Linking Microbial Community Structure to Methane Emissions from Stored Liquid Dairy Manure: Responses to Emission Mitigation Actions

by

Jemaneh Zeleke Habtewold

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements for the degree of Doctor of Philosophy in Environmental Science

Guelph, Ontario, Canada
© Jemaneh Z. Habtewold, May 2018
ABSTRACT

LINKING MICROBIAL COMMUNITY STRUCTURE TO METHANE EMISSIONS FROM STORED LIQUID DAIRY MANURE: RESPONSES TO EMISSION MITIGATION ACTIONS

Jemaneh Habtewold
University of Guelph, 2018
Advisor(s):
Professor Kari Dunfield
Professor Robert Gordon

Stored liquid dairy manure provides a conducive environment to microbial communities for methane (CH$_4$) production. Significant reductions of emissions from these systems have been observed by using different management strategies and treatment technologies. However, knowledge on how microbial communities respond to the mitigation actions is lacking, and is important for developing and refining mitigation strategies. Using lab and meso-scale studies, the responses of microbial communities to varying levels of total solids content, residual slurry, pH, and chemical agents were assessed. Gas chromatography and laser-based trace gas analysis were used to monitor CH$_4$ flux whereas molecular techniques including denaturing gradient gel electrophoresis, quantitative real-time PCR, and next generation sequencing of marker genes were used to study the microbes. With reduction of total solids content (9.5% to 0.3%), we found up to ~23% increases in the mean abundance and activity of methanogens. Cumulative CH$_4$ emissions, however, decreased by ~70% as total solid levels decreased. These results indicated that available carbon substrate, and not methanogen abundance, may be limiting cumulative CH$_4$ emissions at reduced total solids of dairy slurries. In a follow-up study, abundant bacterial and methanogenic
communities were detected, and related to significant increases in cumulative CH$_4$ emissions from stored dairy slurries with 10% and 20% residual slurry. The presence of residual slurry, did not alter the core methanogenic phylotypes, although, *Methanocorpusculum* predominated the methanogen population, RNA data indicated that methanogens related to *Methanosarcina* were the major players in CH$_4$ production.

Microbial communities in residual slurries may be reduced by chemical treatments. In a lab-scale study, significant reductions in the abundance of methanogens (up to ~28%) were observed when sodium persulfate, potassium permanganate, or their combination with sodium hypochlorite. In slurries treated with H$_2$SO$_4$, the abundance of methanogen populations was not impacted. In a subsequent pilot-scale study, the transcriptional activities of methanogen populations and CH$_4$ production were significantly affected by acidification of manure. Taken together, the findings of these studies suggest that the activity responses of slurry microorganisms can help in the refinement and/or development of effective mitigation strategies.
ACKNOWLEDGEMENTS

First, I would like to give my deepest thanks to my advisors, Dr. Kari Dunfield and Dr. Robert Gordon for the invaluable help and guidance during my research. I'm also grateful to my advisory committee, Dr. Andrew VanderZaag and Dr. Claudia Wagner-Riddle, for your vital mentorship in my research. I'm also grateful for the examining committee: Dr. Brandon Gilroyed (internal-external) for the important comments, and Dr. Mario Tenuta (external) for your time and constructive comments for the thesis.

Special thanks go to Kamini Khosla, Jonathan Gaiero, John McCabe, Donna MacLennan, Sean Jordan, Zack DeBruyn, and Vera Sokolov for your incredible technical support in different parts of the study.

I would like to thank Michael Ben-Israel, John Drummelsmith, Andrea Roebuck, Nicola Linton, Tolulope Mafa-Attoye, Travis Mazurek, Pooja Arora, and Micaela Tosi, for the supports and helpful discussions during my research.

I would like to acknowledge the bio-environmental engineering center of Dalhousie University for letting us use its research facility. I would also like to thank the School of Environmental Sciences, University of Guelph, for the financial support through International Graduate Tuition Scholarships. This study has been supported by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA).

At last, but not least, I would like to give deepest thanks to Woinshet for the encouragement you gave me throughout my study. Despite my limited time to you and Nathan, your support was simply the best one can do.
# TABLE OF CONTENTS

Abstract .................................................................................................................................................. ii
Acknowledgements ................................................................................................................................. iv
Table of Contents .................................................................................................................................. v
List of Tables .......................................................................................................................................... viii
List of Figures ......................................................................................................................................... ix
List of Abbreviations ............................................................................................................................. xiv
List of Appendices ................................................................................................................................. xvi

1 General introduction .......................................................................................................................... 1

1.1 Background ....................................................................................................................................... 1

1.2 Methanogenic processes in stored liquid dairy manure ................................................................. 2

1.3 Methanogens in stored dairy manure .............................................................................................. 4

1.4 Mitigation strategies for CH$_4$-emissions from stored liquid dairy manure ............................... 6

1.5 Thesis format, hypotheses, and objectives ...................................................................................... 8

2 Dairy manure total solid levels impact CH$_4$ flux and abundance of methanogenic archaeal communities .................................................................................................................. 11

2.1 Abstract .......................................................................................................................................... 12

2.2 Introduction ....................................................................................................................................... 13

2.3 Materials and Methods .................................................................................................................... 14

2.3.1 Experimental design, CH$_4$ flux and slurry sampling .............................................................. 14

2.3.2 Nucleic acid extractions and PCR amplifications ....................................................................... 15

2.3.3 Phylogenetic analysis ................................................................................................................ 18

2.4 Results ............................................................................................................................................. 18
2.5 Discussion ........................................................................................................................................ 27
2.6 Conclusion ....................................................................................................................................... 29

3 Targeting bacteria and methanogens to understand the role of residual slurry as an
inoculant in stored liquid dairy manure .............................................................................................. 30

3.1 Abstract ........................................................................................................................................... 31
3.2 Introduction ...................................................................................................................................... 32
3.3 Materials and Methods ..................................................................................................................... 36
  3.3.1 Experimental setup and measurement of CH4 flux emissions ....................................................... 36
  3.3.2 Slurry sample collection and analyses .......................................................................................... 38
  3.3.3 Nucleic acid extractions and quantitative real-time PCR ................................................................. 39
  3.3.4 Amplicon library preparation and sequencing .............................................................................. 42
  3.3.5 Sequence data analysis ................................................................................................................ 44
3.4 Results .............................................................................................................................................. 45
  3.4.1 Methane flux and manure characteristics ...................................................................................... 45
  3.4.2 Abundance and activity of methanogens and bacteria ................................................................. 49
  3.4.3 Diversity of archaeal and bacterial communities ....................................................................... 52
  3.4.4 Phylogenetic analysis of archaea and bacteria ............................................................................. 60
3.5 Discussion ........................................................................................................................................ 65
3.6 Conclusion ....................................................................................................................................... 72

4 Sodium persulfate and potassium permanganate inhibit methanogens and
methanogenesis in stored liquid dairy manure .................................................................................... 74

4.1 Abstract ........................................................................................................................................... 75
4.2 Introduction ...................................................................................................................................... 76
4.3 Materials and methods ..................................................................................................................... 79
  4.3.1 Experimental design and manure sampling .................................................................................. 79
LIST OF TABLES

Table 2.1: Stored dairy manure volatile solids (VS), total carbon (TC), and pH at the beginning (20-May) and end (16-Nov) of the storage.........................................................19

Table 3.1: PCR primers and Illumina adaptors used in this study.................................40

Table 3.2: Dry matter contents, volatile solid contents, and pH of stored dairy slurries with 0%, 10%, and 20% residual slurry and filled gradually or in batch a ..................................47

Table 3.3: Volatile fatty acid contents of stored dairy slurries with 0%, 10%, and 20% residual slurry, and filled gradually or in batch.................................................................48

Table 3.4: Diversity indices calculated for archaeal and bacterial 16S rRNA gene sequence reads obtained from residual, fresh, and stored dairy slurries of gradually- and batch-filled tanks..............................................................................55

Table 4.1: Volatile solid (VS) contents and pH of dairy manure before and after (day 120) incubations with different chemical agents. Standard deviations shown were calculated from the duplicate treatments (n=2).................................................................84
LIST OF FIGURES

Figure 1.1: Major microbial groups involving in the degradation of complex organic matter in stored liquid dairy manure.................................................................3

Figure 2.1: Methane (CH\textsubscript{4}) fluxes (kg m\textsuperscript{-2} d\textsuperscript{-1}) (a) and cumulative emissions (kg m\textsuperscript{-2}) from stored dairy slurries having varied total solids (TS) contents (b). Emissions were measured continuously for 180 d using a flow-through steady state chamber method..20

Figure 2.2: Methanogen activity and abundance (Log\textsubscript{10} copies of \textit{mcrA} gene and transcript copies g\textsuperscript{-1} of dry slurry) in dairy slurries containing total solids contents of 9.5\%, 5.8\%, and 0.3\% after about day 30 (a) and Log\textsubscript{10} copies of \textit{mcrA} genes g\textsuperscript{-1} dry slurry after day 120 (b). .................................................................................................................................21

Figure 2.3: Volatile solid (VS) to CH\textsubscript{4} conversion rates in 9.5\%, 5.8\% and 0.3\% TS dairy slurries........................................................................................................................................22

Figure 2.4: Total archaeal abundance (Log\textsubscript{10} copies 16S rRNA genes g\textsuperscript{-1} of dry slurry) in dairy slurries containing TS contents of 9.5\%, 5.8\%, and 0.3\% after day 30 and day 120 of the storage. Standard errors were calculated from the three replicate PCR reactions..................................................................................................................24

Figure 2.5: \textit{mcrA} gene based PCR-DGGE fingerprints of \textit{mcrA} gene in stored dairy slurries. Lanes A through F indicates samples after 30 days (9.5\%TS\_T, 9.5\%TS\_B, 5.8\%TS\_T, 5.8\%TS\_B, 0.3\%TS\_T, and 0.3\%TS\_B, respectively) and G through L indicates after 120 days with same percent of TS and position as A to F...............25
**Figure 2.6:** Phylogenetic tree showing the relationship (at amino acid level) of *mcrA* gene sequences retrieved in this study with *mcrA* gene reference sequences of identified strains. “Band 1” and “Band 2” indicates the two (close) DGGE bands that were consistently present in all samples, and were sequenced……………………26

**Figure 3.1:** Mean daily methane (CH$_4$) flux (g m$^{-2}$ d$^{-1}$) from liquid dairy manure storage tanks containing 0%, 10%, and 20% residual slurry (RS), for gradually or batch filled tanks……………………………………………………………………………………49

**Figure 3.2:** The abundance and activity of bacteria (a) and methanogens (b) in fresh manure, old-slurry (residual slurry), stored dairy slurry (day 65 and day 120), as indicated by *mcrA*/bacterial 16S rRNA genes and transcripts copies (Log$_{10}$ transformed)……………………………………………………………………………………51

**Figure 3.3:** Rarefaction curves, as calculated in Mothur, showing changes in the number of observed OTUs of archaea (a) and bacteria (b) (cut-off=0.03) as sampling intensity (number of sequences sampled) increases from slurry samples…………………54

**Figure 3.4:** The (a) Non-metric multi-dimensional scaling (NMDS) plots of the archaeal (a) and bacterial (b) communities in fresh, residual slurry, and stored slurries…………57

**Figure 3.5:** Dendrograms showing the degree of similarity of dairy slurry samples with respect to archaeal (a) and bacterial (b) community structures…………………………..58

**Figure 3.6:** Venn diagrams displaying the number of shared archaeal OTUs (cut-off=0.03) by residual, fresh, and stored dairy slurries from gradually-filled tanks on day x
65 (a) and day 120 (b) and batch-filled tanks on day 65 (c) and day 120 (d) of storage ........................................................................................................................................59

**Figure 3.7:** Relative proportion of (a) archaeal genera in fresh, residual (old), and stored dairy slurry samples (day 65 and day 120) and (b) active archaeal genera during peak methane (CH₄) flux from each tank .......................................................................................................................... 61

**Figure 3.8:** Relative proportion of bacterial phyla (a) and genera (b) in fresh, residual, and stored dairy slurries. Bars indicate the proportion (%) in each sample, and the paired bars are from the duplicate samples collected from each tank ........................................................................... 64

**Figure 4.1:** Effects of different rates of Na₂S₂O₈ on CH₄ production in stored liquid dairy manure. Bromoethanesulphonate (0.05 mol L⁻¹ slurry)-treated manure was included as negative control. Error bars indicate standard errors of the means of duplicate treatments ...................................................................................................................... 85

**Figure 4.2:** Effects of KMnO₄ on CH₄ production in stored liquid dairy manure. Bromoethanesulphonate (0.05 mol L⁻¹ slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments ...................................................................................................................... 86

**Figure 4.3:** Effects of 3%NaOCl on CH₄ production (as indicated by CH₄/CO₂ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L⁻¹ slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments ...................................................................................................................... 87
Figure 4.4: Effects of chemical combinations (Na$_2$S$_2$O$_8$, KMnO$_4$, and 3%NaOCl at a ratio of 1g:1g:1ml, defined as unit here) on CH$_4$ production (as indicated by CH$_4}$/CO$_2$ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L$^{-1}$ slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments.  

Figure 4.5: Effects of H$_2$SO$_4$ on CH$_4$ production (as indicated by CH$_4}$/CO$_2$ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L$^{-1}$ slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments.  

Figure 4.6: Effects of chemical oxidants on the abundance of bacteria and methanogens in stored liquid dairy manure. Changes (%) in the abundance of bacteria (a-after 60 days; b-after 120 days) and methanogens (c-after 60 days; d-after 120 days) in stored dairy slurries treated with chemical oxidants. Error bars indicate standard error of the means of biological replicates (n=2). A unit is defined here as 1g:1g:1mL of the chemical combinations.  

Figure 4.7: Changes in the relative abundances of methanogens with reference to bacteria as estimated from the changes in the ratios of $mcrA$/bacterial 16S rRNA gene copies. The abundances of bacteria and methanogens, which were used for this calculation, are shown in the appendix Fig. A.2 and A.3. A unit is defined here as 1g:1g:1mL of the chemical combinations.
Figure 5.1: Physicochemical characteristics of stored liquid dairy manure: a) pH and b) volatile solid contents. Values shown for volatile solid contents and pH are mean ± standard deviation of duplicate treatments.

Figure 5.2: Methane flux (a), and cumulative CH$_4$ emissions (b) from stored liquid dairy manure. Values shown are mean ± standard deviation of duplicate treatments. Acid was added a week after of manure loading in the storage tanks.

Figure 5.3: Abundance and activities of total bacteria as indicated by copies (g$^{-1}$ dry manure) of (a) bacterial 16S rRNA genes and (b) transcripts in liquid dairy manure. Values shown are mean ± standard errors of the mean of duplicate treatments.

Figure 5.4: Abundance and activities of methanogens as indicated by copies (g$^{-1}$ dry manure) of (a) mcrA genes, (b) transcripts, and (c) transcript/gene ratios in liquid dairy manure. Values shown are mean ± standard errors of the mean of duplicate treatments.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOVA</td>
<td>Analysis of molecular variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BES</td>
<td>2-Bromoethanesulfonate</td>
</tr>
<tr>
<td>BF</td>
<td>Batch-filled</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CQ</td>
<td>Cycle of quantification</td>
</tr>
<tr>
<td>DFC</td>
<td>Dairy farmers of Canada</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DNA-SIP</td>
<td>Deoxyribonucleic acid-stable isotope probing</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>FM</td>
<td>Fresh manure</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross domestic product</td>
</tr>
<tr>
<td>GF</td>
<td>Gradually-filled</td>
</tr>
<tr>
<td>GHGs</td>
<td>Greenhouse gases</td>
</tr>
<tr>
<td>mcrA</td>
<td>Methyl-Coenzyme Reductase alpha sub-unit A</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acids</td>
</tr>
<tr>
<td>NMDS</td>
<td>Non-metric multidimensional scaling</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic Unit</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-DGGE</td>
<td>PCR followed by DGGE</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acids</td>
</tr>
<tr>
<td>RS</td>
<td>Residual slurry</td>
</tr>
<tr>
<td>Sobs</td>
<td>Species observed</td>
</tr>
<tr>
<td>TC</td>
<td>Total carbon</td>
</tr>
<tr>
<td>TS</td>
<td>Total solids</td>
</tr>
<tr>
<td>VFAs</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

Appendix A: Supplementary data

Figure A.1. CH₄ flux (g m⁻³ slurry) from stored dairy manure that received different levels of residual slurry……………………………………………………………………………142

Figure A.2. Abundance of methanogens (as determined from copy number of mcrA gene) in dairy manure under different chemical treatments………………………………………143

Figure A.3. Abundance of bacteria (as determined from copy number of 16S rRNA gene) in dairy manure under different chemical treatments……………………………………144

Figure A.4. Cumulative CH₄ production from dairy manure treated with Na₂S₂O₈…..145

Figure A.5. Cumulative CH₄ production from dairy manure treated with KMnO₄……..146

Figure A.6. Cumulative CH₄ production from dairy manure treated with 3% NaOCl…147

Figure A.7. Cumulative CH₄ production from dairy manure treated with combinations (1g:1g:1 mL) of Na₂S₂O₈, KMnO₄, and 3% NaOCl…………………………………………………………148

Figure A.8. Cumulative CH₄ production from dairy manure treated with H₂SO₄………149
1 General introduction

1.1 Background

Agricultural practices are among the major sources of atmospheric methane (CH$_4$) (Schaefer et al., 2016), a potent greenhouse gas (GHG) whose atmospheric concentration has increased by 150% since the onset of the industrial revolution (Myhre et al., 2013). In livestock production, enteric fermentation and manure management, are responsible for large proportions of the agricultural CH$_4$ emissions (e.g. ~62% in Canada) (O’Mara, 2011; Environment-Canada, 2014). With the growing demand for dairy products by the ever-increasing human population, intensification of dairy production can increase atmospheric emissions. For instance, by 2030, CH$_4$ emissions from enteric fermentation has been projected to increase by 22% (EPA, 2012). While a number of potential mitigation strategies for enteric CH$_4$ emissions are being developed (Knight et al., 2011; Gerber et al., 2013; Caro et al., 2016), mitigation of CH$_4$ emissions from large volume manure wastes remains one of the challenges in dairy operations.

On many dairy farming operations, manure is collected and stored for extended period of time (6-12 months) (Sheppard et al., 2011). Due to the ease of handling, liquid manure (slurry) has become the preferred form of storage on many dairy farms (VanderZaag et al., 2013; Laubach et al., 2015). However, manure contains large amounts of organic substrates, and creates an ideal environment for anaerobic-degrading microbes and CH$_4$-producing microbes. Consistently, many lab-, pilot- and full-scale studies identified stored dairy slurries as point sources for CH$_4$ emission
For instance, Owen and Silver (2015) reviewed a number of field-based studies on liquid manure storage systems and showed a significantly high level of CH$_4$ emissions from anaerobic lagoons (~368±193 kg hd$^{-1}$ y$^{-1}$) and slurry stores (~101±47 kg hd$^{-1}$ y$^{-1}$). Interestingly, the authors also observed higher (~3×) CH$_4$ emission from anaerobic lagoons than enteric fermentation (~120 kg hd$^{-1}$ y$^{-1}$). Despite variations in the level of CH$_4$ emissions reported in different studies, likely related to secondary factors (e.g. temperature, storage time and conditions) (Amon et al., 2006), the anaerobic conditions and presence of ample methanogenic substrates are among the most important factors making stored liquid manure a major source of CH$_4$ emissions.

1.2 Methanogenic processes in stored liquid dairy manure

Dairy manure contains partially digested plant polymers (cellulose, lignin, proteins and lipids), unabsorbed digestion products (amino acids, sugars and fatty acids), and a number of minerals (Gupta et al., 2016). Thus, stored liquid dairy manure creates a nutrient rich environment for anaerobically-decomposing microorganisms. Anaerobic degradation of organic matter involves four major processes: hydrolysis, acidogenesis, and acetogenesis, and methanogenesis (Liu and Whitman, 2008; Girard et al., 2013; Goswami et al., 2016) (Fig. 1.1).
Figure 1.1: Major microbial groups involving in the degradation of complex organic matter in stored liquid dairy manure; adapted from (Amaya et al., 2013; Manyi-Loh et al., 2013).
Under suitable environmental conditions, hydrolytic bacteria (e.g. Bacteroidetes, Cellulolyticus, Clostridium, and Acetivibrio) produce extracellular enzymes to hydrolyze organic polymers of manure into simpler compounds (monomers) such as glucose, fatty acids, amino acids, alcohols, and gases such as hydrogen sulfide, ammonia, hydrogen, and carbon dioxide (Goswami et al., 2016). Monomeric compounds are further utilized by acidogenic bacteria (e.g. Campylobacter, Peptococcus, and most hydrolytic bacteria), and release by-products such as acetic, propionic, formic, and butyric acids, ethanol, hydrogen and carbon dioxide. While acetic acid, formic acid, hydrogen, and carbon dioxide are direct methanogenic substrates, the higher volatile fatty acids (butyric and propionic acids) need to be further processed to be used by methanogens. Acetogenic bacteria (e.g. Syntrophomonas, Syntrophobacter, Smithella) convert the higher organic acids into acetic acid, hydrogen, and carbon dioxide (Matthies and Schink, 1992; Liu and Whitman, 2008; Goswami et al., 2016). Methanogenesis is the final step in anaerobic degradation of organic matter (Fig. 1.1).

