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Pathogen Reduction and Correlation to Factors Responsible for Pathogen Reduction in Dairy Farm Operations Treating Agricultural Waste

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Abstract. *Approximately 350 million tons of manure is produced annually by agriculture in the U.S. Manure disposal at large concentrated animal feeding operations presents a challenge. Anaerobic digestion of livestock manure to reduce odor and produce biogas for energy generation is a profitable waste management option on farms, but out of reach of most farmers because of high capital costs. For operations reusing solids for bedding, reduction of causative agents of mastitis is a concern. Waste management practices that cause reduction of populations of zoonotic pathogens such as Salmonella spp. and Campylobacter spp. may also reduce public health risks following runoff events. Enteric bacterial reduction in agricultural waste is likely dependent upon operational parameters, moisture, temperature, pH, influent substrate composition and type of medium. Thorough analysis to establish correlations between these different parameters has not been conducted. Our hypothesis is that pathogen concentration in samples is dependent upon physical factors (moisture content and volatile solids content), farm processes and sample type. The objective of this research is to determine factors that control pathogen reduction and/or removal during current dairy manure management practices using Real-time q-PCR techniques. Results indicate that processes including anaerobic digestion and composting cause a significant reduction of pathogens. From regression models it was observed that pathogen concentrations are strongly correlated to moisture content, volatile solids content and sample type. These results will help identify processes and operational parameters that*

yield greater pathogen reduction, allowing better recycling of farm waste solid residuals, increasing economic benefits for dairy farms.

Keywords. Anaerobic digestion, Mastitis, Zoonotic pathogen, Manure management, Real time q-PCR

Introduction

In the U.S., food products such as milk are commonly produced at concentrated animal feeding operations (CAFO). Approximately 42 million tons of dairy manure is produced every year in the U.S. (Li et al., 2009). Manure disposal at CAFOs represents a significant challenge. Traditionally, manure has been land applied making use of its fertilizer value; however, for CAFOs, available cropland for manure application may be limited (Cabrera et al., 2008). According to USDA reports from 2004, direct land application of dairy manure may result in environmental pollution to air and watersheds. In contrast to wastewater biosolids from publicly owned treatment works, pathogen reduction is not required for manure application to land. However, similar regulations for manure biosolids may be developed in the near future (Rogers and Haines, 2005). Therefore, there is a need to quantify the reduction of populations of fecal indicator bacteria such as *Enterococcus spp.* and fecal bacteroides, and enteric bacterial pathogens such as *Salmonella spp.* and *Campylobacter spp.* during manure treatments. This may also reduce public health risks following runoff events especially when treated solids replace raw manures as soil amendments (Smith et al., 1985; Elferink et al., 1998; Mladenovska et al., 2003; Wright et al., 2003).

Anaerobic digestion (AD) of livestock manures to reduce odor and produce usable biogas as a source of energy is of intense interest on U.S. dairy farms (Karim et al., 2005). Anaerobic digestion is a biochemical process that employs a range of complex microbial ecosystems to convert organic wastes into biogas (Wilke et al., 2005). Biological methane production may offset heating and electrical costs on dairies of even modest size, and for larger dairies may yield profits from net metering of electricity to the national grid. AD has long proven to be a successful and beneficial technology for the treatment of municipal waste. However, AD use on farms has generally been less successful (US-EPA Ag-Star, 2007). The cost of building and operating an anaerobic digester on dairy farms is substantial, ranging from $700,000 to $960,000 for a 500 herd dairy farm (US-EPA Ag-Star, 2009). These costs have increased substantially over the past few years, making the implementation of digestion technology out of reach for most farmers (Wright and Graf, 2003; Gooch and Pronto, 2009).

