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Decay of Fecal Indicator Bacterial Populations and Bovine-Associated Source-Tracking Markers in Freshly Deposited Cow Pats

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Understanding the survival of fecal indicator bacteria (FIB) and microbial source-tracking (MST) markers is critical to developing pathogen fate and transport models. Although pathogen survival in water microcosms and manure-amended soils is well documented, little is known about their survival in intact cow pats deposited on pastures. We conducted a study to determine decay rates of fecal indicator bacteria (*Escherichia coli* and enterococci) and bovine-associated MST markers (CowM3, Rum-2-bac, and GenBac) in 18 freshly deposited cattle feces from three farms in northern Georgia. Samples were randomly assigned to shaded or unshaded treatment in order to determine the effects of sunlight, moisture, and temperature on decay rates. A general linear model (GLM) framework was used to determine decay rates. Shading significantly decreased the decay rate of the *E. coli* population ($P < 0.0001$), with a rate of -0.176 day^{-1} for the shaded treatment and -0.297 day^{-1} for the unshaded treatment. Shading had no significant effect on decay rates of enterococci, CowM3, Rum-2-bac, and GenBac ($P > 0.05$). In addition, *E. coli* populations showed a significant growth rate (0.881 day^{-1}) in the unshaded samples during the first 5 days after deposition. UV-B was the most important parameter explaining the decay rate of *E. coli* populations. A comparison of the decay behaviors among all markers indicated that enterococcus concentrations exhibit a better correlation with the MST markers than *E. coli* concentrations. Our results indicate that bovine-associated MST markers can survive in cow pats for at least 1 month after excretion, and although their decay dynamic differs from the decay dynamic of *E. coli* populations, they seem to be reliable markers to use in combination with enterococci to monitor fecal pollution from pasture lands.

Elevated levels of fecal indicator bacteria (FIB) remain the most common cause of impairment in streams and rivers in the United States, with agriculture as the primary source of contamination (1). The federal Clean Water Act defines impaired surface waters as rivers, lakes, or streams that do not meet one or more water quality standards and therefore categorizes these water resources as too polluted for their intended uses. FIB are recommended for water monitoring because of their correlation with gastrointestinal illness (2–4), but they cannot indicate the origin of fecal pollution. This shortcoming is one of the challenges present when attempting to protect and remediate water sources that are impaired due to fecal contamination (5). Emerging library- and culture-independent microbial source-tracking (MST) methods that target host-associated markers and offer information about the sources of fecal contamination are now used widely by state and federal agencies monitoring water resources (6–9). Information can be used for total maximum daily load (TMDL) development and implementation of remediation practices.

Ideally, MST markers and FIB will have similar fates and transport behaviors governed by their concentrations in polluting matrices, extraintestinal survival, growth rates after excretion, and mobility in the environment (10, 11). Of these factors, survival of FIB and MST markers plays an integral role in determining their fate and transport in the environment, since survival can strongly influence prevalence in both fecal sources and water environments. The ability to estimate concentrations of bacteria in feces deposited on a given area of pasture over time will benefit management and mitigation of animal pollution of water, as well as zoonotic pathogen risk assessment (12).

Decay kinetics of FIB and MST markers in waters and manure-amended soils are well documented (13–17), but little is known about the survival of MST markers in bovine feces deposited on pastureland. Many previously published decay rates of FIB, pathogens, and MST markers from bovine feces focused primarily on studies of manure and slurries applied to soil (11, 13, 14). To the best of our knowledge, no study has investigated their decay patterns in naturally occurring fecal deposits. Procedures used in the manure, slurry, and water microcosm studies may enhance microbial inactivation, since fecal materials are subjected to elevated composting temperature, antagonistic microbiota, pH changes, and desiccation associated with soil mixing (12). Mixing of manure also increases aeration, which could decrease survival of the obligately anaerobic *Bacteroidales* cells, the most commonly used MST markers due to their abundance in feces and their superior host specificity. Studies on the survival of FIB and MST markers in freshly deposited cow pats are warranted, therefore, since fecal pollution occurs not only by runoff from applied manure but also from deposited feces.

Contradictory results for factors affecting the survival of FIB and MST markers in bovine manure or slurry and water microcosms have been reported. In water microcosms, sunlight has been reported to decrease the persistence of human-specific MST markers (15) and ruminant-associated markers (16, 17). In contrast, Sokolova et al. (18) reported no signifi-

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cant effect of light on the decay of FIB and MST markers. Temperature has also been shown to correlate strongly with inactivation of *Bacteroidales* spp. in water microcosms (19–23). Positive correlations between moisture and FIB concentrations have been documented in several studies (12, 24–27), but an overall negative relationship between moisture and *Escherichia coli* (14) has been reported.

In our study, the persistence of bovine-associated MST markers, culturable FIB, and their genomes in freshly deposited bovine feces was investigated. Factors that can affect their survival and persistence, such as UV, moisture, and temperature, were also studied under field conditions. With the objective of establishing relationships between each type of measurement under the same environmental conditions, we compared the decay behaviors of MST markers and FIB. The selected quantitative PCR (qPCR) markers used here were the general *Bacteroidales* marker GenBac (28); a cattle-associated marker, CowM3 (29); and a ruminant-specific marker, Rum-2-bac (30).

