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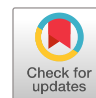

Distribution and Differential Survival of Traditional and Alternative Indicators of Fecal Pollution at Freshwater Beaches

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ABSTRACT Alternative indicators have been developed that can be used to identify host sources of fecal pollution, yet little is known about how their distribution and fate compare to traditional indicators. *Escherichia coli* and enterococci were widely distributed at the six beaches studied and were detected in almost 95% of water samples ($n = 422$) and 100% of sand samples ($n = 400$). Berm sand contained the largest amount of *E. coli* ($P < 0.01$), whereas levels of enterococci were highest in the backshore ($P < 0.01$). *E. coli* and enterococci were the lowest in water, using a weight-to-volume comparison. The gull-associated *Catellicoccus marimammalium* (Gull2) marker was found in over 80% of water samples, regardless of *E. coli* levels, and in 25% of sand samples. Human-associated *Bacteroides* (HB) and *Lachnospiraceae* (Lachno2) were detected in only 2.4% of water samples collected under baseflow and post-rain conditions but produced a robust signal after a combined sewage overflow, despite low *E. coli* concentrations. Burdens of *E. coli* and enterococci in water and sand were disproportionately high in relation to alternative indicators when comparing environmental samples to source material. In microcosm studies, Gull2, HB, and Lachno2 quantitative PCR (qPCR) signals were reduced twice as quickly as those from *E. coli* and enterococci and approximately 20% faster than signals from culturable *E. coli*. High concentrations of alternative indicators in source material illustrated their high sensitivity for the identification of fecal sources; however, differential survival and the potential for long-term persistence of traditional fecal indicators complicate the use of alternative indicator data to account for the levels of *E. coli* and enterococci in environmental samples.

IMPORTANCE *E. coli* and enterococci are general indicators of fecal pollution and may persist in beach sand, making their use problematic for many applications. This study demonstrates that gull fecal pollution is widespread at Great Lakes beaches, whereas human and ruminant contamination is evident only after major rain events. An exploration of sand as a reservoir for indicators found that *E. coli* was ubiquitous, while gull host markers were detected in only 25% of samples. *In situ* sand beach microcosms provided decay rate constants for *E. coli* and enterococci relative to alternative indicators, which establish comparative benchmarks that would be helpful to distinguish recent from past pollution. Overall, alternative indicators are useful for identifying sources and assessing potentially high health risk contamination events; however, beach managers should be cautious in attempting to directly link their detection to the levels of *E. coli* or enterococci.

KEYWORDS beaches, water quality, human-associated indicators, gull-associated indicators, qPCR

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Fecal contamination of recreational waters can be a serious threat to public health. Due to the vast diversity of fecal-borne human pathogens, the USEPA has recommended the use of fecal indicator bacteria (FIB), commonly *Escherichia coli* and entero-

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cocci, to determine if fecal pollution is present. Historically, single-day recreational water advisory thresholds were 235 CFU/100 ml for *E. coli* and 61 CFU/100 ml for enterococci in freshwater (1). The recent Recreational Water Quality Criteria, published by the USEPA in 2012, established a similar range for beach action values of 235 CFU/100 ml for *E. coli* and 70 CFU/100 ml enterococci for freshwater, which relates to an unacceptable health risk to beachgoers of 36 illnesses per 1,000 people (2). The advisory and closure FIB threshold values reflect multiple epidemiological studies that assessed the predictive nature of FIB based on the rate of illness reported by beachgoers (3).

Despite the wide use of FIB in marine and freshwater systems, it has been shown that enterococci and *E. coli* are poorly associated with the presence of human pathogens in environmental waters (4–6). Due to their ubiquitous nature in warm-blooded animals (7–9), fecal indicators can only indicate that fecal contamination may be present; however, certain host sources are more likely than others to carry human pathogens (10). Additionally, *E. coli*, the most commonly used indicator in freshwater systems, has been found to survive in the environment (11–14). Environmental persistence of fecal indicator bacteria undermines the utility for recreational water quality monitoring because the presence of these organisms would not necessarily indicate a recent contamination event, and in some cases, it can lead to an overestimation of the associated public health risk.

To address some of the pitfalls associated with traditional FIB monitoring, alternative indicators have been identified that are host associated and are a major focus of current water quality research. The use of alternative indicators shows promise for the detection of fecal pollution sources and can lead to increased accuracy in identifying health risks to beachgoers as well as aid in the mitigation of pollution sources. Alternative indicator assays commonly target anaerobic fecal bacteria due to their high abundance in animal and human feces and their limited survival in the external environment (15–17). Bacteria within the order *Bacteroidales* are common targets for quantitative PCR (qPCR) assays and have been used for the detection of sewage (18–21), ruminant (19, 22), canine (19), and avian (23, 24) sources of fecal pollution. Certain members within the order *Clostridiales* have also demonstrated host-associated patterns (25). Our lab has developed a qPCR assay that targets the second most abundant human-associated *Lachnospiraceae* (Lachno2) in sewage (26) and has been used to track sewage contamination in environmental waters (27). Gull-associated qPCR assays targeting *Catellicoccus marimammalium* have also been developed (28–30), and field studies have demonstrated that gulls are a common source of degraded water quality at marine and freshwater recreational beaches (31).

Although recreational waters are the primary monitoring focus for beach managers, numerous studies have documented high concentrations of fecal indicators within beach sand (12, 14, 32). Recent research has reported the recovery of bacterial and viral human pathogens from beach sand, providing evidence that sand contact may play an important role in beach-associated gastrointestinal (GI) illness (33–35). Based on a potential bacterial pathogen reservoir in beach sand, the scientific community has called for implementation of a sand monitoring program (35).