One way to improve AD system economics is to reuse solids as bedding material. In order for solids to be recycled, concerns about potentially increased levels of pathogens in the recycled bedding materials need to be addressed. For most farmers, a major concern is the outbreak of mastitis in dairy cattle. Mastitis is an inflammatory response in the udders to pathogenic infection causing decrease in milk production and visibly abnormal milk and is the most common and most costly disease of dairy cattle. Therefore, identification of process and operational parameters that yield greater pathogen reduction will allow for better recycling of solid residuals which may increase economic benefits to U.S. dairy operations.

Pathogen reduction is likely dependent upon operational parameters on farms including storage, treatment processes and influent substrate composition. To date, thorough analysis to establish correlations between these different parameters has not been conducted. (Burke, 2001; Chynoweth et al., 2002; Gerardi et al.; 2003; Koelsch et al., 2003) Indeed, the reduction of bacterial pathogens during manure management in reported scientific literature varies from 1.6 to 3.5 log orders (Horan et al., 2003; Wright et al., 2003). The variability in these findings could be attributed to the different methods used for quantification including plate counts, most probable number, fluorescence in-situ hybridization, or polymerase chain reaction techniques (Rogers et al., 2005). However, with the development and widespread use of molecular biology techniques including real-time q-PCR, the variability in data will likely decrease and more consistency in results will be expected (Valasek and Repa, 2005).

The objective of this research is to determine factors that control pathogen reduction during current dairy manure management practices using real time qPCR techniques. For dairy farms that do not have access to modern molecular biology labs for pathogen identification and quantification in farm samples, this could provide an important insight to relate characterization of sample properties to pathogen reduction. The results from this study will help decision makers on farm operations to adopt simple processes that lead to decrease in pathogen numbers based on the knowledge of various physical factors responsible for the same.

Materials and Methods

Sample collection

Bedding and manure samples were collected in different seasons (January, March, May, and October 2009) in triplicates from three different full-scale dairy operations in Northern New York. All three dairy farms are Concentrated Animal Feeding Operations (CAFOs), but manage their waste differently. Two of the three farms use sand bedding; the third uses recycled organic bedding. Sand is favored for its cleanliness and its apparent comfort for cows. The farm that reuses organic bedding separates their solids prior to anaerobic digestion of the liquid manure, and composts them before reuse for bedding. One farm using sand bedding uses a Mc-Lanahan separator to recycle the sand for reuse and the third farm land-applies its sand-laden dairy manure without any recycling.

Sample characterization

Total solids, volatile solids, COD and pathogen concentrations were tested as described below. Sample analysis for each parameter was performed in triplicate.

Chemical Oxygen Demand

COD was determined using Hach COD digestion vials (High Range Plus COD Reagent, Hach Company, Loveland, CO), following the dichromate COD analysis Method 5220 part D in Standard Methods (APHA, 2005). Standard curves were prepared from 10,000 ppm COD solution (Thermo Electron Corporation, Beverly, MA, USA) with five dilutions of the stock to relate absorbance at 620 nm to concentration ($R^2 > 0.99$), and were used to estimate the COD of the samples. Samples were measured at 620 nm using a spectrophotometer (Shimadzu spectrophotometer, Model # 206-82301-92, Kyoto, Japan). Samples were diluted with de-ionized water so that results would fall within the range of the test kit (200-15,000 mg COD/L).

Total Solids

Total solids were determined as described by Method 2540 part D in Standard Methods (APHA, 2005). In summary, approximately 20 to 30 gram samples by weight were added to clean glass Petri dishes. The weights of the dishes prior to and after the additions were recorded (Scout Pro Scale, Cole Parmer, Vernon Hills, IL, USA) to obtain sample masses. Samples and dishes were placed in a drying oven (Thermo Electron Lindberg, Model # G01305A-1, Asheville, NC, USA) at 103-105ºC for 24 hours, allowing for all moisture to evaporate. Samples and dishes were allowed to cool to room temperature in a desiccator for approximately 12 hours before re-weighing. The dried sample weight was divided by the initial sample weight to obtain solids content in the sample. The moisture content of the sample expressed as a percentage is equal to the percent TS subtracted from 100. The samples were then ignited at 550ºC in a combustion oven (General Signal Lindberg, Model # 51894, Watertown, WI, USA) for 4 hours allowing all the volatile solids to combust. As before, samples and dishes were re-weighed after being allowed to cool to room temperature in a desiccator for approximately 12 hours. The combusted