MATERIALS AND METHODS

Manure collection and study site. Twenty freshly excreted bovine fecal samples were collected from three farms in northern Georgia during the summer of 2012. One farm practices organic beef cattle farming, and the others are traditional beef-producing commercial farms, and the farms handle 20, 50, and >150 heads of cattle, respectively. Cow pats were collected as whole as possible, using 8-in.-diameter, 24-gauge round-end stove caps (Grainger Inc., Lake Forest, IL) and a 24-in. by 12-in. piece of sheet metal-gauge steel (Stanley Hardware, New Britain, CT). Following excretion, the round stove-cap end was placed carefully on the cow pat to avoid disturbing its original structure as much as possible. The metal sheet was slid under the pat, after which the stove cap containing the feces was flipped and covered with a clear plastic bag. Samples were immediately transported to the study site on ice and in the dark.

The study was conducted on a field site located at the U.S. EPA Ecosystems Research Division in Athens, GA. Three cow pats from each farm were randomly assigned to shaded or unshaded treatments, for a total of nine replicate pats per treatment. Cow pat weights ranged from 0.6 to 1.5 kg. Plot covers (2.4 m by 1.7 m) were constructed of polyvinyl chloride (PVC) frames lined with clear acetate films (80% UV transmission; Grafix Plastics, Cleveland, OH) and were placed on top of each treatment set to protect the cow pats from natural rain events. The shaded treatment was created by placing a solid-color tarp (100% UV block) over the clear acetate film structure; the unshaded treatment was covered only by the clear acetate film. Two additional cow pats—one for each treatment—were fitted with a 12-bit smart temperature sensor connected to an onset Hobo U30 data logger (Onset Computer Inc., Bourne, MA). A UV sensor (Satlantic model OCR-504) measuring four different wavelengths (305, 325, 340, and 380 nm) was installed underneath both treatment plot covers and connected to a Stor-X data logger (Satlantic, Halifax, Canada). One additional UV sensor was installed away from the plot covers to monitor full sunlight.

Sample collection. Fecal samples from each cow pat were collected on days 0, 2, 4, 6, 8, 15, 22, 29, 43, and 57, between 9:00 and 10:00 a.m.; they were collected from both the outer crust and the moist interior of the cow pat to obtain representative samples of the entire cow pat. Three or four cores were obtained at various depths from each cow pat, using a sterile V-shaped spatula. Samples were transported to the laboratory within 5 min of collection, and the cores were homogenized in sterile 50-ml centrifuge tubes, with the aid of a sterile spatula. The moisture content (MC) of each homogenous fecal sample was determined gravimetrically by drying 2 to 5 g at 105°C for 24 h. All microbial counts were expressed per gram of dry weight.

Microbiological analysis. Fecal material was suspended in a phosphate buffer solution at a 1:10 ratio. Prior to enumeration, all samples were dispersed by hand shaking and vortexing for 10 min; serial dilutions were performed with sterilized Nanopure water. The concentrations of *E. coli* and enterococci in cow pat samples were enumerated using a Colilert Quanti Tray system (Idexx Lab Inc., Westbrook, ME) according to the manufacturer's instructions and membrane filtration (U.S. EPA method 1600), respectively.

DNA extraction. One hundred milligrams of each homogenized cow pat sample was transferred to powerBead tubes in triplicate (MoBio Laboratories, Carlsbad, CA) and stored at −80°C until extraction, which occurred within 2 weeks of sampling. DNA was extracted using a MoBio Power-Soil DNA isolation kit, with the following modifications to the manufacturer's instructions: (i) bead beating was conducted at 6.5 m s^{-1} for 45 s, using a Fastprep-24 instrument (MP Biomedicals, Solon, Ohio); and (ii) to make the final quantification of the marker more accurate, only half of the bead solution and C1 mixture (405 μl) was transferred after the first step, because it was difficult to accurately carry over all of the supernatant due to absorption by the dried fecal material.

Genomic and plasmid DNA preparation. American Type Culture Collection (ATCC) bacterial strains were used to prepare qPCR standard curves for *E. coli* (ATCC 25922), enterococci (*Enterococcus faecalis* ATCC 29212), and GenBac (*Bacteroides thetaiotaomicron* ATCC 29741). Plasmid DNA standards were synthesized for CowM3 and Rum-2-bac assays by amplifying a segment of the hydrolase domain (HD) superfamily and 16S rRNA loci, respectively, using PCR (Table 1). The amplification product was ligated into a pCR 2.1-TOPO plasmid vector and transformed into One Shot Top10 chemically competent *E. coli*, using a Topo TA kit (Life Technologies, Grand Island, NY). Recombinant bacteria were enumerated on ImMedia ampicillin and kanamycin agar (Life Technologies), and colonies were selected randomly for overnight culture propagation in ImMedia broths (Life Technologies). Plasmids were extracted using a PureLink Quick plasmid miniprep kit (Life Technologies) and then linearized with BamHI-HF enzyme (New England BioLabs, Ipswich, MA). Linearized plasmid DNA was purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and quantified with a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies).