An understanding of the concentrations of alternative host-associated indicators compared with traditional indicators in source fecal samples is needed to establish the prevalence and sensitivity of these indicators and to interpret environmental monitoring results. Benchtop microcosm studies have assessed the survival of *E. coli* and enterococci in beach sand; however, only a few studies have examined the decay of alternative indicators (33, 36–38). The dynamic conditions present in the beach environment cannot be readily replicated in the laboratory; thus, results from previous laboratory survival studies are difficult to directly apply to what might be expected in the environment.

This study employs a comprehensive survey of beaches in urban and rural areas to evaluate the efficacy of molecular methods to assess fecal contamination in sand and water samples. With the goal of providing valuable information to beach managers

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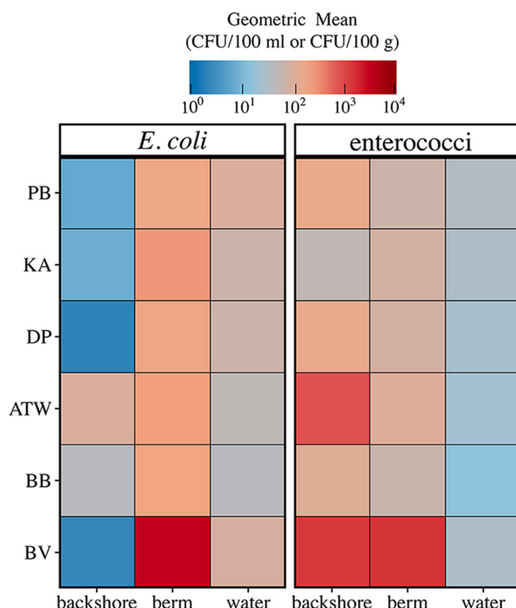

FIG 1 Heat map illustrating the geometric mean concentrations of *E. coli* and enterococci, as measured by membrane filtration, in sand and water samples collected at beaches during 2012 and 2013. Sand samples were compared to water samples on a weight-to-volume basis.

regarding the usefulness of alternative indicators, the present study combined field surveys and microcosm experiments to explore the utility of traditional and alternative fecal indicators within beach sand and water. The present study sought to test the following hypotheses: (i) alternative indicators are more sensitive than *E. coli* or enterococci for detecting fecal pollution; (ii) differential survival can affect the relative concentrations of traditional and alternative fecal indicators in the beach environment; and (iii) sand acts as a long-term reservoir for *E. coli*, and *E. coli* may be detected without evidence of host sources. Overall, this work demonstrates that alternative indicators are useful for detecting recent pollution events from specific sources. However, beach managers are challenged to respond to elevated levels of *E. coli* and enterococci, and the differences in persistence between alternative indicators and traditional indicators, coupled with the probability of multiple ongoing pollution inputs, precludes making inferences about the causes of elevated FIB levels from alternative indicator measurements.

RESULTS

Comparison of levels of *E. coli* and enterococci in the beach environment. We compared the levels of culturable *E. coli* and enterococci across three distinct zones (backshore sand, berm sand, and water) to determine if FIB reservoirs are present in sand and if differences exist between zones. Overall, we found that levels of *E. coli* at the six beaches were significantly higher in berm sand ($P < 0.01$) than in backshore sand, as well as water in a comparison of equal weight to volume (Fig. 1). Densities of enterococci were significantly higher in the backshore sand ($P < 0.01$) than in berm sand and water samples. Overall, some beaches had higher densities of *E. coli* and enterococci in berm and backshore sand than others; however, in water, the mean densities of each indicator were very similar at all beaches. Although berm sand had higher levels of *E. coli* than in water, the concentrations of *E. coli* in paired sand and water samples collected along the same transect were correlated (Spearman's rho = 0.65, $P < 0.01$). The geometric mean concentrations of *E. coli* at the different beaches ranged from 35 to 88 CFU/100 ml for water, and 1.7×10^2 to 3.2×10^3 CFU/100 g of berm sand and 2 to 87 CFU/100 g of backshore sand. Levels of enterococci ranged from 13 to 30 CFU/100 ml for water, 60 to 1.4×10^3 CFU/100 g for berm sand, and 47 to

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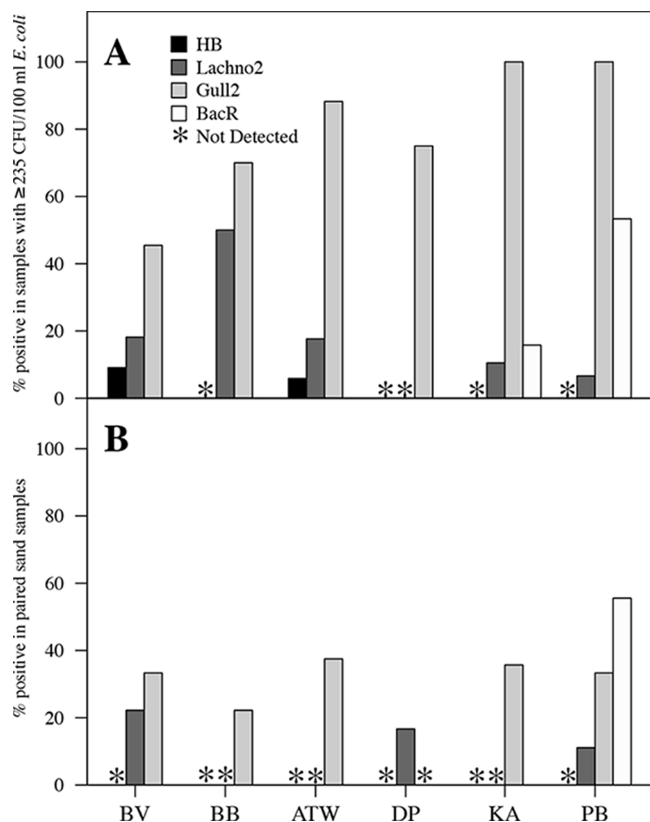

FIG 2 Alternative indicator detection frequencies measured during advisory conditions for water samples with ≥235 *E. coli*/100 ml ($n = 84$) (A) and sand samples paired by date and transect ($n = 69$) (B) collected during 2012 and 2013. Water samples collected under CSO conditions were not included in this figure.