sample weight was divided by the dried sample weight to obtain the ash content of the dry sample (Method 2540 part E, Standard Methods, APHA, 2005). Volatile solids content of the dried sample expressed as a percentage is equal to the percent ash subtracted from 100.

Genomic DNA extraction from pure cultures

Extraction of genomic DNA from bacterial cultures was performed using the MoBio Ultraclean Microbial DNA Isolation kit (MoBio; Carlsbad, CA) as per the manufacturer's protocol. Genomic DNA was extracted from 1.8 mL of overnight bacterial culture cultivated at 37ºC in an incubator (National Appliance, Model # 5100, Portland, OR) in Luria Broth (LB) (Difco; Lawrence, KS) as per protocol given by ATCC (American Type Culture Collection, Manassas, VA).

Genomic DNA from manure and bedding samples

Extraction of bacterial genomic DNA from manure and other samples was carried out using the MoBio PowerSoil DNA Extraction kit (MoBio; Carlsbad, CA) as per the manufacturer's protocol. DNA was extracted from 250 µl or 250 mg of sample. Prior to lysis of cells by bead milling, each sample tube was spiked with Salmon Testes gDNA (Invitrogen; Carlsbad, CA) at a concentration of 2.5 ng/reaction as an exogenous extraction and amplification control. Percent recovery of Salmon Testes gDNA was measured by qPCR as described below, based on expected concentrations in extraction eluents. The percent recoveries for each sample were used to account for potential loss of bacterial gDNA during the extraction process and subsequent PCR inhibition. For the lysis step, the MoBio PowerSoil bead tubes containing the samples were bead milled at 6.0 m/s for thirty seconds using the Fast Prep-24 Bead Beater (MP Biomedicals; Solon, OH).

Determination of DNA Concentration

All DNA concentrations were determined by measuring fluorescence at room temperature using the Quant-iT dsDNA BR Assay kit with the Qubit fluorometer (Invitrogen; Carlsbad, CA) as per manufacturer's protocol. All DNA was stored at -80°C.

Polymerase Chain Reaction (PCR)

All PCR reaction mixes were prepared in a PCR cabinet (ESCO Technologies, Inc., Hatboro, PA) located in a separate laboratory where no template DNA or PCR products were permitted to be stored. Reaction mixes were then taken to a separate room where template DNA was added.

Primers and probes

All primer and probe sets were obtained from Applied Biosystems (ABI; Forster City, CA). Lyophilized primers were re-suspended in TE buffer to a final master stock concentration of 100.0 pmol/µl and stored at -20.0 °C. Probes were received in solution at a master stock concentration of 100.0 pmol/µl and were stored at -20.0 °C. Working concentrations of primers and probes were diluted to 10.0 pmol/µl in TE buffer. Table 1 shows the primer/probe sets and associated bacterial markers used throughout this study.

Table 1. Primer and probe sequences used for Real Time qPCR during the course of this study

