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Relative Decay of *Bacteroidales* Microbial Source Tracking Markers and Cultivated *Escherichia coli* in Freshwater Microcosms[▽]

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Fecal indicator bacteria (FIB), commonly used to regulate sanitary water quality, cannot discriminate among sources of contamination. The use of alternative quantitative PCR (qPCR) methods for monitoring fecal contamination or microbial source tracking requires an understanding of relationships with cultivated FIB, as contamination ages under various conditions in the environment. In this study, the decay rates of three *Bacteroidales* 16S rRNA gene markers (AllBac for general contamination and qHF183 and BacHum for human-associated contamination) were compared with the decay rate of cultivated *Escherichia coli* in river water microcosms spiked with human wastewater. The following five sets of microcosms were monitored over 11 days: control, artificial sunlight, sediment exposure, reduced temperature, and no autochthonous predation. Decay was characterized by estimation of the time needed to produce a 2-log reduction (t_{99}). No treatment-associated differences in the decay of the 4 targets were evident except with reduced predation, where *E. coli*, qHF183, and BacHum markers had lower levels of decay by day 3. However, there were substantial target-associated differences. Decay curves for the AllBac marker indicated a larger persistent population than those of the other targets. Exposure to sunlight, sediment, and reduced predation resulted in more rapid decay of the human-associated markers relative to cultivable *E. coli*, but there were no differences in t_{99} values among the 4 targets under control conditions or at reduced temperatures. Further evaluation of epidemiological relationships will be needed in order to relate the markers directly to health risk. These findings suggest that the tested human-associated markers can complement *E. coli* as indicators of the human impact on sanitary water quality under the constrained conditions described in this paper.

Recreational water quality standards for freshwater are commonly based on the cultivable concentration of the fecal indicator bacterium (FIB) *Escherichia coli* (42). Epidemiological studies demonstrated a correlation between *E. coli* concentration and rates of gastrointestinal illness among swimmers (19, 32, 46), and water quality criteria based on *E. coli* have been established by the U.S. Environmental Protection Agency for the protection of human health (41). However, *E. coli* are found in many hosts, both human and nonhuman, that carry different cohorts of human-pathogenic microorganisms (13), and *E. coli* types have limited host specificity (4, 16, 17). *E. coli* also have been shown to reproduce in the environment under some conditions (7, 12, 23, 37, 49). These limitations of *E. coli* as an indicator of public health risk are well recognized (34) and may result in revision of U.S. recreational water quality criteria (44).

In the pursuit of alternate assessment strategies, researchers in the field of microbial source tracking (MST) have developed and applied host-associated molecular markers of fecal contamination. Prominent among candidate MST protocols are those that detect and quantify the 16S rRNA gene of the order *Bacteroidales* fecal anaerobes. In particular, the human feces-associated (HF) *Bacteroidales* marker HF183, developed by Bernhard and Field (6) for detection by the PCR, is commonly used to detect human-source fecal contamination (14, 18, 20, 38). Several HF quantitative PCR (qPCR) assays (24, 33, 35) that target the same 16S rRNA gene cluster in the genus *Bacteroides* as that used by Bernhard and Field (6) have been designed.

Whereas current standards are based on cultivable *E. coli*, PCR-based MST protocols inherently measure both living and dead cells. Because of this, further validation of these molecular markers for MST in the current regulatory context requires an understanding of marker persistence compared with the persistence of cultivated *E. coli* over time and under various environmental conditions. Although research into live-dead discrimination of *Bacteroidales* shows some promise (2, 3, 48), measurement of total (DNA) marker remains the common method because it requires considerably less time and manipulation and is more easily applied in most laboratories.

Comparison of cultivation- and PCR-based parameters is complicated by potentially different decay characteristics of the targets. The decay of cultivated bacterial targets, such as *E. coli*, is driven by a number of environmental factors. Several studies have shown that *E. coli* decay is more rapid with exposure to sunlight, predation, and salinity and less rapid at colder temperatures (1, 10, 21, 28, 29). Turbidity and association with sediments also have been shown to enhance the survival of *E. coli* in water (10, 15, 27, 31).