1.3×10^3 CFU/100 g for backshore sand. Of the 422 water samples collected, 84 (20%) samples exceeded the USEPA *E. coli* beach action value of 235 CFU/100 ml. We used the average *E. coli* levels across all water sites at a beach to determine advisory days. Similar percentages of advisory days at Bayview Beach (BV; 28%), Bradford Beach (BB; 17%), Atwater Park Beach (ATW; 27%), Doctor's Park (DP; 25%), Kohler-Andrae State Park (KA; 27%), and Point Beach State Park (PB; 18%) were observed at the six beaches.

Alternative indicator qPCR analysis. We examined water samples with *E. coli* concentrations above the advisory threshold ($n = 84$) and below the advisory threshold ($n = 60$) to determine the presence of host-associated indicators, particularly for human or cattle sources, which are known to pose a human health risk, and gulls, which can also pose a health risk and are a common source of fecal pollution at beaches. In addition to the selected water samples, "paired" sand samples (i.e., sand collected on the same date and at the same site as water samples) were also analyzed for the presence of host-associated markers. Of beach samples with high *E. coli* levels, the majority of paired sand samples ($n = 64/69$) also exceeded the advisory criteria on weight-to-volume comparison (i.e., ≥235 CFU *E. coli*/100 g). Of the beach water with low levels of *E. coli*, approximately half of the paired samples ($n = 20/41$) also had <235 CFU/100 ml on a volume-mass basis. Water samples that were collected under combined sewer overflow (CSO) conditions were considered independently ($n = 20$).

The detection frequency of host-associated markers (Gull2, Lachno2, HB, and ruminant-associated *Bacteroidetes* [BacR]) in beach water with high *E. coli* levels and paired sand is shown in Fig. 2. The Gull2 marker was detected more frequently than any other host-associated marker in both water and sand and occurred in 83% of the water samples that had high *E. coli* levels and 28% of the paired sand samples. The Gull2 marker concentrations in water samples with high *E. coli* levels ranged from 1.0×10^2

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to 6.6×10^5 copy number (CN)/100 ml and 1.0×10^4 to 4.3×10^5 CN/100 g in the paired sand samples. The human-associated HB marker was detected in 2.4% of the water samples above the *E. coli* advisory threshold and occurred only at the BV and ATW beaches (which are located in the greater Milwaukee area); HB was absent from the BB, DP, KA, and PB beaches. The HB marker concentrations, when detected, were relatively low compared to those in untreated sewage, ranging from 3.3×10^2 to 9.2×10^2 CN/100 ml in water, and they were absent in all paired sand samples. The second human-associated marker, Lachno2, was detected in 15% of the water samples above the advisory threshold and was found at four of the six beach sites, all at relatively low levels. In paired sand samples, the HB marker was absent, and the Lachno2 marker was detected in 7% of samples; however, a large number of samples ($n = 49/69$) were detected but not quantifiable (DNQ) for Lachno2.

The BacR assay was analyzed only for the two northernmost beaches, PB and KA, due to the close proximity to agricultural operations. For water samples with high *E. coli* levels, the BacR marker was detected in 16% of KA samples but was undetected in paired sand samples. In PB water samples with high *E. coli* levels, the BacR marker was detected in 53% of water samples and in 56% of paired sand samples. For both PB and KA, when BacR was detected, the average concentration was 1.8×10^3 CFU/100 ml in water and 1.5×10^4 CFU/100 g in sand.

We also examined samples with low levels of *E. coli* (<235 CFU/100 ml or g) to assess the occurrence of host markers in cases where water samples would be considered to have acceptable water quality. We found similar distributions and levels of gull contamination in these samples compared to samples with high *E. coli* levels. The Gull2 marker was detected in 82% ($n = 49/60$) of water samples with low *E. coli* levels and 15% ($n = 6/41$) of the paired sand samples. There was not a significant difference in the gull marker levels in water samples with high or low *E. coli* levels ($P = 0.08$). The two human-specific markers were absent in all water samples with <235 CFU of *E. coli*/100 ml; however, there was a small number of samples that had DNQ results ($n = 1$ for HB and $n = 6$ for Lachno2). In contrast, for the sand samples paired with these water samples, the Lachno2 marker was detected in 2/41 of sand samples and DNQ in 20/41 of samples. The HB marker was not detected in any paired sand samples with <235 CFU/100 ml. At the northern beaches potentially impacted by agricultural runoff, the ruminant marker was not detected in any samples with low *E. coli* levels.

