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Differential Decay of Enterococci and *Escherichia coli* Originating from Two Fecal Pollution Sources

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Using *in situ* subtropical aquatic mesocosms, fecal source (cattle manure versus sewage) was shown to be the most important contributor to differential loss in viability of fecal indicator bacteria (FIB), specifically enterococci in freshwater and *Escherichia coli* in marine habitats. In this study, sunlight exposure and indigenous aquatic microbiota were also important contributors, whose effects on FIB also differed between water types.

The fecal indicator bacteria (FIB), enterococci and *Escherichia coli*, are used for the assessment of sanitary quality of recreational waters worldwide. A reasonable correlation between the incidence of gastrointestinal illness in recreational bathers and levels of FIB established in the earlier epidemiological studies (1, 2) continues to be supported by more recent data (3). Despite their long history of use, many uncertainties remain concerning the fate of FIB upon release into the environment and hence their role as a useful predictor of recreator health effects when released from various fecal sources (4).

While sources of FIB are well described and include many mammals and birds, as well as environmental sources (e.g., soils, sediments, and aquatic vegetation) (5–11), few studies have explored survival rates of FIB from animal sources (12). In general, human fecal pollution sources (e.g., sewage and septage) have been investigated more extensively than others (such as domestic farm animals, wildlife, etc.) because of the risk entailed by enteric viral pathogens (13), which are largely assumed to be human specific. However, recent data suggest that risks posed to human health by fresh cattle feces may not be substantially different than those from human sources (14), warranting the need for further research in this area, particularly in relation to the fate of bovine FIB versus those of sewage origin.

Earlier studies report that exposure to ambient sunlight and biotic factors (competition and predation by indigenous aquatic microbiota) are important contributors to FIB decay in ambient waters (15–19). In general, the detrimental effect of sunlight is more pronounced in marine waters than in freshwater (12, 16, 20, 21), while the opposite is the case for biotic interactions (22, 23). However, the majority of FIB decay studies use laboratory-grown control strains and conduct experiments under artificial conditions (18, 24–27), which cannot accurately depict the behavior of FIB originating from "natural" sources and the complexity of interactions in aquatic ecosystems.

The objective of this study was to investigate the effect of select environmental parameters, including (i) water type, (ii) exposure to ambient sunlight, and (iii) the presence of indigenous aquatic microbiota, on the viability (i.e., culturability by standard methods) of *E. coli* and enterococci originating from cattle manure or primary municipal wastewater effluent. Field-deployable submersible mesocosms allowed the assessment of environmental stressors on the decay of FIB by closely mimicking the release of these organisms into a recreational water body.

Two submersible mesocosms measuring approximately 50.8 cm (height) by 101.6 cm (width) by 101.6 cm (depth) were constructed using 1-in. and ¾-in. polyvinyl chloride (PVC) pipes. Small holes were drilled into the commercial-grade, white PVC pipe frame to allow the device to submerge upon deployment. The mesocosm allowed for both light (top half) and dark (bottom half) treatments. In the dark treatment, the lower half was covered by a heavy-duty opaque sheet that effectively blocked sunlight. Sample mixtures with a total volume of 200 ml were contained using 75-mm-flat-width, 13- to 14-kDa-molecular-mass-cutoff regenerated cellulose dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA). Fishing line (24-lb test line) was tied directly onto the PVC mesocosm frames, and dialysis bags containing samples were attached using ¾-in. fishing snap-swivels and 6-lb test line. The sides of the rectangular frame were wrapped in plastic wire mesh to prevent accidental puncture of the bags from floating debris.

The two submersible mesocosms were deployed for a 7-day period at a freshwater site (Riverfront Park at Hillsborough River, Tampa, FL; 28°04′11″N, 82°22′38″W) and at a marine water site (Fort De Soto Park at Gulf of Mexico, Tierra Verde, FL; 27°38′17″N, 82°43′07″W). Study sites were selected based on accessibility and the availability of structures that would allow each mesocosm to be secured (dock or pier) in waters where recreation was considered acceptable.