Method	Target	Locus	Forward and Reverse Primer and Probe	Reference(s)
Zoonotic Bacterial Pathogens and Fecal indicators				
Salmonella, qPCR	*Salmonella* spp.	ttrRSBCA	CTCACCAGGAGATTACAACATGG **(F)** AGCTCAGACCAAAAGTGACCATC **(R)** 6FAM-CACCGACGGCGAGACCGACTTT **(P)**	Malorny et al. (2004)
Enterococcus, qPCR	*Enterococcus* spp.	16S rDNA	AGAAATTCCAAACGAACTTG **(F)** CAGTGCTCTACCTCCATCATT **(R)** 6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA **(P)**	Haugland et al. (2006)
Fecal Bacteroides, qPCR	*Fecal Bacteroides*	16S rDNA	GGGGTTCTGAGAGGAAGGT **(F)** CCGTCATCCTTCACGCTACT **(R)** 6FAM-CAATATTCCTCACTGCTGCCTCCCGTA **(P)**	Dick and Field (2004)
Campylobacter, qPCR	*Campylobacter* spp.	16S rDNA	CACGTGCTACAATGGCATAT **(F)** GGCTTCATGCTCTCGAGTT **(R)** 6FAM-CAGAGAACAATCCGAACTGGGACA **(P)**	Lund et al. (2004)
Mastitis causing Bacterial Pathogens				
Klebsiella pneumoniae, qPCR	*Klebsiella pneumoniae*	16S rDNA	CCTGGATCTGACCCTGCAGTA **(F)** CCGTCGCCGTTCTGTTTC **(R)** CAGGGTAAAAACGAAGGC **(P)**	Shannon et al. (2007)

Quantitative PCR (qPCR)

A hydrolysis probe assay was used for the designated primer/probe sets shown in Table 1. In the PCR assay, each 25.0 µl reaction mixture contained the following: 12.5 µl of 2X LightCycler 480 Probes Master (Roche; Basal, Switzerland), 12.5pmol of each forward and reverse primer, 5.0pmol of fluorogenic probe, 5.0µl template DNA, and PCR-grade water (Roche; Basal, Switzerland). A mixture of all the PCR reagents without template DNA but substituted by PCR grade water was used as a negative control for each PCR assay. A known concentration of genomic DNA (gDNA) was used as a positive control for each PCR assay.

Genomic DNA was extracted from bacterial cells harvested during log-phase growth, the gDNA was measured using the Quant-iT dsDNA BR Assay kit with the Qubit fluorometer as per manufacturer's protocol, and serially diluted to generate standard curves. All qPCR reactions were performed in a LightCycler 480 in 96 Multiwell Plates (Roche; Basal, Switzerland). Each reaction began with an incubation period of 95°C for 10 minutes, followed by 45 cycles of primer/probe dependent cycling parameters. Concentrations of the various gDNA targets shown in Table 1 were extrapolated from the standard curves using the 2nd derivative max calculation in the LightCycler 480 Software 1.5.0 (Roche; Basal, Switzerland). Populations of organisms/g dry sample were estimated by dividing the measured concentration of gDNA by the genome weight of each organism of interest.

Statistical analysis

Statistical analysis using Minitab 15 (State College, PA, USA) was performed to determine the effect of various predictor variables on the concentration of each microbial group: *Campylobacter* spp*, Enterococcus* spp, Fecal Bacteroidales and *Klebsiella pneumoniae.* Log values of bacterial concentrations (#/g dry sample) were calculated. Data was analyzed for samples arranged accordingly for various enteric bacteria and also for the sampling times (January, March, May, and October 2009) to check the affect of the predictor variables on concentrations of the enteric bacteria. The Null Hypothesis was that there was no effect on concentrations of enteric bacteria due to these factors. If p-values found were less than the level of significance ($\alpha = 0.05$, in our case) the Null Hypothesis was rejected. It is to be noted here that bacterial cells being composed of volatile solids, dry matter content of cells contributes a significant amount of volatile solids measured in samples (Bratback and Dundas, 1984; Neidhardt et al., 1990; Loferer-Kro¨ßbacher et al., 1997). In the samples that we tested, bacterial mass makes up to 0.5% dry mass of volatile solids in some samples. Therefore, volatile solids cannot be treated as an independent variable with respect to bacterial concentration but serves as a predictor variable for the same.