Water samples collected at the ATW, BB, and BV beaches 1 to 3 days following a CSO had very low culturable *E. coli* concentrations, ranging between 0 and 23 CFU/100 ml. The human-associated markers were detected in 45% of the post-CSO samples. When detected, concentrations were relatively high and ranged from 2.7×10^3 to 1.4×10^4 CN/100 ml for HB and 1.5×10^3 to 1.2×10^4 CN/100 ml for Lachno2, which is one or more orders of magnitude higher than in water samples collected under non-CSO conditions. The two human-associated markers were highly correlated within samples collected post-CSO (Spearman's rho $= 0.99$, $P < 0.01$).

Concentrations of host-associated markers and ratios to FIB. We assessed the concentrations and variability of markers in gull, untreated sewage, and ruminant fecal sources (Fig. S1). We found that gulls consistently had a high abundance of the Gull2 marker per gram of gull feces. Concentrations of qPCR markers for *E. coli* and enterococci, however, were highly varied in gull fecal samples and ranged from 6.9×10^4 to 1.1×10^{10} CN/g for *E. coli* and 7.0×10^5 to 2.1×10^8 CN/g for enterococci. Gull2 was, on average, three to four orders of magnitude higher than either *E. coli* or enterococci as measured by qPCR. In sewage, concentrations of Lachno2 were approximately 1.5-fold higher than those of HB. These two human-associated markers were at levels similar to those of enterococci but were two orders of magnitude higher than *E. coli*. The ruminant marker BacR was found at concentrations approximately 4-fold higher than the concentrations of *E. coli* and enterococci.

We compared the concentrations of alternative indicators, *E. coli*, and enterococci in fecal sources to their concentrations in environmental samples (Table 1). The ratios

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TABLE 1 Average concentrations and standard deviations of host-associated marker sequences detected in source material and environmental samples, and comparison to fecal indicator abundance

Host-specific assay	Sample type[a]	No.[b]	Host indicator concn (CN/g or 100 ml) (avg ± SD)	qPCR marker ratios (avg ± SD)[c]	
				Host marker per *E. coli*	Host marker per *Enterococcus*
Gull2	Gull fecal	22	$1.2 \times 10^9 \pm 4.6 \times 10^8$	$1.3 \times 10^4 \pm 3.5 \times 10^4$	$1.8 \times 10^4 \pm 4.0 \times 10^4$
	Water	119	$2.6 \times 10^4 \pm 7.5 \times 10^4$	$1.1 \times 10^2 \pm 3.0 \times 10^2$	$6.4 \times 10^2 \pm 2.8 \times 10^2$
	Sand	25	$1.8 \times 10^5 \pm 5.0 \times 10^5$	$1.1 \times 10^2 \pm 1.9 \times 10^2$	$1.9 \times 10^2 \pm 2.9 \times 10^2$
BacR	Ruminant fecal	6	$8.2 \times 10^8 \pm 2.1 \times 10^8$	$2.8 \times 10^3 \pm 8.4 \times 10^2$	$3.9 \times 10^3 \pm 8.9 \times 10^2$
	Water	11	$2.8 \times 10^3 \pm 1.5 \times 10^3$	$1.8 \times 10^0 \pm 2.0 \times 10^0$	$2.7 \times 10^0 \pm 3.0 \times 10^0$
	Sand	8	$1.6 \times 10^4 \pm 6.2 \times 10^3$	$2.8 \times 10^1 \pm 2.4 \times 10^1$	$6.6 \times 10^1 \pm 6.8 \times 10^1$
HB	Sewage influent	43	$3.0 \times 10^7 \pm 4.7 \times 10^6$	$4.3 \times 10^2 \pm 6.6 \times 10^1$	$9.4 \times 10^{-1} \pm 1.4 \times 10^{-1}$
	Water	2	$6.3 \times 10^2 \pm 4.2 \times 10^2$	$2.2 \times 10^0 \pm 2.1 \times 10^0$	$8.2 \times 10^0 \pm 1.5 \times 10^0$
	Post-CSO water	9	$7.2 \times 10^3 \pm 4.3 \times 10^3$	$9.9 \times 10^2 \pm 5.2 \times 10^2$	$9.7 \times 10^2 \pm 1.1 \times 10^3$
Lachno2	Sewage influent	43	$6.3 \times 10^7 \pm 9.8 \times 10^6$	$9.7 \times 10^2 \pm 1.5 \times 10^2$	$1.2 \times 10^0 \pm 2.0 \times 10^{-1}$
	Water	13	$6.3 \times 10^2 \pm 1.4 \times 10^3$	$1.0 \times 10^0 \pm 1.3 \times 10^0$	$1.2 \times 10^1 \pm 2.6 \times 10^1$
	Sand	7	$6.8 \times 10^3 \pm 5.0 \times 10^3$	$8.6 \times 10^0 \pm 1.5 \times 10^1$	$1.0 \times 10^1 \pm 1.1 \times 10^1$
	Post-CSO water	9	$6.6 \times 10^3 \pm 3.9 \times 10^3$	$8.8 \times 10^2 \pm 4.6 \times 10^2$	$9.0 \times 10^2 \pm 9.9 \times 10^2$

[a]Beach water, sand, and post-CSO water samples testing positive for the host-specific indicator assay. The HB marker was not detected in any sand samples.
[b]Number of samples in which host-specific indicators were detected, including samples with high *E. coli* levels (≥235 CFU/100 ml) and low *E. coli* levels (<235 CFU/100 ml).
[c]Ratios for fecal source material (gull fecal, ruminant fecal, and sewage influent) are calculated using qPCR measurements of *E. coli* and enterococci. Ratios for water, sand, and post-CSO water are calculated using culturable measurements of *E. coli* and enterococci.

