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Simultaneous Concentration of Bovine Viruses and Agricultural Zoonotic Bacteria from Water Using Sodocalcic Glass Wool Filters

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Abstract Infiltration and runoff from manured agricultural fields can result in livestock pathogens reaching groundwater and surface waters. Here, we measured the effectiveness of glass wool filters to simultaneously concentrate enteric viruses and bacteria of bovine origin from water. The recovery efficiencies were determined for bovine viral diarrhea virus types 1 and 2, bovine rotavirus group A, bovine coronavirus, poliovirus Sabin III, toxigenic *Escherichia coli*, and *Campylobacter jejuni* seeded into water with three different turbidity levels (0.5, 215, and 447 NTU). Twenty liters of dechlorinated tap water (pH 7) were seeded with the test organisms, and then passed through a glass wool filter using a peristaltic pump (flow rate = 1 liter min^{-1}). Retained organisms were eluted from the filters by passing beef extract-glycine buffer (pH 9.5) in the direction opposite of sample flow. Recovered organisms were enumerated by qPCR except for *C. jejuni*, which was quantified by culture. Mean recovery efficiencies ranged from 55 to 33 % for the bacteria and 58 to 16 % for the viruses. Using bootstrapping techniques combined with Analysis of Variance, recovery efficiencies were found to differ among the pathogen types tested at the two lowest turbidity levels; however, for a given pathogen type turbidity did not affect recovery except for *C. jejuni*. Glass wool filtration is a cost-effective method for concentrating several waterborne pathogens of bovine origin simultaneously, although recovery may be low for some specific taxa such as bovine viral diarrhea virus 1.

Keywords Agricultural runoff · Bovine viruses · Waterborne pathogens · Water sampling · Zoonotic bacteria

Introduction

Because waterborne pathogens are present in water at low concentrations they are usually concentrated from water before analysis. The ability to concurrently concentrate bacterial and viral pathogens is useful to assess the risks from waterborne pathogens and in source tracking (Fong and Lipp 2005). Bovine enteric viruses in particular have been suggested to identify animal sources of fecal pollution (Ley et al. 2002; Bofill-Mas et al. 2011). Most studies in this area have been directed at the concentration of human enteric viruses from water (Ikner et al. 2012), or separate strategies for concentrating bacteria (Goyal and Gerba 1980; Bisha et al. 2011).

Sodocalcic glass wool offers a promising alternative as an adsorptive material for virus concentration. Glass wool, held together by a binding agent and coated with mineral oil, provides both hydrophobic and electropositive sites for adsorption of microorganisms. Viruses are usually negatively charged in water at or near neutral pH and readily adsorb to the positively charged glass wool fibers (Environment Agency 2000). The fibers are inexpensive and require no water conditioning outside of pH adjustment in some circumstances (Wyn-Jones and Sellwood 2001).

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Glass wool has been shown to be effective for the concentration of human enteric viruses from wastewater (Gantzer et al. 1997), drinking water (Grabow et al. 2001; Vivier et al. 2004; Van Heerden et al. 2005; Lambertini et al. 2008), groundwater (Powell et al. 2000, 2003; Van Zyl et al. 2004; Ehlers et al. 2005), river water (Hot et al. 2003; Van Heerden et al. 2005; Albinana-Gimenez et al. 2009), and reservoirs (Van Zyl et al. 2004; Ehlers et al. 2005; Deboosere, et al. 2011).

The present study objective was to evaluate the ability of glass wool filters for simultaneous concentration of bacteria and viruses shed in cattle manure that can be transmitted by waterborne routes, namely, toxigenic *Escherichia coli*, *Campylobacter jejuni*, as well as several bovine viruses. Poliovirus type 3 (Sabin) was used as a surrogate for bovine enteroviruses and as a benchmark for comparison with previous studies that examined poliovirus concentration from water by glass wool filtration.

Materials and Methods

Glass Wool Filter Preparation

The method for constructing glass wool filters is described by Millen, et al. (2012). Ninety grams of washed glass wool Rantigny 725 (Saint Gobain, Isover Orgel, France) was packed into 3.8 cm diameter by 10.2 cm length polyvinylchloride (PVC) threaded pipes with caps using a metal plunger (approximate packed density = 0.5 g cm^{-3} dry weight). The packed columns were flushed with phosphate buffer saline (PBS) pH 7.0 prior to use.

