006). With Supports of CCA results (Fig. III13A-C), positive correlations between methane yield and *Firmicutes* also confirmed observations in this study. The genus, *Turicibacter* belonging to *Firmicutes* is rod-shaped, strictly anaerobic, non-endospore forming, chemiorganotrophic, and fermentative, which followed similar patterns of the genera, *Clostridium* XI and *Clostridium sensu stricto* corresponding to OLR or VFA. It usually produces lactate as the only product of fermentation with optimum temperature and pH for growth, 37 °C and 7.5, respectively (Bosshard et al., 2002).

The dominant species replaced dramatically by phylum *Bacteroidetes* (Fig. III7A) during digester performance collapse with OLR increase (132nd day) was mainly attributed to an increase of the family, *Porphyromonadaceae* or genus, *Petrimonas* (Fig. III7B-C). Members of *Porphyromonadaceae* can produce VFAs from carbohydrates or proteins in acidogenesis (Ziganshin et al., 2011), such as *Petrimonas* generating acetate during sugar fermentation (Grabowski et al., 2005). Under certain circumstance, the increase of OLR promoted fermentation and acidogenesis bacteria growth. The continuous accumulation of VFAs resulting in imbalanced reaction rates between fermentation and methanogenesis, however, influenced the distribution of bacterial populations between *Bacteroidetes* and *Firmicutes* (Fig. III7A). Particularly, several genera affiliated with *Bacteroidetes* may have inhibitive functions in the acetogenic process. One major genus, *Petrimonas*, representing the family *Porphyromonadaceae* affiliated to phylum, *Bacteroidetes*, showed strong correspondence to VFA by CCA (Fig. III12A-C), supporting the speculation that distribution of acidogenic *Bacteroidetes* was related to VFA concentration.

Structure and properties of a microbial community may be influenced by process operation and in return also determine the reactor function in anaerobic digestion (Ramirez et al., 2009). Supported by results from a previous study about the influences of OLR and HRT on microbial community in anaerobic digesters (Rincon et al., 2008), the bacterial community of digesters in this study was more complicated than microbial community diversity in *Archaea* populations revealed previously (Chen et al., 2012). The increasing trend of biodiversity expressed as estimators, Shannon ($H_{Shannon}$) and inverse Simpson ($1/D$) seemed to be more significant after the digesters were recovered from organic overloading (Table III4). Organic overloading increasing the bacterial population diversity from pre to post-overloading suggested the additional function redundancy, which was important for maintaining process stability under performance disturbance (Girvan et al., 2005). Thus, the greater functional diversity as a result of increased bacterial diversity likely improved the capacity of post-overloading bacterial community

to overcome the detrimental impacts of fluctuations of substrate loading rates, usually characterized by the transient accumulation of high concentrations of VFAs (Briones & Raskin, 2003; Hashsham et al., 2000). With β diversity analysis of bacterial community during the entire digester operation period, results from hierarchical cluster analysis (Fig. III9A-B), PCoA (Fig. III11A-B) and Venn diagram (Fig. III10) based on weighed or un-weighted calculations demonstrated the bacterial structure transition from initial setting up status to final steady status recovered from organic overloading in digesters. The stable methanogenic activity of digesters re-achieved after overloading (Chen et al., 2012) also confirmed the adaption of bacterial community to OLR variations. As suggested by this study, adaptation of microorganisms to the inhibitory substance, i.e. high concentration of VFA, can significantly improve methanogenic efficiency, which may result from the selection of more resistant fermentative bacteria already existing in dairy farm waste.

Conclusions

High-throughput sequencing, 454 pyrosequencing of the bacterial communities in mesophilic anaerobic digesters with operation perturbations demonstrated the impact of organic loading rate on the population distributions, especially for relative abundances of *Firmicutes* and *Bacteroidetes*, which dominated the bacterial community. Canonical Corresponding Analysis (CCA) showed that the abundance of *Firmicutes* was related with methanogenesis, while *Bacteroidetes* was linked to the accumulation of volatile fatty acids (VFA) during process imbalance. Consequently, the bacterial community adapted to substrate overloading with greater diversity, likely enhancing the capacity of the anaerobic microbial community to cope with subsequent episodes of substrate overloading.

References

- Ahring, B. 2003. Perspectives for Anaerobic Digestion. in: *Biomethanation I*, (Eds.) B. Ahring, I. Angelidaki, E.C. Macario, H.N. Gavala, J. Hofman-Bang, A.J.L. Macario, S.J.W.H.O. Elferink, L. Raskin, A.J.M. Stams, P. Westermann, D. Zheng, Vol. 81, Springer Berlin Heidelberg, pp. 1-30.
- Angenent, L.T., Karim, K., Al-Dahhan, M.H., Domiguez-Espinosa, R. 2004. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol*, **22**(9), 477-485.
- Bertin, L., Bettini, C., Zanaroli, G., Frascari, D., Fava, F. 2012. A continuous-flow approach for the development of an anaerobic consortium capable of an effective biomethanization of a mechanically sorted organic fraction of municipal solid waste as the sole substrate. *Water Res*, **46**(2), 413-424.
- Bosshard, P.P., Zbinden, R., Altwegg, M. 2002. *Turicibacter sanguinis* gen. nov., sp nov., a novel anaerobic, Gram-positive bacterium. *Int J Syst Evol Microbiol*, **52**, 1263-1266.
- Briones, A., Coats, E., Brinkman, C. 2014. Should we build "obese" or "lean" anaerobic digesters? *PloS one*, **9**(5), e97252-e97252.
- Briones, A., Raskin, L. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr Opin Biotechnol*, **14**(3), 270-276.
- Brosius, J., Palmer, M.L., Kennedy, P.J., Noller, H.F. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, **75**(10), 4801-4805.
- Callaway, T.R., Dowd, S.E., Edrington, T.S., Anderson, R.C., Krueger, N., Bauer, N., Kononoff, P.J., Nisbet, D.J. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *J Anim Sci*, **88**(12), 3977-3983.
- Chao, A. 1984. Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, **11**(4), 265-270.
- Chao, A., Shen, T.-J. 2003. Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. *Environ Ecol Stat*, **10**(4), 429-443.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. *Bioresour Technol*, **99**(10), 4044-4064.
- Chen, Z., Wang, Y., Li, K., Zhou, H. 2014. Effects of increasing organic loading rate on performance and microbial community shift of an up-flow anaerobic sludge blanket reactor treating diluted pharmaceutical wastewater. *J Biosci Bioeng*(0).
- Cirne, D.G., Lehtomäki, A., Björnsson, L., Blackall, L.L. 2007. Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. *J Appl Microbiol*, **103**(3), 516-527.
- Colwell, R.K., Coddington, J.A. 1994. Estimating Terrestrial Biodiversity through Extrapolation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **345**(1311), 101-118.

- Demirel, B., Yenigun, O. 2002. Two-phase anaerobic digestion processes: a review. *J Chem Technol Biotechnol*, **77**(7), 743-755.
- Diamantis, V., Khan, A., Ntougias, S., Stamatelatou, K., Kapagiannidis, A., Aivasidis, A. 2012. Continuous biohydrogen production from fruit wastewater at low pH conditions. *Bioprocess Biosystems Eng*, **36**(7), 965-974.
- Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, **27**(16), 2194-2200.
- Fulthorpe, R.R., Roesch, L.F.W., Riva, A., Triplett, E.W. 2008. Distantly sampled soils carry few species in common. *ISME J*, **2**(9), 901-910.
- Garcia-Pena, E.I., Parameswaran, P., Kang, D.W., Canul-Chan, M., Krajmalnik-Brown, R. 2011. Anaerobic digestion and co-digestion processes of vegetable and fruit residues: Process and microbial ecology. *Bioresour Technol*, **102**(20), 9447-9455.
- Gihring, T.M., Green, S.J., Schadt, C.W. 2012. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. *Environ Microbiol*, **14**(2), 285-290.
- Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A. 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environ Microbiol*, **7**(3), 301-313.
- Grabowski, A., Tindall, B.J., Bardin, V., Blanchet, D., Jeanthon, C. 2005. *Petrimonas sulfuriphila* gen. nov., sp nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. *Int J Syst Evol Microbiol*, **55**, 1113-1121.
- Hashsham, S.A., Fernandez, A.S., Dollhopf, S.L., Dazzo, F.B., Hickey, R.F., Tiedje, J.M., Criddle, C.S. 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *Appl Environ Microbiol*, **66**(9), 4050-4057.
- Jang, H.M., Kim, J.H., Ha, J.H., Park, J.M. 2014. Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater. *Bioresour Technol*, **165**(0), 174-182.
- Jumas-Bilak, E., Roudière, L., Marchandin, H. 2009. Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. *Int J Syst Evol Microbiol*, **59**(5), 1028-1035.
- Kampmann, K., Ratering, S., Kramer, I., Schmidt, M., Zerr, W., Schnell, S. 2012. Unexpected Stability of Bacteroidetes and Firmicutes Communities in Laboratory Biogas Reactors Fed with Different Defined Substrates. *Appl Environ Microbiol*, **78**(7), 2106-2119.
- Kindaichi, T., Ito, T., Okabe, S. 2004. Ecophysiological Interaction between Nitrifying Bacteria and Heterotrophic Bacteria in Autotrophic Nitrifying Biofilms as Determined by Microautoradiography-Fluorescence In Situ Hybridization. *Appl Environ Microbiol*, **70**(3), 1641-1650.
- Krakat, N., Schmidt, S., Scherer, P. 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. *Bioresour Technol*, **102**(10), 5692-5701.
- Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.-H., Krömeke, H., Neuweiger, H., Pühler, A., Runte, K.J., Schlüter, A., Stoye, J., Szczepanowski, R., Tauch, A., Goesmann, A. 2008. Taxonomic composition and gene content of a methane-

- producing microbial community isolated from a biogas reactor. *J Biotechnol*, **136**(1–2), 91-101.
- Liu, W.T., Chan, O.C., Fang, H.H.P. 2002. Microbial community dynamics during start-up of acidogenic anaerobic reactors. *Water Res*, **36**(13), 3203-3210.
- Luo, G., Wang, W., Angelidaki, I. 2013. Anaerobic Digestion for Simultaneous Sewage Sludge Treatment and CO Biomethanation: Process Performance and Microbial Ecology. *Environ Sci Technol*, **47**(18), 10685-10693.
- Mnif, S., Zayen, A., Karray, F., Bru-Adan, V., Loukil, S., Godon, J.J., Chamkha, M., Sayadi, S. 2012. Microbial population changes in anaerobic membrane bioreactor treating landfill leachate monitored by single-strand conformation polymorphism analysis of 16S rDNA gene fragments. *Int Biodeterior Biodegrad*, **73**, 50-59.
- Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., Pettersson, S. 2012. Host-Gut Microbiota Metabolic Interactions. *Science*, **336**(6086), 1262-1267.
- Peet, R.K. 1974. The Measurement of Species Diversity. *Annu Rev Ecol Syst*, **5**, 285-307.
- Pohland, F., Ghosh, S. 1971. Developments in anaerobic stabilization of organic wastes--the two-phase concept. *Environmental Letters*, **1**(4), 255-266.
- Ramirez, I., Volcke, E.I.P., Rajinikanth, R., Steyer, J.P. 2009. Modeling microbial diversity in anaerobic digestion through an extended ADM1 model. *Water Res*, **43**(11), 2787-2800.
- Rincon, B., Borja, R., Gonzalez, J.M., Portillo, M.C., Saiz-Jimenez, C. 2008. Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue. *Biochem Eng J*, **40**(2), 253-261.
- Rincon, B., Raposo, F., Borja, R., Gonzalez, J.M., Portillo, M.C., Saiz-Jimenez, C. 2006. Performance and microbial communities of a continuous stirred tank anaerobic reactor treating two-phases olive mill solid wastes at low organic loading rates. *J Biotechnol*, **121**(4), 534-543.
- Schloss, P.D., Gevers, D., Westcott, S.L. 2011. Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *Plos One*, **6**(12).
- Seo, J., Bakay, M., Chen, Y.-W., Hilmer, S., Shneiderman, B., Hoffman, E.P. 2004. Interactively optimizing signal-to-noise ratios in expression profiling: project-specific algorithm selection and detection p-value weighting in Affymetrix microarrays. *Bioinformatics*, **20**(16), 2534-2544.
- Stams, A.J.M., Plugge, C.M. 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology*, **7**(8), 568-577.
- Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sorensen, S.J., Karlsson, A. 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol*, **85**(3), 612-626.
- Wan, S., Sun, L., Douieb, Y., Sun, J., Luo, W. 2013. Anaerobic digestion of municipal solid waste composed of food waste, wastepaper, and plastic in a single-stage system: Performance and microbial community structure characterization. *Bioresour Technol*, **146**(0), 619-627.
- Wang, H., Lehtomaki, A., Tolvanen, K., Puhakka, J., Rintala, J. 2009. Impact of crop

- species on bacterial community structure during anaerobic co-digestion of crops and cow manure. *Bioresour Technol*, **100**(7), 2311-2315.
- Wang, H., Tolvanen, K., Lehtomaki, A., Puhakka, J., Rintala, J. 2010. Microbial community structure in anaerobic co-digestion of grass silage and cow manure in a laboratory continuously stirred tank reactor. *Biodegradation*, **21**(1), 135-146.
- Yang, Y., Yu, K., Xia, Y., Lau, F.K., Tang, D.W., Fung, W., Fang, H.P., Zhang, T. 2014. Metagenomic analysis of sludge from full-scale anaerobic digesters operated in municipal wastewater treatment plants. *Appl Microbiol Biotechnol*, **98**(12), 5709-5718.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhang, Y., Zhang, X.L., Zhang, H.W., He, Q., Zhou, Q.X., Su, Z.C., Zhang, C.G. 2009. Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis. *Bull Environ Contam Toxicol*, **82**(3), 367-372.
- Ziganshin, A., Schmidt, T., Scholwin, F., Il'inskaya, O., Harms, H., Kleinstaub, S. 2011. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. *Appl Microbiol Biotechnol*, **89**(6), 2039-2052.

Appendix I

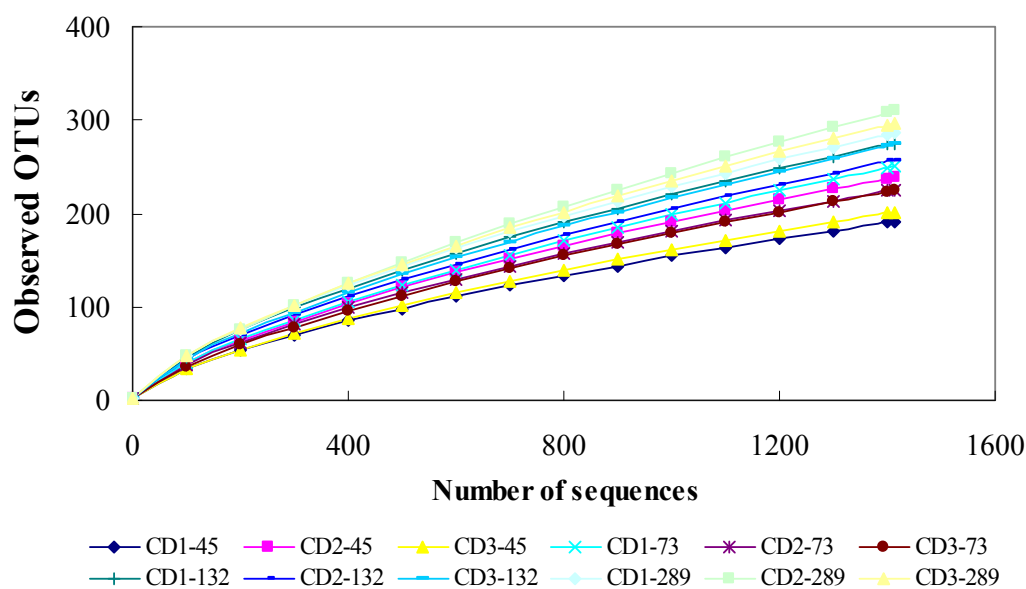


Fig. IIS1. Rarefaction curve of bacterial 16SrRNA gene sequences from individual anaerobic digesters at different sampling times. Number of sequences for each of samples was normalized to be equal.

Table IIS1 List of primers and barcodes used in 454 pyrosequencing for bacterial communities in 12 samples.

Sample ID ^a	Forward primer ^b	Reverse primer ^b	Bar code ^c
CD1-45	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	ATCAGACACG
CD2-45	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	ATATCGCGAG
CD3-45	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	CGTGTCTCTA
CD1-73	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	CTCGCGTGTC
CD2-73	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TAGTATCAGC
CD3-73	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TAGCGCGCGC
CD1-132	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TAGCTCTATC
CD2-132	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TATAGACATC
CD3-132	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TATGATACGC
CD1-289	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TCACTCATAC
CD2-289	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TCATCGAGTC
CD3-289	GTGCCAGCAGCCGCGGTAA	GGACTACCAGGGTATCTAAT	TCGAGCTCTC

^a For sample ID, CD: co-digestion reactors; Numbers '1', '2' and '3': triplicate digesters; '45', '73', '132' and '298': operation days.

^b Sequence for both forward and reverse primers is 5'-3'.

^c 10 base barcodes on forward primers.

Table IIIS2 List of major bacterial OTUs and their relative abundances in each of triplicate semi-CSTRs during different operational periods^a.

Major OTUs ^b	Taxonomy Identification ^c	Relative Abundance (%) ^d of sequences represented by OTUs											
		Operational Day											
		45			73			132			289		
		CD1	CD2	CD3	CD1	CD2	CD3	CD1	CD2	CD3	CD1	CD2	CD3
1	<i>Porphyromonadaceae</i>	0.35	1.20	1.27	3.96	2.83	3.33	20.59	23.21	23.64	6.23	5.17	3.82
2	Unclassified <i>Bacteroidetes</i>	15.50	18.54	21.16	25.05	26.47	32.20	13.59	17.13	15.22	20.17	16.84	19.04
3	Unclassified <i>Bacteria</i>	19.89	16.07	23.43	16.56	17.06	16.99	11.96	13.31	13.09	11.25	12.74	13.94
4	<i>Petrimonas</i>	0.71	0.64	0.21	1.49	2.12	1.42	8.63	8.56	8.42	2.97	2.48	1.49
5	<i>Clostridium_XI</i>	13.80	11.75	11.18	8.49	9.70	8.28	5.17	4.18	4.32	8.70	8.56	7.29
6	<i>Turicibacter</i>	14.51	13.09	10.69	9.62	8.00	8.85	4.81	4.67	5.45	10.33	7.71	6.44
7	<i>Clostridium_sensu_stricto</i>	10.69	10.05	8.99	7.15	7.86	7.57	4.10	3.40	3.11	5.24	4.18	4.39
8	<i>Corynebacterium</i>	2.48	1.77	1.63	3.18	2.97	2.12	2.76	1.98	2.12	2.69	4.74	4.95
9	Unclassified <i>Clostridiales</i>	1.84	2.69	2.55	1.56	1.70	2.19	2.26	1.20	1.91	3.04	1.56	1.70
10	<i>Thermovirga</i>	0.50	0.21	0.07	0.57	0.14	0.42	2.26	2.19	2.62	3.68	9.20	9.98
11	Unclassified <i>Firmicutes</i>	1.49	1.06	1.20	1.84	1.20	1.27	1.98	1.70	1.63	2.76	1.70	1.84
12	<i>Lachnospiraceae</i>	2.55	3.82	2.19	1.49	1.13	1.34	1.77	1.63	2.05	1.63	1.42	1.27
13	<i>Peptostreptococcaceae</i>	1.27	1.27	1.98	1.84	1.34	1.42	1.42	1.56	0.85	1.56	1.42	0.64
14	<i>Trichococcus</i>	1.06	0.57	0.99	0.35	0.71	0.57	1.27	0.57	0.64	0.64	0.35	1.20
15	<i>Saccharofermentans</i>	0.57	0.42	0.64	0.71	1.13	0.85	0.50	0.85	0.99	0.64	0.50	0.42
16	<i>Pseudomonas</i>	0	0.07	0	0.07	0.07	0	1.13	0.78	0.85	0.50	0.92	0.64
17	<i>Janibacter</i>	1.42	1.42	1.27	1.34	1.70	0.57	1.06	0.64	0.92	1.84	2.55	2.34
18	<i>Synergistaceae</i>	0.35	0.64	0.14	0.78	0.00	1.13	0.78	0.64	0.78	0.00	0.92	1.13
19	<i>Ruminococcaceae</i>	0.50	0.85	0.64	0.57	1.42	0.21	0.71	0.64	0.99	1.42	0.21	0.57
20	<i>Anaerolineaceae</i>	0.50	0.99	0.42	0.92	0.14	0.99	0.71	0.57	1.06	0.35	1.70	2.19

^a All sequences for each sample was normalized before OTU determination, as the method applied in biodiversity parameter calculation.

^b In total, the relative percentages of these top 20 OTUs listed for each sample can cover more than 85% of total sequences.

^c OTU taxonomy identification was based on program in Mothur (V1.31.2).

^d Relative abundances (%) of each OUT in total normalized sequences for each triplicate digester (from CD1 to CD3) during different operational phases were summarized.

Chapter IV

Development of Optimized qPCR Assays for Specific Archaeal Populations in Anaerobic Digestion

Part I: Development of *Methanoculleus*-specific Real-time Quantitative PCR Assay for Assessing Methanogen Communities in Anaerobic Digestion

Abstract

Aim: To develop a *Methanoculleus*-specific real-time quantitative PCR (qPCR) assay with high coverage and specificity for the analysis of methanogenic populations in anaerobic digestion.

Methods and Results: A *Methanoculleus*-specific primer/probe set for qPCR was designed in this study based on all *Methanoculleus* 16S rRNA gene sequences in Ribosomal Database Project (RDP) according to TaqMan chemistry. The newly designed primer/probe set was shown to have high coverage and specificity by both in silico and experimental analyses. Amplification efficiency of the *Methanoculleus*-specific primer/probe set was determined to be ideal for qPCR applications. Subsequent field testing on anaerobic digesters showed that results from qPCR were consistent with those from clone library analysis, validating the accuracy of the qPCR assay.

Conclusions: The *Methanoculleus*-specific qPCR assay designed in this study can serve as a rapid and effective tool for the quantification of *Methanoculleus* populations in anaerobic digestion.

Significance and Impact of the Study: *Methanoculleus* populations represent important members of archaeal communities in methanogenic processes, necessitating the need to develop effective tools to monitor *Methanoculleus* population abundance. The qPCR developed in this study provides an essential tool for the quantification of *Methanoculleus* populations in anaerobic digestion and for the understanding of the functions of these methanogens in anaerobic biotransformation.

Introduction

Anaerobic digestion is a proven technology for the treatment of organic wastes, such as food residue and animal manure (Nasir et al., 2012; Zhu et al., 2011). Recent interest in anaerobic digestion has grown significantly due to its potential in producing biogas as a source of renewable energy and reducing greenhouse gas emission (Abbasi et al., 2012). The broader application of anaerobic digestion as a sustainable waste treatment option, however, requires further improvement in treatment efficiency and process stability (Gupta et al., 2012). Given the importance of methanogenic microbial populations in the anaerobic conversion of organic substrates to biogas, the ability to monitor the population dynamics of methanogens is critical for identifying the key indicators of process stability and metabolic pathways controlling anaerobic biotransformation (Appels et al., 2011).

Considerable efforts have been made to developing tools, particularly culture-independent molecular techniques, for the monitoring of methanogens in anaerobic

environments (Narihiro & Sekiguchi, 2007). Real-time quantitative polymerase chain reaction (qPCR) has emerged as a rapid technique to target specific microbial populations with high sensitivity (Smith & Osborn, 2009). Of the two main chemistries used in qPCR, that is, TaqMan and SYBR Green, the former is considered to be capable of providing greater target specificity in microbial ecology studies with the use of an extra oligonucleotide probe in addition to the set of primers used by SYBR Green (Wang & Brown, 1999), leading to the development of a series of TaqMan qPCR assays for methanogen populations by targeting the 16S rRNA genes (Shigematsu et al., 2003; Tang et al., 2005; Yu et al., 2005).

Given the potential metabolic diversity of methanogens, it is often desirable to differentiate and quantify methanogen populations at fine taxonomic resolutions (Garcia et al., 2000; Jetten et al., 1992). *Methanoculleus* like organisms have been frequently identified as the dominant hydrogenotrophic methanogen populations in anaerobic digesters and other methanogenic environments, resulting in considerable interest in monitoring the population dynamics of these organisms (Barret et al., 2012; Cardinali-Rezende et al., 2012; Nettmann et al., 2010). Currently, the only *Methanoculleus*-specific TaqMan qPCR primer/probe set available in the literature targets bases 934–1221 (*E. coli* numbering) of the 16S rRNA gene (Shigematsu et al., 2003), making it difficult to assess the specificity and coverage of the qPCR assay when sequence information for this segment of the 16S rRNA gene is lacking for the target populations. For example, 16S rRNA gene sequences of *Methanoculleus* (Fig. IV1) obtained from clone library analysis of anaerobic digesters cover bases 21–958 (*E. coli* numbering) (Chen et al., 2012; Zhang et al., 2011). Thus, the above-mentioned *Methanoculleus*-specific qPCR primer/probe set could not be applied with confidence for the specific quantification of *Methanoculleus* populations in these anaerobic digesters. Similar issues may also arise when only short reads and partial sequence information are available. Moreover, the current primer/probe set is reported to lack specificity to the genus of *Methanoculleus* (Franke-Whittle et al., 2009), which is of particular concern given the considerable phylogenetic diversity of *Methanoculleus* (Fig. IV1). Therefore, the objective of this study was to design alternative *Methanoculleus*-specific TaqMan qPCR primer/probe set with high sequence coverage and specificity.

Materials and methods

Primer and probe design

The *Methanoculleus*-specific primer/probe set for TaqMan qPCR was designed with 1623 16S rRNA gene sequences of the genus *Methanoculleus* in Ribosomal Database Project (RDP) Release 10 (Cole et al., 2009) as previously described (Yu et al., 2005). Briefly, sequences of primer/probe were determined following guidelines set by Applied Biosystems (Foster City, CA) on parameters including amplicon size, nucleotide position, melting temperature (T_m), complementarity and GC content, using Primer3 (Untergasser et al., 2007). The primer/probe specificity and coverage of the target group, that is, *Methanoculleus*, was first evaluated in silico using the PROBE MATCH programme of RDP II (Cole et al., 2009).

Experimental evaluation of *Methanoculleus*-specific primer/probe set

Primer/probe specificity was further assessed using cloned 16S rRNA genes from *Methanoculleus* (GenBank Accession Number JN052755), *Methanosaeta* (GenBank Accession Number JN052761) and *Methanosarcina* (GenBank Accession Number JN052757), all of which were derived from anaerobic digesters and were representative of methanogen populations common in anaerobic digestion processes (Chen et al., 2012). An additional 16S rRNA gene clone phylogenetically related to *Crenarchaeota* (GenBank Accession Number JN052741) was also included as a potentially significant population in anaerobic digestion (Collins et al., 2005; Zhang et al., 2011). These 16S rRNA gene clones were used as DNA templates for qPCR assays to test the specificity of the *Methanoculleus*-specific primer/probe set. Amplification efficiency (E) was determined with the C_T -Log[Template] plot derived from the quantification of tenfold dilution series of *Methanoculleus* 16S rRNA gene templates (5.53×10^2 – 5.53×10^9 copies) as previously described (Schefe et al., 2006).

Field testing of *Methanoculleus*-specific primer/probe set

The utility of the *Methanoculleus*-specific primer/probe was subsequently tested in six anaerobic digesters treating animal waste, of which triplicate anaerobic mono-digesters were fed with dairy waste only and the other triplicate anaerobic co-digesters were fed with a mixture of dairy waste and poultry waste (Chen et al., 2012). These bench scale digesters (3.6 L) were completely mixed at 35°C with a hydraulic retention time of 20 days. Biomass samples (10 ml) were taken from the effluents of the anaerobic digesters during stable process performance following 400 days of operation. Aliquots of samples were stored at 80°C until analysis. Total DNA from each sample was obtained using the sodium dodecyl sulphate (SDS)-based extraction method as previously described (Zhang et al., 2009). Crude DNA extracts were further purified with the Wizard DNA Clean-Up System (Promega, Madison, WI) following the manufacturer's instructions. The purified DNA was subsequently used for the quantification of *Methanoculleus* and total *Archaea* with qPCR.

TaqMan qPCR procedure

The *Methanoculleus*-specific primers and dual-labelled TaqMan probe, 50-end labelled with 6-carboxyfluorescein (FAM) and 30-end labelled with the Black Hole Quencher (BHQ), were obtained from Biosearch Technologies (Novato, CA). All qPCR assays were performed in 25 µL reaction tubes with 15 pmol of the primers, 5 pmol of the probe and Brilliant II QPCR Master Mix (Agilent, Santa Clara, CA). Thermal cycling consisted of a starting incubation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by up to 45 cycles of 95°C for 30 s and 60°C for 45 s. To determine the relative abundance of *Methanoculleus* populations in the archaeal community, total *Archaea* was quantified using the following TaqMan primer/probe set as previously described (Yu et al., 2005): forward primer Arc 787F-ATTAG ATACCCSBGTAGTCC; probe Arc 915P-AGGAATTGGCGGGGAGCAC and reverse primer Arc1059R-GCCATGCACCWCCTCT. Thermal cycling and fluorescence detection were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Fluorescence

response data were processed with the CFX Manager software provided by the manufacturer (Bio-Rad). For all qPCR assays, standards, controls and samples were run in triplicates. Gene copy numbers were calculated from standard curves based on the log transformation of known concentrations vs the threshold cycle (C_T).

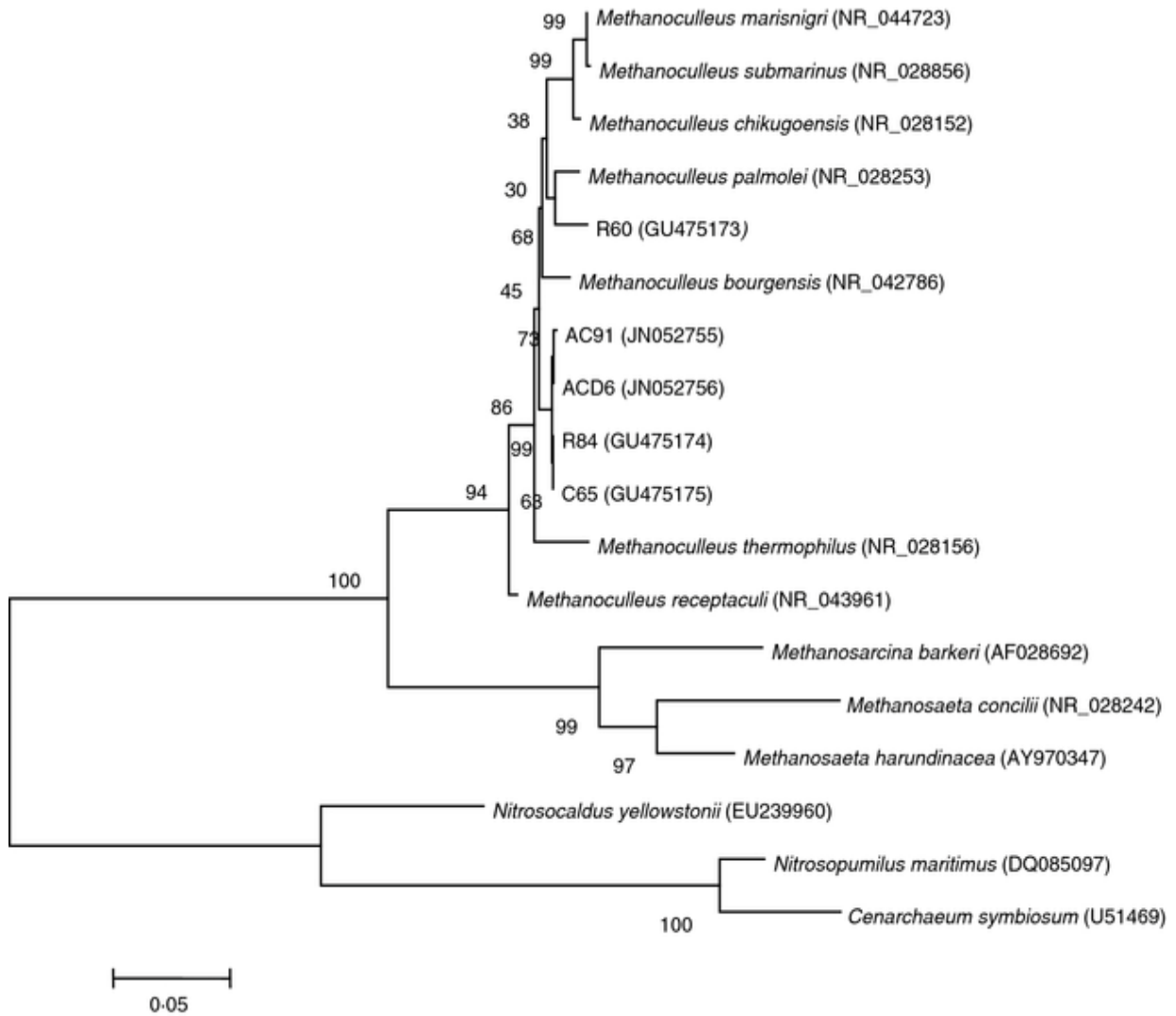


Fig. IV1. Neighbour joining phylogenetic tree showing the grouping of reference *Methanoculleus* cultures and uncultured *Methanoculleus* clones from anaerobic digesters. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position. GenBank accession numbers of the 16S rRNA gene sequences are indicated in the parentheses.

Results

Design of *Methanoculleus*-specific primer/probe set

The set of primers and probe targeting *Methanoculleus* was designed based on the alignment of all 16S rRNA gene sequences classified as *Methanoculleus* in the RDP database (Cole et al., 2009). The resulting *Methanoculleus* specific primer/probe set spans from bases 274 to 497 (*E. coli* numbering) of the 16S rRNA gene, with an amplicon size of 224 bp (Table IV1). The length of the amplicons from the primer/probe set designed in this study is considerably shorter than that of the primer/probe set reported in a previous study (Table IV1), which could potentially improve the amplification efficiency of qPCR assays (Nolan et al., 2006). In addition, the melting temperature (T_m) of the TaqMan probe designed in this study is 69.1°C, precisely within the recommended range of 68–70°C. The difference in T_m between the probe and primers designed in this study is also close to the optimal value of 10°C. All these features could enhance the performance of the *Methanoculleus*-specific qPCR assay developed in this study (Nolan et al., 2006).

Table IV1 Characteristics of primer/probe sets for *Methanoculleus*-specific TaqMan qRT-PCR assays^a

Primer/Probe	Sequence (5'-3')	Position <i>E.coli</i> No.	Amplicon Size (bp)	T_m (°C)	Reference
<i>Forward primer</i>					
Mc-274F	GGAGCAAGAGCCCGGAGT	274-291		60.8	
<i>Probe</i>					
Mc-361P	CGTGATAAGGGAACCTCGAGTGCCT	361-385	224	69.1	This study
<i>Reverse Primer</i>					
Mc-477R	CCAATAAAAGTGGCCACCACT	477-497		59.5	
<i>Forward Primer</i>					
AR934F	AGGAATTGGCGGGGAGCAC	934-953		64.6	
<i>Probe</i>					
MCU1023TAQ	GAATGATTGCCGGGCTGAAGACTC	1000-1023	307	66.9	(Shigema
<i>Reverse Primer</i>					tsu et al.,
MG1200b	CCGGATAATTCGGGGCATGCTG	1200-1221		65.8	2003)

^a Primers/probes designed in this study are shown in boldface type.

In silico evaluation of *Methanoculleus*-specific primer/probe set

A phylogenetic analysis of the *Methanoculleus* 16S rRNA sequences retrieved from RDP indicated that *Methanoculleus* represented a diverse group of methanogens (Fig. IV1). Therefore, sequence coverage and specificity were the primary considerations in our attempts to design qPCR assays with improved performance.

Sequence comparisons showed that the primer/probe set designed in this study had perfect matches to 68, 53 and 63% of the 1623 *Methanoculleus* sequences in RDP with the forward primer, reverse primer and probe, respectively (Table IV2). A similar analysis indicated that the primer/probe set designed by Shigematsu et al. (2003) had perfect matches with 71, 34 and 42% (Table IV2). Thus, except the forward primer Mc-274F, the primer/probe set designed in this study exhibited markedly better coverage than the counterparts designed by Shigematsu et al. (2003). The coverage of the forward primer Mc-274F was slightly lower than that of the forward primer AR934F used by Shigematsu et al. (2003). However, the greater coverage by AR934F was expected, because it was an archaeal domain-specific primer, matching to 62% of all archaeal sequences in RDP (Table IV2). Thus, it is evident that the primer/probe set designed in this study had considerably improved coverage of *Methanoculleus* populations.

More noteworthy is the significant improvement in specificity provided by the primer/probe set designed in this study, which had nonspecific matches to 7, 97 and 9 of the 2285 non-*Methanoculleus* sequences in the family *Methanomicrobiaceae*, with the forward primer, reverse primer and probe, respectively (Table IV2). In comparison, the primer/probe set designed by Shigematsu et al. (2003) had nonspecific matches to 1090, 299 and 57 of the 2285 non-*Methanoculleus* sequences in the family *Methanomicrobiaceae*. Extending the same analysis to *Archaea*, nonspecific matches by the primer/probe set designed by Shigematsu et al. (2003) amounted to 72 583, 947 and 132 of non-*Methanoculleus* archaeal sequences (Table IV2). Apparently, the specificity of the primer/probe set by Shigematsu et al. (2003) was primarily attributable to the specificity of the probe, but not the forward and reverse primers, which is not desirable (Franke-Whittle et al., 2009). In contrast, only 27, 240 and 23 of the 115 750 non-*Methanoculleus* archaeal sequences were matched by the forward primer, reverse primer and probe designed in this study, respectively (Table IV2). Thus, all three sequences of the primer/probe set designed in this study exhibited high specificity in the *Archaea* domain. Indeed, the specificity of the primer/probe set designed in this study is also demonstrated by the high-quality alignments between the primers/probe and 16S rRNA sequences of reference strains and uncultured clones of *Methanoculleus* in RDP (Fig. IV2).

Experimental evaluation of *Methanoculleus*-specific primer/probe set

Amplification efficiency is an important measure of the performance of qPCR assays and was evaluated experimentally using tenfold serial dilutions of *Methanoculleus* 16S rRNA gene templates. Amplification using the primer/probe set designed in this study was highly reproducible with strong correlation ($R^2 = 0.998$) between template concentration and fluorescence response detected as threshold cycle (Fig. IV3). The amplification efficiency was determined to be 92.73%, which is considered to be feasible for qPCR

quantification (Zhang & Fang, 2006). The specificity of the primer/probe set designed in this study was also evaluated with experimental qPCR testing. The DNA templates tested for the *Methanoculleus*-specific qPCR assay were four archaeal 16S rRNA genes from *Methanoculleus*, *Methanosarcina*, *Methanosaeta* and *Crenarchaeota*. Amplification was observed for DNA templates from *Methanoculleus* only and no amplification was detected for DNA templates from any other archaeal population after 45 PCR cycles (Fig. IV4), demonstrating the specificity of the primer/probe set to *Methanoculleus*.

16S rRNA gene	Accession No.	Forward Primer: MC-274F GGAGCAAGAGCCCGGAGT	Probe: MC-361P CGTGATAAGGGAACCTCGAGTGCCT	Reverse Primer: MC-471R CCAATAAAAGTGGCCACCACT
Reference strains of <i>Methanoculleus</i>				
<i>M. bourgensis</i>	NR_042786	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. chikugoensis</i>	NR_028152	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. marisnigri</i>	NR_044723	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. palmolei</i>	NR_028253	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. receptaculi</i>	NR_043961	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. submarinus</i>	NR_028856	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
<i>M. thermophilus</i>	NR_028156	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
Uncultured clones of <i>Methanoculleus</i> from anaerobic digestion				
AC91	JN052755	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
ACD6	JN052756	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
C65	GU475175	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
R60	GU475173	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT
R84	GU475174	GGAGCAAGAGCCCGGAGT	CGTGATAAGGGAACCTCGAGTGCCT	CCAATAAAAGTGGCCACCACT

Fig. IV2. Alignment of representative *Methanoculleus* 16S rRNA gene sequences (5'–3') to the qRT - PCR primer/probe set developed in this study. Mismatches are shown as shaded.

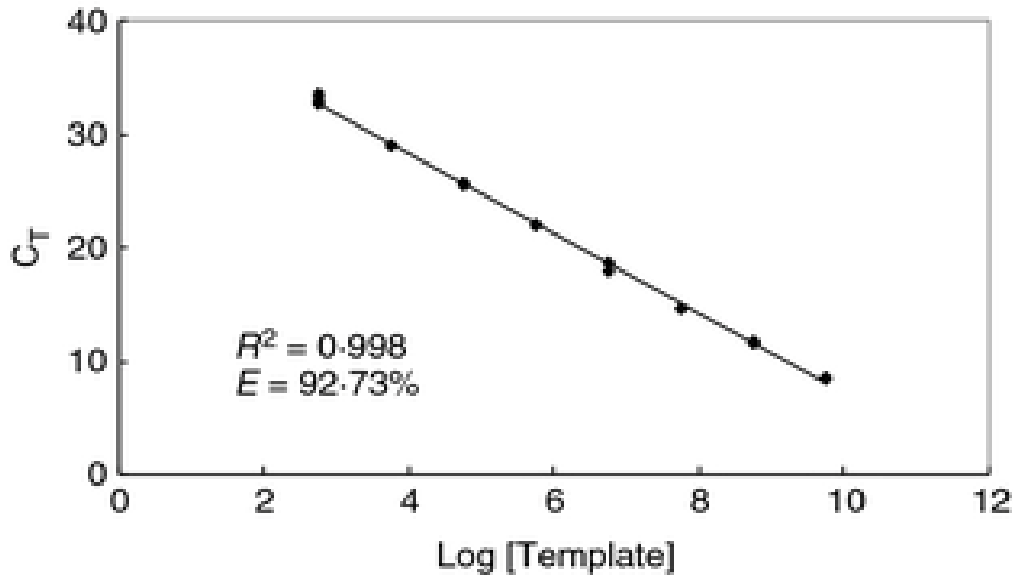


Fig. IV3. Amplification efficiency of *Methanoculleus* - specific qRT - PCR primer/probe set designed in this study as determined with the C_T - Log[Template] plot derived from the quantification of tenfold dilution series of *Methanoculleus* 16S rRNA gene templates (5.53×10^2 – 5.53×10^9 copies).

Field testing of *Methanoculleus*-specific qPCR assay

The utility of the primer/probe set designed in this study was tested in qPCR assays to quantify the population abundance of *Methanoculleus* in anaerobic digesters. Results from qPCR quantification of both *Methanoculleus* and total *Archaea* revealed that the relative abundance of *Methanoculleus* in the overall archaeal community averaged 5.4 and 3.8% in the mono-digesters and co-digesters, respectively (Table IV3). This observation is consistent with the relative abundance of 7 and 5% determined by clone library analysis (100 clones per library) of the same samples (Chen et al., 2012), supporting the effectiveness of the *Methanoculleus*-specific qPCR assay designed in this study for the rapid and accurate quantification of methanogen populations in anaerobic digestion.