were highly varied, particularly for Gull2, as indicated by the high standard deviation (SD) of the mean, which was not unexpected given the high variability in source material. The range of ratios for each alternative indicator is shown in Fig. S2 to S5. The ratio of Gull2 to *E. coli* concentrations was significantly higher in gull fecal samples than in either sand or water samples ($P < 0.01$). The same results were found for ratios of Gull2 to enterococci. There was no significant difference in the ratio of Gull2 to *E. coli* or the ratio of Gull2 to enterococci in a comparison of sand and water beach samples, which could suggest that decay dynamics in these two matrices are similar or that gull fecal droppings constantly deposit these fecal organisms in consistent proportions. Ratios of the BacR marker to *E. coli* or enterococci in cow feces compared to environmental samples were significantly higher ($P < 0.01$). Far fewer environmental samples were positive for the human markers; therefore, trends were difficult to assess. Ratios of human markers to enterococci actually increased in environmental samples compared with the fecal source material.

FIB, gull, and sewage marker decay. In addition to extensive field sampling, we assessed the decay of traditional and alternative indicators in the beach environment using *in situ* microcosm experiments. For gull microcosms, the initial average concentrations ($t = 0$) of culturable *E. coli* and enterococci were 5.0×10^5 and 8.9×10^4 CFU/g, respectively. The initial average concentrations of *E. coli*, enterococci, and Gull2 markers detected by qPCR were 2.4×10^4, 1.0×10^6, and 4.1×10^6 CN/g, respectively. The concentrations of Gull2, *E. coli*, and enterococci detected in the microcosms over time are shown in Fig. 3 and S6. At 35 days following inoculation, qPCR markers and cultured FIB were reduced by four to five orders of magnitude, and further loss past this time point was minimal. Linear regression analysis was carried out using the first-order model of decay for marker concentrations within the linear range of detection (Table 2). The Gull2 marker decay constant was largest ($k = -0.337$ day^{-1}) compared to other qPCR targets and culturable indicators measured in the gull microcosm experiment. The Gull2 decay constant was significantly larger than *E. coli* and enterococci measured by qPCR and enterococci measured by culture ($P < 0.01$). There was no statistical difference in the decay constants for the Gull2 marker and culture-based *E. coli* ($P = 0.029$).

For sewage microcosms, the initial average concentrations of *E. coli* and enterococci were 2.5×10^3 and 2.1×10^3 CFU/g, respectively, as measured by culture methods. The

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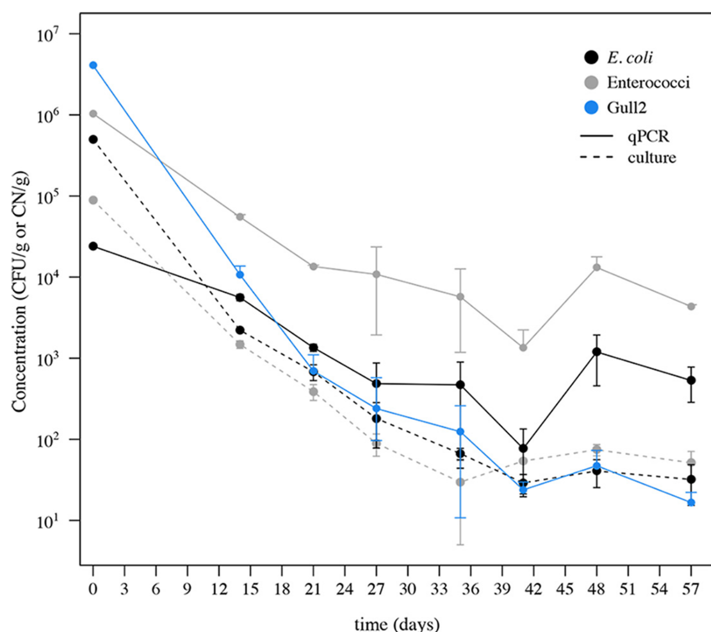

FIG 3 Concentrations of markers and fecal indicators measured over time during the gull microcosm experiment. Circles represent average concentrations for triplicate microcosms. Error bars indicate the standard deviations about the mean concentrations.

initial average concentrations of *E. coli*, enterococci, HB, and Lachno2 were 5.3 × 10^3 CN/g, 1.5 × 10^5 CN/g, 4.7 × 10^4 CN/g, and 6.4 × 10^4 CN/g, respectively, as measured by qPCR. The concentrations of indicators measured over time are shown in Fig. 4 and S7. All indicators by both qPCR and culture were detected throughout the duration of the experiment, with the exception of *E. coli* measured by qPCR, which had concentrations below the limit of quantification after 33 days. The HB marker had a decay constant of −0.175 day^{-1}, which was similar to culture-based enterococci and significantly larger than culture-based *E. coli* and enterococci detected by qPCR ($P < 0.01$). The Lachno2 marker first-order rate constant was significantly larger than that for enterococci detected by qPCR ($P < 0.01$) but similar to the value for culture-based *E. coli*. Most notably, the Lachno2 marker and HB were found to have statistically different decay rate constants, with the HB marker lost at a higher rate than Lachno2; however, concentrations generally remained at an order of magnitude above culturable indicator concentrations throughout the experiment.

