Published January 12, 2017

Journal of Environmental Quality SHORT COMMUNICATIONS

Dairy Manure Total Solid Levels Impact CH_4 Flux and Abundance of Methanogenic Archaeal Communities

J. Habtewold, R. J. Gordon, J. D. Wood, C. Wagner-Riddle, A. C. VanderZaag, and K. E. Dunfield*

Abstract

Stored liquid dairy manures are methane (CH_4) emission hotspots because of the large amount of slurry volatile solids (VS) converted into CH_4 by methanogens under anaerobic conditions. Our research has indicated that a reduction of total solids (TS) of slurries before storage can reduce CH_4 emissions. In the current study, methanogen abundance was characterized in tanks with different CH_4 emissions. Using mesoscale slurry storage facilities equipped for continuous gaseous emission monitoring, we stored dairy slurries having TS from 9.5 to 0.3% for up to 6 mo. Samples were taken after Day 30 and Day 120 of the storage (20 May–16 Nov. 2010) from the upper and bottom layers of the slurries. Methanogenic communities were studied by targeting the gene encoding the α subunit methyl-coenzyme M reductase (*mcrA*), which catalyzes the final step of methanogenesis. Interestingly, mean abundances 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 slurry TS decreased from 9.5 to 0.3%. Nevertheless, compared with Day 30 of storage, mean abundances of methanogens were relatively higher at Day 120 (up to 19%), consistent with an increase in the cumulative CH_4 emissions. Polymerase chain reaction denaturing gel electrophoresis analysis indicated a low methanogen diversity, with most bands sequenced 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.

Core Ideas

- Reduction of total solids levels of stored dairy slurry can reduce cumulative CH_4 flux.
- Abundance and activity of methanogens increase as dairy slurry total solids decrease.
- Volatile solids-to-CH_4 conversion rates appear to be higher as dairy slurry total solids decrease.
- Substrate availability, not methanogen population, limits cumulative CH_4 flux.

J. Environ. Qual. 46:232–236 (2017)
doi:10.2134/jeq2016.11.0451
Received 23 Nov. 2016.
Accepted 5 Dec. 2016.
*Corresponding author (dunfield@uoguelph.ca).

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). The large quantities of organic substrates in liquid manures stored under anaerobic conditions, however, make these environments conducive to methane (CH_4) production. Studies have demonstrated that stored dairy slurries are large CH_4 sources (Külling 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., 2013b). Managing manure-associated methanogenic factors such as TS may therefore 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 6 mo. 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 times more) CH_4 production rates from 4.2% TS compared with 9.2% TS dairy slurries may be due to the higher VS-to-CH_4 conversion rates by the methanogenic communities in lower TS slurries. While much has been published regarding CH_4 emissions from stored dairy slurry, studies that target the methanogenic communities from these environments subjected to mitigation actions such as reduction of TS are limited. A better understanding of the identity, activity, and abundance of methanogens in these systems is critical toward supporting the development and refinement of greenhouse gas mitigation strategies. Reduction of dairy slurry TS levels reduces cumulative

J. Habtewold, C. Wagner-Riddle, and K.E. Dunfield, School of Environmental Sciences, Univ. of Guelph, Canada; R.J. Gordon, Dep. of Geography and Environmental Studies, Wilfrid Laurier Univ., Canada; J.D. Wood, School of Natural Resources, Univ. of Missouri, Columbia, MO, USA; A.C. VanderZaag, Science and Technology Branch, Agriculture and Agri-food Canada, Canada. Assigned to Associate Editor Kim Cook.

Abbreviations: DGGE, denaturing gel electrophoresis; *mcrA*, α subunit of methyl coenzyme M reductase; PCR, polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction; SRA, Short Read Archive; TS, total solids; VS, volatile solids.

CH_4 emission because methanogens can be affected negatively through substrate limitation and, hence, their abundance and activity as slurry TS decrease. Here, we conducted a survey to assess the effects of varying levels of dairy slurry TS on the abundance and activity of methanogenic communities.

