Exhibit H

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Fate of Manure-Borne Pathogens during Anaerobic Digestion and Solids Separation

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Abstract

Anaerobic digestion can inactivate zoonotic pathogens present in cattle manure, which reduces transmission of these pathogens from farms to humans through the environment. However, the variability of inactivation across farms and over time is unknown because most studies have examined pathogen inactivation under ideal laboratory conditions or have focused on only one or two full-scale digesters at a time. In contrast, we sampled seven full-scale digesters treating cattle manure in Wisconsin for 9 mo on a biweekly basis (n = 118 pairs of influent and effluent samples) and used real-time quantitative polymerase chain reaction to analyze these samples for 19 different microbial genetic markers. Overall, inactivation of pathogens and fecal indicators was highly variable. When aggregated across digester and season, log-removal values for several representative microorganisms—bovine *Bacteroides*, *Bacteroidales*-like CowM3, and bovine polyomavirus—were 0.78 ± 0.34, 0.70 ± 0.50, and 0.53 ± 0.58, respectively (mean ± SD). These log-removal values were up to two times lower than expected based on the scientific literature. Thus, our study indicates that full-scale anaerobic digestion of cattle manure requires optimization with regard to pathogen inactivation. Future studies should focus on identifying the potential causes of this suboptimal performance (e.g., overloading, poor mixing, poor temperature control). Our study also examined the fate of pathogens during manure separation and found that the majority of microbes we detected ended up in the liquid fraction of separated manure. This finding has important implications for the transmission of zoonotic pathogens through the environment to humans.

Core Ideas

- Pathogen inactivation is highly variable among full-scale anaerobic digesters.
- Pathogen inactivation by full-scale digesters on cattle farms needs optimization.
- Most microbes end up in the liquid fraction during solids separation of manure.

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Cattle manure is an important reservoir of infectious disease. It contains pathogens that are specific to the cattle themselves (i.e., bovine viruses) as well as zoonotic pathogens that can infect both cattle and humans. Major zoonotic pathogens commonly found in cattle manure include *Campylobacter*, *Escherichia coli* O157:H7, *Salmonella*, *Listeria*, *Cryptosporidium*, and *Giardia* (USEPA, 2013). Although many of these pathogens can be carried asymptomatically in cattle, they cause acute gastrointestinal disease in humans. Symptoms can include diarrhea, vomiting, fever, and nausea, as well as other more severe and/or pathogen-specific conditions (USEPA, 2013).

Most cattle manure is land applied because it is an important source of crop nutrients and there are few alternative disposal options. However, the majority of this manure—520 Tg yr^{-1} in the United States (USEPA, 2013)—typically undergoes little to no treatment prior to land application. Thus, it can contaminate surface water (Corsi et al., 2014) and groundwater (Arnaud et al., 2015) with zoonotic pathogens via runoff and infiltration, respectively. This contamination with zoonotic pathogens can, in turn, spread waterborne disease to humans through both recreational and drinking water (USDA, 2012). Land-applied manure can also contaminate crops, creating the opportunity for disease transmission via food.

Anaerobic digestion is a microbial process that degrades organic matter to produce biogas and digestate. It can also inactivate zoonotic pathogens in cattle manure, but the relevant body of knowledge is limited in several important respects. First, most pathogen inactivation studies related to anaerobic digestion have been conducted at the laboratory scale; only a handful have been conducted on full-scale digesters (Berg and Berman, 1980; Kearney et al., 1993; Gantzer et al., 2001; Massé et al., 2011). Furthermore, the full-scale studies that do exist investigate only one or two digesters each. These studies cannot characterize variation in pathogen inactivation that would be expected to occur as a result of operational differences among farms.

In addition to these limitations, current knowledge is also lacking with respect to the fate of pathogens after solids separation of cattle manure. Solids separation of cattle manure, particularly after anaerobic digestion, is a common practice. This

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Abbreviations: Cq, cycle of quantification; qPCR, quantitative polymerase chain reaction.

practice has important implications for pathogen transmission because the separated solids and liquids are managed independently. Separated solids are commonly reused as bedding material for dairy cattle (Husfeldt et al., 2012); the residual pathogens in separated solids can present a direct health risk to the cattle and might also contaminate raw milk. On the other hand, separated liquids are commonly land applied, and as described above, residual pathogens in land-applied material can contaminate surface water and groundwater.

We pursued two research objectives to address these limitations. First, we sought to quantify the extent of pathogen removal in full-scale anaerobic digesters treating cattle manure, including characterization of variability across digesters. Second, we determined the relative levels of pathogens in separated liquids compared with separated solids.

Our approach focused on enrolling a relatively large number of facilities in frequent sampling over a long time span. The study included seven facilities with full-scale digesters treating cattle manure and other substrates, as well as two facilities with no digesters and only solids separation of raw manure (n = 9 facilities total). We collected samples at these facilities on an approximately biweekly basis for 9 mo, which allowed for characterization of seasonal variation in addition to variation across facilities. Samples were analyzed to determine detection frequencies and concentrations of 19 microbial genetic markers, including bovine pathogens, zoonotic pathogens, and fecal indicators. Finally, for frequently detected microbes, we also calculated the extent of removal achieved by digesters on the basis of both specific digester and season.