Hourly light intensity and temperature measurements were recorded by a Hobo data logger (Bourne, MA). Light intensity was measured approximately 2 to 3 cm below the surface of the water (i.e., at the level of the sunlight-exposed dialysis bags), and it averaged 3,545 and 4,530 lm m^{-2} for freshwater and marine sites, respectively. Average temperatures (± standard deviation [SD]) for the duration of the experiment were 20.8 ± 2.22°C (freshwater site) and 21.2 ± 2.10°C (marine site). Average solar insolation

Received 7 December 2012 **Accepted** 29 January 2013
Published ahead of print 1 February 2013
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doi:10.1128/AEM.03781-12

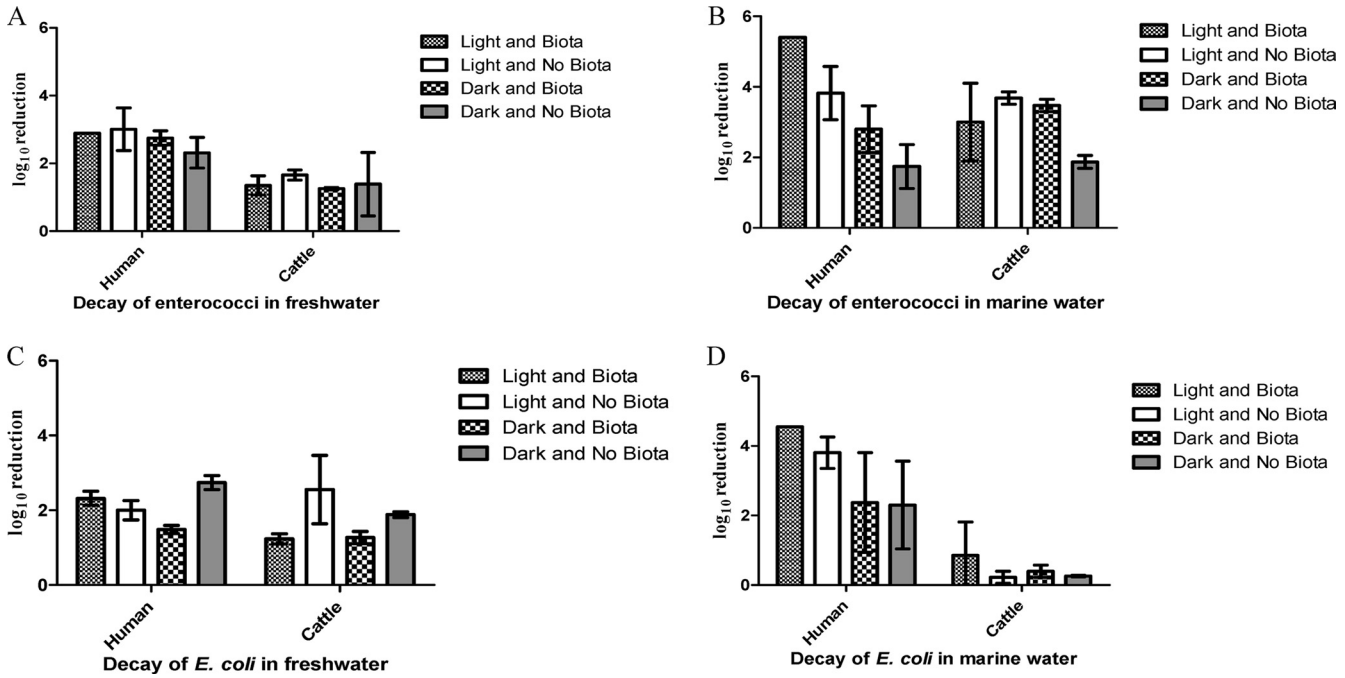

FIG 1 Log_{10} reduction over 7 days of FIB from sewage and cattle manure in marine (B and D) and freshwater (A and C) mesocosms. Shown are the effects of fecal source and treatments (±sunlight and ±aquatic microbiota).

incident on a horizontal surface for the study month of February (5.01 kWh m^{-2} day^{-1}) and clearness index 0.67 (cloud cover normalized on a scale from 0 to 1) data for the region where the mesocosms were installed were obtained from the NASA Langley Atmospheric Science Data Center (http://eosweb.larc.nasa.gov/cgi-bin/sse/sse.cgi).