Materials and Methods

Experimental Design, CH_4 Flux, and Slurry Sampling

This study was conducted from 20 May through 16 Nov. 2010 (173 d) using six mesoscale manure storage tanks located at the Bio-Environmental Engineering Center in Truro, NS, Canada (45°45′ N, 62°50′ W). A detailed description of the site, monitoring, and initial experiment can be found in Wood et al. (2012). Fresh manure that was mixed with urine and milkhouse washwater was obtained from the Dalhousie University Experimental Farm. As the TS content of the fresh dairy slurry was 9.5%, five dilutions (8.5, 5.3, 3.2, 1.3, 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, 5.3, 3.2, 1.3, or 0.3% TS) were batch loaded to six rectangular storage tanks (1.75 m width by 3.90 m length by 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. Methane flux was calculated as described earlier by Wood et al. (2012) (Eq. [1]):

$$F = \frac{C_{out} - C_{in}}{A_s} Q \quad [1]$$

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 (m^2); and Q is the airflow rate (m^3 s^{-1}). After examining the cumulative CH_4 emissions from each TS level, we observed three major categories of TS contents having very close cumulative emissions (≥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). 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. Samples from all six tanks, after 30 and 120 d of storage, were aseptically collected from ~5 cm below the crust–slurry interface and ~20 cm above the bottom (~160 cm below the surface) using a slurry sampler with spring-loaded lid. Replicate samples from each tank and sampling location were pooled and transported to the laboratory in LifeGuard Soil Preservation Solution (MoBio Laboratories Inc.) and then stored in −20°C freezer until nucleic acid extraction.

Nucleic Acid Extractions and Polymerase Chain Reaction 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 α 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 the functional diversities. In this study, mlas-mod F (5′-ggyggtgtmggdttcacmcarta-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′-cggggygcascaggcgcgaa-3′) primers (Walter et al., 2015) to permit a comparison between the methanogens and general archaeal communities.

Quantitative real-time polymerase chain reaction (qPCR) was conducted using a thermal cycler (model 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 polymerase chain reaction (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 values, a pUC plasmid-based inhibition test was conducted. Three PCR 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 were used. Optimized thermal cycling for *mcrA* gene quantification involved initial denaturation at 95°C for 3 min followed by 40 cycles of dissociation (95°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 45 s), and a final step at 72°C for 3 min. For archaeal 16S rRNA gene quantification, the optimized cycling conditions used were initial denaturation at 94°C for 3 min followed by 39 cycles of dissociation (94°C, 10 s), annealing (66°C, 30 s) and extension (72°C, 15 s), and 72°C for 3 min. Plasmid standard curves for *mcrA* (10^8 to 10^1 copies) and archaeal 16S rRNA (10^9 to 10^1 copies) gene quantifications were prepared from *Methanosarcina mazei* (ATCC 43340). Efficiency, r^2, 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 analyze 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 BioRad DCode Universal Mutation Detection System (BioRad Laboratories, Inc.). 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). Bands were excised, and extracted DNA fragments from these bands

were sequenced. Sequences of the *mcrA* fragments retrieved in this study have been deposited into the NCBI short read archive (SRA) under the run accession number SRR3371371.

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. Total carbon and VS levels decreased as slurry TS decreased from 9.5 to 0.3% (Table 1). For all slurries, CH_4 emissions were observed after a lag period of about 4 wk (Fig. 1), implying the onset of methanogenesis. Although cumulative CH_4 emission was higher for 9.5% TS slurry, slightly higher flux rates (up to ~1.3 times) were observed for 0.3% TS just after the assumed start time of methanogenesis, particularly between Day 37 and Day 48. For the 9.5 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. 1a).