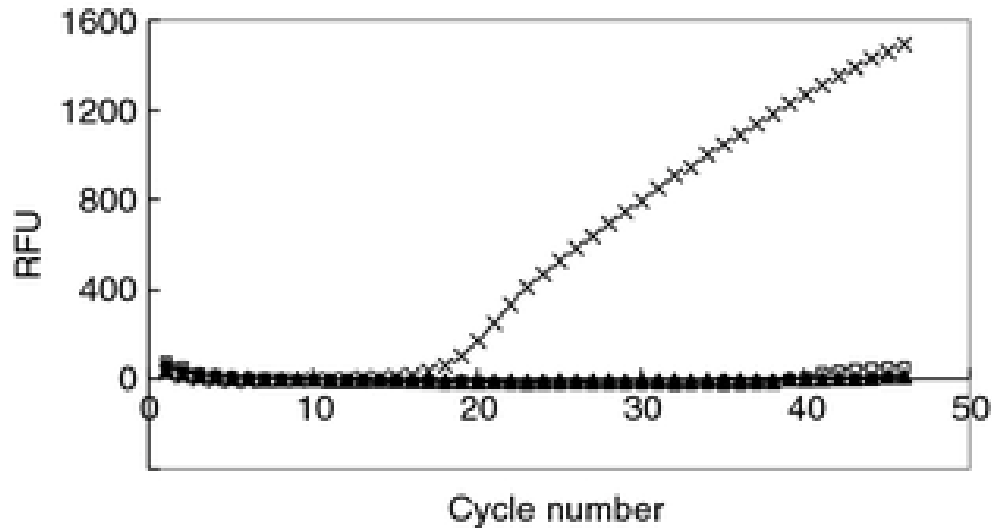


Fig. IV4. Specific detection of *Methanoculleus* as increases in fluorescence using 16S rRNA gene templates of four representative archaeal groups—*Methanoculleus* (Mc), *Methanosarcina* (Msc), *Methanosaeta* (Mst) and *Crenarchaeota* (Cren). All gene templates tested are environmental sequences cloned from anaerobic digesters in a previous study. RFU, Relative fluorescence unit. (—x—) Mc; (—□—) Msc; (—▲—) Mst and (—◆—) Cren.

Table IV2 Comparison of the coverage and specificity of *Methanoculleus*-specific TaqMan qRT-PCR assays.

Primer/probe characteristics			No. of matching sequences [†]		
Primer/probe name*	Sequence (50–30)	<i>E.coli</i> position	<i>Methanoculleus</i> (1623) [‡]	Non- <i>Methanoculleus</i> [§]	
				<i>Methanomicrobiaceae</i> (2285) [¶]	<i>Archaea</i> (115 750) ^{**}
Mc-274F (F)	GGAGCAAGAGCCCGGAGT	274–291	1111	7	27
Mc-477R (R)	CCAATAAAAGTGGCCACCACT	477–497	859	97	240
Mc-361P (P)	CGTGATAAGGGAACCTCGAGTGCCT	361–385	1022	9	23
AR934F (F)	AGGAATTGGCGGGGGAGCAC	934–953	1163	1090	72583
MG1200 (R)	CCGGATAATTCCGGGGCATGCTG	1200–1221	556	299	947
MCU1023TAQ (P)	GAATGATTGCCGGGCTGAAGACTC	1023–1046	677	57	132

*Designations in the parentheses: F, forward primer; R, reverse primer; and P, probe. The primers/probe in italics were designed in this study while the remaining primers/probe were from Shigematsu et al. (2003).

[†]Number of 16S rRNA gene sequences belonging to the target taxon in the Ribosomal Database Project (RDP) database with perfect match to the primer/probe.

[‡]Number in the parenthesis indicates the total number of 16S rRNA gene sequences belonging to the genus *Methanoculleus* in RDP.

[§]Number of non-*Methanoculleus* 16S rRNA gene sequences with perfect match to the *Methanoculleus*-specific primer/probe.

[¶]Number in the parenthesis indicates the number of 16S rRNA gene sequences in the family *Methanomicrobiaceae* which did not belong to the genus *Methanoculleus*.

^{**}Number in the parenthesis indicates the number of 16S rRNA gene sequences in the domain Archaea which did not belong to the genus *Methanoculleus*.

Table IV3 Quantification of *Methanoculleus* abundance with qRT-PCR in anaerobic digesters treating animal waste.

Sample source*	16S rRNA gene targets	No. of copies ml ^{-1†}	Relative abundance (% of total <i>Archaea</i>)
Mono-digesters	<i>Methanoculleus</i>	$(8.3 \pm 1.6) \times 10^6$	5.4 ± 1.0
	Total <i>Archaea</i>	$(1.6 \pm 0.2) \times 10^8$	-
Co-digesters	<i>Methanoculleus</i>	$(4.3 \pm 1.8) \times 10^6$	3.8 ± 0.7
	Total <i>Archaea</i>	$(1.2 \pm 0.4) \times 10^8$	-

*Samples were taken from the treated effluent of anaerobic digesters. Mono-digesters were fed with dairy waste only; co-digesters were fed with a mixture of dairy waste and poultry waste.

[†]The values are the means of triplicate samples $\pm$ standard deviation.

Discussion

Methanoculleus populations are frequently detected in anaerobic digestion processes and are considered as the key members of the microbial community responsible for hydrogenotrophic methanogenesis (Narihiro & Sekiguchi, 2007). However, the abundance of *Methanoculleus* has been reported to vary significantly in anaerobic digestion processes, ranging from being the dominant methanogen population (Cardinali-Rezende et al., 2012; Wirth et al., 2012) to near absence (Riviere et al., 2009; Sundberg et al., 2013). Thus, rapid and accurate quantitative tools, that is, qPCR, are needed to monitor the dynamics of *Methanoculleus* population abundance and its linkage to process performance to gain insight into the functions of these methanogens in anaerobic digestion processes.

A major challenge is that the genus *Methanoculleus* represents a relatively diverse group of hydrogenotrophic methanogens (Fig. IV1). Thus, the specificity of qPCR assays targeting *Methanoculleus* is of particular importance, making qPCR assays based on the TaqMan chemistry more attractive because of greater specificity (Wang & Brown, 1999). Only one TaqMan qPCR assay has been reported in the literature (Shigematsu et al., 2003), which has been shown to lack specificity (Table IV2). One of the concerns of this primer/probe set is the use of an *Archaea* domain-specific primer as the forward primer (Table IV2). Thus, the specificity of the qPCR designed by Shigematsu et al. (2003) relied completely on the probe and the reverse primer only, which is not desirable for the design of highly specific qPCR (Nolan et al., 2006).

In contrast, the *Methanoculleus*-specific qPCR assay designed in this study provides greater specificity from all three sequences of the primer/probe set without sacrificing coverage (Table IV2). The utility of the qPCR assay designed in this study is further supported by experimental and field validation of its amplification efficiency (Fig. IV3) and quantification accuracy (Table IV3). Thus, the *Methanoculleus*-specific qPCR assay designed in this study provides a rapid and effective tool much needed for the specific quantification of *Methanoculleus* populations in anaerobic digestion processes.

References

- Abbasi, T., Tauseef, S.M., Abbasi, S.A. 2012. Anaerobic digestion for global warming control and energy generation-An overview. *Renewable & Sustainable Energy Reviews*, **16**(5), 3228-3242.
- Appels, L., Lauwers, J., Degreve, J., Helsen, L., Lievens, B., Willems, K., Van Impe, J., Dewil, R. 2011. Anaerobic digestion in global bio-energy production: Potential and research challenges. *Renewable & Sustainable Energy Reviews*, **15**(9), 4295-4301.
- Barret, M., Gagnon, N., Morissette, B., Topp, E., Kalmokoff, M., Brooks, S.P.J., Matias, F., Masse, D.I., Masse, L., Talbot, G. 2012. Methanoculleus spp. as a biomarker of methanogenic activity in swine manure storage tanks. *FEMS Microbiol Ecol*, **80**(2), 427-440.
- Cardinali-Rezende, J., Colturato, L., Colturato, T.D.B., Chartone-Souza, E., Nascimento, A.M.A., Sanz, J.L. 2012. Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions. *Bioresour Technol*, **119**, 373-383.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res*, **37**, D141-D145.
- Collins, G., Mahony, T., McHugh, S., Gieseke, A., de Beer, D., O'Flaherty, V. 2005. Distribution, dynamics and in situ ecophysiology of Crenarchaeota in anaerobic wastewater treatment granular biofilms. *Water Science and Technology*, **52**(7), 233-239.
- Franke-Whittle, I.H., Goberna, M., Insam, H. 2009. Design and testing of real-time PCR primers for the quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of uncultured methanogens. *Canadian Journal of Microbiology*, **55**(5), 611-616.
- Garcia, J.L., Patel, B.K.C., Ollivier, B. 2000. Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. *Anaerobe*, **6**(4), 205-226.
- Gupta, P., Singh, R.S., Sachan, A., Vidyarthi, A.S., Gupta, A. 2012. A re-appraisal on intensification of biogas production. *Renewable & Sustainable Energy Reviews*, **16**(7), 4908-4916.
- Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B. 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Lett*, **88**(3-4), 181-197.
- Narihiro, T., Sekiguchi, Y. 2007. Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. *Curr Opin Biotechnol*, **18**(3), 273-278.
- Nasir, I.M., Ghazi, T.I.M., Omar, R. 2012. Anaerobic digestion technology in livestock manure treatment for biogas production: A review. *Eng Life Sci*, **12**(3), 258-269.
- Nettmann, E., Bergmann, I., Pramschufer, S., Mundt, K., Plogsties, V., Herrmann, C., Klocke, M. 2010. Polyphasic Analyses of Methanogenic Archaeal Communities

- in Agricultural Biogas Plants. *Appl Environ Microbiol*, **76**(8), 2540-2548.
- Nolan, T., Hands, R.E., Bustin, S.A. 2006. Quantification of mRNA using real-time RT-PCR. *Nature Protocols*, **1**(3), 1559-1582.
- Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *Isme Journal*, **3**(6), 700-714.
- Schefe, J., Lehmann, K., Buschmann, I., Unger, T., Funke-Kaiser, H. 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene expression’s C T difference” formula. *Journal of Molecular Medicine*, **84**(11), 901-910.
- Shigematsu, T., Tang, Y., Kawaguchi, H., Ninomiya, K., Kijima, J., Kobayashi, T., Morimura, S., Kida, K. 2003. Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. *J Biosci Bioeng*, **96**(6), 547-558.
- Smith, C.J., Osborn, A.M. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol*, **67**(1), 6-20.
- Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sorensen, S.J., Karlsson, A. 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol*, **85**(3), 612-626.
- Tang, Y.Q., Shigematsu, T., Morimura, S., Kida, K. 2005. Microbial community analysis of mesophilic anaerobic protein degradation process using bovine serum albumin (BSA)-fed continuous cultivation. *J Biosci Bioeng*, **99**(2), 150-164.
- Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res*, **35**, W71-W74.
- Wang, T., Brown, M.J. 1999. mRNA quantification by real time TaqMan polymerase chain reaction: Validation and comparison with RNase protection. *Anal Biochem*, **269**(1), 198-201.
- Wirth, R., Kovacs, E., Maroti, G., Bagi, Z., Rakhely, G., Kovacs, K.L. 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. *Biotechnology for Biofuels*, **5**.
- Yu, Y., Lee, C., Kim, J., Hwang, S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*, **89**(6), 670-679.
- Zhang, T., Fang, H.H.P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol*, **70**(3), 281-289.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhang, Y., Zhang, X.L., Zhang, H.W., He, Q., Zhou, Q.X., Su, Z.C., Zhang, C.G. 2009. Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis. *Bull Environ Contam Toxicol*, **82**(3), 367-372.

Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.

Part II: Enrichment of *Methanocalculus* and Development of *Methanocalculus*-Specific qPCR Assay

Abstract

Members of the genus *Methanocalculus* characterized as hydrogenotrophic methanogens present in diverse natural and engineered environments. *Methanocalculus* populations were enriched from anaerobic digesters treating dairy waste using formate as the substrate. *Methanocalculus* sequences retrieved from the enrichment cultures were subsequently used to develop a *Methanocalculus*-specific TaqMan qPCR assay to determine the abundance of *Methanocalculus* populations in the environment, representing the first quantitative tool specifically targeting *Methanocalculus*. The *Methanocalculus*-specific primer/probe set was shown to have high coverage, with perfect match to >80% of all *Methanocalculus* 16S rRNA gene sequences in the Ribosomal Database Project (RDP). High specificity of the qPCR assay was also validated by both *in silico* and experimental analyses. Amplification efficiency of the qPCR assay was determined to be 91.9%, which is satisfactory for quantitative applications. Results from the *Methanocalculus*-specific qPCR analysis of formate-enriched methanogenic cultures were consistent with those from clone library analysis of the same cultures, validating the accuracy of the qPCR assay. Subsequent field application of the qPCR assay found low relative abundance of *Methanocalculus* in anaerobic digesters treating dairy waste, accounting for 0.01% of the archaeal populations. The qPCR results were consistent with the lack of detection of *Methanocalculus* in previous studies of the same anaerobic digesters with clone library analyses, which are less sensitive than qPCR. Thus, the *Methanocalculus*-specific qPCR assay developed in this study is a highly sensitive tool for the rapid and efficient quantification of *Methanocalculus* populations in methanogenic environments and understanding of the ecological functions of these methanogens.

Keywords: *Anaerobic digestion; Formate; Methanocalculus; Methanogen; qPCR*

Introduction

Methanogenesis is of great significance in global carbon cycling in diverse ecosystems (Conrad, 2009). Methanogenesis has also been exploited in anaerobic digestion processes for the treatment of various organic waste streams, including animal manure, food waste, and sludge from wastewater treatment (van Lier et al., 2001). Methanogenic anaerobic digestion of these waste materials has the benefit of producing biomethane as a renewable energy source and reducing greenhouse gas emission, leading to increased effort to enhancing the efficiency and stability of anaerobic digestion processes.

Methanogens as a group of archaeal populations are key members of the microbial communities exclusively involved in the terminal steps of the anaerobic transformation of organic materials into methane (Stams & Plugge, 2009). Given the

importance of methanogens in both natural environments and engineered systems, monitoring the abundance and dynamics of methanogen populations is of great importance to understanding how these microorganisms respond to changing environmental conditions and subsequently affect the performance of the anaerobic food web. While methanogens are characterized by the common trait of methanogenic metabolism, it is well established that methanogen populations differ in substrate spectrum, substrate affinity, and growth kinetics, among others (Liu & Whitman, 2008). Therefore, a number of quantitative tools, such as 16S rRNA gene-based quantitative PCR assays (qPCR), have been developed for the monitoring of specific methanogen populations at fine phylogenetic resolutions (Yu et al., 2005).

Methanocalculus represents a group of hydrogenotrophic mesophilic methanogen populations found in diverse natural habitats in marine, estuarine, terrestrial environments as well as solid waste disposal sites and anaerobic digestion processes (Calderón et al., 2011; Lai et al., 2002; Lai et al., 2004; Mori et al., 2000; Ollivier et al., 1998; Shin et al., 2010; Zhilina et al., 2013), suggesting its potential significance in anaerobic environments. Understanding of the ecological functions of these methanogens, however, is incomplete, hindered by the lack of quantitative tools specifically targeting *Methanocalculus*. Therefore, the objective of this study is to develop a *Methanocalculus*-specific qPCR assay with high sensitivity for the monitoring of these archaeal populations in the environment. Results from this study show that the *Methanocalculus*-specific qPCR assay developed with the sequences of *Methanocalculus* enriched by formate had excellent specificity, coverage, and amplification efficiency, providing a much needed tool for the quantitative monitoring of *Methanocalculus* populations in methanogenic environments.

Materials and methods

Anaerobic digester set-up

Triplicate anaerobic digesters were established as model systems to study methanogen communities and as sources to enrich for specific methanogen populations. These mesophilic continuous bench-scale anaerobic digesters used dairy waste as the feedstock at an organic loading rate of 1.0 gVS/L/day as described previously (Zhang et al., 2011). The temperature of the anaerobic digestion process was maintained at 35 °C and the hydraulic retention time was controlled at 20 days.

Formate enrichment for methanogens

To enrich for hydrogenotrophic methanogen populations including *Methanocalculus*, an anaerobic basal medium was prepared according to the following recipe (per liter) as previously described (He & Sanford, 2002): NaCl, 1.0 g; MgCl₂·6 H₂O, 0.5 g; KH₂PO₄, 0.2 g; NH₄Cl, 0.3 g; KCl, 0.3 g; CaCl₂·2H₂O, 0.015 g; trace element solution, 1.0 mL; Se/Wo solution, 1.0 mL; and resazurin, 1.0 mg. L-cysteine (0.031 g/L) and Na₂S·9 H₂O (0.048 g/L) were added as reductants into the basal medium after it had been boiled and cooled to room temperature under an oxygen-free N₂ atmosphere. NaHCO₃ (2.52 g/L)

was added to the medium as the buffer. The medium was subsequently dispensed into 160-mL serum bottles flushed with pure N₂ and sealed with butyl rubber stoppers and aluminum caps. A sterile vitamin solution (1%) was added after autoclaving (Wolin et al., 1963).

Initial enrichments for methanogens were seeded with a 10% (vol/vol) inoculum of digestate from the continuous bench-scale anaerobic digesters fed with dairy waste along with 50 mM formate as the sole substrate, which was replenished when depleted. At the completion of 10 feedings of formate, secondary enrichments were established by transferring a 10% (vol/vol) inoculum from the initial enrichment cultures into fresh medium followed by repeated feedings of 50 mM formate.

All enrichment cultures were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 °C. Biogas production was measured using a previously described water displacement method (Zhu et al., 2011). Methane content in biogas was analyzed by a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen®-1000; Sigma-Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 mL/min. GC analysis of methane used the following temperature scheme: oven 125 °C, injection port 150 °C and TCD detector 170 °C.

Clone library analysis of formate enrichment cultures

Clone library analysis was performed to examine the composition of methanogen communities enriched by formate and to obtain 16S rRNA gene templates for the design of qPCR assays specific to enriched methanogen populations. Biomass from secondary enrichments was pelleted by centrifugation and stored at –80 °C. Whole community DNA was extracted and purified as previously described (Zhang et al., 2009). DNA extracts from triplicate enrichments were pooled for PCR amplification of the 16S rRNA genes using the following *Archaea*-specific primers: Arch21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch958R (5'-YCCGGCGTTGAMTCCAATT-3') following previously described PCR protocols (DeLong, 1992). Amplicons of 16S rRNA gene sequences were subsequently purified and cloned into plasmid vectors following procedures described previously (Chen et al., 2012). Twenty cloned plasmid inserts were randomly selected for sequencing with the ABI Prism BigDye chemistry (Applied Biosystems, Foster City, CA, USA) using M13 forward and reverse primers. The obtained sequences were checked for chimeric artifacts using the Chimera Check program in the Ribosomal Database Project II (Cole et al., 2003). High quality 16S rRNA gene sequences were deposited at GenBank under the following accession numbers: KJ435044—KJ435059.

Subsequently, phylogenetic analysis was conducted on the 16S rRNA gene sequences retrieved from the formate enrichment cultures by searching the NCBI GenBank database for closely related sequences. The phylogenetic positions of these 16S rRNA gene sequences were assessed by phylogenetic trees constructed with the neighbor-joining algorithm (1000 bootstrap re-samplings) using MEGA 4.0 (Tamura et al., 2007).

Primer/probe design for *Methanocalculus*-specific qPCR assay

Since 16S rRNA sequences belonging to the genus *Methanocalculus* dominated the methanogen community in the formate enrichments (see Results), *Methanocalculus*-specific qPCR assay for the quantification of this group of methanogens was developed based on all of the 376 16S rRNA gene sequences of the genus *Methanocalculus* in RDP Release 11 as well as *Methanocalculus* sequences retrieved from the formate enrichment cultures in this study using primer/probe design procedures previously described for TaqMan chemistry (Chen et al., 2014). The primer/probe sequences were determined following guidelines provided by Applied Biosystems (Foster City, California, USA) on parameters including amplicon size, nucleotide position, melting temperature (T_m), GC content, self-annealing, complementarity, and hairpin formation, according to the Oligo Calculator program (Kibbe, 2007). *In silico* evaluation of the specificity and coverage of the primer/probe set was performed using the ProbeMatch program of RDP II (Cole et al., 2009).

Experimental evaluation and field application of *Methanocalculus*-specific qPCR assay

Primer/probe specificity was further assessed using partial 16S rRNA genes of representative methanogen populations cloned from the continuous bench-scale anaerobic digesters in a previous study (Chen et al., 2012), including *Methanoculleus* (GenBank Accession No. JN052755), *Methanosaeta* (GenBank Accession No. JN052761), and *Methanosarcina* (GenBank Accession No. JN052757), in addition to *Methanocalculus* (GenBank Accession No. KJ435045) derived from the formate enrichment cultures in this study. Moreover, one non-methanogen archaeal 16S rRNA gene clone (Genbank accession JN052741) phylogenetically related to *Crenarchaeota* was also included in the assessment because of the potential significance of *Crenarchaeota* population in anaerobic digestion (Collins et al., 2005; Zhang et al., 2011). These 16S rRNA gene clones were used as DNA templates for qPCR assays to test the specificity of the *Methanocalculus*-specific primer/probe set designed in this study. Amplification efficiency (E) of the qPCR assay was determined with the C_T -Log [Template] plot derived from the quantification of 10-fold dilution series of *Methanocalculus* 16S rRNA gene templates (18 to 1.8×10^9 copies/reaction) as previously described (Scheffe et al., 2006).

To further validate the accuracy of the *Methanocalculus*-specific qPCR assay, quantification of *Methanocalculus* populations was conducted for the secondary formate enrichment cultures. Results from qPCR were compared with those from clone library analysis performed on the same samples. Moreover, the *Methanocalculus*-specific qPCR assay was applied to quantify *Methanocalculus* populations in the archaeal communities in the continuous anaerobic digesters treating dairy waste.

TaqMan qPCR protocol

The *Methanocalculus*-specific primers and dual-labeled TaqMan probe, 5'-end labeled with 6-carboxyfluorescein (FAM) and 3'-end labeled with the Black Hole Quencher

(BHQ), were obtained from Biosearch Technologies (Novato, California, USA). All qPCR assays were performed in 25µL reaction volume with 15 pmol of the primers, 5 pmol of the probe, and Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA). Thermal cycling consisted of a starting incubation at 50 °C for 2 min and an initial denaturation at 95 °C for 10 min, followed by up to 45 cycles at 95 °C for 30 s and 60 °C for 45 s. To determine the relative abundance of *Methanocalculus* populations in the archaeal community, total *Archaea* was quantified with the *Archaea* domain-specific TaqMan primer/probe set as previously described (Yu et al., 2005): forward primer Arc787F-ATTAGATACCCSBGTAGTCC, probe Arc915P-AGGAATTGGCGGGGAGCAC, and reverse primer Arc1059R-GCCATGCACCWCCTCT. Thermal cycling and fluorescence detection were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). For all qPCR assays, standards, controls, and samples were run in triplicates. Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T).

Results

Enrichment of Methanogen populations with formate

Formate was used as the sole substrate to enrich methanogen populations from anaerobic digesters treating dairy waste. The consumption of formate was accompanied with the production of methane gas in the enrichment cultures (Fig. IV5), which could be attributed solely to formate as no methane production was observed in the controls without formate amendment. Methane production averaged 31 mL from each feeding of 50 mM formate, corresponding to a formate/methane molar ratio of 3.9, which is consistent with the stoichiometry of formate-dependent methanogenesis according to the following reaction:



These results suggest that the microbial populations enriched by formate were likely formate-utilizing hydrogenotrophic methanogens.

Methanocalculus populations enriched by formate

To identify the dominant methanogen populations utilizing formate in the enrichment cultures, clone library analysis was conducted. Sequences closely related to *Methanocalculus pumilus* (>97% similarity) dominated the archaeal community, representing 80% of the clones from the formate enrichments (Fig. IV6). Cultures of the genus *Methanocalculus* are known as hydrogenotrophic methanogens capable of using formate for methanogenesis (Zhilina et al., 2013); however, *Methanocalculus* sequences had not been identified in previous microbial community studies of the same anaerobic digesters serving as the source of the formate enrichments in this study (Chen et al., 2012; Zhang et al., 2011). The *Methanocalculus*-like sequences identified by clone library

analysis formed a phylogenetic group distinct from *Methanocorpusculum* and *Methanoculleus* (Fig. IV6). Different from *Methanocalculus*, *Methanocorpusculum* and *Methanoculleus* were detected in previous clone library analyses of the anaerobic digesters as the primary hydrogentrophic methanogen populations (Chen et al., 2012; Zhang et al., 2011).

Since the anaerobic digesters served as the source of inocula for the formate enrichments, the lack of detection of *Methanocalculus* in the anaerobic digesters was inconsistent with the abundance of *Methanocalculus* identified in the formate enrichment, likely the result of the lack of sensitive and accurate quantitative tools specifically targeting *Methanocalculus*. Therefore, there was an important need to develop rapid *Methanocalculus*-specific molecular assays such as qPCR.

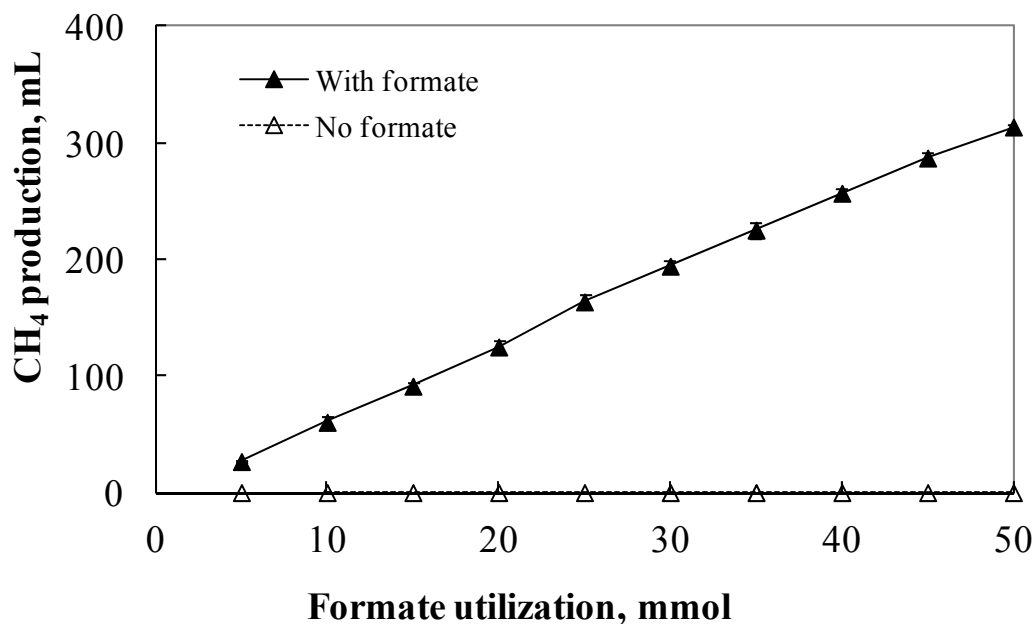


Fig. IV5. Methane production and substrate utilization in secondary enrichment cultures. Controls lacked formate as the substrate. Data points were means of triplicate cultures with the error bars showing standard deviations.

Design of *Methanocalculus*-specific primer/probe set

Phylogenetic analysis of the *Methanocalculus* 16S rRNA sequences retrieved from formate enrichment cultures indicated that *Methanocalculus* represented a relatively diverse group of methanogens (Fig. IV6). Therefore, sequence coverage and specificity were the primary considerations in our attempt to design qPCR assays for the quantification of *Methanocalculus* populations. With increased specificity offered by the probe in addition to the primers, TaqMan chemistry was selected over SYBR Green chemistry for the development of the qPCR assay (Wang & Brown, 1999).

The set of primers and probe targeting *Methanocalculus* was designed based on the alignment of all 16S rRNA gene sequences classified as *Methanocalculus* in the RDP database (Cole et al., 2009) together with *Methanocalculus* sequences obtained from this study. The resulting *Methanocalculus*-specific primer/probe set covers bases 589 to 786 (*E. coli* position) of the 16S rRNA gene, with an amplicon size of 198 bp (Table IV4). The melting temperature (T_m) of the TaqMan probe designed in this study is 69.1 °C and the difference in T_m between the probe and primers designed in this study is 8.6-10.7 °C, precisely within the recommended range for T_m (68-70 °C) and for the difference in T_m between the probe and primers (10 °C) for optimal performance (Nolan et al., 2006). Additionally, no hairpin, self-annealing and complementarity were detected for the primer/probe set. These characteristics suggest that amplification with the *Methanocalculus*-specific primer/probe set would be highly efficient.

***In Silico* evaluation of *Methanocalculus*-specific primer/probe set**

The coverage and specificity of the *Methanocalculus*-specific primer/probe set was first evaluated with sequence analysis, which showed that the primer/probe set designed in this study had perfect matches to 83%, 80%, and 80% of the 376 *Methanocalculus* sequences in RDP with the forward primer, reverse primer, and probe, respectively (Table IV5). Evidently, the primer/probe set exhibited high coverage of *Methanocalculus* populations. More notable is the excellent specificity provided by the primer/probe set designed in this study, which had non-specific matches to 3, 1, and 11 of the 8808 non-*Methanocalculus* sequences in the order *Methanomicrobiales*, with the forward primer, reverse primer, and probe, respectively. Expanding the analysis to the entire *Archaea* domain, only 2 more non-specific matches were found in non-*Methanocalculus* archaeal sequences to the reverse primer (Table IV5). Additionally, no matches to the primer/probe set were found for any sequence belonging to *Bacteria*. Therefore, the primer/probe set designed in this study exhibited both high coverage and high specificity to the *Methanocalculus* populations.

Indeed, the specificity of the primer/probe set designed in this study is also demonstrated by the high quality alignments between the primers/probe and 16S rRNA gene sequences of reference strains from RDP and uncultured clones of *Methanocalculus* retrieved in this study (Fig. IV7).

Table IV4 Characteristics of primer/probe sets for *Methanocalculus*-specific TaqMan qPCR assays^a.

Primer/Probe	Sequence (5'-3')	<i>E. coli</i> Position	Amplicon Size (bp)	T _m (°C)	Hairpin ^b	Self-Annealing/ Complementarity	GC %
<i>Forward Primer</i>							
Mcal-589F	ACCGGGAGAGGTAAGACGTA	589-608		60.5	No	No	55
<i>Probe</i>							
Mcal-628P	ATCTTGTAATCCCCGAGGGACGACC	628-652	198	69.1	No	No	56
<i>Reverse Primer</i>							
Mcal-767R	ACACACCTAACGCACATCGT	767-786		58.4	No	No	50

^aCharacteristics of primer/probe were calculated by Oligonucleotide Properties Calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>).

^bMinimum base pairs required for a hairpin detection: 4.

Table IV5 Coverage and specificity of *Methanocalculus*-specific TaqMan qPCR primer/probe set.

Primer/Probe Characteristics				No. of Matching Sequences ^b		
Primer/Probe Name ^a	Sequence (5'-3')	<i>E. coli</i> position	<i>Methanocalculus</i> (376) ^c	Non- <i>Methanocalculus</i> ^d		
				<i>Methanomicrobiales</i> (8,808) ^e	<i>Archaea</i> (129,854) ^f	<i>Bacteria</i> (2,873,272) ^g
Mcal-589F (F)	ACCGGGAGAGGTAAGACGTA	589-608	311	3	3	0
Mcal-628P (P)	ATCTTGTAATCCCCGAGGGACGACC	628-652	301	1	1	0
Mcal-767R (R)	ACACACCTAACGCACATCGT	767-786	302	11	13	0

^aDesignations in the parentheses: F—forward primer; R—Reverse primer; and P—Probe.

^bNumber of 16S rRNA sequences belonging to the target taxon in the Ribosomal Database Project (RDP) database with perfect match to the primer/probe.

^cNumber in the parentheses indicates the total number of 16S rRNA gene sequences belonging to the genus *Methanocalculus* in RDP.

^dNumber of non-*Methanoculleus* 16S rRNA gene sequences with perfect match to the *Methanocalculus*-specific primer/probe.

^eNumber in the parentheses indicates the number of 16S rRNA gene sequences in the Order *Methanomicrobiales* which did not belong to the genus *Methanocalculus*.

^fNumber in the parentheses indicates the number of 16S rRNA gene sequences in the domain *Archaea* which did not belong to the genus *Methanocalculus*.

^gNumber in the parentheses indicates the number of 16S rRNA gene sequences in the domain *Bacteria*.

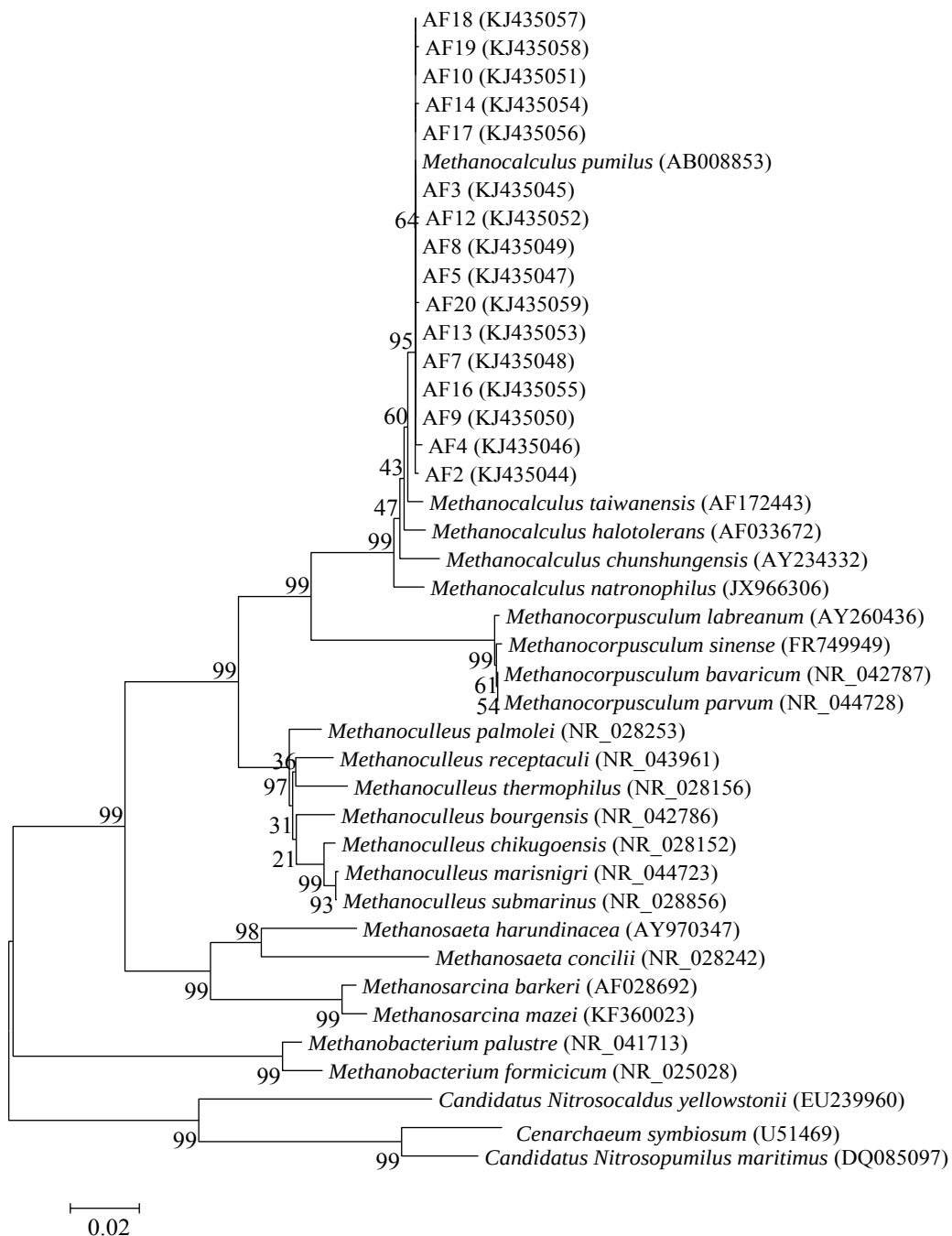


Fig. IV6. Neighbor-joining tree showing the phylogenetic grouping of partial *Methanocalculus* 16S rRNA gene sequences cloned from formate enrichment cultures in relation to sequences of reference *Methanocalculus* cultures and other representative archaeal cultures. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position. Sequence designation: AF—clones from formate enrichments. GenBank accession numbers of the 16S rRNA gene sequences are indicated in the parentheses.

16S rRNA gene	Accession No.	Forward Primer: Mcal-589F ACCGGGAGAGGTAAGACGTA		Probe: Mcal-628P ATCTTGTAATCCCCGAGGGACGACC		Reverse Primer: Mcal-767R ACACACCTAACGCACATCGT
Reference strains of <i>Methanocalculus</i>						
<i>M. chunshungensis</i>	AY234332	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
<i>M. halotolerans</i>	AF033672	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
<i>M. pumilus</i>	AB008853	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
<i>M. taiwanensis</i>	AF172443	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
<i>M. natronophilus</i>	JX966306	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
Uncultured clones of <i>Methanocalculus</i> enriched by formate						
AF2	KJ435044	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF3	KJ435045	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF4	KJ435046	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF5	KJ435047	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF7	KJ435048	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF8	KJ435049	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF9	KJ435050	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF10	KJ435051	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF12	KJ435052	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF13	KJ435053	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF14	KJ435054	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF16	KJ435055	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF17	KJ435056	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF18	KJ435057	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF19	KJ435058	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT
AF20	KJ435059	ACCGGGAGAGGTAAGACGTA		ATCTTGTAATCCCCGAGGGACGACC		ACACACCTAACGCACATCGT

Fig. IV7. Alignment of representative *Methanocalculus* 16S rRNA gene sequences (5'-3') to the *Methanocalculus*-specific primer/probe set developed in this study, with mismatches shown as shaded.

Experimental evaluation of *Methanocalculus*-specific qPCR assay

The specificity of the *Methanocalculus*-specific qPCR assay was further evaluated experimentally using DNA templates of 16S rRNA genes with varying phylogenetic distances to *Methanocalculus*. The DNA templates tested include five archaeal 16S rRNA genes from *Methanocalculus*, *Methanoculleus*, *Methanosarcina*, *Methanosaeta*, and *Crenarchaeota*. Amplification of DNA templates with the *Methanocalculus*-specific primer/probe set was observed for *Methanocalculus* only and no amplification was detected for any other DNA templates after 45 cycles of PCR amplification (Fig. IV8), again demonstrating the high specificity of the primer/probe set to *Methanocalculus*.

Amplification efficiency (E), an important measurement of the performance of qPCR assays, was evaluated experimentally using 10-fold serial dilutions of *Methanocalculus* 16S rRNA gene templates ranging from 1.8×10^1 to 1.8×10^9 copies. The *Methanocalculus*-specific qPCR assay resulted in highly reproducible amplification with strong correlation ($R^2=0.997$) between template concentration and fluorescence response detected as threshold cycle, C_T (Fig. IV9). The amplification efficiency was determined to be 91.9%, which is satisfactory for consistent qPCR quantification (Zhang & Fang, 2006). Moreover, the qPCR assay was able to detect template at 1.8×10^1 copies/mL, indicating the high sensitivity of the qPCR assay for *Methanocalculus* quantification.

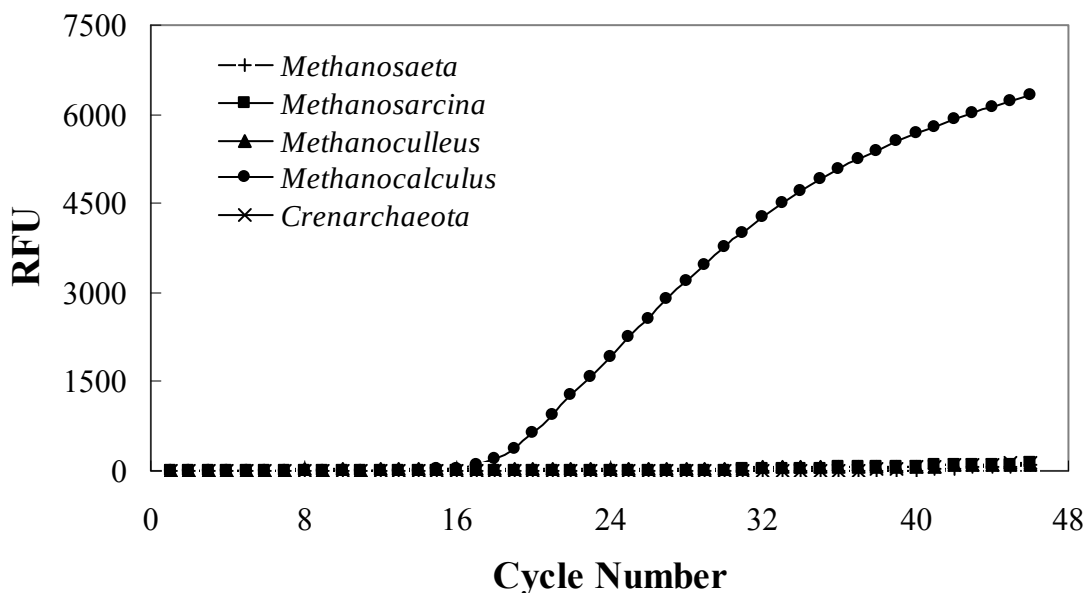


Fig. IV8. Specific amplification of *Methanocalculus* 16S rRNA gene templates of 5 representative archaeal groups—*Methanosaeta*, *Methanosarcina*, *Methanoculleus*, *Methanocalculus*, and *Crenarchaeota*. RFU: relative fluorescence unit.

Field application of *Methanocalculus*-specific qPCR assay

To validate the utility of the *Methanocalculus*-specific qPCR assay, the abundance of *Methanocalculus* populations in the formate enrichment cultures was determined with qPCR and the results were compared with those from clone library analysis. *Methanocalculus* was found to be the dominant archaeal population in the formate enrichments, representing 84% of the entire archaeal community (Table IV6), which is consistent with the 80% relative abundance of *Methanocalculus* estimated by 16S rRNA gene clone library analysis. These results validated the accuracy of the *Methanocalculus*-specific qPCR assay.

The *Methanocalculus*-specific qPCR assay was subsequently applied to quantify the *Methanocalculus* populations in continuous anaerobic digesters treating dairy waste. Results from qPCR quantification of both *Methanocalculus* and total *Archaea* revealed the very low relative abundance of *Methanocalculus*, averaging only 0.01% of the archaeal community in the anaerobic digesters (Table IV6). These results, however, are consistent with the lack of detection of *Methanocalculus* populations in previous studies of the same anaerobic digesters using clone library analysis (Chen et al., 2012; Zhang et al., 2011), which is less sensitive than qPCR. These results suggest that *Methanocalculus* might be present in anaerobic digestion process as minor members of the methanogen communities involved in formate-dependent methanogenesis.

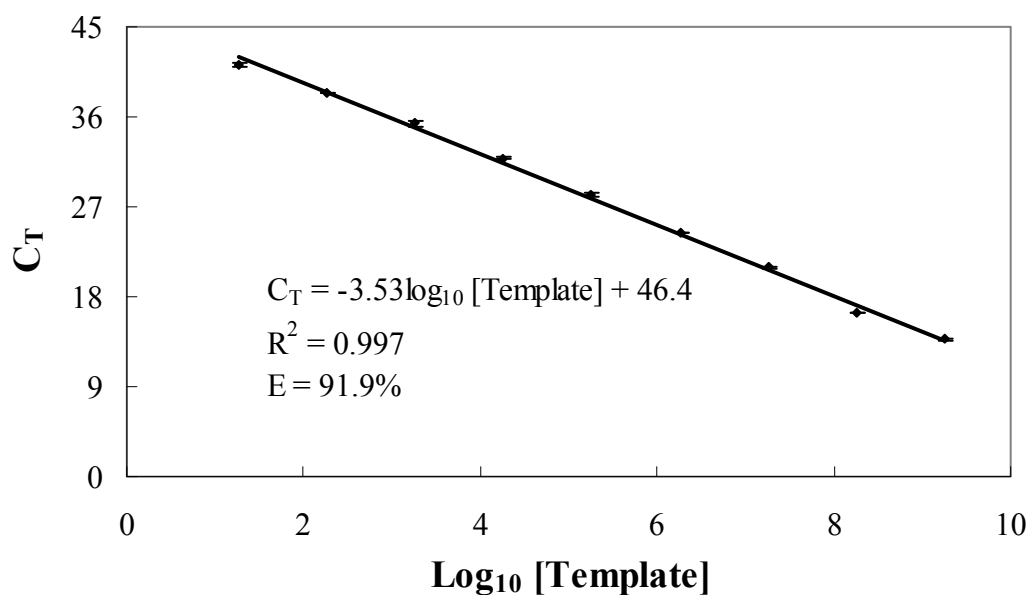


Fig. IV9. Amplification efficiency (E) of the *Methanocalculus*-specific qPCR assay as determined with the C_T-Log[Template] plot derived from the quantification of 10-fold dilution series of *Methanocalculus* 16S rRNA gene templates (1.8×10^1 to 1.8×10^9 copies).