Materials and Methods

Facility Characteristics and Sampling Plan

We sampled at nine facilities (seven dairy farms, one community digester, and one abattoir) in southern and eastern Wisconsin (Table 1). Eight of these facilities processed dairy manure as the main feedstock, whereas the ninth—the abattoir—primarily processed paunch manure. Seven of the nine facilities employed anaerobic digesters; two facilities, both of which were dairy farms, did not. All facilities separated solids. Eight facilities used a screw press, and one used a centrifuge. All facilities with anaerobic digesters separated solids after digestion. In addition to solids separation, three facilities, all of which were dairy farms, also employed some form of secondary solids treatment after separation. Two of these three facilities used an aerobic bedding recovery unit. The third used a blower to dry solids but did not begin using it until the end of May 2012, which was partway through the study (see below for the sampling timeframe).

The seven anaerobic digesters in our study included five plug-flow and two complete-mix designs. All were operated at mesophilic temperature range (~37°C) with nominal residence times of 20 to 30 d. Six digesters treated dairy manure, and one treated paunch manure. Five digesters primarily served individual dairy farms, whereas one was a community digester serving multiple users, including three dairy farms. For digesters serving individual dairy farms, herd sizes varied between 1000 and 5000 head. In addition to manure, two digesters also treated food waste; one treated fats, oils, grease, and ethanol byproducts; and one treated bunker waste.

All facilities were sampled approximately biweekly from December 2011 to August 2012 (n = 17 sampling events per facility). Digesters were sampled for influent (i.e., raw manure) and effluent (i.e., digestate) for each event. Solids separators were sampled for influent, separated liquids, and separated solids for each event. For facilities with digesters, separator influent was the same as digester effluent. For facilities without digesters, separator influent was raw manure. Secondary solids treatment units were sampled for influent (i.e., separated solids) and effluent.

Finally, in addition to the pathogen inactivation study reported here, a related study with a partially overlapping sampling period (September 2011–May 2012) was conducted to characterize the general operating performance of digesters and solids separators at the same nine facilities. This study collected biweekly measurements of total solids, volatile solids, chemical oxygen demand, total phosphorus, total nitrogen, ammonium, and potassium in raw manure, digestate, separated liquids, separated solids, and separated solids having undergone secondary treatment. The results of this related study will be published in detail elsewhere, but we will occasionally refer to them here when they provide useful context.

Sample Collection, Processing, and Analysis

We collected samples in 1-L polypropylene containers, transported them to the laboratory on ice, and stored them refrigerated at 4°C. Samples were processed within 48 to 120 h after collection.

Initial sample processing varied according to sample type. Samples of raw manure, digestate, and separated liquid (50 mg wet mass) were diluted 1:10 in TE buffer (Qiagen). Samples of separated solids or solids having undergone secondary treatment were eluted to remove microorganisms in a manner similar to our procedure for eluting glass wool filters (Lambertini et al., 2008;

Table 1. Summary of sampled facilities.

Facility	Facility type	Digester type	Separator type	Feedstock	No. of animals
1	Dairy farm	Plug flow	Screw press	Dairy manure	4600
2	Dairy farm	None, separator only	Screw press	Dairy manure	700
3	Dairy farm	Complete mix	Screw press with blower	Dairy manure	1400
4	Dairy farm	Plug flow	Screw press	Dairy manure	2600
5	Abattoir	Plug flow	Screw press	Paunch manure, feed wastes	2100, processed daily
6	Dairy farm	Plug flow	Screw press	Dairy manure	2600
7	Dairy farm	Plug flow	Screw press	Dairy manure	1400
8	Community digester serving three dairy farms	Complete mix	Centrifuge	Dairy manure, ethanol byproduct, miscellaneous	Not available
9	Dairy farm	None, separator only	Screw press	Dairy manure	1200

see Supplemental Material for details). This procedure produces a final concentrated sample volume containing the once solid-associated microorganisms.

Nucleic acid extractions were performed using a QIAamp DNA blood mini kit and buffer AVL (Qiagen). For raw manure, digestate, and separated liquid, 140 μL of diluted sample was extracted; for separated solids, 140 μL of final concentrated sample volume was extracted. Additionally, three consecutive freeze-thaw steps using liquid nitrogen and boiling water were added at the beginning of the extraction procedure for samples analyzed for *Cryptosporidium parvum* (Di Giovanni and LeChevallier, 2005).

All microbes were quantified by quantitative polymerase chain reaction (qPCR). The RNA microbes were reverse transcribed in a separate reaction before qPCR (see Supplemental Material for further details). We used published primers and hydrolysis probes for these techniques to quantify all organisms in our study (Table 2). All qPCR assays of extracted samples were performed in duplicate. Standard curves were constructed from 10-fold serial dilutions of gBlocks or Ultramers (depending on target length) (Integrated DNA Technologies) in 0.02% bovine serum albumin. Amplification efficiencies of standard curves varied from 0.78 to 1.01 with mean square error ≤ 0.16.

Laboratory controls included both negative and positive controls. Negative controls for each qPCR assay were used to monitor for contamination at three different steps: extraction, reverse transcription, and qPCR. Extraction negative controls were prepared by extracting nucleic acids from AE buffer (Qiagen) in lieu of sample. Reverse transcription and qPCR negatives consisted of nuclease-free water. A positive control was used for each qPCR assay to ensure amplification was successful; this positive control also served as the reference control relating individual analyses back to their standard curves. Analyses were repeated if any of the three negative controls were positive. They were also repeated if the cycle of quantification (Cq) value of the positive control varied from its expected value by 0.5 cycles or more.