Results and Discussion

Comparison of bacterial populations in the influent and effluent samples from the digester at farm three indicated a two log order reduction of bacterial populations per gram dry sample (Fig. 1). Individual bacterial concentrations in the digester effluent varied significantly. Digester effluent concentrations (in #/gram of dry solids) of *Campylobacter*, *Enterococcus*, Fecal bacteroides and *Klebsiella* were 1.17E+03, 8.54E+01, 6.06E+05, 6.66E+02 respectively.

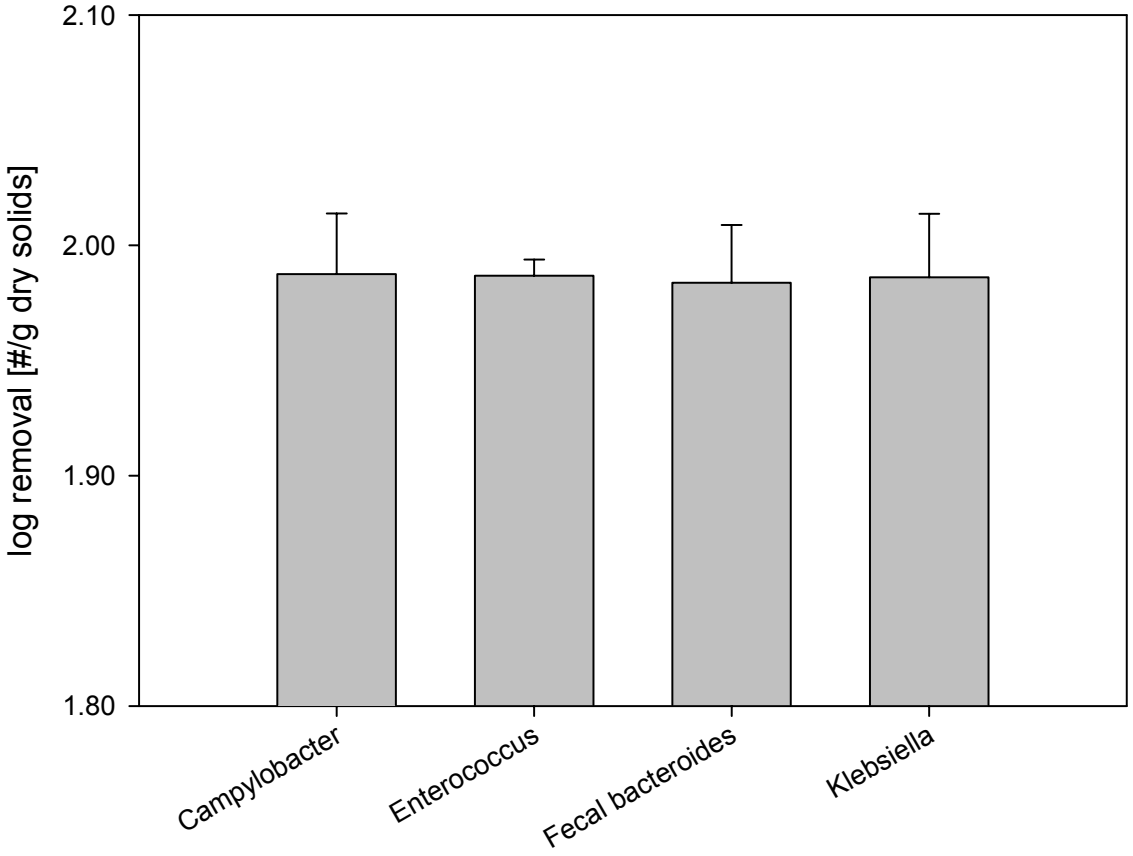

Figure 1. Mean log removal of enteric bacteria during anaerobic digestion. Error bars represent ± one standard deviation of all samples (n=12)

This generally agrees with studies conducted by Bendixen et al. (1992), Horan et al. (2004) and Gooch et al. (2006), who investigated the reduction of *E. coli, Streptococcus, Salmonella, Campylobacter* and *Clostridium* by plate count techniques.