Table IV6 *Methanocalculus* abundance determined with qPCR in anaerobic digesters and formate enrichment cultures.

Sample	16S rRNA Gene Targets	No. of Copies/ ^a	Relative Abundance (% of total <i>Archaea</i>) ^a
Formate	<i>Methanocalculus</i>	$(1.0 \pm 0.0) \times 10^9$	84 $\pm$ 7
Enrichment	Total <i>Archaea</i>	$(1.2 \pm 0.2) \times 10^9$	-
Anaerobic Digester	<i>Methanocalculus</i>	$(4.6 \pm 0.8) \times 10^5$	0.01 $\pm$ 0.00
	Total <i>Archaea</i>	$(4.9 \pm 1.5) \times 10^9$	-

^aValues are the means of triplicates $\pm$ standard deviation.

Discussion

The genus *Methanocalculus* belongs to the order *Methanomicrobiales* and is represented by five known species (Fig. IV6) isolated from diverse habitats: *M. halotolerans* (Ollivier et al., 1998), *M. pumilus* (Mori et al., 2000), *M. taiwanensis* (Lai et al., 2002), *M. chungshingensis* (Lai et al., 2004), and *M. natronophilus* (Zhilina et al., 2013). All cultured *Methanocalculus* strains are characterized as hydrogenotrophic methanogens capable of methanogenesis using formate as the substrate. Sequences of *Methanocalculus* populations were also identified in engineered anaerobic digestion processes treating organic wastes (Shin et al., 2010). However, much remains to be learned about the distribution and ecological functions of these methanogens.

The *Methanocalculus*-specific qPCR assay designed in this study represents the first research tool available in the literature for the specific quantification of *Methanocalculus* populations in the environment. Results from this study have demonstrated the high specificity, coverage, sensitivity and accuracy of the *Methanocalculus*-specific qPCR assay (Table IV5 and 6), providing an important research capability to study the microbial ecology of *Methanocalculus* populations and gain insight to the potential ecological significance of these methanogens.

The application of the *Methanocalculus*-specific qPCR assay revealed the low relative abundance of *Methanocalculus* populations in the anaerobic digesters treating dairy waste, constituting 0.01% of the total archaeal community. However, the amendment of formate as a methanogenesis substrate elevated *Methanocalculus* to the dominant population, representing 84% of the total archaeal community in the enrichment cultures (Table IV6). The dominance of *Methanocalculus* populations was also observed following the addition of xylan as the substrate for biomethane production, where *Methanocalculus* accounted for 63% of the total archaeal populations (Lu et al., 2013). These observations raised important questions on the environmental conditions enhancing the competitiveness of *Methanocalculus* populations in methanogenic processes. The *Methanocalculus*-specific qPCR assay developed in this study will serve as a valuable tool to answer these questions in future research efforts.

References

- Calderón, K., Rodelas, B., Cabirol, N., González-López, J., Noyola, A. 2011. Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment. *Bioresour Technol*, **102**(7), 4618-4627.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Chen, S., Zhu, Z., Park, J., Zhang, Z., He, Q. 2014. Development of Methanoculleus-Specific Real-Time Quantitative PCR Assay for Assessing Methanogen Communities in Anaerobic Digestion. *J Appl Microbiol*, n/a-n/a.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., Tiedje, J.M. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res*, **31**(1), 442-443.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res*, **37**, D141-D145.
- Collins, G., Mahony, T., McHugh, S., Gieseke, A., de Beer, D., O'Flaherty, V. 2005. Distribution, dynamics and in situ ecophysiology of Crenarchaeota in anaerobic wastewater treatment granular biofilms. *Water Science and Technology*, **52**(7), 233-239.
- Conrad, R. 2009. The global methane cycle: recent advances in understanding the microbial processes involved. *Environmental Microbiology Reports*, **1**(5), 285-292.
- Delong, E.F. 1992. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America*, **89**(12), 5685-5689.
- He, Q., Sanford, R.A. 2002. Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. *Biodegradation*, **13**(5), 307-316.
- Kibbe, W.A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res*, **35**, W43-W46.
- Lai, M.-C., Chen, S.-C., Shu, C.-M., Chiou, M.-S., Wang, C.-C., Chuang, M.-J., Hong, T.-Y., Liu, C.-C., Lai, L.-J., Hua, J.J. 2002. *Methanocalculus taiwanensis* sp. nov., isolated from an estuarine environment. *Int J Syst Evol Microbiol*, **52**(5), 1799-806.
- Lai, M.-C., Lin, C.-C., Yu, P.-H., Huang, Y.-F., Chen, S.-C. 2004. *Methanocalculus chunghsingensis* sp. nov., isolated from an estuary and a marine fishpond in Taiwan. *Int J Syst Evol Microbiol*, **54**(1), 183-189.
- Liu, Y., Whitman, W.B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. in: *Incredible Anaerobes: From Physiology to Genomics to Fuels*, (Eds.) J. Wiegel, R.J. Maier, M.W.W. Adams, Vol. 1125, pp. 171-189.
- Lu, X., Rao, S., Shen, Z., Lee, P.K.H. 2013. Substrate induced emergence of different

- active bacterial and archaeal assemblages during biomethane production. *Bioresour Technol*, **148**(0), 517-524.
- Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M., Takamizawa, K. 2000. *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. *Int J Syst Evol Microbiol*, **50**(5), 1723-9.
- Nolan, T., Hands, R.E., Bustin, S.A. 2006. Quantification of mRNA using real-time RT-PCR. *Nature Protocols*, **1**(3), 1559-1582.
- Ollivier, B., Fardeau, M.-L., Cayol, J.-L., Magot, M., Patel, B.K.C., Prensier, G., Garcia, J.-L. 1998. *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well. *Int J Syst Bacteriol*, **48**(3), 821-828.
- Schefe, J., Lehmann, K., Buschmann, I., Unger, T., Funke-Kaiser, H. 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene expression’s C T difference” formula. *Journal of Molecular Medicine*, **84**(11), 901-910.
- Shin, S.G., Lee, S., Lee, C., Hwang, K., Hwang, S. 2010. Qualitative and quantitative assessment of microbial community in batch anaerobic digestion of secondary sludge. *Bioresour Technol*, **101**(24), 9461-9470.
- Stams, A.J.M., Plugge, C.M. 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology*, **7**(8), 568-577.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*, **24**(8), 1596-1599.
- van Lier, J.B., Tilche, A., Ahring, B.K., Macarie, H., Moletta, R., Dohanyos, M., Pol, L.W.H., Lens, P., Verstraete, W. 2001. New perspectives in anaerobic digestion. *Water Science and Technology*, **43**(1), 1-18.
- Wang, T., Brown, M.J. 1999. mRNA quantification by real time TaqMan polymerase chain reaction: Validation and comparison with RNase protection. *Anal Biochem*, **269**(1), 198-201.
- Wolin, E.A., Wolin, M.J., Wolfe, R.S. 1963. Formation of Methane by Bacterial Extracts. *J Biol Chem*, **238**(8), 2882-2886.
- Yu, Y., Lee, C., Kim, J., Hwang, S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*, **89**(6), 670-679.
- Zhang, T., Fang, H.H.P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol*, **70**(3), 281-289.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhang, Y., Zhang, X.L., Zhang, H.W., He, Q., Zhou, Q.X., Su, Z.C., Zhang, C.G. 2009. Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis. *Bull Environ Contam Toxicol*, **82**(3), 367-372.
- Zhilina, T.N., Zavarzina, D.G., Kevbrin, V.V., Kolganova, T.V. 2013. *Methanocalculus natronophilus* sp. nov., a new alkaliphilic hydrogenotrophic methanogenic archaeon from a soda lake, and proposal of the new family Methanocalculaceae. *Microbiology*, **82**(6), 698-706.

Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.

Part III: *Crenarchaeota* in Anaerobic Digestion of Animal Waste

Abstract

Crenarchaeota have been unexpectedly detected in methanogenic communities in anaerobic digestion processes treating organic waste. The ammonia-oxidizing activity of some non-thermophilic *Crenarchaeota* (or *Thaumarchaeota*), however, would not be relevant for the *Crenarchaeota* in anaerobic digestion (*CrenAD*) due to the lack of adequate oxygen in these environments. Phylogenetic analysis revealed that all *CrenAD* sequences clustered into a clade distinct from that of the ammonia-oxidizing *Thaumarchaeota* and were placed in Subgroup 6 of the Miscellaneous *Crenarchaeotal* Group (MCG-6) within Group I.3 *Crenarchaeota*, allowing the development of *CrenAD*-specific qPCR to target these populations with high sensitivity. Population dynamics of *Crenarchaeota* in continuous anaerobic digesters treating animal waste provided indications that the activity of *Crenarchaeota* was linked to process performance, particularly the accumulation of organic acids. *Crenarchaeota* was subsequently shown to be enriched up to ~20 fold from background level as minor constituents of the archaeal community by acetate, propionate, butyrate, or methanol; but not by formate. Since acetate could serve as an intermediate during anaerobic metabolism of the substrates (e.g. propionate and butyrate) that supported *Crenarchaeota*, these results provided the first implication that *Crenarchaeota* in anaerobic digestion might be linked to acetate utilization.

Keywords: Acetate, Anaerobic digestion, *Crenarchaeota*, Miscellaneous *Crenarchaeotal* Group

Introduction

Anaerobic digestion is a methanogenic process with broad applications in the treatment of organic wastes. The benefits of anaerobic digestion as a sustainable waste treatment technology include the production of biomethane as a renewable fuel (Demirel et al., 2010). To enhance the process efficiency and stability of anaerobic digestion, efforts have been made to understand the microbial communities underlying the anaerobic decomposition of organic materials. As a result, significant progress has been made in the quantification and monitoring of specific microbial populations frequently identified in anaerobic digestion processes, such as methanogenic archaea, using molecular techniques with high sensitivity and specificity (Narihiro & Sekiguchi, 2011).

In addition to methanogens, which belong to *Euryarchaeota*, recent molecular surveys of microbial communities in anaerobic digestion processes have unexpectedly detected the presence of 16S rRNA gene sequences related to uncharacterized *Crenarchaeota* populations (Chen et al., 2012; Chouari et al., 2005; Gomez et al., 2011; Leven et al., 2007; Riviere et al., 2009; Zhang et al., 2011). Moreover, *Crenarchaeota* have been reported as the predominant archaeal population in several studies of anaerobic digestion, representing 20-50% of the archaeal community (Acharya et al., 2011; Collins et al., 2005; Enright et al., 2009; Schauer-Gimenez et al., 2010; Siggins et al., 2011),

suggesting the potential significance of these microorganisms in anaerobic decomposition of organic materials. *Crenarchaeota* have also been found globally in many other mesophilic environments and are proposed to play important roles in ammonia oxidation (Francis et al., 2005; Nicol & Schleper, 2006). Indeed, ammonia concentration is typically elevated in anaerobic digestion processes (Yenigun & Demirel, 2013). However, it does not seem plausible for the *Crenarchaeota* populations in anaerobic digestion to carry out ammonia oxidation due to the lack of sufficient oxygen or other oxidized electron acceptors, which is characteristic of methanogenic environments. While some have pointed to the potential linkage between *Crenarchaeota* and specific groups of methanogens in anaerobic digestion (Collins et al., 2005; Enright et al., 2009), other studies of marine sediments have suggested the involvement of *Crenarchaeota* in protein mineralization (Lloyd et al., 2013) or aromatics degradation (Meng et al., 2014). Thus, much remains to be learned about the functions of *Crenarchaeota*, particularly in the unique environment engineered for anaerobic digestion, which is characterized by very high fluxes of carbon and nutrient.

In this study, a phylogenetic analysis was performed on a collection of *Crenarchaeota* 16S rRNA gene sequences from anaerobic digesters, which was shown to form a distinct clade subsequently used for the development of a real-time quantitative PCR (qPCR) assay specifically targeting these *Crenarchaeota* populations. Monitoring the population dynamics of *Crenarchaeota* during process perturbations in anaerobic digesters treating animal waste revealed potential linkages between *Crenarchaeota* abundance and process condition. Subsequent enrichment of *Crenarchaeota* as a minor population with organic acids commonly found in anaerobic digestion provides the first indication of the activity of *Crenarchaeota* in methanogenic environments.

Materials and methods

Anaerobic digester setup and operation

Triplicate continuous mesophilic anaerobic digesters were established prior to this study using dairy waste as the feedstock at an organic loading rate (OLR) of 1.0 gVS/L/day as described previously (Chen et al., 2012). The temperature of the anaerobic digestion process was maintained at 35 °C and the hydraulic retention time was controlled at 20 days. All digesters exhibited stable operation prior to a series of changes in OLR with the addition of poultry waste to induce process perturbations (Fig. IV10). Starting at 1.0 gVS/L/day, the OLR was raised stepwise by 28%, 50% and 77%, before feeding was stopped upon the onset of organic overloading, followed by another series of stepwise increases in OLR to 0.5, 1.0, and 1.5 gVS/L/day, which was maintained until the end of this study (Fig. IV10). Biogas production was monitored with a water displacement method and methane content in biogas was quantified by gas chromatography (GC) with a thermal conductivity detector (TCD) using previously described protocols (Zhu et al., 2011). Concentrations of volatile fatty acids (VFAs) in the digestate were determined by GC with a flame ionization detector (FID) as previously described (Zhu et al., 2011).

Enrichment setup and monitoring

To potentially promote the growth of *Crenarchaeota*, enrichment cultures were established by a 10% (v/v) transfer of the digestate from the continuous anaerobic digesters into an anaerobic basal medium prepared with previously described protocols (He & Sanford, 2002) according to the following recipe (per liter): NaCl, 1.0 g; MgCl₂•6 H₂O, 0.5 g; KH₂PO₄, 0.2 g; NH₄Cl, 0.3 g; KCl, 0.3 g; CaCl₂•2H₂O, 0.015 g; trace element solution, 1.0 mL; Se/Wo solution, 1.0 mL; and resazurin, 1.0 mg, as the redox indicator. NaHCO₃ (2.52 g/L) was added to the medium as the buffer. The enrichment medium was subsequently dispensed into 160-mL serum bottles flushed with pure N₂ and sealed with butyl rubber stoppers and aluminum caps. A sterile vitamin solution (1%) was added after autoclaving to supply potential growth factors (Wolin et al., 1963).

Enrichment cultures were set up with the following compounds as the sole substrate: formate, acetate, propionate, butyrate, or methanol. Subsequent to the inoculation of fresh medium with the digestate from the anaerobic digesters, 10 feedings of the enrichment substrate were delivered into the culture. Each feeding contained 50 mM formate, 20 mM acetate, 20 mM propionate, 20 mM butyrate, or 30 mM methanol. Feedings were initiated when the substrate provided in the previous feeding was depleted. At the completion of 10 feedings of the enrichment substrates, a second 10% (v/v) transfer from the initial enrichments to fresh medium was performed, followed by another 10 feedings of the enrichment substrate.

All enrichment cultures were maintained in triplicates in a shaking incubator at 80 rpm and 35 °C. VFAs, i.e. formate, acetate, propionate, and butyrate, were analyzed with an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Santa Clara, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) using 4 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 ml/min. Prior to HPLC analysis, samples were prepared by filtration and acidification to 0.1 N H₂SO₄ by adding 25 µL of 2 N H₂SO₄ to 475 µL of sample as previously described (He & Sanford, 2004). Detection of VFAs was at 210 nm with a multiple wavelength detector. Methanol was quantified with an Agilent G1888 headspace auto-sampler and an Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a DB-624 capillary column (Agilent, Santa Clara, CA, USA). Prior to GC analysis, filtered samples (1 mL) were transferred into 20-mL headspace glass vials sealed with a Teflon®-lined gray butyl septum secured with aluminum crimp caps. Helium was used as the carrier gas for both the auto-sampler and the GC. The auto-sampler used the following configurations: oven temperature, 70 °C; vial equilibration time using maximum agitation, 15 min; sample loop (1 mL) temperature, 125 °C; transfer line (80 cm) temperature, 125 °C; pressurization time, 0.5 min; loop fill time, 0.03 min; loop equilibration time, 0.05 min; inject time, 0.5 min; carrier gas pressure, 2 psig; vial pressurization, 10 psig; cycle time, 14 min. The GC inlet was maintained at 200 °C and 23.11 psig with a total flow of 5.7 mL/min. The inlet split ratio was 1:10. The oven temperature for the GC was kept at 60 °C for 2 min and then increased at a rate of 25 °C/min until the oven reached 200 °C. The column was operated in the constant flow mode at 3.0 mL/min and 23.11 psig. The FID detector was operated at a temperature of 280 °C with the following gases: hydrogen (30 mL/min), air (400 mL/min), and helium (27 mL/min).

***Crenarchaeota* sequence analysis**

A total of 220 16S rRNA gene sequences of *Crenarchaeota* identified in anaerobic digesters (*CrenAD*) were retrieved from the literature (Table IVS1). The set of *Crenarchaeota* sequences of Miscellaneous Crenarchaeotal Group (MCG) were from the ARB/SILVA 106 non-redundant 16S rRNA database and classified into 17 subgroups as previously described (Kubo et al., 2012). Sequences of other representative archaeal reference strains and clones, including *Crenarchaeota*, *Euryarchaeota*, and *Thaumarchaeota*, were retrieved from NCBI GenBank. The phylogenetic relationships of these 16S rRNA gene sequences were aligned, compared, and phylogenetic trees were constructed with the neighbor-joining algorithm using MEGA 4.0 (Tamura et al., 2007).

Primer/probe design for *CrenAD*-specific qPCR

The grouping of *CrenAD* 16S rRNA gene sequences into a monophyletic clade (Fig. IV11-13) enabled the development of *CrenAD*-specific quantitative polymerase chain reaction (qPCR) assay for the enumeration of this group of *Crenarchaeota* based on all of the 220 *CrenAD* 16S rRNA gene sequences (Table IVS3) using primer/probe design procedures previously described for TaqMan chemistry (Chen et al., 2014). The primer/probe sequences were determined following guidelines provided by Applied Biosystems (Foster City, California, USA) on parameters including amplicon size, nucleotide position, melting temperature (T_m), GC content, self-annealing, complementarity, and hairpin formation. Specificity and coverage of the primer/probe set were evaluated with the ProbeMatch program of RDP II (Cole et al., 2009).

TaqMan qPCR protocol

Biomass from anaerobic digesters and enrichment cultures was pelleted by centrifugation and stored at $-80\text{ }^{\circ}\text{C}$. Whole community DNA was extracted and purified as previously described (Zhang & He, 2013). The *CrenAD*-specific primers and dual-labeled TaqMan probe, 5'-end labeled with 6-carboxyfluorescein (FAM) and 3'-end labeled with the Black Hole Quencher (BHQ), were purchased from Biosearch Technologies (Novato, California, USA). All qPCR assays were performed in 25- μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, and Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA). Thermal cycling consisted of a starting incubation at $50\text{ }^{\circ}\text{C}$ for 2 min and an initial denaturation at $95\text{ }^{\circ}\text{C}$ for 10 min, followed by up to 45 cycles at $95\text{ }^{\circ}\text{C}$ for 30 s and $60\text{ }^{\circ}\text{C}$ for 45 s. To determine the relative abundance of *Crenarchaeota* populations in the archaeal community, total archaea was quantified with the *Archaea* domain-specific TaqMan primer/probe set Arc787F-Arc915P- Arc1059R as previously described (Yu et al., 2005). The acetoclastic methanogen populations were quantified as the sum of *Methanosarcina* and *Methanosaeta* with the following primer/probe sets (Yu et al., 2005): *Methanosaeta*—forward primer, TAATCCTTGAAGGACCACCA; reverse primer, CCTACGGCACCGACAAC; and probe, ACGGCAAGGGACGAAAGCTAGG. *Methanosarcina*—forward primer, CGGTTTGGTCAGTCCTCCG; reverse primer, ACCAGACACGGTCGCGC; and probe, AACGGGTTCGACGGTGAGGGACGA. The

qPCR procedure was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as previously described (Chen et al., 2014). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T).

DNA templates used as the standards for assessing the efficiency and specificity of the *CrenAD*-specific qPCR were partial 16S rRNA genes of representative archaeal populations cloned from the continuous bench-scale anaerobic digesters in a previous study (Chen et al., 2012), including *Crenarchaeota* (GenBank Accession No. JN052741), *Methanoculleus* (GenBank Accession No. JN052755), *Methanosaeta* (GenBank Accession No. JN052761), and *Methanosarcina* (GenBank Accession No. JN052757). Amplification efficiency (E) of the qPCR assay was determined with the C_T -Log[Template] plot derived from the quantification of 10-fold dilution series of *Crenarchaeota* 16S rRNA gene templates (Chen et al., 2014).

Results

Phylogeny of *Crenarchaeota* identified in anaerobic digestion

Crenarchaeota represents a group of globally-distributed archaea found in diverse environments. The identification of *Crenarchaeota* in anaerobic digestion, an engineered process for the treatment of organic wastes, begs the question whether these *Crenarchaeota* populations share physiological traits unique to the environment in anaerobic digestion processes, which are dominated by fermentation and methanogenesis. As the first step, 16S rRNA gene sequences of *Crenarchaeota* were collected from previous studies of anaerobic digestion processes for the treatment of various organic wastes (Table IVS1). Phylogenetic analyses revealed that the *Crenarchaeota* populations found in anaerobic digestion were not closely related to (hyper)thermophilic *Crenarchaeota* strains known to be involved in sulfur metabolism (Fig. IV11). Moreover, all sequences of *Crenarchaeota* from anaerobic digestion formed a cluster separated from those of the ammonia-oxidizing *Crenarchaeota*, recently proposed as a new archaeal phylum *Thaumarchaeota* (Pester et al., 2011). These observations suggest that the *Crenarchaeota* populations identified in anaerobic digestion are phylogenetically distinct from *Thaumarchaeota* populations linked to ammonia oxidation, which would not be plausible as an aerobic metabolism in the strictly anaerobic environment of anaerobic digestion processes.

More detailed analyses of *Crenarchaeota* and *Thaumarchaeota* sequences showed that the sequences identified in anaerobic digestion were exclusively placed within the Group I.3 *Crenarchaeota* lineage (Fig. IV12), which were found in environments such as flooded soils, sediments, and freshwater (Nicol & Schleper, 2006; Ochsenreiter et al., 2003). In contrast, *Thaumarchaeota*, comprising the Group I.1a/b/c lineages (Pester et al., 2011), formed a separate phylogenetic cluster distinct from the *Crenarchaeota* identified in anaerobic digestion. The grouping of all *Crenarchaeota* from anaerobic digestion in a single phylogenetic cluster suggests that these populations may share traits corresponding

to the same ecological niche.

The Group I.3 *Crenarchaeota* lineage is represented by the Miscellaneous Crenarchaeotal Group (MCG), a group of uncultured non-thermophilic archaeal populations with high phylogenetic diversity and broad biogeographical distribution (DeLong, 1998; Inagaki et al., 2003). Since MCG *Crenarchaeota* were recently classified into 17 subgroups (Kubo et al., 2012), the phylogenetic placement of *Crenarchaeota* identified in anaerobic digestion was further examined in relation to the 17 MCG subgroups, revealing that all sequences found in anaerobic digestion were grouped in a single clade, i.e. MCG subgroup 6 (MCG-6) (Fig. IV13). The monophyletic grouping of all *Crenarchaeota* from anaerobic digestion provided the opportunity to design qPCR assays specific to these populations for the detection and monitoring of these organisms in anaerobic digestion processes.

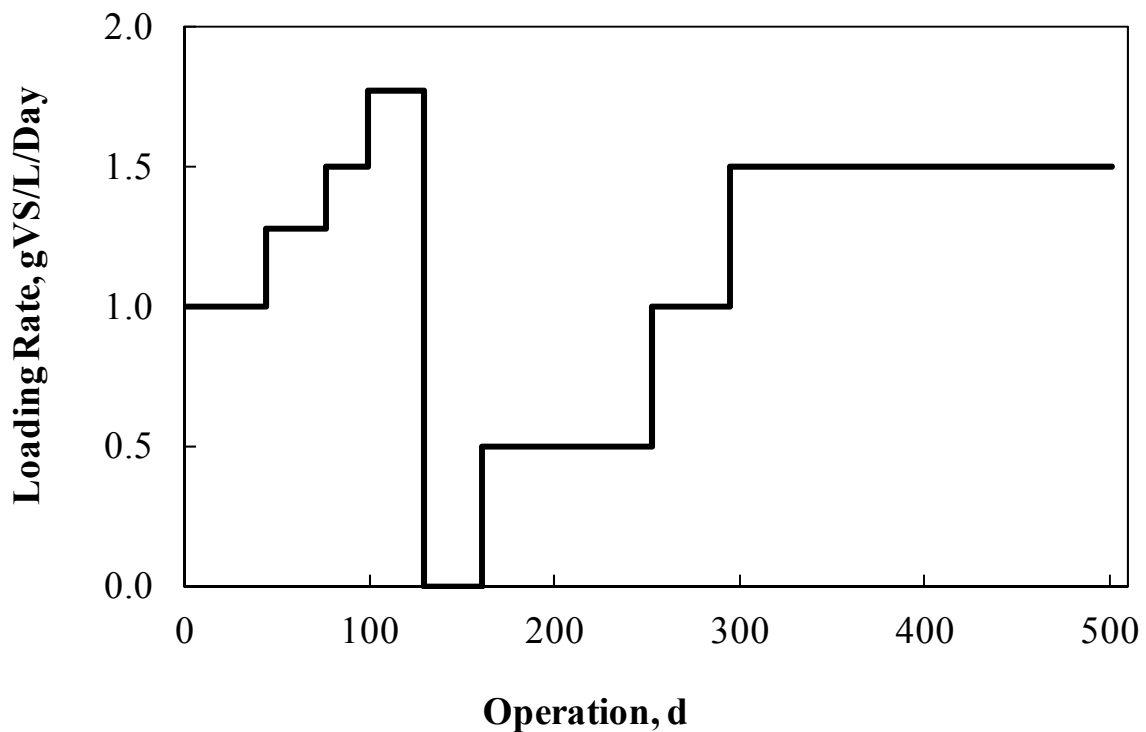


Fig. IV10. Organic loading scheme of the anaerobic digesters treating animal waste.

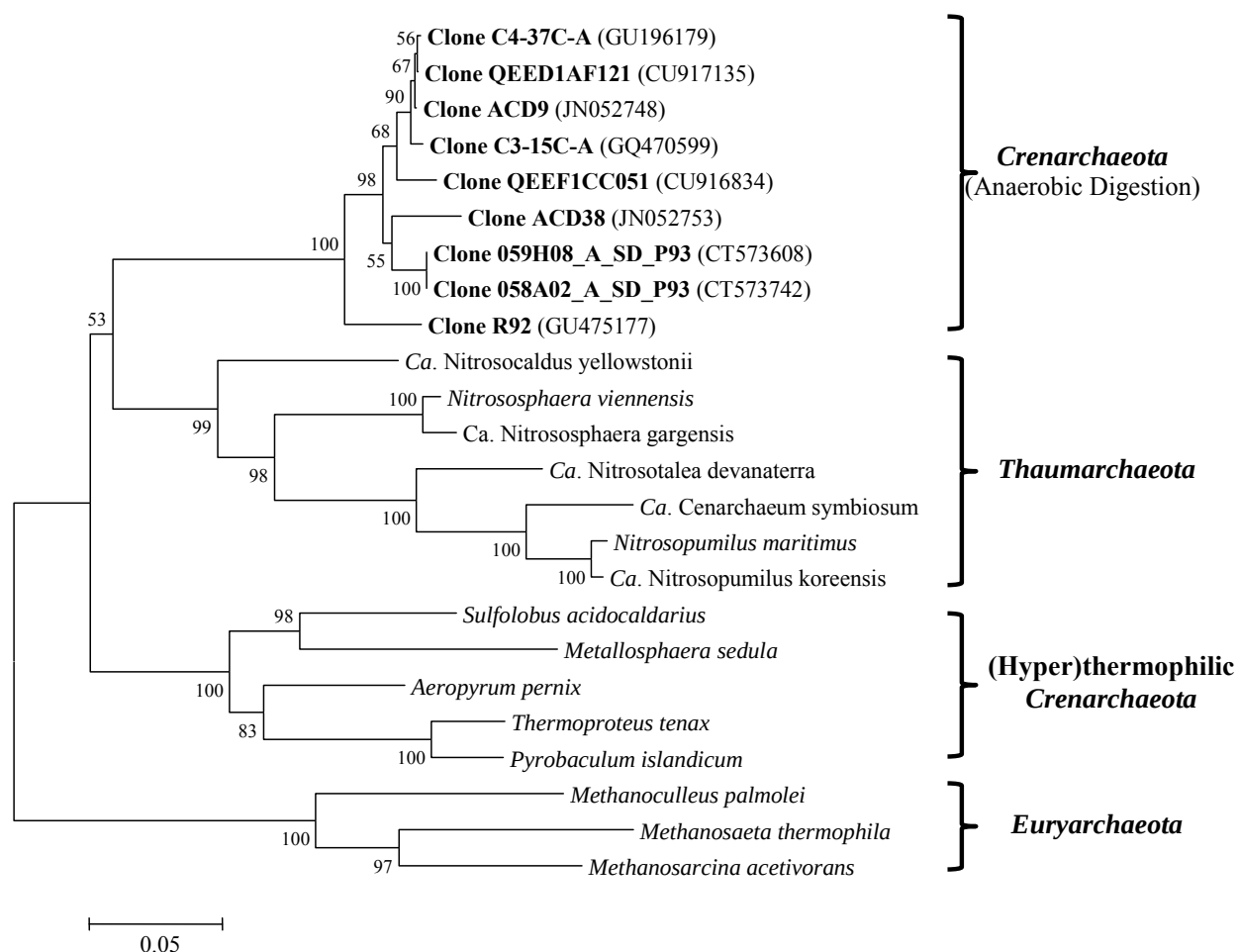


Fig. IV11. Neighbor-joining tree showing the phylogenetic grouping of *Crenarchaeota* 16S rRNA gene sequences cloned from anaerobic dogesters in relation to reference sequences of *Thaumarchaeota*, (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota*. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position. GenBank accession numbers of the 16S rRNA gene sequences are indicated in the parentheses.

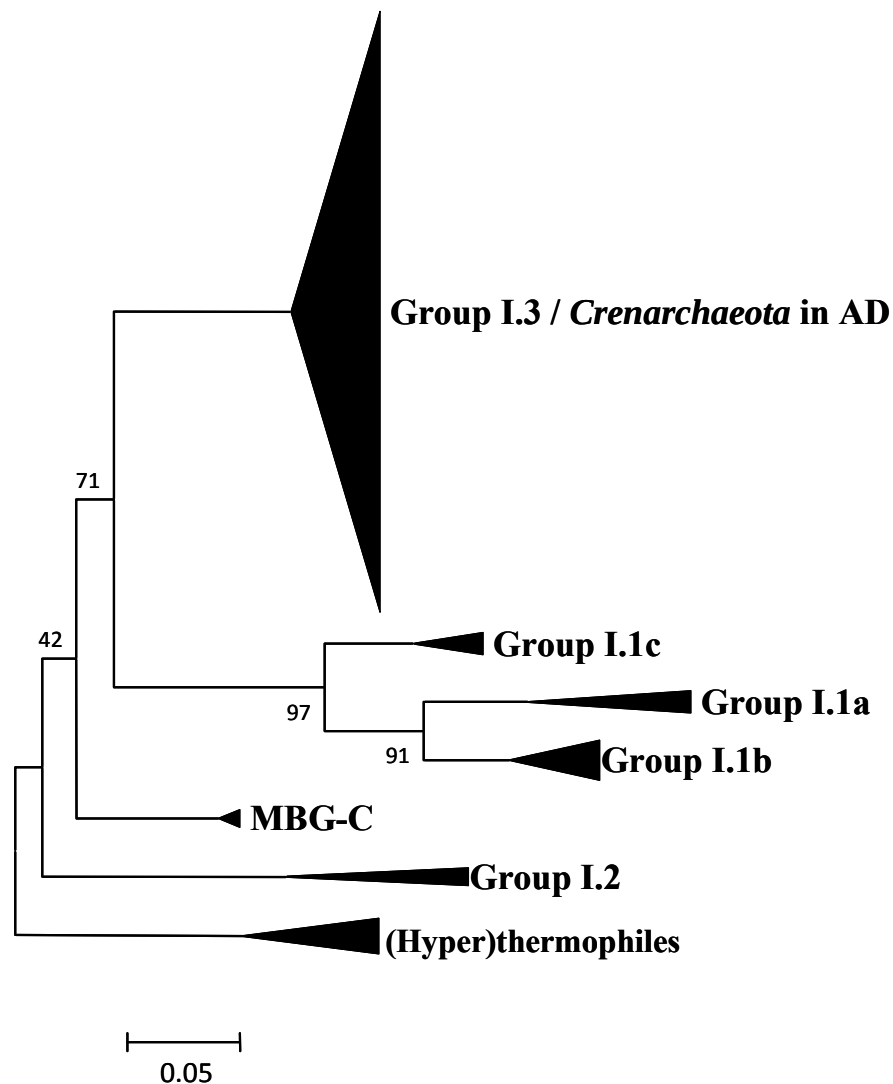


Fig. IV12. Neighbor-joining phylogenetic tree showing the relationships between *Crenarchaeota* identified in anaerobic digestion (AD) processes and select lineages of *Crenarchaeota* found in other environments, according to 16S rRNA gene sequences. The lineage designations follow those of Reference Nicol and Schleper, 2006. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position.

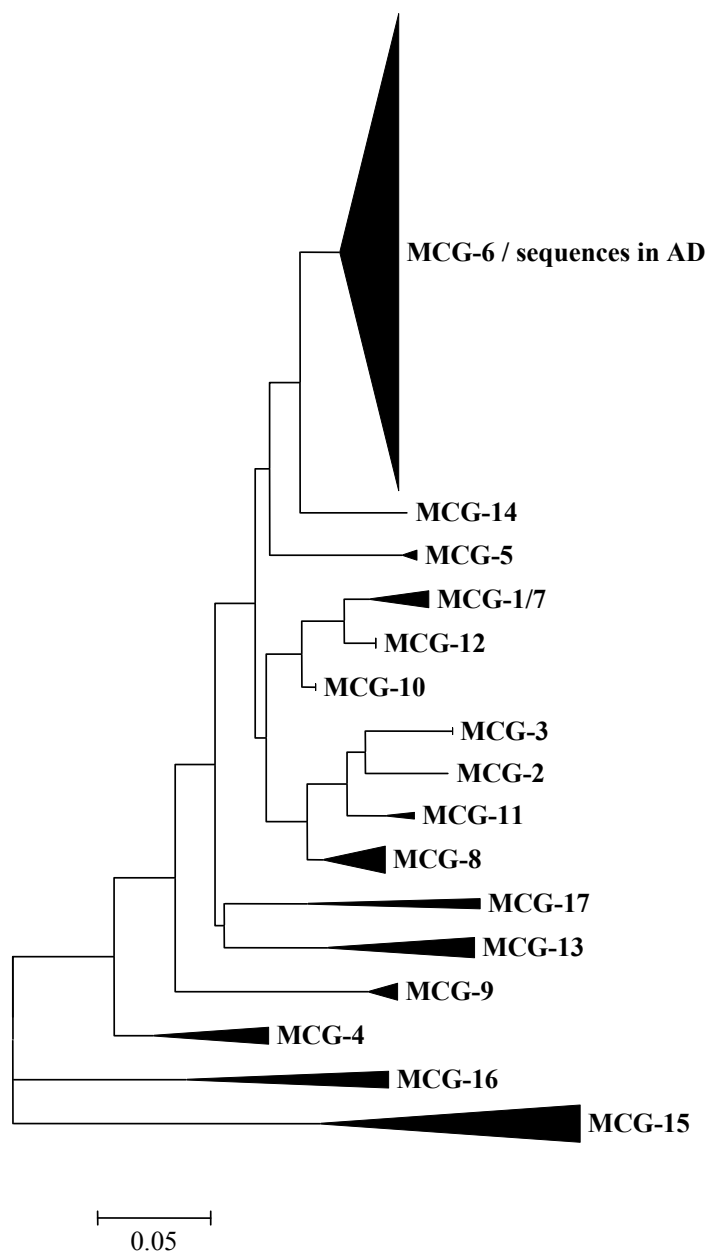


Fig. IV13. Neighbor-joining phylogenetic tree showing the relationships between Miscellaneous Crenarchaeotal Group 6 (MCG-6) identified in anaerobic digestion (AD) processes and other MCG subgroups. Classification of MCG subgroups 1-17 is according to Reference Kubo *et al.*, 2012. The scale bar represents the number of substitutions per sequence position.

Table IV7 Characteristics of TaqMan qPCR primer/probe set targeting *Crenarchaeota* populations in anaerobic digestion (*CrenAD*).

Primer/Probe	Sequence (5'-3')	Position <i>E. coli</i> No.	GC%	T _m (°C)	Amplicon size (bp)
<i>Forward primer</i>					
Cren-460	GAGAGGGCAAGTCTGGTGT	468-486	58	59.5	
<i>Probe</i>					
Cren-490	CGCCGCGGTAATACCAGCTCTC	490-511	64	67.9	91
<i>Reverse primer</i>					
Cren-558	AGCCAGCTACGGATGCTTTA	539-558	50	58.4	

Table IV8 Coverage and specificity of the CrenAD TaqMan qPCR primer/probe set in *Archaea* and *Bacteria*.

Primer/Probe Characteristics		No. of Matching Sequence ^a			
Primer/Probe Name ^a	Sequence (5'-3')	<i>Bacteria</i> (2,636,371) ^b	<i>Crenarchaeota</i> (29,709) ^c	Non- <i>Crenarchaeota</i> ^d	
				<i>Euryarchaeota</i> (76,087) ^e	<i>Archaea</i> (98,670) ^f
Cren-468F (F)	GAGAGGGCAAGTCTGGTGT	0	4,586	3	58
Cren-490P (P)	CGCCGCGGTAATACCAGCTCTC	0	6,636	6	191
Cren-558R (R)	AGCCAGCTACGGATGCTTTA	0	2,201	4	55

^aNumber of 16S rRNA gene sequences belonging to the target taxon in the Ribosomal Database Project (RDP) 2013 database with perfect match to the primer/probe.

^bNumber in the parenthesis indicates the total number of 16S rRNA gene sequences belonging to the domain *Bacteria* in RDP.

^cNumber in the parenthesis indicates the total number of 16S rRNA gene sequences belonging to the genus *Crenarchaeota* in RDP.

^dNumber of non-*Crenarchaeota* 16srRNA gene sequences with perfect match to the primer/probe set.

^eNumber in the parenthesis indicates the number of 16S rRNA gene sequences in the archaeal phylum *Euryarchaeota*.

^fNumber in the parenthesis indicates the number of 16S rRNA gene sequences in the domain *Archaea* which did not belong to the phylum *Crenarchaeota*.

Table IV9 Coverage and specificity of the CrenAD-specific qPCR primer/probe set in Miscellaneous Crenarchaeotal Groups (MCG).

MCG Subgroup ^b	No. of Sequences ^d	Coverage (%) ^a								
		Forward primer			Probe			Reverse primer		
		0 ^e	1 ^e	2 ^e	0 ^e	1 ^e	2 ^e	0 ^e	1 ^e	2 ^e
MCG-1	187	6	49	49	44	44	44	0	0	0
MCG-2	19	0	74	74	16	16	16	0	0	0
MCG-3	118	7	58	58	36	36	36	0	0	0
MCG-4	56	0	45	45	36	36	36	0	0	0
MCG-5	245	50	54	54	31	31	31	2	2	2
MCG-6	1281	59	70	70	37	37	37	37	38	38
MCG-7	80	3	24	24	4	4	4	0	0	0
MCG-8	591	13	61	62	32	32	32	0	0	0
MCG-9	84	21	38	38	45	45	45	0	0	0
MCG-10	75	27	52	52	41	41	41	0	0	0
MCG-11	230	44	50	50	29	29	29	0	0	0
MCG-12	53	60	62	62	25	25	25	0	0	0
MCG-13	120	0	1	1	3	3	3	0	0	0
MCG-14	78	0	0	0	0	0	0	0	0	0
MCG-15	627	0	0	0	0	0	0	0	0	0
MCG-16	28	0	0	0	0	0	0	0	0	0
MCG-17	449	0	0	0	0	0	0	0	0	0
MCG-AD ^c	220	97	100	100	94	100	100	97	100	100

^aPercentage of sequences in the MCG subgroup matching the primer/probe sequences. If sequences information lacked at primer/probe target sites, sequences were excluded from the analysis.

^bClassification of MCG subgroups 1-17 according to Reference Kubo *et al.*, 2012.

^cSequences of MCG Crenarchaeota identified in anaerobic digestion processes (see supplemental material for all sequences).

^dNo. of sequences in SILVA Ref106 NR database with an additional 127 sequences from literature that were identified in anaerobic digestion processes.

^eMismatch allowance set in matching sequences with primer/probe.

Development of CrenAD-specific qPCR

In order to study *Crenarchaeota* populations in anaerobic digestion (CrenAD), a CrenAD-specific qPCR assay was developed. Given the diversity of *Crenarchaeota*, sequence coverage and specificity were the major considerations in our attempt to design the CrenAD-specific qPCR assay. With increased specificity provided by the probe, the TaqMan chemistry was selected and a primer/probe set was designed, which covers bases 468 to 558 (*E. coli* position) of the 16S rRNA gene, with an amplicon size of 91 bp (Table IV7). No hairpin, self-annealing and complementarity were detected for the primer/probe set. These characteristics suggest that amplification with the CrenAD-specific primer/probe set would be efficient, which was validated by the amplification efficiency of 99.1% for the CrenAD-specific primer/probe set using *Crenarchaeota* 16S rRNA gene templates (Fig. IVS1A). Moreover, amplification was highly reproducible with strong correlation ($R^2=0.995$) between template concentration and fluorescence response detected as threshold cycle.

The coverage and specificity of the CrenAD-specific primer/probe set were first evaluated with sequence analysis, which showed that the primer/probe set designed in this study exhibited high specificity to *Crenarchaeota*, having nearly no matches to sequences of *Bacteria* and *Euryarchaeota*, with the latter typically representing the most abundant archaeal populations in anaerobic digestion processes (Table IV8). Expanding the analysis to the entire *Archaea* domain, the CrenAD-specific primer/probe set had non-specific matches to only 0.06%, 0.19%, and 0.10% of the 98,670 non-*Crenarchaeota* archaeal sequences in RDP with the forward primer, reverse primer, and probe, respectively (Table IV8). Among MCGs, the CrenAD-specific primer/probe set had matches exclusively to MCG-6 where all CrenAD were grouped (Table IV9). More importantly, the primer/probe set had excellent matches to nearly all the CrenAD sequences (Table IV9), confirming its utility to monitor *Crenarchaeota* in anaerobic digesters.