Finally, in addition to negative and positive controls, we also used inhibition controls to test for qPCR inhibition in every sample prior to quantification (see Supplemental Material for details). Samples were diluted to mitigate qPCR inhibition according to the difference between measured and expected Cq values for inhibition controls. Samples with measured Cq values less than two cycles higher than expected were not diluted. Samples with measured Cq values two cycles higher were diluted 1:5, and those with Cq values greater than two cycles were diluted 1:10.

Data Analysis

Substantial data analysis was only required to achieve our first objective—quantification of pathogen removal in anaerobic digesters. Digester data were aggregated into 21 unique combinations of season ($n = 3$) and digester ($n = 7$). Season was defined as winter (December, January, or February), spring (March, April, or May), and summer (June, July, or August). Within each combination, digester influent and effluent concentrations were log-transformed (base 10), and log removal was calculated as the mean of transformed effluent concentrations subtracted from the mean of transformed influent concentrations. Log removal values were analyzed using two-way ANOVA with digester and season as factors and an α of 0.05.

Results and Discussion

Digester Performance

Anaerobic digestion inactivated fecal indicators, bovine pathogens, and human pathogens. Detection frequencies of virtually all microbes that we measured were lower in digester effluent than influent (Table 3). In fact, the data for most microbes contained such high proportions of nondetects that log removal could only be estimated robustly (by season and digester) for two fecal indicators and a bovine pathogen: bovine *Bacteroides*, *Bacteroidales*-like CowM3, and bovine polyomavirus. Overall log removal values for these three microbes were 0.78 ± 0.34, 0.70 ± 0.50, and 0.53 ± 0.58, respectively (mean $\pm$ SD, Table 4). These mean log removal values correspond to 83, 80, and 70% removal, respectively (percent removal = $1 - 10^{-\text{log removal}}$).

Although anaerobic digestion inactivated many microbes, the extent of inactivation was also highly variable (Table 4). Bovine *Bacteroides* log removal values varied significantly by both digester and season ($P = 0.02$ for both). Bovine polyomavirus varied only by digester ($P = 0.04$), and *Bacteroidales*-like CowM3 varied only by season ($P < 0.001$), although digester was statistically significant at an α of 0.1 ($P = 0.07$) for the latter. Log removal for bovine *Bacteroides*, *Bacteroidales*-like CowM3, and bovine polyomavirus correlated moderately with each microbe's concentration in digester influent (Pearson's correlation coefficients = 0.62, 0.70, and 0.68, respectively; $P < 0.01$ for all). This could indicate that some of the variability in log removal was due to variability in influent microbe concentrations, rather than to digester performance per se.

Because the other microbes tested were detectable in only 15 to 20% of digester effluent samples (and 50–90% of influent samples), we could only estimate log removal for them by assuming a concentration for nondetections. Rather than assuming one value, we chose to estimate log removal using two values that likely bracket the range in which the true log removal rate most likely exists. First, we assumed that nondetections correspond to microbe concentrations of zero copies per wet gram; this value would most likely underestimate many digester effluent concentrations and thereby cause us to overestimate log removal. Alternatively, we assumed that nondetections were equal to our qPCR detection limits; this value would most likely overestimate many digester effluent concentrations, thereby causing us to underestimate log removal.

Using this approach, minimum log removal—defined by assuming that nondetections are equal to our qPCR detection limit—varied substantially across microorganisms (Table 5). Minimum log removal ranged from −0.01 for *Salmonella* spp. and bovine adenovirus to 0.79 for bovine *Bacteroides* (although caution should be exercised when interpreting log removal values for microbes with particularly low detection frequencies in digester influent, such as bovine adenovirus and enterohemorrhagic *Escherichia coli*). Similarly, maximum log removal—defined by assuming that nondetections are equal to a microbe concentration of zero copies per wet gram—ranged from 0.44 for *Clostridium perfringens* to >2 for bovine coronavirus and enterohemorrhagic *E. coli*. Also of note, the uncertainty in our

Table 2. Target organisms (target gene) and oligonucleotide sequences.