1.3 Methanogens in stored dairy manure

Methanogens are microorganisms that produce CH₄ as a metabolic by-product under anaerobic conditions. Although reports show potential CH₄ production by members of the phylum Verstraetearchaeota and different eukaryotic organisms (Lenhart et al., 2012; Liu et al., 2015; Vanwonterghem et al., 2016; Guo et al., 2017), well-known methanogens are members of the phylum Euryarchaeota. These methanogens are phylogenetically distributed into seven orders (Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanococcales, Methanopyrales,
Methanocellales, and Methanomassiliicoccales). Methanogenic archaea are naturally distributed in various anaerobic environments including sediments, rumen and intestines of animals, stored manure, anaerobic sludge, and landfills (Liu and Whitman, 2008; Barber, 2016). Although several members of methanogenic phylotypes can adapt in different anaerobic environments, factors such as temperature, pH and type of substrate may affect their relative proportions and activities (Zhou et al., 2009). For instance, availability of specific methanogenic substrates in environments may determine the distribution of hydrogenotrophic methanogens (use \( \text{H}_2/\text{CO}_2 \), formate), acetalactic methanogens (ferment acetate), and methylotrophic methanogens (use methylated compounds such as methylamines and methanol) (Liu and Whitman, 2008). Except Methanoseta (strict acetoclastic methanogens), other acetoclastic methanogens are metabolically diverse, capable of performing all of the three methanogenic pathways (Liu and Whitman, 2008).

Many of the methanogens detected so far from stored dairy manure are hydrogenotrophic and/or acetalactic methanogens (e.g. Methanocorpusculum, Methanoculleus, Methanoseta, Methanosarcina, Methanococcus, Methanospirillum, Methanogenium, and Methanobrevibacter) (Barret et al., 2013; Dungan and Leytem, 2013; Duan et al., 2014; Barret et al., 2015; Habtewold et al., 2017). Although the importance of methylotrophic methanogenesis in stored dairy manure have not been reported, the presence of abundant methanol in stored manure (El-Mashad et al., 2011) might suggest the need for further study to understand the role of methylotrophic methanogens in cumulative \( \text{CH}_4 \) emissions from these systems. Indeed, the occurrence
of methanol-utilizing methanogens such as *Verstraetearchaeota* in environments with high CH$_4$ levels (Vanwonterghem et al., 2016) may further suggest the need for wider investigations of methylotrophic methanogens in stored dairy manure where large amount of CH$_4$ is produced. Nevertheless, the presence of methanogens in environments may not always reflect their role in CH$_4$ production, rather their activities can have paramount importance with regard to CH$_4$ emissions. Using incubation-based experiments, Barret et al. (2013) identified the relatively less abundant methanogens (*Methanoculleus* and *Methanosarcina* spp) having strong methanogenic activities in manure slurries. Recently, the authors also conducted $[^{13}\text{C}]-\text{acetate}$ based DNA-stable-isotope probing investigations on stored dairy manure, and identified *Methanoculleus chikugoensis* as the key player in aceticlastic methanogenesis (Barret et al., 2015).

### 1.4 Mitigation strategies for CH$_4$-emissions from stored liquid dairy manure

The fact that dairy manure storage systems are man-made indicates an opportunity to apply best management practices that can reduce its carbon footprints. For instance, management practices such as solid-liquid separation, use of slurry covers, slurry aeration, reducing residual manure, use of bio-filters, and encouraging slurry crust development have been proposed as potential mitigation actions for CH$_4$ emissions from stored liquid dairy manure (Rico et al., 2012; Baldé et al., 2016; Jayasundara et al., 2016). Residual (aged) slurries have been implicated in shortening the lag phase for CH$_4$ production during the new storage (Zeeman et al., 1998). About 26% and ~56% reductions of cumulative CH$_4$ emissions have also been reported after
reduction and complete removal of inoculum manure, respectively (Massé et al., 2008; Wood et al., 2014).

Enhancing the development of slurry surface crusts may create a CH$_4$ sink as it physically blocks the escape of gasses from slurries (Smith et al., 2007), thereby increasing the access to CH$_4$-consuming microorganisms in the aerobic zone (Petersen et al., 2005). Chemical treatments of manure (e.g. acidification, coumarins, narasin, and gypsum) and application of nanoparticles have also been demonstrated to reduce CH$_4$ production in stored manure (Berg and Model, 2008; Petersen et al., 2012; Yang et al., 2012; Andersen and Regan, 2014; Theint et al., 2016; Gautam et al., 2017; Popp et al., 2017). These chemicals and nanoparticles may disinfect slurry and/or alter the characteristics of the manure environment in a way that microbial activity could negatively be impacted (Ottosen et al., 2009; Petersen et al., 2014; Wang et al., 2014). For instance, reduction of slurry pH to 5.5 or 4.5 could reduce methanogenesis (Ottosen et al., 2009; Petersen et al., 2012; Petersen et al., 2014). Indeed, in an earlier study, acid treatment of manure slurries was demonstrated as a potential strategy for mitigating CH$_4$ emissions (Hilhorst et al., 2001). Interestingly, a recent study by Bastami et al. (2016) showed self-acidification, as a result of microbial fermentation of 10% (w/w) brewing sugar supplement, reducing CH$_4$ production up to 99%. Using, zinc oxide and silver nanoparticles, significant reductions of CH$_4$ emissions from stored manure can be achieved (Yang et al., 2012; Gautam et al., 2017); however, the toxicity of nanoparticles-treated manure in agricultural soils may raise concerns (Dinesh et al., 2012; Ben-Moshe et al., 2013; Shen et al., 2015). Hence, in addition to H$_2$SO$_4$ and
nanoparticles, further studies on various chemical agents that are readily available, cheap, and less toxic to farm personnel and the environment may be necessary.

As many of the emission mitigation actions proposed so far impact the functioning of CH$_4$-producing microbes, the efficiencies of the strategies lie on the extent slurry microbial communities may be impacted. For instance, how the functioning of microbial communities in stored slurries respond to reductions in available substrates, residual inoculum, manure acidification, and application of chemical oxidants are important questions that may lead to a solid understanding of the extent the mitigation action affects the microbes, which is critical in refining or developing effective mitigation strategies.

1.5 Thesis format, hypotheses, and objectives

This thesis is presented in a manuscript-style which have been published (Chapters 2, 3, and 4), or in preparation for peer reviewed journal submission (Chapter 5). This thesis work was part of a project that aimed to explore and understand the key factors that make stored liquid dairy manure point sources for GHG gas emissions, and develop effective mitigation strategies that can reduce environmental footprints of dairy farming operations. The overall goal of this thesis was to understand the dynamics of microbial communities in stored liquid dairy manure under different mitigation actions. To achieve this goal, the following specific objectives were addressed.

1. Reduction of available substrates in stored liquid dairy manure (for instance by solid-liquid separation or using sand as bedding material) are assumed to negatively
impact methanogenic communities, resulting in reduced CH$_4$ emissions. Thus, we aimed to understand the relationship between total solids content of liquid dairy manure and abundance and activity of slurry microbial communities (Chapter 2).

2. Residual slurry that is left after removal of ‘aged’ manure for land application can increase CH$_4$ emissions during new storage. The inoculant effects of well-adapted microbial communities have been hypothesized for the increase in CH$_4$ emissions. We aimed to investigate the abundance, diversity, and activity of bacterial and methanogenic communities in residual slurries and stored slurries with and without residual inoculants (Chapter 3).

Many of the lab- and pilot-scale studies conducted so far used batch-incubation while the actual storage conditions mainly involve gradual filling of open tanks. We hypothesized that batch- and gradual-filling of storage tanks can have different effects on the stability of slurry environment and CH$_4$ emissions. Thus, to simulate the actual conditions, we also aimed to investigate the differences in CH$_4$ flux and microbial dynamics when liquid dairy manure is filled in batch and gradually (Chapter 3).

3. There is a need to develop effective mitigation strategies that can minimize GHG emissions from stored liquid dairy manure. Hence, we assessed the mitigation potentials of some readily available chemical oxidants that are commonly used in environmental bioremediation (Chapter 4).

4. Slurry acidification has been described as a potential mitigation strategy for GHGs from stored liquid dairy manure. We hypothesized that the mechanism of slurry
acidification to reduce CH₄ emissions is due to lower pH impacting the transcriptional activity of methanogenic communities. Hence, we aimed to investigate the abundance and activity responses of bacterial and methanogenic communities in an acidified slurry (Chapter 5).

The final chapter of this thesis (Chapter 6) summarizes the findings of Chapters 2 to 5, and discusses their implications for overall CH₄ mitigation.
2 Dairy manure total solid levels impact CH$_4$ flux and abundance of methanogenic archaeal communities

J. Habtewold$^1$, R. J. Gordon$^2$, J. D. Wood$^3$, C. Wagner-Riddle$^1$, A. C. VanderZaag$^4$, K. E. Dunfield$^1$

$^1$School of Environmental Sciences, University of Guelph, Canada; $^2$Department of Geography and Environmental Studies, Wilfrid Laurier University, Canada; $^3$School of Natural Resources, University of Missouri, Columbia MO, USA; $^4$Science and Technology Branch, Agriculture and Agri-food Canada, Ottawa, Canada

This manuscript has been published in the Journal of Environmental quality:


doi:10.2134/jeq2016.11.0451

Author Contributions:

J Habtewold was the primary researcher and was responsible for the lab work, data analysis, and manuscript preparation. J Wood was credited for the design of the study and sample collection. C. Wagner-Riddle and A VanderZaag were credited for the valuable advices on the experimental design, statistical analyses and manuscript review. R. Gordon and K. E. Dunfield were credited for their invaluable mentorship on lab-work and trouble-shooting on data analysis, manuscript preparation, and data interpretation.
2.1 Abstract

Stored liquid dairy manures are methane (CH$_4$) emission point sources, due to the large amount of slurry volatile solid (VS) converted into CH$_4$ by methanogens under anaerobic conditions. Our research has indicated that a reduction of total solid (TS) of slurries prior to storage can reduce CH$_4$ emissions. In the current study, methanogen abundance in tanks with different CH$_4$ emissions were characterized. Using mesoscale manure storage tanks equipped for continuous gaseous emission monitoring, dairy slurries having TS from 9.5% to 0.3% were stored and monitored for up to 6 months. Manure samples were taken after day 30 and day 120 of the storage (20 May through 16 Nov 2010) from upper and bottom layers of the slurries. Methanogenic communities studied by targeting the gene encoding the alpha subunit methyl-coenzyme M reductase (mcrA), which catalyzes the final step of methanogenesis. Mean abundance of methanogens increased by ~8% and 23% at the top and bottom sections, respectively, as slurry TS decreased from 9.5% to 0.3%. Cumulative CH$_4$ emissions, however, decreased by ~70% as TS slurry TS decrease from 9.5% and 0.3%. Compared to the day 30 of storage, mean abundance of methanogens was relatively higher at day 120 (up to 19%), consistent with an increase in the cumulative CH$_4$ emissions. PCR-DGGE analysis indicated a low methanogen diversity, with most bands sequenced being closely related to the genus *Methanocorpusculum* (>95% amino acid sequence similarity), the hydrogenotrophic methanogens. Results suggest that available carbon substrate and not methanogen abundance may be limiting cumulative CH$_4$ emissions at reduced TS levels of dairy slurries.
Keywords: climate change, dairy farming, manure management, methanogen, total solid

2.2 Introduction

Liquid storage of dairy manure has become the predominant practice for manure management in many farms due to ease of handling (VanderZaag et al., 2013; Laubach et al., 2015). However, the large quantities of organic substrates in liquid manures under anaerobic conditions make these environments conducive to methane (CH$_4$) production. Studies have demonstrated that stored dairy slurries are large CH$_4$ sources (Kulling et al., 2003; Amon et al., 2006; Rodhe et al., 2009). This is because during storage, the successive actions of microorganisms on dissolved and suspended solids of the slurries typically accumulate by-products such as H$_2$, CO$_2$, formate and acetate, the methanogenic substrates (Liu and Whitman, 2008). The level of total solids (TS) of dairy slurries may determine the amount of volatile solids (VS), a proxy for CH$_4$ production (Wood et al., 2012), although other factors such as length of storage, manure temperature and atmospheric conditions can have important implications (Barret et al., 2013). Managing manure-associated methanogenic factors, such as TS, therefore may be important in mitigating CH$_4$ emissions from stored dairy slurry.

Wood et al. (2012) demonstrated the relationships of cumulative CH$_4$ emissions and TS contents of dairy slurries stored for about six months. The authors described relatively higher VS to CH$_4$ conversion rates in slurries having lower TS, suggesting VS as an important source of methanogenic substrates in dairy slurries (Dziewit et al., 2015). Massé et al. (2003) also found that higher (~11.8× more) CH$_4$ production rates
from 4.2% TS than 9.2% TS dairy slurries, indicating the higher VS to CH$_4$ conversion rates by the methanogenic communities in lower TS slurries. While several studies have been conducted regarding CH$_4$ emissions from stored dairy slurry, studies that target the methanogenic communities from these environments are limited. A better understanding of the identity, activity and abundance of methanogens in these systems is critical towards supporting the development and refinement of GHG mitigation strategies. Reduction of dairy slurry TS levels reduce cumulative CH$_4$ emission because methanogens can be affected negatively through substrate limitation, and, hence their abundance and activity as slurry TS decrease. Here, the current study was conducted to assess the effects of varying levels of dairy slurry TS on the abundance and activity of methanogenic communities.

2.3 Materials and Methods

2.3.1 Experimental design, CH$_4$ flux and slurry sampling

This study was conducted from 20 May through 16 Nov 2010 (173 days) using six meso-scale manure storage tanks located at the Bio-Environmental Engineering Center in Truro, Nova Scotia, Canada (45°45′ N, 62°50′ W). A detailed description of the site and monitoring experiment can be found in Wood et al. (2012). Fresh manure that was mixed with urine and milk-house washwater was obtained from the Dalhousie University Experimental Farm. As the TS content of the fresh dairy slurry was 9.5%, 5 dilutions (8.5%TS, 5.3%TS, 3.2%TS, 1.3%TS, and 0.3%TS) were prepared using well water. Approximately 10.4 m$^3$ of fresh slurry (9.5%TS) or diluted slurries (8.5%TS, 5.3%TS, 3.2%TS, 1.3%TS, or 0.3%TS) were batch loaded to six rectangular storage
tanks (1.75 m width ×3.90 m length ×1.80 m depth), each of which was covered by a flow-through steady state chamber. Methane flux densities from each tank were monitored continuously from the start to end of the storage period. CH$_4$ flux was calculated as described earlier by Wood et al. (2012) (Eq. 1):

$$ F = \frac{C_{out} - C_{in}}{A_s} Q $$  \hspace{1cm} (Eq. 1)

where $F$ is CH$_4$ flux (g m$^{-2}$s$^{-1}$); $C_{out}$ and $C_{in}$ are chamber’s outlet and inlet CH$_4$ concentrations (g m$^{-3}$), respectively; $A_s$ is the surface area of the storage tank (in m$^2$), and $Q$ is the airflow rate (m$^3$ s$^{-1}$). After examining the cumulative CH$_4$ emissions from each TS level, three major categories of TS contents having similar cumulative emissions (i.e. ≥ 8.2% TS, 1.3–5.8% TS and 0.3% TS having 12–13, 6–9, and <3 kg CH$_4$ m$^{-2}$, respectively) were observed. Hence, manure samples from three TS levels (9.5%, 5.8% and 0.3%) were selected for a more in-depth assessment of the abundance and activity of methanogenic archaeal communities. Slurry samples were obtained at day 30 and 120, 5 cm below the crust-slurry interface and 20 cm from the floor of each tank. Samples were collected ascetically using a spring-loaded sampler. Replicate samples from each tank and sampling location were pooled and transported to the laboratory in LifeGuard® Soil Preservation Solution (MoBio Laboratories Inc., Carlsbad CA, USA), and then stored in -20 °C freezer until nucleic acid extraction.

2.3.2 Nucleic acid extractions and PCR amplifications

DNA and RNA were extracted from the slurry samples obtained after day 30 of storage and only DNA was extracted from samples obtained after day 120 using RNA
PowerSoil® Total RNA Isolation with DNA Elution Accessory Kits (MoBio Laboratories Inc) following the manufacturer’s protocol. Total RNA from each sample was reverse transcribed into complementary DNA (cDNA) using high capacity cDNA reverse transcription kit (Applied Biosystems) following the recommended protocol. Both DNA and cDNA were then stored in -80 °C freezer until further analysis. As phylogenetic analysis of methanogens using the functional gene encoding the alpha subunit of methyl-coenzyme M reductase (mcrA), an enzyme that catalyzes the final step in methanogenesis (Thauer, 1998), is correspondent with 16S rRNA analysis results (Luton et al., 2002), targeting mcrA genes may highlight both the phylogenetic and functional diversities. In this study, mlas-mod F (5’_GGYGGTGTMGDTCACMCARTA_3’) and mcrA-rev-mod R (5’_CGTTCATBGCGTAGTTVGGRTAGT_3’) primers (Steinberg and Regan, 2009; Angel et al., 2012) were used to target mcrA genes and transcripts (cDNA). Archaeal 16S rRNA genes were quantified using A934bR (5’_GTGCTCCCCCGCCAATTCCT_3’) and A364aF (5’_CGGGGYGCASCAGGCGCGA_3’) primers (Walter et al., 2015) to permit a comparison between methanogen and general archaeal communities.

Quantitative real-time PCR (qPCR) was conducted using a thermal cycler (CFX96; BioRad Laboratories Inc.). The 20 µl qPCR reaction mix contained 10 µl Ssofast EvaGreen Supermix (BioRad laboratories Inc.), 0.4 µl (10 pM) of each of the forward and reverse primers, 8.2 µl of PCR grade water, and 1 µl template DNA (1 to 10 ng µl⁻¹). To assess potential inhibitory effects, qPCR reactions were performed with a 5 to 100× range of diluted template DNA from each sample. After selecting two
consecutive dilutions with reasonable transition of quantification cycle (Cq) values, a pUC plasmid-based inhibition test was conducted. Three PCR reaction tubes containing template DNA (genomic DNA + pUC, genomic DNA + PCR grade water or pUC + PCR grade water) were amplified using M13 primers. As the selected dilutions did not show any effect on the amplification of pUC (data not shown), the dilution that resulted in 1 to 10 ng µl⁻¹ of template DNA was selected for further PCR analysis. In the qPCR, three replicate reaction tubes for each sample was employed. Optimized thermal cycling for mcrA gene quantification involved initial denaturation at 95°C for 3 min followed by 40 cycles of dissociation (95°C, 30s), annealing (55°C, 30s) and extension (72°C, 45s), and a final step at 72°C for 3 min. For archaeal 16S rRNA gene quantification, the optimized cycling conditions employed were initial denaturation at 94°C for 3 min followed by 39 cycles of dissociation (94°C, 10s), annealing (66°C, 30s) and extension (72°C, 15s), and 72°C for 3 min. Plasmid standard curves for mcrA (10⁸ to 10¹ copies) and archaeal 16S rRNA (10⁹ to 10¹ copies) gene quantifications were prepared from Methanosarcina mazei (ATCC 43340). Efficiency, r², and slope of the standard curve for mcrA gene quantification were 95.5%, 0.98, and -3.43 whereas for 16S rRNA gene quantification, these values were 99.4%, 0.99 and -3.34, respectively. CFX Manager™ software version 3.1 (Bio-Rad Laboratories, Inc.) was used to obtain the qPCR data.

To identify the abundant methanogens, mcrA gene fragments (~470 bp) obtained after PCR amplification using the above primers but with the GC-clamp on the forward primer were separated using the BioRad DCode™ Universal Mutation Detection System (BioRad Laboratories, USA). Denaturing gradient gel electrophoresis (DGGE) analysis
was performed on 8% polyacrylamide gel having 20% to 35% denaturant gradient (84/147 g L\(^{-1}\) and 80/140 ml L\(^{-1}\) urea and formamide, respectively). Two bands that were consistently present in all samples were excised and extracted DNA fragments from these bands were sequenced at the Genomic Facility Laboratory at the University of Guelph.

2.3.3 Phylogenetic analysis

After Blastn-analysis of the partial \textit{mcrA} gene sequences obtained from the two strong and close DGGE bands against the NCBI’s non-redundant nucleotide collection database, the closest and known \textit{mcrA} reference sequences downloaded from the FunGene database (Fish et al., 2013) were included for aligning the newly retrieved sequences using the BioEdit software version 7.2.5 (Hall, 1999). After translating the nucleotide sequence into its amino acid sequence using the FrameBot section of the FunGene pipeline, phylogenetic analysis was conducted in MEGA6 (Tamura et al., 2013) using the neighbor-joining method (Saitou and Nei, 1987). Sequences of the \textit{mcrA} fragments retrieved in this study have been deposited into the NCBI short read archive (SRA) under the accession number SRR3371371.

2.4 Results

Reduction of TS levels of stored dairy slurry lowered cumulative CH\(_4\) emission; however, the abundance and activity of CH\(_4\)-producing microbial communities did not decrease. VS and TC levels reduced as slurry TS decreased from 9.5% to 0.3% (Table 2.1). For the 9.5%TS and 5.8%TS slurries, peak fluxes occurred after day 60 of storage,
with the 9.5% TS displaying a second broader peak with lower flux magnitudes at day 120 of storage (Fig. 2.1a).

Interestingly, the abundance of methanogenic communities did not show concurrent reductions with slurry TS (Fig. 2.2). At day 30 of storage, \( \log_{10} \) copies \( mcrA \) genes \( g^{-1} \) of dry slurry at the top section of the 9.5% TS and 0.3% TS slurries were (Mean ± SEM) 8.4±0.02 and 9.1±0.03, ~8.1% increase in 0.3% slurry. At the same location, at day 120 of storage, 9.02±01 and 10.12±0.03 \( \log_{10} \) copies of \( mcrA \) gene were detected from 9.5%TS and 0.3% TS slurries, respectively, ~12.3% increase in 0.3% slurry.

Table 2.1: Stored dairy manure volatile solids (VS), total carbon (TC), and pH at the beginning (20-May) and end (16-Nov) of the storage.