The specificity of the primer/probe set designed in this study was also evaluated with experimental qPCR testing. The DNA templates tested for the CrenAD-specific qPCR assay were *Crenarchaeota* along with *Methanosarcina*, *Methanosaeta*, and *Methanoculleus*, representing the three most abundant archaeal 16S rRNA gene sequences in the anaerobic digesters of this study (Chen et al., 2012). Amplification was observed for DNA templates of *Crenarchaeota* only and no amplification was detected for any other DNA templates after 40 PCR cycles (Fig. IVS1B), demonstrating the specificity of the primer/probe set to *Crenarchaeota*.

Indeed, the specificity of the primer/probe set designed in this study is also supported by the high quality alignments between the primers/probe and CrenAD 16S rRNA gene sequences (Fig. IV14). Multiple mismatches were evident between the CrenAD-specific primer/probe set and 16S rRNA gene sequences of reference strains of *Thaumarchaeota* and representative clones of all MCG subgroups, except MCG-6, which is consistent with the exclusive grouping of CrenAD sequences in MCG-6 (Fig. IV13).

These results validated the utility of the qPCR assay developed in this study for the specific monitoring of *Crenarchaeota* in anaerobic digestion.

16S rRNA gene	Accession No.	Forward Primer: Cren-468F <u>GAGAGGGCAAGTCTGGTGT</u>	Probe: Cren-490P <u>CGCCGCGGTAATACCAGCTCTC</u>	Reverse Primer: Cren-558R <u>TAAAGCATCCGTAGCTGGCT</u>
<i>Crenarchaeota</i> clones from anaerobic digestion				
C4-37C-A	GU196179	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
QEED1AF121	CU917135	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
ACD9	JN052748	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
C3-15C-A	GQ470599	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
QEEF1CC051	CU916834	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
ACD38	JN052753	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
R92	GU475177	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
<i>Crenarchaeota</i> clones of MCG				
¹ FeSO ₄ _A_130	GQ356866	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCAT <u>TCGTAGCCT</u> GCT
² H2_E07	DQ301965	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
³ ZES-54	EF367492	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTCGTAGC</u> CGGCT
⁴ 242mbsf_1_3C	EU915227	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTCGTAGC</u> CGGC
⁵ KM35A-12	AB300143	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	<u>T</u> AAAGCATCCGTAGC <u>CA</u> GCT
⁶ R92	GU475177	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGCATCCGTAGCTGGCT
⁷ TG_BD0.2	HM483669	GAGAGGGCAAG <u>CCG</u> GGTGT	CGCCGCGGTAATACC <u>GCT</u> CTC	TAAAGCATCCGTAGC <u>CGGC</u>
⁸ ArchSlurry_12C	FR695336	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
⁹ ZES-119	EF367556	GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
¹⁰ MSASA-A6	EF125508	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
¹¹ ARCHDPK31	DQ190101	<u>G</u> GAGAGGGCAAGTCTGGTGT	CGCCGCGG <u>T</u> AATACCAGCTCTC	TAA <u>A</u> AGC <u>TCCGTAGCCG</u> ACT
¹² MKCSB-A8	DQ363756	GAGAGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
¹³ MKCSB-H10	DQ363825	<u>G</u> GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGCT <u>CC</u>	TAAAGC <u>GTC</u> CGTAGC <u>CTGCT</u>
¹⁴ MKCSB-E9	DQ363766	<u>G</u> GAGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTA <u>A</u> ACCAGCTCTC	TAAAGC <u>GTC</u> CGTAGCTGGCT
¹⁵ JMYA30	FJ810534	<u>A</u> GGGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTA <u>A</u> ACCAGC <u>CCCT</u>	TAAAGC <u>GTC</u> CGTAGCTGGCT
¹⁶ LPBBA71	FJ902705	GGGAGGGCAAG <u>A</u> TGGTGT	CGCCGCGGTA <u>A</u> ACC <u>A</u> CTCC	TAAAGC <u>GTCGTAGCT</u> <u>TGCC</u>
¹⁷ pIR3AB04	AY354118	<u>G</u> GGGAGGGCAAG <u>CT</u> GGTGT	CGCCGCGGTAATACCAGC <u>CCC</u>	TAAAGC <u>GTC</u> CGTAGC <u>CGGC</u>
<i>Thaumarchaeota</i>				
<i>Ca. N. yellowstonii</i>	EU239960	<u>G</u> GGTGGGCAAGT <u>T</u> TGGTGT	CGCCGCGGTAATACCAGC <u>ACC</u>	TAAAGCATCCGTAGC <u>CGGC</u>
<i>Ca. N. gargensis</i>	NR102916	<u>G</u> GGTGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGC <u>ACC</u>	TAAAGCATCCGTAGCT <u>TGTT</u>
<i>Ca. N. devanaterri</i>	JN227488	GGGTGGGCAAGTCTGGTGT	CGCCGCGGTAATACCAGC <u>ACT</u>	TAAAGCATCCGTAGC <u>CGGC</u>
<i>Ca. C. symbiosum</i>	U51469	GGGTGGGCAAGTCTGGTGT	CGCCGCGGTA <u>AA</u> ACCAGC <u>ACT</u>	TAAAGCATCCGTAGC <u>TGCC</u>
<i>N. maritimus</i>	NR102913	<u>G</u> GGTGGGCAAGT <u>T</u> CTGGTGT	CGCCGCGGTA <u>AA</u> ACCAGC <u>ACT</u>	TAAAGCATCCGTAGC <u>CGGC</u>
<i>Ca. N. koreensis</i>	NR102904	<u>G</u> GGTGGGCAAGT <u>T</u> CTGGTGT	CGCCGCGGTA <u>AA</u> ACCAGC <u>ACT</u>	TAAAGCATCCGTAGC <u>CGGC</u>

Fig. IV14. Alignment of representative *Crenarchaeota* and *Thaumarchaeota* 16S rRNA gene sequences (5'-3') to the qPCR primer/probe set developed in this study. For *Crenarchaeota* clones of MCG, the superscript numerical value for each clone indicates its classification to MCG subgroups 1-17 according to Reference Kubo *et al.*, 2012. The alignment of MCG subgroup 6 is underlined as the target of the qPCR primer/probe set. Mismatches are shown as shaded.

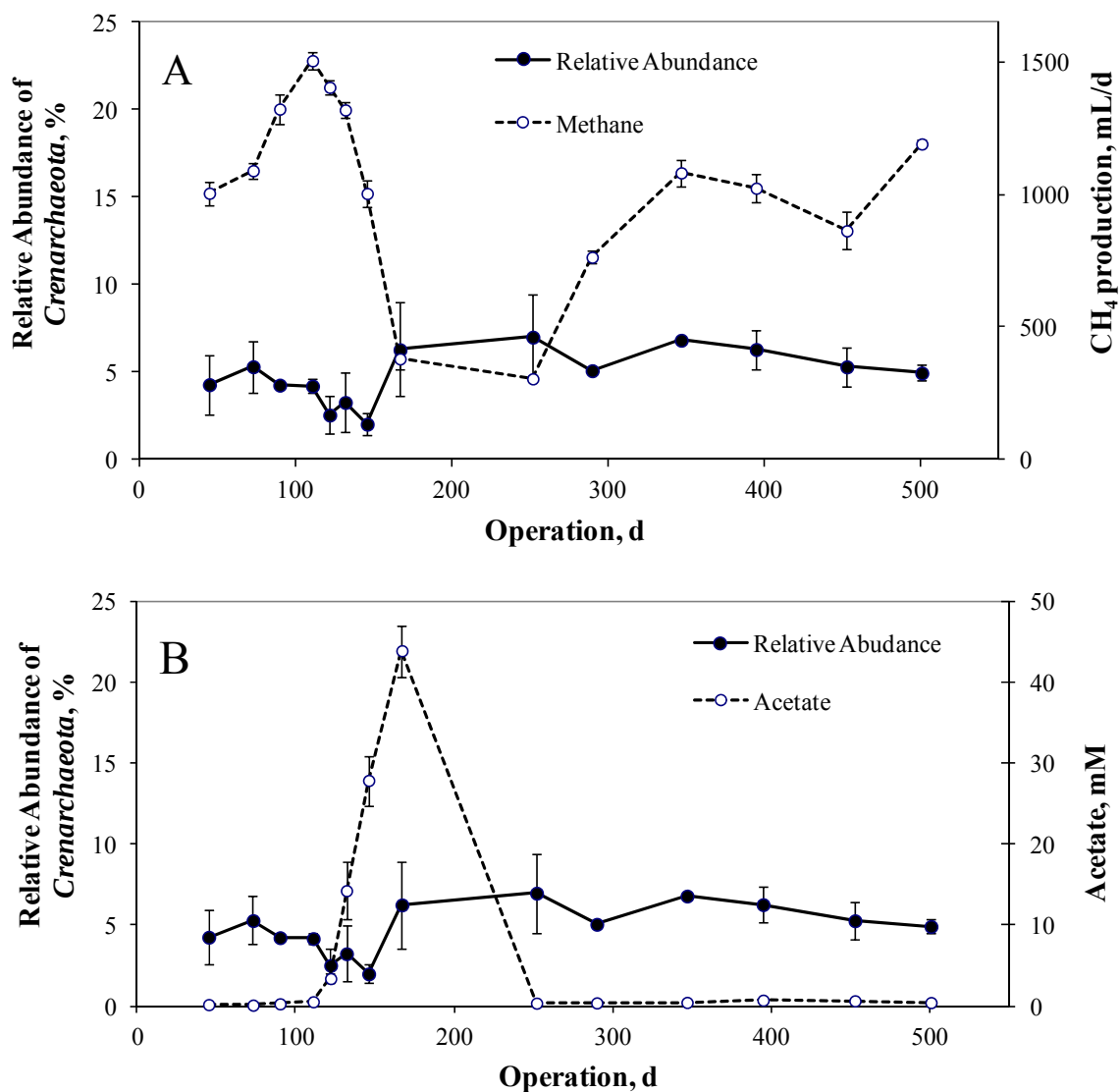


Fig. IV15. Changes in *Crenarchaeota* relative abundance in anaerobic digesters treating animal waste in relation to A) methane production, and B) acetate concentration. *Crenarchaeota* relative abundance was the percentage of archaeal 16S rRNA gene copies belonging to *Crenarchaeota*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations.

Linkage of *Crenarchaeota* abundance to anaerobic digestion performance

The *CrenAD*-specific qPCR was used to monitor the dynamics of *Crenarchaeota* in triplicate anaerobic digesters treating animal waste. In order to evaluate potential responses of *Crenarchaeota* populations to changes in process conditions, the organic loading rate (OLR) of the digesters was adjusted periodically (Fig. IV10) to induce process perturbations characterized by significant fluctuations in methane production and organic acids accumulation (Fig. IV15).

Despite process perturbations, the relative abundance of *Crenarchaeota* quantified by the *CrenAD*-specific qPCR remained relatively constant in the anaerobic digesters, ranging from 2.0% to 7.0% of the archaeal population. A closer examination of process performance revealed that *Crenarchaeota* abundance trended lower in periods of higher methane production and climbed when methane production fell (Fig. IV15A). For example, *Crenarchaeota* abundance peaked when methane production declined to the lowest level between 167 and 252 days of operation (Fig. IV15A), which coincided with the accumulation of acetate to the highest level in the anaerobic digesters (Fig. IV15B). These observations pointed to a potential linkage between *Crenarchaeota* abundance and process conditions, particularly acetate, as the decline in methane production during this period of operation could be attributed to the accumulation of organic acids such as acetate.

Enrichment of *Crenarchaeota* as a minor population

Given the linkage between *Crenarchaeota* and organic acids in the anaerobic digesters, four short-chain volatile fatty acids (VFAs) commonly detected in anaerobic digestion processes were used to set up methanogenic enrichment cultures as the sole substrate using biomass from the anaerobic digesters as the inoculum. Methanol was also tested as a substrate to study the activity of *Crenarchaeota* under methylotrophic methanogenic condition. *CrenAD*-specific qPCR analysis showed that *Crenarchaeota* was detected in all enrichments, albeit at different abundance (Table IV10). The highest abundance of *Crenarchaeota* was found in the acetate enrichments, accounting for 1.21% of the archaeal populations. Evidently, *Crenarchaeota* was enriched as a minor archaeal population because its abundance was much greater than what would be expected from simple dilution during the enrichment process, i.e. ~0.05%.

In contrast, the formate enrichments exhibited the lowest relative abundance of *Crenarchaeota*, merely 0.02% of the archaeal populations (Table IV10), which was lower than the 0.05% expected from dilution, indicative of the lack of growth with formate as the substrate. Other substrates, including propionate, butyrate, and methanol, all supported *Crenarchaeota* as a minor archaeal population in the enrichments, providing evidence for the activity of *Crenarchaeota* in anaerobic digestion.

Correlation between *Crenarchaeota* and acetoclastic methanogens

Since previous studies suggested the association of *Crenarchaeota* with acetoclastic methanogens in anaerobic digestion (Collins et al., 2005), *Methanosaeta* and

Methanosarcina populations were quantified with qPCR as the primary acetoclastic methanogens in the enrichment cultures. With the only exception of the formate enrichments, acetoclastic methanogens were the dominant archaea in all enrichments, comprising greater than 50% of the archaeal populations (Fig. IV16). In particular, the acetate and butyrate enrichments had the highest abundance of acetoclastic methanogens, accounting for >80% of total archaea. In comparison, the acetoclastic methanogen abundance in the formate enrichments was negligible (Fig. IV16), which coincided with the very low counts of *Crenarchaeota* in the same formate enrichments (Table IV10). Thus, the presence of *Crenarchaeota* and acetoclastic methanogens appeared to be concurrent, even though the acetoclastic methanogens were much more abundant than *Crenarchaeota* in the enrichment cultures.

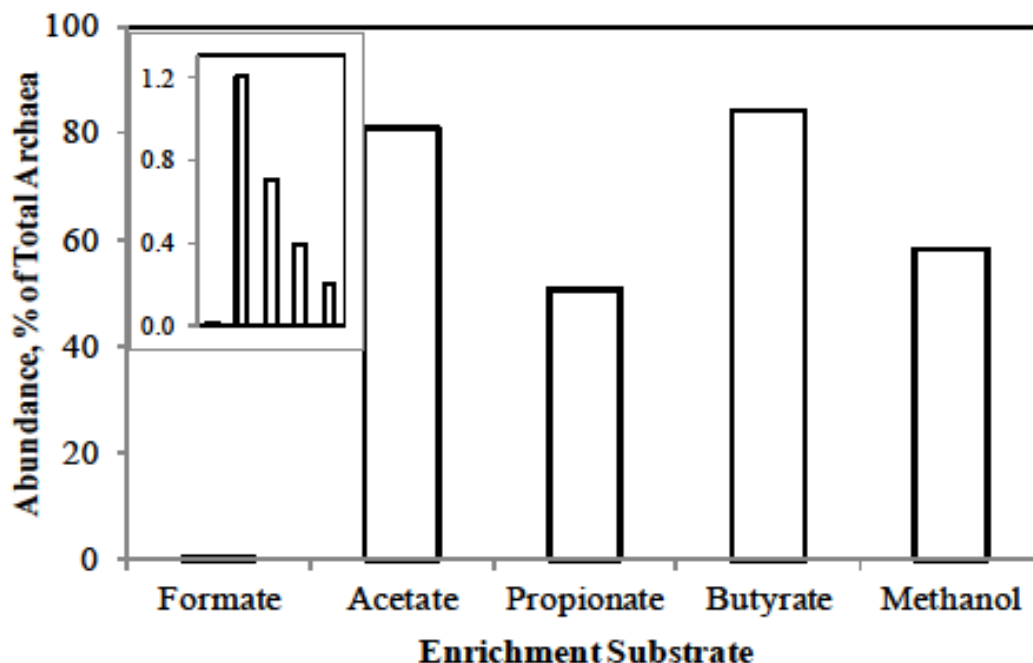


Fig. IV16. Abundance of acetoclastic methanogens in methanogenic enrichment cultures amended by various organic acids or methanol as the sole substrate. The inset shows the abundance of *Crenarchaeota* in the same enrichment cultures for comparison. Abundance of acetoclastic methanogens was determined as the sum of 16S rRNA genes copy numbers of *Methanosarcina* and *Methanosaeta* by qPCR.

Table IV10 Quantification of *Crenarchaeota* abundance in methanogenic enrichments with *CrenAD*-specific qPCR.

Enrichment ^a	No. of 16S rRNA Gene Copies/mL ^b		Relative Abundance (% of Total <i>Archaea</i>)
	Total <i>Archaea</i>	<i>Crenarchaeota</i>	
Formate	(1.2±0.1)×10 ⁹	(1.9±0.7)×10 ⁵	0.02±0.00
Acetate	(1.1±0.1)×10 ⁹	(1.3±0.1)×10 ⁷	1.21±0.19
Propionate	(1.7±0.2)×10 ⁹	(1.2±0.3)×10 ⁷	0.70±0.19
Butyrate	(2.7±0.1)×10 ⁹	(1.1±0.1)×10 ⁷	0.39±0.03
Methanol	(5.4±0.7)×10 ⁹	(1.1±0.3)×10 ⁷	0.20±0.06

^aEnrichments were established by a 10% (v/v) transfer of anaerobic digestate to fresh medium followed by 10 feedings of the enrichment substrate and subsequently a 2nd 10% (v/v) transfer to fresh medium followed by another 10 feedings of the enrichment substrate. Each feeding contained 50 mM of formate, 20 mM acetate, 20 mM propionate, 20 mM butyrate, or 30 mM methanol.

^bThe values are the means of triplicate samples ± standard deviation.

Discussion

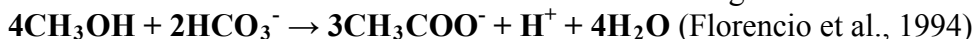
Mesophilic *Crenarchaeota* has received much attention due to its abundance in diverse habitats in both terrestrial and marine environments (DeLong, 1998). Some of these organisms are characterized by the ammonia oxidation metabolism, supporting the proposal of a novel phylum *Thaumarchaeota* to distinguish these archaea from other crenarchaeotes (Nicol & Schleper, 2006; Stahl & de la Torre, 2012). *Crenarchaeota* populations have also been frequently identified in the methanogenic environments of anaerobic digesters (Acharya et al., 2011; Chen et al., 2012; Chouari et al., 2005; Collins et al., 2005; Enright et al., 2009; Gomez et al., 2011; Leven et al., 2007; Schauer-Gimenez et al., 2010; Siggins et al., 2011; Zhang et al., 2011). Given the strictly anaerobic condition characteristic of anaerobic digestion, a lifestyle of ammonia oxidation metabolism, which requires oxygen, may not be likely for these organisms. Indeed, our phylogenetic analyses of all *Crenarchaeota* sequences identified in anaerobic digestion (*CrenAD*) showed that these populations formed a cluster distinct from that of *Thaumarchaeota* (Fig. IV11 and 12), consistent with the possibility of divergent ecological functions between *CrenAD* and *Thaumarchaeota*. Another important finding from these analyses was the grouping of all *CrenAD* in MCG-6 (Fig. IV13), allowing the development of *CrenAD*-specific qPCR (Fig. IV14 and Table IV9).

The population dynamics of *CrenAD* in anaerobic digesters treating animal waste (Fig. IV15), as revealed by qPCR quantification, provided the first indication of the activity of *Crenarchaeota* in these environments. The linkage between *CrenAD* and process conditions, such as acetate accumulation (Fig. IV15), further implicated the potential involvement of *CrenAD* in the anaerobic food web. The enrichment of *CrenAD* as a minor archaeal population using acetate as the sole substrate, by more than 20 fold from background level (Table IV10), adds to the evidence supporting the possible involvement of *CrenAD* in the utilization of organic acids.

Another interesting observation is that formate appeared as the only enrichment substrate that did not support *CrenAD* (Table IV10). Accordingly, formate was also the only substrate that did not support acetoclastic methanogens (Fig. IV16), which is expected given that formate is a substrate for hydrogenotrophic methanogenesis. In all other enrichments, concurrent presence of *Crenarchaeota* and acetoclastic methanogens was observed, which appears to be consistent with the suggested association between *Crenarchaeota* and acetoclastic methanogens, possibly as a form of symbiotic partnership in anaerobic digestion (Collins et al., 2005). Among the substrates tested in this study, propionate and butyrate are known to serve as substrates for acetate production in anaerobic metabolism following reactions such as:



Methanol can also be fermented to acetate with the following reactions as examples:





Thus, acetate would be available as an intermediate in enrichment cultures amended with propionate, butyrate, or methanol, and might subsequently link potential metabolic interactions between *Crenarchaeota* and acetoclastic methanogens. It has to be noted, however, that the acetoclastic methanogens identified in the methanol enrichments were dominated by *Methanosarcina* (data not shown). Since *Methanosarcina* is metabolically versatile (De Vrieze et al., 2012), it is likely that methylotrophic methanogenesis, instead of acetoclastic methanogenesis, was the primary metabolic pathway carried out by the methanogens in the methanol enrichments. Accordingly, metabolic interactions via acetate would be reduced, which might explain the relatively lower abundance of *Crenarchaeota* in the methanol enrichments as compared to the propionate or butyrate enrichments (Table IV10), with the assumption that *Crenarchaeota* and acetoclastic methanogens were metabolically related via acetate.

It should be noted that even though *CrenAD* experienced a substantial increase in abundance following enrichment, it remained a rather minor population, accounting for only 1.2% of the archaeal community in the acetate enrichments (Table IV10). Thus, instead of serving as a keystone population directly involved in acetate utilization, *CrenAD* might be more likely to be a minor constituent of the acetate-utilizing community, or involved in the metabolism of secondary metabolites from acetate-utilizing populations. Therefore, functions recently proposed for other MCG populations, such as the degradation of proteins and aromatics (Lloyd et al., 2013; Meng et al., 2014), may also be relevant to *CrenAD* given the potential availability of these substrates in anaerobic digestion. The relatively low abundance of *Crenarchaeota* in anaerobic digestion also assures the advantage of the *CrenAD*-specific qPCR over other tools with its inherited sensitivity for the monitoring of these organisms.

Some studies have reported *Crenarchaeota* as the predominant archaeal populations in anaerobic digestion, representing 20-50% of the archaeal abundance (Acharya et al., 2011; Collins et al., 2005; Enright et al., 2009; Schauer-Gimenez et al., 2010; Siggins et al., 2011). Maintaining such high levels of population abundance would require a major shift in the carbon and energy fluxes toward *Crenarchaeota*, which would be quite different from the anaerobic digesters examined in this study. To advance our understanding of the elusive ecophysiology of *Crenarchaeota*, efforts are need to identify process conditions that control the abundance of *Crenarchaeota* in anaerobic digestion processes, which can be aided by the *CrenAD*-specific qPCR developed in this study.

References

- Abram, F., Enright, A.M., O'Reilly, J., Botting, C.H., Collins, G., O'Flaherty, V. 2011. A metaproteomic approach gives functional insights into anaerobic digestion. *J Appl Microbiol*, **110**(6), 1550-60.
- Acharya, B.K., Pathak, H., Mohana, S., Shouche, Y., Singh, V., Madamwar, D. 2011. Kinetic modelling and microbial community assessment of anaerobic biphasic fixed film bioreactor treating distillery spent wash. *Water Res*, **45**(14), 4248-4259.
- Balk, M., Weijma, J., Stams, A.J.M. 2002. *Thermotoga lettingae* sp nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. *Int J Syst Evol Microbiol*, **52**, 1361-1368.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Chen, S., Zhu, Z., Park, J., Zhang, Z., He, Q. 2014. Development of Methanoculleus-Specific Real-Time Quantitative PCR Assay for Assessing Methanogen Communities in Anaerobic Digestion. *J Appl Microbiol*, n/a-n/a.
- Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., Sghir, A. 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. *Environ Microbiol*, **7**(8), 1104-1115.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res*, **37**, D141-D145.
- Collins, G., Kavanagh, S., McHugh, S., Connaughton, S., Kearney, A., Rice, O., Carrigg, C., Scully, C., Bhreathnach, N., Mahony, T., Madden, P., Enright, A.M., O'Flaherty, V. 2006. Accessing the black box of microbial diversity and ecophysiology: recent advances through polyphasic experiments. *J Environ Sci Health A Tox Hazard Subst Environ Eng*, **41**(5), 897-922.
- Collins, G., Mahony, T., McHugh, S., Gieseke, A., de Beer, D., O'Flaherty, V. 2005. Distribution, dynamics and in situ ecophysiology of Crenarchaeota in anaerobic wastewater treatment granular biofilms. *Water Science and Technology*, **52**(7), 233-239.
- Collins, G., Woods, A., McHugh, S., Carton, M.W., O'Flaherty, V. 2003. Microbial community structure and methanogenic activity during start-up of psychrophilic anaerobic digesters treating synthetic industrial wastewaters. *FEMS Microbiol Ecol*, **46**(2), 159-70.
- De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W. 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. *Bioresour Technol*, **112**(0), 1-9.
- DeLong, E.F. 1998. Everything in moderation: Archaea as 'non-extremophiles'. *Curr Opin Genet Dev*, **8**(6), 649-654.
- Demirel, B., Scherer, P., Yenigun, O., Onay, T.T. 2010. Production of Methane and Hydrogen from Biomass through Conventional and High-Rate Anaerobic Digestion Processes. *Crit Rev Environ Sci Technol*, **40**(2), 116-146.
- Enright, A.M., McGrath, V., Gill, D., Collins, G., O'Flaherty, V. 2009. Effect of seed sludge and operation conditions on performance and archaeal community

- structure of low-temperature anaerobic solvent-degrading bioreactors. *Syst Appl Microbiol*, **32**(1), 65-79.
- Florencio, L., Field, J.A., Lettinga, G. 1994. Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. *Appl Environ Microbiol*, **60**(1), 227-234.
- Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(41), 14683-14688.
- Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F., Moletta, R. 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol*, **63**(7), 2802-2813.
- Gomez, E., Martin, J., Michel, F.C. 2011. Effects of organic loading rate on reactor performance and archaeal community structure in mesophilic anaerobic digesters treating municipal sewage sludge. *Waste Manage Res*, **29**(11), 1117-1123.
- He, Q., Sanford, R.A. 2004. Acetate threshold concentrations suggest varying energy requirements during anaerobic respiration by *Anaeromyxobacter dehalogenans*. *Appl Environ Microbiol*, **70**(11), 6940-6943.
- He, Q., Sanford, R.A. 2002. Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. *Biodegradation*, **13**(5), 307-316.
- Huang, L.N., Chen, Y.Q., Zhou, H., Luo, S., Lan, C.Y., Qu, L.H. 2003. Characterization of methanogenic Archaea in the leachate of a closed municipal solid waste landfill. *FEMS Microbiol Ecol*, **46**(2), 171-177.
- Inagaki, F., Suzuki, M., Takai, K., Oida, H., Sakamoto, T., Aoki, K., Nealson, K.H., Horikoshi, K. 2003. Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. *Appl Environ Microbiol*, **69**(12), 7224-7235.
- Kubo, K., Lloyd, K.G., Biddle, J.F., Amann, R., Teske, A., Knittel, K. 2012. Archaea of the Miscellaneous Crenarchaeotal Group are abundant, diverse and widespread in marine sediments. *Isme Journal*, **6**(10), 1949-1965.
- Leven, L., Eriksson, A.R.B., Schnurer, A. 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *FEMS Microbiol Ecol*, **59**(3), 683-693.
- Li, T., Mazeas, L., Sghir, A., Leblon, G., Bouchez, T. 2009. Insights into networks of functional microbes catalysing methanization of cellulose under mesophilic conditions. *Environ Microbiol*, **11**(4), 889-904.
- Lloyd, K.G., Schreiber, L., Petersen, D.G., Kjeldsen, K.U., Lever, M.A., Steen, A.D., Stepanauskas, R., Richter, M., Kleindienst, S., Lenk, S., Schramm, A., Jorgensen, B.B. 2013. Predominant archaea in marine sediments degrade detrital proteins. *Nature*, **496**(7444), 215-218.
- McKeown, R.M., Scully, C., Enright, A.M., Chinalia, F.A., Lee, C., Mahony, T., Collins, G., O'Flaherty, V. 2009. Psychrophilic methanogenic community development during long-term cultivation of anaerobic granular biofilms. *Isme Journal*, **3**(11), 1231-1242.
- Meng, J., Xu, J., Qin, D., He, Y., Xiao, X., Wang, F. 2014. Genetic and functional

- properties of uncultivated MCG archaea assessed by metagenome and gene expression analyses. *Isme Journal*, **8**(3), 650-659.
- Narihiro, T., Sekiguchi, Y. 2011. Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea. *Microbial Biotechnology*, **4**(5), 585-602.
- Nicol, G.W., Schleper, C. 2006. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol*, **14**(5), 207-212.
- Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L., Schleper, C. 2003. Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol*, **5**(9), 787-797.
- Pester, M., Schleper, C., Wagner, M. 2011. The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. *Curr Opin Microbiol*, **14**(3), 300-306.
- Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *Isme Journal*, **3**(6), 700-714.
- Schauer-Gimenez, A.E., Zitomer, D.H., Maki, J.S., Struble, C.A. 2010. Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. *Water Res*, **44**(12), 3555-3564.
- Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev*, **61**(2), 262-&.
- Siggins, A., Enright, A.-M., O'Flaherty, V. 2011. Low-temperature (7 degrees C) anaerobic treatment of a trichloroethylene-contaminated wastewater: Microbial community development. *Water Res*, **45**(13), 4035-4046.
- Stahl, D.A., de la Torre, J.R. 2012. Physiology and Diversity of Ammonia-Oxidizing Archaea. *Annual Review of Microbiology*, Vol 66, **66**, 83-101.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*, **24**(8), 1596-1599.
- Wolin, E.A., Wolin, M.J., Wolfe, R.S. 1963. Formation of Methane by Bacterial Extracts. *J Biol Chem*, **238**(8), 2882-2886.
- Yenigun, O., Demirel, B. 2013. Ammonia inhibition in anaerobic digestion: A review. *Process Biochem*, **48**(5-6), 901-911.
- Yu, Y., Lee, C., Kim, J., Hwang, S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*, **89**(6), 670-679.
- Zhang, Y., He, Q. 2013. Characterization of bacterial diversity in drinking water by pyrosequencing. *Water Science and Technology-Water Supply*, **13**(2), 358-367.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.
- Ziganshin, A., Schmidt, T., Scholwin, F., Il'inskaya, O., Harms, H., Kleinsteuber, S. 2011. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. *Appl Microbiol Biotechnol*, **89**(6), 2039-2052.

Appendix II

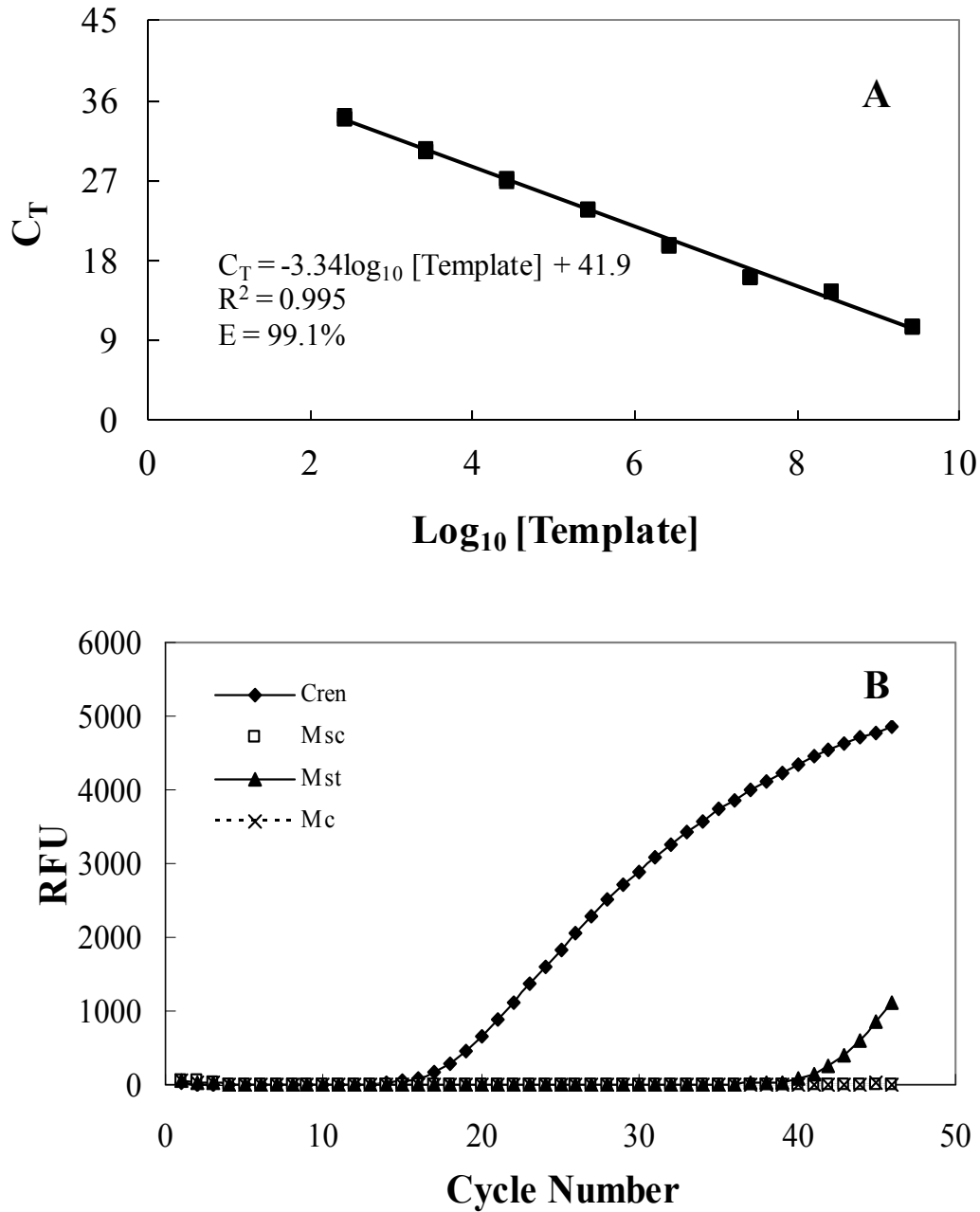


Fig. IVS1. Amplification efficiency (E) and specificity of the *CrenAD*-specific qPCR. A) Amplification efficiency as determined with the C_T -Log[Template] plot derived from the quantification of 10-fold dilution series of *CrenAD* 16S rRNA gene templates; and B) Specific detection of *CrenAD* as increases in fluorescence using 16S rRNA gene templates of 4 representative archaeal groups—*Methanoculleus* (Mc), *Methanosarcina* (Msc), *Methanosaeta* (Mst) and *Crenarchaeota* (Cren). RFU: relative fluorescence unit.

Table IVS1 List of *Crenarchaeota* 16S rRNA gene sequences from anaerobic digestion processes.

No	GeneBank Accession #	Reference
1	AJ576215	(Huang et al., 2003)
2	AY161252	(Collins et al., 2003)
3	AY161253	(Collins et al., 2003)
4	AY161254	(Collins et al., 2003)
5	AY161257	(Collins et al., 2003)
6	AY161258	(Collins et al., 2003)
7	AY228683	(Collins et al., 2006)
8	AY835784	(Collins et al., 2005)
9	AY835787	(Collins et al., 2005)
10	AY835789	(Collins et al., 2005)
11	AY835793	(Collins et al., 2005)
12	AY835803	(Collins et al., 2005)
13	CT573519	(Chouari et al., 2005)
14	CT573562	(Chouari et al., 2005)
15	CT573588	(Chouari et al., 2005)
16	CT573607	(Chouari et al., 2005)
17	CT573608	(Chouari et al., 2005)
18	CT573612	(Chouari et al., 2005)
19	CT573626	(Chouari et al., 2005)
20	CT573631	(Chouari et al., 2005)
21	CT573639	(Chouari et al., 2005)
22	CT573651	(Chouari et al., 2005)
23	CT573661	(Chouari et al., 2005)
24	CT573665	(Chouari et al., 2005)
25	CT573667	(Chouari et al., 2005)
26	CT573676	(Chouari et al., 2005)
27	CT573677	(Chouari et al., 2005)
28	CT573708	(Chouari et al., 2005)
29	CT573709	(Chouari et al., 2005)
30	CT573719	(Chouari et al., 2005)
31	CT573721	(Chouari et al., 2005)
32	CT573737	(Chouari et al., 2005)
33	CT573742	(Chouari et al., 2005)
34	CT573757	(Chouari et al., 2005)
35	CT573764	(Chouari et al., 2005)
36	CT573765	(Chouari et al., 2005)
37	CU915923	(Riviere et al., 2009)
38	CU915991	(Riviere et al., 2009)
39	CU916006	(Riviere et al., 2009)
40	CU916119	(Riviere et al., 2009)
41	CU916196	(Riviere et al., 2009)
42	CU916729	(Riviere et al., 2009)
43	CU916735	(Riviere et al., 2009)
44	CU916756	(Riviere et al., 2009)
45	CU916760	(Riviere et al., 2009)

Table IVS1. Continued.		
No	GeneBank Accession #	Reference
46	CU916767	(Riviere et al., 2009)
47	CU916771	(Riviere et al., 2009)
48	CU916785	(Riviere et al., 2009)
49	CU916788	(Riviere et al., 2009)
50	CU916834	(Riviere et al., 2009)
51	CU916855	(Riviere et al., 2009)
52	CU916859	(Riviere et al., 2009)
53	CU916860	(Riviere et al., 2009)
54	CU916870	(Riviere et al., 2009)
55	CU916895	(Riviere et al., 2009)
56	CU916897	(Riviere et al., 2009)
57	CU916900	(Riviere et al., 2009)
58	CU916913	(Riviere et al., 2009)
59	CU916918	(Riviere et al., 2009)
60	CU916920	(Riviere et al., 2009)
61	CU916921	(Riviere et al., 2009)
62	CU916926	(Riviere et al., 2009)
63	CU916928	(Riviere et al., 2009)
64	CU916930	(Riviere et al., 2009)
65	CU916944	(Riviere et al., 2009)
66	CU916960	(Riviere et al., 2009)
67	CU916962	(Riviere et al., 2009)
68	CU916967	(Riviere et al., 2009)
69	CU916979	(Riviere et al., 2009)
70	CU917024	(Riviere et al., 2009)
71	CU917031	(Riviere et al., 2009)
72	CU917038	(Riviere et al., 2009)
73	CU917078	(Riviere et al., 2009)
74	CU917080	(Riviere et al., 2009)
75	CU917084	(Riviere et al., 2009)
76	CU917089	(Riviere et al., 2009)
77	CU917091	(Riviere et al., 2009)
78	CU917124	(Riviere et al., 2009)
79	CU917128	(Riviere et al., 2009)
80	CU917135	(Riviere et al., 2009)
81	CU917155	(Riviere et al., 2009)
82	CU917175	(Riviere et al., 2009)
83	CU917186	(Riviere et al., 2009)
84	CU917205	(Riviere et al., 2009)
85	CU917213	(Riviere et al., 2009)
86	CU917217	(Riviere et al., 2009)
87	CU917220	(Riviere et al., 2009)
88	CU917246	(Riviere et al., 2009)
89	CU917251	(Riviere et al., 2009)
90	DQ386714	(McKeown et al., 2009)
91	DQ386716	(McKeown et al., 2009)
92	DQ386726	(McKeown et al., 2009)

Table IVS1. Continued.		
No	GeneBank Accession #	Reference
93	DQ386728	(McKeown et al., 2009)
94	DQ661687	(Leven et al., 2007)
95	DQ679927	(McKeown et al., 2009)
96	DQ679928	(McKeown et al., 2009)
97	EF552166	(Li et al., 2009)
98	EU118565	Unpublished
99	EU118566	Unpublished
100	EU118570	Unpublished
101	EU118573	Unpublished
102	EU118575	Unpublished
103	EU118579	Unpublished
104	EU118580	Unpublished
105	EU118583	Unpublished
106	EU118586	Unpublished
107	EU118587	Unpublished
108	EU662668	Unpublished
109	EU662672	Unpublished
110	EU662680	Unpublished
111	EU662686	Unpublished
112	EU662691	Unpublished
113	EU662693	Unpublished
114	EU662694	Unpublished
115	EU912847	Unpublished
116	EU912851	Unpublished
117	FJ005026	Unpublished
118	GQ406367	Unpublished
119	GQ406371	Unpublished
120	GQ406372	Unpublished
121	GQ465434	Unpublished
122	GQ465435	Unpublished
123	GQ470578	(Schauer-Gimenez et al., 2010)
124	GQ470579	(Schauer-Gimenez et al., 2010)
125	GQ470580	(Schauer-Gimenez et al., 2010)
126	GQ470581	(Schauer-Gimenez et al., 2010)
127	GQ470582	(Schauer-Gimenez et al., 2010)
128	GQ470583	(Schauer-Gimenez et al., 2010)
129	GQ470584	(Schauer-Gimenez et al., 2010)
130	GQ470594	(Schauer-Gimenez et al., 2010)
131	GQ470595	(Schauer-Gimenez et al., 2010)
132	GQ470596	(Schauer-Gimenez et al., 2010)
133	GQ470597	(Schauer-Gimenez et al., 2010)
134	GQ470598	(Schauer-Gimenez et al., 2010)
135	GQ470599	(Schauer-Gimenez et al., 2010)
136	GU196153	(Schauer-Gimenez et al., 2010)
137	GU196155	(Schauer-Gimenez et al., 2010)
138	GU196159	(Schauer-Gimenez et al., 2010)
139	GU196161	(Schauer-Gimenez et al., 2010)

Table IVS1. Continued.		
No	GeneBank Accession #	Reference
140	GU196165	(Schauer-Gimenez et al., 2010)
141	GU196170	(Schauer-Gimenez et al., 2010)
142	GU196171	(Schauer-Gimenez et al., 2010)
143	GU196173	(Schauer-Gimenez et al., 2010)
144	GU196174	(Schauer-Gimenez et al., 2010)
145	GU196175	(Schauer-Gimenez et al., 2010)
146	GU196176	(Schauer-Gimenez et al., 2010)
147	GU196177	(Schauer-Gimenez et al., 2010)
148	GU196179	(Schauer-Gimenez et al., 2010)
149	GU196182	(Schauer-Gimenez et al., 2010)
150	GU196186	(Schauer-Gimenez et al., 2010)
151	GU196189	(Schauer-Gimenez et al., 2010)
152	GU196191	(Schauer-Gimenez et al., 2010)
153	GU475150	(Zhang et al., 2011)
154	GU475151	(Zhang et al., 2011)
155	GU475152	(Zhang et al., 2011)
156	GU475153	(Zhang et al., 2011)
157	GU475154	(Zhang et al., 2011)
158	GU475155	(Zhang et al., 2011)
159	GU475156	(Zhang et al., 2011)
160	GU475157	(Zhang et al., 2011)
161	GU475158	(Zhang et al., 2011)
162	GU475159	(Zhang et al., 2011)
163	GU475160	(Zhang et al., 2011)
164	GU475161	(Zhang et al., 2011)
165	GU475162	(Zhang et al., 2011)
166	GU475163	(Zhang et al., 2011)
167	GU475164	(Zhang et al., 2011)
168	GU475165	(Zhang et al., 2011)
169	GU475166	(Zhang et al., 2011)
170	GU475167	(Zhang et al., 2011)
171	GU475168	(Zhang et al., 2011)
172	GU475169	(Zhang et al., 2011)
173	GU475170	(Zhang et al., 2011)
174	GU475171	(Zhang et al., 2011)
175	GU475172	(Zhang et al., 2011)
176	GU475177	(Zhang et al., 2011)
177	GU475179	(Zhang et al., 2011)
178	GU475180	(Zhang et al., 2011)
179	GU946456	(Abram et al., 2011)
180	GU982665	Unpublished
181	GU982672	Unpublished
182	HM218930	(Acharya et al., 2011)
183	HM218932	(Acharya et al., 2011)
184	HM218933	(Acharya et al., 2011)
185	HM218934	(Acharya et al., 2011)
186	HM218935	(Acharya et al., 2011)

Table IVS1. Continued.		
No	GeneBank Accession #	Reference
187	HM218937	(Acharya et al., 2011)
188	HM218938	(Acharya et al., 2011)
189	HM218947	(Acharya et al., 2011)
190	HM218948	(Acharya et al., 2011)
191	HM749806	(Siggins et al., 2011)
192	HM749807	(Siggins et al., 2011)
193	HM749811	(Siggins et al., 2011)
194	HM749818	(Siggins et al., 2011)
195	HM749823	(Siggins et al., 2011)
196	HM749824	(Siggins et al., 2011)
197	HQ008060	Unpublished
198	HQ008069	Unpublished
199	HQ008071	Unpublished
200	HQ270538	(Siggins et al., 2011)
201	HQ270541	(Siggins et al., 2011)
202	HQ270545	(Siggins et al., 2011)
203	HQ290289	(Ziganshin et al., 2011)
204	HQ315890	(Siggins et al., 2011)
205	HQ315896	(Siggins et al., 2011)
206	JN052741	(Chen et al., 2012)
207	JN052742	(Chen et al., 2012)
208	JN052743	(Chen et al., 2012)
209	JN052744	(Chen et al., 2012)
210	JN052745	(Chen et al., 2012)
211	JN052746	(Chen et al., 2012)
212	JN052747	(Chen et al., 2012)
213	JN052748	(Chen et al., 2012)
214	JN052749	(Chen et al., 2012)
215	JN052750	(Chen et al., 2012)
216	JN052751	(Chen et al., 2012)
217	JN052752	(Chen et al., 2012)
218	JN052753	(Chen et al., 2012)
219	JN052754	(Chen et al., 2012)
220	U81774	(Godon et al., 1997)

Chapter V

Dominance of *Methanosaeta* at High Acetate Concentrations in Anaerobic Digestion

Part I: Dynamics of Major Methanogenic Populations in Anaerobic Digesters with Organic Loading Fluctuation

Abstract

Two sets of triplicate CSTR anaerobic digesters, one set of control digester fed with dairy farm waste as sole substrate and another set of co-digesters fed with dairy farm and poultry wastes were set up in mesophilic conditions (35°C). Throughout the whole operational period, performance of control digesters was kept stable with constant organic loading rate (OLR). For co-digesters, OLR increased stepwise to enhance the biogas production until the performance was deteriorated by acetate accumulation. To recover the co-digester performance due to organic overloading, OLR was stopped at first and then maintained at lower level. Finally, the stable performance of co-digesters was reached under optimum OLR after the co-digesters were fully recovered from OLR perturbation. To study dynamics of methanogenic populations in both sets of digesters during whole operational period, there were total 15 time points were selected for both types of digester to cover different stages of performances. Revealed by 16S rRNA specific qPCR assays, *Methanosaeta* was persistent and predominant in both sets of digesters throughout the whole operational period even under co-digester performance collapse with acetate accumulation, which seemed to be inconsistent with previous studies and unexplainable based on conventional acetoclastic methanogenesis model. Although relative abundances of *Methanosarcina* and *Methanoculleus* among total archaeal populations increased during co-digester performance collapse with considerably high acetate concentration, they still can not compete over *Methanosaeta*. Moreover, the relatively small percentages of *Methanoculleus* may indicate inconsiderable amount of CH₄ attributed to hydrogenotrophic methanogenesis. As expected, stable compositions of *Methanosaeta*, *Methanosarcina* and *Methanoculleus* in control digesters were in according with their stable performance and operational conditions.