Less is known about the persistence of *Bacteroidales* DNA under various environmental conditions. In a laboratory microcosm study, Kreader (25) detected *Bacteroides distasonis* in river water spiked with human feces for up to 2 weeks at 4°C by conventional PCR but for only 1 to 2 days at 24°C. In addition to temperature, predation was shown to be associated

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with more rapid marker decay. Seurinck et al. (35) used qPCR to quantify the HF marker (qHF183) in freshwater over time, and a 2-log reduction in the copy number was reached after 6 days at 28°C, a 1-log reduction was seen after 10 days at 12°C, and no significant reduction was seen in 28 days at 4°C. Okabe and Shimazu (30) investigated the decay of various *Bacteroidales* markers in microcosms spiked with feces by qPCR and found longer persistence with lower temperature and higher salinity. Bell et al. (5) spiked horse feces into stream water microcosms and used the AllBac general *Bacteroidales* qPCR assay (26) to compare the effects of temperature, fecal aggregate size, and concentration on decay rates in unfiltered (normal community) and filtered (reduced biological activity, including predation) water. As in the report by Kreader (25), temperature and predation were shown to be the controlling factors, while aggregate size and initial concentration had no significant effects on decay rates.

Reports on the effects of sunlight on *Bacteroidales* decay have been variable. Bae and Wuertz (3) found no difference in the times needed to produce a 2-log reduction (t_{99}) for either the general or HF *Bacteroidales*-based DNA markers BacUni and BacHum (24) in sunlight-exposed or dark seawater microcosms incubated at 14°C (t_{99} was approximately 7 days for both markers). This was in contrast to a seawater microcosm study by Walters et al. (48), in which the HF DNA marker BacHum exhibited significantly faster decay in natural sunlight (t_{90} = 1.77 days) than in the dark (t_{90} = 8.72 days) at 17°C.

In a freshwater application, Walters and Field (47) used qPCR to estimate HF marker concentrations in microcosms spiked with feces, incubated them at 13°C, and compared them with cultivated FIB. They found that exposure to natural sunlight did not significantly affect the persistence of either total (from both live and dead cells) HF *Bacteroidales* DNA markers or cultivable *E. coli*. However, measurement of rRNA by reverse transcriptase qPCR, rather than measurement of DNA by qPCR, indicated that exposure to sunlight did result in the more rapid decay of HF markers associated with living cells.

The objective of this study was to measure the relative decay of cultivated *E. coli* and general and HF *Bacteroidales* 16S rRNA gene copy numbers in wastewater-spiked river water as it ages under different conditions. Microcosms were constructed to simulate environmental stresses, spiked with sewage, and monitored over time. One potential application of these results is the use of an HF marker-to-AllBac ratio to help interpret the contribution of human sources to overall fecal load or of an HF marker-to-*E. coli* ratio to interpret the extent to which human sources contribute specifically to *E. coli* density. If decay rates among the general marker, HF markers, and *E. coli* are similar, these ratios could be considered stable over time and take the science one step closer to this eventual use.

MATERIALS AND METHODS

Microcosms and treatments. Microcosm experiments were conducted using constant-temperature walk-in incubators at the Soil Microbial Ecology Laboratory in the School of Environment and Natural Resources at The Ohio State University. Fresh river water from the Olentangy River near the Ohio State University was collected into autoclaved, 20-liter polypropylene carboys (Nalgene Nunc, Rochester, NY), using a sump pump after flushing with river water. Sediment was collected from the river bottom using a presterilized petite Ponar sediment sampler. Large organic debris (leaves and sticks) was picked from the sediment manually before addition to selected microcosms.

Each of the 15 microcosms consisted of 15 liters of river water spiked with 1% (vol/vol) settled, untreated wastewater from the Olentangy Environmental Control Center (Delaware, OH). The series of microcosms was divided in sets of 3 among 4 test treatments and 1 set of controls. The four test treatments were the following: (i) artificial sunlight, with typical distribution of UV (UVA and UVB) and photosynthetically active radiation (PAR) (12 h on/12 h off); (ii) reduced temperature (15°C); (iii) removal of autochthonous predation (autoclaved river water); and (iv) exposure to sediment (10 g/liter added and settled). All microcosms except those in the reduced temperature treatment were incubated at 25°C, and the experiment was carried out for 11 days. The initial turbidity of source river water was 102 nephelometric turbidity units (NTU); by the first day of the experiment, turbidities fell to between 45 and 69 NTU (the sediment microcosms had higher values than the others), and by the end of the experiment, turbidities dropped to between 22 and 47 NTU. The potential effect of the settling observed through this reduction in turbidity was addressed by shaking each microcosm to resuspend, and resampling after the 11-day sampling was collected and processed.

For all treatments except artificial sunlight, carboy lids were fitted with aquarium tubing and air release valves through rubber stoppers, and incubations took place in the dark under constant mixing using an air stone and aquarium air pump. Serological pipets (25 ml) were inserted through the rubber stoppers to allow for sampling. For the artificial sunlight experiments, 5-gallon buckets were bleached (6% hypochlorite), rinsed with distilled water, and covered with 0.05-mm-thick FEP-Teflon film, which is 96% transmissive to UVA and UVB light (Dupont Corporation, Wilmington, DE). Submersible, recirculating pumps were placed in the buckets to minimize condensation on the UV-transparent film, which was observed with the aeration method of mixing. The microcosms were acclimated in the incubators overnight at treatment temperatures prior to being spiked with wastewater. Submersible temperature loggers (Hobo Pendant; Onset Computer Corporation, Bourne, MA) were placed in 2 of the 3 light microcosms, the darkened control that was held in the same room, an additional control that was held in a 25°C room, and one test microcosm that was held in a 15°C room.