Microbial Stocks

Seven different virus and bacteria taxa were tested: bovine viral diarrhea virus (BVDV) type 1, BVDV type 2, bovine rotavirus group A, bovine coronavirus, poliovirus type 3 (Sabin), *E. coli* O157:NM, and *C. jejuni*. Pathogens were seeded to the final concentrations reported in Table 1; *C. jejuni* recovery experiments were conducted at two concentrations, 5 and 500 colony forming units (CFU) l^{-1}. Commercial vaccine preparations were the source for the four bovine viruses. BVDV type 1 and 2 viruses were obtained from Bovi-Shield Gold-5 Vaccine (Pfizer, New York, NY), bovine rotavirus A, and bovine coronavirus from Calf Guard Bovine Rota-Coronavirus Vaccine (Pfizer, New York, NY). A concentrated stock of poliovirus type 3 (Sabin) was obtained by growing the virus in the BGM cell line. After cytopathic effects destroyed 90 % of the cell monolayer, the cultures were freeze thawed three times, followed by removal of cell debris by centrifugation at 900×g for 10 min. The working stocks of poliovirus and vaccine preparations were frozen at −80 °C and thawed prior to seeding. Working stocks of bacteria were obtained by growing *E. coli* O157:NM (American Type Culture Collection (ATCC), Manassas, VA, Catalog # 700378) on MacConkey overnight at 37 °C and *C. jejuni* (ATCC Catalog # 33560) overnight on CVA agar under microaerophilic conditions at 42 °C.

Experimental Design

An independent experimental trial consisted of seeding the seven test organisms into 20 l dechlorinated tap water from a groundwater source and pumping the seeded water by peristaltic pump at a flow rate of 1 l min^{-1} from carboys through a glass wool filter. The bacteria test organisms were not included in every trial. All tubing and containers had previously been disinfected with 0.5 % chlorine for at least 30 min followed by chlorine neutralization with 0.05 mol l^{-1} Na$_2$S$_2$O$_3$ and rinsed with sterile 18 Mohm water. Three water turbidity levels were evaluated; these were prepared by mixing into the 20-l test water volumes dried agricultural soil (surface horizon of Withee silt loam) at: 0 g l^{-1} (0.5 NTU), 1.27 g l^{-1} (215 NTU), and 2.75 g l^{-1} (447 NTU). Ambient water pH was 7.6 and all samples were adjusted to pH 7.0 before glass wool filtration by the addition of 1 mol l^{-1} HCl. A 10 μm nominal pore size polypropylene prefilter (MacMaster-Carr, Elmhurst, IL) was used for 215 NTU and 447 NTU water matrices to prevent clogging of the glass wool filter.

Filter Elution and Flocculation

Glass wool filters were eluted in the direction opposite to the original flow with two 80 ml elutions of 3 % beef extract-glycine buffer (BEG) at pH 9.5 (Goyal and Gerba 1980). The first eluent was allowed to soak the filter for 15 min before adding the second eluent, which was immediately pushed through the filter by air. Eluates containing suspended sediments were centrifuged at 1,000×g for 5 min, and the solution was saved. The soil pellet was resuspended in BEG (pH 9.5) and centrifuged again at 1,000×g for 5 min. The solutions were added to the original eluate and adjusted to pH 7.0–7.5 with 1 mol l^{-1} HCl and then flocculated by the addition of polyethylene glycol 8000 [8 % (wt vol^{-1})] and NaCl (final concentration, 0.2 mol l^{-1}). This mixture was stirred for 1 h at 4 °C, incubated overnight at 4 °C, and centrifuged at 4,200×g for 45 min at 4 °C. The pellet was resuspended in sterile 0.15 mol l^{-1}·Na$_2$HPO$_4$ solution (pH 7.0). Prefilters were placed into a plastic bag with 200 ml BEG, pH 9.5, massaged and soaked for 15 min, and the eluate poured off and further concentrated as described for the glass wool filter eluates. These final concentrated sample volumes

Table 1 Glass wool filter recovery of bovine-origin pathogens in water and ANOVA results for differences in recovery among pathogen types for a given water turbidity level