Organism or target name	Sequence (5′–3′)	Reference
Bacteroidales-like CowM3 (HD super family hydrolase)		Shanks et al., 2008
Forward primer	CCTCTAATGGAAAATGGATGGTATCT	
Reverse primer	CCATACTTCGCCTGCTAATACCTT	
Probe	TTATGCATTGAGCATCGAGGCC	
Bovine adenovirus (hexon)		Wong and Xagoraraki, 2010
Forward primer	CRAGGGAATAYYTGTCTGAAAATC	
Reverse primer	AAGGATCTCTAAATTTYTCTCCAAGA	
Probe	TTCATCWCTGCCACWCAAAGCTTTTTT	
Bovine *Bacteroides* (16s rRNA†)		Mieszkin et al., 2010
Forward primer	ACAGCCCGCGATTGATACTGGTAA	
Reverse primer	CAATCGGAGTTCTTCGTGAT	
Probe	ATGAGGTGGATGGAATTCGTGGTGT	
Bovine coronavirus (M protein)		Abd-Elmaksoud et al., 2014
Forward primer	ATTAGAACTGGAAGTTGGTGGA	
Reverse primer	TCACATAAGCTGGCAAATCT	
Probe	ACAATAATACGTGGTCATCTTTACATGCAAG	
Bovine enterovirus (5′ noncoding region)		Gibson and Schwab, 2011
Forward primer	GCCGTGAATGCTGCTAATCC	
Reverse primer	GTAGTCTGTTCCGCCTCCACCT	
Probe	CGCACAATCCAGTGTTGCTACGTCGTAAC	
Bovine rotavirus (*VP7*)		Chang et al., 1999
Forward primer	TGCCACACTGTTGTCAATATTA	
Reverse primer	TCCTCTGCTGTTGGGAAAAGTT	
Probe	GGTAAGCCGCTAGAAGCAGATTTGACAGTG	
Bovine rotavirus C (*VP6*)		Chang et al., 1999
Forward primer	GAAGCTGTATGTGATGATGA	
Reverse primer	AGAATATATGAATTTCTATATTCAAA	
Probe	CAACGTTAATCGCATTAGCTTCA	
Bovine polyomavirus (*VP1*)		Wong and Xagoraraki, 2011
Forward primer	TGGCTTTCTGACTCAGCCAAA	
Reverse primer	TCTCTTCCTGAGAGTCACAGACATG	
Probe	ACCAACAGCAATTTAGAGGCCTTCCCAG	
BVDV 1 and 2‡ (5′ noncoding region)		Brooks et al., 2007
Forward primer	TAGCCATGCCCTTAGTAGGAC	
BVDV1 reverse primer	GACGACTACCCTGTCCTCAGG	
BVDV2 reverse primer	GACGACTCCCCTGTACTCAGG	
BVDV1 probe	CAGTGGTGAGTTCGTTGGATGGCT	
BVDV2 probe	AGGGGACTAGCGGTAGCAGTGAGTTC	
Campylobacter jejuni (*mapA*)		Best et al., 2003
Forward primer	CTGGTGGTTTTGAAGCAAAGATT	
Reverse primer	CAATACCAGTGTCTAAAGTGCGTTTAT	
Probe	TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT	
Clostridium perfringens (*cpA*)		Gurjar et al., 2008
Forward primer	TGCACTATTTTGGAGATATAGATAC	
Reverse primer	CTGCTGTGTTTATTTTATACTGTTC	
Probe	TCCTGCTAATGTTACTGCCGTTGA	
Cryptosporidium parvum (*hsp-70*)		Di Giovanni and LeChevallier, 2005
Forward primer	TCCTCTGCCGTACAGGATCTCTTA	
Reverse primer	TGCTGCTCTTACCAGTACTCTTATCA	
Probe	TGTTGCTCCATTATCACTCGGTTTAGA	
Escherichia coli O157:H7 (*eae*)		Ibekwe et al., 2004
Forward primer	GTAAGTTACACTATAAAAGCACCGTCG	
Reverse primer	TCTGTGTGGATGGTAATAAATTTTTG	
Probe	AAATGGACATAGCATCAGCATAATAGGCTTGCT	

Table 2. Continued.

Organism or target name	Sequence (5′–3′)	Reference
Giardia lamblia (beta giardin gene)		Baque et al., 2011
Forward primer	GGCCCTCAAGAGCCTGAAC	
Reverse primer	GGGCGATCGTCTCCTTCTC	
Probe A	AGACGGGCATTGCCA	
Probe B	CTCGAGACAGGCATC	
Mycobacterium avium subsp. *paratuberculosis* (*MAP-900*)		Beumer et al., 2010
Forward primer	CCGCTAATTGAGAGATGCGATTGG	
Reverse primer	ATTCAACTCCAGCAGCGCGGCCTC	
Probe	TCCACGCCCGCCCAGACAGG	
Salmonella spp. (*invA*)		Hoorfar et al., 2000
Forward primer	TCGTCATTCCATTACCTACC	
Reverse primer	AAACGTTGAAAAACTGAGGA	
Probe	TCTGGTTGATTTCCTGATCGCA	
Staphylococcus spp. (16s rRNA)		Hansen et al., 2010
Forward primer	TCCTACGGGAGGCAGCAGT	
Reverse primer	GGACTACCAGGGTATCTAATCCTGTT	
Probe	AATCTTCCGCAATGGGCGAAAGC	
Streptococcus spp. (16s rRNA)		Hansen et al., 2010
Forward primer	TCCTACGGGAGGCAGCAGT	
Reverse primer	GGACTACCAGGGTATCTAATCCTGTT	
Probe	CCAGAAAGGGACSGCTAACT	

† rRNA, ribosomal RNA.
‡ BVDV, bovine viral diarrhea virus.

Table 3. Microbe detection frequencies and concentrations in digester influent and effluent samples. For detection frequencies, *n* refers to the number of samples. Unless otherwise noted, concentrations are the minimum, maximum, and geometric mean of detected concentrations (i.e., nondetects have been excluded).

