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Biotic Interactions and Sunlight Affect Persistence of Fecal Indicator Bacteria and Microbial Source Tracking Genetic Markers in the Upper Mississippi River

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The sanitary quality of recreational waters that may be impacted by sewage is assessed by enumerating fecal indicator bacteria (FIB) (*Escherichia coli* and enterococci); these organisms are found in the gastrointestinal tracts of humans and many other animals, and hence their presence provides no information about the pollution source. Microbial source tracking (MST) methods can discriminate between different pollution sources, providing critical information to water quality managers, but relatively little is known about factors influencing the decay of FIB and MST genetic markers following release into aquatic environments. An *in situ* mesocosm was deployed at a temperate recreational beach in the Mississippi River to evaluate the effects of ambient sunlight and biotic interactions (predation, competition, and viral lysis) on the decay of culture-based FIB, as well as molecularly based FIB (Entero1a and GenBac3) and human-associated MST genetic markers (HF183 and HumM2) measured by quantitative real-time PCR (qPCR). In general, culturable FIB decayed the fastest, while molecularly based FIB and human-associated genetic markers decayed more slowly. There was a strong correlation between the decay of molecularly based FIB and that of human-associated genetic markers (r^2, 0.96 to 0.98; $P < 0.0001$) but not between culturable FIB and any qPCR measurement. Overall, exposure to ambient sunlight may be an important factor in the early-stage decay dynamics but generally was not after continued exposure (i.e., after 120 h), when biotic interactions tended to be the only/major influential determinant of persistence.

Contamination of environmental waters by sewage poses a serious threat to human health; hence, identifying sources of fecal pollution is vital for water quality management, remediation efforts, and estimation of their impacts, aided by quantitative microbial risk assessment (QMRA) (1–3). Combined sewer overflows (CSOs), sanitary sewer overflows (SSOs), sewage spills, and faulty septic systems are all common sources through which high concentrations of untreated or partially treated sewage can enter environmental waters.

Recreational water quality is typically assessed using culture-based enumeration of fecal indicator bacteria (FIB) (e.g., *Escherichia coli* and enterococci) worldwide and, more recently, quantitative real-time PCR (qPCR) in the United States (4), neither of which provides information about the pollution source, since these organisms are commensal inhabitants of the gastrointestinal tracts of mammals, birds, and various insects (5–14). Microbial source tracking (MST) has emerged in response to the increasing need to identify and manage sources of fecal pollution, and it aims to remedy the situation through identification of contributing fecal sources by targeting genetic markers thought to be closely associated with particular animal hosts (15). A large subset of human-associated MST methods target genetic markers harbored in *Bacteroides* spp. (recently reviewed in reference 15) via endpoint PCR and qPCR. These methods have been successfully employed to identify likely human fecal pollution in many different water types (16–22).

However, relatively little is known about the fate of various MST genetic markers in the environment. Most decay studies to date either have been conducted in the laboratory under simulated environmental conditions or have used closed-microcosm systems (e.g., glass beakers or aquarium tanks) *ex situ* to mimic ambient conditions (23–27). Nonetheless, the decay of various *Bacteroides* host-associated genetic markers has been reported to increase with higher temperatures representative of summer conditions (23, 26, 28, 29), and it appears to be more rapid in freshwater compared to marine waters (25, 26, 30). The effect of ambient sunlight, however, remains ambiguous. Some researchers report no significant difference in the decay of *Bacteroides* host-associated genetic markers with or without sunlight exposure (24, 25, 31), while others note that decay under light conditions is significantly faster (32). It has also been suggested that exposure to sunlight is more detrimental to live cells (as assessed by propidium monoazide treatment) than it is to DNA, implying that the disagreement in the reported literature may be due to the physiological state rather than to a direct effect of sunlight (33). Considerably less is known about the impact of biotic interactions (e.g., predation, competition, and viral lysis); existing laboratory-scale and mesocosm studies suggest that these interactions can play an important role (23, 24, 26, 28), but this assertion has not been confirmed in field studies.

Successful management of recreational waterways requires a thorough understanding of FIB, MST genetic marker, and pathogen decay dynamics in aquatic habitats, as well as the effects that