TABLE 2 First-order decay coefficients with standard deviations calculated for qPCR markers and fecal indicator bacteria in gull and sewage microcosm experiments

Microcosm type[a]	Marker assay	Decay rate constant $k \pm$ SD ($days^{-1}$)	R^2
Gull	Gull2	−0.337 ± 0.029	0.96
	E. coli	−0.131 ± 0.013	0.97
	E. coli (culture)	−0.287 ± 0.021	0.98
	Enterococcus spp.	−0.176 ± 0.014	0.97
	Enterococci (culture)	−0.25 ± 0.012	0.99
Sewage	HB	−0.175 ± 0.017	0.96
	Lachno2	−0.130 ± 0.011	0.97
	E. coli	−0.141 ± 0.009	0.99
	E. coli (culture)	−0.114 ± 0.011	0.96
	Enterococcus spp.	−0.083 ± 0.006	0.98
	Enterococci (culture)	−0.183 ± 0.018	0.95

[a]Linear regressions were calculated over 35 days for gull microcosm calculations and 51 days for sewage microcosm calculations.

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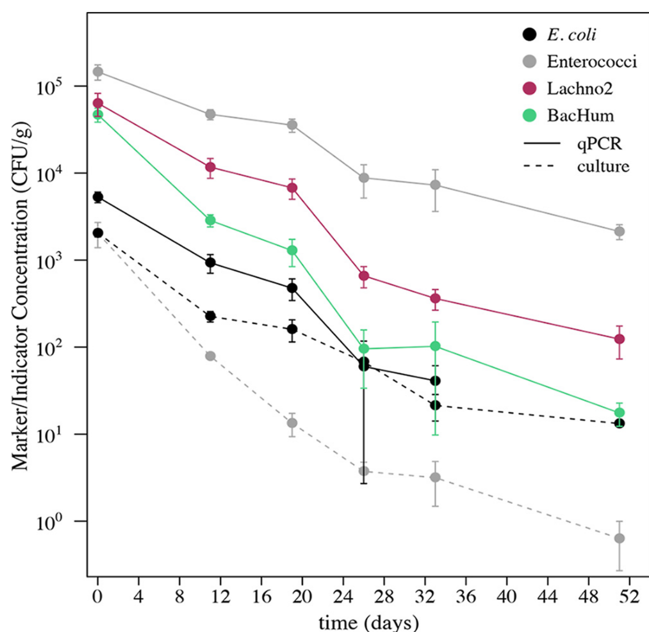

FIG 4 Concentrations of markers and fecal indicators measured over time during the sewage microcosm experiment. Circles represent average concentrations for triplicate microcosms. Error bars indicate the standard deviations about the mean concentrations. *E. coli*, as measured by qPCR, was analyzed at every time point and was detected but not quantified after 33 days. BacHum, human-associated bacteria.

DISCUSSION

Fecal contamination of recreational water poses a threat to beachgoer health, and the resulting beach advisories and/or closures can have serious economic consequences (39). Beach water quality monitoring practices, which typically rely on culture-based enumeration of *E. coli* and/or enterococci, fall short in their ability to provide beach managers with timely and detailed information concerning sources of fecal pollution that could pose a health risk for beachgoers. This study examined the use of alternative indicators for identifying sources in instances where elevated levels of fecal indicators are detected in water and explored how alternative indicators persist in sand compared to *E. coli* and enterococci.

Evidence of gull contamination in water was widespread at all beaches, consistent with previous reports in marine regions (31, 40), with the Gull2 marker detected in 83% of samples with high *E. coli* levels and 82% of samples with low *E. coli* levels. Traditional FIB concentrations in gull feces have been reported to be highly varied, with concentrations of *E. coli* and enterococci reported to range between 10^2 and 10^8 CFU/g (41) and between 10^2 and 10^{10} CFU/g (42–44), respectively. We found similar variability in the gull fecal samples we analyzed, with concentrations of FIB ranging over six orders of magnitude for *E. coli* and three orders of magnitude for enterococci (Fig. S1). The large range of *E. coli* and enterococci in gull feces has not been shown to vary by season, geography, or age of gull (42, 45); the omnivorous diet and scavenging tendencies of gull populations may explain much of this variation. The Gull2 marker was much more consistent and, on average, was four orders of magnitude higher than FIB. Next-generation sequencing has revealed that *Catellicoccus* is the most abundant genus in gull feces, representing, on average, 55% of the total community, which illustrates the utility of *Catellicoccus* marker assays to detect gull waste (43). The same study showed that *Enterococcus* spp. and *Escherichia* spp. represent a smaller fraction of the population, at ~10% (43). Gull feces can also contain some human pathogens, such as *Campylobacter* and *Salmonella* (46). Compared to human sewage, the presence of gull fecal pollution poses a comparatively lessened risk of illness, yet can lead to an excessive number of beach closings (10).

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In contrast, human sources were rarely detected in beach water but, when detected, were at only two of the urban beaches that have nearby stormwater outfalls. Stormwater outfalls have been reported to be frequently contaminated with sanitary sewage (47, 48), and these discharges can act as local sewage sources in the absence of known contamination events, such as sewage overflows. When human indicators were detected, the Lachno2 marker was detected more frequently. This result could reflect differences in marker survival and/or differences in initial marker concentrations. Newton et al. found that the concentration of HB was significantly correlated with Lachno2 ($R^2 = 0.86$) in water samples collected in the Milwaukee inner harbor (26). In that study and the present study, Lachno2 was found at 1.5-fold-higher levels in sewage than HB. The two human markers, compared to traditional FIB alone, provide greater reliability in the detection of human fecal pollution, a finding that was exemplified in the assessment of post-CSO samples. CSOs occur 1 to 3 times per year in Milwaukee and are a regional rather than local source of fecal pollution to beaches. Previous studies in this system reported that levels of *E. coli* and enterococci are generally low during and after CSO/sanitary sewer overflow (SSO) events, but human markers have been detected at $>10^5$ CN/100 ml in the open waters of Lake Michigan adjacent to the BB and BV beach sites (27, 49). In this study, 1 day following release of a CSO, the HB and Lachno2 markers produced a robust signal in the swimming area, while *E. coli* was well below the limit for water quality advisories and in some samples, was absent. These data demonstrate that alternative indicators, such as Lachno2 and HB, are very useful to assess serious water quality concerns where dilute pollution could present a serious health risk to beachgoers.