<table>
<thead>
<tr>
<th>Tanks</th>
<th>Sampling date</th>
<th>Sampling position</th>
<th>VS (%)</th>
<th>TC (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5%TS</td>
<td>20-May</td>
<td>Mixed</td>
<td>7.8</td>
<td>3.30</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>16-Nov</td>
<td>Top</td>
<td>3.7</td>
<td>1.98</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom</td>
<td>6.6</td>
<td>3.32</td>
<td>7.4</td>
</tr>
<tr>
<td>5.8%TS</td>
<td>20-May</td>
<td>Mixed</td>
<td>4.8</td>
<td>2.03</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>16-Nov</td>
<td>Top</td>
<td>1.2</td>
<td>0.73</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom</td>
<td>4.7</td>
<td>2.43</td>
<td>7.3</td>
</tr>
<tr>
<td>0.3%TS</td>
<td>20-May</td>
<td>Mixed</td>
<td>0.2</td>
<td>0.10</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>16-Nov</td>
<td>Top</td>
<td>0.7</td>
<td>0.22</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom</td>
<td>3.6</td>
<td>2.56</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Figure 2.1: Methane (CH$_4$) fluxes (kg m$^{-2}$ d$^{-1}$) (a) and cumulative emissions (kg m$^{-2}$) from stored dairy slurries having varied total solids (TS) contents (b). Emissions were measured continuously for 180 d using a flow-through steady state chamber method.

Similarly, mean Log$_{10}$ mcrA gene copies g$^{-1}$ of dry slurry in the bottom sections of the slurries increased by ~23.7% and ~11.3% at day 30 and 120, respectively, as slurry TS reduced from 9.5% to 0.3%. Interestingly, these increases in the copies of mcrA genes as slurry TS decreased from 9.5% TS to 0.3% TS were not consistent with the changes in cumulative CH$_4$ emissions (Fig. 2.1b), although they did follow the trends seen in the VS to CH$_4$ conversion rate (Fig. 2.3). The activities of methanogens (as
determined from \textit{mcrA} transcript copies g\textsuperscript{-1} dry manure) were also higher (~5.7% and ~28%) in 0.3% TS slurries (Fig 2.2a) at the top and bottom sections of the slurries, respectively.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{chart.png}
\caption{Methanogen activity and abundance (Log\textsubscript{10} copies of \textit{mcrA} gene and transcript copies g\textsuperscript{-1} of dry slurry) in dairy slurries containing total solids contents of \textit{mcrA} gene and \textit{mcrA} transcript.}
\end{figure}

\textbf{Figure 2.2}: Methanogen activity and abundance (Log\textsubscript{10} copies of \textit{mcrA} gene and transcript copies g\textsuperscript{-1} of dry slurry) in dairy slurries containing total solids contents of
9.5%, 5.8%, and 0.3% after about day 30 (a) and Log$_{10}$ copies of $mcrA$ genes g$^{-1}$ dry slurry after day 120 (b).

**Figure 2.3:** Volatile solid (VS) to CH$_4$ conversion rates in 9.5%, 5.8% and 0.3% TS dairy slurries.

Cumulative CH$_4$ fluxes and abundance of methanogens varied with sampling date. The increase in cumulative CH$_4$ production during the storage time was consistent with the abundance and activity of methanogens (Fig. 2.1a and b). The abundance of the total archaeal community, as targeted by their 16S rRNA gene, followed a similar trend as the methanogens (Fig. 2.4). For instance, 6-17% and 7-10% increase in total
archaeal 16S rRNA genes g⁻¹ of dry slurry at about day 30 and 120, respectively, were detected as TS decreased from 9.5% to 0.3%TS. However, consistent with methanogens, the abundances of total archaea increased (from ~0.5 to 18.7%) as storage time increased.

In PCR-DGGE analysis, only a few bands were observed (Fig. 2.5), indicating a low methanogen diversity. Moreover, the disappearance of the faint band (Band 3) as the storage time increased might suggest succession of the methanogenic phylotypes. The partial mcrA gene sequences obtained from the two strong and very close DGGE fingerprints were identical. Both Blastp and Blastn analyses suggested that the newly retrieved sequences were nearly identical to the mcrA gene sequences of an uncultured archaea obtained from stored swine manure slurry in Sherbrooke, Quebec, Canada (Barret et al., 2013). Members of the genus *Methanocorpusculum* might be the closest known relatives (>99% at amino acid levels) to the newly retrieved sequences (Fig. 2.6).
Figure 2.4: Total archaeal abundance (Log$_{10}$ copies 16S rRNA genes g$^{-1}$ of dry slurry) in dairy slurries containing TS contents of 9.5%, 5.8%, and 0.3% after day 30 and day 120 of the storage. Standard errors were calculated from the three replicate PCR reactions.
Figure 2.5: *mcrA* gene based PCR-DGGE fingerprints of *mcrA* gene in stored dairy slurries. Lanes A through F indicates samples after 30 days (9.5%TS_T, 9.5%TS_B, 5.8%TS_T, 5.8%TS_B, 0.3%TS_T, and 0.3%TS_B, respectively) and G through L indicates after 120 days with same percent of TS and position as A to F.
Figure 2.6: Phylogenetic tree showing the relationship (at amino acid level) of mcrA gene sequences retrieved in this study with mcrA gene reference sequences of identified strains. “Band 1” and “Band 2” indicates the two (close) DGGE bands that were consistently present in all samples, and were sequenced.
2.5 Discussion

With the increasing concerns about the contribution of manure management associated CH\textsubscript{4} fluxes to global climate change, studies are focusing on potential mitigation strategies (Wood et al., 2012; Baldé et al., 2016). If efficient mitigation strategies of CH\textsubscript{4} emission from liquid dairy manure storage systems (the significant sources of CH\textsubscript{4}) are to be designed, understanding the microbial communities that are responsible for production of CH\textsubscript{4} in slurries would be critical. This study revealed the higher abundance and activity of methanogens as TS levels of slurries decreased.

In this study, the lag period of about 30 days during with low CH\textsubscript{4} fluxes from all slurries (Fig. 3.1) could be due to substrate limitation for methanogenesis. This is because methanogenesis requires accumulation of sufficient amounts of suitable substrates such as acetate, H\textsubscript{2}/CO\textsubscript{2}, or formate (Liu and Whitman, 2008). Thus, a short storage time could be a potential mitigation strategy to reduce CH\textsubscript{4} emissions from these point sources, although the volume of manure and land availability may challenge short storages. The abundance of methanogenic communities responded differently to the level of slurry TS and length of manure storage time. Increases in the abundance and activity of methanogens as slurry TS reduced from 9.5% to 0.3% contradicted the cumulative CH\textsubscript{4} emissions (Fig. 2.1b and 2.2). Although cumulative CH\textsubscript{4} emissions seemed limited by substrate availability (e.g. \(~3.2\times\) more from 9.5% than 0.3% TS slurries) (Fig. 2.1), methanogenic communities might have been favored in lower TS slurries where CH\textsubscript{4} production rates could be higher. Higher TS slurries could have large amount of by-products such as propionic acid, inhibitory compounds to
methanogenesis (Barredo and Evison, 1991; Barret et al., 2013; Zhang et al., 2014). Moreover, the abundance of total archaeal communities also showed similar trends as methanogens to the total solids levels, suggesting the importance of reducing excess substrate loads for the functioning of most archaeal communities. Increased abundance of methanogenic and total archaeal communities with storage time might be due to the availability of more methanogenic substrates generated from the available TS of slurries (Park et al., 2006).

The few DGGE fingerprints observed for mcrA gene fragments in all of the tanks, particularly after day 120 of storage might indicate that most methanogenic phylotypes that are likely occurring in the fresh manure could not adapt in the stored slurries. DGGE bands observed in 9.5%TS but not in 5.8% and 0.3%TS slurries were likely due to reduction of available specific methanogenic substrates (e.g. CO₂/H₂ or acetate) as TS decreased. In 9.5%TS slurries where methanogenic substrates are expected to be relatively higher, these methanogens could have sufficient amount of the specific substrates, hence might play a significant role in contributing to the cumulative flux. The predominance of Methanocorpusculum related phylotypes, detected in this study, coincided with previous reports where close relatives have often been detected in stored dairy slurries (Gagnon et al., 2011). These methanogens are known to reduce CO₂ into CH₄ using hydrogen, indicating the predominance hydrogenotrophic methanogenesis regardless of the dairy TS level in storage systems.
2.6 Conclusion

Total solids are the main sources of VS which is a proxy for microbial CH$_4$ production. Earlier work showed a positive relationship between TS content of dairy slurries and cumulative CH$_4$ emission (Wood et al., 2012). The current study demonstrated that the abundance and activity of methanogenic communities per gram dry manure were higher in tanks containing lower TS slurries. Therefore, the amount of CH$_4$ emitted is likely limited by the level of TS and hence the available carbon substrate and not methanogen population at reduced TS levels of dairy slurries.
3 Targeting bacteria and methanogens to understand the role of residual slurry as an inoculant in stored liquid dairy manure

Jemaneh Habtewold\textsuperscript{a}, Robert Gordon\textsuperscript{b}, Vera Sokolov\textsuperscript{b}, Andrew VanderZaag\textsuperscript{c}, Claudia Wagner-Riddle\textsuperscript{a} and Kari Dunfield\textsuperscript{a}

\textsuperscript{a.} School of Environmental Sciences, University of Guelph, Guelph, N1G 2W1, ON, Canada
\textsuperscript{b.} Department of Geography & Environmental Studies, Wilfrid Laurier University, Waterloo, N3T 2W2, ON, Canada
\textsuperscript{c.} Agriculture and Agri-Food Canada, Ottawa, K1A 0C5, ON, Canada

This manuscript has been published in Applied and Environmental Microbiology:


Author Contributions:

J Habtewold was the primary researcher and was responsible for the experimental design, manure sampling, lab work, data analysis, and manuscript preparation. V Sokolov was credited for the role in the design of the study and sample collection. C. Wagner-Riddle and A VanderZaag were credited for the valuable advices on the experimental design, statistical analyses and manuscript review. R. Gordon and K. Dunfield were credited for their invaluable mentorship on lab-work and trouble-shooting on data analysis, manuscript preparation, and data interpretation.
3.1 Abstract

Microbial communities in residual slurry left after removal of stored liquid dairy manure have been presumed to increase methane emission during new storage, but these microbes have not been studied. While actual manure storage tanks are filled gradually, pilot- and farm-scale studies on methane emissions from such systems often use a batch approach. In this study, six pilot-scale outdoor storage tanks with (10% and 20%) and without residual slurry were filled (gradually- or in batch) with fresh dairy manure, and methane and methanogenic and bacterial communities were studied during 120 days of storage. Regardless of filling type, increased residual slurry levels resulted in higher abundance of methanogens and bacteria after 65 d of storage. However, stronger correlation between methanogen abundance and methane flux was observed in gradually-filled tanks. Despite some variations in the diversity of methanogens or bacteria with the presence of residual slurry, core phylotypes were not impacted. In all samples, phylum Firmicutes (~57-70%) predominated bacteria where >90% were members of Clostridia. Methanocorpusculum dominated (~57-88%) archaeal phylotypes while Methanosarcina gradually increased with storage time. During peak flux of methane, Methanosarcina was the major player in methane production. Results suggest that increased levels of residual slurry have little impact on the dominant methanogenic or bacterial phylotypes, but large population sizes of these organisms may result in increased methane flux during the initial phases of storage.

Importance: Methane is the major GHG emitted from stored liquid dairy manure. Residual slurry left after removal of stored manure from tanks has been implicated in
increasing methane emissions in new storages, and well-adapted microbial communities in it are the drivers to the increase. Linking methane flux to the abundance, diversity, and activity of microbial communities in stored slurries with different levels of residual slurry can help to improve the mitigation strategy. Meso- and lab-scale studies conducted so far on methane flux from manure storage systems used batch-filled tanks while the actual condition in many farms involves gradual filling. Hence, this study provides important information towards determining levels of residual slurry that result in significant reduction of well-adapted microbial communities prior to storage thereby reducing methane emissions from manure storage tanks filled under farm conditions.

**Keywords**: dairy manure, greenhouse gas, methane, methanogen, residual slurry

### 3.2 Introduction

Dairy farming is responsible for large amount of non-CO$_2$ GHG emissions, mainly from enteric fermentation and manure management. In large dairy farms, manure is usually stored as slurry for extended periods (i.e. months to one year) (Sheppard et al., 2011), creating a medium that supports the growth of microbial communities involved in anaerobic degradation and CH$_4$ production (VanderZaag et al., 2011). During pumping out of slurry tanks for land application, complete removal is practically impossible and residual slurries (RS) of varying amounts are usually left in storages. In view of the potential CH$_4$ emission mitigation strategies from these systems, the inoculum effects of RS in new storages have become the focus of several studies (Sommer et al., 2007; Massé et al., 2008; Wood et al., 2014; Balde et al., 2016; Massé et al., 2016; Ngwabie et al., 2016). Different levels of RS left in storage tanks, especially with actual farm
conditions where manure is transferred to tanks gradually throughout the storage period, results in varied RS to fresh manure ratios throughout the storage period. Thus, understanding the effects of these ratios on the dynamics of microbial communities that derive CH$_4$ emission and linking them with CH$_4$ flux during storage is critical towards developing effective mitigation strategies. For instance, accelerated starts of CH$_4$ production from liquid dairy manure has been observed after supplementing with 7.6% inoculum material (manure that has been stored for 45-60 d at 20°C) (Sommer et al., 2007). About 56 and 97% reductions of CH$_4$ emission have also been demonstrated by complete removal (Massé et al., 2016) and reduction of RS levels from ~26% to 13% (Massé et al., 2008), respectively. Using pilot-scale outdoor storage tanks, ~56% reduction of cumulative CH$_4$ emission by complete removal of old manure compared to partial emptying was demonstrated (Wood et al., 2014). Using the same tanks, ~26% lower cumulative CH$_4$ emission was also reported after lowering old residual manure from 15% to 5% (Ngwabie et al., 2016). Interestingly, these decreases in CH$_4$ emissions were presumed to be explained by reductions of well-adapted consortium of microorganisms in the RS that might readily start functioning in new storages, although no study so far investigated these microbial communities.

Although total solids contents (hence volatile solids contents) of dairy manure might suggest potential CH$_4$ output from the storage system (Habtewold et al., 2017), the activities of methanogenic communities in the manure are critical as they are responsible for producing CH$_4$. Their abundance is not only important in the stored manure but also during the new storage. This is because the residual slurry left after
removal for land application, which is presumed to have abundant and well-adapted microbial communities, can have significant inoculant effects during the new storage and hence increase CH$_4$ emissions. In this Chapter, the structure of bacterial and methanogenic communities in residual, fresh and stored dairy manure was investigated, and the effects of different levels of residual slurry was related to CH$_4$ emissions.

With long-term storage of dairy manure, bacterial and methanogenic communities of different sources (e.g. rumen, soil, water, bedding material) may be enriched and acclimatized to storage conditions (Li et al., 2014; Steinmetz et al., 2016). All microbial communities functioning in rumen environments may not be able to establish in manure storage systems as these environments can differ with a number of parameters such as temperature and substrate composition. Thus, after long-term storage of manure, a consortium of microbial communities well-adapted to existing storage conditions may readily breakdown manure organic polymers (polysaccharides, proteins, lipids), an important step towards CH$_4$ production. To convert complex polymers into CH$_4$, four physiological groups of microorganisms are important: hydrolytic, acidogenic, acetogenic, and methanogenic microbial communities (Liu and Whitman, 2008). First, hydrolytic bacteria degrade residues of organic polymers into simple compounds such as glucose, fatty acids, amino acids, alcohols, H$_2$, and CO$_2$. As efficiency of the hydrolysis step determines the rate of anaerobic degradation (Tomei et al., 2009) particularly when the ratio of hydrolytic bacteria to methanogenic archaea is high (Ma et al., 2013), the relative composition of these bacteria in RS may be critical. By-products of hydrolysis are substrates for acidogenic bacteria that further convert
them into different organic acids (e.g. acetic, lactic, propionic, formic, and butyric acids), ethanol, H$_2$ and CO$_2$. Acetogenic bacteria then converts higher organic acids such as butyric and propionic acids into acetic acid, H$_2$, and CO$_2$, which are important substrates for methanogenesis (Liu and Whitman, 2008). As long-term storage of dairy slurry may enrich these hydrolytic, acidogenic, acetogenic, and methanogenic microorganisms, investigating these communities in RS and their effects on CH$_4$ emission in stored manure is an important step towards the refinement and/or development of efficient mitigation strategies.

It is critical to assess the effects of RS on CH$_4$ emission and structure of slurry microbial communities using dairy manure that is stored and managed similar to farm conditions. Slurry storage systems of dairy farms are usually filled gradually (i.e. daily to weekly loadings) throughout the storage period by transferring barn collections or slurries from temporary storages (Sheppard et al., 2011). Compared to batch-filled tanks that have often been used to study CH$_4$ flux from manure storage systems (Wood et al., 2014; Ngwabie et al., 2016), repeated loading in gradually-filled tanks may affect the stability of slurry environments and microbial communities. For instance, with repeated loadings, crusts that have been presumed to be the potential locations where aerobic oxidation of CH$_4$ may occur (Ambus and Petersen, 2005; Petersen et al., 2005; Nielsen et al., 2013; Duan et al., 2014), could be impacted. Although available substrates for microbial communities in batch-filled tanks can be high in the initial phases of storage, gradual filling continually supplies fresh substrates. Thus, the impact of frequency of filling on CH$_4$ flux and microbial communities needs to be assessed.
Advanced molecular methods such as quantitative real-time PCR (qPCR) and massively parallel sequencing fragments of universal marker genes such as 16S rRNA are revealing the structure of microbial communities from complex environments (St-Pierre and Wright, 2013; St-Pierre and Wright, 2014). These techniques have also been successfully used to study functional groups of microorganisms such as methanogens by targeting a gene or transcript related to their function (Aydin et al., 2015; Wilkins et al., 2015; Krakova et al., 2016). Three different physiological groups of methanogens are known (hydrogenotrophic, aceticlastic, and methylotrophic), and have the gene encoding the alpha subunit of methyl coenzyme A reductase (mcrA) that catalyzes the last step of methanogenesis (Steinberg and Regan, 2009). Since 16S rRNA gene- and mcrA gene-based phylogenies of methanogens are congruent (Luton et al., 2002), targeting the mcrA genes or transcripts may provide both phylogenetic and functional information about the methanogen community.

The objective of this study was to assess the effects of different RS levels (0%, 10%, and 20%) on CH4 emissions and the structure of methanogenic and bacterial communities in stored dairy slurries filled gradually and in batch.

### 3.3 Materials and Methods

#### 3.3.1 Experimental setup and measurement of CH4 flux emissions

The study was conducted from 02 June-24 September 2016 (120 d) at Dalhousie University’s Bio-Environmental Engineering Center (BEEC) in Truro, NS, Canada (45°45' N, 62°50' W). Six pilot-scale (3.9 m × 1.75 m × 1.8 m) manure storage tanks enclosed by flow-through steady-state chambers were used. This site has been
previously described by Wood et al. (2012). The RS was obtained from a previous study conducted at the site and consisted of dairy slurry stored for about 1-year. Dairy slurry (freshly excreted to 2 weeks old) was obtained from a nearby farm that had about 185 dairy animals (lactating and dry cows, calves, and heifers), used washed sand as bedding material, and stored dairy waste (washwater, dung, urine and bedding material) in a concrete lagoon. Tanks with two levels of RS (10% or 20%) and without any RS were filled with fresh dairy slurry either in batch (on day 1 to 100% volume, 10.6 m$^3$) or gradually with incremental manure additions on day 1 (~33.3% volume), day 20 (~33.3% volume) and day 40 (~33.3% volume). Gas samples were drawn continuously from each tank’s outlet and ambient air, and CH$_4$ concentrations were measured at the site using model TGA 100A tunable diode laser trace gas analyzer (Campbell Scientific Inc., Logan, UT). Methane flux (F, g m$^{-2}$ s$^{-1}$) was calculated as described earlier (Wood et al., 2012):

$$ F = \frac{C_{\text{out}} - C_{\text{in}}}{A_s} Q $$

Where $C_{\text{out}}$ and $C_{\text{in}}$ are chamber’s outlet and inlet CH$_4$ concentrations (g m$^{-3}$), respectively; $A_s$ is the surface area of the storage tank (m$^2$); and Q is the airflow rate (m$^3$ s$^{-1}$) as determined from the wind velocity in each tank’s venturi using cup anemometers (Davis Instruments Corp, Hayward CA). Fluxes were then calculated into daily averages.
3.3.2 Slurry sample collection and analyses

Prior to storage, two grams of slurry samples were collected in duplicate from fresh and 1-year old slurries using 15 mL Falcon tubes containing 5 mL LifeGuard™ Soil Preservation Solution (MoBio Laboratories Inc., Carlsbad, CA). During storage, slurry from the top (~5 cm from surface) and bottom (~10 cm from tank’s floor) sections of each tank was sampled every 15 days for 120 days. From a preliminary test on a few samples, significant shifts in the abundance of methanogenic communities were observed on day 65 (when peak CH₄ emissions for most of the tanks were detected), hence samples from fresh manure, old slurry, and day 65 and day 120 samples were selected for further microbial analysis. On each date, two samplings were made from each tank (top and bottom). During each sampling, nine samples were collected from distinct locations of a tank and homogenized in a clean bucket using a sterile metal rod. Sub-samples (2 g each) were then collected in duplicate sterile 15 mL Falcon tubes each containing 5 mL LifeGuard™ Soil Preservation Solution. Samples were then transported on ice to the lab and stored at -20°C freezer until nucleic acid extractions. Further sub-samples of appropriate volume were also collected to analyze for pH, dry matter (DM), volatile solid (VS) content, and volatile fatty acids (VFAs). Dry matter, VS, and pH were determined at the Nova Scotia Department of Agriculture’s Laboratory Services (Harlow Institute, Bible Hill, NS) using standard methods. The VFA analysis was performed by InnoTech Alberta (Vegreville, AB) via headspace gas chromatography.
3.3.3 Nucleic acid extractions and quantitative real-time PCR