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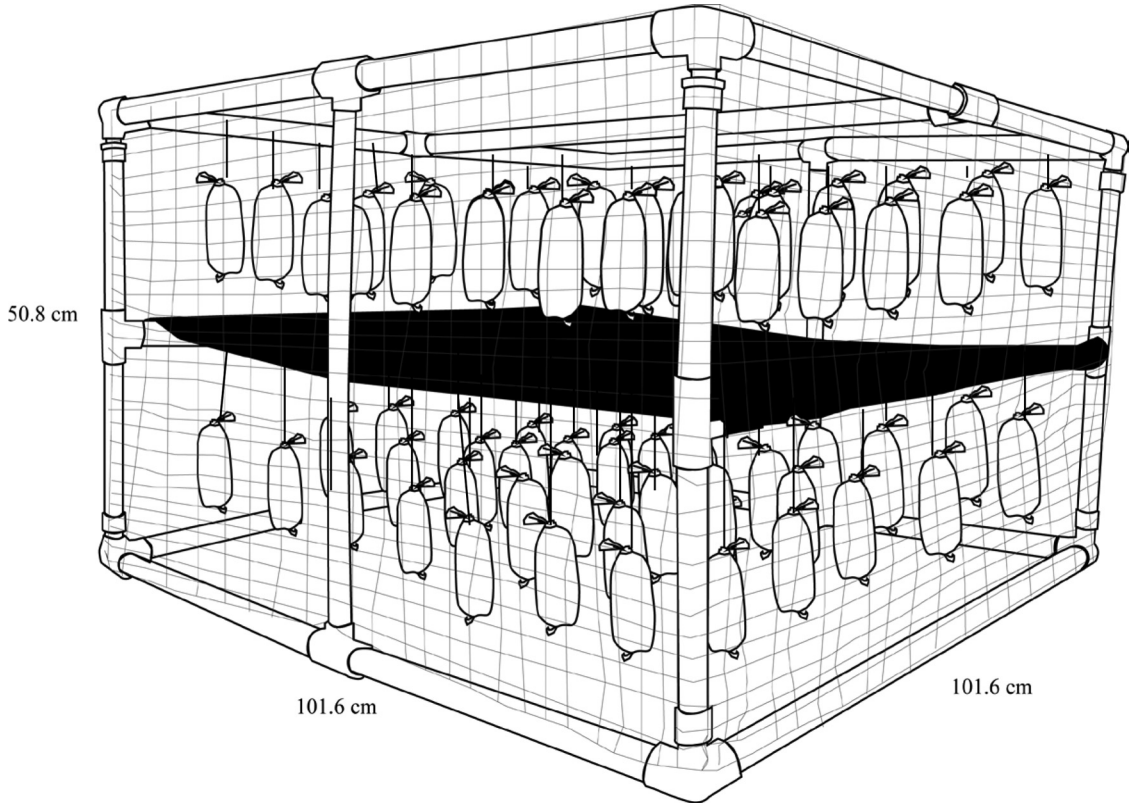

FIG 1 Schematic diagram of the submersible aquatic mesocosm.

different environmental factors have on their decline. Here we present the effects of ambient sunlight and biotic interactions on decay of sewage-born FIB, measured by both culture and qPCR techniques, as well as selected human-associated MST genetic markers. In order to simulate accidental release of high concentrations of untreated sewage and capture the complexities of the changing environmental conditions, we utilized an *in situ* mesocosm device consisting of multiple diffusion bags filled with mixtures of primary treated sewage and river water (34).

MATERIALS AND METHODS

Site description. Experiments were conducted on the Upper Mississippi River at Buffalo Shores Beach (GPS coordinates 41°27′11.23″N and 90°44′33.33″W) during October 2011. The average water temperature and turbidity range during the study were 14.1 ± 0.7°C and 14.5 to 19.8 nephelometric turbidity units (NTU), respectively. The monthly average solar insolation incident on a horizontal surface (3.08 kW h m^{-2} day^{-1}) and monthly average daylight cloud (61.9%) data for the Buffalo Shores Beach were obtained from NASA Langley Research Center (http://eosweb.larc.nasa.gov/).

Submersible aquatic mesocosm. A submersible mesocosm device (Fig. 1) was constructed as previously described (34). Briefly, the device consisted of a polyvinyl chloride (PVC) pipe (diameter, 3/4 to 1 in.) frame wrapped in plastic mesh wire to prevent floating debris from damaging the encased 200-ml dialysis tubing diffusion bags. The mesocosm was designed to evaluate the effect of sunlight irradiation, so the lower half of the device was covered using a heavy-duty black plastic sheet (dark treatments) and the top half of the device left uncovered (light treatments) (Fig. 1). The mesocosm was deployed so that the light treatment dialysis bags were submerged approximately 5 to 10 cm below the water surface. The potential sunlight attenuation by the dialysis bag was evaluated by measuring the strength of UV light (solar power meter by Ambient Weather, Chandler, AZ, and UVX radiometer by UVP, LLC, Upland, CA) with and without the dialysis bag cover. The difference in UV readings was minor (i.e., <10%).

Sample mixtures (200 ml) were contained within dialysis bags consisting of 75-mm-flat-width, 13- to 14-kDa-pore-size regenerated cellulose

TABLE 1 Summary of master calibration curve characteristics and TaqMan probes and primers used

Assay	Target(s)	Equation	*E*	r^2	LLOQ[b]	Reference(s)
Entero1a[a]	Enterococcal 23S rRNA gene and internal amplification control	$y = 38.4 - 3.38x$	0.94	0.99	0.84	67, 68
GenBac3	*Bacteroidales* 16S rRNA gene	$y = 38.9 - 3.50x$	0.94	0.99	0.89	29
HF183	Human-associated *Bacteroidales* 16S rRNA gene	$y = 38.4 - 3.30x$	0.94	0.98	0.98	69
HumM2	*Bacteroidales*-like putative σ factor	$y = 39.6 - 3.30x$	0.94	0.98	0.84	70

[a] Multiplex format.
[b] The lower limit of quantification (LLOQ), expressed as a $\log_{10}$ copy number, represents the upper bound of the 95% confidence interval for the average of 10 copy dilutions from six independent standard curves ($n = 18$).