Keywords: Anaerobic co-digestion, Acetate concentration, Organic overloading, qPCR, *Methanosaeta*, *Methanosarcina*, *Methanoculleus*

Introduction

Anaerobic digestion of organic wastes such as dairy farm and poultry wastes (Chen et al., 2012; Zhang et al., 2011) is a well-established technology and has been applied in sustainable management worldwide (Verstraete et al., 2005). This technology not only reduces the waste volume, but also produces biogas containing mainly CH₄ and CO₂, which has the potential to become a critical renewable energy source (Panwar et al., 2011). The biomethane is mainly produced by acetoclastic and hydrogenotrophic methanogens (Demirel & Scherer, 2008b) by utilizing H₂, CO₂ and short chain fatty acids such as acetate, formate, which are generated as the byproducts by fermentative bacteria through a series of reactions of organic substrate degradation including acidogenesis and acetogenesis (Appels et al., 2008).

Typically, it is estimated that approximately two third or more of total CH₄ generated from natural environments or engineering systems via the acetoclastic pathway while the rest of it produced by hydrogenotrophic methanogenesis (Jeris & McCarty, 1965; Parkin & Owen, 1986; Smith & Mah, 1966). There are two representative acetoclastic methanogens, *Methanosaeta* and *Methanosarcina*. Due to the lower maximum specific growth rate ($\mu_{\max}$) and half saturation constant (K_S) or higher acetate affinity (Conklin et al., 2006; Smith & Ingram-Smith, 2007; Thauer et al., 2008), the obligate acetoclastic methanogen, *Methanosaeta* were found to be predominant in anaerobic digesters with stable performance and low acetate concentration (less than 1mM) (Conklin et al., 2006; McHugh et al., 2003; McMahon et al., 2004). Having higher $\mu_{\max}$ and K_S (Conklin et al., 2006; De Vrieze et al., 2012), *Methanosarcina*, with versatile methanogenic pathways (De Vrieze et al., 2012) usually competed over *Methanosaeta* during digester starting up or unstable performance with fatty acid accumulation (Conklin et al., 2006; De Vrieze et al., 2012; Griffin et al., 1998; McMahon et al., 2004). Moreover, under some circumstances, it was observed that *Methanosarcina* may shift its methanogenic mechanism from acetoclastic to hydrogenotrophic pathway associated with syntrophic acetate oxidation under high acetate concentrations (De Vrieze et al., 2012; Hattori, 2008; Weimer & Zeikus, 1978; Winter & Wolfe, 1979).

Besides acetoclastic methanogens, the typical hydrogenotrophic methanogen, *Methanoculleus* was widely distributed into anaerobic digesters (Chen et al., 2012; Chen et al., 2014; Zhang et al., 2011). Its relative abundances reported in anaerobic digestion process varied significantly from dominant to rare populations (Cardinali-Rezende et al., 2012; Sundberg et al., 2013; Wirth et al., 2012) indicating the pathways shift of methanogenesis. Under high acetate concentrations, *Methanoculleus* was observed to be dominant in association with syntrophic acetate oxidation (Kim et al., 2014; Schnürer et al., 1999).

In this study, two sets of laboratory scale CSTR anaerobic digesters were set up in mesophilic condition (35°C). One set of control digesters fed with dairy farm wastes was maintained under constant operational conditions. Another set treated as co-digesters was added with poultry wastes as the co-substrate. OLR was stepwise elevated by increasing the fraction of poultry waste until performance of co-digesters collapsed. To recover the performance from organic overloading, OLR was stopped at first and then maintained at low level with only dairy farm waste feeding. Finally, the enhanced biogas production from co-digester was re-achieved with optimum OLR with poultry waste adding, when co-digesters were fully recovered from OLR perturbation. To reveal the dynamics of relative abundances of typical methanogens including *Methanosaeta*, *Methanosarcina* and *Methanoculleus* in both sets of digesters, there were total 15 time points selected to cover the entire operational phase under different OLRs. The 16S rRNA specific qPCR assays were implemented to quantify the methanogenic populations.

Materials and Methods

Anaerobic digesters operation and biomass sampling

One set of triplicate mesophilic continuous anaerobic digesters were established with dairy waste as the sole substrate and all operational conditions were kept constant over the whole experimental period. Another set of triplicate co-digesters were fed with dairy farm waste as the primary substrate and poultry waste as the co-substrate following the configuration described previously (Chen et al., 2012; Zhang et al., 2011). Due to the higher solids content of poultry waste as compared with the primary substrate dairy waste, the enhancement of biogas production can be fulfilled in the co-digestion process (Chen et al., 2012). All six digesters had a working volume of 3.6 L and were operated in a constant temperature room at 35°C and with hydraulic retention time at 20 days.

Initially, all digesters were initiated with inoculum from an operating laboratory scale dairy manure anaerobic digester and established using dilute dairy waste as the only feed. After the stable anaerobic digestion performance was achieved, poultry waste was added as an organic-rich co-substrate into co-digesters to achieve enhanced methane production. Prior to stepwise increases of poultry waste loading, all co-digesters should exhibit stable performance with consistent pH, methane yield and volatile fatty acids (VFAs). In the beginning, the organic loading rate (OLR) of poultry waste was 0.3 g VS L⁻¹day⁻¹. With the biogas production enhancement, the OLR of poultry waste was increase stepwise to 0.5 g VS L⁻¹day⁻¹ and subsequently to 0.8 g VS L⁻¹day⁻¹, at which stable performance was disrupted by substrate overloading due to the organic overloading at excessive levels. Meanwhile, the OLR of the dairy farm waste was kept constant for all six digesters at 1.0g VS L⁻¹day⁻¹. To recover the performance of co-digesters from disturbance, feeding of co-digesters was then stopped and later restored stepwise from 0.5, to 1.0 g VS L⁻¹day⁻¹ when stable operation was again achieved. Dairy waste was the only substrate during the recovery process. Subsequently, poultry waste was added into co-digesters again to raise the total OLR from 1.0 to 1.5 g VS L⁻¹day⁻¹. During the whole process, another set of triplicate digesters considered as control digesters was operated with constant conditions and only fed with dairy farm waste at OLR, 1.0 g VS L⁻¹day⁻¹.

To analyze the microbial community dynamics in response to OLR perturbations during the whole digester operational period (500 days approximately), there were mainly 15 time points chosen to include all digester operational stages under different OLRs. For each time point, samples from both of the control and co-digesters were preserved. The scheme of the digestate sampling was summarized in Table V1.

Chemical analysis

Biogas production from the anaerobic digesters was used as the primary parameter to monitor digestion performance (Michaud et al., 2002) and was determined using a water-displacement method described previously (Zhu et al., 2011). VFAs in the digestate were quantified using a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and a Restek Stabilwax ®-DA column as previously described (He et al., 2009). Methane content in biogas was analyzed using a Hewlett Packard 5890

Series II gas chromatograph equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen[®]-1000; Sigma-Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 ml min⁻¹ and the following temperature scheme: oven 125°C, injection port 150°C and detector 170°C.

Table V1 Time points for both of the control and co-digester samplings.

Sample ID	OLR (Control) ^a	OLR (co-digester) ^a	Operation day	Operational status (co-digester) ^b
1	1.0	1.0	45	Stable performance before perturbation
2	1.0	1.3	73	
3	1.0	1.5	90	
4	1.0	1.8	111	Optimum biogas production
5	1.0	1.8	122	Performance collapsed
6	1.0	1.8	132	
7	1.0	0	146	
8	1.0	0.5	167	Performance recovering
9	1.0	0.5	252	
10	1.0	1.0	290	
11	1.0	1.5	326	Performance stabilization
12	1.0	1.5	347	
13	1.0	1.5	395	Performance fully stabilized
14	1.0	1.5	453	
15	1.0	1.5	501	

^a OLR, Organic loading rate, gVS/L/day.

^b The status for control digesters is constant stable performance during the whole operation period due to the constant OLR.

Table V2 List of the primer and probe sets for TaqMan real time PCR assays in this study.

Name	Target group	Sequence (5'-3')	<i>E. coli</i> No ^d	T _m (°C) ^e	GC (%)	Amplicon size (bp)	Reference
Arc-787F ^a	<i>Archaea</i>	ATTAGATACCCSBGTAGTCC	787-806	61.0	45	273	[45]
Arc-915P ^b		AGGAATTGGCGGGGGAGCAC	915-934	70.1	65		
Arc-1059R ^c		GCCATGCACCWCCTCT	1044-1059	62.3	63		
Mst-702F ^a	<i>Methanosaeta</i>	TAATCCTTGAAGGACCACCA	702-721	61.0	45	161	[45]
Mst-753P ^b		ACGGCAAGGGACGAAAGCTAGG	753-774	70.0	59		
Mst-862R ^c		CCTACGGCACCACCAAC	846-862	62.0	65		
Msc-586F ^a	<i>Methanosarcina</i>	CGGTTTGGTCAGTCCTCCG	586-604	61.6	63	257	Design for this study
Msc-743P ^b		AACGGGTTCGACGGTGAGGGACGA	743-766	70.6	63		
Msc-842R ^c		ACCAGACACGGTCGCGC	826-842	59.8	71		
Mc-274F ^a	<i>Methanoculleus</i>	GGAGCAAGAGCCCCGGAGT	274-291	60.8	67	223	[206]
Mc-361P ^b		CGTGATAAGGGAACCTCGAGTGCCT	361-385	69.1	56		
Mc-477R ^c		CCAATAAAAGTGGCCACCACT	477-497	59.5	48		

^a Forward primers.^b 6-carboxyfluorescein (FAM)-BHQ1 probes.^c Reverse primers.^d Position in the 16S rRNA of *E. coli*.^e Melting temperatures calculated with the nearest-neighbor thermodynamic values method using the Oligo Calculator.**Table V3** Summary of TaqMan real time PCR standard curves with primer and probe sets applied in this study.

Sets	Linear range (copy/μL) ^a	Slope ^a	R ² ^a	Intercept ^a	Efficiency (%) ^{a, b}	The closest relatives in GenBank ^c
Arc	8.08×10 ² – 8.08×10 ⁹	-3.548 ± 0.017	0.995 ± 0.002	44.245 ± 0.465	91.36 ± 0.60	<i>Methanosaeta concilii</i> (CP002565) (98%)
Mst	3.58×10 ² – 3.58×10 ⁹	-3.382 ± 0.100	0.996 ± 0.002	41.084 ± 0.617	97.56 ± 4.00	<i>Methanosaeta concilii</i> (CP002565) (98%)
Msc	5.55×10 ² – 5.55×10 ⁹	-3.351 ± 0.119	0.988 ± 0.007	41.505 ± 1.357	98.81 ± 5.09	<i>Methanosarcina barkeri</i> (AF028692) (98%)
Mc	5.53×10 ² – 5.53×10 ⁹	-3.490 ± 0.112	0.994 ± 0.002	42.919 ± 0.830	93.42 ± 4.05	<i>Methanoculleus palmolei</i> (NR_028253) (98%)

^a All the values are expressed as average ± standard deviation.^b The amplification efficiency is calculated by the equation, Efficiency= [10^(-1/slope)-1]×100%.^c The representative 16S rRNA sequences with highest similarities in GeneBank (<http://blast.ncbi.nlm.nih.gov>). The accession numbers and the similarity are indicated in the parentheses.

TaqMan qPCR analysis

Biomass from anaerobic digesters and enrichment cultures was pelleted by centrifugation and stored at -80°C . Whole community DNA was extracted and purified as previously described (Zhang & He, 2013). The major acetoclastic and hydrogenotrophic methanogen populations, *Methanosarcina*, *Methanosaeta* and *Methanoculleus* were quantified with the primer/probe sets listed in Table V2. To determine the relative abundance of methanogen populations in the archaeal community, total archaea was quantified with the *Archaea* domain-specific TaqMan primer/probe set Arc787F-Arc915P - Arc1059R as previously described (shown in Table V1). All primers and dual-labeled TaqMan probe, 5'-end labeled with 6-carboxyfluorescein (FAM) and 3'-end labeled with the Black Hole Quencher (BHQ1), were purchased from Biosearch Technologies (Novato, California, USA). All qPCR assays were performed in 25- μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, and Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA). Thermal cycling consisted of a starting incubation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by up to 45 cycles at 95°C for 30 s and 60°C (for all primer/probe sets) for 45 s. The qPCR procedure was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as previously described (Chen et al., 2014). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T).

DNA templates used as the standards for assessing the efficiency and specificity of the methanogen specific qPCR were partial 16S rRNA genes of representative archaeal populations cloned from the continuous bench-scale anaerobic digesters in a previous study (Chen et al., 2012), including, *Methanosaeta* (GenBank Accession No. JN052761), *Methanosarcina* (GenBank Accession No. JN052757) and *Methanoculleus* (GenBank Accession No. JN052755). Amplification efficiency (E) of the qPCR assay was determined with the $\text{CT-Log}[\text{Template}]$ plot derived from the quantification of 10-fold dilution series of 16S rRNA gene templates (Chen et al., 2014). To eliminate the influences of the DNA extraction efficiency and biomass sampling biases on 16SrRNA gene quantification, the relative percentage of each methanogen population among total archaeal population was converted instead of applying absolute number.

Results

Digester performance

As expected, biogas productions increased gradually for both sets of digesters under the same OLR, $1.0\text{gVS L}^{-1}\text{day}^{-1}$ during starting up period (0 ~ 45days) (Fig. V1). After the steady state of the biogas production reached, 1000 mL/day, the OLR of co-digesters was increased stepwise from 1.3 to $1.8\text{ gVS L}^{-1}\text{day}^{-1}$ by adding poultry litters (Fig. V1). Correspondingly, the biogas from co-digesters increased from 1500 to 2500 mL/day. Before the elevation of the OLR, the stable biogas producing rate under the previous level should be achieved. Under the maximum OLR, $1.8\text{ gVS L}^{-1}\text{day}^{-1}$, however, the

continuous feeding organic rich substrate, poultry litter into co-digesters interfered the reaction balance between fermentation and methanogenesis. The biogas production from co-digesters, therefore, was deteriorated due to the accumulation of volatile fatty acids, which may become toxic to methanogens (Chen et al., 2012; Zhang et al., 2011).

In order to recover the methanogenic performance of co-digesters, the organic loading of co-digesters was ceased and sodium hydroxide solution was spiked into co-digesters to neutralize the pH inside. After 10 days, the OLR of co-digesters was restored at half of the control digesters, $0.5 \text{ gVS L}^{-1}\text{day}^{-1}$. Fortunately, biogas production was resumed and the rates became stable at $450 \sim 550 \text{ mL/day}$ in proportion to the biogas production rates of control digesters fed with constant OLR, $1.0 \text{ gVS L}^{-1}\text{day}^{-1}$ (Fig. V1). After more than three months of recovering process with relatively low OLR, the OLR of co-digesters was increased to the initial level during digesters setting up, $1.0 \text{ gVS L}^{-1}\text{day}^{-1}$. The identical biogas production rate of co-digesters with control digesters indicated the fully recovered methanogenic activities through the deterioration of the microbes with organic loading perturbations. To promote biogas production through co-digestion, organic rich substrate, poultry litter was added into dairy farm waste and reloaded into co-digesters at the optimum loading rates, $1.5 \text{ gVS L}^{-1}\text{day}^{-1}$ after maintained at $1.0 \text{ gVS L}^{-1}\text{day}^{-1}$ for almost 40 days. As compared to the biogas production rate of control digesters with only dairy farm waste feeding, it increased by $30\% \sim 50\%$ from co-digesters, ranged from 1500 to 1800 mL/day (Fig. V1). Moreover, there was no inhibitive effect of co-digestion at the optimum OLR, $1.5 \text{ gVS L}^{-1}\text{day}^{-1}$.

During the whole operational period, the triplicate control digesters were fed by the sole substrate, dairy farm waste and the OLR was maintained constant at $1.0 \text{ gVS L}^{-1}\text{day}^{-1}$. After digester setting up stage, the biogas production of control digesters became stable ranging from 1000 to 1200 mL/day (Fig. V1), which may indicate the well balanced reaction rates between biopolymer degradation, fermentation and methanogenesis.

qPCR assays

To quantify the methanogenic populations in anaerobic digesters, the standard curves for different 16S rRNA genes of methanogens were determined by ten fold serial dilutions of 16S rRNA gene templates ($\times 10^2 \sim \times 10^9$ copies) screened from previous study (Chen et al., 2012), *Methanosaeta*, *Methanosarcina* and *Methanoculleus* respectively. With primer/probe sets designed in this or previous studies (listed in Table V2), amplifications of the 16S rRNA genes were all highly reproducible with strong correlations (≥ 0.99) between template concentrations and fluorescence response detected as threshold cycle numbers, C_T (Table V3). Moreover, as the important parameter of the performance of qPCR assays, the amplification efficiencies for all assays ranged between 91% and 98% , which could be considered to be feasible for qPCR quantifications (Zhang & Fang, 2006).

Methanosaeta in anaerobic digestion

Similar with the previous studies about the methanogenic populations in anaerobic

digesters (Chen et al., 2012; Demirel & Scherer, 2008a; McHugh et al., 2003; Zhang et al., 2011; Zheng & Raskin, 2000), *Methanosaeta* in control anaerobic digesters was dominant among total archaeal communities with stable performance (Fig. V2B). Although its relative percentages varied along the whole operational period ranging from 45% to 68% approximately, *Methanosaeta* was always the predominant methanogen species and for most of time, its fractions were above 50% and increased with operational days except the setting up periods (Fig. V2B). Moreover, the relatively low acetate concentration maintained during whole period in control digesters (less than 1mM) can also explain the phenomena based on pervious theory that *Methanosaeta* became more competitive than *Methanosarcina* under conditions with low acetate concentration due to its lower K_S and μ_{max} values in growth kinetics and its higher acetate affinity (Conklin et al., 2006; Raskin et al., 1995).

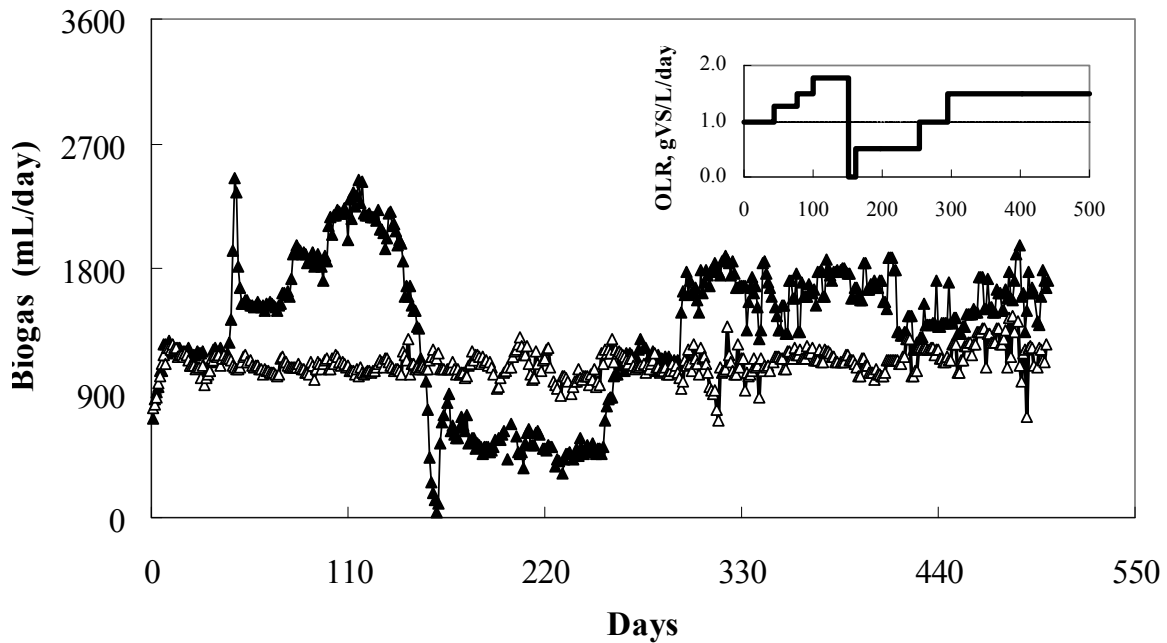


Fig. V1. Biogas production of anaerobic control and co-digesters. Inset indicates the organic loading rates (OLR) of control (dash line) and co-digesters (solid line) along the experimental period. Biogas of control digesters (-Δ-); Biogas of co-digesters (-▲-).

For co-digesters, however, the dynamics of the relative fractions of *Methanosaeta* in archaeal compositions seemed to be different from previous observations. Initially, with the poultry waste adding, the relative percentage of *Methanosaeta* was 30% averagely, which was less than that in control digesters. Its fractions begun to increase significantly from 30% to 65% when the biogas production was promoted and increased gradually with stepwise increase of OLR (Fig. V2A). With the performance collapse due to continuous poultry waste feeding at maximum level, however, its fractions decreased dramatically to 35% approximately. Through the fluctuation of its relative percentages during digester performance recovering period, the relative fractions of *Methanosaeta* increased from 35% to 60% under low organic loading rate (Fig. V2A). Although its percentages decreased to 40% at the beginning of OLR increase from 1.0 to 1.5 gVS L⁻¹ day⁻¹, they restored gradually and maintained at stable level at 50% when performance of co-digesters was fully resumed (Fig. V2A), which was similar to the relative percentages of *Methanosaeta* in control digesters (Fig. V2B).

Different from observations of previous studies, there was no significant correlation between relative percentages of *Methanosaeta* and acetate concentrations in digesters observed (Fig. V3A). Surprisingly, the relative percentage of *Methanosaeta* increased rather than decreased with the rapid acetate accumulation (up to 85mM) during co-digester performance perturbations, which was inconsistent with observations from previous studies that *Methanosarcina* rather than *Methanosaeta* usually became the dominant methanogens during digester setting up period or unstable performance with relatively high acetate acid concentrations (Griffin et al., 1998; McMahon et al., 2001; McMahon et al., 2004; Stroot et al., 2001).

***Methanosarcina* in anaerobic digestion**

For *Methanosarcina* in control digester under stable performance and much lower acetate concentrations, its relative percentages kept constant within the range from 10% to 15% (Figs. V4B and V5B), which was almost consistent with observations from previous studies (Chen et al., 2012; Zhang et al., 2011). While, in co-digesters, relative amounts of *Methanosarcina* and *Methanosaeta* in archaeal populations were identical, 25% ~ 30% in the beginning of co-substrate, poultry waste adding. With biogas production promotion with stepwise increase of OLR, however, fractions of *Methanosarcina* was replaced by *Methanosaeta* gradually and its relative percentages decreased significantly to only 7% ~ 8% (Fig. V4A). Dissimilar with the dynamics of *Methanosaeta* during co-digester performance collapse, the fractions of *Methanosarcina* increased up to 30% averagely under high acetate concentration due to organic acid accumulation (Fig. V5A), which could be explained by the conventional theory that *Methanosarcina* became more competitive for acetate utilization under high concentration (> 1mM) due to its higher K_s and μ_{max} (Conklin et al., 2006; Gujer & Zehnder, 1983; Qu et al., 2009). Even under the highest relative percentages of *Methanosarcina*, i.e. 30%, *Methanosaeta* was still the predominant among archaeal species, at 45% approximately (Fig. V2A and V3A). When the performance of co-digester was fully recovered from OLR perturbations, the percentages of *Methanosarcina* in co-digesters decreased gradually and maintained at

stable levels, i.e. 15% ~ 18% (Figs. V4A and V5A), which was comparable with that in control digesters (Figs. V4B and V5B).

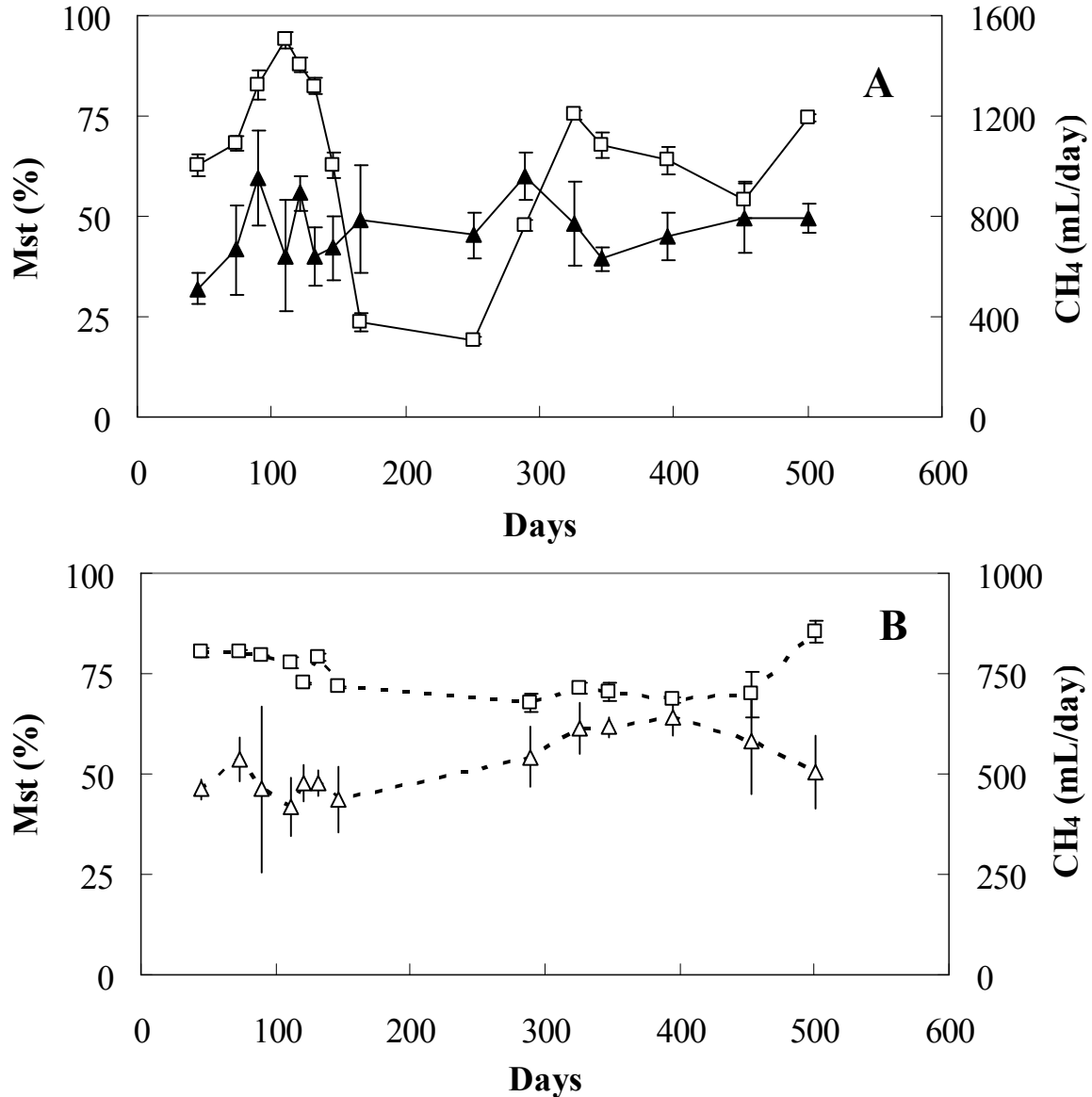


Fig. V2. Changes in *Methanosaeta* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to methane production. *Methanosaeta* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanosaeta*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Mst, % - co-digesters (-▲-); Mst, % - control digesters (···Δ···); CH₄ production - co-digesters (-□-); CH₄ production - control digesters (···□···).

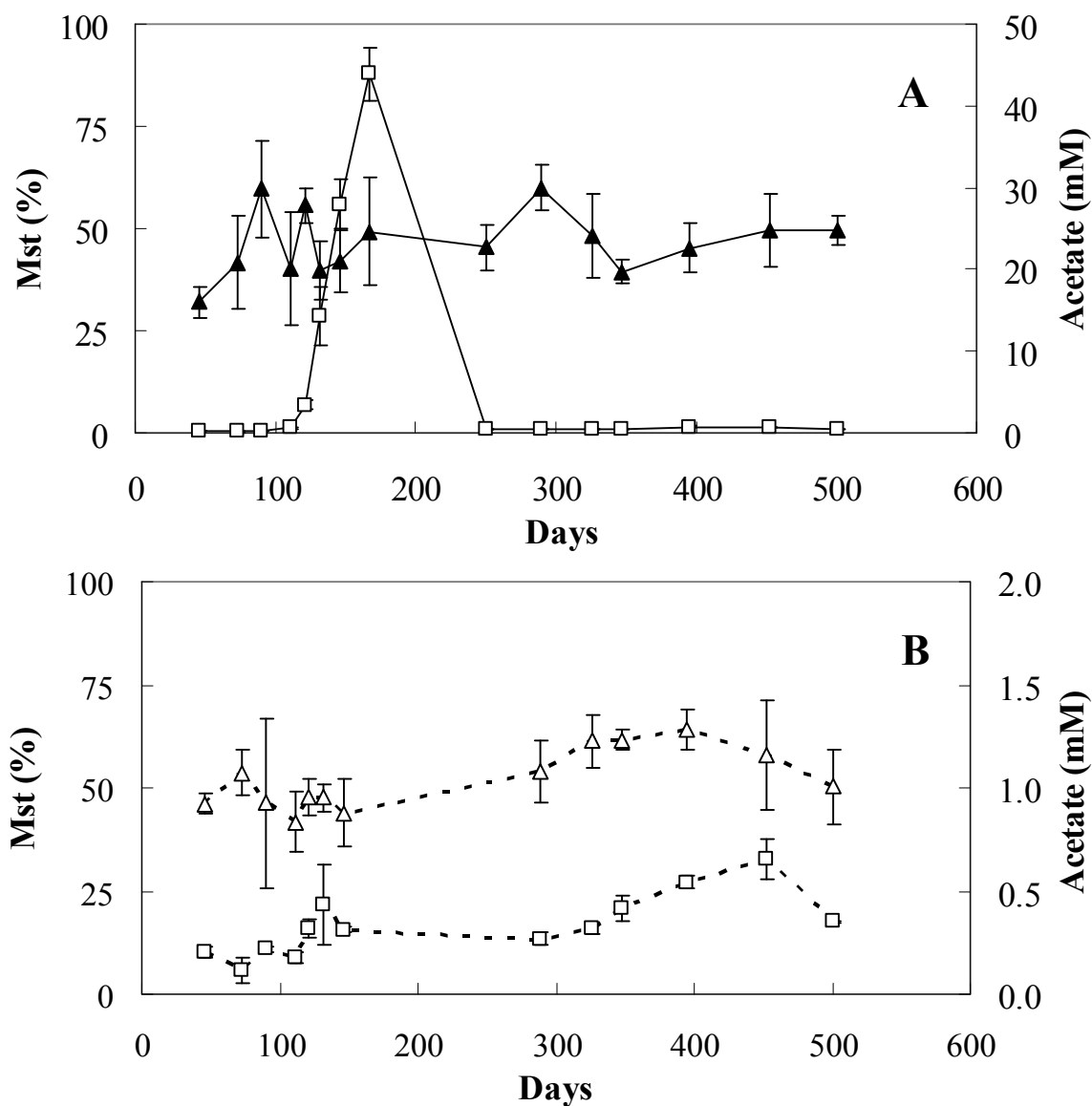


Fig. V3. Changes in *Methanosaeta* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to acetate concentration. *Methanosaeta* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanosaeta*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Mst, % - co-digesters (-▲-); Mst, % - control digesters (···Δ···); Acetate - co-digesters (-□-); Acetate - control digesters (···□···).

***Methanoculleus* in anaerobic digestion**

As compared to the acetoclastic methanogens including *Methanosaeta* and *Methanosarcina*, the hydrogenotrophic methanogens belonging to *Methanoculleus* were the minor populations composing no more than 10% of total archaeal populations. In control digesters, the relative percentages of *Methanoculleus* were kept constant at 3% averagely with the first 350 day operation. Then they increased to 5.5% during the last 150 days in this study (Fig. V6B). As shown in Fig. V7B, the dynamics of *Methanoculleus* seemed to be correlated with the acetate concentrations in control digesters although concentrations of acetate were maintained very low (less than 1mM). Although relative fractions of *Methanoculleus* increased along the operational period, its relatively small fractions indicated that the hydrogenotrophic pathway may not be the mainstream metabolism such as acetoclastic methanogenesis.

In anaerobic co-digesters with poultry wastes added as the co-substrates, however, the scenario of the relative percentages of *Methanoculleus* was apparently dissimilar with that in control digesters fed by the sole substrate, dairy farm wastes. Its percentages increased from less than 3% to 6% synchronized with stepwise OLR increase during initial operational stages (Fig. V6A). Under the continuous highest OLR, however, the relative fractions of *Methanoculleus* started to decline with accumulation of acetate (Fig. V7A), which indicated that the imbalance reaction rates between fermentation and methanogenesis deteriorated the hydrogenotrophic methanogenic activities of *Methanoculleus*. During co-digester performance recovering, the relative percentages of *Methanoculleus* increased dramatically up to nearly 9% under relatively high acetate concentration (Fig. V7A). As suggested by some previous studies that the pathway of anaerobic acetate oxidation probably became feasible in the presence of the hydrogenotrophic methanogen, *Methanoculleus* (Hattori, 2008; Sasaki et al., 2011; Schnürer et al., 1999), the increasing trend of *Methanoculleus* composition among total archaeal populations may predict the promotion of syntrophic acetate oxidation processes during recovering period after co-digester performance collapse although more direct and confident evidence are necessary in future studies. Sequentially, its fractions decreased and became stable (3% ~ 4%) along the rest operation period when the performance of co-digesters fully recovered from OLR perturbation (Figs. V6A and V7A).

Discussion

In accord with previous studies (Chen et al., 2012; McHugh et al., 2003; McMahon et al., 2001; Williams et al., 2013; Zhang et al., 2011), the dominance of acetoclastic methanogen, *Methanosaeta* was also observed in both of control and co-digesters as results of relative low acetate concentration (<1 mM) under stable performance. Surprisingly, *Methanosaeta* rather than *Methanosarcina*, however, was found dominant and persistent among total archaeal populations even under co-digester performance collapse with dramatic acetate accumulation, which was inconsistent with the observations from some studies about the microbial communities in anaerobic reactors that *Methanosarcina* could replace *Methanosaeta* under high acetate concentrations or unstable digester performance due to OLR perturbations (Conklin et al., 2006; McMahon et al., 2004; Steinberg & Regan, 2011).

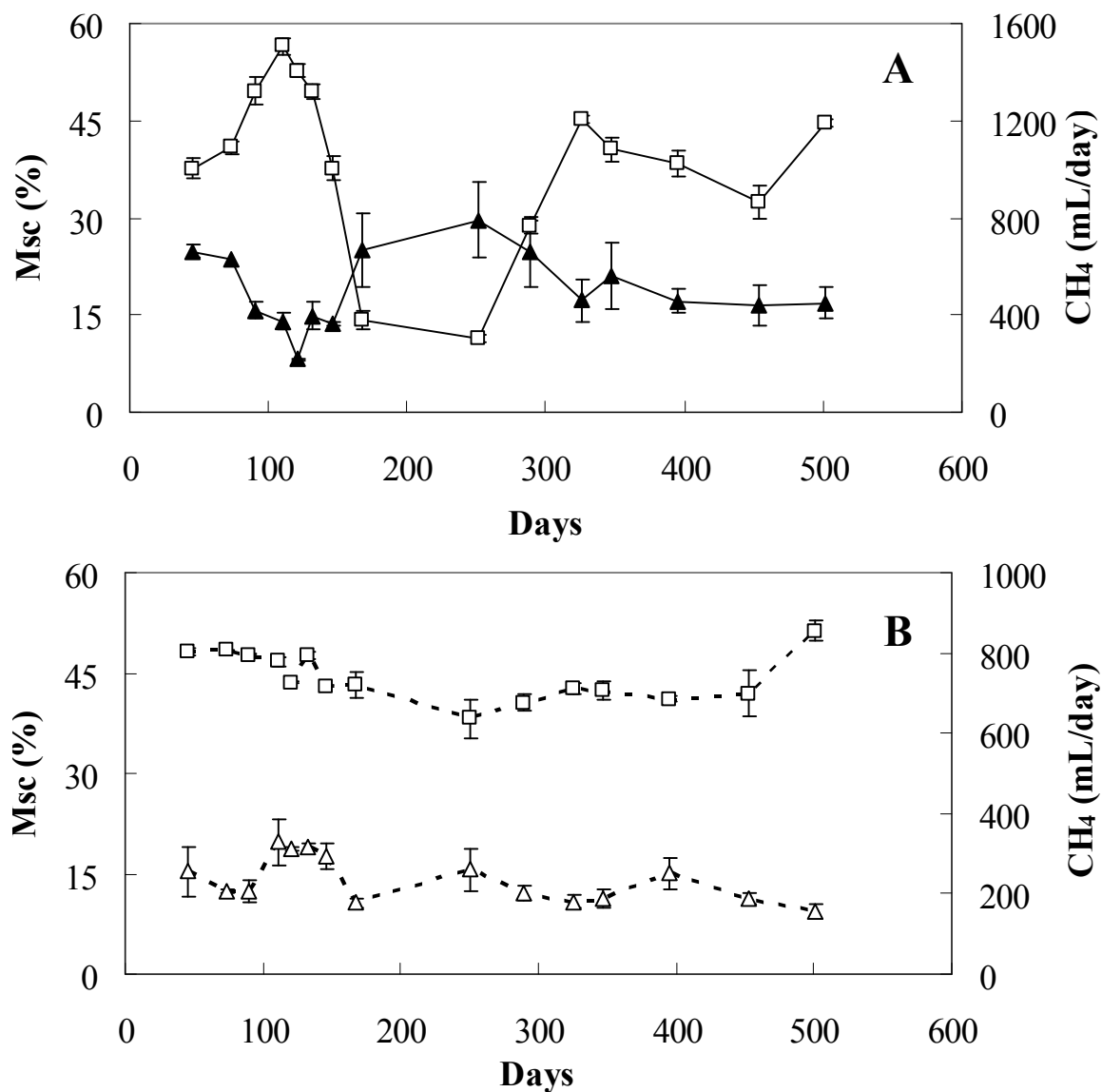


Fig. V4. Changes in *Methanosarcina* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to methane production. *Methanosarcina* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanosarcina*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Msc, % - co-digesters (-▲-); Msc, % - control digesters (···Δ···); CH₄ production – co-digesters (-□-); CH₄ production – control digesters (···□···).

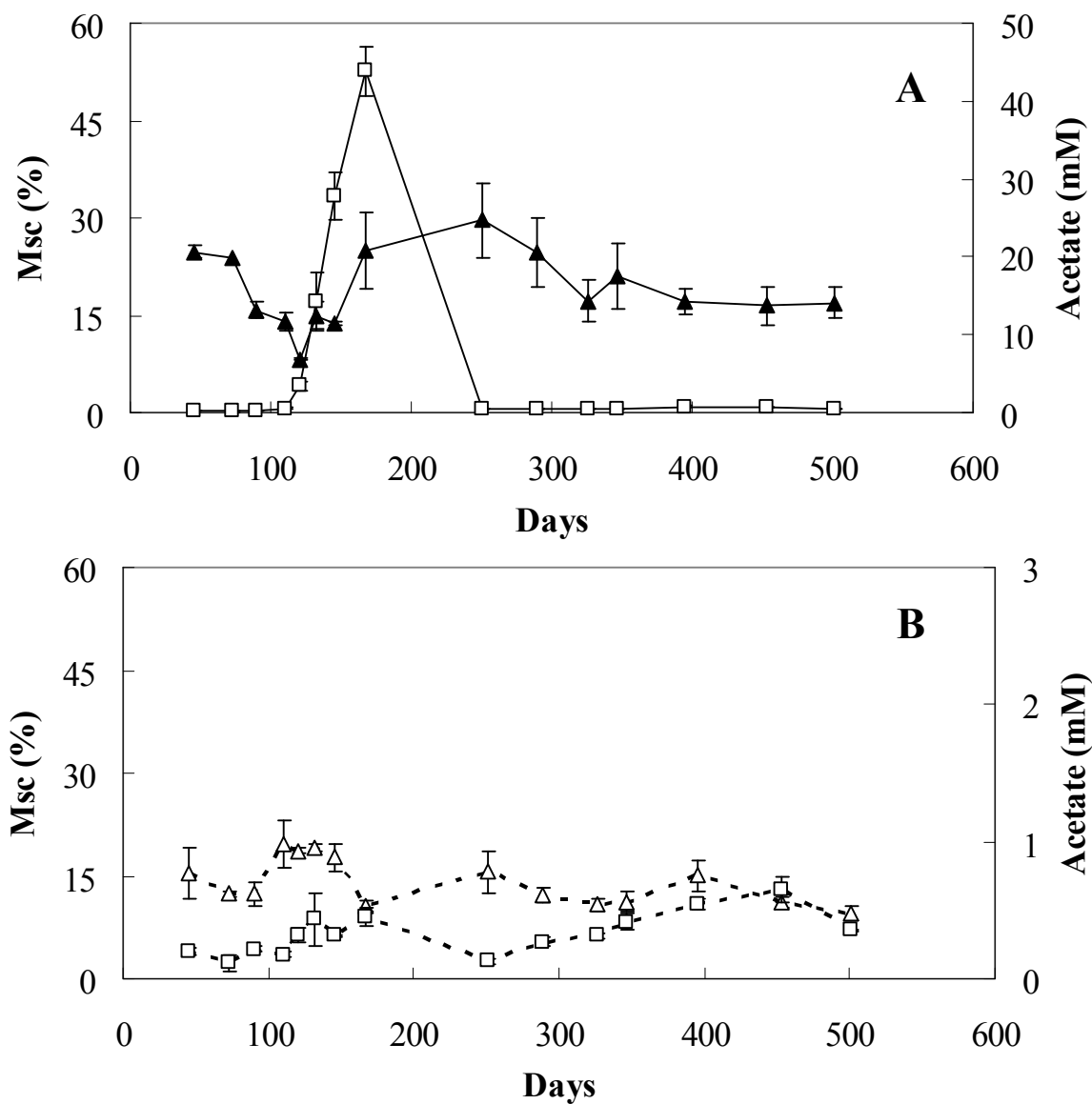


Fig. V5. Changes in *Methanosarcina* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to acetate concentration. *Methanosarcina* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanosarcina*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Msc, % - co-digesters (-▲-); Msc, % - control digesters (···Δ···); Acetate - co-digesters (-□-); Acetate - control digesters (···□···).