Artificial sunlight. A 400-W Philips MSR 400 HR hydrargyrum medium-arc iodide (HMI) lamp (Koninklijke Philips Electronics, Netherlands) was used to approximate the levels of natural UVA, UVB, and PAR, based on the American Society for Testing and Materials (ASTM) standard terrestrial solar spectral irradiance distributions for U.S. latitude 37°N (1a). The lamp's spectral power distribution is similar to that of sunlight (continuous spectrum from 250 to 800 nm; power plateau from 400 to 700 nm). It was tested prior to and during the experiment using radiometers to measure output in the UVA (320- to 400-nm) and UVB (280- to 320-nm) ranges (Solarmeter 5.0 and 6.2; Solartech, Inc., Harrison Township, MI) and the PAR (400- to 700-nm) range (Field Scout quantum light meter; Spectrum Technologies, Inc., Plainfield, IL).

Target intensities for UVA, UVB, and PAR (from ASTM typical summertime values) were 8.9 mW/cm^2 (UVA+UVB), 0.3 mW/cm^2 (UVB), and 1,950 μmol/m^2/s (PAR). The intensities of irradiation that the experimental apparatus delivered to the microcosms were 0.68 mW/cm^2 (UVA+UVB), 0.06 mW/cm^2 (UVB), and 240 μmol/m^2/s (PAR). These intensities, approximately 1/10 of the target intensities, delivered 17 to 69% (mean, 26%) of the daily total PAR (mmol/m^2) measured in a separate study at Huntington Beach on Ohio's Lake Erie shore during the summer of 2008 (our unpublished data).

Sampling. Sampling was carried out 1 h after inoculation, with 10 ml/liter wastewater, and again at the same times on days 1, 3, 5, and 11. For each sample, volumes of 40 ml were removed to 50-ml polypropylene Oak Ridge centrifuge tubes (Nalgene Nunc, Rochester, NY) for marker DNA analysis. Increasing volumes (10 ml to 100 ml) were removed for *E. coli* analysis over time, as different volumes were required to remain within assay limits of detection. A 10-ml sample was collected separately from each microcosm for turbidity analyses.

Enumeration of *E. coli*. The Colilert assay using the Quanti-Tray/2000 was used to measure cultivable *E. coli* in each microcosm sample, according to the manufacturer's protocol (IDEXX Laboratories, Inc., Westbrook, ME). Dilutions were made as needed, with 10^{-1} and 10^{-2} dilutions on day 0 and with direct analysis of 100-ml volumes by day 5.

Preparation of internal DNA processing control. A pure culture of *Pantoea stewartii* was prepared as described previously (39). Estimates of concentrations were made by plate counting, and 1-ml culture aliquots were stored at −80°C. This Gram-negative-control species, with morphological characteristics similar to those of the *Bacteroidales*, was spiked into each of the microcosm samples prior to processing and DNA extraction to monitor recovery efficiency. Percent recovery of the spike was used to adjust marker concentrations. The genetic target for detection of *P. stewartii* was the exopolysaccharide synthesis gene (*cpsD*), carried as a single copy on the chromosome (9). Previous work (39) demonstrated that

TABLE 1. Primer and probe identities, target genes, sequences, and amplicon lengths for general fecal, HF and internal control qPCR assays used in this study[a]

Primer/probe	Target gene	Sequence (5′–3′)	Size (bp)	Reference
AllBac296F	General *Bacteroidales* 16S rRNA	GAGAGGAAGGTCCCCCAC	106	26
AllBac412R		CGCTACTTGGCTGGTTCAG		
AllBac-Bhq		CCATTGACCAATATTCCTCACTGCTGCCT		
BacHum160F	HF *Bacteroidales* 16S rRNA	TGAGTTCACATGTCCGCATGA	81	24
BacHum241R		CGTTACCCCGCCTACTATCTAATG		
BacHum193FBhq		TAGGGGTTCTGAGAGGAAGGTCCCCC		
HF183F	HF *Bacteroidales* 16S rRNA	ATCATGAGTTCACATGTCCG	82	35
Bac242R		TACCCCGCCTACTATCTAATG		
cpsRT74F	*P. stewartii cpsD*	TGCTGATTTTAAGTTTTGCTA	82	40
cpsRT177R		AAGATGAGCGAGGTCAGGATA		
cps133-Bhq		TCGGGTTCACGTCTGTCCAACT		

[a] Internal control, *P. stewartii*.

adjustment of observed qPCR data according to the level of spike recovery reduced variability in replicate analyses, suggesting that it could be used to enhance precision in studies involving trends analysis and treatment comparisons. Each of the 40-ml samples from the microcosms was spiked with approximately 1.5×10^6 CFU of *P. stewartii* at the time of collection.