Slurry samples in LifeGuard™ Soil Preservation Solution were centrifuged and pellets were used to co-extract total RNA and DNA using RNA PowerSoil Total RNA Isolation with DNA Elution Accessory Kits (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer’s protocol. As the massive research projects such as Earth Microbiome Project that aimed to characterize microbial life on this planet also use nucleic acid extraction kits from MoBio, efficient extractions of nucleic acids were expected following the optimized and recommended protocol. Duplicate RNA and DNA samples per tank and sampling date were prepared by pooling extractions made from the top and bottom sections of individual samplings. Using triplicate reaction tubes, 8 µL RNA samples were treated with RQ RNase-Free DNase (Promega) to remove contaminant DNA and then reverse transcribed into complementary DNA (cDNA) using high-capacity cDNA reverse transcription kit with RNase Inhibitor (Applied Biosystems) following the recommended protocols. Before further downstream analyses, both cDNA and DNA samples were diluted and assessed for potential inhibitory effects as described earlier (Habtewold et al., 2017) (Chapter 2). Appropriate dilutions were then selected to conduct quantitative real-time qPCR and amplicon sequencing.
Table 3.1: PCR primers and Illumina adaptors used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Used for</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mlas-mod F</td>
<td>ggyggtgmgmgtacmcarta</td>
<td>qPCR</td>
<td>(methanogens)</td>
</tr>
<tr>
<td>mcrA-rev-mod R</td>
<td>cgttcagtggttgtagtgtagtgtagt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bac338F</td>
<td>actcctacggcgccagcag</td>
<td>qPCR (bacteria)</td>
<td>(Fierer et al., 2005)</td>
</tr>
<tr>
<td>Bac518R</td>
<td>attacggcgctctgg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>341F</td>
<td>cctacgggnggwcag</td>
<td>Amplicon preparation</td>
<td>(Herlemann et al., 2011)</td>
</tr>
<tr>
<td>805R</td>
<td>gactachvgggtatctaat</td>
<td>(bacteria)</td>
<td></td>
</tr>
<tr>
<td>Arch349F</td>
<td>gycascagkgcmgaaw</td>
<td>Amplicon preparation</td>
<td>(Takai and Horikoshi, 2000)</td>
</tr>
<tr>
<td>Arch806R</td>
<td>ggtacvsgggtatctaat</td>
<td>(archaea)</td>
<td></td>
</tr>
<tr>
<td>Adaptor A</td>
<td>gtctcgtggctggtgggtgtgtagtggagtggagtggagagagagagagag</td>
<td>Illumina MiSeq sequencing</td>
<td></td>
</tr>
<tr>
<td>Adaptor B</td>
<td>tgcgtcggcagcgcgtcatgtgtagtggagtgtgtagtggagagagagagag</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clear Multiplate™ 96-Well PCR Plates (BioRad Laboratories, Inc., Hercules, CA) were used to prepare duplicate reaction mixes from each sample, and qPCR was performed using a CFX96™ Real-Time System on C1000 Touch™ Thermal Cycler (BioRad Laboratories, Inc., Hercules, CA). Methyl coenzyme A reductase (mcrA) genes and transcripts were quantified using mlas-mod F and mcrA-rev-mod R primers (Steinberg and Regan, 2009; Angel et al., 2012) (Table 3.1). Bacterial population size and activities were also estimated by targeting 16S rRNA genes and transcripts using Bac 338F and Bac 518R primers (Fierer et al., 2005) (Table 3.1). The 20-µl qPCR reaction mix contained 10 µl Ssofast EvaGreen Supermix (BioRad Laboratories, Inc.), 1 µl (10 pM) of each primer, 2 µl template DNA or cDNA (1 to 10 ng µl⁻¹), and 6 µl of PCR-grade water. Thermal cycling for mcrA gene and transcript quantifications involved initial
denaturation at 98°C for 2 min followed by 40 cycles of dissociation (95°C, 5 s), annealing (57°C, 10 s) and extension (72°C, 15 s), and a final step at 72°C for 30 s before increasing the temperature by 0.5°C from 65°C to 95°C for 5 s to analyze melting curves. For bacterial 16S rRNA gene and transcript quantification, the optimized cycling conditions used were initial denaturation at 98°C for 2 min followed by 34 cycles of dissociation (98°C, 5 s), annealing (55°C, 5 s) and extension (65°C, 5 s), and a final extension 72°C for 3 min before increasing the temperature by 0.5°C from 65°C to 95°C for 5 s. Known copies of plasmid standard curves for \textit{mcrA} \((10^8\) to \(10^1\)) and bacterial 16S rRNA \((10^9\) to \(10^1\) copies) genes and transcripts quantifications were prepared from \textit{Methanosarcina mazei} (ATCC 43340) and pure culture of \textit{Clostridium thermocellum}, respectively. Efficiency, \(r^2\), and slope of the plasmid standard curve for \textit{mcrA} gene were 93.6±2.8%, 0.99, and -3.4 ±0.1, whereas for 16S rRNA gene, these values were 98.0±0.7%, 0.99, and -3.30±0.01, respectively. Cycle of quantifications (CQs) for the highest diluted standard point of \textit{mcrA} \((10^1\)) and no template controls (NTCs) were 36.1±0.4 and 39.1±1.1, respectively whereas for bacterial 16S rRNA gene, these values were 28.1±0.2 and 32±0.0, respectively. CFX Manager software version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA) was used to analyze the qPCR data. To determine the effects of RS on the abundance of methanogens and bacteria, one-way analysis of variance (ANOVA) was carried out using log-transformed copy numbers of \textit{mcrA} and 16S rRNA genes and transcripts on day 65 and 120 slurries. When statistically significant variation (\(p<0.05\)) was observed among means of gene copies in slurries that
received 0%, 10%, and 20% RS, Tukey’s post-hoc test was conducted using GraphPad prism v.7 (GraphPad Software, Inc.) to determine the sample that resulted in variation.

3.3.4 Amplicon library preparation and sequencing

Amplicon libraries of archaeal and bacterial 16S rRNA genes were prepared from DNA samples obtained from RS, fresh slurry, and stored slurries (0% and 20%). To identify the key players during peak flux of CH$_4$ from each tank, a library of archaeal 16S rRNA transcript was also constructed from RNA samples. As CH$_4$ flux from tanks with 10% and 20% RS of both filling types did not show significant variation, only tanks with 0%Rs and 20% RS were used to determine the identities and relative proportions of bacterial and archaeal phylotypes. For bacteria, amplicons were prepared using 341F and 805R primers that target the V3-V4 region of 16S rRNA gene ([Herlemann et al., 2011](#)) whereas primer pairs Arch349F and Arch806R were used to prepare archaeal 16S rRNA gene and transcript libraries ([Takai and Horikoshi, 2000](#)). On both archaeal and bacterial primers, Illumina adapter sequences A and B (Table 3.1) were added to the 5’-ends of the forward and reverse primers, respectively.

For both genes, amplicons for MiSeq sequencing were prepared in two PCR steps with a total of 33 (bacteria) or 37 (archaea) cycles. First, archaeal and bacterial 16S rRNA genes were amplified for 25 PCR cycles using the above modified primer sets. For each sample, the 25 µL PCR reaction mix contained 5 µL of 5X Phusion HF buffer, 0.25 µL of Thermo Scientific™ Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 0.5µL of 10mM dNTPs (Thermo Scientific), 0.5 µL of each primer (10 µM), 2 µL of diluted DNA (10 to 50 ng/µL) or cDNA, and 16.25 µL
nuclease free water. Thermal cycling for both gene targets were as follows: initial
denaturation at 98°C for 3 min, followed by 25 cycles of dissociation at 98°C for 10 s,
primer annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension for 5
min. Duplicate PCR reaction tubes were pooled and products were cleaned using silica
spin columns (Wizard® SV Gel and PCR Clean-Up System; Promega) following the
recommended protocol. The second step PCR was performed for 8 (bacteria) or 12
(archaea) cycles to attach Illumina index tags to the ends of the amplicons that were
obtained from the first-step PCR. For each sample, a different combination of the Index
primers 1 (N7xx) and Index primers 2 (S5xx) of Illumina’s Nextera® XT DNA Library
Preparation Kit (Illumina Inc., San Diego, CA) were used to perform PCR. This was
performed in a single 50 µL reaction mix per sample, and used same proportion of
reagents and similar thermal cycling conditions as the first-step PCR except the 4 µL
purified amplicons used as template. PCR products were then purified by magnetic
beads (Agencourt AmPure XP; Beckman Coulter, Brea, CA) and re-suspended in 25
µL. Purified PCR products were tested for correct amplicon length using gel
electrophoresis, and submitted to the University of Guelph Advanced Analysis Centre,
Genomic Facility (Guelph, ON) for sequencing. Prior to sequencing, libraries were
normalized by Sequalprep (Thermo Fisher Scientific, Hampton, NH) and library quality
was assessed from a random sample of 12 samples using Bioanalyzer DNA1000 chip
(Agilent, Santa Clara, CA). Multiplexed sample sequencing was conducted using MiSeq
Reagent Kit v2 (500-cycles) (Illumina Inc., San Diego, CA) producing paired end reads
of 250 bp in length. Unprocessed FASTQ files were received for subsequent analysis.
3.3.5 **Sequence data analysis**

Raw sequence data of archaeal and bacterial 16S rRNA genes and transcripts were processed and analyzed in Mothur v.1.39.5 (Schloss et al., 2009) following the recommended pipeline for MiSeq 16S rRNA gene sequences (Kozich et al., 2013) with some modifications. Briefly, after the forward and reverse reads of each sample was merged, target specific primer sequences removed and sequences were screened for ambiguity and length. Then, sequences were aligned against the Silva gold reference file v128, further screened for length and homopolymer, overhangs and common gaps filtered, and pre-clustered to further denoise sequencing errors. After removal of potential chimeric sequences, Mothur-formatted version of the RDP’s 16S rRNA reference (version 16) was used to classify sequences into phylotypes at 80% cut-off in which undesirable targets that might have been picked by primers were filtered. Finally, purified sequences were clustered into operational taxonomic units (OTUs) at 0.03 cut-off (97% similarity), phylotypes of OTUs identified using the RDP’s 16S rRNA reference database, and rarefaction curves calculated. For OTU-based alpha diversity (e.g. number of OTUs, coverage, Chao1, and Inverse Simpson diversity estimate) and beta diversity (e.g. non-metric multidimensional scaling and shared OTUs) analyses, sequence reads were subsampled and rarefied. Phylogenetic trees generated using the Yue and Clayton theta distance matrices (calculated by relaxed neighbor joining algorithm of clearcut in mothur) (Chao, 1984; Sheneman et al., 2006; Kozich et al., 2013) were used to construct dendrograms showing community structure-based
variations between samples, and significance of variations were assessed using
AMOVA and parsimony analyses.

Sequence accession

The unprocessed sequence reads of bacterial 16S rRNA gene, archaeal 16S
rRNA gene, and archaeal 16S rRNA transcripts obtained in this study have been
deposited in NCBI’s short read archives as FASTQ files with the accession numbers
from SRR6132422-SRR6132441, SRR6132442-SRR6132461, SRR6132462-
SRR6132469, respectively.

3.4 Results

3.4.1 Methane flux and manure characteristics

Absence of RS (0%) had an impact only in gradually-filled tanks where peak flux
occurred about 3 weeks later than the tanks with 10% or 20% RS (Fig. 3.1). In the
absence of RS, the maximum mean daily flux reached was ~43-47% lower than the
tanks with 10% and 20% RS. This resulted in up to 85% reductions of the cumulative
emissions compared to the tanks with RS. There was little variation (~11.25 ± 0.53 kg
CH₄ m⁻²) in cumulative fluxes from tanks with 10% and 20% RS. In batch-filled tanks, no
significant difference in CH₄ flux or cumulative emissions (~9.09±0.23 kg CH₄ m⁻² slurry)
were detected due to the presence or absence of RS. Similar trends were observed
when CH₄ flux was calculated per volume of manure at different time points (Appendix
Fig. A.1). Manure characteristics (e.g. dry matter and VS contents, VFAs, and pH)
varied with storage time. Compared to RS, dry matter and VS contents of fresh dairy
slurries were higher (~8.7- and 8.4-fold, respectively), thus different levels of RS combined with heterogeneity of the farm manure, resulted in varied contents of these parameters in stored slurries on day 1 of storage (Table 3.2). Regardless of RS levels and filling type, these initial contents reduced with storage time. On day 65, relatively higher levels of VFAs and hence lower pH were observed in slurries without RS compared to slurries that contained 10% or 20% RS (Table 3.2 and 3.3). Nevertheless, pH of slurries from most treatments decreased initially (until day 18) and then increased gradually after, reaching 7.8±0.2 on day 120. Most of the VFAs detected during peak flux of CH₄ (day 65) were not detected in day 120 samples, which corresponded in up to 5.3-fold reduction of CH₄ flux.
**Table 3.2**: Dry matter contents, volatile solid contents, and pH of stored dairy slurries with 0%, 10%, and 20% residual slurry and filled gradually or in batch

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dry matter (%)</th>
<th>Volatile solids (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>18</td>
<td>65</td>
</tr>
<tr>
<td><strong>FM</strong></td>
<td>20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RS</strong></td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>0% GF</strong></td>
<td>19.6</td>
<td>13.8</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>10% GF</strong></td>
<td>9.5</td>
<td>13.2</td>
<td>10.6</td>
</tr>
<tr>
<td><strong>20% GF</strong></td>
<td>5.8</td>
<td>8.92</td>
<td>10.2</td>
</tr>
<tr>
<td><strong>0% BF</strong></td>
<td>14.1</td>
<td>13</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>10% BF</strong></td>
<td>10.7</td>
<td>17.3</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>20% BF</strong></td>
<td>11.8</td>
<td>12.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*FM, fresh manure; RS, residual slurry; GF, gradually filled; BF, batch filled.*
### Table 3.3: Volatile fatty acid contents of stored dairy slurries with 0%, 10%, and 20% residual slurry, and filled gradually or in batch.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volatile fatty acids (g L⁻¹ dairy slurry; mean ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formic</td>
</tr>
<tr>
<td><strong>Day 65</strong></td>
<td></td>
</tr>
<tr>
<td>0%_GF</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>10%_GF</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>20%_GF</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>0%_BF</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>10%_BF</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>20%_BF</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td><strong>Day 120</strong></td>
<td></td>
</tr>
<tr>
<td>0%_GF</td>
<td>0.02 (T)</td>
</tr>
<tr>
<td>10%_GF</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>20%_GF</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>0%_BF</td>
<td>ND</td>
</tr>
<tr>
<td>10%_BF</td>
<td>0.02 (T)</td>
</tr>
<tr>
<td>20%_BF</td>
<td>0.05±0.04</td>
</tr>
</tbody>
</table>

aValues are averages of concentrations obtained from the bottom (~10 cm from tank floor) and top (~5 cm from surface) samples of each tank and time point. A “T” or “B” in parentheses indicates that a value from either the bottom or top of the tank was not detectable (ND).
Figure 3.1: Mean daily methane (CH₄) flux (g m⁻² d⁻¹) from liquid dairy manure storage tanks containing 0%, 10%, and 20% residual slurry (RS), for gradually or batch filled tanks.

3.4.2 Abundance and activity of methanogens and bacteria

There were more (between ~11 and 117%) copies per gram of dry manure (Log₁₀ transformed) of mcrA and bacterial 16S rRNA genes and transcripts in the one-year old residual slurry (RS) compared to fresh dairy slurry used in this study (Fig. 3.2a and 3.2b). During the initial 65 days of storage, these abundance differences resulted in significantly varied (one-way ANOVA, p<0.05) abundances of methanogens and
bacteria in stored slurries that received different levels of RS. In samples collected on day 65, the presence of 10% and 20% RS in gradually-filled tanks increased the mcrA gene copies between ~7-12% (as calculated from Log_{10}-transformed values) compared to the control (Fig. 3.2a). Consistently, the activities of methanogens in these slurries, as observed from Log_{10} copies of mcrA transcripts, increased up to ~12% (Fig. 3.2a). On day 65, in slurry samples from batch-filled tanks, 10% and 20% RS also resulted in ~6.5-8% increase of mcrA gene copies (Log_{10} transformed) (Fig. 3.2a). Mean copies (Log_{10} transformed) of mcrA transcripts were also increased between ~2.5-6%. In both gradually- and batch filled tanks, most of RS-related increases in the copies of mcrA genes and transcripts were statistically significant (Tukey’s post hoc test using Log_{10} transformed values, p<0.05). However, positive correlations between daily flux of CH_{4} during the week of day 65 and mcrA gene (r^2=0.6; p<0.05) or transcript (r^2=0.53; p<0.05) copies were observed only in gradually-filled tanks.

Day 65 slurry samples collected from batch-filled tanks had more copies (g^{-1} dry manure) of mcrA genes (~5-10%) and transcripts (~4-9%) compared to slurries from gradually-filled tanks (Fig. 3.2a). While these increases were statistically significant (Tukey’s p<0.05), they were not reflected in CH_{4} flux. This is because, during the week of day 65, daily mean flux of CH_{4} from gradually-filled tanks with RS were ~23.2% higher compared to the corresponding batch-filled tanks. However, in the absence of RS, the gradually-filled tank showed the lowest CH_{4} flux during the week of day 65 (Fig. 3.1).
Figure 3.2: The abundance and activity of bacteria (a) and methanogens (b) in fresh manure, old-slurry (residual slurry), stored dairy slurry (day 65 and day 120), as
indicated by mcrA/bacterial 16S rRNA genes and transcripts copies (Log$_{10}$ transformed). Results shown are mean and standard errors of the duplicated biological samples. Letters shown on the bars indicate significance levels obtained after Tukey’s post hoc test using Log$_{10}$ transformed copies; different letters on bars indicate statistically significant differences at $p<0.05$). FM and RS denotes fresh manure and residual slurry, whereas GF, and BF denotes gradually- and batch-filled, respectively.

Except the tank with 10% RS and filled in batch, RS-related increases in the abundance of bacteria coincided with the changes in methanogen abundance (Fig. 3.2b). In day 65 samples, slurries with 20% RS of both filling types had more copies (up to 5%), as calculated using Log$_{10}$ transformed copy numbers, of 16S rRNA genes and transcripts (Fig. 3.2b). On day 120, the abundance and activities of methanogens and bacteria did not show clear trends between RS levels or filling types, indicating little effects of RS in later stages of storage. Thus, qPCR results suggested that increased levels of RS could increase the number methanogen and bacterial populations during the initial phases of storage, which may have increased CH$_4$ flux.

### 3.4.3 Diversity of archaeal and bacterial communities

After a series of quality inspections and denoising of raw sequences, on average, 35,074 (1255 unique) and 8549 (2056 unique) quality reads per sample were obtained for archaeal and bacterial 16S rRNA amplicons, respectively. From the RNA samples that were obtained from slurries during peak flux of CH$_4$, 9841 (6,88 unique) quality reads per sample of archaeal 16S rRNA transcripts were also retrieved. Pre-aligned sequence lengths of archaeal and bacterial 16S rRNA amplicons were 348-360 bp and
379-405bp, respectively. The final quality reads of archaeal and bacterial 16S rRNA amplicons were used to define OTUs at 0.03 cut-off and to perform further OTU-based analyses. Two samples with low number of amplicon sequences were excluded from OTU-based analysis in archaea. Rarefaction curves (Fig. 3.3a and 3.2b), particularly for archaeal 16S rRNA gene amplicon library, indicated reasonable sampling efforts to capture most of the species in slurry samples. This was consistent with the Good’s coverage estimates that indicated sufficient capture of archaeal (>99%) and bacterial (80-92%) species from these samples (Table 3.4).
Figure 3.3: Rarefaction curves, as calculated in Mothur, showing changes in the number of observed OTUs of archaea (a) and bacteria (b) (cut-off=0.03) as sampling intensity (number of sequences sampled) increases from slurry samples. RS, FM, GF, and BF represent residual slurry, fresh manure, gradually-filled and batch-filled, respectively. D65 and D120 represent day 65 and day 120, whereas a and b are replicates.
Table 3.4: Diversity indices calculated for archaeal and bacterial 16S rRNA gene sequence reads obtained from residual, fresh, and stored dairy slurries of gradually- and batch-filled tanks. Numbers presented for Chao1 and Inverse Simpson are means of the minimum and maximum values. RS, FM, GF, and BF represent residual slurry, fresh manure, gradually- and batch-filled, respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cov.</th>
<th>sobs</th>
<th>Chao</th>
<th>Invsimpson</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh manure</td>
<td>0.99</td>
<td>98±9</td>
<td>182±24</td>
<td>1.7±0.06</td>
</tr>
<tr>
<td>RS</td>
<td>0.99</td>
<td>108±17</td>
<td>210±37</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Day 65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0_BF</td>
<td>0.99</td>
<td>128±17</td>
<td>242±56</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>0_GF</td>
<td>0.99</td>
<td>106±12</td>
<td>240±11</td>
<td>2±0.2</td>
</tr>
<tr>
<td>20_BF</td>
<td>0.99</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>20_GF</td>
<td>0.99</td>
<td>128±2</td>
<td>274±7</td>
<td>2.2±0.02</td>
</tr>
<tr>
<td>Day 120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0_BF</td>
<td>0.99</td>
<td>112±8</td>
<td>227±3</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>0_GF</td>
<td>0.99</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>20_BF</td>
<td>0.99</td>
<td>116±4</td>
<td>249±2</td>
<td>2.8±0.02</td>
</tr>
<tr>
<td>20_GF</td>
<td>0.99</td>
<td>100±3</td>
<td>225±12</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh manure</td>
<td>0.82</td>
<td>1904±47</td>
<td>6096±230</td>
<td>69.4±2.6</td>
</tr>
<tr>
<td>RS</td>
<td>0.80</td>
<td>2055±30</td>
<td>7018±94</td>
<td>80.9±5.3</td>
</tr>
<tr>
<td>Day 65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0_BF</td>
<td>0.85</td>
<td>1451±18</td>
<td>5268±81</td>
<td>20.5±2.1</td>
</tr>
<tr>
<td>0_GF</td>
<td>0.86</td>
<td>1434±5</td>
<td>4573±143</td>
<td>24.4±0.3</td>
</tr>
<tr>
<td>20_BF</td>
<td>0.86</td>
<td>1297±41</td>
<td>5092±805</td>
<td>19.6±0.6</td>
</tr>
<tr>
<td>20_GF</td>
<td>0.88</td>
<td>1167±108</td>
<td>4050±447</td>
<td>21.4±1.6</td>
</tr>
<tr>
<td>Day 120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0_BF</td>
<td>0.89</td>
<td>1087±83</td>
<td>3459±606</td>
<td>18.8±0.87</td>
</tr>
<tr>
<td>0_GF</td>
<td>0.92</td>
<td>908±19</td>
<td>2425±204</td>
<td>12.9±1.9</td>
</tr>
<tr>
<td>20_BF</td>
<td>0.91</td>
<td>905±64</td>
<td>2608±453</td>
<td>20.5±0.2</td>
</tr>
<tr>
<td>20_GF</td>
<td>0.91</td>
<td>925±30</td>
<td>2905±10</td>
<td>13.2±2.5</td>
</tr>
</tbody>
</table>