qPCR assays and quantification. Primers and probes used in this study are shown in Table 1. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA) and rehydrated to concentrations of 500 μM and 100 μM, respectively, in nuclease-free water. qPCR assays were performed with a model 7500 HT Fast real-time sequence detector (Applied Biosystems). Reaction mixtures (20 μl) for all assays contained 1× TaqMan Fast universal PCR master mix with No AmpErase uracil-*N*-glycosylase (Life Technologies), 0.02 mg/ml bovine serum albumin (BSA) (Life Technologies), 1 μM (each) primers, 80 nM 6-carboxyfluorescein (FAM)- or VIC-labeled TaqMan probe, and 4 μl of either genomic DNA (fecal samples), 40 to 1×10^7 target sequence copies (CowM3 and Rum-2-Bac), or 5 to 4×10^4 target gene copies (*E. coli*, enterococci, and GenBac). All reactions were duplicated in MicroAmp Fast 96-well reaction plates covered with MicroAmp optical adhesive film (Life Technologies). Thermal conditions for all assays except CowM3 assays were 95°C for 20 s (initial denaturation), followed by 40 cycles of short denaturation at 95°C for 3 s and a combined annealing and primer extension phase at 60°C for 30 s. The initial and short denaturation durations for the CowM3 assay were 2 min and 5 s, respectively. Data were analyzed with Sequence Detector Software (SDS), set to start and end cycles of 3 and 15, respectively, and a threshold determination of 0.2 for the salmon and Entero1 assays; otherwise, the automatic baseline and threshold were used. Threshold cycle (C_T) values were exported to Microsoft Excel for further statistical analysis. To prevent cross-contamination, dedicated equipment and separate laboratories were used for every step from DNA extraction to qPCR amplification. In addition, a minimum of two no-template controls and two DNA standards were included for each assay performed in a 96-well qPCR plate.

Inhibition. To monitor qPCR inhibition from the fecal matrix, salmon DNA (Sigma, St. Louis, MO) was used as an exogenous internal

TABLE 1 Primers and probes used for qPCR assays

Assay name	Primer or probe	Primer or probe sequence (5′–3′)[a]	Target organism	Reference
Salmon	Forward primer	GGTTTCCGCAGCTGGG	Salmon (*Oncorhynchus keta*) testes	56
	Reverse primer	CCGAGCCGTCCTGGTCTA		
	Probe	FAM-AGTCGCAGGCGGCCACCGT-TAMRA		
Entero1	Forward primer	AGAAATTCCAAACGAACTTG	*Enterococcus*	57
	Reverse primer	CAGTGCTCTACCTCCATCATT		
	Probe	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA		
EPA-EC 23S	Forward primer	GGTAGAGCACTGTTTTGGCA	*E. coli*	58
	Reverse primer	TGTCTCCCGTGATAACTTTCTC		
	Probe	FAM-TCATCCCGACTTACCAACCCG-TAMRA		
Genbac	Forward primer	GGGGTTCTGAGAGGAAGGT	*Bacteroidales*	28
	Reverse primer	CCGTCATCCTTCACGCTACT		
	Probe	VIC-CAATATTCCTCACTGCTGCCTCCCGTA-Iowa Black		
CowM3	Forward primer	CCTCTAATGGAAAATGGATGGTATCT	Cattle-associated microbial population	29
	Reverse primer	CCATACTTCGCCTGCTAATACCTT		
	Probe	FAM-TTATGCATTGAGCATCGAGGCC-TAMRA		
Rum-2-bac	Forward primer	ACAGCCCGCGATTGATACTGGTAA	Ruminant-associated microbial population	30
	Reverse primer	CAATCGGAGTTCTTCGTGAT		
	Probe	FAM-ATGAGGTGGATGGAATTCGTGGTGT- TAMRA		

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

positive control (IPC). Four microliters of DNA extracted from each sample was added to a qPCR mix consisting of salmon primers and probe, BSA, and 0.05 ng/μl salmon DNA. Extracted DNA from a pure culture of *Enterococcus faecalis* was included as a positive control for every 96-well qPCR. Mean uninhibited salmon C_T values were obtained by adding salmon DNA to duplicate control samples containing extracted DNA from pure *Enterococcus faecalis* cultures. Reactions were deemed inhibited if the salmon C_T value was 1 unit higher than the average salmon C_T observed for the positive controls. All samples from cow pats collected at the commercial farms showed inhibition, so DNAs were diluted 5, 10, and 25 times with autoclaved Nanopure water and rechecked for inhibition. Twenty-five-fold dilution resulted in C_T values close to the detection limits of our assays, so the 10-fold dilution was selected.