Turbidity (NTU)	Pathogen	Concentration seeded[a,b]	N	% Recovery		P value
				Arithmetic mean (%)	Standard deviation (%)	
0.5	Bovine Coronavirus	250	9	25.8	21.3	**0.0012**
	Bovine Rotavirus A	2500	9	21.0	9.4	
	BVDV 1	250	9	12.9	5.4	
	BVDV 2	25	9	22.6	14.5	
	C. jejuni	500	7	58.1	16.2	
	C. jejuni	5	7	31.4	15.9	
	E. coli O157:NM	25	6	45.0	12.0	
	Poliovirus	25000	9	60.1	45.6	
215	Bovine Coronavirus	250	9	9.2	2.4	**<0.0005**
	Bovine Rotavirus A	2500	9	21.6	8.8	
	BVDV 1	250	9	12.9	13.5	
	BVDV 2	25	9	23.1	20.6	
	C. jejuni	500	6	33.7	12.3	
	C. jejuni	5	7	28.3	13.3	
	E. coli O157:NM	25	3	72.7	74.0	
	Poliovirus	25000	9	70.2	32.3	
447	Bovine Coronavirus	250	8	19.5	27.1	0.0554
	Bovine Rotavirus A	2500	9	23.8	22.7	
	BVDV 1	250	9	21.1	26.2	
	BVDV 2	25	9	13.6	11.9	
	C. jejuni	500	8	22.1	5.3	
	C. jejuni	5	8	37.6	17.7	
	E. coli O157:NM	25	6	55.7	64.5	
	Poliovirus	25000	9	43.2	26.8	

Bold P values indicate statistically different recovery efficiencies

[a] Final seeded concentrations are in genomic copies l^{-1}, except *C. jejuni* concentrations are in CFU l^{-1}

[b] Pathogen working stock concentrations (genomic copies ml^{-1} except *C. jejuni* concentrations are in CFU ml^{-1}) were as follows: Coronavirus, 2×10^4; Rotavirus A, 2×10^5; BVDV type 1, 2×10^4; BVDV type 2, 2×10^3; *C. jejuni*, 1×10^4; *E. coli* O157:NM, 2×10^3; Poliovirus, 2×10^6

(FCSV) from both the glass wool filter and prefilter were then assayed separately and the results summed to obtain the recovered quantity of test organism.

Microbial Enumeration

Campylobacter jejuni was enumerated by spreading 100 µl of serial tenfold dilutions of FCSV on duplicate plates of CVA agar (Remel, Lenexa, KS) and counting the appropriate colony forming units. *Escherichia coli* O157:NM and viruses were enumerated by quantification of the genomic copies using qPCR and two step RT-qPCR, respectively, and interpolation from the standard curves created for each organism type. RT-qPCR inhibition was evaluated by hepatitis G virus (HGV) armored RNA (Asuragen Inc., Austin, TX). These methods were identical to those described in Lambertini et al. (2008), except amplification of cDNA occurred in a 96-well microplate using the Roche Light-Cycler 480 System (Roche Diagnostics, Mannheim, Germany). The LightCycler Probes Master Kit (Roche Diagnostics) was used to prepare the PCR mixes.

Table 2 lists the final concentrations and sequences of the primers (Integrated DNA Technologies, Coralville, Iowa) and TaqMan probes (TIB MOLBIOL, Berlin, Germany) for each pathogen. Amplification conditions started with a hot start polymerase activation step for 10 min at 95 °C, followed by 45 cycles of 15 s at 94 °C and 1 min at 60 °C.

Recovery Calculation

A 20 l negative control unseeded water sample was processed for each trial. No background pathogens were detected at any time in the water or soil-amended water samples. In addition, for each recovery trial an unseeded 20-l water sample was passed through a glass wool filter, eluted, and the resulting negative eluate then seeded with the same concentration of pathogens as the corresponding water sample. Pathogens in the seeded negative eluate were enumerated with the same method as the water samples, and these values were used as the devisors when calculating percent recovery. Quantifying the seeded pathogens in a negative eluate takes into consideration differences in pathogen enumeration that could result from matrix differences created by the glass wool filter. The importance of this step when quantifying pathogens by qPCR is discussed in Lambertini et al. (2008) and Borchardt et al. (2013).