In comparison of bacterial populations in non-composted solids versus composted solids also indicated a two log order reduction of bacterial populations per gram dry sample due to composting (Fig. 2). As with the digester effluent individual bacterial concentrations varied significantly. Composter effluent concentrations (in #/gram of dry solids) of *Campylobacter*,

Enterococcus, Fecal bacteroides and *Klebsiella* were 1.32E+02, 1.41E+02, 1.09E+06, 2.1E+02 respectively.

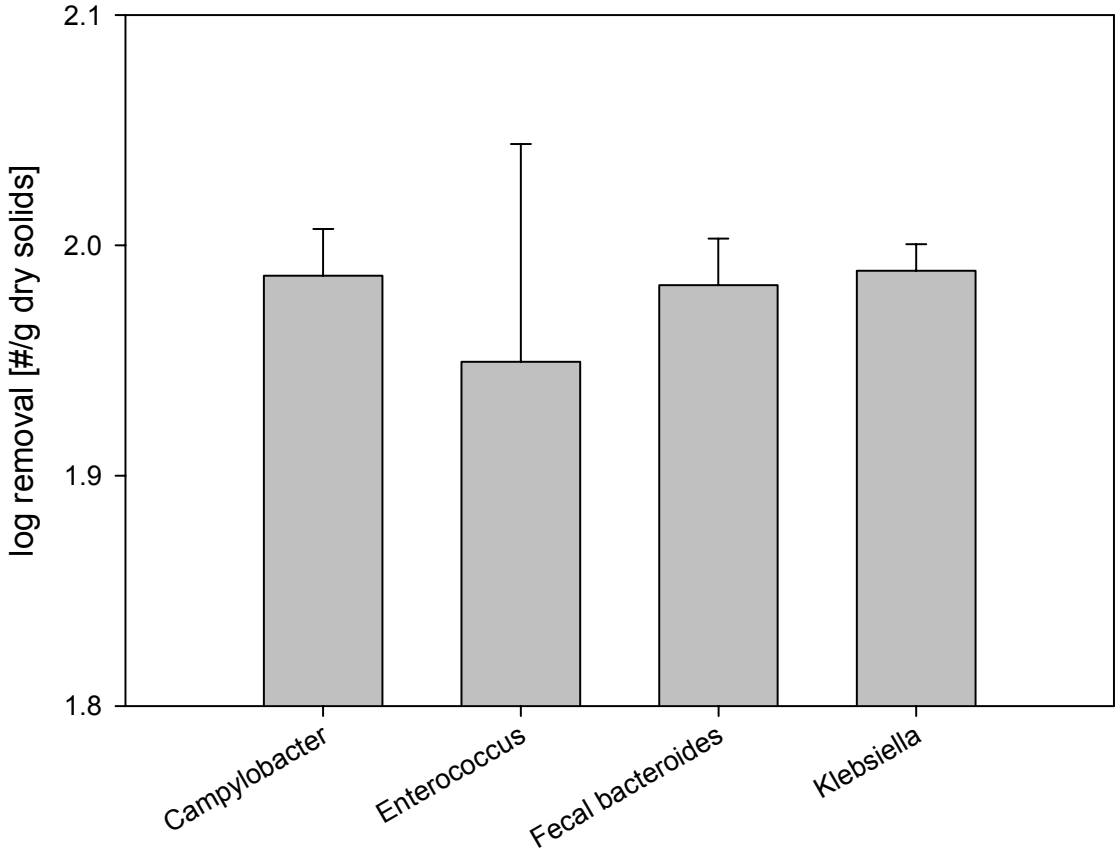

Figure 2. Mean log removal of enteric bacteria during composting. Error bars represent ± one standard deviation of all samples (n=12)

Similar reductions in *Listeria, Salmonella* and *E. coli* during composting have been reported by Connor and Blake (1990) and Sikora (2002).