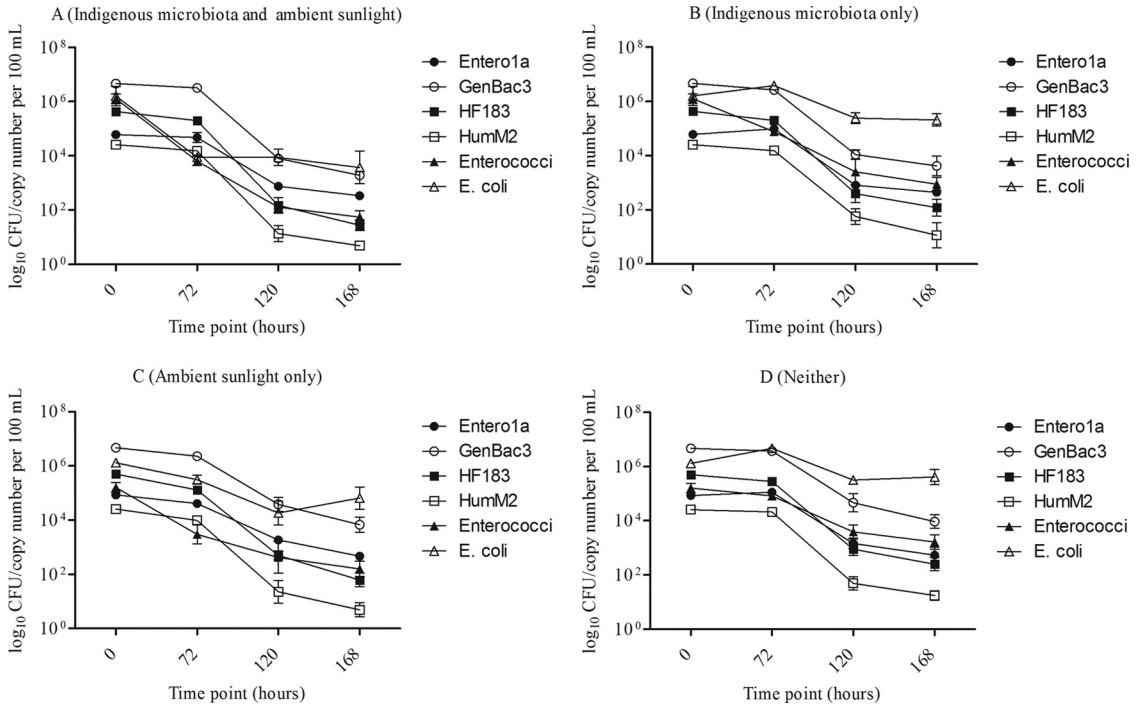

FIG 2 Changes in concentration estimates over time for culture-based and molecularly based FIB, as well as genetic human-associated MST markers, when exposed to different treatment variables. Data are averages from replicate dialysis bags ($n = 3$); error bars represent standard deviations.

dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA); bags were affixed to the device using fishing line and fishing snap swivels. Three replicate dialysis bags for each treatment (treatments 1 to 4, described below) were collected approximately every other day for a period of 7 days. Upon harvest, dialysis bags were placed into marked ziplock bags, filled partially with ambient water at the site (to avoid desiccation), and transported on ice within an hour to Iowa State Hygienic Laboratory located in Coralville, IA. Dialysis bags were vigorously shaken to mix the contents and opened using sterile scissors.

Study design. In order to determine the effects of environmental variables on the decay of culture-based and molecularly based FIB, as well as human-associated MST genetic markers, experimental treatments were as follows: 1, exposure to ambient sunlight and indigenous river microbiota; 2, exposure to indigenous river microbiota only; 3, exposure to ambient sunlight only; and 4, exposure to neither ambient sunlight nor indigenous river microbiota. For all of the treatments, dialysis bags were filled with 100 ml of Mississippi River water and 100 ml of primary sewage effluent from a local wastewater treatment plant (City of Davenport Water Pollution Control Plant; GPS coordinates 41°29′31.83″N and 90°37′41.71″W). A 1:1 ratio was selected to mimic sewage spills such as CSO events, where estimated loads from sanitary sewage can be greater than 50% and pollutants such as fecal indicator organisms are reported to be up to 10 times higher than in treated sewage discharges (35–40), and is consistent with earlier decay studies (23–25, 34, 41, 42). Both river water and primary sewage effluent were collected in the morning on the same day that the mesocosm was deployed. For treatments 3 and 4, indigenous microbiota were removed by filtering the river water through 0.45-μm and then 0.22-μm nitrocellulose membranes, followed by processing through an electropositive 6-inch NanoCeram cartridge filter (2 to 3 μm; Argonide, Stanford, FL) for virus removal (43). Residual culturable enterococci, *E. coli*, and aerobic/facultatively anaerobic heterotrophs in filtered river water were tested on mEI (44), modified mTEC (45), and tryptic soy agar (TSA), respectively, with negligible detection (i.e., <10 CFU per 1 ml of river water.