Key: sobs=observed species richness; Cov. = coverage; Chao = Chao 1 calculator; invsimp=Inverse Simpson index

Diversity indices (sobs, Chao1, and Inverse Simpson index) calculated for archaeal 16S rRNA amplicon sequences indicated little difference in the richness and
diversity of archaea between RS and fresh dairy manure (Table 3.4). With the presence of 20% RS, the diversity of archaea in slurries from both gradually- and batch-filled tanks were slightly increased. These diversity differences gradually intensified with storage time, resulting in groupings of slurry samples with and without RS (Fig. 3.4a). Despite some overlap, the observed separation of slurries with and without RS in the NMDS plots (Stress = 0.18; $r^2 = 0.95$) were significant (AMOVA, $p<0.001$). A consistent result (Parsimony, $p= 0.038$) was also observed in a tree that was generated using Yue and Clayton theta distance matrices of archaeal 16S rRNA amplicon sequences (Fig. 3.5a). Analysis of shared OTUs indicated that (Fig. 3.6a-d), RS and fresh manure shares more than 50% of their total OTUs. Most (25-33) of these shared OTUs were also present in stored slurries from all tanks. These shared OTUs include the most dominant ones in all tanks, which might govern microbial processes in slurries. Thus, RS-related differences in the diversity of archaea might be less important as the dominant OTUs were consistently present among all slurries.
Figure 3.4: The (a) Non-metric multi-dimensional scaling (NMDS) plots of the archaeal (a) and bacterial (b) communities in fresh, residual slurry, and stored slurries. Yue and Clayton theta distance matrix of archaeal and bacterial 16S rRNA gene amplicons were used to calculate the respective NMDS axis values. In the legend, FM, RS, D65, and D120, represent fresh manure, residual slurry, day 65, and day 120, respectively. The dark green, blue, red, and black colors represented samples from fresh manure, residual slurry, gradually-filled (GF), and batch-filled (BF) tanks, respectively.
Figure 3.5: Dendrograms showing the degree of similarity of dairy slurry samples with respect to archaeal (a) and bacterial (b) community structures. Trees were generated using Thetayc distance matrices of archaeal and bacterial 16S rRNA gene amplicon sequences. FM, and RS, GF, and BF respectively show fresh manure, residual slurry, gradual filling, and batch filling, whereas a and b show the duplicate samples.
Figure 3.6: Venn diagrams displaying the number of shared archaeal OTUs (cut-off=0.03) by residual, fresh, and stored dairy slurries from gradually-filled tanks on day 65 (a) and day 120 (b) and batch-filled tanks on day 65 (c) and day 120 (d) of storage. As the duplicate samples of each treatment showed almost the same number of OTUs, these diagrams only represented one of duplicates. FM, RS, GF, and BF represent fresh manure, residual slurry, gradually-filled, and batch-filled, respectively.

The diversity of bacteria in RS and fresh slurries showed little variation. Unlike archaea, stored slurries with and without RS of both filling types did not show noticeable variation in day 65 or day 120 samples (Table 3.4). However, when day 65 and day 120
samples compared, the number of OTUs (sobs and Chao1 estimates) and Inverse
Simpson Diversity Indices reduced, indicating gradual reduction of diversity. In NMDS
analysis (Stress = 0.06; $r^2 = 0.99$), there was significant separation (AMOVA, p<0.001)
of day 65 and day 120 samples (Fig. 3.4b). The Yue and Clayton theta distance
matrices-based tree of slurry samples further confirmed the differences (Parsimony, p =
0.002) between day 65 and day 120 samples (Fig. 3.5b). Even though the diversity of
bacteria reduced with storage time, analysis of shared OTUs among slurry samples
indicated little change in proportion of the predominant OTUs (data not shown). Hence,
like archaea, changes in the diversity of bacteria due to the presence of RS or storage
time might not be important as the predominant OTUs, which might govern the microbial
processes in slurries, were consistently present in all samples.

3.4.4 Phylogenetic analysis of archaea and bacteria

Almost all (>99.9%) of the archaeal 16S rRNA gene and transcript reads were
phylogenetically assigned to methanogenic archaea, indicating the suitability of stored
dairy slurry for these organisms. Twenty-eight archaeal genera were identified from all
slurry samples, which were dominated by the genus *Methanocorpusculum* accounting
for ~ 56.6-88% of the reads. The genus *Methanosarcina* also represented a significant
proportion of the reads (up to ~36.7%). Other major methanogenic genera identified in
this study were *Methanobrevibacter, Methanomassiliicoccus, Methanoculleus*, and
several uncultured members, all representing up to 17.5% of the reads in each sample
(Fig. 3.7a). On the other hand, RNA samples obtained during peak flux of CH$_4$ were
dominated by the genus *Methanosarcina*, accounting for ~57% of the reads (Fig. 3.7b).
Figure 3.7: Relative proportion of (a) archaeal genera in fresh, residual (old), and stored dairy slurry samples (day 65 and day 120) and (b) active archaeal genera during peak methane (CH$_4$) flux from each tank. Each bar indicates the proportion (%) of archaeal genera in each sample, and the paired bars are from the duplicate samples collected from storage tank. Data outputs from shared OTUs and taxonomic analysis of
OTUs were used to generate the graph. FM, RS, GF, and BF represent fresh manure, residual slurry, gradual filling, and batch filling, respectively.

Regardless of RS levels, no significant variation in the relative abundances of major methanogenic genera were observed (Fig. 3.7a). The genus *Methanocorpusculum*, which was predominant in all samples, represented ~8.5% more reads in RS than fresh slurry. However, this difference did not result in noticeable variation in stored slurries of both filling types. *Methanosarcina* and *Methanobrevibacter* were relatively abundant in fresh dairy slurry representing about ~1.2% and ~10% more reads, respectively (Fig. 3.7a). The relative proportion of *Methanosarcina*, the second most abundant methanogens in stored slurries (~7-28 %), varied more with storage time than presence or absence of RS. Its mean relative abundance increased from ~14.3% (on day 65) to 36.4% (on day 120) (Fig. 3.7a). While *Methanocorpusculum* dominated 16S rRNA gene sequences of all samples, *Methanosarcina* predominated in the RNA samples obtained during peak flux of CH$_4$ in each tank (Fig. 3.7b). It represented 42.4-57.6% of the reads in day 65 samples of most tanks. In gradually-filled tank without RS, which had peak flux of CH$_4$ on day 87, *Methanosarcina* represented ~75.3% of the reads. This indicated a gradual increase of this genera with storage time. The lower relative proportion of *Methanosarcina* in one of the day 65 samples from a batch-filled tank with 20% RS might be due to the spatial heterogeneity of VFA levels in the tank (Table 3.3). Thus, in addition to *Methanocorpusculum*, both DNA and RNA data indicated the importance of methanogens related to the genus *Methanosarcina* as key players in CH$_4$ production.
A total of 27 phyla were identified from bacterial 16S rRNA amplicon sequences. Phylum Firmicutes predominated in all slurry samples, representing 57-70% of the reads (Fig. 3.8a). While 16 of the 27 phyla represented <0.5% of the reads in each sample, phylum Bacteroidetes, Synergistetes, Spirochaetes, Actinobacteria, Proteobacteria, and Chloroflexi represented between ~1-15% of the total reads in each sample (Fig. 3.8a). *Romboutsia* and *Clostridium XI*, in day 65 samples and *Sedimentibacter* and *Syntrophomonas* in day 120 samples were the predominant genera of phylum Firmicutes (Fig. 3.8b). As the relative abundances of different bacterial phyla in fresh and RS were not significantly different (Fig. 3.8a), the presence of 20% RS did not alter the relative proportions of these phyla in stored slurries of both filling types. During storage, however, the relative abundance of Bacteroidetes in all slurries gradually decreased while Synergistetes showed the reverse.
**Figure 3.8:** Relative proportion of bacterial phyla (a) and genera (b) in fresh, residual, and stored dairy slurries. Bars indicate the proportion (%) in each sample, and the paired bars are from the duplicate samples collected from each tank. Data outputs from shared OTUs and taxonomic analysis of OTUs were used to make the graph. In the
graph, FM, RS, GF, and BF denote fresh manure, residual slurry, gradual filling, and batch filling, respectively.

Despite the difference in the relative proportion of *Sedimentibacter* between fresh manure and one-year old RS (1.4% and 3.3%, respectively), no significant difference was observed with the presence of RS in both day 65 and day 120 slurry samples (Fig. 3.8b). However, its relative proportion increased with storage time, reaching ~20.3% on day 120. Similar trends were observed for *Cloacibacillus* and *Syntrophomonas* where gradual increases (up to ~11% and 6%, respectively) in relative proportions were observed in day 120 slurries. The relative proportions of the genus *Romboutsia* in fresh manure and RS were ~4% and 1%, respectively, which did not result in significant variation in stored slurries of both day 65 and day 120 samples. However, its relative proportion in day 65 (12-15.5%) was higher than in day 120 slurries (~8%) (Fig. 3.8b). Similarly, the relative proportion of *Clostridium* XI was higher in fresh manure (~2.4%) than in RS (~0.8%), and showed gradual reduction between day 65 (~9.5-11.6%) to ~4.5% in day 120 slurries (Fig. 3.8b).

### 3.5 Discussion

In dairy farming, which is a source of large amount of non-CO$_2$ GHGs (i.e. CH$_4$ and N$_2$O), liquid manure storage systems have been identified as point sources for CH$_4$ emission (VanderZaag et al., 2011). In these storage systems, the inoculum effect of RS in stimulating anaerobic degradation processes and CH$_4$ production has been evident from early studies (Zeeman, 1991; Zeeman, 1994). The authors demonstrated shorter lag phases for CH$_4$ productions from stored animal manure when about 15%
old-manure is left after emptying storage tanks. In the current study, despite the RS-related increases in the abundances of bacterial and methanogenic communities in day 65 slurries, impacts on CH$_4$ flux was observed only in gradually-filled tanks. With gradual filling, a practice that represents the actual manure storage conditions in many dairy farms, the longer lag phase (~3 weeks) and lower peak flux of CH$_4$ from 0% RS tank resulted in up to 85% reduction of cumulative emission. With the presence of 10% or 20% RS, increases in CH$_4$ flux and cumulative emission did not vary, indicating 10% RS might be sufficient to stimulate the start-up of anaerobic degradation and CH$_4$ production. As complete removal of residual manure often difficult, future studies need to examine range between 0% RS and 10% RS for levels that have minimal inoculum effect during storage. In other studies, linear increases in CH$_4$ production with the presence of up to 20-30% inoculum (digested old-manure slurry) in stored liquid dairy manure (Ngwabie et al., 2016) or food wastewater (Pathak and Srivastava, 2007) have been reported; however, the source of inoculum, storage condition, and characteristics of fresh manure may determine the activities of microbial inoculants. In this study, low CH$_4$ production in the tank without RS was coincided with low abundance of methanogens and bacteria in day 65 samples.

Gradual filling of tanks means increasing volumes of manure throughout the storage period, thus considering surface area to volume ratio in CH$_4$ flux calculation was necessary to assess if available volume of slurry resulted the observed flux levels. However, CH$_4$ flux per cubic meter of slurries had similar trends with the flux calculated for surface area (data not shown). The influence of RS-derived microbial abundance on
CH$_4$ production can also be realized from the positive correlation that occurred between daily CH$_4$ fluxes during the week of day 65 and RS level-related increases in the abundance ($r^2=0.6$; $p<0.05$) and activity ($r^2=0.53$; $p<0.05$) of methanogens.

Unlike in previous batch-based studies (Wood et al., 2014; Massé et al., 2016; Ngwabie et al., 2016), in this study, no significant difference in CH$_4$ flux and cumulative emission was observed among batch-filled tanks with and without RS. After complete removal of old-manure sludge (RS) from batch-filled tanks, ~97% reduction of fugitive CH$_4$ emissions (Massé et al., 2016) and ~56% reduction of cumulative CH$_4$ emissions (Wood et al., 2014) have been reported. In the current study, what obscured the expected differences in CH$_4$ flux among batch-filled tanks (with/without RS) is not yet clear. Unlike the small difference in CH$_4$ flux from these tanks, there was a positive correlation between RS levels and bacterial or methanogen abundance. However, abundance of these organisms and CH$_4$ flux was poorly correlated during the week of day 65. Thus, with high abundance and activity of bacteria and methanogens at high RS levels, some unknown factors rather than RS might have played a role in reducing CH$_4$ emission from these tanks. Undisturbed crusts that could physically block gaseous emissions and potential methanotrophy (Petersen et al., 2005; Hansen et al., 2009; Nielsen et al., 2013) are among the factors that might impact CH$_4$ emission from these tanks. In different storage systems, characteristics of crust (e.g. thickness) and methanotrophy may vary with the nature of manure (e.g. total solid level) (Wood et al., 2012).
Regardless of filling type, the influences of RS on the abundance and activities of bacterial and methanogenic communities were not important after day 120, thus only playing a significant role during the initial phases of storage when population sizes rather than available substrates may be limiting. Using different sources of inoculum material (wetland sediment, landfill leachate, and mesophilic digestate), a recent study (Witarsa et al., 2016) indicated poor correlations between methanogen population size and biochemical methane potential. However, in different storage systems, these correlations may vary with factors that may affect flux such as presence of crust and hence potential methanotrophy (Husted, 1994; Misselbrook et al., 2005; Petersen et al., 2005; Hansen et al., 2009; Aguerre et al., 2012; Nielsen et al., 2013) and activity of methanogens (e.g. substrate level, pH, and moisture level) (Kim et al., 2015). Thus, in dairy farms where liquid manure is gradually loaded in open storage tanks or lagoons, reduction or complete removal of RS can lower the number of well-adapted bacteria and methanogens that may serve as inoculants. It is important to note, however, that RS removal should occur as close as possible to the time when high emissions are expected; therefore late-spring RS removal is recommended and fall RS removal is unlikely to be effective (Balde et al., 2016).

In all slurry samples, lower diversity of archaea compared to bacteria was observed, a finding inline with previous reports from manure-based mesophilic anaerobic digesters (Zakrzewski et al., 2012; St-Pierre and Wright, 2014; Town et al., 2014; Sun et al., 2015). Differences in the diversity of archaea among all samples were small. As archaeal communities in rumen and manure environments are generally
dominated by methanogens (Janssen and Kirs, 2008) and many of them can establish in both environments (Liu and Whitman, 2008), storage of dairy slurry might not result in significant shifts in the diversity of archaea or methanogens (Bouity-Voubou et al., 2008). Indeed, an earlier study (Leite et al., 2016) identified similar methanogenic archaeal communities from fresh dairy manure and a mixed inoculum obtained from different biogas reactors, although higher abundance in the later, which is consistent with the current study. The relatively higher diversity of bacteria in RS compared to fresh manure was not expected, but the source of RS might have influenced the diversity. Nevertheless, the stability of core communities throughout the storage, as observed from the stability of dominant bacterial OTUs in all samples, could be an indication that diversity differences might not be important. A study (Bouity-Voubou et al., 2008) also showed little difference in the diversity of bacteria between several-month-adapted inoculum and swine slurry treated with anaerobic digesters and supplemented with the inoculum. Hence, RS might be more important in contributing to the starting number of methanogen and bacterial populations than adding new phylotypes to dairy slurry storage systems.

The dominant bacterial phyla identified in this study (i.e. Firmicutes, Bacteriodetes, and Synergistetes) were typical of anaerobic digester and rumen environments (St-Pierre and Wright, 2014; Abendroth et al., 2015; Jewell et al., 2015; Sun et al., 2015; Snelling and Wallace, 2017; Zhou et al., 2017). The dominance of Firmicutes throughout the storage might suggest members of this phylum as key players in the anaerobic degradation of organic substrates in stored dairy manure. Dairy
manure contains large amount of lignocellulose biomass (e.g. celluloses, hemicellulose, lignin, and pectin) and some amounts of protein, which are substrates for hydrolytic microorganisms. Thus, these substrates might have supported the hydrolytic members of Firmicutes such as *Clostridium* XI and *Romboutsia* (Kuribayashi et al., 2017) that were dominant in day 65 slurries. While the reduction in the relative proportion of these bacteria in day 120 samples might indicate gradual limitation of available substrates, their by-products (i.e. organic acids) might have supported acidogenic bacteria such as *Sedimentibacter* (Imachi et al., 2016) that were predominant in day 65 and day 120 samples.

The predominance of hydrolytic and acidogenic bacteria especially in day 65 and day 120 slurries might suggest accumulation of methanogenic substrates (CO\(_2\)/H\(_2\) and acetate), coinciding with the exclusive dominance (>99.9%) of methanogen-related archaeal OTUs. Although methanogens that are closely related to the genus *Methanocorpusculum* have rarely been reported from rumen environments (Daquiado et al., 2014; Oren, 2014; Singh et al., 2015), we identified these methanogens as predominant in all samples. The predominance particularly in fresh dairy slurry that was expected to be the main source of these organisms might indicate these methanogens as members of rumen archaea. Moreover, the predominance of these methanogens in RS and stored dairy slurries with 10% and 20% RS might suggest stored liquid dairy manure as suitable habitats for these methanogens. Members of *Methanocorpusculum* are hydrogenotrophic, and have been isolated from anaerobic systems where fermentative by-products such as H\(_2\)/CO\(_2\) or formate may be accumulated (Zhao et al.,
1989; Liu and Whitman, 2008; Oren, 2014). Although these substrates are also used by the genus *Methanobrevibacter*, its relative proportion showed gradual reduction with storage. This might be due to its poor adaptability to storage conditions; this methanogen was commonly reported as dominant in rumen environments (Liu et al., 2012; Daquiado et al., 2014; Henderson et al., 2015).

Despite the low abundance of *Methanosarcina* in fresh and RS, its relative abundance gradually increased with storage time. Moreover, during peak flux of CH$_4$ from each tank, the most active methanogens were members of this genus. This might be explained by its versatile metabolic capability as it uses a range of substrates including CO$_2$/H$_2$, acetate, methylated compounds, and methanol (Liu and Whitman, 2008; Oren, 2014). Moreover, this methanogen has relatively higher tolerance to different environmental stresses (e.g. high ammonium concentration, high VFA contents, and wider pH range) (Demirel and Scherer, 2008; De Vrieze et al., 2012). Particularly, its gradual increase with storage time was coincided with the gradual increase in the proportion of Synergistetes that was dominated by the genus *Cloacibacillus*. This bacterium uses different amino acids (e.g. arginine, lysine, histidine, and serine) and releases direct (acetate, H$_2$, and CO$_2$) and indirect (propionate, butyrate, and valerate) methanogenic substrates (Ganesan et al., 2008; Militon et al., 2015). Moreover, accumulation of these methanogenic substrates could be contributed by members of Firmicutes such as *Sedimentibacter*, an amino-acid-utilizing bacterium (Imachi et al., 2016) that increased with storage time. Moreover, higher organic acids such as propionic acid could be converted into acetate, formate, H$_2$ and CO$_2$, by
acetogenic bacterial communities such as *Syntrophomonas* (Xiao et al., 2015) that were relatively abundant in day 120 samples. While acetate was detected in both day 65 and day 120 samples, OTUs related to the genus *Methanosaeta* (methanogens that can grow solely on acetate) (Liu and Whitman, 2008; Oren, 2014) were detected in very low proportion (<0.5%). *Methanosaeta* has high affinity to acetate (Demirel and Scherer, 2008) but has often been described to be less competitive at high acetate concentrations (Witarsa et al., 2016), although a recent study found strong competitiveness of these methanogens in animal wastewater with elevated acetate level (Chen et al., 2017).

### 3.6 Conclusion

On many farms, complete removal of dairy slurry from storage tanks is difficult. Residual slurry may contain large numbers of well-adapted bacterial and methanogenic communities. As a result, the inoculum effect is significant. This study demonstrated the effects of different levels of RS on CH₄ emissions and abundance and diversity of methanogenic and bacterial communities. Regardless of filling type (gradual or batch), increased RS levels resulted in higher abundance of methanogens and bacteria in day 65 slurry samples. However, the increased abundances of these populations were significantly correlated with CH₄ emissions only in gradually-filled tanks that represent the typical slurry storage practices in many dairy farms. Regardless of RS and slurry filling method, little variations in diversity of bacteria and methanogens were observed among slurry samples. In all samples, Firmicutes and *Methanocorpusculum* were the predominant bacteria and methanogen, respectively. In addition to its gradual increase
with storage, *Methanosarcina* represented the most active methanogen during peak flux of CH$_4$ from each tank, indicating its significant role to the overall CH$_4$ emitted. Overall, this study revealed the importance of RS in providing newly stored manures with large number of active and well-adapted bacteria and methanogens that may accelerate start of anaerobic degradation and methanogenesis, and complete removal of RS through alternative pumping systems may provide a low-cost option in reducing CH$_4$ emissions from dairy slurry storage systems.