Solids characterization of samples indicated that sand laden manure streams had ~50-70% moisture per total weight and 6-8% volatile solids per dry weight basis. Similarly manure streams without sand had 90-95% moisture per total weight and 8-10% volatile solids per dry weight. Organic solids retained 35-45% moisture per total weight and 30-40% of their dry weight was volatile solids. Sand samples had 6-9% moisture and 0.5-2% volatile solids. Statistical analysis on predictor and affecting variables showed that moisture, volatile solids content, sample type (manure, sand or organic bedding) and waste treatment processes (anaerobic digestion, composting etc.) have significant effects on concentration of enteric bacteria whereas sample location had little or no effect on enteric bacteria concentration (Table 2).

Table2. P-values from Kruskal-Wallis test to determine the effect of various parameters on concentration of selected bacterial species

Bacteria	Farm	Process on Farm	Sample Type	Moisture	Volatile Solids
Campylobacter	0.798	0.018	0.004	0.028	0.011
Enterococcus	0.762	0.017	0.028	0.011	0.003
Fecal bacteroides	0.981	0.027	0.032	0.05	0.029
Klebsiella	0.852	0.031	0.019	0.023	0.049

$P \leq 0.05$ → Strong effect on bacterial concentrations
$P > 0.05$ → Weak/no effect on bacterial concentrations

Bedding type had a significant effect on enteric bacterial concentration; sand bedding had a one to two log order lower enteric bacterial concentration which confirms to studies by Hogan et al. (1999), Harner et al. (2005) and Kristula (2005). Also, as expected, moisture content and volatile solids content are important factors on enteric bacteria concentrations also shown by Hogan et al. (1989) and Kristula et al. (2005).

Statistical analyses confirm a significant decrease in bacterial populations due to anaerobic digestion and composting. Also, sand harbors one to two log orders fewer bacteria than organic bedding. Unused virgin sand has the least number of enteric bacteria followed by recycled sand and bedding sand, respectively. Organic bedding solids similarly contain a half to one log order higher concentration of enteric bacteria after bedding than before bedding. Taking parameters that affect bacterial concentrations such as moisture and volatile solids content one at a time as a predictor variable, correlation and linear regression analysis was done for the response (log values of enteric bacteria). Moisture content of samples shows a strong positive correlation to enteric bacterial populations (Pearson's correlation constant r = 0.47). However, out of the four enteric bacterial species, no effect of moisture content was observed for *Enterococcus* populations (r=0) within the range of this study; the other three bacterial species exhibited strong positive correlations. This result needs further investigation.

It was found that sand laden samples showed a strong effect to variation in volatile solids content of samples with respect to enteric bacterial populations. This is evident in Fig. 3 which shows the regression plot of *Campylobacter* spp concentrations (log values) with volatile solids content (% dry weight) [R^2 =0.48] Similar effects were seen for all the other enteric bacterial populations, tested. Concentrations of enteric bacterial pathogens in organic bedding samples did not demonstrate strong correlations to volatile solids content; because of the high initial volatile solids content of organic bedding, treatment had little effect on total volatile solids.