The ruminant marker was only tested for at rural beaches and was detected in 16% and 53% of water samples with elevated *E. coli* at KA and PB, respectively. Both beaches are near river discharge points (Fig. 5), with PB closer to its river. All of the samples in which the ruminant marker was detected had elevated *E. coli* levels and were collected on the same date; considering these sites span almost 2 km of shoreline, this demonstrates that there was widespread contamination on this day. When the BacR marker was detected in water, *E. coli* levels averaged 1,500 CFU/100 ml, and levels of enterococci averaged 1,900 CFU/100 ml, with ratios of alternative indicators to traditional FIB lower than what was found in source material (Table 1), suggesting the pollution could be attenuated in the environment. Alternatively, these ratios could have also been affected by the presence of other sources of additional traditional FIB. Compared to either *E. coli* or enterococci, the high abundance and low variability of BacR in ruminant fecal samples underscore the reliability of BacR for the detection of fecal pollution from agricultural runoff.

Sand has been widely considered an intermittent source of fecal indicator bacteria to water (12, 50–52). At all beaches examined, the highest *E. coli* densities (on a per-weight basis) were found within berm sand samples, while densities of enterococci were found to be higher in backshore sand. Previous work has noted high levels of *E. coli* in wash-zone beach sand (12, 53); however, few studies have compared multiple beaches concurrently for both indicators, allowing us to benchmark one against the other. These findings suggest that *E. coli* in beach sand is favored under high-moisture conditions, and enterococci are favored under low-moisture conditions, irrespective of source inputs. Water samples harbored lower concentrations of *E. coli* and enterococci per 100 ml, compared to 100-g berm or backshore sand samples, consistent with a recent study by Staley et al. (54). Although a determination of bacterial transfer dynamics between sand and water are not within the aims of this study, the high correlation of FIB between berm sand and water suggests that the sand FIB-carrying capacity is large and has the potential to seed FIB to the nearshore water.

The distribution and decay of alternative indicators in sand were examined to assess how alternative indicator persistence compared with traditional indicators that are commonly used in water quality monitoring programs. Despite >80% of all water samples showing evidence of gull waste, only 25% of sand samples from the six beaches were positive for the Gull2 marker, but all had *E. coli* and enterococci present.

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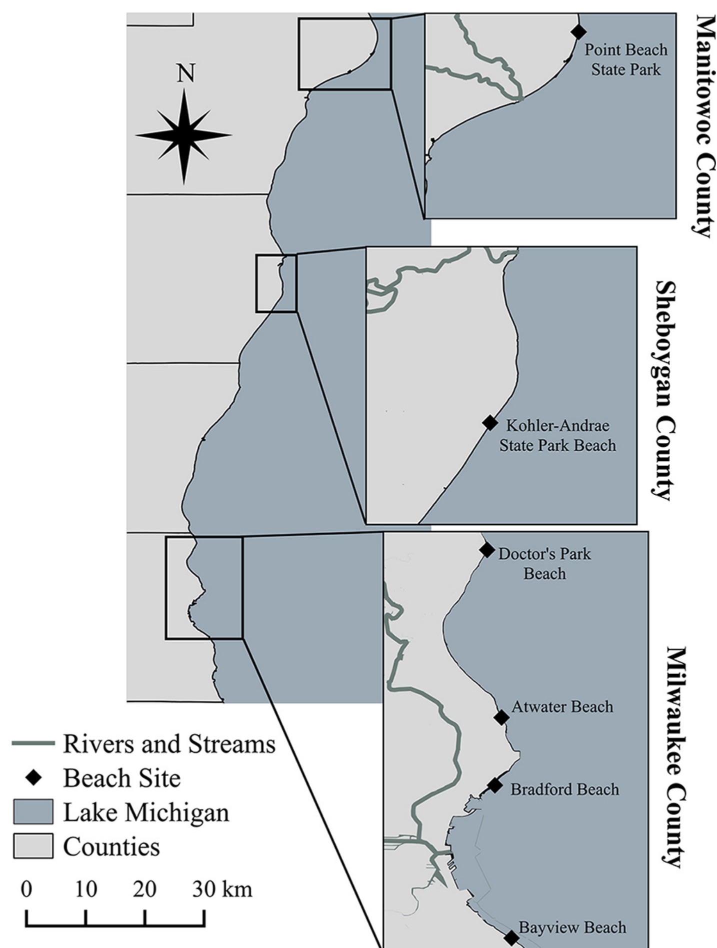

FIG 5 Beach sites: Manitowoc County: Point Beach State Park; Sheboygan County: Kohler-Andrea State Park Beach; Milwaukee County: Doctor's Park Beach, Atwater Beach, Bradford Beach, and Bayview Beach. (Map created using QGIS version 2.10.1-Pisa.)

The results from water samples support the conclusion that the main external source of *E. coli* to the beach environment was likely gulls, with the exception of occasional widespread regional contamination.