Sample processing and DNA extraction. Immediately after spike addition, samples were vortexed and then concentrated at $2{,}880 \times g$ for 10 min in a Sorvall RC2-B centrifuge (Thermo Electron Corp., Asheville, NC), and supernatant was decanted. After the addition of 500 μl of MoBio PowerSoil DNA extraction kit lysis buffer (MoBio, Inc., Carlsbad, CA), cells were vortexed and stored at −80°C prior to DNA extraction. The MoBio kit protocol was modified to use all the supernatant, as described by Stoeckel et al. (39), and extraction controls using PCR-grade water were included each day. The DNA was eluted in 250 μl of C6 reagent, and purified DNA was stored at −20°C.

qPCR assays. Genomic (*P. stewartii*) and plasmid (all other assays) DNA for qPCR standards was prepared according to the work of Stoeckel et al. (39). All assays were performed according to published protocols (24, 26, 35, 40). Sequences for primers and probes used in the study are listed in Table 1. Reactions were run on the Bio-Rad iQ5 instrument (Bio-Rad Laboratories, Inc., Hercules, CA) in 25-μl volumes, using 5 μl of the 250-μl extracts as a template. No-template controls containing PCR-grade water, along with 6-point standard curves, were run in triplicate with each set of reactions. Test reactions were carried out in duplicate. Unamended river water and sewage samples were processed as negative controls for the internal spike and as positive controls for the background occurrence of the markers in the sample matrices. Limits of detection (LOD) for each assay were defined as the fifth percentile among observed threshold cycles (C_T) across all blanks and negative-control reactions. Inhibition of the qPCR was monitored by making 10^{-1} and 10^{-2} extract dilutions and was considered significant when the difference in C_T between dilutions was ≤3. Inhibition was not detected in any sample.

Normalization of standard curves. Variability among qPCR standards was minimized by the generation of composite standard curves, in which acceptance parameters were established based on efficiency between 0.80 and 1.10 and R^2 values of >0.99 (Sigma qPCR technical guide). The C_T for every observation was adjusted according to the average difference in C_T between the run standard curve and the composite standard curve (ΔC_T). When an additional standard curve was added to the composite, 100 iterations were performed to reposition the composite standard curve and recalculate ΔC_T values for each constituent qPCR run.

Data analyses. Analytical replicates were expressed as the geometric mean marker copy number per 100 ml or *E. coli* most probable number (MPN) per 100 ml, and experimental replicates were expressed as the mean $\log_{10}$ reductions and t_{99} ± standard deviations. Decay was characterized by the $\log_{10}$ reduction for each experimental parameter at each time point. Using the following equations, decay rates were calculated based on a first-order decay model, $N_t/N_0 = 10^{-kt}$ (11), and t_{99} values were calculated using the decay constant (k) in the following equation, $t_{99} = -2/k$.

In many cases, the full time scale of data collected did not fit the first-order decay model; hence, those data that were approximately linear, plus all data relevant to 99% reduction whether linear or not, were used in the calculations (as described further in Results). Differences among the mean values were evaluated at a 95% confidence interval using Tukey's honestly significant difference (HSD) post-hoc multiple-comparisons test in conjunction with analysis of variance (ANOVA) in SPSS 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

In this study, 15 river water microcosms were sampled on days 0, 1, 3, 5, and 11 after being spiked with human wastewater to compare the decay of *Bacteroidales* general and HF markers against cultivated *E. coli* under the effects of artificial sunlight, exposure to sediment, reduced temperature, and reduced predation. Decay ($\log_{10}$ reduction) at each sampling time was compared for each of the four targets within (Table 2) and across (Table 3) treatments, and t_{99} were compared across treatments (Table 4). Temperature, monitored continuously over the duration of the study of the five representative microcosms, remained stable. Within the three constant-temperature rooms used for this experiment, means and standard deviations were 24.2 ± 0.1°C, 25.2 ± 0.6°C, and 15.4 ± 0.0°C at reduced temperature (data not shown). Irradiation by the lamp used to create artificial sunlight caused an increase in temperature for treated microcosms compared to that of the control in the same room (28.3 ± 1.3°C for the treated microcosm compared to 25.2 ± 0.6°C for the control that was shielded from light).