Microbe†	Detection frequency (*n*)		Concentration	
	Influent	Effluent	Influent	Effluent
	% (no.)		copies wet g^{-1}	
Bacteroidales-like CowM3	100 (118)	95 (119)	3.1×10^4–1.4×10^9, 6.5×10^6	1.9×10^4–2.8×10^7, 1.3×10^6
Bovine *Bacteroides*	99 (118)	97 (119)	2.4×10^3–1.5×10^8, 3.0×10^6	2.6×10^3–7.0×10^6, 5.0×10^5
Bovine polyomavirus	99 (118)	100 (119)	7.3×10^3–3.0×10^8, 9.2×10^5	6.4×10^3–7.1×10^7, 2.8×10^5
Bovine enterovirus	92 (118)	14 (118)	1.1×10^4–1.0×10^7, 2.6×10^5	1.4×10^4–1.6×10^5, 4.4×10^4
Clostridium perfringens	69 (118)	19 (119)	6.0×10^2–2.4×10^6, 2.2×10^4	2.4×10^3–3.8×10^5, 2.9×10^4
Campylobacter jejuni	65 (118)	19 (118)	8.8×10^2–2.4×10^5, 1.0×10^4	9.1×10^2–2.7×10^4, 3.6×10^3
Bovine coronavirus	59 (118)	0 (116)	9.1×10^3–5.6×10^9, 3.8×10^5	Not detected
Group A rotavirus	50 (118)	17 (119)	2.8×10^3–8.1×10^7, 1.1×10^5	8.7×10^3–7.2×10^6, 1.0×10^5
Salmonella spp.	29 (118)	3 (77)‡	3.7×10^2–1.9×10^5, 6.3×10^3	3.1×10^3–1.8×10^4, 7.6×10^3
Bovine adenovirus	8 (118)	1 (77)‡	2.9×10^3–2.6×10^4, 5.0×10^3	3.2×10^3 ($n = 1$)
Enterohemorrhagic *Escherichia coli*	4 (118)	0 (56)‡	1.4×10^3–8.5×10^4, 8.2×10^3	Not detected
Cryptosporidium parvum	0.8 (118)	0 (35)‡	4.4×10^3 ($n = 1$)	Not detected
Giardia lamblia	0.8 (118)	0 (35)‡	1.3×10^4 ($n = 1$)	Not detected

† Group C rotavirus, bovine viral diarrhea virus 1 (BVDV1), bovine viral diarrhea virus 2 (BVDV2), *Mycobacterium avium* subsp. *paratuberculosis*, *Staphylococcus* spp., and *Streptococcus* spp. were never detected.

‡ Because these organisms were rare in influent, fewer effluent samples were analyzed for them.

estimates of log removal using this approach tended to increase with decreasing detection frequencies in digester influent and effluent, as illustrated by the difference between minimum and maximum log removal values for each microbe.

Digester Performance Compared to Expectations from Literature

There is no clear consensus in the scientific literature on the extent of inactivation that should occur in full-scale anaerobic digesters, so we conducted a comprehensive literature review in an attempt to identify one. Our literature search returned 23 papers investigating >20 different microorganisms at both the laboratory and full scales over a wide range of temperatures (see Supplemental Material, p. S6). These papers also investigated microbial inactivation over a wide range of residence times and reactor configurations, both of which can confound comparisons among papers that are based on values for extent of removal (e.g., log removal).

To account for this, we extracted exponential decay coefficients from the data presented in each paper. For batch reactors, we determined decay coefficients from the slopes of survival curves and the exponential decay equation, $C/C_0 = \exp(-kt)$, where t is time, C is the microbe concentration at time t, C_0 is

Table 4. Log removal calculated by digester and season for bovine *Bacteroides*, *Bacteroidales*-like CowM3, and bovine polyomavirus.

Digester	Season	Bovine *Bacteroides*	*Bacteroidales*-like CowM3	Bovine polyomavirus
1	Spring	0.62	0.90	0.04
3	Spring	1.06	1.38	1.03
4	Spring	0.83	0.70	−0.14
5	Spring	0.59	0.80	0.82
6	Spring	0.99	1.04	0.41
7	Spring	0.90	1.17	0.24
8	Spring	1.34	1.50	0.67
1	Summer	0.24	−0.16	−0.66
3	Summer	0.64	0.28	0.47
4	Summer	0.90	0.42	0.40
5	Summer	−0.16	−0.48	0.95
6	Summer	0.55	0.08	0.51
7	Summer	0.48	0.14	0.50
8	Summer	1.15	0.64	0.30
1	Winter	0.68	0.93	0.32
3	Winter	0.55	0.68	0.14
4	Winter	0.74	0.60	−0.07
5	Winter	0.94	0.93	2.14
6	Winter	1.06	0.99	0.62
7	Winter	0.91	0.98	1.07
8	Winter	1.28	1.24	1.39
Mean		0.78	0.70	0.53
SD		0.34	0.50	0.58

the microbe concentration at time zero, and k is the exponential decay coefficient (Tchobanoglous et al., 2003). For complete-mix reactors, we determined decay coefficients from microbe concentrations in digester influent (C_{inf}), microbe concentrations in digester effluent (C_{eff}), and digester residence times (θ) using Eq. [1] (Tchobanoglous et al., 2003):

$$k = \left[\left(C_{inf} / C_{eff}\right) - 1\right] / \theta \quad [1]$$

Similarly, we determined decay coefficients for plug-flow reactors using C_{inf}, C_{eff}, and θ with Eq. [2] (Tchobanoglous et al., 2003):

$$k = \ln\left(C_{inf} / C_{eff}\right) / \theta \quad [2]$$

Finally, we determined decay coefficients for cyclic batch reactors (batch reactors where a small fraction of the volume is replaced once each day) using Eq. [3] (Ginnivan et al., 1980):

$$k = \ln\left[\left(1 - R + RC_{inf}\right) / C_{eff}\right] / R\theta \quad [3]$$

where R is the fractional replacement volume and other variables are as defined earlier. All three equations listed above are derived from mass balances assuming exponential microbial decay. Their use produces decay coefficients that are independent of residence time and reactor configuration, thereby allowing for straightforward comparison among inactivation studies that vary in these parameters.