Culture-based FIB enumeration. Membrane filtration on mEI and modified mTEC agar was used to enumerate culturable enterococci and *E. coli* according to standard protocols (44, 45). In the earlier stages of the experiment (e.g., 0 h and 72 h), decimal dilution series were used, while 10-ml dialysis bag aliquots were processed for the later time points (e.g., 120 h and 168 h). All data were $\log_{10}$ transformed and expressed as $\log_{10}$ CFU 100 ml^{-1}.

qPCR assays. A 50-ml aliquot from each dialysis bag was filtered through a 0.45-μm nitrocellulose membrane, and the filter was stored at −80°C until further processing (<6 months). In order to minimize the holding time, samples were divided into two sets (or batches), each containing an even number of filters. DNA was extracted from filters using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA). The manufacturer's recommended protocol was used except for the following: (i) an additional 10-min incubation of bead beating tube containing filter and C1 reagent at 65°C followed by (ii) utilization of a FastPrep homogenizer (MP Biomedicals, Santa Ana, CA) for 1 min at 60 ms^{-1} instead of vortexing. Extracted DNA was stored at 4°C for no longer than 24 h prior to qPCR testing. The total mass of extracted DNA was quantified using the Quant-iT Pico Green double-stranded DNA (dsDNA) assay kit (Life Technologies, Grand Island, NY) on a SpectraMax Paradigm Multi-Mode microplate detection platform (Molecular Devices, LLC, Sunnyvale, CA) according to the manufacturer's instructions.

GenBac3, HF183, HumM2, and Sketa22 qPCR assays were performed in simplex format, while Entero1a was performed in multiplex format (the targets were the enterococcal 23S rRNA gene and an internal amplification control [IAC]), as described previously (46). All qPCR assays were performed within 24 h after DNA extraction. Table 1 lists the TaqMan probes (Life Technologies, Grand Island, NY) and primers used. Environmental MasterMix (Life Technologies, Grand Island, NY) was used for qPCRs. Simplex reaction mixtures contained 0.2 mg ml^{-1} bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 μM each forward and reverse primers, 80 nM 6-carboxyfluorescein (FAM)-labeled TaqMan probe (Life Technologies, Grand Island, NY), and 2 μl of template DNA, and the