Primary wastewater effluent from a local municipal wastewater treatment plant (Howard F. Curren Advanced Wastewater Treatment Plant, Tampa, FL) was collected on the day of the experiment (time 0 [T_0]), and fresh cattle manure was collected from 10 different animals on the day prior to the experiment and held at 4°C overnight to minimize changes in microbial populations. Dialysis bags representing a "human" source were filled with 100 ml of primary effluent and 100 ml of either untreated (e.g., not filter-sterilized) or filter-sterilized ambient water, depending on the biotic treatment (described below). A composite source ("cattle") was created by mixing 10 g of cattle manure from each animal, followed by resuspension (1:10 ratio) in sterile phosphate-buffered water (0.0425 g · $liter^{-1}$ KH_2PO_4 and 0.4055 g · $liter^{-1}$ of $MgCl_2$; pH 7.2) (28). Fecal suspensions were vigorously vortexed until there were no visible clumps, and solids were allowed to settle for 5 min. One hundred milliliters of the supernatant was then mixed with ambient water as described above.

In order to determine the effect of environmental variables on the loss of viability of FIB, treatments included (i) exposure to ambient sunlight and aquatic indigenous microbiota (treatment a), (ii) exposure to ambient sunlight only (treatment b), (iii) exposure to aquatic indigenous microbiota only (treatment c), and (iv) exposure to neither variable (treatment d) (see Table 2). This experimental design was replicated for both FIB sources (primary effluent and cattle manure) and water types (freshwater and marine water). For treatments that contained no aquatic indigenous microbiota (i.e., treatments b and d), ambient water was successively filter sterilized by 0.45-μm and 0.22-μm nitrocellulose membrane filters and finally through a NanoCeram filter (Argonide, Stanford, FL) to remove virus-sized particles. The removal efficiency for bacteria was evaluated using tryptic soy agar and mEI and mTEC media for aerobic/facultatively anaerobic heterotrophs, enterococci, and *E. coli*, respectively. All three types of media demonstrated negligible concentrations of bacteria (i.e., <5 CFU/100 ml). Triplicate dialysis bags per treatment were collected at the beginning of the experiment (T_0), after 24 h (T_1), and every other day for 7 days.

Harvested dialysis bags were placed into marked ziplock bags filled with fresh ambient water (to avoid sample desiccation) and were transported on ice back to the lab for analysis. Dialysis bags were mixed by being inverted several times to evenly mix the contents prior to sample analyses. In the early stages of the experiment (e.g., T_0 and T_1), decimal dilution series were performed, and FIB

TABLE 1 Schematic of the experimental design

Source	Sunlight	Indigenous microbiota	Treatment designation
Human	Present	Present	a
		Absent	b
	Absent	Present	c
		Absent	d
Cattle manure	Present	Present	a
		Absent	b
	Absent	Present	c
		Absent	d

TABLE 2 The effect of bovine versus sewage fecal source on decay of enterococci and *E. coli* in two water types under four treatment regimens[a]

FIB	Water type	Source of variation	% of total variation	*P* value[b]
Enterococci	Freshwater	Fecal source	70.8	<0.0001
		Treatment	5.00	0.33
		Interaction	2.35	0.64
	Marine	Fecal source	3.31	0.085
		Treatment	56.9	<0.0001
		Interaction	24.0	0.0016
E. coli	Freshwater	Fecal source	10.8	0.015
		Treatment	39.3	0.0009
		Interaction	26.9	0.0052
	Marine	Fecal source	69.1	<0.0001
		Treatment	11.2	0.021
		Interaction	5.78	0.13

[a] The treatments were with or without sunlight and with or without aquatic microbiota. See Table 3 for individual effects.
[b] Two-way factorial ANOVA.

were enumerated by culture on mEI and mTEC media using standard membrane filtration protocols (29, 30). In the later stages of the experiment (e.g., T_5 and T_7), dilutions were not performed as levels of FIB decreased; 10 ml of undiluted sample was processed instead. All FIB densities were $\log_{10}$ transformed and normalized to a 100-ml volume. "Decay" (loss in viability) is expressed as $\log_{10}$ reduction over 7 days, calculated by subtracting the density on day 7 from that at the start of the experiment (T_0).