Our literature review confirmed that published estimates of microbial inactivation in anaerobic digesters are highly variable (Table 6). Standard deviations of exponential decay coefficients were large compared with their means. The distributions of coefficients were also skewed to the right. For example, the median coefficient at mesophilic temperatures was 1.1 d^{-1}, whereas the mean was 2.1 d^{-1}. Similarly, the median at thermophilic temperatures was 6.1 d^{-1}, with a mean of 250 d^{-1}.

Table 5. Log removal calculated using the data from Table 3 and two different assumptions about the concentration of nondetects.

Microbe†	Log removal	
	Nondetects = 95% LOD‡	Nondetects = 0 copies wet g^{-1}
Bacteroidales-like CowM3	0.72	0.72
Bovine *Bacteroides*	0.79	0.79
Bovine polyomavirus	0.51	0.51
Bovine enterovirus	0.78	1.59
Clostridium perfringens	0.19	0.44
Campylobacter jejuni	0.15	0.98
Bovine coronavirus	0.78	>2.00
Group A rotavirus	0.17	0.51
Salmonella spp.	−0.01	0.90
Bovine adenovirus	−0.01	1.10
Enterohemorrhagic *Escherichia coli*	0.00	>2.00

† *Cryptosporidium parvum* and *Giardia lamblia* are excluded because each was only detected once in digester influent.

‡The 95% limit of detection (LOD) is assumed to equal its theoretical value for quantitative polymerase chain reaction of three copies per reaction, which corresponds to 7×10^3 copies wet g^{-1} for microbes with DNA genomes and 4×10^4 copies wet g^{-1} for microbes with RNA genomes.

Table 6. Exponential decay coefficients (*n* = 320 total) for microorganisms in anaerobic digesters based on review of the scientific literature (*n* = 23 papers). The majority of coefficients (*n* = 220) represent total coliforms, fecal coliforms, *Escherichia coli*, *Salmonella* spp., or *Streptococcus* spp.

Condition	Decay coefficient (mean ± SD)	*n*
	d^{-1}	
Psychrophilic	0.38 ± 0.28	58
Mesophilic	2.1 ± 3.3	160
Escherichia coli	3.8 ± 4.8	40
Streptococcus spp.	0.65 ± 0.38	16
Laboratory-scale	2.6 ± 3.8	103
Full-scale	1.2 ± 1.8	57
Thermophilic	250 ± 1600	102

Our literature review also confirmed several often-quoted trends related to microbial inactivation in anaerobic digesters. There was a clear trend of increasing inactivation rates with temperature, and laboratory-scale digesters generally achieved better microbial inactivation than full-scale digesters (Table 6). Furthermore, inactivation also varied according to microbe. For instance, exponential decay coefficients for *Escherichia coli*, a Gram-negative microorganism, were generally larger than those for *Streptococcus* spp., a Gram-positive microorganism, at mesophilic temperatures (Table 6).

In our own study, microbe inactivation during anaerobic digestion was poor when initially compared with expectations from the literature. From Table 6, a typical exponential decay coefficient for microbes in full-scale, mesophilic anaerobic digesters appears to be ~1.2 d^{-1} (half-life = 14 h). For 20-d residence times, this equates to log removal values of 1.4 for an ideal complete-mix digester [obtained by solving Eq. [1] for $\log(C_{inf}/C_{eff})$] and 10

for an ideal plug-flow digester [obtained by solving Eq. [2] for $\log(C_{inf}/C_{eff})$]. Our mean log removal values for bovine *Bacteroides* and *Bacteroidales*-like CowM3 are substantially lower than these ideal values.

The difference between our log-removal values and those calculated using literature data is largely due to our use of qPCR to quantify inactivation. The vast majority of inactivation estimates in our literature review are based exclusively on culture methods (300 of 320 values). The few based on qPCR in this group tend to rank quite low in terms of magnitude—in the lowest 28% on average. Furthermore, the only study in our literature review that used *both* approaches (Chen et al., 2012) found that qPCR tended to produce inactivation estimates that were anywhere from 7 to 300 times lower than those produced using culture measurements. This difference could be due to a limitation in the qPCR measurements because, unlike culture methods, qPCR cannot distinguish between live and dead microbial cells. More specifically, our use of qPCR could have caused the number of live cells in effluent samples to be overestimated, thus causing us to underestimate inactivation.

However, it is possible that the difference in inactivation estimates is more likely attributable to limitations in the culture measurements, rather than in the qPCR measurements, based on two lines of evidence. First, it seems unlikely that free DNA from dead microbes would be readily available for qPCR amplification in anaerobic digesters. The digesters are designed to promote a high level of microbial metabolism, and free DNA would likely be degraded quickly because of its value as a substrate to support that metabolism. Second, culture methods are known to have their own limitation. Specifically, bacterial culture methods tend to underestimate numbers of live cells due to the phenomenon of the viable-but-nonculturable (VBNC) state (Roszak and Colwell, 1987). Moreover, it is possible for numbers of culturable bacteria to decline with time in a given environment at a much faster rate than viable bacteria (Oliver, 2005). In an anaerobic digester, this could cause inactivation estimates based on culture measurements to be overestimated by a substantial degree.