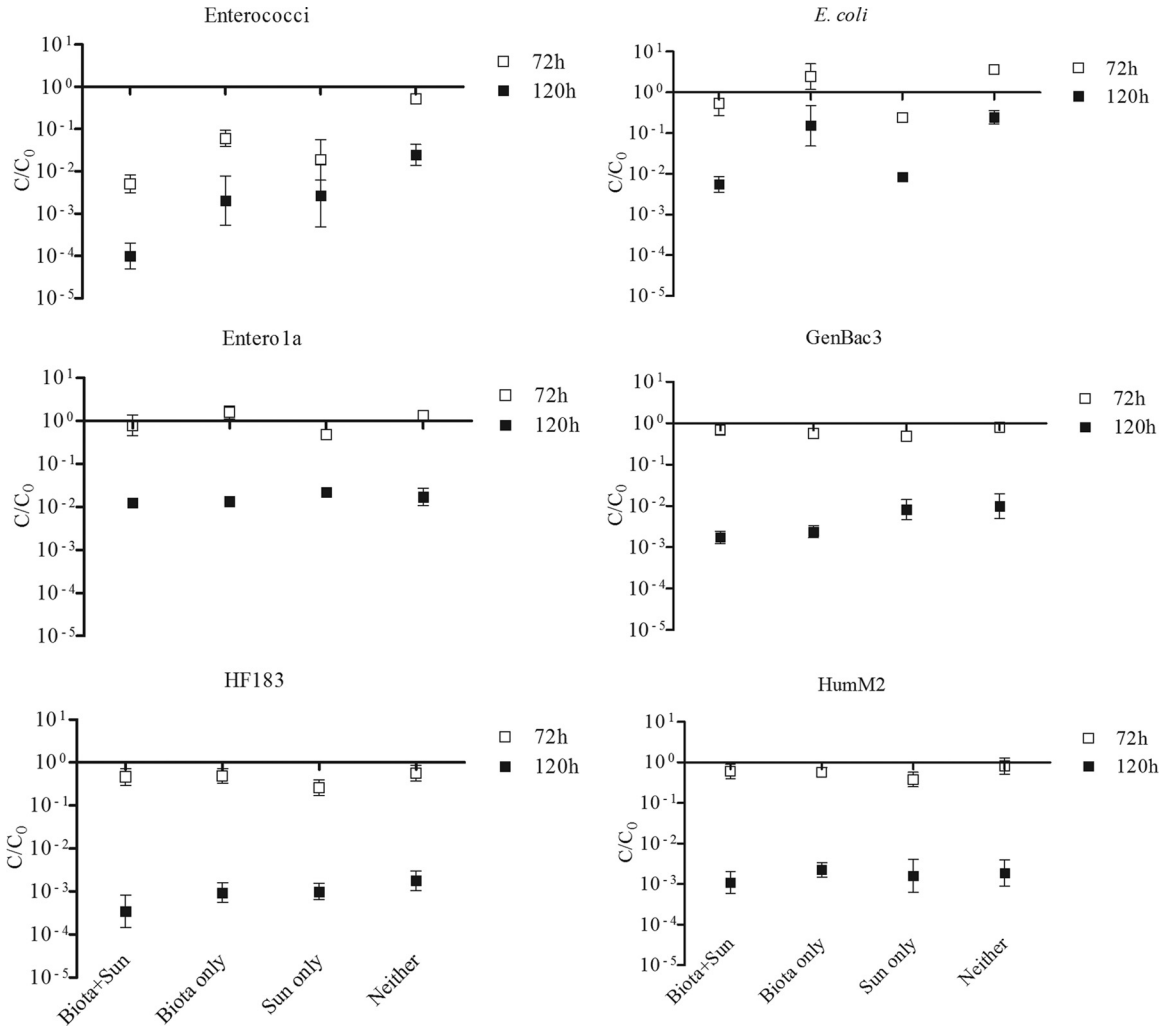

FIG 3 Effects of treatment variables on decay rates of culture- and molecularly based FIB, as well as human-associated MST genetic markers, after 72 h and 120 h. Data are averages from replicate dialysis bags ($n = 3$); error bars represent standard deviations.

volume was brought up to 25 μl using ultrapure H_2O (Sigma-Aldrich, St. Louis, MO). Multiplex reaction mixtures were prepared using the same reaction conditions as described above except with the addition of 80 nM VIC-labeled UCP1 TaqMan probe, and 2 μl of IAC template (50 copies). For calibration curve reactions, 2 μl of linearized plasmid constructs ranging from 10 to 10^6 copies per reaction were used as templates. All qPCRs were performed in triplicate using a 7900 HT Fast real-time sequence detector (Life Technologies, Grand Island, NY). The thermal cycling profile for all assays was as follows: 2 min at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The threshold was adjusted manually to either 0.03 (GenBac3, Entero1a, HF183, and Sketa22) or 0.08 (HumM2) and quantification cycle (C_q) values were exported to Microsoft Excel for further analyses.

Quality assurance/quality controls (QA/QC). A new working stock of the salmon DNA sample processing control (final concentration, 0.2 μg ml^{-1}) was prepared for each DNA extraction batch and added to each bead beating tube containing nitrocellulose filter and to extraction blanks (five replicates per DNA extraction batch). The concentration of salmon DNA was estimated using the Sketa22 qPCR assay (4, 46–48). Acceptance thresholds were calculated for each batch as extraction blank mean $C_q + 3$, as previously used (46, 49).

Potential amplification interference was specifically estimated by spik-

TABLE 2 Effect of environmental variables on decline of culture-based FIB

Assay	Time point (h)	Biotic interactions		Sunlight		Interaction	
		% Contribution	*P* value	% Contribution	*P* value	% Contribution	*P* value
Enterococci	72	24	0.002	66	<0.0001	1.4	0.30
	120	44	0.003	36	0.005	0.8	0.60
E. coli	72	0.8	0.51	80	<0.0001	6.4	0.08
	120	1.6	0.27	89	<0.0001	0.01	0.93

TABLE 3 *Post hoc* tests of effect of biotic interactions in the presence or absence of sunlight for culture-based FIB

Assay	Time point (h)	Sunlight	*P* value
Enterococci	72	Present	>0.05
		Absent	<0.01
	120	Present	<0.05
		Absent	>0.05
E. coli	72	Present	>0.05
		Absent	>0.05
	120	Present	>0.05
		Absent	>0.05

ing 50 copies of the IAC into Entero1a test reaction mixtures as previously described (47). The interference threshold was established by repeated readings of the IAC ($n = 20$) in buffer only. Evidence of amplification interference was defined as C_q values exceeding the control IAC mean $C_q \pm 1.5$ (49–52). In order to estimate contamination by extraneous DNA, a minimum of five no-template controls (NTCs) were included with each instrument run.

Data analyses. Master calibration curves (generated from six independent standard curves), lower limit of quantification (LLOQ), and concentrations estimates of qPCR genetic markers were calculated using a Bayesian Markov chain Monte Carlo (MCMC) approach on the publicly available software WinBUGS, version 1.4.1 (53). The upper bound of the 95% confidence interval for the highest dilution (i.e., 10 copies) of the plasmids used to generate master calibration curves served as the LLOQ. Amplification efficiencies (E) were calculated using the formula $E = 10^{(-1/\text{slope})} - 1$, and concentration estimates are reported as $\log_{10}$ copy number 100 ml^{-1}.