Recovery of internal processing control. The internal control spike, *P. stewartii*, was monitored to measure recovery efficiency for each sample through the steps of sample processing and DNA extraction (39). Recovery of the internal control ranged from 2.5% to 73% of the estimated 1.5×10^6 CFU spike concentration. Concentrations of the *Bacteroidales* targets were estimated by dividing the observed concentration by the proportional spike recovery. Coefficients of variation (CV) for marker concentrations in triplicate microcosms ranged from 1.2% to 870% (mean overall CV = 57%) before adjustment for spike recovery and from 0.53% to 58% (mean overall CV = 16%) after adjustment. The reduced CV in adjusted measurements indicated that use of the internal processing control enhanced measurement precision and validated its use in this application.

Starting concentrations and assay LOD. Following equilibration and spiking of microcosms, mean concentrations of the four targets were 2.12×10^4 *E. coli* MPN/100 ml, 8.38×10^8 AllBac copies/100 ml, and 3.76×10^7 and 1.02×10^7 copies/

TABLE 2. Level of significance for $\log_{10}$ reduction values among microbial targets for each treatment and day[a]

Target	Treatment day	Level of significance[b]				
		Control	Light	Sediment	Reduced temp	Reduced predation
E. coli	1	a	a	a	a	a
qHF183		a	b	c	a	bc
BacHum		a	b	c	a	ab
AllBac		a	b	b	a	c
E. coli	3	b	a	b	a	a
qHF183		ab	<LOD	c	a	b
BacHum		b	<LOD	c	a	ab
AllBac		a	a	a	a	ab
E. coli	5	b	<LOD	b	a	a
qHF183		<LOD	<LOD	c	<LOD	c
BacHum		<LOD	<LOD	<LOD	<LOD	bc
AllBac		a	Detected	a	a	b

[a] Log_{10} reduction values were plotted in Fig. 1.
[b] <LOD, one or more observations below the LOD and statistical comparisons not done; detected, observation was above the LOD but below the lowest standard curve value. Lowercase letters denote statistically significant differences in the remaining concentration in each treatment on each day, as evaluated by Tukey's HSD test ($P < 0.05$).

100 ml for qHF183 and BacHum, respectively. The LOD for BacHum and qHF183 qPCR assays were 4 and 6 copies/5-μl extract. The LOD for AllBac was approximately 450 copies/5 μl, or about 5.6×10^4 copies/100 ml, and the LOD for *E. coli* was 1 MPN/100 ml. All HF marker measurements were at or near the LOD under each treatment by day 5. AllBac was detectable up to day 11 in all treatments. *E. coli* was detectable up to day 5 in all treatments except light and in reduced predation up to day 11. In all but the reduced predation treatment, *E. coli* density was below the U.S. EPA single-sample water quality criterion (235 MPN/100 ml) (42) by day 3.

TABLE 3. Level of significance for $\log_{10}$ reduction values among treatments within each microbial target and day[a]

Treatment	Target day	Level of significance[b]			
		E. coli	qHF183	BacHum	AllBac
Control	1	a	ab	a	a
Light		ab	abc	ab	a
Sediment		a	a	ab	a
Red temp		a	bc	bc	a
Red predation		b	c	c	a
Control	3	a	a	a	a
Light		a	<LOD	<LOD	a
Sediment		a	a	a	a
Red temp		a	a	a	a
Red predation		b	b	b	a
Control	5	a	<LOD	<LOD	a
Light		<LOD	<LOD	<LOD	a
Sediment		a	<LOD	<LOD	a
Red temp		a	<LOD	<LOD	a
Red predation		b	Detected	Detected	a

[a] Log_{10} reduction values were plotted in Fig. 1.
[b] <LOD, one or more observations below the LOD and statistical comparisons not done; detected, observation was above the LOD but below the lowest standard curve value. Lowercase letters denote statistically significant differences in the remaining concentration of each target on each day, as evaluated by Tukey's HSD test ($P < 0.05$).

Decay curves. Fig. 1 shows the mean decay curves for the four targets in each treatment. Statistical differences evaluated by mean separation of the rates of target decay within and across each treatment and each sample time are shown in Tables 2 and 3, to be used in conjunction with Fig. 1. There were no differences in decay among any targets in control and reduced temperature treatments on the first day (Table 2). Among the other three treatments, decay of *E. coli* at day 1 was always the same or less than decay of the other targets. Decay of the two human markers was not different under any treatments on day 1. The decay response of AllBac compared with the other targets varied on day 1.

By the third day of the experiment, the decay of AllBac was always the same or less than that of the other targets. *E. coli* decay remained the same as or less than the decay of the HF markers. The reduced temperature treatment was the only one for which there remained no difference among the decay of targets. The decay of the two HF targets was not significantly different for any of the treatments. The HF markers dropped to below the LOD in the light treatment, and the decay was greater than those of *E. coli* and AllBac in the sediment treatment.