Interestingly, the abundance of methanogenic communities did not show concurrent reductions with slurry TS (Fig. 2). At Day 30 of storage, Log10 copies *mcrA* genes g^{-1} of dry slurry at the top section of the 9.5 and 0.3% TS slurries were 8.4 ± 0.02 (mean ± SEM) 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 Log10 copies of *mcrA* gene were detected from 9.5 and 0.3% TS slurries, respectively, ~12.3% increase in 0.3% slurry. Similarly, mean Log10 *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 Day 120 of the storage period, respectively, as slurry TS decreased from 9.5 to 0.3%. Interestingly, the increases in the copies of *mcrA* genes as slurry TS decreased from 9.5 to 0.3% TS were not consistent with the changes in cumulative CH_4 emissions (Fig. 1b), although they did follow the trends seen in the VS-to-CH_4 conversion rate (Fig. 3).

Cumulative CH_4 fluxes and abundance of methanogens changed as the storage time increased. The increase in cumulative CH_4 production during the storage time was consistent with the abundance and activity of methanogens (Fig. 1b and 2a). At Day 120 of storage, Log10 copies of *mcrA* genes g^{-1} of dry slurry increased up to 19 and 12.5% in the top and bottom sections of the slurry, respectively (Fig. 2b). The abundance of the total archaeal community, as targeted by its 16S rRNA gene, followed a similar trend as the methanogens (Fig. 4). For instance, 6 to 10% and 7 to 17% increase in total archaeal 16S rRNA genes g^{-1} of dry slurry at about Day 30 and Day 120 of storage, 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 of *mcrA* gene fragments were observed (data not shown), indicating a low diversity. Moreover, a faint band appeared only in 9.5% TS slurries might indicate the effects of slurry dilution on methanogenic

Table 1. Stored dairy manure volatile solids, total carbon, and pH at the beginning (20 May) and end (16 Nov.) of the storage.

Tanks	Sampling date	Sampling position	Volatile solids	Total carbon	pH
			%		
9.5% total solids	20 May	Mixed	7.8	3.3	7
	16 Nov.	Top	3.7	1.98	7.4
		Bottom	6.6	3.32	7.4
5.8% total solids	20 May	Mixed	4.8	2.03	6.9
	16 Nov.	Top	1.2	0.73	7.4
		Bottom	4.7	2.43	7.3
0.3% total solids	20 May	Mixed	0.2	0.1	6.8
	16 Nov.	Top	0.7	0.22	7.2
		Bottom	3.6	2.56	7.2

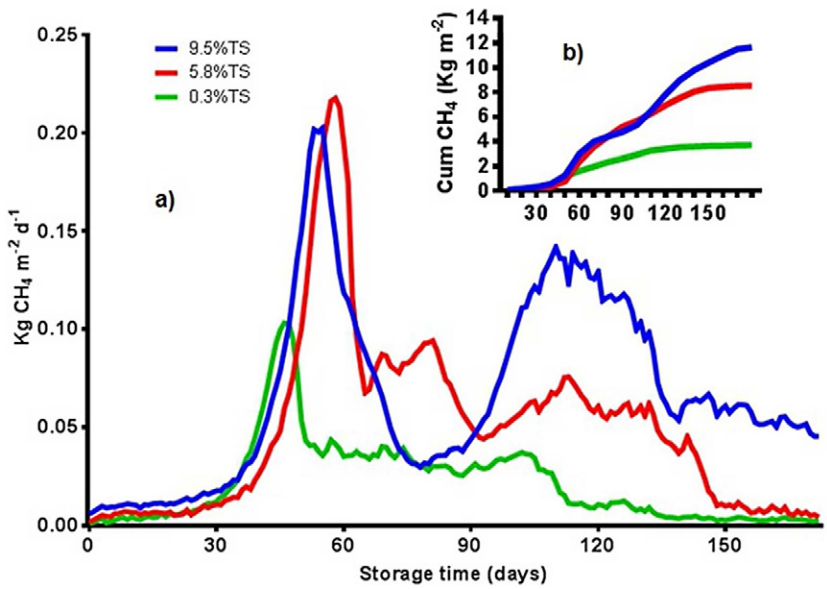

Fig. 1. (a) Methane (CH_4) fluxes (kg m^{-2} d^{-1}) and (b) cumulative emissions (kg m^{-2}) from stored dairy slurries having varied total solids (TS) contents. Emissions were measured continuously for 180 d using flow-through steady-state chamber method.