Changes in target bacteria estimates were calculated as $\log_{10}(C/C_0)$, where C_0 was the estimate at the beginning of the experiment and C was the estimate remaining after 72 or 120 h. The 72-h time point was selected to represent early stages of the decay, and 120 h was selected as the final time point because estimates for some of the targets at the 168-h time point were below the respective LLOQ.

Two-way analysis of variance (ANOVA) (StatMate version 2.0 for Windows; GraphPad, San Diego, CA) was used to characterize effects of exposure to indigenous river microbiota or ambient sunlight on culture-based FIB, qPCR-based FIB, and human-associated MST measurements. Data for the analyses were organized in a 2-by-2 block design with "indigenous microbiota" and "sunlight" as fixed factors, each with two levels (i.e., presence and absence). The number of data points used in each two-way ANOVA ranged from 12 (culture based) to 36 (qPCR). The Pearson correlation coefficient (StatMate version 2.00 for Windows; GraphPad, San Diego, CA) was used to determine whether statistically significant relationships between decay patterns existed for the different indicator types.

RESULTS

QA/QC and performance metrics. Master calibration curve equations and associated performance metrics are listed in Table 1. The total DNA mass per reaction ranged from 5.5 ng to 45 ng, depending on the sample. Addition of salmon DNA and subsequent Sketa22 qPCR analysis was used as a sample processing control, and all samples were within acceptance values for the first and second batches of DNA extractions, being 24 ± 3.0 C_q and 28 ± 3.0 C_q, respectively (46, 49). DNA extraction batch-specific thresholds were determined for the IAC to identify potential amplification interferences, and these values were 31.1 + 1.5 C_q and 33.9 + 1.5 C_q for the first and second batches, respectively (49, 50, 52). No amplification interference was detected. A total of 108 qPCR amplifications (sum of NTCs and extraction blanks) were used to assess potential contamination by extraneous DNA, and none contained C_q values within the quantification range of the qPCR assays (Table 1). Variability between triplicate qPCR measurements was calculated as percent coefficient of variation (CV), and it ranged from 0.12 to 4.06 at 0 h, from 0.09 to 3.82 at 72 h, and from 0.54 to 19.7 at 120 h for all assays. For the last time point (168 h), the percent CV for the GenBac3 and Entero1a assays ranged from 0.34 to 5.18, as these two targets remained within our range of quantification, but it was considerably higher for HF183 and HumM2 (1.57 to 71.0), since a number of samples were below the LLOQ at this time point.

Culturable enterococci and *E. coli*. Standard membrane filtration methods were used to enumerate enterococci and *E. coli* for each time point and treatment. Both groups exhibited a largely biphasic decay pattern, with slight decreases in concentrations observed within 72 h followed by greater reductions between 72 and 120 h and apparent leveling off during the last 48 h (Fig. 2). Overall, the highest reductions of culturable FIB occurred when dialysis bags were exposed to both indigenous microbiota and ambient sunlight, while selective exclusion of either factor generally resulted in prolonged survival (Fig. 2 and 3; Tables 2 and 3). For enterococci, both factors were important in early decay dynamics (i.e., after 72 h), although sunlight appeared to be a more important contributor (66% of total variation) than microbiota (24%) (Tables 2 and 3). At later time intervals (i.e., after 120 h), the importance of sunlight decreased (36% of total variation), while the contribution from microbiota became more important (43%) (Fig. 3; Tables 2 and 3).

In contrast to the case for culturable enterococci, exposure to ambient sunlight was the only significant factor impacting culturable *E. coli* and contributing more than 80% to total variability, irrespective of the time point (Fig. 3; Tables 2 and 3). Interaction of variables was not statistically significant for either enterococci or *E. coli* (Tables 2 and 3).

FIB (Entero1a and GenBac3) measured by qPCR. FIB measured by qPCR followed biphasic decay patterns similar to those described for culturable enterococci and *E. coli* (Fig. 2), although the decrease in concentrations was less pronounced. Further-

TABLE 4 Effect of environmental variables on decline of molecularly based FIB

Assay	Time point (h)	Biotic interactions		Sunlight		Interaction	
		% Contribution	*P* value	% Contribution	*P* value	% Contribution	*P* value
Entero1a	72	8.0	0.01	56	<0.0001	1.9	0.20
	120	34	0.0002	1.5	0.38	5.0	0.11
GenBac3	72	0.04	0.89	5.7	0.09	35	0.0001
	120	70	<0.0001	2.0	0.14	0.1	0.70

TABLE 5 *Post hoc* tests of effect of biotic interactions in the presence or absence of sunlight for molecularly based FIB

Assay	Time point (h)	Sunlight	*P* value
Entero1a	72	Present	<0.05
		Absent	>0.05
	120	Present	<0.001
		Absent	>0.05
GenBac3	72	Present	<0.01
		Absent	<0.05
	120	Present	<0.0001
		Absent	<0.0001

TABLE 7 *Post hoc* tests of effect of biotic interactions in the presence or absence of sunlight for human-associated MST genetic markers

Assay	Time point (h)	Sunlight	*P* value
HF183	72	Present	<0.05
		Absent	>0.05
	120	Present	<0.01
		Absent	>0.05
HumM2	72	Present	<0.05
		Absent	>0.05
	120	Present	>0.05
		Absent	>0.05

more, both Entero1a and GenBac3 appeared to decrease less than the human-associated genetic MST markers (Fig. 2). Exposure to ambient sunlight was a significant factor in the early decay of Entero1a signal, contributing 56% to the variation, but its importance diminished as time progressed (Fig. 3; Tables 4 and 5). The opposite was the case in the presence of indigenous aquatic microbiota; while only a marginally important contributor to early stages of decay (8.0% contribution to variability), it became the only significant contributor after 120 h, accounting for 34% of the variability (Fig. 3; Tables 4 and 5). There was no statistically significant interaction between variables in this data set (Tables 4 and 5).