By the fifth day of the experiment, one or both HF markers were below the LOD in all but the reduced predation treatment. *E. coli* and AllBac remained similar in decay under reduced temperature, and *E. coli* had greater decay than AllBac under control conditions and with sediment and showed less decay than AllBac under reduced predation.

When decay data among the treatments were evaluated (Table 3), the reduced predation treatment resulted in less decay of targets (*E. coli*, qHF183, and BacHum) than the control at day 1 and day 3. The only other treatment effect noted was less decay of BacHum at a reduced temperature than that under the control conditions on day 1. No differences in decay for AllBac among the treatments up to day 5 were observed.

Evaluation of time needed to produce 99% decay. Changes in the slopes of the curves suggestive of biphasic decay occurred in

TABLE 4. Mean t_{99} for all targets and time needed to achieve 235 *E. coli* MPN/100 ml under different treatments, as estimated from $\log_{10}$ reductions

Treatment (temp [°C])	Mean t_{99} (SD) (days)[a]				Mean time (days) needed to achieve 235 *E. coli* MPN/100 ml (SD)
	E. coli	qHF183	BacHum	AllBac	
Control (25)	2.02 (0.28) a	2.16 (0.47) a	1.74 (0.16) a	3.28 (1.26) a	1.98 (0.28)
Light (25)	2.18 (0.18) b	1.71 (0.23) a	1.54 (0.42) b	2.55 (0.23) b	2.13 (0.18)
Sediment (25)	2.80 (0.42) b	1.88 (0.47) a	2.08 (0.10) a	4.44 (0.34) c	2.74 (0.41)
Reduced temp (15)	3.01 (1.07) a	2.53 (0.36) a	2.35 (0.53) a	2.73 (0.84) a	2.94 (1.04)
Reduced predation (25)	7.14 (0.34) b	2.78 (0.03) a	3.03 (0.23) a	3.75 (0.86) a	6.98 (0.33)

[a] Letters denote statistically significant differences among the t_{99} of microbial targets within treatments, as evaluated by Tukey's HSD test ($P < 0.05$).

all treatments (per Bae and Wuertz [3]) and were most pronounced for AllBac (Fig. 1). Nonlinear target reduction over time, compounded by differences in curve shapes for various treatments and targets, indicated that a simple first-order decay model up to day 11 was not appropriate in most cases. However, 99% reduction was achieved within the near-linear area of the decay curve in most cases, so the decay constants and resulting t_{99} values were generated from the linear portion of each curve. In