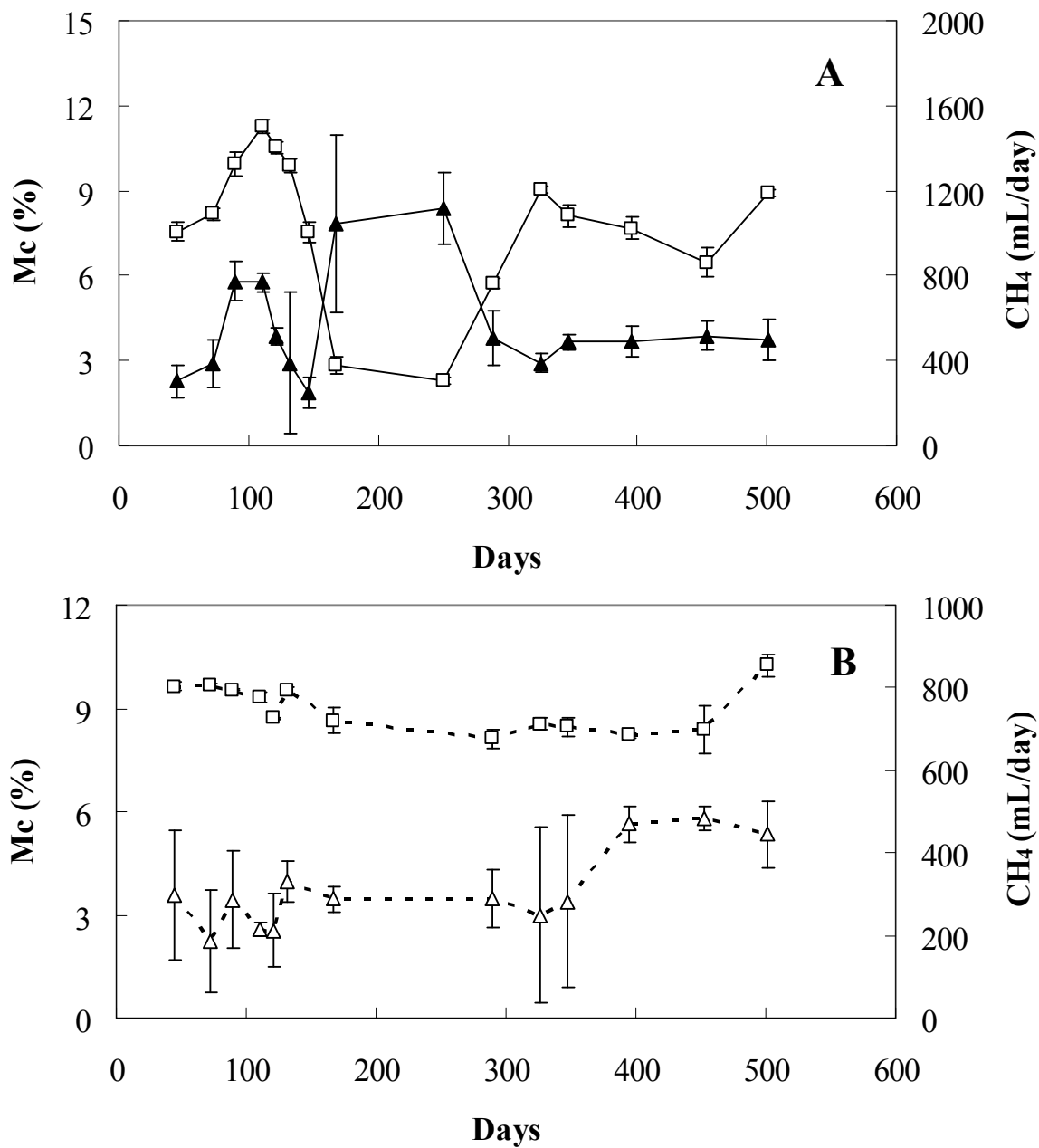


Fig. V6. Changes in *Methanoculleus* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to methane production. *Methanoculleus* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanoculleus*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Mc, % - co-digesters (-▲-); Mc, % - control digesters (···Δ···); CH₄ production - co-digesters (-□-); CH₄ production - control digesters (···□···).

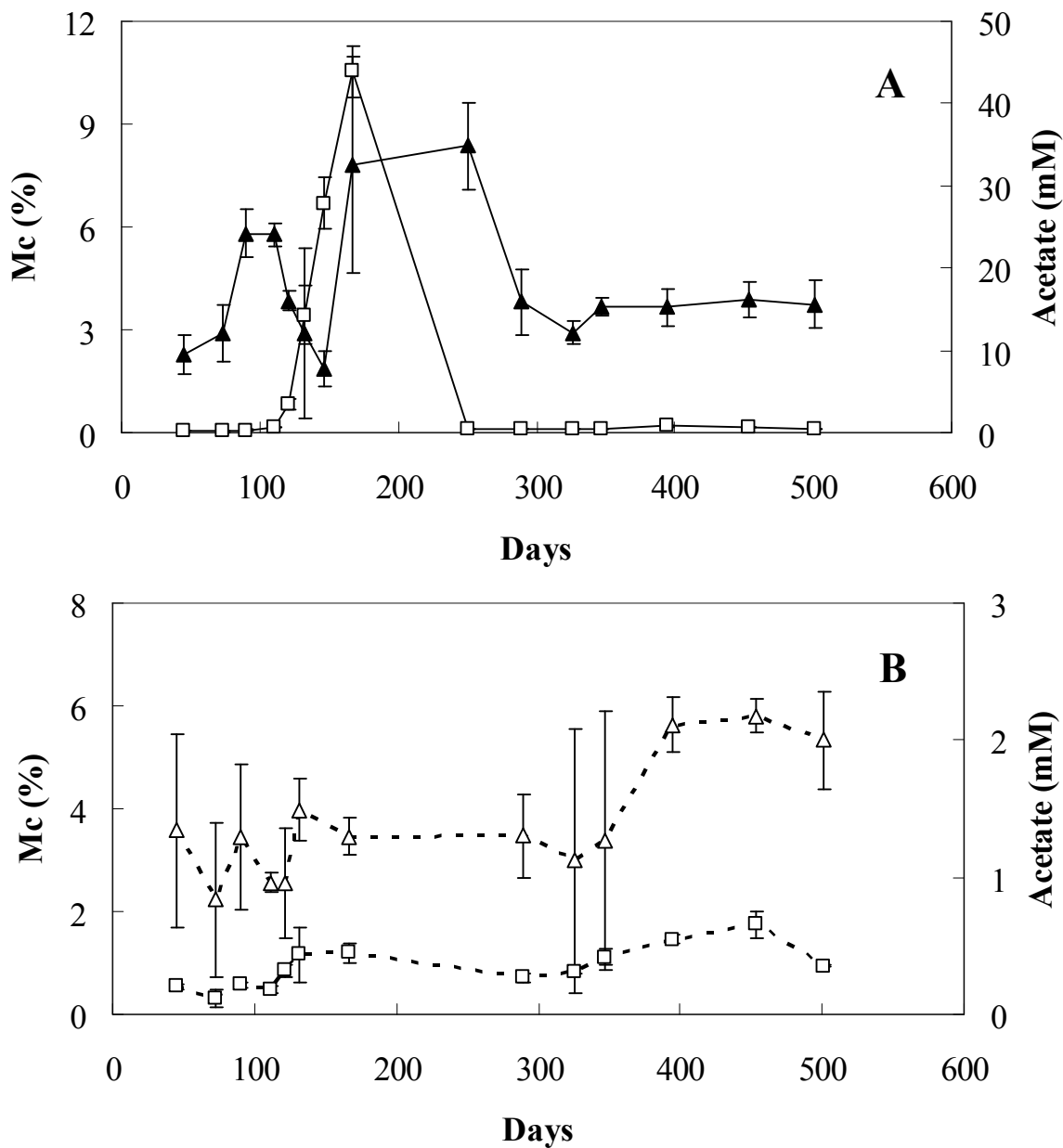


Fig. V7. Changes in *Methanoculleus* relative abundance in anaerobic co-digesters (A) and control digesters (B) in relation to acetate concentration. *Methanoculleus* relative abundance was the percentage of total archaeal 16S rRNA gene copies belonging to *Methanoculleus*, as determined by qPCR. Data points were means of triplicates with the error bars showing standard deviations. Mc, % - co-digesters (-▲-); Mc, % - control digesters (···△···); Acetate - co-digesters (-□-); Acetate - control digesters (···□···).

As described by the conventional acetoclastic methanogenesis model, *Methanosarcina* is more competitive than *Methanosaeta* under high acetate concentration because of its higher K_S and μ_{max} (Conklin et al., 2006; De Vrieze et al., 2012), while *Methanosaeta* becomes dominant under low acetate concentrations due to its lower K_S and high affinity of acetate (Conklin et al., 2006; Smith & Ingram-Smith, 2007). However, the scenario of relative fractions of these two acetoclastic methanogens, *Methanosaeta* and *Methanosarcina* among total archaeal communities in co-digesters under performance collapse with high acetate concentration (up to 85mM averagely) seemed to be unexplainable with this conventional theory. Although this model has been widely applied to interpret many observations of the acetoclastic methanogens distributions in anaerobic digesters under various operational conditions, some unexpected observations that the dominancy of *Methanosaeta* in anaerobic digestion process even under high acetate concentrations (Moertelmaier et al., 2013; van Haandel et al., 2013; Yilmaz et al., 2014) may suggest other growth factors influencing the growth competition between these two species, in which the conventional kinetics model could not explain (Yilmaz et al., 2014).

Consistent with previous studies of the microbial communities in anaerobic digestion by 16S rRNA clone library (Chen et al., 2012; Zhang et al., 2011), the relative percentages of *Methanosarcina* in both of control and co-digesters under stable performance ranged between 15% and 18% of total archaeal populations. Although its fractions in co-digesters increased up to 30% under OLR perturbation with acetate accumulation, it still didn't become the dominant acetoclastic methanogens, which was inconsistent previous observations that *Methanosarcina* could replace *Methanosaeta* and became dominant under unstable performance or high acetate concentration (Conklin et al., 2006; De Vrieze et al., 2012; McMahon et al., 2004). Additionally, due to the versatile methanogenic pathways of *Methanosarcina* (De Vrieze et al., 2012; Thauer et al., 2008), it can not exclude the possibility that partial methane was produced by *Methanosarcina* through hydrogenotrophic pathway under digester performance collapse with acetate accumulation. As suggested by previous studies (Hao et al., 2010; Qu et al., 2009; Winter & Wolfe, 1979), the syntrophic acetate oxidation could become a significant metabolism and the products, $CO_2 + H_2$ can be utilized in hydrogenotrophic methanogenesis by *Methanosarcina* when acetate concentration was elevated in high level (up to 85mM in this study). Although it is out of the scope of this study, it is still meaningful to prove this speculation by future investigations.

Methanoculleus populations were frequently detected in anaerobic digestion processes considered as the key members of the microbial community responsible for hydrogenotrophic methanogenesis (Narihiro & Sekiguchi, 2007). Unlike some studies reported that *Methanoculleus* was predominant in anaerobic digestion process (Cardinali-Rezende et al., 2012; Wirth et al., 2012), its relative fractions measured in this study were only ranged from 3% to 5% under the stable performance of digesters, which was consistent with previous studies about these digesters (Chen et al., 2012; Chen et al., 2014; Zhang et al., 2011). As compared to relative percentages of *Methanosarcina* and *Methanosaeta* in archaeal populations, the small percentages of *Methanoculleus* in both

types of digesters indicated that the methane produced by *Methanoculleus* following the hydrogenotrophic pathway was the major resource although they increased from 3% to 9% in co-digesters under performance collapse with dramatic acetate accumulation. As speculated that *Methanosarcina* may shift the acetoclastic pathway to hydrogenotrophic metabolism in association with syntrophic acetate oxidation under acetate accumulation, the increase of relative percentages of *Methanoculleus* may indicated the occurrence of syntrophic acetate oxidation in co-digester during performance collapse due to OLR elevation.

Conclusion

The methanogen specific qPCR applied in this study for sampling collected from anaerobic digesters revealed that the acetoclastic methanogen, *Methanosaeta* rather than *Methanosarcina* was predominant among total archaeal populations in both of control and co-digesters throughout the operational period even under co-digester performance collapse with acetate accumulation. Although relative abundance of *Methanosarcina* increased from 15% to 30% approximately, under high acetate concentration in co-digesters due to OLR perturbation, it still can not compete over *Methanosaeta*. The relatively small percentages of the typical hydrogenotrophic methanogen, *Methanoculleus* (typically 3% ~ 5%) indicated the inconsiderable CH₄ production attributed to hydrogenotrophic metabolism as compared to acetoclastic pathway. The percentages of *Methanoculleus* increase, from 3% to 9% in co-digesters under performance collapse with acetate accumulation may indicate the syntrophic acetate oxidation which needs more investigations in future studies. As expected from previous studies, the relative abundances of typical methanogens, *Methanosaeta* (45% ~ 50%) *Methanosarcina* (15% ~ 18%) and *Methanoculleus* (3% ~ 5%) were kept stable in the control digesters with stale performance.

References

- Appels, L., Baeyens, J., Degreè, J., Dewil, R. 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in Energy and Combustion Science*, **34**(6), 755-781.
- Cardinali-Rezende, J., Colturato, L., Colturato, T.D.B., Chartone-Souza, E., Nascimento, A.M.A., Sanz, J.L. 2012. Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions. *Bioresour Technol*, **119**, 373-383.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol*, **113**(6), 1371-1379.
- Chen, S., Zhu, Z., Park, J., Zhang, Z., He, Q. 2014. Development of Methanoculleus-Specific Real-Time Quantitative PCR Assay for Assessing Methanogen Communities in Anaerobic Digestion. *J Appl Microbiol*, n/a-n/a.
- Conklin, A., Stensel, H.D., Ferguson, J. 2006. Growth kinetics and competition between Methanosarcina and Methanosaeta in mesophilic anaerobic digestion. *Water Environ Res*, **78**(5), 486-496.
- De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W. 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. *Bioresour Technol*, **112**(0), 1-9.
- Demirel, B., Scherer, P. 2008a. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. *Reviews in Environmental Science and Bio/Technology*, **7**(2), 173-190.
- Demirel, B., Scherer, R. 2008b. Production of methane from sugar beet silage without manure addition by a single-stage anaerobic digestion process. *Biomass & Bioenergy*, **32**(3), 203-209.
- Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L. 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. *Biotechnol Bioeng*, **57**(3), 342-355.
- Gujer, W., Zehnder, A.J.B. 1983. Conversion processes in anaerobic-digestion. *Water Science and Technology*, **15**(8-9), 127-167.
- Hao, L.-P., Lü, F., He, P.-J., Li, L., Shao, L.-M. 2010. Predominant Contribution of Syntrophic Acetate Oxidation to Thermophilic Methane Formation at High Acetate Concentrations. *Environ Sci Technol*, **45**(2), 508-513.
- Hattori, S. 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ*, **23**(2), 118-127.
- He, Q., Lokken, P.M., Chen, S., Zhou, J.Z. 2009. Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. *Bioresour Technol*, **100**(23), 5955-5965.
- Jeris, J.S., McCarty, P.L. 1965. The Biochemistry of Methane Fermentation Using [image] Tracers. *Journal (Water Pollution Control Federation)*, **37**(2), 178-192.
- Kim, S., Bae, J., Choi, O., Ju, D., Lee, J., Sung, H., Park, S., Sang, B.-I., Um, Y. 2014. A pilot scale two-stage anaerobic digester treating food waste leachate (FWL): Performance and microbial structure analysis using pyrosequencing. *Process Biochem*, **49**(2), 301-308.
- McHugh, S., Carton, M., Mahony, T., O'Flaherty, V. 2003. Methanogenic population

- structure in a variety of anaerobic bioreactors. *FEMS Microbiol Lett*, **219**(2), 297-304.
- McMahon, K.D., Stroot, P.G., Mackie, R.I., Raskin, L. 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—II: microbial population dynamics. *Water Res*, **35**(7), 1817-1827.
- McMahon, K.D., Zheng, D., Stams, A.J.M., Mackie, R.I., Raskin, L. 2004. Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. *Biotechnol Bioeng*, **87**(7), 823-834.
- Michaud, S., Bernet, N., Buffiere, P., Roustan, M., Moletta, R. 2002. Methane yield as a monitoring parameter for the start-up of anaerobic fixed film reactors. *Water Res*, **36**(5), 1385-1391.
- Moertelmaier, C., Li, C., Winter, J., Gallert, C. 2013. Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. *Bioresour Technol*(0).
- Narihiro, T., Sekiguchi, Y. 2007. Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. *Curr Opin Biotechnol*, **18**(3), 273-278.
- Panwar, N.L., Kaushik, S.C., Kothari, S. 2011. Role of renewable energy sources in environmental protection: A review. *Renewable and Sustainable Energy Reviews*, **15**(3), 1513-1524.
- Parkin, G., Owen, W. 1986. Fundamentals of Anaerobic Digestion of Wastewater Sludges. *J Environ Eng*, **112**(5), 867-920.
- Qu, X., Vavilin, V.A., Mazeas, L., Lemunier, M., Duquennoi, C., He, P.J., Bouchez, T. 2009. Anaerobic biodegradation of cellulosic material: batch experiments and modelling based on isotopic data and focusing on aceticlastic and non-aceticlastic methanogenesis. *Waste Manag*, **29**(6), 1828-37.
- Raskin, L., Zheng, D., Griffin, M.E., Stroot, P.G., Misra, P. 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes. *Antonie Van Leeuwenhoek*, **68**(4), 297-308.
- Sasaki, D., Hori, T., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y. 2011. Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste. *J Biosci Bioeng*, **111**(1), 41-6.
- Schnürer, A., Zellner, G., Svensson, B.H. 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. *FEMS Microbiol Ecol*, **29**(3), 249-261.
- Smith, K.S., Ingram-Smith, C. 2007. Methanosaeta, the forgotten methanogen? *Trends Microbiol*, **15**(4), 150-155.
- Smith, P.H., Mah, R.A. 1966. Kinetics of Acetate Metabolism during Sludge Digestion. *Applied Microbiology*, **14**(3), 368-&.
- Steinberg, L.M., Regan, J.M. 2011. Response of lab-scale methanogenic reactors inoculated from different sources to organic loading rate shocks. *Bioresour Technol*, **102**(19), 8790-8798.
- Stroot, P.G., McMahon, K.D., Mackie, R.I., Raskin, L. 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—I. digester performance. *Water Res*, **35**(7), 1804-1816.

- Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sorensen, S.J., Karlsson, A. 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol*, **85**(3), 612-626.
- Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W., Hedderich, R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews Microbiology*, **6**(8), 579-591.
- van Haandel, A., De Vrieze, J., Verstraete, W., dos Santos, V.S. 2013. Methanosaeta dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. *Journal of Chemical Technology & Biotechnology*, n/a-n/a.
- Verstraete, W., Morgan-Sagastume, F., Aiyuk, S., Waweru, M., Rabaey, K., Lissens, G. 2005. Anaerobic digestion as a core technology in sustainable management of organic matter. *Water Science and Technology*, **52**(1-2), 59-66.
- Weimer, P.J., Zeikus, J.G. 1978. Acetate metabolism in *Methanosarcina barkeri*. *Arch Microbiol*, **119**(2), 175-182.
- Williams, J., Williams, H., Dinsdale, R., Guwy, A., Esteves, S. 2013. Monitoring methanogenic population dynamics in a full-scale anaerobic digester to facilitate operational management. *Bioresour Technol*, **140**, 234-242.
- Winter, J., Wolfe, R. 1979. Complete degradation of carbohydrate to carbon dioxide and methane by syntrophic cultures of *Acetobacterium woodii* and *Methanosarcina barkeri*. *Arch Microbiol*, **121**(1), 97-102.
- Wirth, R., Kovacs, E., Maroti, G., Bagi, Z., Rakhely, G., Kovacs, K.L. 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. *Biotechnology for Biofuels*, **5**.
- Yilmaz, V., Ince-Yilmaz, E., Yilmazel, Y.D., Duran, M. 2014. Is acetoclastic methanogen composition in full-scale anaerobic processes related to acetate utilization capacity? *Appl Microbiol Biotechnol*, **98**(11), 5217-26.
- Zhang, T., Fang, H.H.P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol*, **70**(3), 281-289.
- Zhang, Y., He, Q. 2013. Characterization of bacterial diversity in drinking water by pyrosequencing. *Water Science and Technology-Water Supply*, **13**(2), 358-367.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z.W., Linville, J.L., Chen, S., He, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol*, **102**(2), 779-785.
- Zheng, D., Raskin, L. 2000. Quantification of *Methanosaeta* Species in Anaerobic Bioreactors Using Genus- and Species-Specific Hybridization Probes. *Microb Ecol*, **39**(3), 246-262.
- Zhu, Z.W., Hsueh, M.K., He, Q. 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*, **36**(6), 1802-1807.

Part II: Dominance of *Methanosaeta* Populations at High Acetate Concentrations in Anaerobic Digestion

Abstract

Instead of *Methanosarcina*, another acetoclastic methanogen, *Methanosaeta* was found to be predominant in both acetate enrichments with high concentration (20mM) and lab-scale mesophilic anaerobic digesters under high acetate concentrations (25 ~ 36mM) with extra concentrated acetate spiking in this study. Moreover, the enrichment of *Methanosarcina* observed in enrichment cultures and digesters with methanol loading may indicate that *Methanosarcina* in this system functioned in methylotrophic pathway more favorably than acetoclastic metabolism. To determine the growth kinetics of these two species from digesters in acetate utilization, the enrichment of *Methanosarcina* was obtained by series of transferring of methanol feeding cultures then following series of transferring of acetate feeding cultures. After enrichment process, *Methanosaeta* and *Methanosarcina* became dominant among total archaeal populations (>90%) in enrichment cultures, respectively. As expected, values of maximum specific growth rate, $\mu_{\max}$ and half-velocity constant, K_S of *Methanosaeta* determined in this study were within the range of reported values. The maximum growth rate of *Methanosarcina*, however, was much lower than *Methanosaeta*, which was inconsistent with values determined by previous studies. For mixed enrichment cultures after incubation in batch reactors with 20mM acetate, dominance of *Methanosaeta* was always achieved even in mix cultures inoculated with larger fractions of *Methanosarcina* initially, which can be explained by growth kinetics of these two species determined in this study.

Keywords: *Methanosaeta*, *Methanosarcina*, Anaerobic digestion, Growth kinetics, Competition, Aceticlastic pathway, Methylotrophic pathway

Introduction

In anaerobic digestion process, acetoclastic pathway is predominant in methanogenesis (Ferry, 1992; Jeris and McCarty, 1965; Smith and Mah, 1966) and more than 60% of the CH_4 generated from natural environments is following this mechanism (Bridgham et al., 2013; Ferry, 1992; Penning et al., 2006). There are two well known genera of acetoclastic methanogens, i.e. *Methanosaeta* and *Methanosarcina* transforming acetate to methane and carbon dioxide. The growth kinetics of the acetate utilization in methanogenesis of these two genera was intensively studied for both pure and enrichment cultures (Conklin et al., 2006; Fukuzaki et al., 1990; Huser et al., 1982; Jetten et al., 1992; Min and Zinder, 1989; Mladenovska and Ahring, 2000; Ohtsubo et al., 1992). As well known, *Methanosarcina* has higher maximum growth rate, $\mu_{\max}$ and half-saturation coefficient, K_S than *Methanosaeta* (Conklin et al., 2006; De Vrieze et al., 2012). Thus, *Methanosarcina* is expected to be predominant under high acetate concentration and short hydraulic retention time (HRT) of anaerobic digesters. Under relatively low acetate concentration or longer HRT conditions, however, *Methanosaeta* becomes predominant acetoclastic methanogens due to its higher acetate affinity (De Vrieze et al., 2012; Hulshoff Pol et al., 2004; Liu and Whitman, 2008; Smith and Ingram-Smith, 2007).

Methanosaeta and *Methanosarcina*, therefore, provide a classic example of the coexistence of two microorganisms competing for the same substrate. Generally, acetate concentration at around 1mM was commonly applied as a threshold to predict the dynamics or distribution of *Methanosaeta* and *Methanosarcina* in natural or engineered systems. Following conventional acetoclastic methanogenesis modeling, anaerobic digestion process dominated by *Methanosarcina* could indicate the enhanced abilities of acetate utilization, which may improve the process stability (De Vrieze et al., 2012). On the other hand, *Methanosaeta* usually becomes dominant in many typical municipal anaerobic digesters operated steadily at low acetate concentration and long HRT (Jetten et al., 1990; Smith and Ingram-Smith, 2007). So far, this theory has been applied to explain many experimental observations successfully. Under digester stable performance, *Methanosaeta* was usually found to be the predominant acetoclastic methanogens due to low acetate concentration (<1mM) (Lee et al., 2014; McHugh et al., 2003; McMahon et al., 2001; Raskin et al., 1995; Zheng and Raskin, 2000). Meanwhile, with accumulation of acetate during digester start up or high organic loading rates (OLR), the other typical acetoclastic methanogens, *Methanosarcina* could compete over *Methanosaeta* and become dominant (De Vrieze et al., 2012; Griffin et al., 1998; Leclerc et al., 2004; Lins et al., 2014; Ma et al., 2013; McMahon et al., 2004).

Besides many observations described above following the established theory, however, there were many unexpected results from pervious studies, which could not be explained by current acetoclastic methanogenesis model. Unlike the dominance of acetoclastic methanogens, *Methanosarcina* in digesters with high acetate concentration, *Methanosaeta* persisted at acetate concentration exceeding 10mM in anaerobic migrating blanket reactor (Angenent et al., 2002). Similarly, dominance of *Methanosarcina* can not be achieved in a CSTR during startup period with relatively high acetate concentrations (10 ~ 20mM) (Moertelmaier et al., 2013) or an upflow anaerobic sludge bed reactor (UASB) despite the favorable conditions for *Methanosarcina* growth (van Haandel et al., 2013). Unexpectedly, with biomass samples collected from both the municipal and industrial anaerobic digesters, the statistically insignificant correlations between acetate utilization rates and acetoclastic methanogen population compositions reported by a recently study also suggested that there might be factors other than acetate utilization kinetics to determine the dominant acetoclastic methanogens in anaerobic digestion process (Yilmaz et al., 2014). Additionally, from anaerobic digester sludge, a novel acetate utilizing bacteria, *Synergistes* group 4, instead of *Methanosarcina*, was firstly reported to have higher K_S and μ_{max} and compete over *Methanosaeta* for acetate under high concentrations (up to 10mM) (Ito et al., 2011). Although more evidence and further studies are still needed in the future, it could alter the knowledge of the classic theory of acetate utilization or competition in methanogenesis process established previously.

In this study, the unconventional phenomenon of the predominance and persistence of *Methanosaeta* was observed in lab-scale semi-CSTR digesters during organic over loading with acetate accumulations (Chen et al., 2012) or the enrichment cultures fed with high acetate concentration (20mM). Instead of acetate enrichment, *Methanosarcina* seemed to be enriched by methanol. Further investigations, therefore, were implemented to focus on the characterization of physiological features of

Methanosaeta and *Methanosarcina* in our anaerobic digesters including: (1) predominant methanogens in digesters under high acetate concentration; (2) community structure alternation with methanol feeding; (3) growth kinetics of acetate utilization.

Materials and Methods

Anaerobic digester setup and operation

Two sets of triplicate continuous mesophilic anaerobic digesters were established prior to this study using dairy waste as the feedstock at an organic loading rate (OLR) of 1.0 gVS/L/day as described previously (Chen et al., 2012). The temperature of the anaerobic digestion process was maintained at 35 °C and the hydraulic retention time was controlled at 20 days.

Anaerobic medium preparation

To enrich methanogen populations, an anaerobic basal medium was prepared according to the following recipe (per liter) as previously described (He and Sanford, 2002): NaCl, 1.0 g; MgCl₂·6 H₂O, 0.5 g; KH₂PO₄, 0.2 g; NH₄Cl, 0.3 g; KCl, 0.3 g; CaCl₂·2H₂O, 0.015 g; trace element solution, 1.0 mL; Se/Wo solution, 1.0 mL; and resazurin, 1.0 mg. L-cysteine (0.031 g/L) and Na₂S·9 H₂O (0.048 g/L) were added as reductants into the basal medium after it had been boiled and cooled to room temperature under an oxygen-free N₂ atmosphere. NaHCO₃ (2.52 g/L) was added to the medium as the buffer. The medium was subsequently dispensed into 160-mL serum bottles flushed with pure N₂ and sealed with butyl rubber stoppers and aluminum caps. A sterile vitamin solution (1%) was added after autoclaving (Wolin et al., 1963).

Acetate and methanol enrichment for methanogens in initial stages

Initial enrichments for methanogens were seeded with a 10% (vol/vol) inoculum of digestate from the continuous bench-scale anaerobic digesters fed with dairy waste along with 20 mM acetate or 30mM methanol as the sole substrate respectively, which were replenished when depleted. At the completion of 10 feedings of substrates, secondary enrichments were established by transferring a 10% (vol/vol) inoculum from the initial enrichment cultures into fresh medium followed by repeated feedings of 20mM acetate or 30mM methanol. All enrichment cultures were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 °C. Methane production was measured periodically.

Clone library analysis of enrichment cultures

Clone library analysis was performed to examine the composition of methanogen communities enriched by acetate or methanol. Biomass from secondary enrichments was pelleted by centrifugation (at 14000g for 15min) and stored at -80 °C. Whole community DNA was extracted and purified as previously described (Zhang et al., 2009). DNA extracts from triplicate enrichments were pooled for PCR amplification of the 16S rRNA genes using the following *Archaea*-specific primers: Arch21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch958R (5'-YCCGGCGTTGAMTCCAATT-3') following previously described PCR protocols (DeLong, 1992). Amplicons of 16S rRNA

gene sequences were subsequently purified and cloned into plasmid vectors following procedures described previously (Chen et al., 2012). Twenty cloned plasmid inserts were randomly selected for sequencing with the ABI Prism BigDye chemistry (Applied Biosystems, Foster City, CA, USA) using M13 forward and reverse primers. The obtained sequences were checked for chimeric artifacts using the Chimera Check program in the Ribosomal Database Project II (Cole et al., 2003). High quality 16S rRNA gene sequences were deposited at GenBank under the following accession numbers: KJ504229—KJ504247, KJ504248-KJ504265.

Subsequently, phylogenetic analysis was conducted on the representative 16S rRNA gene sequences retrieved from acetate and methanol enrichment cultures by searching the NCBI GenBank database for closely related sequences. The phylogenetic positions of these 16S rRNA gene sequences were assessed by phylogenetic trees constructed with the neighbor-joining algorithm (1000 bootstrap re-samplings) using MEGA 4.0 (Tamura et al., 2007).

Quantifications of *Methanosaeta*, *Methanosarcina* in secondary enrichment cultures

Quantifications of *Methanosaeta*, *Methanosarcina* and total *Archaea* populations in secondary acetate and methanol enrichment cultures were assessed by TaqMan real time PCR assays. For each enrichment culture, relative abundances of *Methanosaeta* and *Methanosarcina* in archaeal populations were compared. Results from qPCR were also used to compare with those from clone library analysis performed on the same samples.

Anaerobic digester with high concentration of acetate spiking

Following the establishment of stable anaerobic digestion performance, high concentration of sodium acetate solution (5M) was spiked into one set of triplicate digesters to accelerate acetate concentration inside digesters to ~ 25mM initially. Sequentially, extra acetate was mixed with slurry dairy farm waste and loaded into triplicate treatment digesters daily. Meanwhile, digester performance parameters, pH, methane production, total solid (TS) concentration, volatile solid (VS) concentration and acetate concentration were monitored. To maintain the high concentration of acetate in treatment digesters, the daily extra acetate loading rate was increased stepwise until it reached stable performance of digesters and high acetate concentration (>20mM) (Fig. V11). The acetate spiking was ceased after 5 days of stable performance and maintained digester normal operational conditions afterwards (Chen et al., 2012).

To verify the reproducibility of digester performance under high acetate concentration condition, the acetate spiking was repeated following the stable anaerobic digestion performance after previous acetate loading. Similar with the first acetate spiking, acetate concentration in triplicate treatment digesters were increase to ~ 25mM at one time. The extra acetate mixed with dairy farm waste was loaded into digesters as the first time to maintain high acetate concentration (Fig. V11). The same parameters of digester performances were monitored periodically. After 6 days of acetate loading, the stable digester performance was achieved. Subsequently, extra acetate loading was stopped and normal digester operational conditions were maintained to make digester performance restored to original status.

Table V4 Quantification of 16SrRNA gene abundances of major methanogens with qPCR in acetate and methanol enrichment cultures from anaerobic digesters.

Enrichment ^a	16S rRNA Gene Targets	No. of Copies/mL ^d	Relative Abundance (% of Total <i>Archaea</i>) ^d
Acetate (20mM) enrichment culture ^b	<i>Methanosaeta</i>	$(8.92 \pm 0.73) \times 10^8$	80.39 ± 13.50
	<i>Methanosarcina</i>	$(3.55 \pm 0.87) \times 10^6$	0.31 ± 0.04
	Total <i>Archaea</i>	$(1.12 \pm 0.14) \times 10^9$	-
Methanol (30mM) enrichment culture ^c	<i>Methanosaeta</i>	$(4.98 \pm 2.99) \times 10^7$	0.88 ± 0.44
	<i>Methanosarcina</i>	$(3.19 \pm 1.50) \times 10^9$	57.30 ± 20.68
	Total <i>Archaea</i>	$(5.42 \pm 0.65) \times 10^9$	-

^a Enrichments were established by a 10% (v/v) transfer of anaerobic digestate to anaerobic medium followed by 10 feedings of substrates and subsequently a 2nd 10% (v/v) transfer to anaerobic medium followed by another 10 feedings of substrates.

^b Each feeding contained 20mM acetate.

^c Each feeding contained 30mM methanol.

^d Values are the means of triplicate samples $\pm$ standard deviation.

During the whole process, another set of triplicate digesters treated as control reactors were maintained constantly under the same operational conditions as described previously (Chen et al., 2012). The digester performance parameters were also monitored. For both treatment and control digesters, effluent samples were collected daily for future analysis.

Anaerobic digester with methanol spiking

After acetate loading into digesters was ceased, methanol mixed with dairy farm waste was loaded into triplicate treatment digesters following stable anaerobic digestion performance achieved. Since there was no immediate increase of methane production with initial methanol loading at 15mM/day, it was decreased to 5.5mM/day for 5 days until the significant increase of methane was monitored. Methanol loading rate was then increased stepwise from 5.5 to the 15mM/day with stable performance (Fig.V14). The same parameters of digester performance were also monitored during the whole experimental process. The first methanol spiking was stopped after maintained at loading rate at 15mM/day for 4 days. As the similar scheme applied in previous acetate loading, the second methanol loading were implemented at loading rate, 15mM/day for 9 days to reach stable performance (Fig. V14). Then, methanol loading was stopped and digesters were maintained under normal conditions until they reached stable status.

During the whole process, another set of triplicate digesters treated as control reactors were maintained constantly. Meanwhile, digester performance was also monitored. Effluent samples from both of treatment and control digesters were collected daily for future analysis.

Quantifications of *Methanosaeta* and *Methanosarcina* in anaerobic digesters with acetate and methanol spiking

For acetate or methanol loading, samples of two time points of stable digester performances with acetate or methanol optimum loadings (B and D for acetate or II and IV for methanol shown in Fig. V9 and Fig. V14 respectively) were chosen for quantitative analyses for *Methanosaeta* and *Methanosarcina* populations by TaqMan qPCR assays. Under stable performance without acetate or methanol loading, samples of three time points, i.e. before acetate or methanol spiking between two acetate or methanol spiking events and after second acetate or methanol loading (A, C and E for acetate or I, III and V for methanol shown in Fig. V9 and Fig. V14 respectively) were also taken to quantify these two populations. To provide the background quantitative information of *Methanosaeta* and *Methanosarcina* populations without acetate or methanol loading, 5 time points, the same as those chosen in acetate or methanol loading in treatment digesters were also chosen from control digesters.

To investigate relative abundances of *Methanosaeta* and *Methanosarcina* in total archaeal populations in all digester samples, absolute copy numbers of total *Archaea* were determined by TaqMan qPCR assay. Relative abundances of *Methanosaeta* and *Methanosarcina* in archaeal populations were compared.

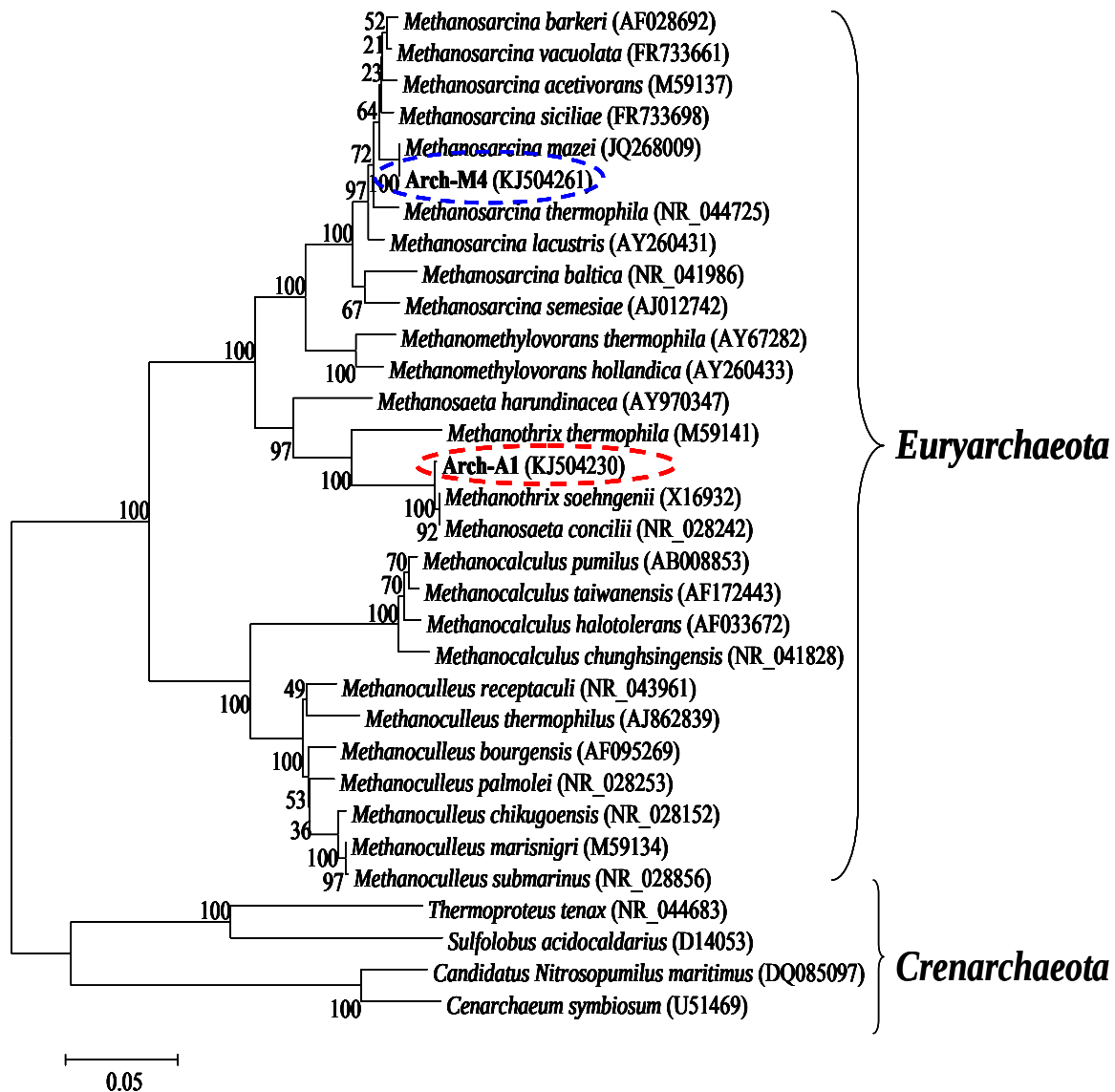


Fig. V8. Neighbour joining phylogenetic tree showing the grouping of reference *Methanosaeta* and *Methanosarcina* cultures and representative clones of uncultured *Methanosaeta* (Arch-A1) and *Methanosarcina* (Arch-M4) from acetate (20mM) and methanol (30mM) enrichment cultures respectively with inoculums from anaerobic digesters. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position. GenBank accession numbers of the 16S rRNA gene sequences are indicated in the parentheses.

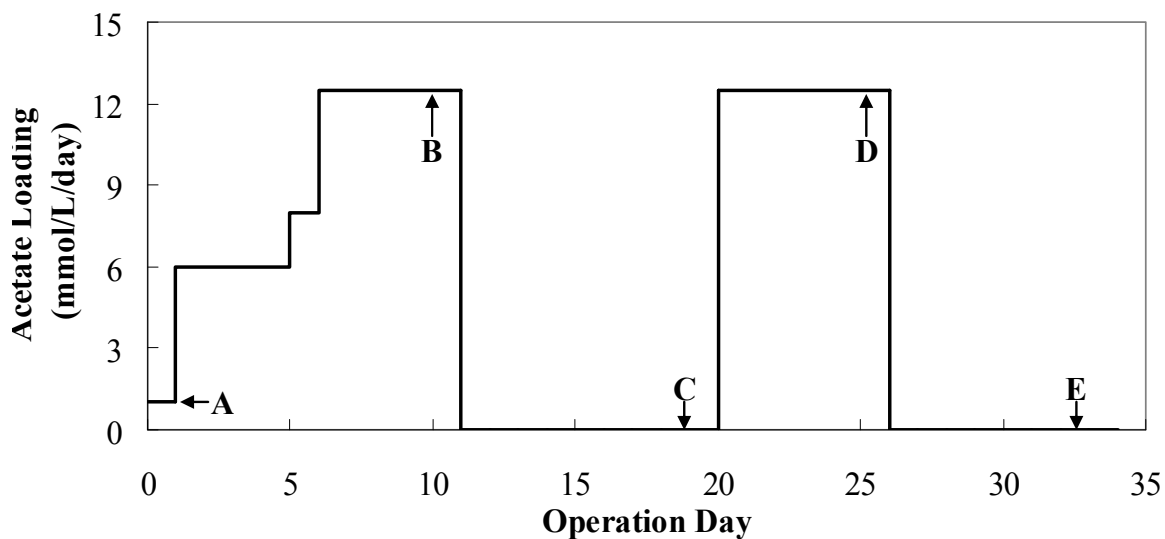


Fig. V9. Changes in the acetate loading rate of the treatment anaerobic digesters during the period of study. Letters and arrows indicate the sampling points of biomass from digesters.