Therefore, we believe that inactivation estimates based on qPCR are likely closer to the true rate of inactivation for viable pathogens. In this case, our own inactivation estimates should only be compared with those from previous studies that have also used qPCR (Narula et al., 2011; Slana et al., 2011; Chen et al., 2012). Among these, a typical exponential decay coefficient at mesophilic temperature appears to be $\sim$0.2 d^{-1} (half-life = 3.5 d). Calculated with a 20-d residence time as before, this equates to log removal values of 0.70 for an ideal complete-mix digester and 1.7 for an ideal plug-flow digester. Given the large number of plug-flow digesters in our study (five of seven) and the similarity of the lower value for an ideal complete-mix digester to our own log removal values, we conclude that the digesters in our study are operating at a relatively inefficient level.

This conclusion is supported by two further lines of evidence. First, the high degree of variability in microbe inactivation across both digester and season is itself indicative of poor digester performance. Second, the volatile solids destruction achieved by these digesters that was measured in the related study provides corroborating evidence for their generally poor performance. Volatile solids destruction in this related study was 30 $\pm$ 14% when aggregated by season and digester (n = 21, mean $\pm$ SD). In contrast, volatile solids destruction by full-scale anaerobic digesters at municipal wastewater treatment plants might be expected to average 50% (Speece, 1988).

Distribution of Microbes in Solid and Liquid Fractions after Manure Separation

We observed a substantial difference in the distribution of microbes between the liquid fraction and solid fraction of separated manure. Detection frequencies and concentrations of nearly every microbe that we could detect were substantially higher in the liquid fraction compared with the solids fraction (Table 7). This included *Bacteroidales*-like CowM3, bovine *Bacteroides*, bovine polyomavirus, bovine enterovirus, *Clostridium perfringens*, *Campylobacter jejuni*, and Group A rotavirus. Furthermore, a mass balance on total solids in the solids separators indicates that, on average, 92% of the manure entering the separators exits in the liquid fraction (see Supplemental Material, p. S9). Thus, for most of the microbes that we could detect in manure, the majority of their numbers ended up in the liquid fraction, as opposed to the solid fraction, after separation.

This conclusion could be an artifact of our approach for processing samples; the liquid fraction was extracted directly for nucleic acids, whereas microorganisms in the solids fraction had to be first eluted from the solids, then nucleic acids were extracted. The extra elution step for the solids fraction could have resulted in loss of microorganisms, yielding less nucleic acid (Robe et al., 2003). However, mass balances on bovine *Bacteroides* and bovine polyomavirus indicated no systematic losses of either microbe in the separators that we sampled. All gene copies of each organism that entered the separators in unseparated manure, which was also processed using the direct extraction technique, were fully accounted for by summing the number of gene copies in the separated liquid and solid fractions. Furthermore, our conclusion that most microbes end up in the liquid fraction is consistent with previous studies showing that high concentrations of dissolved organic matter, such as in manure, decrease microbe attachment to solid surfaces (Guber et al., 2005), although the mechanism by which dissolved organic matter creates unfavorable attachment conditions is still debated (Bradford et al., 2013).

Although the majority of microbes that we detected ended up in the liquid fraction of separated manure, bovine and zoonotic pathogens were also still present in the solid fraction (Table 7). They were even present—although at relatively low levels—in solids having undergone secondary treatment for use as bedding. Thus, these solids should not be regarded as pathogen free, and their use as bedding material for dairy cattle may deserve closer scrutiny with respect to herd health.

Implications for Future Research

Three factors likely account for the variability in microbe inactivation and generally poor digester performance that we observed: overloading, poor mixing, and poor temperature control. Overloading is the practice of increasing a digester's flow rate beyond that which it was designed for; this is often the result of increasing herd sizes or accepting off-farm feedstocks. Overloading a digester reduces its effective residence time, thereby providing less time to inactivate pathogens.

Table 7. Microbe detection frequencies and concentrations in unseparated manure, separated liquids, separated solids, and separated solids after secondary treatment. For detection frequencies, *n* refers to the number of samples. Unless otherwise noted, concentrations are the minimum, maximum, and geometric mean of detected concentrations (i.e., nondetects have been excluded).