**Funding**

This work was supported by Ontario Ministry of Agriculture, Food and Rural Affairs.
Sodium persulfate and potassium permanganate inhibit methanogens and methanogenesis in stored liquid dairy manure

Jemaneh Habtewold\textsuperscript{a}, Robert Gordon\textsuperscript{b}, Paul Voroney\textsuperscript{a}, Vera Sokolov\textsuperscript{b}, Andrew VanderZaag\textsuperscript{c}, Claudia Wagner-Riddle\textsuperscript{a} and Kari Dunfield\textsuperscript{a}

\textsuperscript{a}School of Environmental Sciences, University of Guelph, Guelph, N1G 2W1, Canada
\textsuperscript{b}Wilfrid Laurier University, Waterloo, N3T 2W2, Canada
\textsuperscript{c}Agriculture and Agri-Food Canada, Ottawa, K1A 0C5, Canada

This manuscript has been accepted by the Journal of Environmental Quality.

Author Contributions:

J Habtewold was the primary researcher and was responsible for the experimental design, manure sampling, lab work, data analysis, and manuscript preparation. P Voroney and V Sokolov were credited for their valuable advices on the experiment. C. Wagner-Riddle and A VanderZaag were credited for the valuable advices on the experimental design, statistical analyses and manuscript review. R. Gordon and K. E. Dunfield were credited for their invaluable mentorship on lab-work and trouble-shooting on data analysis, manuscript preparation, and data interpretation.
4.1 Abstract

Stored liquid dairy manure is a point sources for methane (CH\(_4\)) emission, thus effective mitigation strategies are required. We assessed sodium persulfate (Na\(_2\)S\(_2\)O\(_8\)), potassium permanganate (KMnO\(_4\)), and sodium hypochlorite (NaOCl) for impacts on the abundance of microbial communities and CH\(_4\) production in liquid dairy manure. Liquid dairy manure treated with different rates (1, 3, 6, and 9 g or ml L\(^{-1}\) slurry) of these chemicals or their combinations were incubated under anoxic conditions at 22.5±1.3°C for 120 days. Untreated and sodium 2-bromoethanesulfonate (BES)-treated manures were included as negative and positive controls, respectively, whereas sulfuric acid (H\(_2\)SO\(_4\))-treated manure was used as a reference. Quantitative real-time PCR was used to quantify the abundances of bacteria and methanogens on days 0, 60, and 120. Headspace CH\(_4\)/CO\(_2\) ratios were used as a proxy to determine CH\(_4\) production. Unlike bacterial abundance, methanogen abundance and CH\(_4\)/CO\(_2\) ratios varied with treatments. Addition of 1-9 g L\(^{-1}\) slurry of Na\(_2\)S\(_2\)O\(_8\) and KMnO\(_4\) reduced methanogen abundance (up to ~28%) and peak CH\(_4\)/CO\(_2\) ratios (up to 92-fold). Except at the lowest rate, chemical combinations also reduced the abundance of methanogens (up to ~17%) and CH\(_4\)/CO\(_2\) ratios (up to 9-fold). It is likely that 3%NaOCl contributed less to these reductions, however, as no significant impacts were observed on methanogen abundance and CH\(_4\)/CO\(_2\) ratios in 3%NaOCl-treated slurries. With slurry acidification, the ratios reduced up to two-fold whereas methanogen abundance unaffected. Results suggest that Na\(_2\)S\(_2\)O\(_8\) and KMnO\(_4\) may offer alternative options to reduce CH\(_4\) emission.
from stored liquid dairy manure, but this warrants further assessments at larger scales for environmental impacts and characteristics of the treated manure.

**Keyword:** chemical oxidants; dairy manure; greenhouse gas; methanogens

### 4.2 Introduction

Large volumes of liquid manure are stored annually from intensive dairy farming operations due to mandatory storage capacity required for nutrient management planning (e.g. 240 days in Ontario, Canada) prior to land application (Hilborn, 2010; Sheppard et al., 2011). Liquid manure storage is increasing for handling purposes (VanderZaag et al., 2013), but creates a conducive environment to methane (CH$_4$) production (Jayasundara et al., 2016; Leytem et al., 2017). Potential mitigation strategies related to management practices (e.g. solid-liquid separation, covers, biofilters, removal of residual inoculum, slurry aeration, and feed management) or chemical-based treatments (e.g. acidification, nanoparticles, coumarins, narasin, and gypsum) have been proposed (Berg and Model, 2008; Petersen et al., 2012; Yang et al., 2012; Andersen and Regan, 2014; Theint et al., 2016; Gautam et al., 2017; Popp et al., 2017). However, there remains a need to evaluate other mitigation strategies.

Compared to management related CH$_4$ emission mitigation strategies, chemical-based treatments can be more efficient as the chemicals directly impact methanogens or alter the manure environment in a way that microbial activity could be impacted (Ottosen et al., 2009; Petersen et al., 2014; Wang et al., 2014). For instance, H$_2$SO$_4$-based acidification of dairy slurry can inhibit the activities of most methanogens,
although certain acidophilic methanogens have been shown to be favored (e.g. *Thermoplasmata*) (Petersen et al., 2014). These methanogens may drive the system towards methylotrophic methanogenesis (Poulsen et al., 2013) but have less contribution to the overall methanogenic process in manure storage tanks (Barret et al., 2013). The toxicity of H$_2$SO$_4$ to humans and the environment can also make handling difficult. Application of zinc oxide nanoparticles to swine manure (3 g L$^{-1}$) and silver nanoparticles to municipal solid waste (10 mg kg$^{-1}$) have also been demonstrated to inhibit methanogens and CH$_4$ production (Yang et al., 2012; Gautam et al., 2017). However, the toxicity of H$_2$SO$_4$ and application of nanoparticles-treated manure in agricultural soils may raise concerns for farm personnel and disrupt natural soil processes (Dinesh et al., 2012; Ben-Moshe et al., 2013; Shen et al., 2015). Chemical oxidants with less harm such as sodium persulfate (Na$_2$S$_2$O$_8$), potassium permanganate (KMnO$_4$), and sodium hypochlorite (NaOCl), which are commonly used in the removal of organic matter from hydrocarbon-contaminated soils and wastewaters (Fayad et al., 2013; Wu et al., 2014; Jakubauskaite et al., 2016), may oxidize slurry organic matter including microorganisms, and hence can inhibit methanogenesis. These oxidants may also be used to treat residual slurry which contain large number of active bacterial and methanogenic communities (Habtewold et al., 2018). Thus, inhibitory effects of chemical oxidants (Na$_2$S$_2$O$_8$, KMnO$_4$, and NaOCl) on CH$_4$ production and growth of methanogens in stored liquid dairy manure need to assessed.

Sodium persulfate, KMnO$_4$, and NaOCl are readily available chemical oxidants that have often been used in soil remediation, water treatment processes, industrial
processes, and as antimicrobial agents (Vianna et al., 2004; Karpenko et al., 2009; Tsitonaki et al., 2010). The ability of these chemicals to oxidize organic matter is the main reason for these applications. For instance, Na$_2$S$_2$O$_8$, which dissolves and releases strong oxidant anions in wet environments, can treat soils contaminated with different petroleum hydrocarbons (Yen et al., 2011; Wu et al., 2016) and disinfect water (Eder, 1985). Potassium permanganate has also been used in drinking water treatment (Guan et al., 2010) and in the paper industry, where plant polymers are major constituents (Fahmy et al., 2008). Moreover, with application of KMnO$_4$ in sewage treatment, Wu et al. (2014) demonstrated the disintegration of sludge floc and subsequent increase of polymeric substances in supernatants. As microorganisms are composed of organic matter and KMnO$_4$ can oxidize them, accumulated polymeric substances might also be contributed from organisms in the sludge. Similarly, NaOCl can also oxidize and remove organic carbon from soil (Zimmermann et al., 2007). Thus, in stored liquid dairy manure where abundant organic matter is available, chemical oxidants may negatively influence available substrates and microbial communities, thereby reducing CH$_4$ production.

The objective of this study was to assess the potential to reduce the abundance of slurry microbes (bacteria and methanogens) and reduce CH$_4$ production in stored liquid dairy manure by using Na$_2$S$_2$O$_8$, KMnO$_4$, NaOCl, individually or in combination.
4.3 Materials and methods

4.3.1 Experimental design and manure sampling

Prior to this study, a preliminary incubation experiment was conducted using KMnO₄ (≥99%, Sigma-Aldrich) and household bleach (3%NaOCl) to determine the range of concentrations where CH₄ production may be impacted. Results indicated that KMnO₄ can reduce CH₄ production from 1 g KMnO₄ L⁻¹ slurry whereas little impacts were observed for 3%NaOCl (data not published). By including Na₂S₂O₈ (98%, Fisher Scientific), the current incubation experiment was set using different rates (1, 3, 6, or 9 g or ml L⁻¹ slurry) of KMnO₄, Na₂S₂O₈, 3%NaOCl, or their combinations (1:1:1, g, g, ml) to assess potential impacts on the abundance of methanogens and bacteria in stored liquid dairy manure. In addition to untreated manure (negative control), manure treated with 0.05 mol L⁻¹ sodium 2-bromoethanesulfonate (BES) (98%, Sigma-Aldrich), which is known to stop both hydrogenotrophic and aceticlastic methanogenesis (Zinder et al., 1984), was used as a positive control. As a reference point, an acidification treatment was also included. Acidification (pH 6.5, 6.0, and 5.5) was done using H₂SO₄ (95.08%, Sigma-Aldrich), which has been demonstrated in several studies to reduce CH₄ production from stored liquid manure (Ottosen et al., 2009; Petersen et al., 2014).

Fresh manure was obtained from an in-barn short-term (<1 day) storage pit of the Elora Livestock Research and Innovation Centre Dairy Facility, Ontario, Canada. The pit receives diluted manure (due to washwater and urine, 5.5% dry matter content) from a combination of lactating and dry cows, heifers and calves. After thorough homogenization in a clean bucket, 60 ml slurry was dispensed to each 1-L Mason jars
with different rates of the chemical agents. Each treatment was incubated in duplicate and there were 42 jars in total. Slurries in jars were then stirred to mix with the chemical agents. No inoculum was added. The headspace of each jar was flushed (~3 mins) with pure N\textsubscript{2} gas (99%, Praxair Canada Inc.) and immediately sealed with aluminium lids fitted with butyl rubber stoppers and stored at room temperature (22.5 ±1.3 °C) to simulate peak manure temperature during the Canadian summer (Baldé et al., 2016). Temperature of the incubation room was continuously monitored using a thermometer. Dry matter contents of fresh dairy slurry samples and loss-on-ignition based determination of volatile solid contents of slurry samples at the start and end of storage were conducted at the University of Guelph laboratory services, agriculture and food laboratory. Manure pH was measured in the laboratory at the start and end of incubation using a pH meter (Fischer Scientific).

4.3.2 Estimation of methane production

Gas samples were drawn every two weeks from each jar’s headspace using a 5-ml syringe and immediately injected into a gas chromatograph (8610C, SRI Instruments, California, USA) that was fitted with flame ionization detector and calibrated. Serially diluted CH\textsubscript{4} (99.9%, Praxair Canada Inc.) and CO\textsubscript{2} (50%, Praxair Canada Inc.) gases, each bracketing sample concentrations, were used to make the respective linear standard curves. Dilutions were performed using pure N\textsubscript{2}. For each gas sample or standard gas, signal peak area was obtained using Peaksimple software (v. 4.49). Duplicate gas measurements were made from each jar and concentrations in
parts per million (ppm) of CH$_4$ and CO$_2$ were calculated using the linear regression equation of the standard curves.

After each headspace sampling, the jar was opened to release accumulated gas and re-flushed with N$_2$. Therefore, headspace pressures for all jars were low at the time of sampling based on the apparent pressure observed during sampling with the syringe. Thus, to convert ppm into mass (g) of CH$_4$ or CO$_2$ using the Ideal Gas Equation, we assumed headspace pressure equaled atmospheric pressure at the time of headspace sampling, although pressure was not measured. We recognize this is not the recommended procedure for assessing cumulative biogas production, however, it simplified the process of obtaining samples for microbial analysis. We consider the approach adequate to assess the inhibitory effects of the oxidizing agents, given the focus of the study being on microbial dynamics, and effects relative to the negative and positive control.

4.3.3 DNA extractions and quantitative real-time PCR

Two grams of slurry samples on day 0 (untreated fresh manure before incubation), day 60 (when peak CH$_4$/CO$_2$ ratios detected in most jars), and day 120 (end of incubation) were used to assess changes in the abundance of bacteria and methanogens. Microbial analyses were conducted for treatments that received the lowest and highest rates of chemicals. DNA extractions were made using PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc.) following the manufacturer’s protocol. Prior to qPCR, DNA samples were diluted and assessed for potential inhibitory effects as described previously (Habtewold et al., 2017).
Clear Multiplate™ 96-Well PCR Plates (BioRad Laboratories, Inc.) were used to prepare triplicate reaction mixes from each sample, and qPCR was performed using CFX96™ Real-Time System on C1000 Touch™ Thermal Cycler (BioRad Laboratories, Inc.). To quantify methanogens and bacteria, the α sub-unit of methyl coenzyme A reductase (mcrA) and bacterial 16S rRNA genes were targeted using mlas-mod F/mcrA-rev-mod R and Bac16S 338F/518R primers, respectively (Steinberg and Regan, 2009). The 20-µL qPCR reaction mix contained 10 µL Ssofast EvaGreen Supermix (BioRad Laboratories, Inc.), 1 µL (10 pM) of each primer, 2 µL template DNA (1 to 10 ng µL⁻¹), and 6 µL of PCR-grade water. Thermal cycling for mcrA gene quantification involved initial denaturation at 98°C for 2 min followed by 40 cycles of dissociation (95°C, 5 s), annealing (57°C, 10 s) and extension (72°C, 15 s), and a final step at 72°C for 30 s before increasing the temperature by 0.5°C from 65°C to 95°C for 5 s to analyze melting curves. For bacterial 16S rRNA gene quantification, the optimized cycling conditions used were initial denaturation at 98°C for 2 min followed by 34 cycles of dissociation (98°C, 5 s), annealing (55°C, 5 s) and extension (65°C, 5 s), and a final extension 72°C for 3 min before increasing the temperature by 0.5°C from 65°C to 95°C for 5 s. Known copies of plasmid standard curves for mcrA (10⁸ to 10⁴) and bacterial 16S rRNA (10⁹ to 10¹) genes quantifications were prepared from Methanosarcina mazei (ATCC 43340) and a pure culture of Clostridium thermocellum, respectively. Efficiency, r², and slope of the plasmid standard curves of both genes were 100%, 0.99, and -3.32, respectively. CFX Manager software version 3.1 (Bio-Rad Laboratories, Inc.) was used to analyze the qPCR data.
4.3.4 Statistical analysis

At each sampling time, gas measurements were performed two times whereas microbial gene abundance determinations were performed in triplicate. Thus, means of these technical replicates were used to calculate standard errors (indicated in each graph) of the biological replicates. To assess if CH$_4$ production was affected by the addition of different rates of chemicals, the significance of changes in CH$_4$/CO$_2$ ratios as rates (1-9 g ml$^{-1}$ slurry) of chemicals increased were analyzed using ANOVA and Tukey’s post hoc analysis. For methanogen abundance, one-way ANOVA with pairwise comparisons of each treatment rates against the untreated slurries were performed. Differences were considered significant at $p<0.05$, and statistical analyses were performed using GraphPad prism v.7 (GraphPad Software, Inc.).

4.4 Results

4.4.1 Methane production and slurry characteristics

Apart from the gradual increases observed over time, the pH of slurries did not show significant variations among treatments (Table 4.1). Methane/CO$_2$ ratios at each sampling time were used as a proxy for CH$_4$ production (Yvon-Durocher et al., 2014; Poulsen et al., 2017). In jars with untreated dairy slurry, CH$_4$/CO$_2$ ratios started to peak from day 45 and reached maximum (1.9±0.1) on day 60 (Fig. 4.1). After day 60, mean ratios gradually reduced, reaching 1.2±0.1 on day 120. This loss of carbon to gaseous products was consistent with the reduction in VS contents of slurries by ~11% (t test, $p=0.0106$) (Table 4.1). As a point of reference, after 120 days of storage, the untreated slurry produced 127±3 ml CH$_4$ g$^{-1}$VS — slightly more than half of the IPCC B$_0$ value —
which is reasonable considering the incubation was done without inoculum and at 22.5°C (Zeeman and Gerbens, 2002). From these jars, ~119±10 ml CO₂ g⁻¹VS were also detected. Methane concentrations in the headspace of BES-treated slurries were negligible, although ~71±3 ml CO₂ g⁻¹VS were detected. This was indeed observed from the negligible peak area detected in the GC measurements of the gas samples.

**Table 4.1:** Volatile solid (VS) contents and pH of dairy manure before and after (day 120) incubations with different chemical agents. Standard deviations shown were calculated from the duplicate treatments (n=2).

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Treatment</th>
<th>VS (%) (mean ±SD)</th>
<th>Slurry pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td>Fresh manure</td>
<td>86±1.6</td>
<td>7.40±0.00</td>
</tr>
<tr>
<td></td>
<td>Untreated manure</td>
<td>75±0.1</td>
<td>8.20±0.06</td>
</tr>
<tr>
<td></td>
<td>Na₂S₂O₈ (1 g L⁻¹ slurry)</td>
<td>73±1.0</td>
<td>8.42±0.11</td>
</tr>
<tr>
<td></td>
<td>Na₂S₂O₈ (9 g L⁻¹ slurry)</td>
<td>68±1.3⁺</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KMnO₄ (1 g L⁻¹ slurry)</td>
<td>68±3.7⁺</td>
<td>8.03±0.18</td>
</tr>
<tr>
<td></td>
<td>KMnO₄ (9 g L⁻¹ slurry)</td>
<td>65±1.0⁺</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3%NaOCl (1 mL L⁻¹ slurry)</td>
<td>75±1.5</td>
<td>8.04±0.086</td>
</tr>
<tr>
<td></td>
<td>3%NaOCl (9 mL L⁻¹ slurry)</td>
<td>76±0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Combination (1 g L⁻¹ slurry)</td>
<td>74±0.7</td>
<td>8.32±0.15</td>
</tr>
<tr>
<td></td>
<td>Combination (9 g L⁻¹ slurry)</td>
<td>69±0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (pH 6.5)</td>
<td>76±0.3</td>
<td>8.3±0.21</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (pH 5.5)</td>
<td>78±1.6</td>
<td>8.38±0.08</td>
</tr>
</tbody>
</table>

⁺significantly different (Tukey’s test, p<0.05) from untreated slurries

Compared to the untreated slurries that had peak CH₄/CO₂ ratios on day 60, the ratios in Na₂S₂O₈-treated slurries were relatively low (Fig. 4.1). The highest rate tested (9 g L⁻¹ slurry) had similar effects as BES (CH₄/CO₂ ratios, 0.02±0.02), the remaining jars with 1, 3, and 6 g L⁻¹ slurry also showed significant reductions (Tukey’s p<0.0001)
in CH₄/CO₂ ratios (0.73±0.04, 0.68±0.01, and 0.15±0.03, respectively) (Fig. 4.1). Unlike the untreated controls, in jars with 1 and 3 g L⁻¹ slurry, CH₄/CO₂ ratios peaked on day 75 (1.1±0.6 and 1.0±0.1, respectively) while jars with 6 g L⁻¹ slurry the ratios increased with storage time. Thus, with the addition of Na₂S₂O₈ in stored liquid dairy manure, peak CH₄/CO₂ ratios could be reduced and delayed, an indication that CH₄ production can be reduced (Appendix Fig. A.4).

**Figure 4.1:** Effects of different rates of Na₂S₂O₈ on CH₄ production in stored liquid dairy manure. Bromoethanesulphonate (0.05 mol L⁻¹ slurry)-treated manure was included as negative control. Error bars indicate standard errors of the means of duplicate treatments.

Except for jars with 1 g L⁻¹ slurry, significant reductions in CH₄ production occurred with the presence of KMnO₄ (Fig. 4.2, Appendix Fig. A.5). At the lowest rate,
maximum CH₄/CO₂ ratios detected were on day 60 (1.8±0.1), which were comparable with the ratios in untreated slurries (1.9±0.1). Thus, the resulting reductions in CH₄ productions might not be significant. Although peak CH₄/CO₂ ratios for jars with 3 and 6 g L⁻¹ slurry (1.9±0.5 and 1.8±0.5, respectively) were also comparable with the untreated controls, they were detected 45 days after peaks observed in untreated slurries (Fig. 4.2). At the highest rate tested, CH₄/CO₂ ratios were consistently low (<0.2) throughout the storage period.

---

**Figure 4.2:** Effects of KMnO₄ on CH₄ production in stored liquid dairy manure. Bromoethanesulphonate (0.05 mol L⁻¹ slurry)-treated manures were included as
negative control. Error bars indicate standard errors of the means of duplicate treatments.

Figure 4.3: Effects of 3%NaOCl on CH$_4$ production (as indicated by CH$_4$/CO$_2$ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L$^{-1}$ slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments.

Unlike slurries that were treated with Na$_2$S$_2$O$_8$ or KMnO$_4$, no reductions in CH$_4$ production might have occurred with the presence of up to 9 mL L$^{-1}$ slurry of 3%NaOCl (Fig. 4.3 and Appendix Fig. A.6). Except at the highest rate, NaOCl-treated slurries had
peak CH₄/CO₂ ratios (2.4±0.3) on day 60, which were higher than the ratios observed from untreated slurries. Despite the 2-weeks delay for peak CH₄/CO₂ ratios, the ratios for jars with 9 mL L⁻¹ slurry were higher (2.2±0.2) than the untreated controls. Thus, by adding up to 9 ml L⁻¹ of 3%NaOCl in stored dairy slurries, reduction in CH₄ production might not be possible (Appendix Fig. A.6). However, when combined with Na₂S₂O₈ and KMnO₄ (at the ratio of 1g:1g:1ml), the higher application rates significantly reduced CH₄/CO₂ ratios (Fig. 4.4, Appendix Fig. A.7).

Figure 4.4: Effects of chemical combinations (Na₂S₂O₈, KMnO₄, and 3%NaOCl at a ratio of 1g:1g:1ml, defined as unit here) on CH₄ production (as indicated by CH₄/CO₂ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L⁻¹
slurry)-treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments.