Statistical analysis. Concentrations of FIB and MST markers were transformed by taking the natural logarithm (log). Decay rates and effects of environmental factors (e.g., UV-B) were estimated by appropriately parameterized linear models. To explore the effects of environmental factors, temperature and UV-B data were analyzed by temporal synchronization analysis (TSA) (31), where the sum or average of the independent variables is examined over a temporal window rather than relying on instantaneous values taken at the time of sampling. Cumulative UV-B data 5 days before sampling and temperature data a day prior to sampling were determined to be significant temporal windows that improved the model's ability to fit the data set. The general form of the linear model for the concentration of a given FIB or MST marker is as follows:

$$\log(C_{i,t}) = \beta_{i,0} + \beta_1 t + \beta_2 x_{i,t} t + \varepsilon_{i,t} \quad (1)$$

where $C_{i,t}$ is the concentration in cow pat i at time t, $\beta_{i,0}$ is the initial log concentration, β_1 is the overall decay rate (i.e., effect due to time t), β_2 is the difference in decay rate due to the environmental variable $x_{i,t}$ (i.e., the effect due to the interaction between $x_{i,t}$ and time t), and $\varepsilon_{i,t}$ is a normally distributed error term with mean zero and variance σ^2. We assume that errors are independent of each other. This general statistical model follows the decay model framework described by McCullough and Nelder (32).

This model has several notable features. Because multiple cow pats were used during the experiment, we included a separate $\beta_{i,0}$ for each, allowing different initial concentrations; each FIB or MST marker was given a single decay rate (β_1) and interaction parameter (β_2), however. The parameter β_2 represents the differential decay rate induced by the environmental factor $x_{i,t}$. Statistical significance of the parameter β_2 provides evidence that changes in the environmental factor $x_{i,t}$ induce changes in microbial decay. Thus, our hypothesis tests of environmental factors were conducted by testing for significance of the appropriate interaction term. The logarithmic transformation of the concentrations $C_{i,t}$ and corresponding linear model also follow the assumption of exponential decay of the microorganisms.

For FIB experiencing an initial regrowth phase, a piecewise linear regression was used. The general form of the piecewise regression, where we assumed regrowth up until a time (t) of 5 days, was as follows:

$$\log(C_{i,t}) = \beta_{i,0} + \beta_1 t + \varepsilon_{i,t} \quad \text{for } t \leq 5 \quad (2)$$

$$\log(C_{i,t}) = [\beta_{i,0} + \beta_1(5)] + \beta_2(t-5) + \varepsilon_{i,t} \quad \text{for } t > 5 \quad (3)$$

In this case, β_1 represents the initial exponential growth rate of microorganism i, which occurs through time 5 (with time being in days), and β_2 represents the exponential decay experienced after time 5. To detect environmental effects for microbes experiencing regrowth, we appropriately combined the model in equation 1 with the model in equations 2 and 3.

Finally, a pairwise correlation was performed to access the relationship among FIB and MST markers, using STaTa-12 (StataCorp LP, College Station, TX). All other statistical analyses were performed with the publicly available R software (33). For each test, the acceptable level of significance (α) was 0.05.

RESULTS

There were no significant differences in decay rates of FIB and MST markers among collection sites ($P > 0.05$), so comparisons of results are not shown. After comparing treatments (shading versus no shading), results showed that treatment affected only the decay rates of *E. coli* populations, that is, *E. coli* had separate decay rates for shaded and unshaded samples (Table 2). Decay rates for all others were determined using combined shaded and

TABLE 2 Growth and decay rates of FIB and MST markers in cow pats ($n = 18$)[a]

Organism or genetic marker	Treatment	Growth rate (β_1)	Decay rate (β_2)	T_{90} (days)[b]	T_{99} (days)[b]	Model r^2
Escherichia coli	Unshaded	0.881*	−0.297*	7.75	15.51	0.82[d]
	Shaded	0.0544	−0.176	13.08	26.16	0.82[d]
EPA-EC23S		ND	−0.0621	37.08	74.15	0.80
Enterococcus[c]		NA	−0.163	14.13	28.25	0.76
Entero1[c]		NA	−0.0434	53.06	106.12	0.61
CowM3[c]		NA	−0.126	18.27	36.54	0.69
Rum-2-bac[c]		NA	−0.111	20.74	41.48	0.74
GenBac[c]		NA	−0.128	17.99	35.97	0.74

[a] Log concentrations were modeled within the general linear model framework. Asterisks indicate statistical significance between the shaded and unshaded treatments ($P < 0.05$). ND, not determined; NA, not available.
[b] Derived from decay rate (β_2).
[c] Since shading had no effect on decay rates, decay rates are not presented as a function of treatment.
[d] The model includes shaded and unshaded treatments, since it was a significant term.

unshaded data. FIB and MST markers were monitored for 49 and 57 days, respectively.

FIB. The initial average concentrations (arithmetic means) of culturable enterococci and genome copies (Entero1) were 1.74×10^7 CFU g^{-1} dry weight (coefficient of variation [CV] = 1.40) and 4.25×10^5 gene copies (GC) g^{-1} dry weight (CV = 0.56), respectively. A slight increase in culturable enterococcus concentrations was observed during the first 2 days after deposition in 33% of shaded and 66% of unshaded samples. Likewise, enterococcal genomic concentrations also showed an increase of 1.25 log for the first 2 days after deposition, followed by a slow decline until day 57. The slight increase in enterococcus concentration was not significantly different from the starting concentration (Fig. 1). Since regrowth was not significant, a first-order decay model was used. There were no statistically significant differences between the decay rate coefficients of shaded and unshaded treatments for culturable and genomic enterococci.