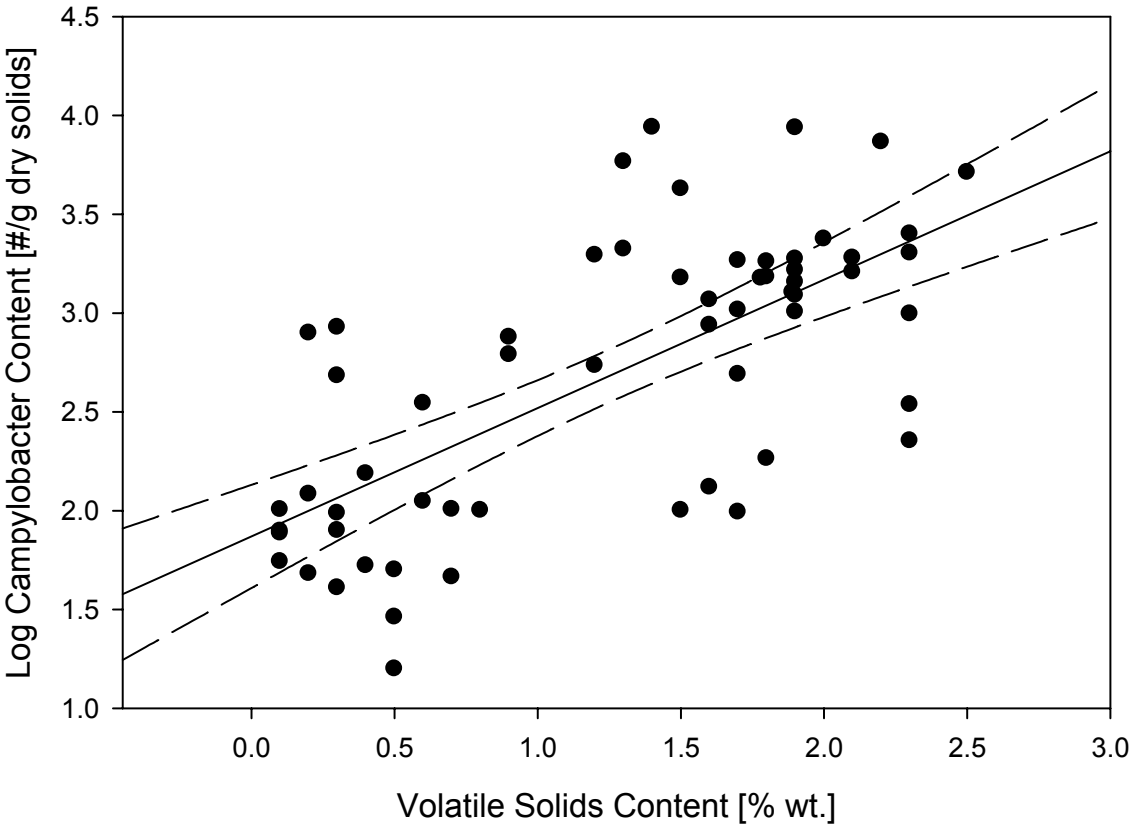

Figure 3. Regression plot of *Campylobacter* populations (log values) versus volatile solids (% weight) in sand samples showing that volatile solids content effect concentration of bacterial populations to a significant extent in inorganic media like sand

Conclusion

Anaerobic digestion and composting at high temperatures were shown to be good methods to achieve bacterial and pathogen reduction in dairy waste samples. A positive correlation was observed between the moisture level and volatile solids content of the samples and their bacterial concentrations, individually and for all enteric bacteria. Bedding solids harbored enteric bacteria irrespective of being organic or inorganic. However, bacterial concentrations in inorganic bedding samples were significantly lower than organic bedding samples. Sand recycle by Mc-Lanahan separator on farm two helped in achieving bacterial reduction. Enteric bacterial concentrations in recycled sand were about 1 log order less than in used bedding sand (before treatment), but were still 1 to 1.5 log orders greater than fresh (unused) sand. Individual bacterial concentrations tested in the samples were variable. This suggests a need to test for more than fecal indicator bacterium or only few selected pathogens for prevention of disease outbreaks on dairy farms. Using all enteric bacterial concentrations together for statistical analysis does not show strong effects of the predictor variables on the response variable. However, when data is arranged according to sample type (for example, sand laden samples and organic samples), the affect of the predictor variables is well marked. Moreover, these factors play a greater role on sand bedding samples rather than organic bedding samples.

More in-depth research with larger sample sizes needs to be completed in order to derive improved models for predicting the affects of the physical parameters and type of medium on pathogen reduction in manure treatment processes. Results from this research will be helpful in laying the guidelines for such future work.

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