Prior to beginning the experiment, power analysis was conducted using pilot data to determine the appropriate number of replicates needed (e.g., dialysis bags per treatment) using GraphPad (StatMate version 2.00 for Windows; GraphPad, San Diego CA). The effect of fecal source (cattle manure or primary effluent), water type (freshwater or marine) and treatments (exposure to ambient sunlight and indigenous aquatic microbiota) was assessed by two-way factorial analysis of variance (ANOVA) (GraphPad Prism software version 5.00 for Windows).

In general, cattle manure FIB persisted considerably longer than their sewage counterparts, irrespective of the water type or treatment, and the difference was generally significant (Fig. 1 and Table 1). This finding is consistent with previous reports showing long-term persistence and even initial growth of *E. coli* and enterococci in cow fecal patties (31) and with bovine manure incorporated into soil (32). Extended persistence of FIB in cattle manure was especially evident for enterococci in freshwater and *E. coli* in marine waters, where densities decreased 2 to 3 orders of magnitude less than that of sewage FIB (Fig. 1A and D). Fecal source was the most important determinant of survival for enterococci in freshwater and for *E. coli* in marine water ($P < 0.0001$), contributing ~70% to the total observed variation (Table 2). Interaction of variables was not significant for either one of these data sets ($P > 0.05$), suggesting that the influence of fecal source is not dependent on the treatment effects (i.e., presence/absence of indigenous aquatic microbiota or light/dark condition) but rather is a result of intrinsic properties of the FIB from different fecal sources.

At least one study suggested that decay rates of FIB are not source dependent, when the sources considered were limited to sewage influent/effluent and urban storm drain runoff (33). In contrast, our data indicate that FIB from bovine manure may exhibit markedly different decay patterns compared to sewage-derived FIB, presumably because of the nature of fecal sources (e.g., higher particulate matter content of cattle manure, providing potential nutrients and surface for attachment and different selective pressures on FIB in host gastrointestinal systems) as well

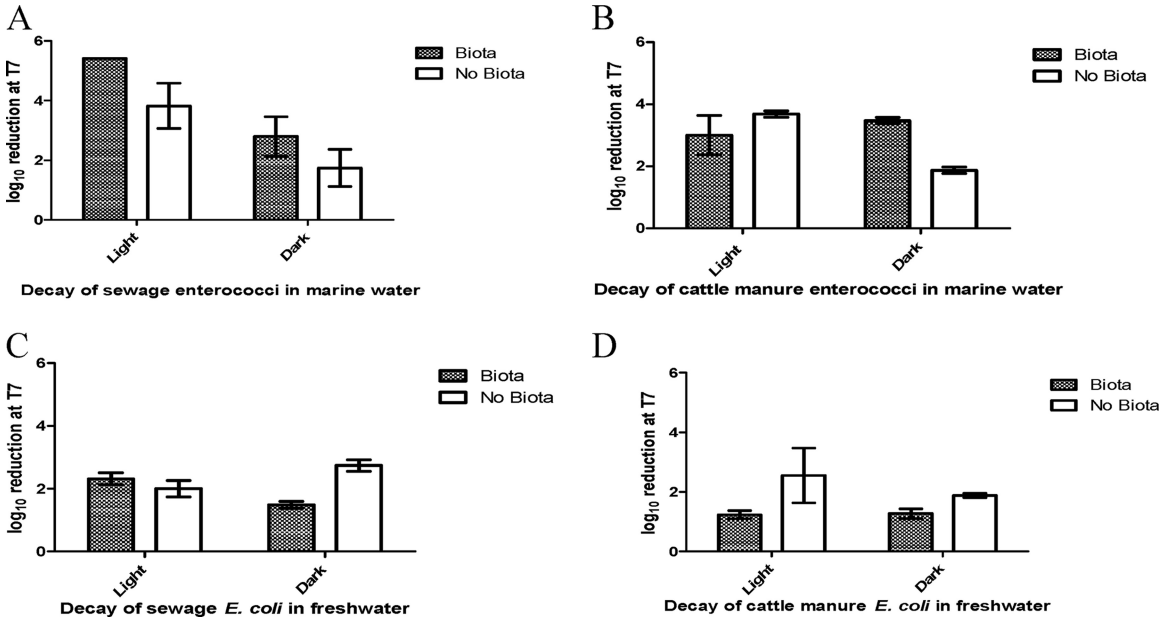

FIG 2 Log_{10} reduction over 7 days of enterococci in marine waters (A and B) and *E. coli* in freshwater (C and D) mesocosms. Shown are the effects of sunlight exposure and the presence of indigenous aquatic microbiota.