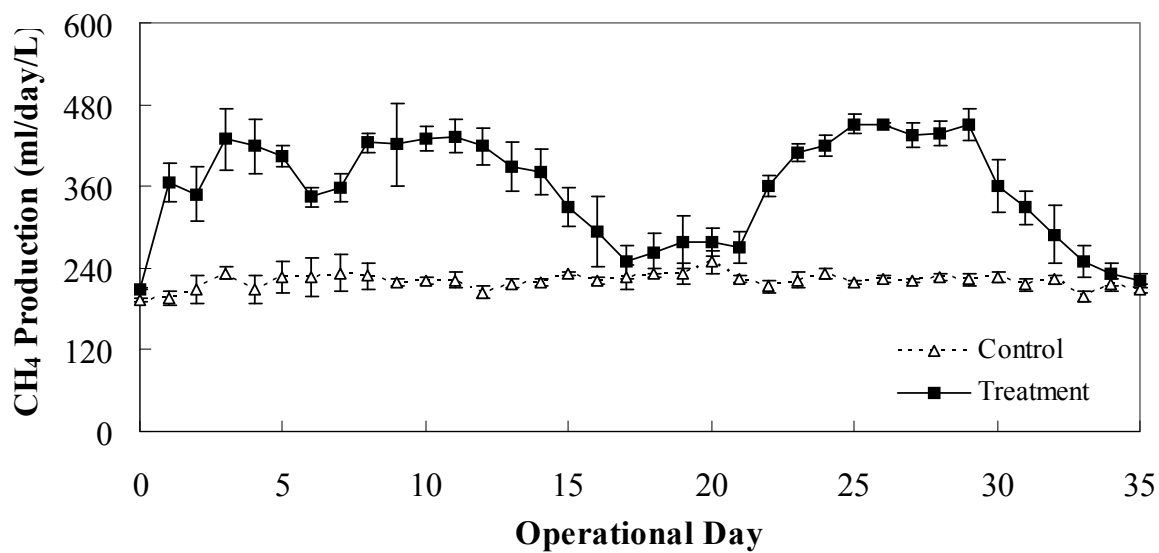


Fig. V10. Methane production of control and treatment digesters with acetate loading. Data are means of triplicate digesters, with the error bars indicating the standard deviation. Control digesters ($\cdots\Delta\cdots$); Treatment digesters ($\blacksquare$).

Enrichments of *Methanosaeta* and *Methanosarcina* from anaerobic digesters

To obtain relatively purified enrichment culture of *Methanosaeta*, a 1% (vol/vol) inoculum of the secondary acetate enrichment culture was transferred into fresh anaerobic medium fed with 20mM acetate, which was described in details previously. Continually, a series of 1% (vol/vol) inoculum transfer from previous generations of acetate enrichment cultures into fresh medium with 20mM acetate was processed.

Different from the scheme applied for *Methanosaeta* enrichment with single substrate, acetate, it is necessary to use both methanol and acetate in separate steps sequentially, due to versatile metabolisms of *Methanosarcina* (De Vrieze et al., 2012). The secondary methanol enrichment culture was used as inoculum (1%, vol/vol) transferred into fresh medium with 30mM methanol initially. Similar with procedures applied for *Methanosaeta* enrichment, inoculums from previous enrichments were transferred into 30mM methanol medium continually until acetoclastic methanogens, *Methanosaeta* became extinct. In order to prevent bacterial growth, ampicillin was added into methanol medium with final concentration of 100mg/L. After 10 times transfers with methanol, inoculum was transferred (1%, vol/vol) into 20mM acetate media subsequently. The transfer process was continued 5 times to obtain *Methanosarcina* enrichment finally.

In all transfer processes, methane generated from acetate or methanol enrichment cultures was monitored. The inoculum (1%, vol/vol) of the enrichment culture was transferred into the next fresh medium after accumulative methane completion indicating the depletion of acetate or methanol.

Quantifications of *Methanosaeta* and *Methanosarcina* in enrichment cultures

Quantifications of *Methanosaeta*, *Methanosarcina* and total *Archaea* populations in acetate cultures were assessed by TaqMan real time PCR assays when the enrichment processes were completed. For each enrichment culture, relative abundances of *Methanosaeta* and *Methanosarcina* in archaeal populations were determined.

Competition between *Methanosaeta* and *Methanosarcina* under high acetate concentration

After determinations of the quantifications of *Methanosaeta* and *Methanosarcina* in enrichment cultures, two enrichments were mixed with three different ratios and inoculated into fresh anaerobic media with 20mM acetate, which were 70/30, 50/50 and 30/70 respectively. For each reactor, 1mL mixed inoculum was transferred into a 160mL serum bottle with 100mL anaerobic medium. After 20mM acetate loading, methane and acetate concentrations in batch reactors were monitored periodically until acetate was depleted. *Methanosaeta* and *Methanosarcina* enrichment cultures without mixing were treated as controls. All reactors were set up in duplicate. After incubation, biomass samples from reactors were collected for further studies.

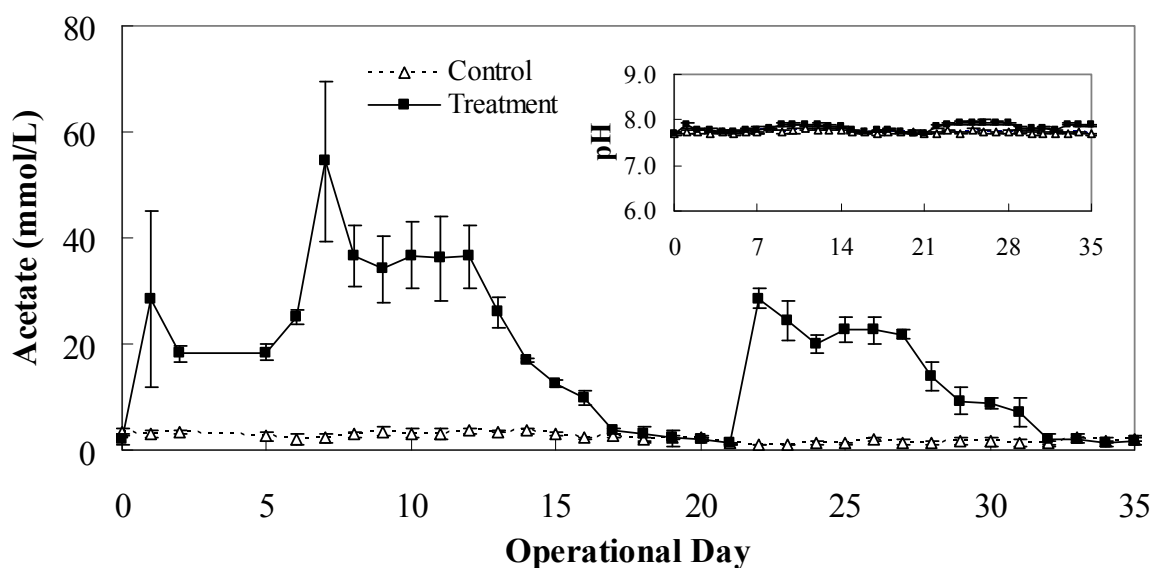


Fig. V11. Concentrations of acetate in both of control ($\cdots\Delta\cdots$) and treatment ($\blacksquare$) anaerobic digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation. Inset indicates the pH values of both of the control and treatment digesters during acetate spiking period.

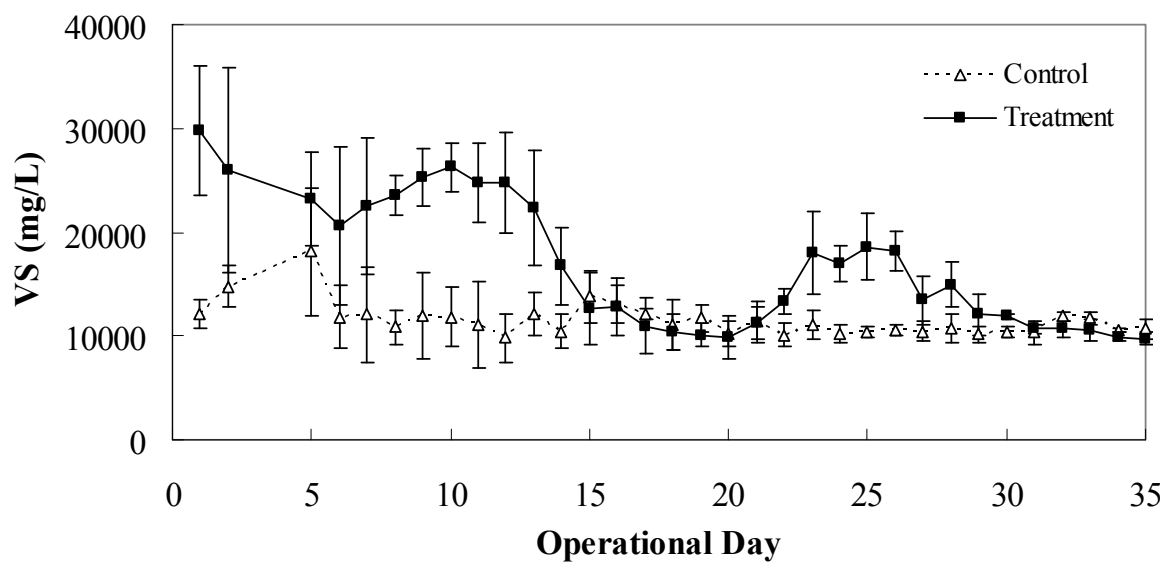


Fig. V12. Volatile solid concentrations of both of the control ($\cdots\Delta\cdots$) and treatment ($\blacksquare$) anaerobic digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation.

Specific growth kinetics of *Methanosaeta* and *Methanosarcina* enrichment cultures

The methane production of *Methanosaeta* and *Methanosarcina* enrichments with acetate was monitored. To eliminate the bias in growth rates estimation because of the dramatic biomass increase in exponential growth phase, the method of resting cell suspensions was applied in this study (Robinson and Tiedje, 1984). Initially, inoculums from both enrichments were transferred into multiple serum bottles (~ 20 for each) with 100mL anaerobic medium fed with 20mM acetate. Methane production was monitored till acetate was depleted. Biomass concentrations of two enrichments were determined by filtering 10mL liquid samples through 0.2 μ m polycarbonate membrane filters (Millipore, Billerica, MA, USA). The weight of biomass was measured after dried in a 102°C oven for 4 hours. Sequentially, the growth yields of two enrichments, Y (mg VSS/mg COD or mg VSS/mmol_{acetate}) were determined.

Methane accumulated in all bottles was purged by N₂ for 15min under anaerobic conditions at first. The N₂ purging was repeated after bottles were set under mesophilic conditions overnight in order to eliminate the influences of the residual methane on methane production rate estimation in further experiments. For both of the enrichments, different acetate concentrations, 0.1, 0.2, 0.5, 1, 2, 5 10 and 20mM were reloaded into serum bottles with resting cell suspensions achieved by 20mM acetate feeding in previous step respectively. Duplicate reactors were set for each acetate concentration. To prevent the significant biomass increase from utilizing acetate, methane productions under different acetate concentrations were only monitored during relatively short period. The slope of the methane production verse the short period of time was considered as the methane production rate (mM/hr) under certain acetate concentration.

The specific growth rates, μ (h⁻¹), under different acetate concentrations were calculated from the methane production rates divided by biomass concentrations and timed by growth yields, Y finally. Based on Monod equation (Monod, 1949), specific growth curves of the two enrichment cultures were plotted. Additionally, maximum specific growth rate, $\mu_{\max}$ (hr⁻¹) and half velocity constant, K_S (mM) of these two enrichment cultures were estimated by Woolf plots (Powell, 1983).

Microscopic analysis of enrichment cultures

Due to distinguished morphologies between *Methanosaeta* and *Methanosarcina*, the acridine orange direct counting (AODC) method (Fischer and Velimirov, 2000) was applied to exam relative abundances of *Methanosaeta* or *Methanosarcina* in enrichments or mixed cultures after incubation. 10 μ L liquid samples were placed on microscope slides and dried under room temperature. After heat fixed on flame rapidly, 25 μ L acridine orange (25mg/mL) was dropped onto each dry pellet to dye the sample in dark room for 2 min. Then, acridine orange was rinsed out by DI water and dried under room temperature again. Cover glass was put on top of microscope slides with mineral oil for microscopic analysis. The samples on microscope slides were observed at magnification of 1000 $\times$ with a Carl Zeiss AXIO Scope A1 fluorescence microscope (excitation wavelength at 502nm and emission wavelength at 526nm) equipped with an Axiocam ERc 5s microscope camera (Carl Zeiss, Thornwood, NY, US).

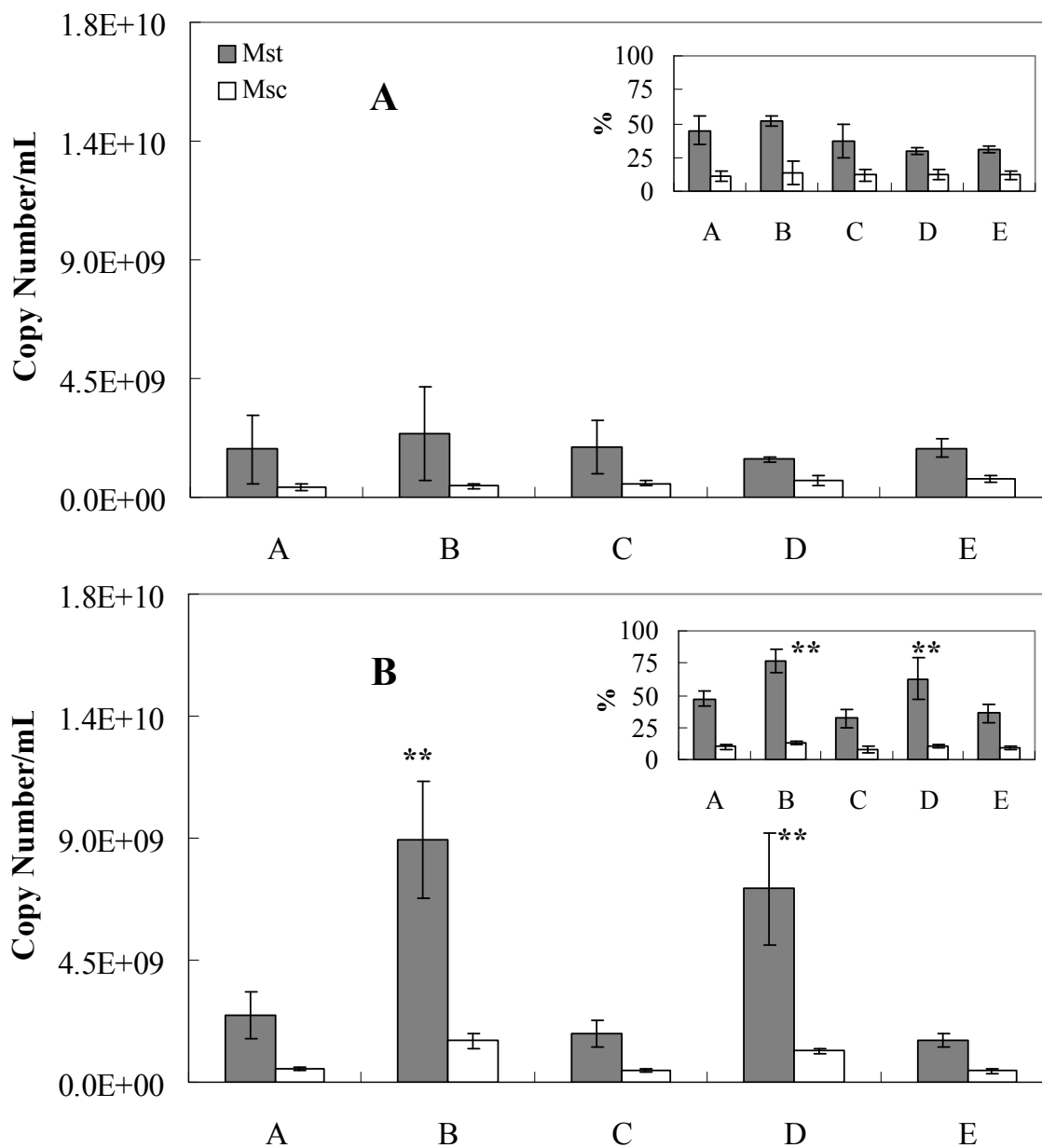


Fig. V13. 16SrRNA gen copy numbers of *Methanosaeta* (gray bar) and *Methanosarcina* (white bar) in both of control (A) and treatment digesters with acetate spiking (B) during whole experimental period. Insets in both of figures indicate the relative percentages of *Methanosaeta* (gray) and *Methanosarcina* (white) in total *Archaea* population. Data are means of triplicate digesters, with the error bars indicating the standard deviation. “***” indicates significant difference between control and treatment digesters ($p < 0.01$).

DNA extraction from enrichment cultures or anaerobic digesters

For digestate samples, 0.25mL liquid samples were transferred into 2mL sterilized eppendorf tubes after homogenization. Biomasses were pelleted by centrifugation (at 14000g for 15min) and stored at -80°C for future analysis. For enrichment or mixed enrichment cultures, biomass were obtained by filtering 1mL liquid samples through sterilized polycarbonate membrane filters with $0.2\mu\text{m}$ pore size (Millipore, Billerica, MA, USA). All filtration apparatuses were autoclaved before use. After filtration, biomasses were collected for DNA extraction immediately. Whole community DNA of all samples was extracted and purified as previously described (Zhang et al., 2009).

TaqMan qPCR protocol

All qPCR assays were performed in 25- μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA) and 1 μL DNA sample. *Methanosarcina* and *Methanosaeta* were quantified with the following primer/probe sets (5' - 3'): *Methanosaeta*—forward primer, TAATCCTTGAAGGACCACCA; reverse primer, CCTACGGCACCGACAAC; and probe, ACGGCAAGGGACGAAAGCTAGG. *Methanosarcina*—forward primer, CGGTTTGGTCAGTCCTCCG; reverse primer, ACCAGACACGGTCGCGC; and probe, AACGGGTTTCGACGGTGAGGGACGA. The relative abundances of *Methanosaeta* and *Methanosarcina* populations in the archaeal community, total archaea were quantified with the *Archaea* population measurement with archaeal domain-specific TaqMan primer/probe set Arc787F-Arc915P- Arc1059R as previously described (Yu et al., 2005). Thermal cycling consisted of a starting incubation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by up to 45 cycles at 95°C for 30 s and 60°C (total *Archaea*) or 57°C (*Methanosaeta*) or 61°C (*Methanosarcina*) for 45 s. The qPCR procedures were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as previously described (Chen et al., 2012). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T).

DNA templates used as the standards for qPCR were partial 16S rRNA genes of representative archaeal populations cloned from the continuous bench-scale anaerobic digesters in a previous study (Chen et al., 2012), including *Methanosaeta* (GenBank Accession No. JN052761) and *Methanosarcina* (GenBank Accession No. JN052757). Standard curves and amplification efficiencies (E) of the qPCR assays were determined with the C_T -Log[Template] plots derived from the quantification of 10-fold dilution series of 16S rRNA gene templates (Chen et al., 2014).

Chemical analysis

Biogas production from the anaerobic digesters was used as the primary parameter to monitor digestion performance (Michaud et al., 2002) and was determined using a water-displacement method described previously (Zhu et al., 2011). Methane content in biogas was analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carboxen®-1000; Sigma-Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a

flow rate of 5 ml min⁻¹ and the following temperature scheme: oven 125°C, injection port 150°C and detector 170°C. Total solids (TS) and volatile solids of the digesters were monitored according to standard methods: 2540 B and E respectively (APHA, 2005).

Acetate in digesters or enrichment cultures was analyzed with an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Santa Clara, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) using 4 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 ml/min. Prior to HPLC analysis, samples were prepared by filtration and acidification to 0.1 N H₂SO₄ by adding 25 µL of 2 N H₂SO₄ to 475 µL of sample as previously described (He and Sanford, 2004). Detection of VFAs was at 210 nm with a multiple wavelength detector. Methanol in digester effluents was quantified with an Agilent G1888 headspace auto-sampler and an Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a DB-624 capillary column (Agilent, Santa Clara, CA, USA). Prior to GC analysis, filtered samples (1 mL) were transferred into 20-mL headspace glass vials sealed with a Teflon®-lined gray butyl septum secured with aluminum crimp caps. Helium was used as the carrier gas for both the auto-sampler and the GC. The auto-sampler used the following configurations: oven temperature, 70 °C; vial equilibration time using maximum agitation, 15 min; sample loop (1 mL) temperature, 125 °C; transfer line (80 cm) temperature, 125 °C; pressurization time, 0.5 min; loop fill time, 0.03 min; loop equilibration time, 0.05 min; inject time, 0.5 min; carrier gas pressure, 2 psig; vial pressurization, 10 psig; cycle time, 14 min. The GC inlet was maintained at 200 °C and 23.11 psig with a total flow of 5.7 mL/min. The inlet split ratio was 1:10. The oven temperature for the GC was kept at 60 °C for 2 min and then increased at a rate of 25 °C/min until the oven reached 200 °C. The column was operated in the constant flow mode at 3.0 mL/min and 23.11 psig. The FID detector was operated at a temperature of 280 °C with the following gases: hydrogen (30 mL/min), air (400 mL/min), and helium (27 mL/min).

Results

Acetate and methanol enrichments with secondary transfer

After acetate or methanol enrichment, the methanogenic populations were studied with qPCR and 16S rRNA gene clone library methods. All clones screened from acetate enrichment cultures were affiliated to *Methanosaeta*, while approximately 40% of sequences screened from methanol enrichments belonged to *Methanosarcina*. Others belonged to *Methanomethylovorans* (data not shown). There was no *Methanosaeta* sequences detected in methanol enrichment cultures. Sequences representing *Methanosaeta* and *Methanosarcina* were selected for 16S rRNA gene phylogenetic tree building with other typical acetoclastic and hydrogentrophic methanogens (Fig. V8). Two typical sequences, Arch-A1 and M4 (Fig. V8), have the closest relationships with *M. concilii* or *M. soehngenii* and *M. mazei* in phylogenetic tree, respectively. As shown, the distance between *Methanosarcina* and *Methanomethylovorans* was closer than *Methanosaeta*.

As compared to quantitative results of *Methanosaeta* and *Methanosarcina* in two enrichment cultures with qPCR assays, observations from clone library studies were

consistent with the quantitative results. As shown (Table V4), obligate acetoclastic methanogen, *Methanosaeta* was enriched after acetate enrichment process. Averagely, 80% of total *Archaea* populations belonging to *Methanosaeta* indicated its dominance under relatively high acetate concentration (20mM). Another acetoclastic methanogen, *Methanosarcina* with versatile metabolisms (De Vrieze et al., 2012; Liu et al., 2011; Thauer et al., 2008) was a minor species only comprising less than 1% of *Archaea* populations (Table V4). Through methanol enrichment process (30mM), however, *Methanosarcina* became a major population (57%). Inconsistent with observations from previous studies that *Methanosarcina* was more competitive than *Methanosaeta* in acetate utilization under high concentration due to its advantageous metabolisms (De Vrieze et al., 2012; Smith and Mah, 1980; Thauer et al., 2008; Wandrey and Aivasidis, 1983; Weimer and Zeikus, 1978), dominance of *Methanosaeta* rather than *Methanosarcina* observed in acetate enrichment cultures with high concentration is unexpected.

Acetate loading into digesters with high concentration

Because of changes of acetate concentration during operational time in batch incubation, semi-continuous reactors were applied to achieve a constant high acetate concentration. Initially, triplicate digesters were spiked with concentrated sodium acetate solution to elevate acetate concentration above 20mM. Sequentially, extra acetate loading into treatment digesters with dairy farm waste slurry was increased stepwise up to the maximum, 12.5mmol/L/day under stable digester performance (Fig. V9). After 5 days of feeding, acetate loading was ceased. During the first acetate loading, CH₄ generation rate was increased by 100%, from ~200 to 420 mL_{CH₄}/L_{digester}/day (Fig. V10). Without acetate loading, CH₄ production recovered to initial status as compared to control digesters. To monitor the reproducibility of CH₄ production increase in response to acetate loading, the second acetate loading was implemented. In this time, the maximum rate, 12.5mmol/L/day was directly loaded into digesters for 6 days. Similarly, CH₄ production rate increased from 270 to 450 mL_{CH₄}/L_{digester}/day (Fig. V10). As expected, three control digesters had stable CH₄ production at rate, 220 mL_{CH₄}/L_{digester}/day throughout the whole experimental.

Correspondingly, extra acetate utilization rates in treatment reactors increased from 2 to 12mmol/L/day during the first acetate loading event. The similar trend was also observed in second acetate spiking events, which was basically consistent with the stoichiometry of acetate in methanogenesis (Thauer et al., 1977). With extra acetate loadings in both events, the relatively high acetate concentration (20 ~ 36mM) was maintained (Fig. V11).

Additionally, during the whole experiment period, pH values in digesters with high acetate loadings seemed not to be disrupted significantly and kept constant as same as the pH in control digesters (7.6 ~ 7.7). Moreover, the dramatic increase of the volatile solid concentrations in digesters with acetate loading, from 1.5 to 2 times higher than control digesters, indicated the biomass accumulations (Fig. V12).

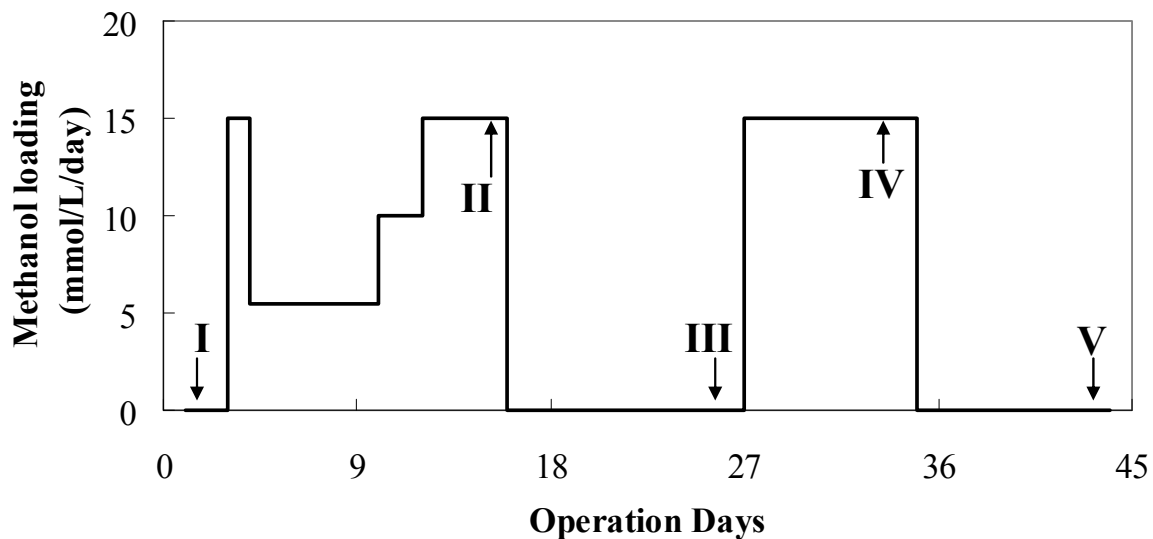


Fig. V14. Changes in the methanol loading rate of the treatment anaerobic digesters during the period of study. Letters and arrows indicate the sampling points of biomass from digesters.

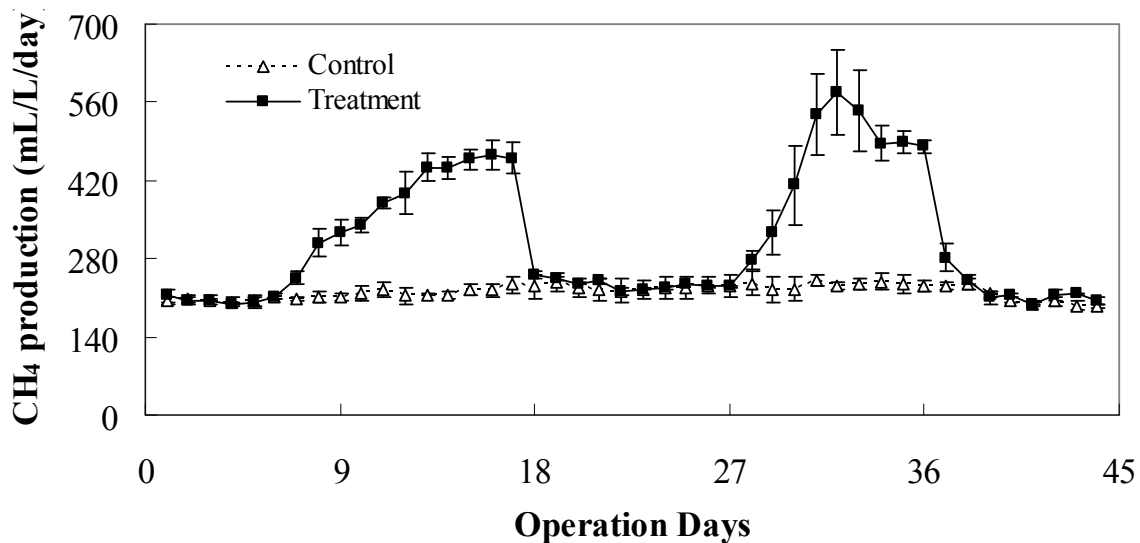


Fig. 15. Methane production of control and treatment digesters with methanol spiking. Data are means of triplicate digesters, with the error bars indicating the standard deviation. Control digesters ($\cdots\Delta\cdots$); Treatment digesters ($\blacksquare$).

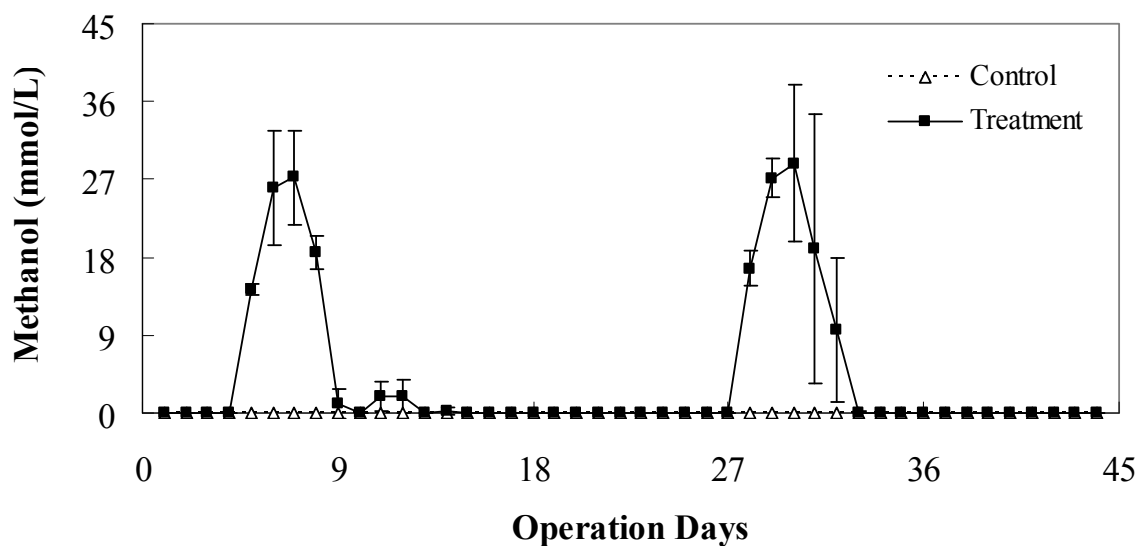


Fig. V16. Concentrations of methanol in both of control ($\cdots\Delta\cdots$) and treatment ($\blacksquare$) anaerobic digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation.

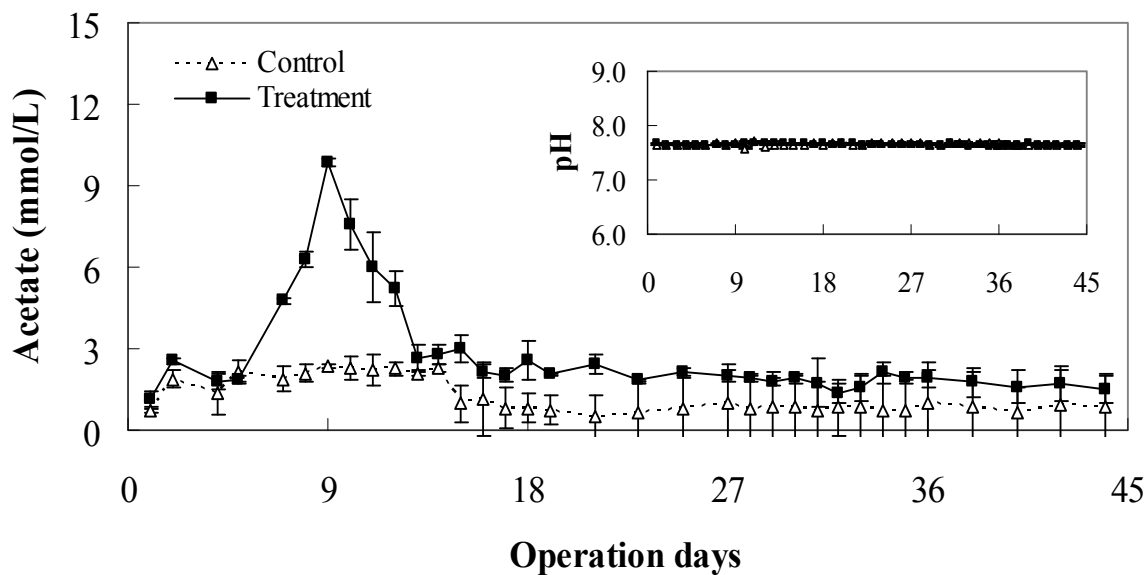


Fig. V17. Concentrations of acetate in both of the control ($\cdots\Delta\cdots$) and treatment ($\blacksquare$) anaerobic digesters. Data are means of triplicate digesters, with the error bars indicating the standard deviation. Inset indicates the pH values of both of the control and treatment digesters during methanol spiking period.

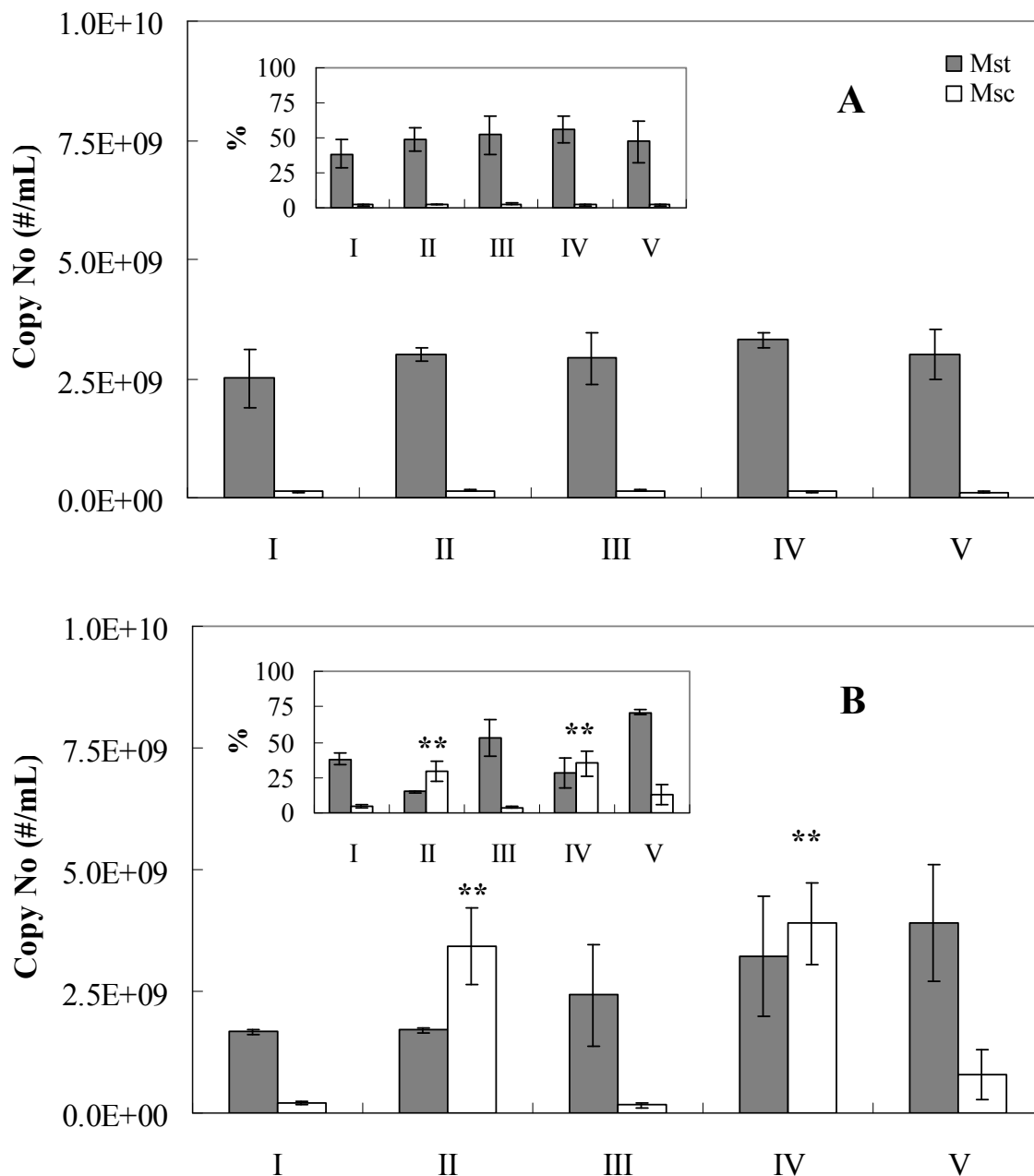


Fig. V18. 16SrRNA gen copy numbers of *Methanosaeta* (gray bar) and *Methanosarcina* (white bar) in both of control (A) and treatment digesters with metahnlol spiking (B) during whole experimental period. Insects in both of figures indicate the relative percentages of *Methanosaeta* (gray) and *Methanosarcina* (white) in total *Archaea* population. Data are means of triplicate digesters, with the error bars indicating the standard deviation. “***” indicates significant difference between control and treatment digesters ($p < 0.01$).

Table V5 Quantification of 16SrRNA gene abundances of major methanogens with qPCR in acetate enrichment cultures.

Enrichment ^a	16S rRNA Gene Targets	No. of Copies/mL ^d	Relative Abundance (% of Total <i>Archaea</i>) ^d
Acetate ^b	<i>Methanosaeta</i>	$(1.42 \pm 0.07) \times 10^8$	91.32 ± 4.39
	<i>Methanosarcina</i>	$(1.43 \pm 0.17) \times 10^6$	0.92 ± 0.11
	Total <i>Archaea</i>	$(1.55 \pm 0.01) \times 10^8$	-
Acetate inoculated with methanol enrichment ^c	<i>Methanosaeta</i>	NA ^e	0
	<i>Methanosarcina</i>	$(1.05 \pm 0.02) \times 10^8$	91.00 ± 0.10
	Total <i>Archaea</i>	$(1.15 \pm 0.02) \times 10^8$	-

^a Enrichments were achieved by a series of transfers of anaerobic cultures from previous generation to next serum bottles with fresh anaerobic medium.

^b Acetate enrichment was achieved by a series of transfers (10%, v/v) of anaerobic digestates with feeding of 20mM acetate.

^c Acetate enrichment was achieved by a series of transfers (1%, v/v) of methanol enrichment cultures with feeding of 20mM acetate.

^d Values are the means of triplicate samples $\pm$ standard deviation.

^e Not detected by qPCR assay.

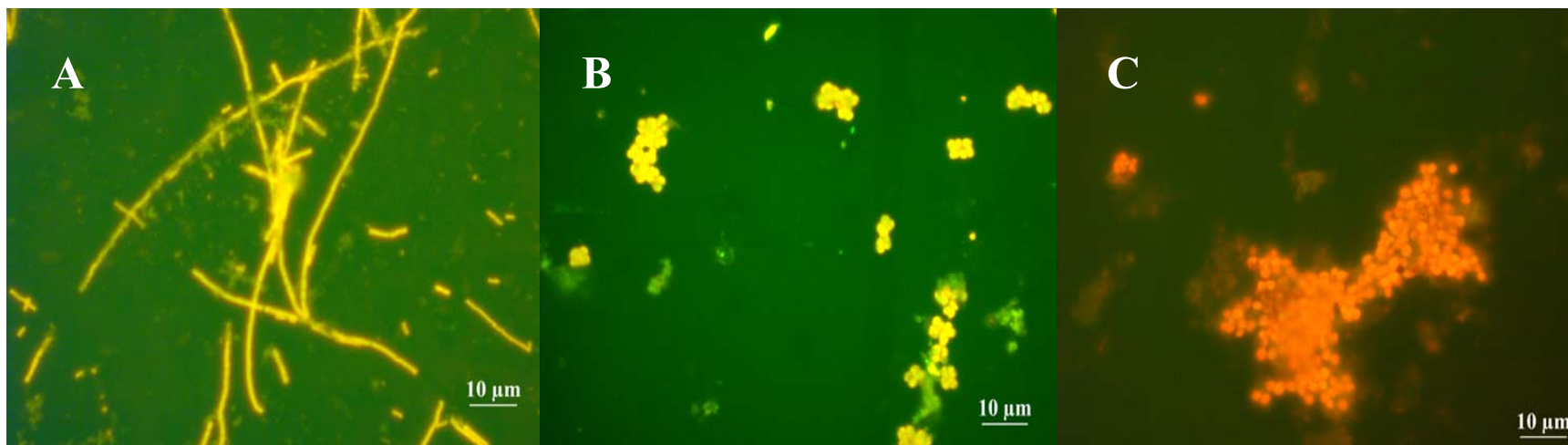


Fig. V19. Epifluorescence micrographs of acetate or methanol enrichment cultures from anaerobic digesters with acridine orange direct counting method (AODC). (A) acetate (20mM) enrichment culture from anaerobic digestates; (B) methanol (30mM) enrichment culture; (C) acetate (20mM) enrichment culture inoculated with cultures from methanol enrichment (B). Bar, 10µm.

Relative abundances of *Methanosaeta* and *Methanosarcina* in response to acetate loading

As shown in Fig. V9, samples at 5 time points were collected from both treatment and control digesters for methanogenic population quantifications with qPCR assays. Concentrations of *Methanosaeta* or *Methanosarcina* were $(1.88 \pm 0.35) \times 10^9$ copy/mL and $(4.95 \pm 1.11) \times 10^8$ copy/mL under digester stable performance without acetate loading, respectively (Fig. V13A-B). Relative abundances of *Methanosaeta* and *Methanosarcina* were (38.62 ± 8.26) % and (11.03 ± 1.71) % of the total *Archaea* respectively, which was consistent with observations from previous studies (Chen et al., 2012; Zhang et al., 2011). As expected, relatively low acetate concentrations, 1 ~ 2 mM (Fig. V11), under the steady state of the digester performance without acetate loading could make *Methanosaeta* dominant or more competitive than *Methanosarcina* due to its higher affinity of acetate and lower K_S and μ_{max} (Conklin et al., 2006; De Vrieze et al., 2012; Huser et al., 1982; Jetten et al., 1990; Min and Zinder, 1989; Ohtsubo et al., 1992; Patel, 1984).