Neither variable was a statistically significant contributor to the early decay dynamics of GenBac3; however, after 120 h, the importance of the presence of indigenous aquatic microbiota became more apparent, as it was the only statistically significant contributor to decay for that time point, accounting for 70% of the variation (Fig. 3; Tables 4 and 5). Interaction of variables was statistically significant in the early stages of decay, but not after 120 h, suggesting that the effect of the presence of indigenous aquatic microbiota on GenBac3 was not dependent on sunlight in the later stages of the decay.

Human-associated MST genetic markers (HF183 and HumM2). Overall, the decay patterns of human-associated MST markers were similar to those observed for qPCR-based FIB (Fig. 2 and 3). This was especially evident for HF183, where early decay dynamics were significantly impacted by sunlight only (17% contribution to variation), with the importance of indigenous river microbiota presence becoming more evident after 120 h (28% contribution to variability) (Fig. 3; Tables 6 and 7). Interaction of variables was significant only for decay patterns observed after 72 h, suggesting that the impact of sunlight within that time frame was dependent on the presence of indigenous aquatic microbiota.

Similar to the early decay pattern of HF183, sunlight was the only statistically significant factor impacting decline of the HumM2 marker after 72 h of exposure, and statistically significant interaction of variables suggests that the observed effect of sunlight was dependent on the presence of indigenous aquatic microbiota (Fig. 3; Tables 6 and 7). However, unlike for HF183, neither environmental variable was an important contributor to decay after 120 h of exposure (Fig. 3; Tables 6 and 7). It is important to note that the HumM2 marker was near the LLOQ for some of the treatments at this time point, which could introduce higher variability.

Correlations in decay of different indicator types. There was a moderately strong, positive correlation between decay of the two culturable FIB examined, irrespective of the treatment type, but there was no statistically significant correlation between culturable FIB and qPCR-based FIB/human-associated genetic marker decay patterns (Table 8). The decay of qPCR-based FIB (Entero1a and GenBac3) and human-associated genetic markers (HF183 and HumM2) was strongly correlated among each other and between the two categories (Table 8).

DISCUSSION

Earlier epidemiological studies established a reasonable correlation between levels of culturable FIB and incidence of gastrointestinal illness in recreational bathers (54, 55), but more recent data support improved health risk prediction from qPCR targets (Entero1a more so than GenBac3) in municipal wastewater-impacted freshwater (56) and marine water (57) settings. Our *in situ* decay study was designed to investigate the effects of ambient sunlight and biotic interactions on culture-based enterococci and *E. coli*, FIB measured by qPCR, and select human-associated MST genetic markers.

Both exposure to ambient sunlight and biotic interactions (viral lysis, predation, and/or competition) are factors known to adversely affect survival of culturable FIB (enterococci and *E. coli*) in

TABLE 6 Effect of environmental variables on decline of human-associated MST genetic markers

Assay	Time point (h)	Biotic interactions		Sunlight		Interaction	
		% Contribution	*P* value	% Contribution	*P* value	% Contribution	*P* value
HF183	72	4.5	0.15	17	0.008	14	0.02
	120	28	0.0002	23	0.0006	1.5	0.33
HumM2	72	0.3	0.71	15	0.01	19	0.006
	120	0.6	0.71	9.5	0.13	3.7	0.33

TABLE 8 Correlations in decay patterns of culture-based/qPCR-based FIB and human-associated genetic MST markers

Indicator	Correlation (*P* value) with:					
	Enterococci	*E. coli*	Entero1a	GenBac3	HF183	HumM2
Enterococci		0.76 (0.005)	0.50 (0.05)	0.55 (0.04)	0.53 (0.04)	0.47 (0.06)
E. coli			0.64 (0.02)	0.62 (0.02)	0.60 (0.01)	0.65 (0.02)
Entero1a				0.96 (<0.0001)	0.98 (<0.0001)	0.98 (<0.0001)
GenBac3					0.98 (<0.0001)	0.96 (<0.0001)
HF183						0.99 (<0.0001)

the environment (58–60). However, recent studies have shown that the magnitude of that effect is likely to vary among FIB in different habitats and even in different locations within the same habitat (i.e., water column and sediments) (58, 60). Our results indicate that persistence of culturable, sewage-borne enterococci in the Mississippi River water was affected by both ambient sunlight and biotic interactions. Sunlight was a more important determinant of survival in the early stages, while biotic interactions became more influential in the later stages. This finding is similar to the results of Wanjugi and Harwood (60), who showed that the survival of culturable enterococci was negatively affected by indigenous river microbiota; however, they used a single strain of *Enterococcus faecalis*, while we utilized enterococci present in primary sewage effluent. The effect of sunlight on culturable, sewage-borne enterococci also corroborates a previous report indicating a significantly greater reduction when exposed to sunlight compared to dark conditions (42).