Culturable *E. coli* and its genomic marker (EPA-EC23S) had average initial concentrations of 2.20×10^6 most probable number (MPN) g^{-1}dry weight (CV = 2.0) and 3.80×10^7 GC g^{-1} dry weight (CV = 2.2), respectively. The culturable *E. coli* concentration increased significantly from days 0 to 5 in unshaded cow pats ($P < 0.05$), but no significant increase was observed in shaded cow pats ($P > 0.05$) (Fig. 1 and Table 2). The genomic concentration of *E. coli* also increased for unshaded samples during the first 5 days, by 1.51 log, and for shaded samples during the first 2 days, by 1.11 log (Fig. 1).

MST markers. The MST markers exhibited fairly consistent

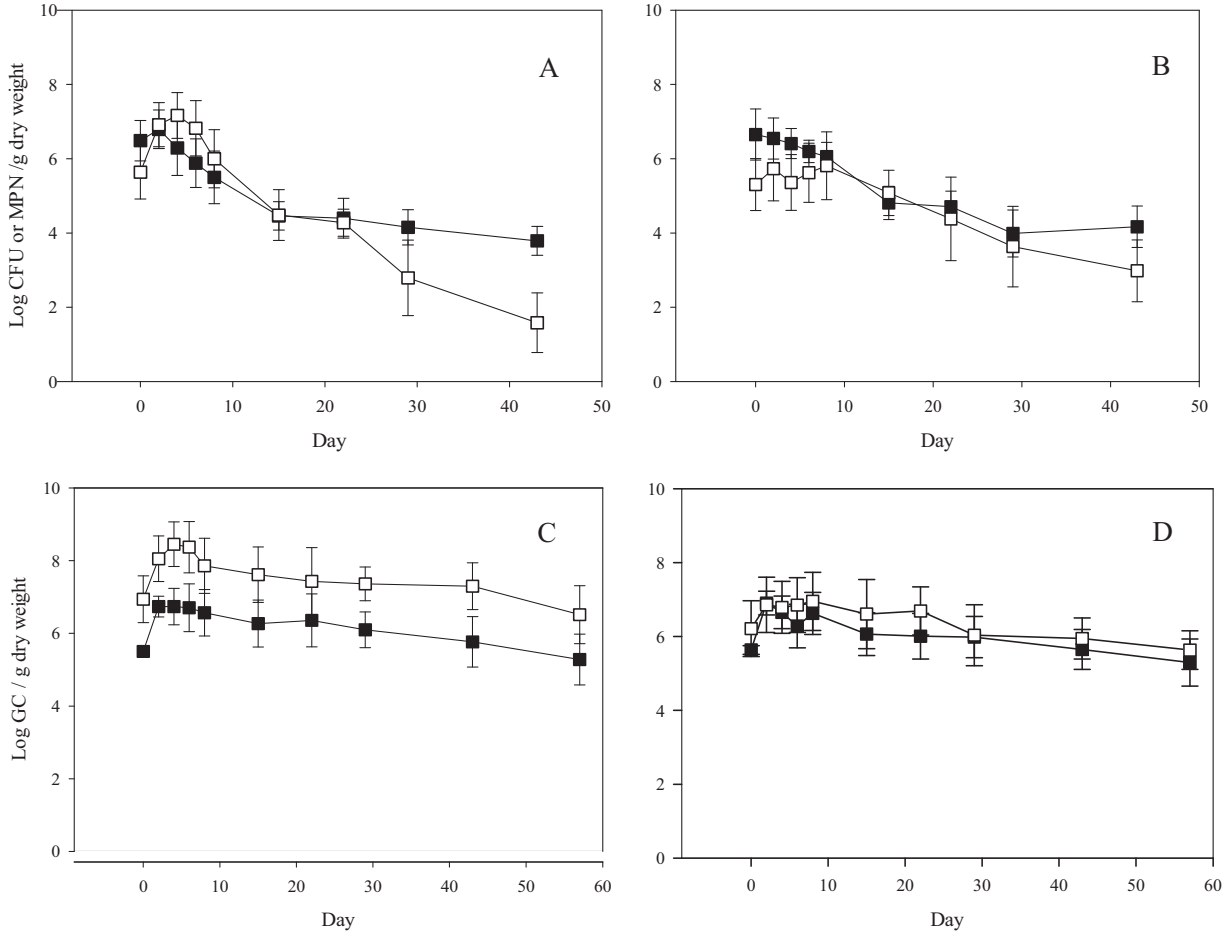

FIG 1 Decay curves for FIB as a function of treatment ($n = 18$). Levels of culturable *E. coli* (□) and enterococci (■) in unshaded (A) and shaded (B) plots and of their corresponding qPCR markers in unshaded (C) and shaded (D) plots are shown. Error bars represent 95% confidence intervals.