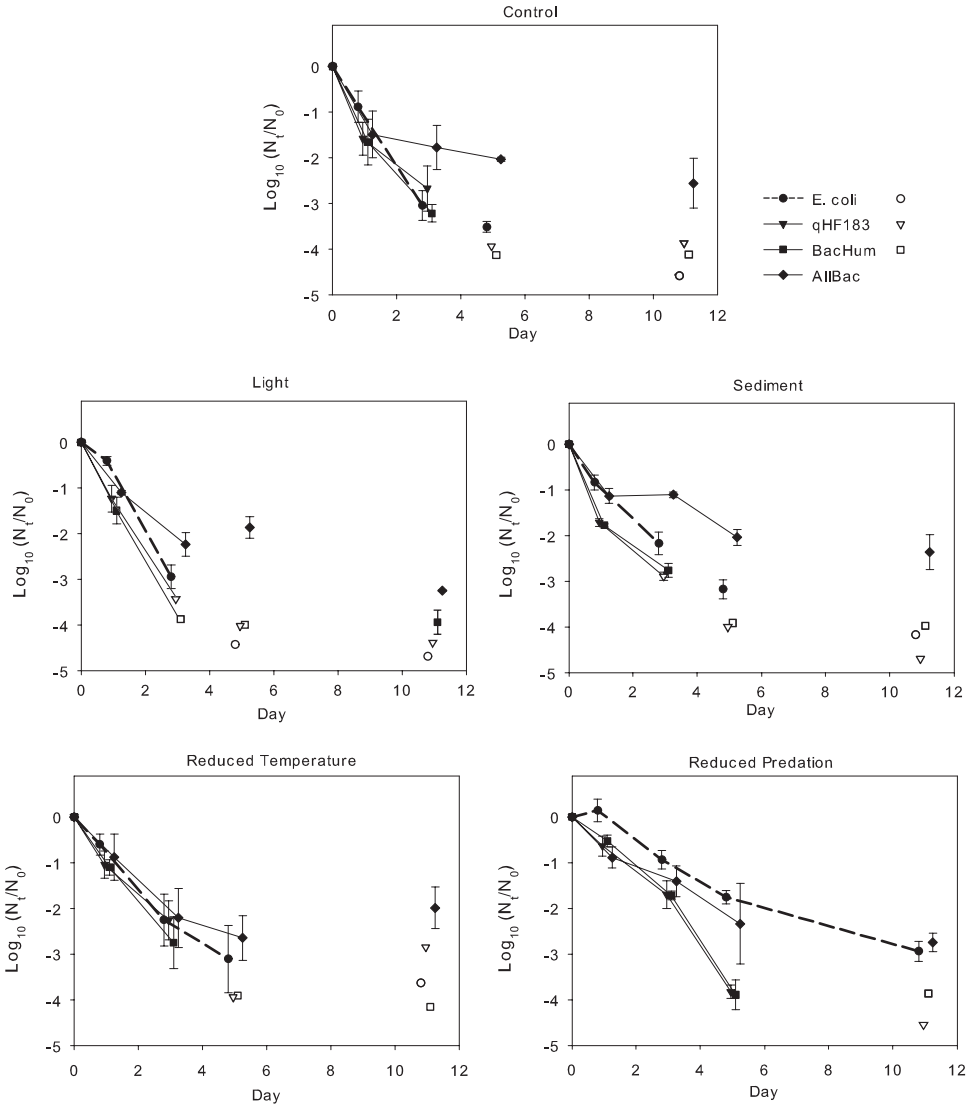

FIG. 1. Mean decay curves for *E. coli* and markers in microcosms exposed to different conditions, measured as the remaining concentration [$\log_{10}(N_t/N_0)$] over time (t = days). Data points represent the mean values obtained among triplicate microcosms. Error bars indicate standard deviations. Filled symbols represent data points for which the target could be measured in all three microcosms. Open symbols represent data points for which at least one observation was below the limit of detection. Points used for the calculation of decay constants are connected by lines.

the light treatment, the HF markers had decayed to below the LOD by day 3 in some microcosms. In these microcosms, a value representing one-half of the LOD was used to estimate t_{99}.

No significant differences were observed among t_{99} values for any target in the control or the reduced temperature treatments (Table 4). Similarly, t_{99} values for the HF markers were not significantly different from each other under any treatment. As might be expected from evaluation of the decay curves (Fig. 1), both HF markers reached t_{99} earlier than *E. coli* under conditions of light, sediment, and reduced predation. Under control, light, and reduced temperature treatments, the t_{99} values for AllBac were not significantly different from those of *E. coli*.

It was of special interest to note that AllBac in sediment had a t_{99} that was longer than that under any other condition and longer than those of the other targets in the sediment treatment. Following collection of the last sample on day 11, sediments were resuspended, and an additional sample was collected and analyzed from each treatment. The AllBac marker recovered nearly all of its initial concentration (0.28-log reduction from the initial log concentration of 8.54) in the sediment treatment, while the HF markers remained at or below the LOD after mixing (approximately 4-log reduction from the initial log concentration of 7.29) (data not shown). Cultivable *E. coli* remained 2.7 logs below their initial log concentration of 4.28 in the resuspended sample.

In order to evaluate these results in terms of the consequence for water quality monitoring, the time needed to achieve 235 *E. coli* MPN/100 ml was also calculated (Table 4). Because the starting concentration of *E. coli* in the microcosms was 2.10×10^4 MPN/100 ml, nearly 100 times the criterion, the time needed to achieve 235 MPN/100 ml was very similar to the corresponding t_{99} values. Except for grossly contaminated waters (beyond 10^5 MPN/100 ml), the t_{99} values presented are relevant to most scenarios encountered in recreational water monitoring.

DISCUSSION

A primary goal of this research was to evaluate the relative rates of decay of genetic markers and cultivated *E. coli.* Among other uses, this information is necessary in order to substitute a genetic marker for cultivated *E. coli*, the current standard for public health risk in recreational waters, as considered by USEPA (43). Alternately, analysis for human-associated genetic markers might be used in conjunction with *E. coli* to evaluate the relative contribution from humans to the overall level of *E. coli*. A major finding of this study was that HF marker decay was consistent with, or significantly faster than, that of *E. coli* under all treatments. This indicates that the HF markers might be useful as conservative estimators of human-origin *E. coli* even as fecal contamination ages in the environment.

The complex interactions of various environmental parameters with bacterial decay are difficult to model (11). The scope of relevance for the decay evaluations reported in this paper is limited to the time needed to produce 99% decay because it was impossible to extrapolate the first-order decay model beyond this point. A water body would have to be contaminated beyond a level of 23,500 MPN/100 ml to retain significant levels of contamination (above 235 MPN/100 ml) past the t_{99} level of decay. For this reason, interpretations from this experiment, while limited in scope, are applicable under conditions found in most environmental settings.