In two events with acetate loading, acetoclastic methanogenes including *Methanosaeta* and *Methanosarcina* increased significantly (Fig. V13B). For *Methanosaeta*, the concentration increased to $(8.04 \pm 1.30) \times 10^9$ copy/mL, more than four times of that without acetate loading. The relative abundance in total *Archaea* populations also increased from 40% to 70% approximately. For *Methanosarcina*, its relative percentage was still kept constant at 10%, averagely, although its concentration increased by 2 ~ 3 times to $(1.33 \pm 0.26) \times 10^9$ copy/mL (Fig. V13B). It was understandable that both of acetoclastic methanogens, i.e. *Methanosaeta* and *Methanosarcina* increased with acetate spiking. Surprisingly, however, *Methaosaeta* but not *Methanosarcina* became dominant in high acetate concentration (>20mM) under steady state of semi-continuous digesters. To investigate the metabolisms between these two species in anaerobic digestion, further studies were necessary to provide solid evidence.

Methanol loading into digesters

Due to enrichment effects of *Methanosarcina* with methanol in batch reactors (Table V4), methanol spiking into semi-continuous digesters were implemented. Initially, methanol was loaded into triplicate digesters at 15mmol/L/day. Since there was no immediate response of CH_4 production increase, the loading rate was then reduced to 5.5mmol/L/day and increased to maximum rate, 15mmol/L/day stepwise again (Fig. V14). After 5 days of feeding at maximum rate, methanol loading was stopped. Similar with the scheme of the acetate feeding, the second methanol loading at rate 15mmol/L (for 9 days) was repeated in order to verify the reproducibility of CH_4 production increase in response to methanol spiking.

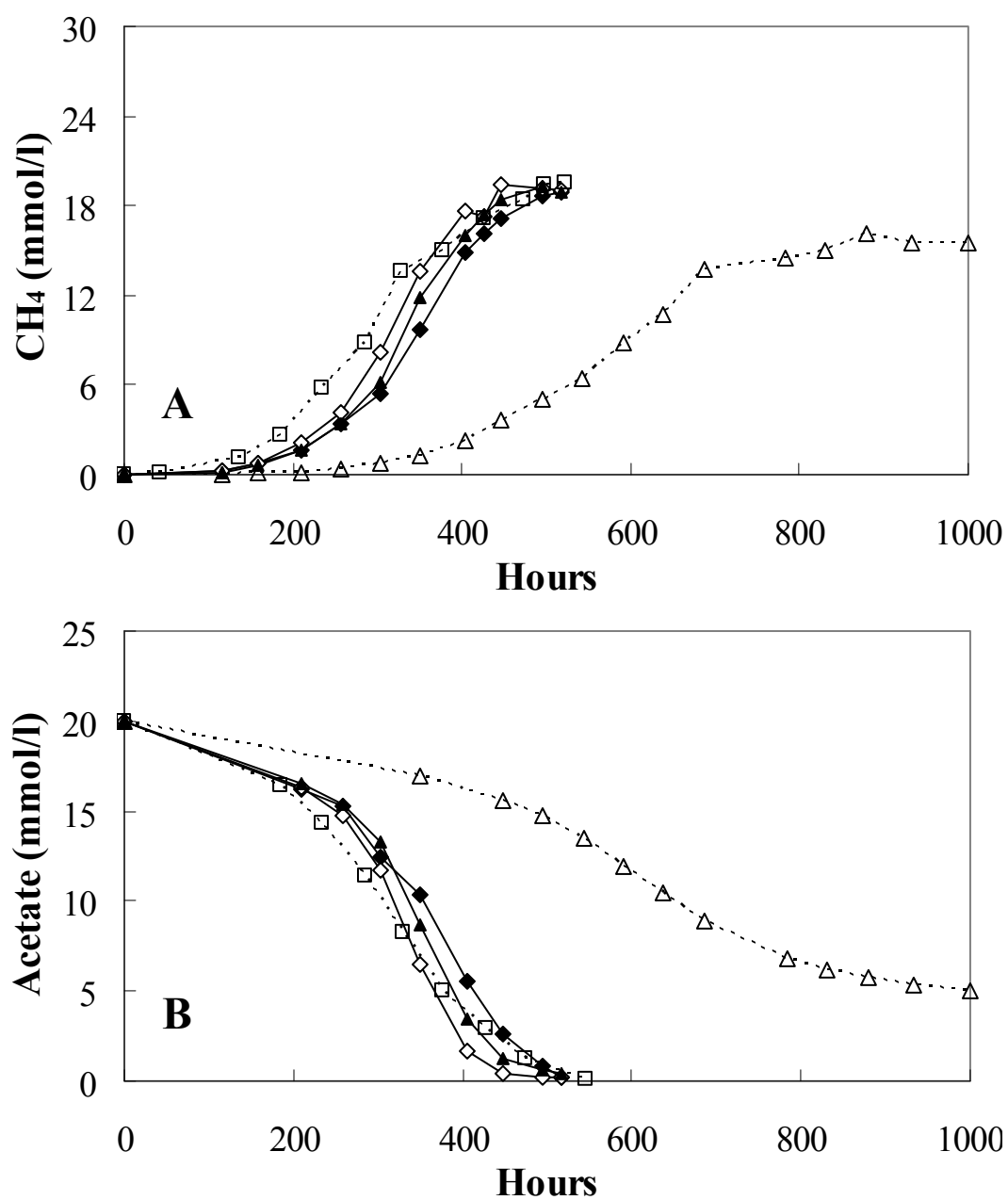


Fig. V20. Concentration changes of acetate (A) and methane (B) in anaerobic batch reactors with inocula of mixed cultures of *Methanosaeta* (Mst, enriched from digesters) and *Methanosarcina* (Msc, enriched from digesters) under different initial ratios during incubation period. Mst / Msc = 70/30 (-◆-); Mst / Msc = 50/50 (-◇-); Mst / Msc = 30/70 (-▲-); 100% Msc (--Δ--), 100% Mst (--□--).

Under steady state of triplicate digesters with methanol loading, methane production rates were 450 and 490 mL_{CH₄}/L_{digester}/day during these two events respectively, which were almost two times higher than those without methanol loading (Fig. V15). When methanol feedings were ceased, CH₄ production rates decreased to original status at 200 mL_{CH₄}/L_{digester}/day. As compared to methane production rates in control reactors in acetate loading experiment, the stable CH₄ generation from control digesters at rates ranging from 200 ~ 230 mL_{CH₄}/L_{digester}/day in methanol spiking experiment seemed to be consistent (Fig. V15). In the beginning of these two methanol loading events, methanol was accumulated initially. With the metabolic altering and new methanogenic functionalism development, methanol was rapidly utilized and no considerable concentration was detected (Fig. V16). Additionally, acetate concentration in both treatment and control digesters was also monitored. Generally, it was maintained constant, at 1 ~ 2 mM for both sets of digesters except the first methanol loading event, in which acetate increased up to 9mM initially and then reduced to normal level immediately (Fig. V17).

Relative abundances of *Methanosaeta* and *Methanosarcina* in response to methanol loading

As shown in Fig. V14, samples of 5 time points were collected from both treatment and control digesters for methanogenic population quantifications. As observed in methanol enrichment cultures, *Methanosarcina* was enriched and became dominant in total *Archaea* populations (Fig. V18B). In average, *Methanosaeta* varied from 2.5 to 3.0 × 10⁹ copy/mL in both of the treatment and control digesters without methanol feeding. Although there were some variations of *Methanosaeta* concentrations, they didn't have significant differences, while *Methanosarcina* concentrations responded to methanol loading dramatically, increased by almost 20 times, from (1.82 ± 0.22) × 10⁸ to (3.88 ± 0.32) × 10⁹ copy/mL (Fig. V18B). Meanwhile, total *Archaea* populations also increased 2.5 times from (4.81 ± 0.64) × 10⁹ to (1.14 ± 0.02) × 10¹⁰ copy/mL. In control digesters, generally, relative percentages of *Methansaeata* and *Methanosarcina* among total *Archaea* populations were (48.52 ± 6.58) % and (2.22 ± 0.27) %, respectively (Fig. V18A). Similar patterns were also observed in treatment digesters without methanol loading (Fig. V18B). With methanol lading, however, the relative abundance of *Methanosaeta* decreased to (21.48 ± 9.50) % and increased to normal level when the methanol feeding was stopped. For *Methanosarcina*, however, the relative abundance increased significantly to (32.31 ± 3.49) % with methanol loading. When methanol feeding was ceased, its abundance decreased to the same level as control digesters (Fig. V18B). It is understandable that the relative abundance of *Methanosaeta* decreased with methanol loading due to considerable increase of *Methanosarcina*. Although *Methanosarcina* is metabolic versatile in methanogenesis (Thauer et al., 2008), the promotion of growth with methanol rather than acetate may indicate the more preferable methylotrophy than acetotrophy of *Methanosarcina* in anaerobic digestion process.

***Methanosaeta* and *Methanosarcina* enrichment with acetate and methanol**

After enrichment processes, higher percentages (all above 90%) of *Methanosaeta* and *Methansarcina* among total *Archaea* populations achieved with acetate (20mM) or

methanol (30mM), indicated the relatively purified cultures for future investigations (Table V5). After enrichment, less than 1% of archaeal sequences belonging to *Methanosarcina* indicated its less advantageous competition in acetate utilization than *Methanosaeta*. No *Methanosaeta* sequence, however, was detected by qPCR assay in *Methanosarcina* enrichment culture proving the extinction of *Methanosaeta* in methanol enrichment before the acetate enrichment. On the other hand, microscopic images with AODC method of enrichment cultures supported qPCR results. As shown in Fig. V19A, filament cells indicated the dominance of *Methanosaeta*. Instead, cultures with methanol enrichment (Fig. V19B) and acetate enrichments of cultures transferred from methanol enrichment (Fig. V19C) showed the coccoid cells or large aggregates, which indicated that *Methanosaeta* became extinct and *Methanosarcina* was predominant.

Competition between *Methanosaeta* and *Methanosarcina* in mixed cultures

After enrichment, both *Methanosaeta* and *Methanosarcina* cultures mixed with three ratios, 7:3, 1:1 and 3:7 were inoculated into batch reactors with 20mM acetate. During incubation period, the acetate and methane concentrations were monitored. As shown in Fig. V20A-B, the acetate utilization rate or CH₄ production rate, equivalently, were much higher than *Methanosarcina* enrichment culture without *Methanosaeta*. For all mixed cultures inoculated with different initial ratios, their kinetics in methanogenesis seemed to be similar. Within no more than 500 hours, reactions can be completed. The equal reaction rates of acetate utilization and methane production were consistent with the stoichiometry of aceticlastic methanogenesis. Surprisingly, without *Methanosaeta* mixed, however, methane production rate of *Methanosarcina* enrichment was much slower under relatively high acetate concentration (20mM), in which more than 1000 hours were needed to complete the reactions. When acetate concentrations decreased to 4 ~ 5 mM, reaction rates begun to decelerate dramatically. During a relatively long period of time, acetate or methane concentrations almost kept constant (Fig. V20A-B).

After incubation, microcosms of mixed cultures were collected for AODC analysis. As compared to initial statuses of mixed cultures with different ratios (Fig. V21A-C), dominance of filament cells of all cultures after incubation (Fig. V21D-F) also indicated the advantageous competition of *Methanosaeta* in acetate utilization rather than *Methanosarcina*.

Growth kinetics of *Methanosaeta* and *Methanosarcina* enrichments

For both enrichments obtained from digesters, growth kinetics of acetoclastic methanogenesis was determined under various acetate concentrations with resting cell methods. As shown in Fig. V22, the specific growth curve of *Methanosaeta* enrichment was all above that of *Methanosarcina* enrichment up to the maximum acetate concentration, 20mM in this study, which was inconsistent previous observations that *Methanosaeta* was usually dominant and became more competitive in acetate utilization than *Methanosarcina* under low acetate concentration (<1mM) (Conklin et al., 2006; Jetten et al., 1990; Min and Zinder, 1989; Schmidt et al., 2000). Similar with growth kinetics parameters, i.e. growth yield, Y (mgVSS/mgCOD), maximum specific growth rate, $\mu_{\max}$ (hr⁻¹) and half-velocity constant, K_s (mM) of *Methanosaeta* determined by previous studies (Fukuzaki et al., 1990; Huser et al., 1982; Jetten et al., 1990; Ohtsubo et

; Patel, 1984), these values of *Methanosaeta* enriched in this study were almost within ranges expect the growth yield value, Y , which was higher in study (Table V6). For *Methanosarcina* enriched in this study, results of growth yield and K_s seemed to be similar with values determined previously (Fukuzaki et al., 1990; Min and Zinder, 1989; Smith and Mah, 1980; Wandrey and Aivasidis, 1983; Weimer and Zeikus, 1978). For the maximum specific growth rate, $\mu_{\max}$, however, the value measured in this study, was more than 10 times lower than previous', which may explain the phenomena observed in digesters with high acetate concentration loading and mix cultures with two species that *Methanosaeta* was dominant and more competitive in acetoclastic methanogenesis than *Methanosarcina* even under high acetate concentration (>20mM). Although mechanisms resulting in the opposite observations in this study were still elusive, more studies were still needed in the future.

Discussion

Observation that *Methanosaeta* population was dominant and more preferable in acetoclastic metabolisms than *Methanosarcina* under high acetate concentration (>20mM) was inconsistent with previous studies (Conklin et al., 2006; De Vrieze et al., 2012; Fukuzaki et al., 1990; Thauer et al., 2008). Although it seemed striking that results from this study didn't agree with conventional opinions about growth competition between these two populations in acetoclastic metabolism, there were still some studies having the opposite observations recently (Ito et al., 2011; Moertelmaier et al., 2013; van Haandel et al., 2013). In studies of Moertelmaier et al. 2013 and van Haandel et al., 2013, *Methanosaeta* was always the dominant acetoclastic methanogens in USAB or CSTR in restart session, despite the favorable conditions for *Methanosarcina* development or relative high acetate concentration in reactors (10 ~ 20mM) during restart period. Although it was speculated that it may be attributed to the reduced tendency to incorporate into sludge granules for *Methanosarcina*, more strong evidence are still needed to support this. Additionally, Yilmaz et al., (2014) evaluated the relationship between acetoclastic methanogen composition and acetate utilization capacity by incubating biomass samples into batch serum bottles, which were collected from 15 full-scale anaerobic digesters. With one-way ANOVA and PCA analyses, however, there was no statistically significant correlation between acetate utilization capacities and acetoclastic methanogene community composition. Besides kinetics of acetate utilization determining the predominant acetoclastic methanogenes, alternatively, there were other factors influencing the methanogene community structures, e.g., ammonia concentration (Calli et al., 2005; Karakashev et al., 2005) or trace metals, particularly Fe, Ni and Co (Qiang et al., 2013; Rothman et al., 2014; Takashima et al., 2011). It is critical for future studies to investigate the roles of ammonia or trace metals playing in acetoclastic methanogenesis, although these are beyond the topic in this study.

Although it was commonly considered that *Methanosarcina* had high maximum specific growth rate, $\mu_{\max}$ and K_s , which made it more competitive than *Methanosaeta* in acetoclastic metabolism (Conklin et al., 2006), there was a wide range of growth kinetic values for both species reported by previous studies (Yilmaz et al., 2014). For $\mu_{\max}$, it ranged from 0.003 to 0.03hr⁻¹ for *Methanosaeta* and from 0.008 to 0.06hr⁻¹ for

Methanosarcina (Fukuzaki et al., 1990; Huser et al., 1982; Hutten et al., 1980; Jetten et al., 1990; Min and Zinder, 1989; Mladenovska and Ahring, 2000; Ohtsubo et al., 1992; Zinder and Mah, 1979; Zinder et al., 1985). Based on these values reported, these two ranges of $\mu_{\max}$ for *Methanosaeta* and *Methanosarcina* species shared the common interval. It can not exclude the possibility that *Methanosaeta* has higher values of $\mu_{\max}$ than *Methanosarcina* under some circumstance and *Methanosaeta* became more competitive in acetate utilization even under relatively high acetate concentrations. Surprisingly, the $\mu_{\max}$ of *Methanosarcina* enrichment culture, only 0.0003hr^{-1} estimated in this study was much lower than previous values reported (Table V6). It can explain the dominance of *Methanosaeta* in anaerobic digesters even with high concentration of acetate loading (Fig. V13). Another reason to explain this phenomenon could be the acetate concentration range applied in digesters or batch reactors in this study. The highest acetate concentration used in batch study was 20mM and was 25~36mM in semi-CSTR digesters. Also, the $\mu_{\max}$ of *Methanosarcina* enrichment determined under the highest concentration, 20mM could be underestimated because the specific growth rate could increase with acetate concentration increase from 20mM to 100mM or even higher. Within certain acetate concentration range (0-20mM in this study), therefore, it is possible that *Methanosaeta* could be more competitive under a wider acetate concentration range than *Methanosarcina*, which may be inconsistent with the acetate concentration threshold established previously, 1mM approximately (Conklin et al., 2006; De Vrieze et al., 2012; Jetten et al., 1992).

Instead of the acetoclastic metabolism of *Methanosarcina*, its methylotrophic methanogenesis was also verified by the performance of the methanol enrichment and digesters with methanol loading. As compared to the abundance of *Methanosarcina* in digesters with acetate loading (Fig. V13), its concentration increased dramatically in response to methanol loading (Fig. V18). It indicated that methanol was more preferable to be utilized than acetate by *Methanosarcina* from digesters, which can be confirmed by previous studies about the growth kinetics of *Methanosarcina* with different substrates or mixed substrates. It was found that the doubling time for *Methanosarcina* was shortened with methanol than acetate (Zinder and Mah, 1979) and the methanogenesis rate with methanol was approximately 5 times higher as compared to the substrate, acetate (Hutten et al., 1980). When the methanol catabolism was induced, acetate catabolism may be repressed (Zinder and Elias, 1985), which was also proved by the stable carbon isotope fractionation analysis of the methanogenesis from different substrates. It was revealed that the isotopic fractionation for methane derived from acetate was only one-third of that with methanol (Krzycki et al., 1987). Due to the versatile metabolic pathways of *Methanosarcina* in methanogenesis (De Vrieze et al., 2012), digesters with methanol loading showed a potential metabolism of *Methanosarcina* in whole anaerobic digestion food web besides acetotrophy and provided one possible explanation of the dominance of *Methanosaeta* in digesters with high acetate concentration rather than *Methanosarcina*. Therefore, functionalism of *Methanosarcina* in anaerobic digesters and the distributions of its three major pathways, i.e. acetotrophy, hydrogentrophy and methylotrophy in methanogenic process are still needed to be investigated in the future.

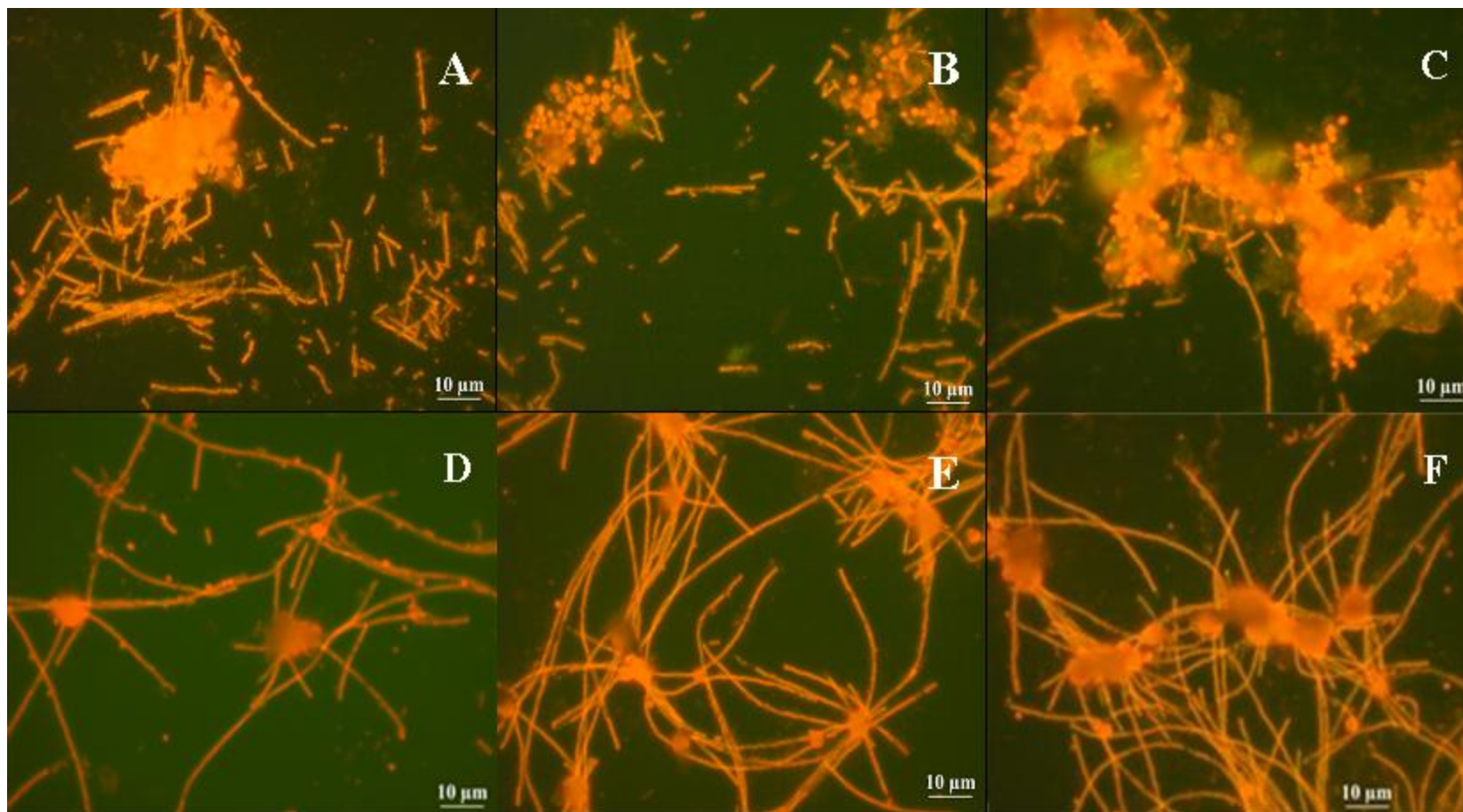


Fig. V21. Epifluorescence micrographs of mixed cultures of *Methansaeta* sp. (enriched) and *Methanosarcina* sp. (enriched) in anaerobic batch reactors with acridine orange direct counting method (AODC). Cells with morphologies of nonmobile rods forming filaments and individual coccoid cells or large aggregates represent *Methansaeta* sp. and *Methanosarcina* sp. respectively. Figures from (A) to (C) indicate initial ratios between *Methansaeta* sp. and *Methanosarcina* sp., 70/30, 50/50 and 30/70 approximately, at the beginning of anaerobic digester setting. Figures from (D) to (F) indicate final statuses of mix cultures of *Methansaeta* sp. and *Methanosarcina* sp. in anaerobic batch reactors after incubation, which were inoculated by mix cultures of *Methansaeta* sp. and *Methanosarcina* sp. with ratios, 70/30, 50/50 and 30/70 respectively. Bar, 10μm.

Table V6 Summary of growth kinetics parameters of enrichment cultures of *Methanosaeta* and *Methanosarcina* utilizing acetate.

Growth Kinetics Parameters	Enriched <i>Methanosaeta</i> ^b	Enriched <i>Methanosarcina</i> ^b
$\mu_{\max}(\text{hr}^{-1})^a$	$(3.76 \pm 0.12) \times 10^{-3}$	$(3.03 \pm 0.95) \times 10^{-4}$
$K_s (\text{mM})^a$	0.65	5
Y (mg VSS/mg COD)	$(2.58 \pm 0.22) \times 10^{-2}$	$(3.15 \pm 0.27) \times 10^{-2}$

^a Values were estimated by specific growth curves approximately. Values of $\mu_{\max}$ were estimated based on the highest acetate concentration, 20mM applied in this study.

^b Data are means of triplicate anaerobic batch reactors, with the error bars indicating the standard deviation.

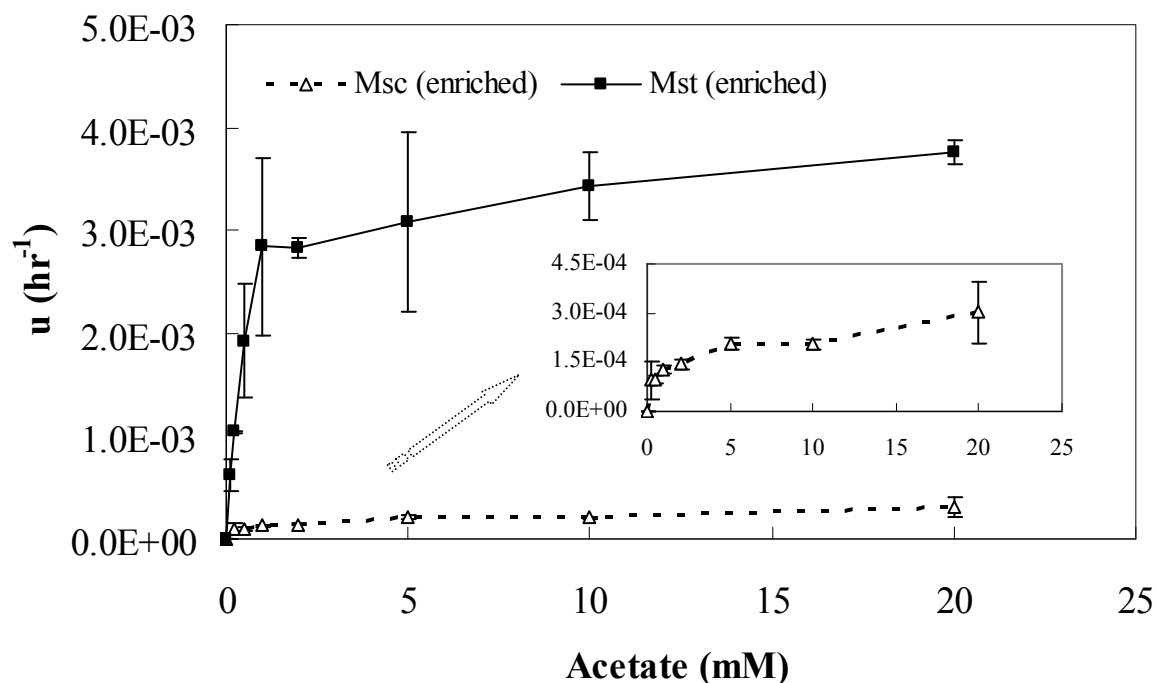


Fig. V22. Specific growth curves of enrichment cultures of *Methanosaeta* (Mst, ■-) and *Methanosarcina* (Msc, ··Δ··) under different acetate concentrations. Inset is the specific growth curve of Msc enrichment culture zoomed in. Data are means of triplicate anaerobic batch reactors, with the error bars indicating the standard deviation.

Conclusion

Dominance of *Methanosaeta* was observed in both enrichment cultures with high acetate loading (20mM) and lab-scale mesophilic anaerobic digesters with high acetate concentrations (25 ~ 36mM). Instead of acetate, the enrichment of *Methanosarcina* proved by methanol enrichment cultures and semi-CSTR digesters with methanol loading indicated the preferable methylotrophic methanogenesis of *Methanosarcina* from the digesters in this study despite other pathways. Dissimilar with previous results, the much lower maximum specific growth rate, μ_{max} of *Methanosarcina* enrichment culture determined in this study can be proved by final ratios of *Methanosaeta* to *Methanosarcina* in mixed enrichment cultures after 20mM acetate incubation, in which *Methanosaeta* was always the dominant acetoclastic methanogens even in mix cultures inoculated with more *Methanosarcina* initially.

References

- Angenent, L.T., Zheng, D., Sung, S., Raskin, L., 2002. Microbial community structure and activity in a compartmentalized, anaerobic bioreactor. *Water environment research : a research publication of the Water Environment Federation*. 74, 450-61.
- APHA. 2005. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.
- Bridgham, S.D., Cadillo-Quiroz, H., Keller, J.K., Zhuang, Q., 2013. Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. *Global Change Biology*. 19, 1325-1346.
- Calli, B., Mertoglu, B., Inanc, B., Yenigun, O., 2005. Community Changes During Start-up in Methanogenic Bioreactors Exposed to Increasing Levels of Ammonia. *Environmental Technology*. 26, 85-91.
- Chen, S., Zamudio Cañas, E.M., Zhang, Y., Zhu, Z., He, Q., 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *Journal of Applied Microbiology*. 113, 1371-1379.
- Chen, S., Zhu, Z., Park, J., Zhang, Z., He, Q., 2014. Development of Methanoculleus-specific real-time quantitative PCR assay for assessing methanogen communities in anaerobic digestion. *Journal of Applied Microbiology*. 116, 1474-1481.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., Tiedje, J.M., 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research*. 31, 442-443.
- Conklin, A., Stensel, H.D., Ferguson, J., 2006. Growth kinetics and competition between Methanosarcina and Methanosaeta in mesophilic anaerobic digestion. *Water Environment Research*. 78, 486-496.
- De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W., 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. *Bioresource Technology*. 112, 1-9.
- DeLong, E.F., 1992. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences*. 89, 5685-5689.
- Ferry, J.G., 1992. Methane from acetate. *Journal of Bacteriology*. 174, 5489-5495.
- Fischer, U.R., Velimirov, B., 2000. Comparative study of the abundance of various bacterial morphotypes in an eutrophic freshwater environment determined by AODC and TEM. *Journal of Microbiological Methods*. 39, 213-224.
- Fukuzaki, S., Nishio, N., Nagai, S., 1990. Kinetics of the Methanogenic Fermentation of Acetate. *Applied and Environmental Microbiology*. 56, 3158-3163.
- Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L., 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. *Biotechnology and Bioengineering*. 57, 342-355.
- He, Q., Sanford, R., 2002. Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. *Biodegradation*. 13, 307-316.
- He, Q., Sanford, R.A., 2004. Acetate Threshold Concentrations Suggest Varying Energy Requirements during Anaerobic Respiration by *Anaeromyxobacter dehalogenans*. *Applied and Environmental Microbiology*. 70, 6940-6943.

- Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. *Water Research*. 38, 1376-1389.
- Huser, B., Wuhrmann, K., Zehnder, A.B., 1982. *Methanotherx soehngeni* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. *Arch. Microbiol.* 132, 1-9.
- Hutten, T.J., Bongaerts, H.C.M., van der Drift, C., Vogels, G.D., 1980. Acetate, methanol and carbon dioxide as substrates for growth of *Methanosarcina barkeri*. *Antonie van Leeuwenhoek*. 46, 601-610.
- Ito, T., Yoshiguchi, K., Ariesyady, H.D., Okabe, S., 2011. Identification of a novel acetate-utilizing bacterium belonging to Synergistes group 4 in anaerobic digester sludge. *The ISME journal*. 5, 1844-56.
- Jeris, J.S., McCarty, P.L., 1965. The Biochemistry of Methane Fermentation Using [image] Tracers. *Journal (Water Pollution Control Federation)*. 37, 178-192.
- Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B., 1990. Acetate threshold values and acetate activating enzymes in methanogenic bacteria. *FEMS Microbiology Letters*. 73, 339-344.
- Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B., 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanotherx soehngeni* and *Methanosarcina* spp. *FEMS Microbiology Letters*. 88, 181-198.
- Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors. *Applied and Environmental Microbiology*. 71, 331-338.
- Krzycki, J.A., Kenealy, W.R., DeNiro, M.J., Zeikus, J.G., 1987. Stable Carbon Isotope Fractionation by *Methanosarcina barkeri* during Methanogenesis from Acetate, Methanol, or Carbon Dioxide-Hydrogen. *Applied and Environmental Microbiology*. 53, 2597-2599.
- Leclerc, M., Delgènes, J.-P., Godon, J.-J., 2004. Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. *Environmental Microbiology*. 6, 809-819.
- Lee, J., Hwang, B., Koo, T., Shin, S.G., Kim, W., Hwang, S., 2014. Temporal variation in methanogen communities of four different full-scale anaerobic digesters treating food waste-recycling wastewater. *Bioresource Technology*.
- Lins, P., Reitschuler, C., Illmer, P., 2014. *Methanosarcina* spp., the key to relieve the start-up of a thermophilic anaerobic digestion suffering from high acetic acid loads. *Bioresource Technology*. 152, 347-354.
- Liu, H., Wang, J., Wang, A., Chen, J., 2011. Chemical inhibitors of methanogenesis and putative applications. *Applied microbiology and biotechnology*. 89, 1333-40.
- Liu, Y., Whitman, W.B., 2008. Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea. *Annals of the New York Academy of Sciences*. 1125, 171-189.
- Ma, J., Zhao, B., Frear, C., Zhao, Q., Yu, L., Li, X., Chen, S., 2013. *Methanosarcina* domination in anaerobic sequencing batch reactor at short hydraulic retention time. *Bioresource Technology*. 137, 41-50.

- McHugh, S., Carton, M., Mahony, T., O'Flaherty, V., 2003. Methanogenic population structure in a variety of anaerobic bioreactors. *FEMS Microbiology Letters*. 219, 297-304.
- McMahon, K.D., Stroot, P.G., Mackie, R.I., Raskin, L., 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—II: microbial population dynamics. *Water Research*. 35, 1817-1827.
- McMahon, K.D., Zheng, D., Stams, A.J.M., Mackie, R.I., Raskin, L., 2004. Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. *Biotechnology and Bioengineering*. 87, 823-834.
- Michaud, S., Bernet, N., Buffière, P., Roustan, M., Moletta, R., 2002. Methane yield as a monitoring parameter for the start-up of anaerobic fixed film reactors. *Water Research*. 36, 1385-1391.
- Min, H., Zinder, S.H., 1989. Kinetics of Acetate Utilization by Two Thermophilic Acetotrophic Methanogens: *Methanosarcina* sp. Strain CALS-1 and *Methanotherix* sp. Strain CALS-1. *Appl Environ Microbiol*. 55, 488-91.
- Mladenovska, Z., Ahring, B.K., 2000. Growth kinetics of thermophilic *Methanosarcina* spp. isolated from full-scale biogas plants treating animal manures. *FEMS Microbiology Ecology*. 31, 225-229.
- Moertelmaier, C., Li, C., Winter, J., Gallert, C., 2013. Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. *Bioresource Technology*.
- Monod, J., 1949. The Growth of Bacterial Cultures. *Annu. Rev. Microbiol*. 3, 371-394.
- Ohtsubo, S., Demizu, K., Kohno, S., Miura, I., Ogawa, T., Fukuda, H., 1992. Comparison of acetate utilization among strains of an aceticlastic methanogen, *Methanotherix soehngenii*. *Appl Environ Microbiol*. 58, 703-5.
- Patel, G.B., 1984. Characterization and nutritional properties of *Methanotherix concilii* sp. nov., a mesophilic, aceticlastic methanogen. *Canadian Journal of Microbiology*. 30, 1383-1396.
- Penning, H., Claus, P., Casper, P., Conrad, R., 2006. Carbon Isotope Fractionation during Acetoclastic Methanogenesis by *Methanosaeta concilii* in Culture and a Lake Sediment. *Applied and Environmental Microbiology*. 72, 5648-5652.
- Powell, G.E., 1983. Interpreting gas kinetics of batch cultures. *Biotechnol. Lett*. 5, 437-440.
- Qiang, H., Niu, Q., Chi, Y., Li, Y., 2013. Trace metals requirements for continuous thermophilic methane fermentation of high-solid food waste. *Chemical Engineering Journal*. 222, 330-336.
- Raskin, L., Zheng, D., Griffin, M.E., Stroot, P.G., Misra, P., 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes. *Antonie van Leeuwenhoek*. 68, 297-308.
- Robinson, J., Tiedje, J., 1984. Competition between sulfate-reducing and methanogenic bacteria for H₂ under resting and growing conditions. *Arch. Microbiol*. 137, 26-32.
- Rothman, D.H., Fournier, G.P., French, K.L., Alm, E.J., Boyle, E.A., Cao, C., Summons, R.E., 2014. Methanogenic burst in the end-Permian carbon cycle. *Proceedings of the National Academy of Sciences*.

- Schmidt, J., Mladenovska, Z., Lange, M., Ahring, B., 2000. Acetate conversion in anaerobic biogas reactors: Traditional and molecular tools for studying this important group of anaerobic microorganisms. *Biodegradation*. 11, 359-364.
- Smith, K.S., Ingram-Smith, C., 2007. Methanosaeta, the forgotten methanogen? *Trends in Microbiology*. 15, 150-155.
- Smith, M.R., Mah, R.A., 1980. Acetate as Sole Carbon and Energy Source for Growth of *Methanosarcina* Strain 227. *Applied and Environmental Microbiology*. 39, 993-999.
- Smith, P.H., Mah, R.A., 1966. Kinetics of Acetate Metabolism during Sludge Digestion. *Applied Microbiology*. 14, 368-&.
- Takashima, M., Shimada, K., Speece, R.E., 2011. Minimum requirements for trace metals (iron, nickel, cobalt, and zinc) in thermophilic and mesophilic methane fermentation from glucose. *Water environment research : a research publication of the Water Environment Federation*. 83, 339-46.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution*. 24, 1596-1599.
- Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological reviews*. 41, 100-80.
- Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews Microbiology*. 6, 579-591.
- van Haandel, A., De Vrieze, J., Verstraete, W., dos Santos, V.S., 2013. Methanosaeta dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. *Journal of Chemical Technology & Biotechnology*, n/a-n/a.
- Wandrey, C., Aivasidis, A., 1983. Continuous Anaerobic Digestion with *Methanosarcina barkeri*. *Annals of the New York Academy of Sciences*. 413, 489-500.
- Weimer, P.J., Zeikus, J.G., 1978. Acetate metabolism in *Methanosarcina barkeri*. *Arch. Microbiol.* 119, 175-182.
- Wolin, E.A., Wolin, M.J., Wolfe, R.S., 1963. Formation of Methane by Bacterial Extracts. *Journal of Biological Chemistry*. 238, 2882-2886.
- Yilmaz, V., Ince-Yilmaz, E., Yilmazel, Y.D., Duran, M., 2014. Is acetoclastic methanogen composition in full-scale anaerobic processes related to acetate utilization capacity? *Applied microbiology and biotechnology*. 98, 5217-26.
- Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnology and Bioengineering*. 89, 670-679.
- Zhang, Y., Zamudio Canas, E.M., Zhu, Z., Linville, J.L., Chen, S., He, Q., 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresour Technol.* 102, 779-85.
- Zhang, Y., Zhang, X., Zhang, H., He, Q., Zhou, Q., Su, Z., Zhang, C., 2009. Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis. *Bull Environ Contam Toxicol.* 82, 367-372.

- Zheng, D., Raskin, L., 2000. Quantification of Methanosaeta Species in Anaerobic Bioreactors Using Genus- and Species-Specific Hybridization Probes. *Microbial ecology*. 39, 246-262.
- Zhu, Z., Hsueh, M.K., He, Q., 2011. Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. *Renewable Energy*. 36, 1802-1807.
- Zinder, S.H., Elias, A.F., 1985. Growth substrate effects on acetate and methanol catabolism in *Methanosarcina* sp. strain TM-1. *Journal of Bacteriology*. 163, 317-323.
- Zinder, S.H., Mah, R.A., 1979. Isolation and Characterization of a Thermophilic Strain of *Methanosarcina* Unable to Use H₂-CO₂ for Methanogenesis. *Applied and Environmental Microbiology*. 38, 996-1008.
- Zinder, S.H., Sowers, K.R., Ferry, J.G., 1985. NOTES: *Methanosarcina thermophila* sp. nov., a Thermophilic, Acetotrophic, Methane-Producing Bacterium. *International Journal of Systematic Bacteriology*. 35, 522-523.

Chapter VI

Conclusion

Conclusion

Anaerobic digestion is an important biological waste treatment process capable of simultaneous pollution mitigation and renewable energy recovery. The broader adoption of anaerobic digestion technology for waste treatment has been hindered by concerns of potential process instability resulting from the susceptibility of bacterial and methanogenic microbial populations to changes in process conditions, such as fluctuations in organic loading rates, which are frequently encountered in anaerobic digestion processes. Therefore, insight into the linkages between process performance and microbial community is valuable for developing efficient methanogenic processes.

Focusing on a set of six continuous bench-scale anaerobic digesters in operation for more than five years, results from microbial ecology, physiology, and process analyses in this study show that the use of proper co-substrate could significantly enhance the process efficiency of anaerobic digestion and that increased diversity of the anaerobic microbial community may contribute to the enhanced functional stability of the anaerobic digestion process, which are consistent with findings in previous studies on the benefits of anaerobic co-digestion and microbial community diversity.

An important finding of this study is the identification of *Methanosaeta* as the dominant populations in anaerobic digestion at higher acetate levels, outcompeting *Methanosarcina*, which is unexpected. Acetoclastic methanogenesis is a key pathway for biological methane production, representing two thirds of all biogenic CH₄ generated on earth. Only two genera of methanogens, i.e. *Methanosaeta* and *Methanosarcina*, are known to carry out this pathway, converting acetate to methane. It is considered that *Methanosaeta* and *Methanosarcina* have contrasting physiology and occupy different niches in the anaerobic food web: *Methanosarcina* has higher maximum growth rate, $\mu_{\max}$, and half-saturation coefficient, K_s , than those of *Methanosaeta*, rendering competitiveness to *Methanosarcina* at high acetate levels and to *Methanosaeta* at low acetate levels. Findings from this study suggest that this may not always be the case, consistent with previously unexplained observations of the dominance of *Methanosaeta* in some anaerobic digestion processes experiencing elevated acetate levels. This finding sheds new light on the linkage between methanogen population dynamics and process conditions, which could potentially provide an updated framework for understanding the drivers of anaerobic microbial community assembly and determinants of methanogenic process performance.

Additionally, it is found in this study that mesophilic *Crenarchaeota* populations were minor but active members of the microbial community in anaerobic digestion. Mesophilic *Crenarchaeota* has received much attention due to its abundance in diverse habitats in both terrestrial and marine environments. Some of these organisms are characterized by the ammonia oxidation metabolism, supporting the proposal of a novel phylum *Thaumarchaeota* to distinguish these archaea from other crenarchaeotes. Given the strictly anaerobic condition characteristic of anaerobic digestion, a lifestyle of ammonia oxidation metabolism, which requires oxygen, may not be likely for these organisms in anaerobic digestion. Indeed, phylogenetic analyses of all *Crenarchaeota* sequences identified in anaerobic digestion show that these populations form a cluster

distinct from that of *Thaumarchaeota*, consistent with the possibility of divergent ecological functions between *Crenarchaeota* identified in anaerobic digestion and *Thaumarchaeota*. While this study provides indications that these *Crenarchaeota* populations may be linked to the utilization of organic acids, further efforts are needed to understand the function and significance of these populations in methanogenic processes.

VITA

Si Chen was born in November 1983 in Zhengzhou, China. He began his academic studies at the Northeastern University at Shenyang, China in 2003 where he attained his Bachelor of Engineering in Environmental Engineering in 2007. After graduation, he began his graduate study in Environmental Engineering at the University of Kansas at Lawrence, KS, USA. After one year of study, he transferred from the University of Kansas to the University of Tennessee in Knoxville, TN, USA in 2008. After one and a half year's graduate study, he completed a thesis entitled "Export of Carbon, Nutrients and Microbiological Indicators in Beaver Creek Watershed, Tennessee" under the direction of his advisor, Dr. Qiang He. In December 2009, he attained his Master of Science in Environmental Engineering at the University of Tennessee, Knoxville. He started his Ph.D study at the University of Tennessee, Knoxville under the direction of Dr. Qiang He in January, 2010. He will attain his Doctor of Philosophy in Civil Engineering (Environmental Engineering concentration) with a focus on Microbial Ecology and interactions in anaerobic digestion in December 2014.