Unlike enterococci, culturable sewage-borne *E. coli* did not appear to be affected by biotic interactions. Instead, exposure to sunlight was the only significant factor in decay in both early and later stages of the experiment. The effect of sunlight exposure on *E. coli* is not surprising (42, 61, 62), but the lack of an effect from biotic interactions was unexpected. Recent studies (34, 58, 60) have shown that the indigenous aquatic microbiota is an important determinant of *E. coli* survival in both marine and freshwater habitats. While some of these studies utilized laboratory-grown strains and were conducted in outdoor mesocosms (58, 60), which may overestimate the effects of experimental variables, others were conducted using the same *in situ* mesocosm and primary sewage effluent as the inoculum (34). The most notable differences between these studies were temperate versus subtropical rivers (Mississippi River, IA, versus Hillsborough River, FL) used as receiving waters and different (local) primary sewage effluents, suggesting that associated variables such as indigenous microbial populations (from sewage and ambient water) may play an important role. In the current study, we noted a much stronger effect of sunlight exposure, which was not apparent in our earlier subtropical river work (34), possibly due at least in part to the higher turbidity of the latter (17.0 versus 66.6 NTU). Previous studies have shown that bacterivorous protozoa prefer metabolically active prey over damaged or dead cells (63–65), which suggests that the lack of a biotic interaction effect in the current study may be partly due to the cellular damage caused by sunlight exposure.

As previously reported (25, 27), we also observed extended persistence of the Entero1a qPCR signal compared to that of culturable enterococci and slower decay of qPCR-based FIB (Entero1a and GenBac3) than of human-associated MST genetic markers (24, 25) but relatively similar overall decay profiles among all qPCR targets (25, 30), at least partly due to comparable responses to treatments tested in this study. The detrimental effects of sunlight and biotic treatments on the culturable fecal indicator bacteria compared to DNA molecules targeted by qPCR suggest that the underlying mechanisms of decay were different. However, similarities in decay trends across all qPCR assays suggest that the mechanism for deterioration of the primer/probe region may be the same. Additional research is needed to characterize how robust these trends are under other conditions and how these differences help or hinder water quality applications such as recreation water quality monitoring, fecal source allocation, and subsequent QMRA modeling.

The existing literature on the effect of ambient sunlight on decay of genetic MST markers in surface freshwaters (groundwater and lake, creek, and river water) generally supports the point that sunlight is not an important factor in decay (25, 27, 30, 66). These studies employed modeling of the decay rates and usually did not examine decay patterns at early time points. Our work supports the idea to describe *in situ* decay profiles during the period when the effect of experimental variables appeared to be the strongest. Thus, while our results suggesting that ambient sunlight does not appear to be an important contributor after 120 h of wastewater release agree with previous findings, the significant effect of sunlight on early-stage decay dynamics appears to be less well understood.

Only a limited number of studies have examined the effects of biotic interactions (predation, viral lysis, and competition) on the persistence of (q)PCR targets, such as *Bacteroides* and/or enterococci in freshwater environments (23, 24, 26, 28). The earlier work supports the view that survival of a laboratory strain of *Bacteroides fragilis* (23) and feces-derived *Bacteroides distasonis* (28) was negatively impacted by the combined effects of predation and elevated temperatures. Extended persistence of sewage- and/or fecally derived indicators (measured by qPCR) and human-associated MST genetic markers is documented for autoclaved (24) and filter-sterilized (26) river water. Our study expands on these findings, as it was not conducted either under laboratory conditions (24) or in a fully closed outdoor mesocosm (23, 26); rather, we attempted greater environmental interaction by using *in situ* submersed diffusion bags and primary sewage effluent as an inoculum.

Although these experiments advance our understanding of fecal indicator decay in a freshwater system, it is important to note that this study focused on pollution events where high concentrations of untreated (or partially treated) sewage are typical and may not be representative of pollution events from treated sewage. Furthermore, it is also likely that biotic effects will be influenced by the proportion of pollution source to ambient river water. Additional research is warranted to determine if the trends identified in this study are consistent under these different scenarios.

In summary, our results indicate that biotic interactions and

exposure to ambient sunlight are both important factors in the decay of sewage-borne culturable and qPCR-based FIB, as well as human-associated MST genetic markers. In general, sunlight-induced decay was often a key factor in the early stages of decomposition (<72 h after wastewater release), while biotic interactions played a larger role during the later stages. Future studies are needed to explore the range of influence that different water types and associated indigenous microbiota can have on the decay of fecal pollution markers, combined with characterization of microbial community members to identify key taxa involved in these important biotic interactions.

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