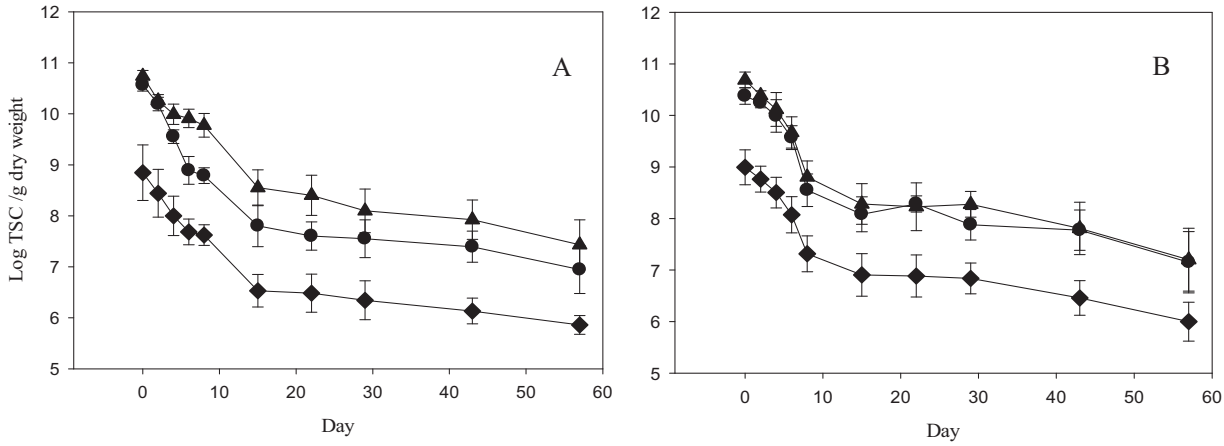

FIG 2 Decay curves for MST markers as a function of treatment ($n = 18$). Levels of CowM3 (◆), Rum-2-Bac (●), and GenBac (▲) in unshaded (A) and shaded (B) plots are shown. Error bars represent 95% confidence intervals.

concentrations between replicate cow pats for each sampling point, with narrow confidence intervals (Fig. 2). CowM3 had an average initial concentration of 1.69×10^9 target sequence copies (TSC) g^{-1} dry weight (CV = 1.1), Rum-2-bac had an initial concentration of 3.36×10^{10} TSC g^{-1} dry weight (CV = 0.46), and GenBac had an initial concentration of 5.70×10^{10} TSC g^{-1} dry weight (CV = 0.42). There was no significant difference in decay rates between treatments for each marker or between markers ($P > 0.05$) (Table 2).

Correlation among FIB and MST markers. A pairwise correlation analysis was performed to determine how significantly concentrations of FIB and MST markers correlated. Culturable *E. coli* and enterococci had a strong correlation coefficient of 0.68 ($P < 0.001$) relative to each other but slightly lower correlation coefficients (0.49 to 0.57) with their corresponding genomic markers (Table 3). In addition, culturable enterococci had higher correlation coefficients with each MST marker than did *E. coli*. The correlation coefficients among MST markers were >0.9 (Table 3); FIB genomic concentrations had only moderate correlations with MST markers (0.37 to 0.44).

Environmental parameter effects on FIB and MST marker decay rates. The average cow pat temperature 1 day prior to sampling was 31.4 ± 3.0°C for the unshaded treatment and 26.1 ± 1.8°C for the shaded one. Decay rate coefficients increased as temperature increased for CowM3 ($P < 0.001$) and Rum-2-bac ($P < 0.05$) (Table 4); temperature had no significant effect on *E. coli* and enterococci or their genomes. UV-B (305.9 nm) was below the detectable range for the shaded plot, and the cumulative 5-day average UV-B for the unshaded plot, measured prior to sampling, was 1.73 ± 0.41 μW cm^{-2}. UV-B had a positive correlation only with the decay rate of culturable *E. coli* ($P < 0.001$) (Table 4), i.e., as UV-B increased, the *E. coli* concentration decreased. The percentages of moisture content of all fecal samples averaged 89% ± 1.2% on day 0 and decreased to 14% ± 3.0% and 19% ± 2.7% by day 57 for unshaded and shaded samples, respectively. The culturable *E. coli* population decayed faster, with a decrease in moisture content ($P < 0.001$), but the host-specific MST markers (CowM3 and Rum-2-bac) had a slower decay ($P < 0.05$) as moisture content decreased (Table 4). Moisture content had no effect on the enterococcal decay rate.

DISCUSSION

The aim of this study was to determine the persistence of FIB and bovine-associated MST markers in undisturbed cow pats. We characterized the decay rates of various FIB and molecular markers under a representative agricultural scenario where feces are surface deposited as cow pats and not incorporated into the soil. Our results show that *E. coli* concentrations were significantly higher ($P < 0.05$) than concentrations observed at 0 days in the unshaded treatments for the first 5 days after deposition. Regrowth of *E. coli* for up to 7 days has been well documented in the literature (14, 34–36). Meays et al. (37) observed *E. coli* increases on days 1 and 7 under 40% and 0% shading, respectively, which suggests that *E. coli* can replicate in the environment. Sinton et al.

TABLE 3 r^2 values based on pairwise correlations among FIB and MST markers[a]

Organism or genetic marker	r^2 value						
	Enterococcus	*E. coli*	Entero1	CowM3	Rum-2-bac	GenBac	EPA-EC23S
Enterococcus	1.0000						
E. coli	0.6858	1.0000					
Entero1	0.4944	0.4307	1.0000				
CowM3	0.7859	0.6085	0.4297	1.0000			
Rum-2-bac	0.7402	0.5453	0.395	0.9129	1.0000		
GenBac	0.8156	0.5602	0.452	0.9032	0.9387	1.0000	
EPA-EC23S	0.3410	0.5661	0.6337	0.4429	0.3684	0.339	1.0000

[a] *P* values are <0.001.