Compared with the untreated controls, CH$_4$/CO$_2$ ratios in acidified slurries were also relatively low (1.2-1.5), and peak ratios were observed after day 75 or later (Fig. 4.5). For instance, on day 75, the ratios for pH 6.5, 6.0, and 5.5 were 1.53±0.04, 0.92±0.75, and 1.16±0.26, respectively. However, when compared with the chemical oxidants, slurry acidification had lower impacts on CH$_4$/CO$_2$ ratios, but the delays for peak ratios could still result in significant reduction of CH$_4$ production.

Figure 4.5: Effects of H$_2$SO$_4$ on CH$_4$ production (as indicated by CH$_4$/CO$_2$ ratios) in stored liquid dairy manure. Sodium 2-bromoethanesulphonate (0.05 mol L$^{-1}$ slurry)-
treated manures were included as negative control. Error bars indicate standard errors of the means of duplicate treatments.

4.4.2 Effects of chemical oxidants on the abundances of bacteria and methanogens

As estimated from the copy numbers of 16S rRNA genes g⁻¹ dry manure, after 60 and 120 days of storage, mean abundance of bacteria in untreated slurries increased slightly (~2%) from abundances in fresh manure (~2×10¹¹). However, mean abundance of methanogens in untreated slurries increased by ~23% (calculated from Log₁₀ transformed copies g⁻¹ slurry of mcrA genes) when compared with fresh manure (~6.8×10⁷). In treated slurries, however, methanogens were more sensitive to the chemicals added when compared to bacteria (Fig. 4.6a-d). After 60 and 120 days of storage, copy numbers (Log₁₀ transformed) of bacterial 16S rRNA genes g⁻¹ dry manure showed little variation with chemical treatments (Fig. 4.6a). The only exceptions where total bacterial abundance had significant reductions (Tukey’s p<0.05) were in KMnO₄ (at the highest rate) and BES-treated slurries on day 120 (Fig. 4.6a). Unlike bacterial 16S rRNA gene copies, copy numbers of mcrA genes (Log₁₀ transformed) g⁻¹ dry manure varied with treatments and storage period (Fig. 4.6c-d). Compared to untreated slurries, slurries treated with KMnO₄ at the highest rate and BES had the lowest copies (7.1±0.2 and 6.8±0.1, respectively, calculated from Log₁₀ transformed copies) of mcrA genes g⁻¹ dry manure. On day 60 and day 120, Na₂S₂O₈ at 1 g L⁻¹ slurry reduced mcrA gene copies (Log₁₀) by ~5% and ~8%, respectively, whereas at 9 g L⁻¹ slurry, copies on day 60 and day 120 were both ~13% lower when compared with untreated slurries (Fig. 4.6c-d). Unlike Na₂S₂O₈, the population number of methanogens were significantly
impacted (Tukey’s p<0.05) by KMnO₄. For instance, when compared with copies from untreated slurries, mcrA gene copies (Log₁₀) reduced by up to ~28% at 9 g L⁻¹ slurry of KMnO₄, consistent with its strong impacts on volatile solid contents of manure (Table 4.1). By contrast, 3%NaOCl had little impact on the abundance of methanogens (Fig. 4.6c-d), coinciding its impacts on CH₄/CO₂ ratios and volatile solid contents (Table 4.1, Fig. 4.3). When combined with KMnO₄ and Na₂S₂O₈ (in 1ml:1g:1g ratio), however, mcrA gene copies (Log₁₀) reduced up to ~14% (Fig. 4.6c-d). On the other hand, mcrA and bacterial 16S rRNA gene copies did not vary significantly with slurry acidifications (pH 4.5) (Fig. 4.6a-d).
Figure 4.6: Effects of chemical oxidants on the abundance of bacteria and methanogens in stored liquid dairy manure. Changes (%) in the abundance of bacteria (a-after 60 days; b-after 120 days) and methanogens (c-after 60 days; d-after 120 days) in stored dairy slurries treated with chemical oxidants. Error bars indicate standard error of the means of biological replicates (n=2). A unit is defined here as 1g:1g:1mL of the chemical combinations.
With less impacts of chemical treatments on the total abundance of bacteria, we were also interested to see the changes in the abundance of methanogens with reference to bacterial abundance. The ratio of \textit{mcrA} gene to bacterial 16S rRNA gene copies (\textit{mcrA/bac 16S rRNA genes}) in fresh manure was 0.69 (Fig. 4.7). During incubation, \textit{mcrA/bac 16S rRNA gene ratios} increased to ~0.85. However, treatments that significantly impacted methanogen abundance (BES, \textit{Na}_2\textit{S}_2\textit{O}_8, \textit{KMnO}_4, and combinations) showed low \textit{mcrA/bac 16S rRNA gene ratios}. The higher \textit{mcrA/bac 16S rRNA gene ratios} with 1 g L\textsuperscript{-1} slurry (\textit{Na}_2\textit{S}_2\textit{O}_8 and combinations), 3%\textit{NaOCl}, and pH, also coincided with \textit{CH}_4/\textit{CO}_2 ratios and abundance of methanogens.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_4_7.png}
\caption{Changes in the relative abundances of methanogens with reference to bacteria as estimated from the changes in the ratios of \textit{mcrA/bacterial 16S rRNA gene}}
\end{figure}
copies. The abundances of bacteria and methanogens, which were used for this calculation, are shown in the appendix Fig. A.2 and A.3. A unit is defined here as 1g:1g:1mL of the chemical combinations.

4.5 Discussion

A large amount of CH₄ is usually produced from stored liquid dairy manure, which is rich in volatile solid that can be converted into methanogenic substrates through the action of different physiological groups of bacteria (Barret et al., 2013). Chemical oxidants such as Na₂S₂O₈ and KMnO₄, and NaOCl may disinfect the slurry environment and oxidize organic substrates (Hamilton, 1974; Mikutta et al., 2005; Siregar et al., 2005), which may release by-products such as MnO₂, CO₂, and/or complex organic intermediates. Thus, with disinfectant and organic substrate removal capabilities of these chemical oxidants (Hoag et al., 2000; Siregar et al., 2005; Chen et al., 2015), microorganisms that produce CH₄ in stored liquid dairy manure can be inhibited.

The current lab-scale study demonstrated that 1 g L⁻¹ slurry (and perhaps lower) rates of Na₂S₂O₈ could significantly reduce CH₄ production when compared to untreated slurries. The highest rate (9 g L⁻¹ slurry) of Na₂S₂O₈ tested had almost similar effects with 0.05 mole L⁻¹ slurry of BES, a known inhibitor that can stop both hydrogenotrophic and aceticlastic methanogenesis (Zinder et al., 1984). Despite its impressive effect in mitigating CH₄ production, BES is highly expensive (e.g. $0.92 for 1g L⁻¹ slurry) when compared with the chemical oxidants (e.g. <$0.06 for 1g L⁻¹ of Na₂S₂O₈ or KMnO₄) (ebiochem, China). Although the effects of Na₂S₂O₈ on the abundance of bacteria were not significant, reductions in the abundance of methanogens coincided with reductions
in CH₄/CO₂ ratios which can be a proxy for CH₄ production (Yvon-Durocher et al., 2014; Poulsen et al., 2017). As a strong disinfectant (EPA, 2015), bacterial and methanogenic communities in slurries were expected to be impacted as rates of Na₂S₂O₈ increased. However, as has been demonstrated in a soil environment, concentrations ≤10 g L⁻¹ might not impact all bacterial communities (Tsitonaki et al., 2008). Indeed, in most of the incubations, pH increases observed with storage time might have been related to loss of volatile solids (e.g. acetate, propionate, and butyrate) due to bacterial consumptions (Sorensen, 1998) that have been impacted minimally by the chemical agents. However, as one of the major methanogenic substrates, acetate consumption by bacteria and perhaps methanogens might have been impacted as rates of Na₂S₂O₈ increased (Tsitonaki et al., 2008). The authors explained its potential impacts on proton motive force which could influence acetate utilization. As an important methanogenic substrate in stored dairy manure (Barret et al., 2013), any impacts on acetate synthesis or utilization might result in significant reduction of CH₄ production. For instance, a 25% reduction in CH₄ production has been demonstrated in a biogas reactor upon inhibition of~72% of acetate producing bacteria by application of 1 g L⁻¹ coumarin (Popp et al., 2017). Thus, at rates ≤9 g L⁻¹ slurry of Na₂S₂O₈, methanogens that can use CO₂/H₂ (hydrogenotrophic methanogens) might have contributed more to the total abundance than those that use acetate (acetoclastic methanogens). Sodium persulfate is a readily available reagent that has environmentally benign by-products (e.g. CO₂) (Hori et al., 2005; Huling and Pivetz, 2006). Thus, its strong impacts on CH₄/CO₂ ratios (hence impacts on potential CH₄ production) while indigenous bacterial communities remain
unaffected might warrant further large-scale assessment of this chemical for potential use in full-scale dairy farms.

Reductions of CH$_4$ production in KMnO$_4$-treated slurries (as observed from reduced CH$_4$/CO$_2$ ratios) were significant (up to ~14-fold), but not as in Na$_2$S$_2$O$_8$-treated slurries (up to ~92-fold). However, volatile solid contents of slurries treated with KMnO$_4$ were relatively lower when compared with Na$_2$S$_2$O$_8$-treated slurries. This might not be related to microbial consumption as methanogen abundance also reduced more with increased rates of KMnO$_4$ than Na$_2$S$_2$O$_8$. As a strong oxidizing agent, KMnO$_4$ is commonly used to remove organic matter from soil and water systems (Fayad et al., 2013; Wu et al., 2014; Naceradska et al., 2017; Rissanen et al., 2017). And, its removal efficiency might also be high when compared with Na$_2$S$_2$O$_8$ (Usman et al., 2017), thus it is reasonable that KMnO$_4$ might have oxidized more slurry organic matter (source of methanogenic substrates), and inactivated microorganisms. This could explain the relatively higher reductions in volatile solid contents; however, it was not clear how CH$_4$/CO$_2$ ratios were relatively higher in KMnO$_4$-treated slurries under similar application rates. Particularly, with higher impacts on the total abundance of methanogens (also showed low mcrA/bac16S rRNA gene ratios), studies that focus on the identities and activities of methanogens in slurries treated with KMnO$_4$ may be necessary. Like Na$_2$S$_2$O$_8$, KMnO$_4$ is readily available but its potential impact on indigenous microbial communities in slurries and perhaps soil microorganisms after manure is applied on agricultural lands might need further study.
Sodium hypochlorite is also a strong oxidizing agent that is commonly used to remove organic matter from soil samples (Siregar et al., 2005). However, in the current study, addition of up to 9 ml L\(^{-1}\) slurry of 3%NaOCl did not result in significant variation of microbial abundance and volatile solid contents when compared with untreated slurry. As indicated in previous studies, NaOCl is a general disinfectant at concentrations ≥4% (Guan et al., 2014; Jadhav et al., 2014; Han et al., 2016) that can destroy microbial proteins (Winter et al., 2008). Thus, in the current study, the low concentration (final concentration <3%) of NaOCl might have been insufficient to significantly impact slurry microbial communities. It should also be noted that bleach can decompose over time, specifically at high temperature and with exposure to light, thus its stability might also have been compromised during storage in the store where it was purchased. The unexpected increase in CH\(_4\) production in 3%NaOCl-treated slurries might be related to some possible impacts on the stability of slurry organic matter, which can increase available substrates to slurry microorganisms that were not been affected by the treatment. When 3%NaOCl was combined with Na\(_2\)S\(_2\)O\(_8\) and KMnO\(_4\), however, significant reductions of CH\(_4\)/CO\(_2\) ratios were observed except at the lowest rate tested. The lowest rate that contained 0.33 g L\(^{-1}\) each of Na\(_2\)S\(_2\)O\(_8\) and KMnO\(_4\) and 0.33 ml L\(^{-1}\) 3%NaOCl did not impact the ratio, but when rates were increased to a level where Na\(_2\)S\(_2\)O\(_8\) or KMnO\(_4\) alone could inhibit CH\(_4\) production, relatively stronger impacts were observed. Thus, combination of these oxidizing chemicals appeared to have a synergistic impact on methanogenesis. Consistent results were observed on the
abundance of methanogens, where significantly low abundance of methanogens was detected at 9 units L\(^{-1}\) slurry.

Acid treatment of stored manure can affect the functioning of most methanogens that otherwise are active at a pH of around neutrality (El-Mashad et al., 2011; Petersen et al., 2014). In the current study, the population number of methanogens were not impacted with acidification, which is consistent with a previous study (Petersen et al., 2014) where the authors found little variation in the total abundance of methanogens on reduction of slurry pH to 5.5. Reductions in CH\(_4\)/CO\(_2\) ratios, which might indicate potential reduction in CH\(_4\) production, observed in the current study were consistent to previous reports (Ottosen et al., 2009; Petersen et al., 2012; Petersen et al., 2014). Slurry acidification may be considered as a potential strategy in mitigating CH\(_4\) emissions from stored liquid manure (Hilhorst et al., 2001), but compared to slurries treated with chemical oxidants, impacts on CH\(_4\) production due to acidification seemed low. Particularly, between days 60-90 (peak CH\(_4\)/CO\(_2\) ratios in most incubations), slurries treated with Na\(_2\)S\(_2\)O\(_8\), KMnO\(_4\), and combinations had stronger impacts on CH\(_4\)/CO\(_2\) ratios (\(p<0.001\); Tukey’s test), and hence on potential CH\(_4\) production. In addition to reducing CH\(_4\) emissions, slurry acidification also reduces NH\(_3\) emissions from stored liquid dairy manure (Petersen et al., 2012; Petersen et al., 2014; Wang et al., 2014; Bastami et al., 2016), thus further studies of slurries treated with chemical oxidants for potential reduction of NH\(_3\) emissions may provide important options in mitigating GHG emissions from these systems.
4.6 Conclusion

Results indicated that Na$_2$S$_2$O$_8$ and KMnO$_4$, strong chemical oxidants that are readily available and commonly used in environmental remediation, reduced CH$_4$/CO$_2$ ratio and negatively impacted the growth of methanogens in stored liquid dairy manure at application rates of ≥1 g L$^{-1}$ slurry, thus could reduce CH$_4$ production. Methanogen abundances and CH$_4$/CO$_2$ ratios did not reduce with addition of up to 9 ml L$^{-1}$ slurry 3%NaOCl, but when combined with Na$_2$S$_2$O$_8$ and KMnO$_4$, strong synergistic effects on both methanogen abundance and CH$_4$/CO$_2$ ratios were observed. When compared with slurry acidification, which is gaining more attention as a potential strategy to abate CH$_4$ emission from stored liquid dairy manure, the chemical agents tested in this study (Na$_2$S$_2$O$_8$, KMnO$_4$, or their combinations) appeared to have more impacts on methanogens and CH$_4$ production. Thus, these chemical agents may offer options for emission mitigation from stored liquid dairy manure. However, to use these strong oxidants for slurry treatment at the farm-scale, further studies to determine their environmental impacts, agronomic value and physico-chemical characteristics of the treated manure need to be assessed.

Acknowledgements

The authors acknowledge Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) for the financial assistance.
5 Acidification of liquid dairy manure affects transcriptional activities of methanogens and methane emissions

Jemaneh Habtewold\textsuperscript{a}, Robert Gordon\textsuperscript{b}, Vera Sokolov\textsuperscript{b}, Andrew VanderZaag\textsuperscript{c}, Claudia Wagner-Riddle\textsuperscript{a} and Kari Dunfield\textsuperscript{a}

\textsuperscript{a}. School of Environmental Sciences, University of Guelph, Guelph, N1G 2W1, ON, Canada
\textsuperscript{b}. Department of Geography & Environmental Studies, Wilfrid Laurier University, Waterloo, N3T 2W2, ON, Canada
\textsuperscript{c}. Agriculture and Agri-Food Canada, Ottawa, K1A 0C5, ON, Canada

This manuscript is in preparation for submission to a peer reviewed journal.

Author Contributions:

J Habtewold was the primary researcher and was responsible for the experimental design, lab work, data analysis, and manuscript preparation. V Sokolov was credited for the role in the design of the study and sample collection. C. Wagner-Riddle and A VanderZaag were credited for the valuable advices on the experimental design, statistical analyses and manuscript review. R. Gordon and K. E. Dunfield were credited for their invaluable mentorship on lab-work and trouble-shooting on data analysis, manuscript preparation, and data interpretation.
5.1 Abstract

Acidification of stored liquid dairy manure can reduce methane (CH\textsubscript{4}) emissions; however, little is known about abundance and activity responses of microbial communities in acidified slurries. Liquid dairy manure treated with sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) or water (control) were stored in six pilot-scale storage tanks (~10.5 m\textsuperscript{3}), and CH\textsubscript{4} emissions and changes in the abundances and activities of bacterial and methanogenic communities were monitored for 120 days. Methane emissions were measured continuously at the site using a laser-based trace gas analyzer whereas quantitative real-time polymerase chain reaction (qPCR) was employed to quantify bacterial 16S rRNA and Methyl-coenzyme M Reductase A (mcrA) genes and transcripts. Compared to untreated slurries, cumulative CH\textsubscript{4} emissions from acidified slurries with a mean pH of ~5.9 and ~6.5 were reduced by ~76±7% and ~78±6%, respectively. Acidification did not result in significant shifts in the abundances and activities of bacteria (p<0.05).

While the abundances (Log\textsubscript{10} mcrA gene copies) of methanogens reduced only up to ~6%, the activities of methanogens (Log\textsubscript{10} mcrA transcript copies) reduced up to ~20%. Similarly, mcrA transcript/gene ratios were also lower (up to ~21%) in acidified slurries. The results suggest that acidification of liquid dairy manure has a stronger impact on transcriptional activities than methanogen populations which results in reduced CH\textsubscript{4} emissions.
Keywords: dairy manure, greenhouse gas, manure acidification, methane, methanogens

5.2 Introduction

Livestock production is an important source of methane (CH$_4$) emissions (e.g. 119.1± 18.2 Tg in 2011) (Wolf et al., 2017), mainly from enteric fermentation and manure management of dairy farming operations (Laubach et al., 2015; Jayasundara et al., 2016; Wolf et al., 2017). The large volumes of manure produced annually from intensive dairy farming operations are usually stored in liquid form (VanderZaag et al., 2013), which create conducive environments for CH$_4$ production (Grant et al., 2015; Petersen, 2017). Thus, there is a need for effective mitigation strategies for CH$_4$ emissions from dairy manure storage systems.

Sulfuric acid (H$_2$SO$_4$)-based acidification of liquid dairy manure (pH up to ~5.0) that has primarily been used to abate ammonia (NH$_3$) emissions, but has been shown to reduce CH$_4$ emissions (Ottosen et al., 2009; Petersen et al., 2012; Fangueiro et al., 2015; Sommer et al., 2017). For instance, 57 days after acidification (pH 5.5) of liquid cattle manure, Sommer et al. (2017) demonstrated ~68% reduction of CH$_4$ emissions. More than 90% reduction of CH$_4$ emissions from acidified pig slurry were also been reported by Petersen et al. (2014). Indeed, acidification of stored liquid dairy manure have already been implemented at farm-scale in some countries such as Denmark. However, knowledge about the abundance and activity responses of these microbes to slurry acidification is limited. Using chemical oxidants and H$_2$SO$_4$, we detected significant inhibition of methanogens and reduction of CH$_4$ production from dairy

102
manure (Habtewold et al., 2018), although larger scale assessments of these chemical agents may be needed.

In stored liquid dairy manure, complete degradation of complex organic matter involves a consortium of microbial communities (hydrolytic, acidogenic, and acetogenic bacteria, and methanogens). The activities of the bacterial groups (particularly hydrolytic and acidogenic bacteria) typically occur at a slurry pH of 5.0-7.0 (Pind et al., 2003), whereas most methanogens have a narrower pH optima between 6.8 and 7.8 (Lay et al., 1997; Whitman et al., 2006; Weiland, 2010; Angelidaki et al., 2011). This may indicate that, in liquid dairy manure where pH is usually around 7.0, pH deviations (e.g. due to slurry acidification) can have an impact on the functioning of these microbial communities, which may result in reduced CH\textsubscript{4} production. Thus, with slurry acidification, understanding how the functioning of these microbes is affected may be an important question that may help to improve the mitigation efficiency of slurry acidification.

The functioning of environmental microbial communities may not always be determined from total population abundance as dead and dormant cells can have significant contributions to total genomic DNA (Blagodatskaya and Kuzyakov, 2013). Particularly, with slurry acidification where significant number of bacterial and methanogenic communities could be dormant or dead, DNA-based abundances of these microbes may not reflect activities. However, changes in the transcriptional levels of 16S rRNA genes or mRNA of functional genes are often related to growth and activity of microbial communities (Ma et al., 2012; Blagodatskaya and Kuzyakov, 2013; Wilkins
et al., 2015). Hence, in the current study, we aimed to investigate activity responses of bacterial and methanogenic communities in acidified liquid dairy manure by targeting bacterial 16S rRNA and mcrA genes and transcripts. Despite some biases due to dead and dormant cells, transcript/functional gene ratios may also provide an important indication of the microbial activity (Freitag and Prosser, 2009; Blagodatskaya and Kuzyakov, 2013).