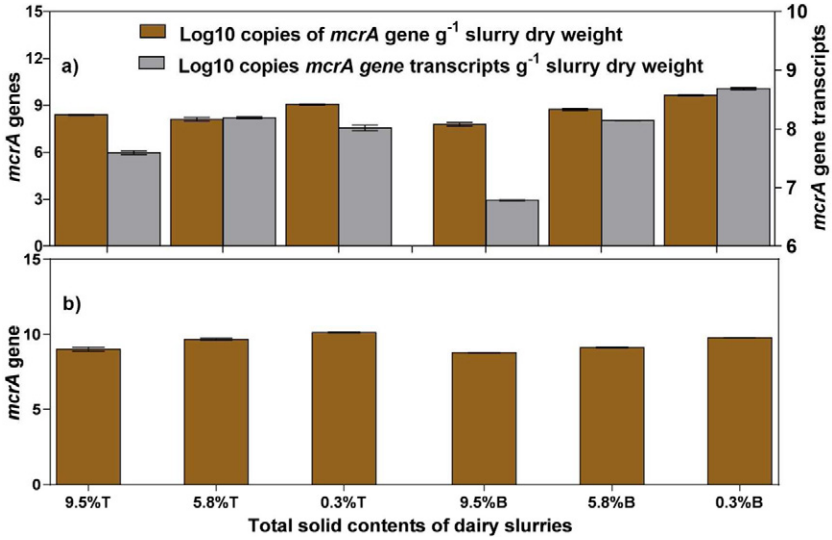

Fig. 2. (a) Methanogen activity and abundance (Log10 copies of *mcrA* transcript and gene copies g^{-1} of dry slurry) in dairy slurries containing total solids contents of 9.5, 5.8, and 0.3% after about Day 30 and (b) Log10 copies of *mcrA* genes g^{-1} dry slurry after Day 120. Standard errors were calculated from the three replicate polymerase chain reactions. "T" and "B" represent the top and bottom sections of the slurries, respectively.

phylotypes. On the other hand, 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, QC, Canada (Barret et al., 2013a). Members of the genus *Methanocorpusculum* may be the closest known relatives (>99% at amino acid levels) to the newly retrieved sequences.

Discussion

With the increasing concerns about the contribution of manure management-associated CH_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_4 emission from liquid dairy manure storage systems, large sources of CH_4, are to be designed, understanding the microbial communities responsible for production of CH_4 in slurries is critical. This study reveals the higher abundance and activity of methanogens as TS levels of slurries decreased.

In this study, the lag period of about 30 d, during which low CH_4 fluxes from all slurries were observed (Fig. 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_2/CO_2, or formate (Liu and Whitman, 2008). Thus, a short storage time could be a potential mitigation strategy to reduce CH_4 emissions from these hotspots, although large productions of manure and limitation of land for application may not allow for short storages. Interestingly, 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 decreased from 9.5 to 0.3% contradicted the cumulative CH_4 emissions (Fig. 1b and 2). Although cumulative CH_4 emissions seemed limited by substrate availability (e.g., ~3.2 times more from 9.5% than 0.3% TS slurries) (Fig. 1), methanogenic communities may have been favored in lower TS slurries where CH_4 production rates could be higher. Higher TS slurries could be saturated with by-products such as NH_3 and propionic acid, inhibitory compounds to methanogenesis (Barredo and Evison, 1991; Barret et al., 2013b; Zhang et al., 2014). Moreover, the abundance of total archaeal communities also showed similar trends as methanogens, suggesting the importance of reducing excess substrate loads for the functioning of most archaeal communities. On the other hand, increased abundance of methanogenic and total archaeal communities with storage time could be due to the availability of more methanogenic substrates generated from the available TS of slurries (Park et al., 2006).