TABLE 4 Regression coefficients for statistically significant environmental parameters

Parameter	Organism or genetic marker	Regression coefficient	*P* value
Moisture	*Escherichia coli*	0.0015	0.001
	Enterococcus		>0.05
	CowM3	−0.0017	0.003
	Rum-2-bac	−0.0014	0.005
	GenBac		>0.05
Temperature	*Escherichia coli*		>0.05
	Enterococcus		>0.05
	CowM3	−0.0027	0.001
	Rum-2-bac	−0.0021	0.02
	GenBac	−0.0012	0.04
UV-B	*Escherichia coli*	−0.075	0.001
	Enterococcus		>0.05
	CowM3		>0.05
	Rum-2-bac		>0.05
	GenBac		>0.05

(12) reported that growth was determined primarily by manure water content and secondarily by temperature, while Muirhead and Littlejohn (34) concluded that temperature was the responsible factor. On day 5 of our study, we had only limited data and could not make statistical inferences from the effects of temperature, moisture, and UV on the growth rate of *E. coli*. It is possible that the observed growth of *E. coli* was affected by complex interactions of many variables, including photoreactivation, which was not determined herein (38, 39). Furthermore, microbes in freshly excreted feces are in the logarithmic growth phase, which may partly explain the frequently observed growth of fecal *E. coli* in fresh cow pats (12, 34, 35). In contrast to *E. coli*, enterococci did not exhibit the same regrowth dynamics during the first weeks of our study. This behavior is not unusual for enterococci. Using composited cow pats, Soupir et al. (35) observed no regrowth of enterococci during a summer sampling, the same period as in our study. In contrast, Sinton et al. (12) observed an increase in enterococcus concentration between the first and second sampling times during the summer season, but they did not find any statistical significance.

Exposure to sunlight significantly decreased survival of *E. coli* but not enterococci. After initial regrowth, *E. coli* populations decayed faster than enterococci in unshaded cow pats. Similar results were reported by Meays et al. (37), who indicated that shading was the only significant factor enhancing survival of *E. coli* from day 17 to day 45. In contrast, Van Kessel et al. (36) reported minimal differences in die-off rates between shaded and unshaded treatments; however, their shaded cow pats were placed under a tree, which would not have shielded them completely from sun or rain, resulting in a statistically insignificant die-off difference between both treatments. The most significant effect of shading, which is considered important to enhancing survival of FIB in cow pats, can be attributed to protection from UV (12, 15, 37). Furthermore, UV-B has been reported to have a more lethal effect on bacterial DNA inactivation than that of UV-A (15, 16, 40–45). In our study, a higher decay rate of the *E. coli* population was significantly associated with higher UV-B irradiance, but UV-B had no effect on enterococcal decay. Previous research suggested that enterococci may require a higher dose (i.e., intensity × residence time) of UV-B to achieve inactivation similar to that of *E. coli* (46). For instance, at a UV-B maximum of 21.3 μW cm^{-2}, the time required for 99% decay or 2-log reduction (T_{99}) values for pure cultures of *E. coli* and *E. faecalis* in sterile water were 45 min and 100 min, respectively (46). In other words, *Enterococcus* required an approximately 2.2-fold increase in the UV-B dose to attain *E. coli*'s die-off rate. In our study, we calculated that a 1.8-fold increase in UV-B dose would be required for enterococci to exhibit a die-off rate similar to that of *E. coli* (Table 2). In considering the germicidal effect of UV light, previous studies reported that enterococci required an ~1.5 times higher dose of UV-C for the same level of inactivation (99.9%) as that achieved in *E. coli* (39, 47). Furthermore, the authors of a review of UV disinfection of viruses, bacteria, and protozoa (48) calculated the microbial inactivation credit (MIC) for 1-, 2-, 3-, or 4-log inactivation for environmental *E. coli* and *Streptococcus faecalis*. For a 4-log inactivation, *E. coli* requires 18 mJ/cm^2 and *S. faecalis* demands 30 mJ/cm^2, a 1.6-fold increase in UV dose. The different responses to UV may be attributed to cell wall structures. A thick, uniform peptidoglycan layer forms 90% of the cell wall of enterococci, while *E. coli* has a multilayered cell wall structure with a relatively thin inner peptidoglycan layer (only 10% of the cell wall) and an outer membrane of lipopolysaccharide and proteins. It has been observed that the peptidoglycan layer is the most resistant membrane wall component (49, 50) for protection against UV-induced damage. Based on our study results, we conclude that the major factor responsible for the decay of *E. coli* populations is UV-B, with an estimated decay of 0.075 log per day for every unit increase in UV-B (Table 4).