Table 2 qPCR primers and probes used in this study

Organism	Primer or probe	Primer or probe sequence (5′-3′)	Optimum Concentration (nM)	Amplicon Size (bp)	Reference
BVDV 1 and 2	BVD-fwd	TAGCCATGCCCTTAGTAGGAC	700	94	Brooks et al. 2007
	BVD 1-rev	GACGACTACCCTGTCCTCAGG	700		
	BVD-2-rev	GACGACTCCCCTGTACTCAGG	700		
	BVD 1-probe	CAGTGGTGAGTTCGTTGGATGGCT	100		
	BVD-2-probe	AGGGGACTAGCGGTAGCAGTGAGTTC	100		
Bovine Rotavirus A	RVA-fwd	TGCCACACTGTTGTCAATATTA	300	168	Chang et al. 1999
	RVA-rev	TCCTCTGCTGTTGGGAAAAGTT	300		Chang et al. 1999
	RVA-probe	GGTAAGCCGCTAGAAGCAGATTTGACAGTG	100		This study
Bovine Coronavirus	CoV-fwd	ATTAGAACTGGAAGTTGGTGGA	500	199	This study
	CoV-rev	TCACATAAGCTGGCAAATCT	500		
	CoV-probe	ACAATAATACGTGGTCATCTTTACATGCAAG	100		
Enterovirus-Poliovirus Sabin III	EV-fwd	CCTCCGGCCCCTGAATG	300	196	De Leon et al. 1990
	EV-rev	ACCGGATGGCCAATCCAA	900		
	EV-probe	CGGAACCGACTACTTTGGGTGTCCGT	100		
E.coli O157:NM	eae-fwd	GTAAGTTACACTATAAAAGCACCGTCG	700	106	Ibekwe et al. 2004
	eae-rev	TCTGTGTGGATGGTAATAAATTTTTG	700		
	eae-probe	AAATGGACATAGCATCAGCATAATAGGCTTGCT	100		

Percent recovery was calculated as the number of genomic copies (or CFUs) of the test organism recovered after filtration of the water sample divided by the number of genomic copies (or CFUs) of the test organism seeded into the eluate of the unseeded water sample multiplied by 100.

Results and Discussion

Glass wool filters were effective in concentrating pathogens in water with a wide range of turbidity levels. Mean recovery efficiencies and standard deviations by turbidity level and by pathogen type are reported in Table 1. Recoveries across the three water matrices ranged from 22.1 to 72.7 % for the bacteria and 9.2 to 70.2 % for the viruses. Bacteria had higher recoveries than the viruses, and among the viruses poliovirus had the highest recovery.

Among nine independent recovery experiments, nondetects (i.e., no recovery) were observed for BVDV type 1 and BVDV type 2 once each at 0.5 NTU turbidity, three times for BVDV 1 and twice for BVDV 2 at 215 NTU turbidity, and twice for BVDV 2 at 447 NTU turbidity. These non-detects were assigned a zero value and included in the mean recovery calculation. All other seeded test organisms were recovered every time an experiment was performed.

Percent recoveries, the outcome measure for each pathogen type and turbidity level, were tested for normality, outliers, and homogeneity of variance. The tests indicated

that the data were not normally distributed, outliers were present in the datasets, and the datasets have significantly different variances. The datasets were transformed using the arcsine trigonometric function to attain normality to fulfill the requirements for conducting a classical analysis of variance (ANOVA). However, even after transformation, the same tests showed none of the requirements were met.

Alternatively, we applied bootstrapping techniques for conducting the ANOVA (Mooney and Duval, 1993) with at least 5,000 iterations, where recovery percent was the dependent variable and pathogen type and turbidity level were the independent variables. Table 1 shows the P value for each of the bootstrapping ANOVA tests with the region for rejecting the null hypothesis set at 5 %. The null hypothesis stated there is no significance difference between the means of percent recoveries for the different pathogen types under the three turbidity levels.

Comparing within a turbidity level, percent recoveries were significantly different among pathogen types at 0.5 NTU and 215 NTU, whereas at the 447 NTU turbidity level the difference in recoveries among pathogens was minor (Table 1). This suggests when turbidity is high the interaction of soil particles with the filter masks the differences in adsorption and size exclusion characteristics of the pathogens that normally results in differences in filter retention.