Plots of decay as a function of time indicated that *E. coli* and AllBac tended to decay with a biphasic pattern rather than a first-order model of continuous decay. The shapes of the curves varied, indicating that the various treatments affected subpopulations of targets differently. Possible explanations for this phenomenon include population heterogeneity or differences in growth phase at the time of inoculation (1, 22). In this study, since wastewater was introduced into river water, either factor could contribute to the exhibited patterns; potential rapid initial decay of newly introduced cells and DNA may have been followed by a reduction in the decay rate due to the presence of a more adapted or protected background population. In all cases, however, the biphasic nature of the decay curve came into effect below the 99% (2-log) reduction level.

In one epidemiological study, Wade et al. found that a marker of general fecal contamination was not consistently correlated with health risk (45). The general fecal contamination marker used in the present study (AllBac) exhibited a pronounced biphasic decay curve, likely due to the greater heterogeneity (the number of different phylotypes) this marker represents compared with that of the other targets. Some of these groups were more persistent than the HF markers or *E. coli*. The biphasic curve suggests that a reservoir of persistent AllBac markers could be present in previously contaminated waters, such that AllBac could not be used as an alternate indicator of health risk (in place of *E. coli*) or in ratio with the HF markers to estimate the source contribution. Indeed, when sediments were resuspended at the end of this experiment, the final concentration of the AllBac marker returned to about 50% of its original concentration, while the other markers all remained at less than 1% of the original concentration. The failure of the other general *Bacteroidales* marker (GenBac) to predict health risk under some conditions in the prior study (45) may be explained, in part, by these findings.

While it has been suggested that sunlight is the most important controlling factor for the survival of *E. coli* in water (29, 36), the present study, as in that of Walters and Field (47), found no significant effect of exposure to sunlight on either the survival of *E. coli* or the persistence of the HF DNA markers. One plausible explanation for the finding in the current study is the turbidity in the microcosm source water (102 NTU)—turbidity plays a role in limiting the effects of light on decay rates (8). Another explanation is that the source of artificial sunlight was not as strong as natural sunlight, providing only about one-fourth of the daily irradiance measured throughout the summer at a Lake Erie beach (our unpublished data). The source of sunlight, temperature, and likely other environmental conditions were quite different in the Walters and Field study (47), which took place outdoors in Oregon, thus making direct comparisons difficult. The current study showed more similarity in the decay of *E. coli* and the HF markers in the sunlight treatment than the Oregon study.

Microcosms containing sediment had significantly higher mean levels of turbidity than the other microcosms over the course of the 11-day experiment ($P = 0.016$); however, at each time step, the decay of all targets in the sediment microcosms

was not significantly different from the decay observed in the control microcosms (Table 3). Reduced temperature, as in other studies, resulted in longer persistence for all targets, and t_{99} values showed no significant differences among the targets at 15°C. However, the t_{99} values for qHF183 were considerably lower than those reported by Seurinck et al. (35) (6 days at 28°C versus 2.16 days at 25°C in this study; 10 days at 12°C versus 2.53 days at 15°C in this study).

Although reduced predation had the greatest effect on decay for all of the targets, the significance of this parameter may not be great in fresh recreational water where this is not a typical state of the environment. The t_{99} value for *E. coli* under reduced predation was significantly higher than those for the *Bacteroidales* targets, which suggests that in applications such as drinking water management, the molecular markers may not correlate well with *E. coli*.

Better understanding of decay characteristics of the regulatory FIB, *E. coli*, and MST molecular markers of fecal contamination was needed to evaluate the markers as potential substitutes for, or complements to, *E. coli* density measurements in water quality management (43). If the marker-to-*E. coli* ratio is consistent over time, it may be possible to (i) use AllBac as a rapidly measured indicator in place of *E. coli* or (ii) use the human-associated markers to describe the relative amount of *E. coli* contamination due to human sources. In general, the genetic markers had t_{99} values that were similar to or higher than those of *E. coli* under all tested conditions. However, AllBac showed evidence of a persistent subpopulation below the 99% removal level; HF markers had 4-log or greater removal by day 5 of the experiment, while less than 3-log removal of AllBac had been achieved by day 11. This reservoir of persistent AllBac makes it unlikely that the AllBac marker could be used as a rapidly measured surrogate for *E. coli* contamination in recreational waters. The qHF183 and BacHum markers, however, may be useful as conservative measures to indicate the prevalence of human-origin *E. coli* in contaminated waterways. Further evaluation of the cooccurrence of HF markers and *E. coli* in sources of human and nonhuman fecal contamination is required to derive the necessary models.

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