Moisture content (MC) had a minimal but significant effect on persistence of *E. coli*, with a coefficient of 0.0015 (Table 4). Reported results on the effect of moisture content on *E. coli* survival have been mixed. Some reports indicate that MC has a positive correlation with FIB concentration (12, 24–27); however, Wang et al. (14) reported a higher overall reduction in *E. coli* levels at 83% MC at 27°C, but no effect at 55% and 30% MC. Meays et al. (37) showed that the MC of fecal pats at sampling time was not correlated with the concentration of *E. coli*.

Another factor we addressed is the potential effect of temperature on persistence of host-specific and general *Bacteroides* sp. markers in cow pats. The influence of temperature on *Bacteroidales* inactivation has been reported elsewhere (18, 20–22), but no study has reported the coefficient associated with a decreased concentration. Our results indicate that for every unit increase in temperature, there was an ~0.002-log decrease per day in the bovine-associated MST markers (CowM3 and Rum-2-bac) and a 0.0012-log decrease per day in the GenBac concentration (Table 4). This fractional decrease in marker concentration due to temperature is negligible compared to the overall decay rate, suggesting that temperature is not the dominant factor affecting the persistence of these markers in undisturbed cow pats.

FIB genomic markers exhibited lower decay rates than their culturable forms. One explanation is the ability of qPCR to detect DNAs from cells undergoing various metabolic stages, such as cultivable cells, viable but not culturable cells (VBNC), nonviable intact cells, and extracellular-free DNA (11). This was evident in our results for both EPA-EC23S and Entero1, which had a final

concentration on day 57 that was not much different from the starting concentration.

Conversely, MST markers (CowM3, Rum-2-bac, and GenBac) persisted in cow pats, with similar decay rates: they did not grow in the environment, and shading had no effect. The effects of shading on bovine-associated MST markers have been reported in water microcosm studies (17, 18). Two ruminant-specific markers (CF 193 and BacR) were monitored in freshwater microcosms spiked with fresh cow feces and incubated under light and dark conditions. The authors reported no effect of light on decay rates of these markers. In another study (16), however, exposure to light resulted in faster decay of a cow-specific marker (BacCow-UCD), suggesting that under certain conditions (e.g., presence of oxygen), light could speed decay of these markers in the environment.

Our results indicate that quantification of MST markers was possible up to day 57, suggesting that these markers can persist in undisturbed cow pats long after deposition. To our knowledge, this is the first study to report decay constants of MST markers in this type of environmental matrix. Long persistence in dry cow pats (MC was down to about 15% by the end of our study) has implications for the impact of dry fecal material as a source of contamination to surface waters. These results suggest that pasturelands containing large amounts of dry cow pats may contribute high concentrations of both FIB and MST markers for extended periods after deposition during runoff-producing rain events. Therefore, in assessing contamination of surface waters in agricultural watersheds, pastures need to be taken into consideration as sources of contamination even when cattle are not actively grazing at the site. The persistence of bovine-associated MST markers in water microcosms or manure-amended soils (15–18, 51, 52) was lower than that reported here, suggesting that when fecal material reaches aquatic environments or is incorporated into the soil, MST markers do not persist as long as in intact cow pats. The shorter survival times (2 to 15 days) reported in these studies can be attributed to various physical and biological factors, including dissolved oxygen and predation, which have been implicated in shortening the persistence of strictly anaerobic *Bacteroidales* spp. (16, 19–22, 53–55). Balleste and Blanch (22) suggested that environmental *Bacteroides* strains may be more sensitive to dissolved oxygen than pure cultures of *Bacteroidales* spp. By sampling individual, undisturbed surface-deposited cow pats without prior mixing, our experimental design preserved a more intact atmospheric condition, which may have helped to protect environmental *Bacteroidales* cells against the toxic effect of oxygen and offered a more accurate estimation of their survival in agricultural settings. It is noteworthy that anoxic conditions may also enhance survival of enterococci in cow pats: for instance, Marti et al. (54) reported T_{90} values of ~24 for *E. coli* and >43 days for enterococci under microaerophilic conditions at 20°C. Moreover, the persistence of enterococci was comparable to that of a universal *Bacteroidales* marker (AllBac) and two pig-specific *Bacteroidales* markers (Pig-1-bac and Pig-2-Bac) reported in the same study under the same conditions as the FIB, with T_{90}s of >43 days. Because the decay of *Bacteroidales* markers could not be explained by any of the physical parameters tested herein, we suggest that more studies on the effect of UV-B on *Bacteroidales* markers in fecal matrices are warranted. Comparing MST markers and FIB decay behaviors revealed that MST markers seemed to persist at a rate similar to that of enterococci rather than that of *E. coli* populations. While the decay rate of *E. coli* populations was decreased by sunlight exposure, neither the MST markers nor enterococcal populations exhibited significant effects on their decay rates when exposed to sunlight. These results suggest that it is necessary to pay close attention to the type of indicator used to assess impairment of water resources in relation to the MST markers used to identify the potential sources of contamination. For instance, the difference in decay rates suggests that while a water body might not show impairment due to *E. coli*, bovine MST markers might still be present, indicating an impact by cattle, especially if dry fecal material is present in the area affecting the stream. The close relationship between enterococci and bovine MST markers could make them reliable markers to be used simultaneously to assess the water quality of surface waters in this type of scenario.

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