Comparing within a pathogen type, turbidity did not affect recovery except for *Campylobacter* seeded at 500 CFU l^{-1} (Table 1) (P value < 0.0005). For the other

Table 3 All pairwise comparisons of glass wool filter recovery efficiencies by pathogen type averaged across turbidity levels

Pathogen 1			Pathogen 2			P value
Name	Arithmetic mean (%)	Standard deviation (%)	Name	Arithmetic mean (%)	Standard deviation (%)	
Bovine Rotavirus A	**22.1**	**14.5**	**Bovine Coronavirus**	**18.1**	**20.1**	**<0.00005**
BVDV 1	15.6	17.1	Bovine Coronavirus	18.1	20.1	0.992
BVDV 1	**15.6**	**17.1**	**Bovine Rotavirus A**	**22.1**	**14.5**	**<0.00005**
BVDV 2	19.7	16.1	Bovine Coronavirus	18.1	20.1	0.978
BVDV 2	**19.7**	**16.1**	**Bovine Rotavirus A**	**22.1**	**14.5**	**<0.00005**
BVDV 2	19.7	16.1	BVDV 1	15.6	17.1	1.000
C. jejuni (high)[a]	37.4	19.4	Bovine Coronavirus	18.1	20.1	0.666
C. jejuni (high)	37.4	19.4	Bovine Rotavirus A	22.1	14.5	0.054
C. jejuni (high)	37.4	19.4	BVDV 1	15.6	17.1	0.197
C. jejuni (high)	37.4	19.4	BVDV 2	19.7	16.1	0.140
C. jejuni (low)[b]	32.7	15.6	Bovine Coronavirus	18.1	20.1	0.201
C. jejuni (low)	32.7	15.6	Bovine Rotavirus A	22.1	14.5	0.288
***C. jejuni* (low)**	**32.7**	**15.6**	**BVDV 1**	**15.6**	**17.1**	**0.02522**
***C. jejuni* (low)**	**32.7**	**15.6**	**BVDV 2**	**19.7**	**16.1**	**0.01548**
C. jejuni (low)	32.7	15.6	*C. jejuni* (high)	37.4	19.4	0.997
E. coli O157:NM	54.8	49.3	Bovine Coronavirus	18.1	20.1	1.000
***E. coli* O157:NM**	**54.8**	**49.3**	**Bovine Rotavirus A**	**22.1**	**14.5**	**0.00021**
E. coli O157:NM	54.8	49.3	BVDV 1	15.6	17.1	1.000
E. coli O157:NM	54.8	49.3	BVDV 2	19.7	16.1	0.999
E. coli O157:NM	54.8	49.3	*C. jejuni* (high)	37.4	19.4	0.645
E. coli O157:NM	54.8	49.3	*C. jejuni* (low)	32.7	15.6	0.234
Poliovirus	**57.9**	**36.2**	**Bovine Coronavirus**	**18.1**	**20.1**	**0.00088**
Poliovirus	57.9	36.2	Bovine Rotavirus A	22.1	14.5	0.992
Poliovirus	**57.9**	**36.2**	**BVDV 1**	**15.6**	**17.1**	**<0.00005**
Poliovirus	**57.9**	**36.2**	**BVDV 2**	**19.7**	**16.1**	**<0.00005**
Poliovirus	57.9	36.2	*C. jejuni* (high)	37.4	19.4	0.317
Poliovirus	57.9	36.2	*C. jejuni* (low)	32.7	15.6	0.783
Poliovirus	**57.9**	**36.2**	***E. coli* O157:NM**	**54.8**	**49.3**	**0.00332**

Bold table rows indicate statistically different recovery efficiencies between pathogen pairs

[a] Seeded at 500 CFU l^{-1} final concentration

[b] Seeded at 5 CFU l^{-1} final concentration

pathogens, namely bovine coronavirus, bovine rotavirus A, BVDV Type 1, BVDV Type 2, *E. coli* O157:NM, poliovirus, and *Campylobacter* seeded at 5 CFU l^{-1} turbidity did not affect recovery as indicated by ANOVA P values of 0.22, 1, 0.56, 0.32, 0.68, 0.37, and 0.69, respectively. It appears that for most of the pathogen types and concentrations tested factors other than turbidity level are more important in determining recovery efficiency by glass wool filtration.