TABLE 3 The effect of light/dark treatments and the presence/absence of indigenous microbiota on decay of enterococci in marine and *E. coli* in freshwater habitats

FIB	Water type	Fecal source	Source of variation	% of total variation	*P* value[a]
Enterococci	Marine	Human	Light vs dark	66.7	0.0001
			Presence of biota	21.2	0.0048
			Interaction	0.83	0.47
		Cattle	Light vs dark	15.8	0.077
			Presence of biota	7.50	0.20
			Interaction	45.8	0.0087
E. coli	Freshwater	Human	Light vs dark	0.22	0.70
			Presence of biota	23.4	0.0031
			Interaction	65.7	0.0001
		Cattle	Light vs dark	5.78	0.23
			Presence of biota	53.1	0.0076
			Interaction	7.23	0.23

[a] Two-way factorial ANOVA.

as likely differences in the strains involved. This finding agrees with the differential survival of *E. coli* isolates from dog, sewage, and soil sources found by Anderson et al. (12); however, to the best of our knowledge, direct comparisons of FIB decay from cattle manure and sewage effluents have not previously been reported. This finding is important for several reasons, including the possibility that zoonotic (bacterial) pathogens from cattle manure may share the ability to survive in secondary habitats. Conversely, longer persistence of bovine FIB compared to likely zoonotic pathogens (4) would make them a more conservative marker of health risk.

This observed trend of extended survival of FIB from cattle manure was also evident for enterococci in marine waters and for *E. coli* in freshwater, which also included pronounced treatment effects (Fig. 1B and C and Fig. 2). Specifically, the presence/absence of indigenous aquatic microbiota and exposure to light/dark conditions contributed nearly 60% and 40% to observed variations in enterococcal ($P < 0.0001$) and *E. coli* ($P = 0.0009$) densities, respectively (Table 2). Interestingly, for enterococci originating from cattle manure, neither treatment variable was independently significant, but the presence of indigenous marine aquatic microbiota enhanced decay under the dark conditions (Fig. 2B and Table 3). In contrast, enterococci originating from sewage were impacted by both treatment variables (indigenous aquatic microbiota and sunlight), although exposure to sunlight was a more important determinant than it was for isolates from cattle (Fig. 2A and Table 3). Irrespective of the fecal source, the presence of indigenous aquatic microbiota was the only significant contributor to decay of *E. coli* in freshwater (Fig. 2C and D and Table 3). Many studies have reported the detrimental effect of sunlight on FIB survival (19, 34, 35), but the impact of indigenous aquatic microbiota is rarely considered in the same context. Our findings suggest that the commonly overlooked influence of biological interactions may bias results, leading to inaccurate assessments of the complex interplay of environmental variables on the fate of FIB (and possibly other zoonotic pathogens). In support of our conclusion, a recent FIB decay study conducted in Florida indicated that both predation and competition are important contributors to *E. coli* decay in subtropical freshwater systems (27).

In summary, we employed submersible *in situ* mesocosms to estimate the role of fecal pollution source, exposure to ambient light, and influence of microbial predation and competition on FIB decay. Although exposure to sunlight and the presence of aquatic microbiota under certain conditions were influential on FIB decay, the fecal source seemed to be the more consistent factor influencing FIB survival under the conditions employed in this study. Hence the extrapolation of health risks (implied by FIB) from sewage impacted recreational waters to those impacted by cattle manure requires additional comparisons to potential pathogens before it can be deemed valid.

ACKNOWLEDGMENTS

The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to Agency's administrative review and approved for publication. The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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