Microbe†	Detection frequency				Concentration			
	Unseparated‡	Liquids	Solids	Solids, secondary	Unseparated	Liquids	Solids	Solids, secondary
	% (no.)				copies wet g^{-1}			
Bacteroidales-like CowM3	96 (153)	93 (150)	86 (148)	57 (42)	1.9×10^4–2.9×10^8, 2.1×10^6	1.3×10^4–3.3×10^7, 1.1×10^6	4.8×10^1–8.5×10^5, 6.1×10^3	1.4×10^2–2.2×10^4, 1.8×10^3
Bovine *Bacteroides*	98 (153)	95 (150)	89 (148)	79 (42)	2.6×10^3–4.3×10^7, 9.3×10^5	2.4×10^3–3.0×10^7, 5.0×10^5	4.8×10^1–4.1×10^6, 3.2×10^3	9.9×10^1–2.0×10^4, 1.0×10^3
Bovine polyomavirus	100 (153)	97 (150)	76 (148)	17 (42)	2.4×10^3–7.1×10^7, 2.3×10^5	3.6×10^3–9.7×10^7, 2.6×10^5	8.6×10^1–1.8×10^6, 5.8×10^3	3.0×10^1–5.2×10^4, 3.2×10^3
Bovine enterovirus	30 (152)	16 (150)	17 (116)	3 (30)	1.4×10^4–9.0×10^7, 1.6×10^5	1.7×10^4–4.2×10^5, 7.2×10^4	7.4×10^2–2.1×10^4, 2.0×10^3	6.0×10^3 ($n = 1$)
Clostridium perfringens	23 (153)	7 (130)	15 (115)	3 (29)	1.1×10^3–3.8×10^5, 1.7×10^4	2.5×10^3–7.8×10^4, 1.5×10^4	1.6×10^2–8.3×10^3, 1.3×10^3	1.1×10^3 ($n = 1$)
Campylobacter jejuni	26 (152)	10 (134)	12 (125)	6 (32)	9.1×10^2–1.2×10^5, 5.8×10^3	2.5×10^3–1.0×10^6, 3.5×10^4	5.5×10^1–5.2×10^3, 4.0×10^2	2.0×10^2–4.3×10^3, 9.4×10^2
Bovine coronavirus	12 (150)	1 (149)	1 (136)	0 (38)	1.1×10^4–1.4×10^7, 5.1×10^5	1.9×10^4 ($n = 1$)	1.9×10^4 ($n = 1$)	Not detected
Group A rotavirus	22 (153)	15 (150)	5 (136)	0 (38)	8.7×10^3–7.6×10^7, 1.5×10^5	2.5×10^4–3.0×10^7, 3.2×10^5	8.3×10^2–6.8×10^3, 3.0×10^3	Not detected
Salmonella spp.	4 (111)	0 (144)	1 (125)	0 (35)	3.1×10^3–2.2×10^4, 1.2×10^4	Not detected	7.1×10^1 ($n = 1$)	Not detected

† Bovine adenovirus, enterohemorrhagic *Escherichia coli*, *Cryptosporidium parvum*, and *Giardia lamblia* were never detected in separated liquid or solid samples, so they are not included in this table.

‡ For facilities with digesters, unseparated manure samples are the same as digester effluent. For facilities without digesters, unseparated manure samples are untreated manure.

Similarly, poor mixing can lead to shorter effective residence times by allowing for formation of dead zones in digesters. These dead zones can average nearly half of the total volume in full-scale systems (Ontario Ministry of the Environment, 1978). Finally, poor temperature control results in many time periods during which temperatures are at less-than-optimum values, and these lower temperatures cause slower rates of microbe inactivation.

Field studies to investigate the impacts of these three factors on the performance of full-scale anaerobic digesters would be valuable to the livestock industry. Such studies should have two general goals. First, they should seek to establish the relative effect sizes of overloading, poor mixing, and temperature control on pathogen inactivation. Studies designed to achieve this goal would help prioritize these three factors; they might also identify other factors at the root of poor pathogen inactivation in full-scale digesters. Second, field studies should investigate optimal design criteria for pathogen inactivation by relating measures of temperature control and mixing performance to inactivation over a range of loading conditions. Achieving these two goals would not only improve anaerobic digester performance with respect to pathogen inactivation, but would also likely improve performance with respect to methane production. Thus, these field studies could simultaneously contribute to protecting human health and improving the profitability of anaerobic digesters on cattle and dairy farms.

Further studies investigating the inactivation of bovine viruses during anaerobic digestion might also be valuable to the livestock industry. Previous studies have only investigated two bovine viruses during anaerobic digestion: bovine enterovirus and bovine parvovirus (Monteith et al., 1986; Lund et al., 1996). Our study adds data on four previously uninvestigated bovine viruses: bovine polyomavirus, bovine coronavirus, Group A rotavirus, and bovine adenovirus. However, we could only produce robust estimates of inactivation for bovine polyomavirus because the other three viruses were uncommon in manure. Laboratory experiments might be more appropriate for these uncommon bovine viruses because they would allow digester influent to be spiked with consistently high concentrations of the viruses in question. The results from experiments like these would provide livestock producers with information on the benefits of anaerobic digesters with respect to reducing the on-farm prevalence of bovine viruses.

Conclusion

We investigated the extent of pathogen inactivation in full-scale anaerobic digesters treating cattle manure. Unlike previous studies, ours examined inactivation in many digesters with frequent, biweekly sampling over several seasons and measured a large selection of fecal indicators, bovine pathogens, and zoonotic pathogens using qPCR. This approach allowed for characterization of digester-to-digester and seasonal variability. Anaerobic digesters inactivated pathogens and fecal indicators, but the extent of inactivation for fecal indicators was generally poor compared with expectations based on the literature. It was also highly variable from digester to digester. More research is needed to determine the source of these performance deficiencies and to optimize the design and operation of full-scale digesters with respect to pathogen inactivation.

We also investigated the distribution of microbes in separated liquids and solids after separation of manure. Some pathogens

were present in the solid fraction, even in solids undergoing additional secondary treatment, which has implications for herd health when separated solids are used as bedding for cattle. However, most microbes that we detected ultimately ended up in the liquid fraction. Pathogens in this liquid fraction of separated manure can contaminate crops, groundwater, and surface water when land applied, thus contributing to transmission of zoonotic disease from cattle to humans.

Supplemental Material Available

Supplemental material for this article is available online.

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