5.3 Materials and Methods

5.3.1 Methane measurements and manure samplings

The study was conducted during the warm season (25 June through 23 October 2017) at a research facility at Dalhousie University’s Bio-Environmental Engineering Center (BEEC) in Truro, NS, Canada (45°45’ N, 62°50’ W). Six pilot-scale (10.6 m$^3$) rectangular outdoor manure storage tanks. This site has been previously described by Wood et al. (2012). About 10.5 m$^3$ fresh dairy slurry obtained from a commercial farm was loaded to each tank. Using duplicate tanks, ~1.4L or ~2.4L m$^{-3}$ slurry 70%H$_2$SO$_4$ was injected across the depth (with simultaneous mixing) of slurries. The remaining two tanks were used as controls (injected with 2.4L m$^{-3}$ slurry of water). Gas samples were drawn continuously from each tank’s outlet and ambient air, and CH$_4$ concentrations were measured at the site using tunable diode laser trace gas analyzer (TGA 100A; Campbell Scientific Inc., Logan, UT). Methane flux (g m$^{-2}$ s$^{-1}$) was calculated as described by Wood et al. (2012) and emissions were then converted into daily averages.
For the microbial study, duplicate slurry samples before and after acidification were collected. Before acidification, fresh manure samples that could represent the starting manure for all tanks were collected. After acidification, slurry samples were collected ~10 cm from the top and ~80 cm from bottom section of each tank on day 21 (14 July), day 50 (14 August; when peak CH$_4$ flux for the untreated tanks were detected), and day 103 (06 October, when CH$_4$ fluxes from the control tanks decreased). From each sampling location, nine slurry samples were collected from random points across the surface, and pooled in a clean bucket. Then, two grams subsamples (in duplicate) were collected from each pool in 15 mL Falcon tubes containing 5 mL LifeGuard$^\text{TM}$ Soil Preservation Solution (MoBio Laboratories Inc., Carlsbad, CA). Samples were then transported in cold to the lab, and stored in -20°C freezer until nucleic acid extractions (at the end of storage). Sub-samples (of appropriate volume) were also collected to analyze pH, dry matter (DM), and volatile solid (VS) contents at the Nova Scotia Department of Agriculture’s Laboratory Services (Harlow Institute, Bible Hill, NS) using standard methods.

5.3.2 Nucleic acid extractions and quantitative real-time PCR

Slurry samples in LifeGuard$^\text{TM}$ Soil Preservation Solution were centrifuged (4000×g for 10 min) and pellets were used to co-extract total RNA and DNA using RNA PowerSoil Total RNA Isolation with DNA Elution Accessory Kits (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer’s protocol. As there was little difference in the abundances of bacteria and methanogens between the top and bottom sections of slurries, DNA or RNA samples from these locations were pooled to have one
representative sample per tank. Before further downstream analyses, both cDNA and DNA samples were diluted and assessed for potential inhibitory effects as described earlier (Habtewold et al., 2017) (Chapter 2). RNA samples were reverse transcribed into complementary DNA (cDNA) using Maxima™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific™) following the manufacture’s protocol with few modifications. Briefly, 1 µl each of 10× dsDNase Buffer and dsDNase were added to 2µl (~0.3-1.0 µg) RNA, gently mixed and span, and incubated at 37 °C for 5 minutes in a preheated thermocycler with lid temperature adjusted to 37 °C. After chilled on ice and briefly centrifuged, 4 µl Maxima cDNA H Minus Master Mix (5×) and 6 µl nuclease-free water were added, and gently mixed and centrifuged. Complementary DNA synthesis reactions, which were performed in a thermocycler with lid temperature adjusted to 50 °C, thermal conditions were: 25°C for 10 minutes, 50 °C for 15 minutes, and 85 °C for 5 minutes. In addition to DNA (diluted 50×), cDNA (diluted 100×) were then used as template in quantitative real-time polymerase chain reaction (qPCR). Reaction ingredients, conditions, and thermal cycling of qPCR was conducted as described previously (Habtewold et al., 2017) (Chapter 2). Known copies of plasmid standard curves for mcrA (10^7 to 10^1) and bacterial 16S rRNA (10^8 to 10^1 copies) genes and transcripts quantifications were prepared from Methanosarcina mazei (ATCC 43340) and pure culture of Clostridium thermocellum, respectively. Efficiency, r^2, and slope of the plasmid standard curve for mcrA gene were 98.5±2.8%, 0.99, and -3.34 ±0.04, whereas for 16S rRNA gene, these values were 98.5±2.7%, 0.99, and -3.36±0.07,
respectively. CFX Manager software version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA) was used to analyze the qPCR data.

To understand the link between the abundances of slurry microbial communities and reductions in CH$_4$ emission from acidified slurries, correlating their changes with acidification was necessary. Thus, using untreated slurries as reference, reduction in cumulative CH$_4$ emission and abundance of mcrA gene/transcripts due to slurry acidification were correlated using GraphPad prism v.7 (GraphPad Software, Inc.).

5.4 Results

5.4.1 Manure characteristics and CH$_4$ flux

Initially, the pH of fresh dairy manure used in the current study was ~7.5. Twenty-one days after addition (and mixing) of ~1.4L and ~2.4L 70% H$_2$SO$_4$ per cubic meter of dairy slurry, mean pH of slurries was 6.5±0.1 and 5.9±0.0, respectively (Fig. 5.1a), while pH of untreated slurries was 6.8±0.07. After day 50, however, slurry pH gradually increased in all tanks by 0.35±0.2. Volatile solid contents of dairy slurries from all tanks reduced with storage, although more reductions (by up to ~12%) were observed in untreated slurries (Fig. 5.1b). Despite some variability in CH$_4$ flux between tanks that contained untreated slurries, peak CH$_4$ fluxes occurred between day 50-60 (~76-52 g m$^{-2}$ d$^{-1}$) (Fig. 5.2a). However, CH$_4$ flux from all acidified slurries were consistently low (<10 g m$^{-2}$ d$^{-1}$) throughout the storage period. When compared with untreated slurries, low fluxes from acidified slurries (treated with ~1.4L and ~2.4L 70% H$_2$SO$_4$ m$^{-3}$ slurry) resulted in ~76±7% and 78±6% reductions in cumulative CH$_4$ emission, respectively (Fig. 5.2b).
**Figure 5.1:** Physicochemical characteristics of stored liquid dairy manure: a) pH and b) volatile solid contents. Values shown for volatile solid contents and pH are mean ± standard deviation of duplicate treatments.
Figure 5.2: Methane flux (a), and cumulative CH$_4$ emissions (b) from stored liquid dairy manure. Values shown are mean ± standard deviation of duplicate treatments. Acid was added a week after of manure loading in the storage tanks.
5.4.2 Abundance and activities of bacteria and methanogens

Fresh manure (<1-day after being transferred from the dairy farm) used in the current study had large number of bacteria, where copy number (Log$_{10}$) of 16S rRNA genes and transcripts were ~10.3 and ~13.3 g$^{-1}$ dry manure, respectively (Fig. 5.3a). The abundance of total bacteria in stored liquid dairy manure were minimally impacted with acidification, showing up to ~4% lower copies (Log$_{10}$) of 16S rRNA genes when compared to untreated slurries (Fig. 5.3a). Similarly, the activities of bacteria, as estimated from rRNA transcript copies g$^{-1}$ dry manure, had little change with slurry acidification (Fig. 5.3b), where Log$_{10}$ copy numbers were only ~0.5% (on day 50) and ~1% (on day 103) lower when compared to the untreated slurries.

![Graphs showing changes in bacterial abundance and activities over time with and without acidification.](image-url)
**Figure 5.3:** Abundance and activities of total bacteria as indicated by copies (g\(^{-1}\) dry manure) of (a) bacterial 16S rRNA genes and (b) transcripts in liquid dairy manure. Values shown are mean ± standard errors of the mean of duplicate treatments.

Slurry acidification had stronger effects on the activities than total abundances of methanogens (Fig. 5.4a and 5b). Initially, there were 6.8±0.1 copies (Log\(_{10}\)) of mcrA genes g\(^{-1}\) dry manure in fresh dairy manure (Fig. 5.4a). After 21 days of storage, mcrA gene copies g\(^{-1}\) dry manure increased by ~30% and ~25% in untreated and acidified slurries, respectively. The effects of slurry acidification, however, were not noticeable on day 21 when mcrA gene copies in all slurries were almost similar (Fig. 5.4a). Even after day 50 or day 103, only up to ~6% lower mcrA gene copies (Log\(_{10}\)) were detected from acidified slurries when compared with untreated slurries. With slurry acidification, a positive correlation was observed between methanogen abundance and CH\(_4\) emissions (\(r^2=0.93, \alpha=0.16\)). Unlike mcrA gene copies, mcrA transcript copies (Log\(_{10}\)) g\(^{-1}\) dry manure showed higher reductions with slurry acidification (Fig. 5.4b). After day 21, slurries that received ~2.4L 70% H\(_2\)SO\(_4\) m\(^{-3}\) slurry resulted in ~8% lower copies of mcrA transcripts while mcrA transcript/gene ratios reduced by ~1.7% (Fig. 5.4c). During peak flux time for untreated slurries (days 50-60), all acidified slurries had ~20% lower copies (Log\(_{10}\)) of mcrA transcript g\(^{-1}\) dry manure. These reductions were significantly correlated (\(r^2=0.99, \alpha=0.01\)) with CH\(_4\) emissions. On days 50 and 103, mcrA transcript/gene ratios in acidified slurries reduced by ~16.5% and 21%, respectively (Fig. 5.4c). During peak flux (day 50), mcrA transcript/gene ratios across treatments were also positively correlated (\(r^2=0.99, \alpha=0.02\)) with cumulative CH\(_4\) emissions.
Figure 5.4: Abundance and activities of methanogens as indicated by copies (g⁻¹ dry manure) of (a) mcrA genes, (b) transcripts, and (c) transcript/gene ratios in liquid dairy manure. Values shown are mean ± standard errors of the mean of duplicate treatments.
5.5 Discussion

Stored liquid dairy manure emits large amounts of CH₄, thus effective mitigation strategies are in need. Studies have demonstrated that H₂SO₄-based acidification of liquid dairy manure (pH up to ~5.5) can reduce CH₄ emissions between ~67-90% (Petersen et al., 2012; Petersen et al., 2014; Misselbrook et al., 2016; Sommer et al., 2017). Consistently, in the current study, ~69-84% reductions (per-tank basis) of cumulative CH₄ emissions were detected after acidification of liquid dairy manure to mean pH of ~5.9. This indicates that a small change in slurry pH can disrupt methanogenesis which has pH optima between 6.8 and 7.8 (Lay et al., 1997; Liu et al., 2008; Weiland, 2010; Mao et al., 2017). Slurry acidification are presumed to negatively impact active microbial groups that derive CH₄ production.

With slurry acidification (pH ~6.5 or pH ~5.9), shifts in the abundance and activities of bacterial communities, as estimated from 16S rRNA gene and transcript copies, respectively, were not significant. Thus, with minimal impacts on the growth and metabolic activities of bacterial groups (hydrolytic, acidogenic, and acetogenic), reductions in CH₄ emission observed after slurry acidification might not be related to limitations of methanogenic substrates. Rather, the activities of these bacteria might have been favoured as slurry pH reduced (down to ~5.5) (Lin et al., 2013; Kuruti et al., 2017). Gradual reductions of VS contents observed in acidified slurries might also be related to the activities of bacteria that can tolerate lower pH. Acidification may increase separation of slurry particles (Fangueiro et al., 2015; Gomez-Munoz et al., 2016;
Regueiro et al., 2016), thereby increasing available substrates to hydrolytic and acidogenic bacteria. The action of these groups of bacteria then result in accumulation of organic acids (e.g. acetic, propionic, and butyric acids), which coincide with pH reduction of slurries in the first three weeks of storage. Regardless of treatments, however, slurry pH gradually increased after day 50 of storage time, although the changes in untreated slurries were relatively higher. This is consistent with previous studies (Patni and Jui, 1985; Sommer et al., 2017), which might be due to microbial consumptions of accumulated organic acids.

Acidification had little effect on the abundance of methanogenic populations. A study on stored pig slurries by Petersen et al. (2014) also indicated negligible effects of slurry acidification (pH up to 5.5) on the abundance of methanogens while >90% reduction in CH₄ emission was detected. An incubation-based study by Ottosen et al. (2009) indicated >98% reductions of microbial processes (oxygen consumption rate, methanogenesis and sulphate reduction) in acidified pig slurries where methanogenesis was estimated from the linear increase in headspace concentration during storage. Consistent with those studies, little impact was observed on the population sizes of methanogens but significant reductions in CH₄ production were observed in the current study. While CH₄ production is related to the abundance of active methanogens, mcrA gene-based quantification of methanogens targets all the active, dormant, and dead cells. Hence, it was important to target mRNA of the mcrA genes that are present whenever methanogenic microbes are physiologically active. Unlike the abundance of methanogens, a strong positive correlation ($r^2=0.99$, $\alpha=0.01$) between mcrA transcripts
and CH₄ emissions was observed, suggesting the activities (not abundance) of methanogens were negatively impacted (inhibited) by acidification.

In conclusion, H₂SO₄-based acidification of stored liquid dairy manure to pH ~6.5 and ~5.9 reduced cumulative CH₄ emissions by ~76±7% and 78±6%, respectively. Acidification significantly impacted the activities of methanogens while minimal impact was observed on the abundance of total methanogen populations. Thus, reduced transcriptional activities of methanogens reduced CH₄ emissions from acidified liquid dairy manure, not total methanogen abundance.
6 General discussion and conclusions

Stored liquid dairy manure is a point sources for CH$_4$ emissions. To abate CH$_4$ emissions from such systems, various mitigation strategies have been developed (Petersen et al., 2012; Wood et al., 2014; Ngwabie et al., 2016). Most of these strategies impact the functioning of microbial communities that are directly or indirectly involved in CH$_4$ production. Understanding the abundance, activity, and diversity responses of these communities is important to fine-tune and/or developing effective mitigation strategies, although studies so far overlooked the importance of understanding these responses. This work explored the responses of bacterial and methanogenic communities in stored dairy slurries where different mitigation actions were assessed. In particular, we investigated the responses of slurry microbial communities to varying levels of manure total solids, residual slurry, slurry pH, and chemical oxidants.

First, we found that the abundance and functional activity of methanogenic communities increased as slurry total solids content decreased from 9.5% to 0.3% (Chapter 2). As liquid dairy manure contains large amount of dissolved organic matter, diluted (low total solids levels) slurries can still contain sufficient amounts of substrates to support the growth of bacterial and methanogenic communities. As slurry substrate levels decrease, low levels of some toxic intermediates (e.g. propionic acid) and hence increased activities of methanogens may occur (Barret et al., 2013). Indeed, we observed higher volatile solids to CH$_4$ conversion rates as total solids contents decreased. However, higher cumulative CH$_4$ emissions were observed in tanks that
contained higher total solids levels. Thus, availability of substrates, not methanogen population, may be limiting cumulative CH$_4$ production from the tanks with reduced total solids levels. It is important to note, however, that reduction of total solids levels by dilution of slurries with water cannot be considered as a potential mitigation strategy. This is because, dilution creates additional volumes (of diluted slurries) where the activities of methanogenic communities increased, which may result in increased cumulative CH$_4$ production. It is also important to note that reduction of manure total solids by simple dilution differs from solid-liquid separation of dairy slurries prior to storage, in which the latter reduces total solids levels and coarse solids while CH$_4$ production per volume of slurry can increase in the liquid fraction (Rico et al., 2007; Rico et al., 2012). This is because the liquid fraction can supply the microbes with large amount of undiluted dissolved nutrients. Thus, unlike in diluted slurries, the abundance and activities of microbial communities in typical manure storage systems (where substrate is not limiting) can be positively correlated with CH$_4$ emissions. In these nutrient-rich environments, the amount of CH$_4$ emitted during storage could also be related to the presence of well-adapted bacterial and methanogenic inoculants. With long-term storage of dairy manure, microbial communities that adapt to the storage conditions (e.g. temperature and substrate) could be inoculants for the new storage, when residual (aged) slurries are left in the tank. Indeed, a number of studies show longer lag phases of methanogenesis and reduced cumulative CH$_4$ emissions from stored dairy slurries at lower residual slurry levels (Wood et al., 2014; Ngwabie et al., 2016). Consistently, with the presence of residual slurries in stored liquid dairy manure,
we found increased abundances and activities of bacteria and methanogens during the initial phases of storage (Chapter 3). This was reflected in CH₄ flux and cumulative emissions. Irrespective of residual slurry level, however, the abundances and activities of microbial communities were not important at the later stages of storage period. This further indicates the importance of residual slurry in accelerating the onset of methanogenesis and hence increasing cumulative CH₄ emissions. Thus, in countries like Canada, residual slurry may need to be completely removed for summer storage, during which the abundant and well-adapted microbial communities in residual slurries may be favored with the warm season and readily start methanogenesis. On many dairy farming operations, however, complete removal of residual slurry is practically difficult. Other strategies such as treatment of residual slurries using chemicals (e.g. acids and oxidants) prior to tank filling may help to reduce the inoculant microbial communities. At lab-scale, we assessed the effects of some chemical agents for potential use in mitigating CH₄ productions in stored liquid dairy manure (Chapter 4). Interestingly, Na₂S₂O₈ and KMnO₄ that are relatively cheaper and easily available oxidants showed significant reductions in CH₄ production in stored liquid dairy manure. When these chemical oxidants were combined with NaOCl, impacts on methanogens and CH₄ productions became stronger. These chemical oxidants disinfect the slurry environment and can also oxidize organic substrates (Hamilton, 1974; Mikutta et al., 2005; Siregar et al., 2005) as they have often been used in removing soil organic matter (Hoag et al., 2000; Siregar et al., 2005; Chen et al., 2015). Sodium persulfate, in particular, showed strong negative effects on the abundance of methanogens and CH₄ production while
the abundance of total bacteria unaffected. At the same incubation condition, acidified slurries (pH 5.5) showed a relatively lower impact on methanogens and CH$_4$ production, indicating the stronger impacts of these chemical oxidants in reducing emissions from stored slurries, although further large-scales may be needed. Thus, the negative impacts of these chemicals on the abundance of microorganisms in slurries suggest their potential to be used in the treatment of residual slurry in reducing the inoculant microbial community. Consistent with the current lab-scale study, reduced methanogenesis and CH$_4$ emissions have been observed after dairy slurry acidification in pilot- and full-scale storage tanks (Lay et al., 1997; Liu et al., 2008; Weiland, 2010; Mao et al., 2017). Most methanogens functions at a pH of around neutrality (El-Mashad et al., 2011; Petersen et al., 2014), acidification of dairy slurry is expected to impact methanogenesis. Using pilot-scale tanks filled with acidified slurries, we detected stronger positive correlation ($r^2=0.99$, $\alpha=0.01$) between $mcrA$ transcript copies and cumulative CH$_4$ emissions (Chapter 5), indicating the inhibitory effects of acidification on the activities of methanogens. Despite the significant effects on CH$_4$ production, methanogenesis may still occur in low pH (~5.0-6.0) slurries as it may favour some methanogens (Taconi et al., 2008). The toxicity of H$_2$SO$_4$ that is used for slurry acidification may also be a concern for farm personnel.

In lab- and meso-scale CH$_4$ emission studies, simulating storage conditions to the actual manure storage conditions is critical for translating the findings into farm scales. For instance, on many dairy farming operations, storage tanks are filled gradually (i.e. daily to weekly loadings) throughout the storage period by transferring
fresh barn collections or slurries from temporary storages (Sheppard et al., 2011). However, many small-scale studies often use batch-filled tanks (Wood et al., 2014; Ngwabie et al., 2016) where findings may not reflect the actual conditions and hence can lead to inaccurate conclusions. For instance, we demonstrated significant difference in cumulative CH$_4$ emissions between batch- and gradually-filled tanks (Chapter 3). These differences might be related to surface stability of slurries where the relatively thicker surface crusts in batch-filled tanks can physically block gaseous emissions, thereby increasing the access to CH$_4$-consuming aerobic bacteria (Ambus and Petersen, 2005; Petersen et al., 2005). Stability of slurry surface crusts can be impacted negatively by frequent top-loadings of slurries. Thus, solid understanding of the biological and physicochemical characteristics of stored manure, and simulating the actual storage conditions is critical in developing effective mitigation strategies.

To estimate CH$_4$ emissions from manure storage systems, many of the mathematical models developed so far mainly consider factors such as manure characteristics, storage conditions, and environmental conditions (VanderZaag et al., 2013; Petersen et al., 2016; Leytem et al., 2017; Rotz, 2017). While microorganisms in slurries are responsible for CH$_4$ production, the importance of microbial information in CH$_4$ flux estimations have been overlooked. It might be assumed that those factors could control the functioning of microbial communities; however, the huge functional diversity of microorganisms under different environmental conditions can still lead to CH$_4$ production (Taconi et al., 2008; Nolla-Ardevol et al., 2015). Hence, considering the microbial data (e.g. abundance, activity, identity of methanogens) as important inputs in
model development and/or focusing to what extent a mitigation strategy affects the functioning of microbial communities in slurries with low impacts on the environment is vital.

Development of effective and environmentally benign mitigation strategies for CH$_4$ emissions from manure storage systems reduces the carbon footprint of the dairy farming industry, and may contribute to the economic viability of the sector. For instance, the Canadian dairy farming sector contributed up to $19.9 billion in 2016 (DFC, 2016). However, with the growing demand and increasing international competitiveness for dairy products, environmental footprints of dairy wastes (e.g. through GHGs and pollutions) can negatively impact the public view of the industry, which in turn can impact its economic contribution to the country. Thus, for a sustainable production of dairy farming and hence to have a positive image from the public, development of effective and environmentally benign mitigation strategies of GHG emissions, which needs to take all key factors (including the microbial data) into account, is invaluable.
References


Environment-Canada. 2014. National inventory report: Greenhouse gas sources and sinks in Canada; the Canadian government’s submission to the UN framework convention on climate change.


Figure A.1. CH$_4$ flux (g m$^{-3}$ slurry) from stored diary manure that received different levels of residual slurry.
Figure A.2. Abundance of methanogens (as determined from copy number of mcrA gene) in dairy manure under different chemical treatments.
Figure A.3. Abundance of bacteria (as determined from copy number of 16S rRNA gene) in dairy manure under different chemical treatments.
Figure A.4. Cumulative CH$_4$ production from dairy manure treated with Na$_2$S$_2$O$_8$. 
Figure A.5. Cumulative CH$_4$ production from dairy manure treated with KMnO$_4$. 
**Figure A.6.** Cumulative CH$_4$ production from dairy manure treated with 3% NaOCl.
Figure A.7. Cumulative CH₄ production from dairy manure treated with combinations (1g:1g:1 mL) of Na₂S₂O₈, KMnO₄, and 3% NaOCl.
Figure A.8. Cumulative CH$_4$ production from dairy manure treated with H$_2$SO$_4$. 