Aggregating recovery data across turbidity levels and examining all pairwise comparisons between pathogen types, percent recoveries differed among virus types and between bacteria and viruses but not between bacterial species (Table 3). However, these comparisons need to be interpreted cautiously because a key limitation of the

present study is the quantity of pathogen seeded varied with pathogen type, not allowing a clear separation between the effects of seed quantity and pathogen type on percent recovery. Previously, Lambertini et al. (2008) showed glass wool filter recovery efficiencies did differ among species and serotypes of human enteric viruses but not by the quantity of viruses seeded.

Among pathogen types poliovirus (57.9 %) and *E. coli* O157:NM (54.8 %) had the greatest recovery efficiency, while BVDV-1 (15.6 %) had the lowest (Table 3).

Simultaneous concentration of waterborne bacteria and viruses has been previously reported using filtration (Payment et al. 1989) ultrafiltration (Hill et al. 2005; Liu et al. 2012), and continuous flow ultracentrifugation (Bisha et al. 2011), however, these methods often require costly

equipment or filters. Glass wool filters have been used by several groups (Lambertini et al. 2008; Environment Agency 2000; Vilaginès et al. 1993) to concentrate human enteric viruses from a variety of water sources. Here we report the use of these filters for concentrating both viral and bacterial pathogens associated with cattle manure from water at turbidity levels simulating agricultural runoff. The filters are advantageous in that they are inexpensive, highly portable, usable in a wide range of water matrices, and effective for simultaneous concentration of both bacterial and viral waterborne pathogens. They can be constructed to any size, depending on volumes to be sampled. After disinfection, filter housings are reusable.

Glass wool filters, however, do have limitations. As with any virus concentration method that relies on electro-positively charged media for virus adsorption (e.g., 1MDS filter, CUNO Inc., Meriden, CT), filter effectiveness depends on ambient water pH. In our laboratory, we have selected pH 7.5 as the cut-off, above which the water pH is adjusted downward by continuously pumping 0.25 mol l^{-1} HCl into the filter input line during sampling.

Virus recovery measured in the present study compares favorably with values observed in previous glass wool validation studies with human viruses. The average poliovirus recovery rate across the three water matrices was 58 %, near the ranges of 62–77 % and 60–83 % reported by Vilaginès et al. (1993, 1997), 70–91 % range noted in the UK Environment Agency study (Environment Agency 2000), and 17–155 % reported by Lambertini et al. (2008), and 38–81 % range noted by Millen et al. (2012). The first three previous studies cited adopted working parameters different from those used here, such as filtration rate, water source, and filter dimensions, making direct comparison of recovery efficiencies equivocal. It is also important that the virus enumeration techniques used were not identical; specifically Vilaginès et al. (1993) and the UK Environment Agency (Environment Agency 2000) used a plaque assay. The studies completed by Lambertini et al. (2008) and Millen et al. (2012) used the same filtration and virus enumeration techniques as employed here.

Our recovery data for E. coli O157:NM and C. jejuni as well as the recovery data for Salmonella enterica reported in Millen et al. (2012) demonstrate that the glass wool filtration is an effective for concentrating waterborne bacteria. Even with C. jejuni concentrations as low as five CFU per liter in highly turbid water glass wool filtration recovered about a quarter to a third of the bacteria (Table 1). Payment et al. (1989) examined fiberglass filters for the recovery efficiency of Clostridium perfringens and Legionella pneumophila and found efficiencies were 83 and 55 %, respectively.

Hill et al. (2005) tested ultrafiltration for recoveries of E. coli, Enterococcus faecalis, S. enterica, Bacillus globigii and found mean recovery efficiencies were from 70 to 93 % for all bacterial species.

Bisha et al. (2011) evaluated modified Moore swabs and continuous flow centrifugation for concentrating S. enterica and E. coli O157:H7. Liu et al. (2012) tested the recovery of C. perfringens spores, and E. coli by ultrafiltration, and the recovery efficiencies were greater than 50 %.

Having one simple method for concentrating multiple types of waterborne pathogens is advantageous for assessing pathogen levels in water and the associated health risk. We demonstrated that inexpensive and easily constructed glass wool filters are effective for concentrating a variety of waterborne viral and bacterial pathogens typically found in runoff from agricultural fields with applied dairy manure. The method is applicable to water quality assessment and source tracking.

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