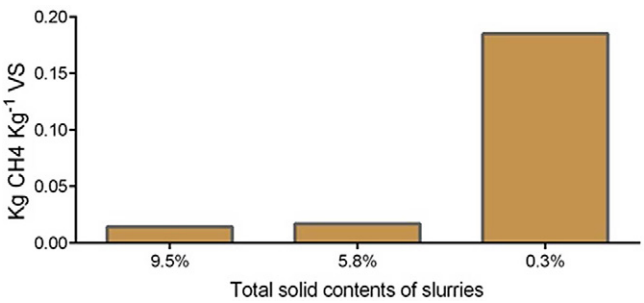

Fig. 3. Volatile solid (VS)-to-CH_4 conversion rates in 9.5, 5.8, and 0.3% total solid dairy slurries.

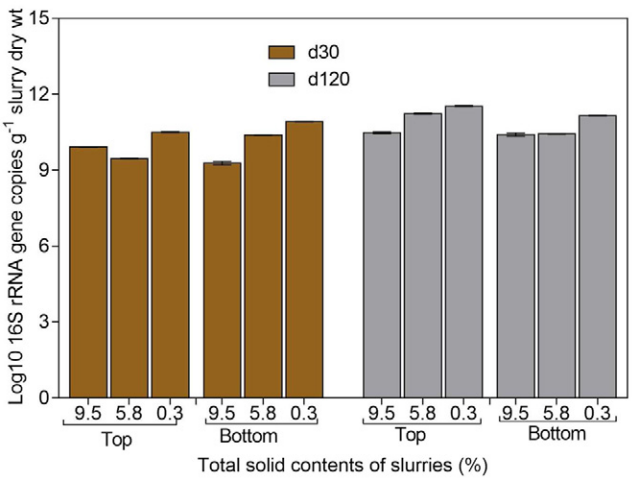

Fig. 4. Total archaeal abundance (Log10 copies 16S rRNA genes g^{-1} of dry slurry) in dairy slurries containing total solid contents of 9.5, 5.8, and 0.3% after Day 30 and Day 120 of the storage. Standard errors were calculated from the three replicate polymerase chain reactions.

The few DGGE fingerprints observed for *mcrA* gene fragments in all of the tanks, particularly after Day 120 of storage, may indicate that most methanogenic phylotypes that are likely occurring in the fresh manure could not adapt in the stored slurries. The DGGE bands observed in 9.5% TS but not in 5.8 and 0.3% TS slurries were likely due to a 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 a sufficient amount of the specific substrates that they depend to thrive and hence may play a significant role in contributing to the cumulative flux. On the other hand, the predominance of *Methanocorpusculum*-related phylotypes, detected in this study, might not be surprising as their close relatives in the order *Methanomicrobiales* have often been detected in stored dairy slurries (Gagnon et al., 2011). These methanogens are known to reduce CO_2 into CH_4 using hydrogen, indicating the predominance hydrogenotrophic methanogenesis regardless of the dairy TS level in storage systems.

Conclusions

In the current survey of CH_4 flux and abundance of methanogenic communities from stored liquid dairy manure, the importance of slurry TS which is the source of VS (a proxy for microbial CH_4 production) was demonstrated. Earlier work showed a positive relationship between TS content of dairy slurries and cumulative CH_4 emission (Wood et al., 2012). Our study demonstrated that the abundance and activity of methanogenic communities were higher in tanks containing lower TS slurries, resulting in higher VS-to-CH_4 conversion rates. Therefore, cumulative CH_4 flux 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.

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