We examined the time frame that it would take to reduce the Gull2 marker below the levels of *E. coli* and enterococci in sand. This study is one of the first to deploy *in situ* microcosms in the beach environment to mimic environmental conditions. The vast majority of previous studies that reported the survival of indicators in beach sand have been performed using controlled benchtop experiments (33, 36–38, 55, 56), which, by design, cannot reproduce the range of interrelated conditions in the natural environment, including daily temperature fluctuations, UV radiation, and humidity cycles. Our microcosm experiments were designed to act as chambers that were subjected to natural temperature variations, allowing for the passage of water and nutrients, while ensuring the microbial integrity of the inoculated sand contained inside. Due to the large variation of FIB in gull fecal samples, microcosm inocula derived from pooled gull droppings were used to mimic average initial concentrations of all the indicators.

Although the Gull2 marker decay constant was greater than culturable *E. coli* or enterococci, this marker was consistently detected at higher concentrations for approximately 30 days before concentrations dropped below cultured traditional indicator levels. Because the microcosms mimicked beach conditions, this time frame could be a useful benchmark for beach managers when assessing gull sources at beaches. Interestingly, the decay constant for the Gull2 marker was similar to rates obtained for

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lab benchtop microcosms containing sand and water from Santa Cruz, CA, that utilized seawater (36). The Gull2 assay used in this study is reported to have a high level of sensitivity and specificity during a multilaboratory study of gull-associated assays (29). The only host species shown thus far to cross-react with Gull2 is pigeons. Genetic markers that target *Catellicoccus*, such as Gull2, appear to be very robust for source detection and could be useful for evaluating the success of gull deterrent programs that are aimed at reducing this nuisance bird loafing on beaches, which can result in accumulation of FIB in the sand.

Sand microcosms with sewage as a fecal source demonstrated that the human-associated markers HB and Lachno2 also had a higher decay rate constant than culturable *E. coli*. Similar to the gull microcosms, we used untreated sewage as the inoculum; thus, the relative proportions of each indicator at the start of the experiment were similar to an actual contamination event. The human-associated markers were detected at levels above culturable *E. coli* and enterococci in sand for ~50 days, suggesting that these markers could give indications of sewage impacts to beaches over this time frame. Since humans are reservoirs for many human pathogens, human sources create a serious health risk to beachgoers, and an assessment of residual contamination in sand might be useful since inputs may be sporadic and rain driven, making it difficult to detect these sources in water.

Lachno2 was detected but not quantified in a high number (69/110) of sand samples. The HB marker decay constant was larger than that of the Lachno2 marker. Differences in the human marker decay patterns suggest that old pollution may result in the sole detection of Lachno2, given similar initial concentrations of the two markers; however, there are several other alternative explanations for this result. Samples with trace amounts of DNA template, due to environmental dilution and/or attenuation, can lead to DNQ results. Specifically for microbial source tracking studies, DNQ results may be the result of the presence of old fecal pollution. Additionally, amplification below the limit of quantification may indicate low-level cross-reactivity with nontarget organisms, such as those indigenous to the natural microbial community. We observed a greater-than-expected number of DNQ samples in sand, but very few were observed in water samples for Lachno2. Environmental interference has been reported with alternative indicator assays, targeting *Bacteroides* (57, 58), which highlights that previously uncharacterized organisms could interfere with assays targeting fecal bacteria. Low levels of amplification, such as with the DNQ samples, can occur when there is a large amount of similar but nontarget sequence (59). Large numbers of DNQ results were reported in a recent study of 41 microbial source tracking markers, where the authors suggested that detection thresholds were very important in determining if a source is present or absent (8). Cross-reactivity with canine feces could be another possible explanation, as Lachno2 has been detected in some canine fecal samples (60); however, testing with an established canine marker (61) produced negative results (data not shown). Further validation of this marker in the sand matrix is needed.

Understanding the dynamics of both traditional and alternative fecal indicators in the beach environment is essential for effectively identifying fecal pollution sources and evaluating potential health risks. This is especially important as beach managers move toward implementing molecular testing methodologies. From this study and others, it is clear that it is virtually impossible to interpret single-day or even extensive multiday survey data to identify sources of *E. coli* observed based on the presence of alternative indicators. Contamination scenarios are complex and involve repeated deposition, differential survival of indicators, and interchange between sand, water, and other matrices, such as wrack. Despite this, the high abundance and consistency of alternative indicators in source fecal material demonstrate that beach managers could reliably employ alternative indicators to detect specific suspected sources from recent pollution events, such as human sources from stormwater or sewage overflows, or impacts from nearby agricultural watersheds. The lack of correlation between elevated *E. coli* levels and identification of sources with alternative indicators in both water and sand

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suggests that elevated *E. coli* levels should not be the only criterion for choosing samples for testing by qPCR methods.

Growing evidence, including the results put forth in this study, substantiates that persistence of fecal indicators in beach sand is a major confounder of monitoring programs. The absence of alternative indicators of the most probable sources for a beach may be a result of differential decay and could be considered evidence that pollution is from a past rather than recent pollution event. Our microcosm studies suggest that source-associated indicators will be at higher concentrations than the culturable FIB associated with that source when inputs occurred >30 days prior for the Gull2 marker and >50 days prior for sewage markers. Alternatively, *E. coli* and enterococci that occur with no other evidence of fecal pollution could represent strains that are naturalized, a phenomenon worth further exploration (62). Overall, with proper interpretation of monitoring results, the use of alternative indicators can improve the breadth of beach pollution assessments and aid in source identification at recreational beaches.

MATERIALS AND METHODS

Study area and sample collection. This study was conducted during the summer months of 2012 to 2013 along the western coastline of Lake Michigan at six Wisconsin beaches. Beaches included Point Beach State Park (PB), Kohler-Andrae State Park (KA), Doctor's Park (DP), Atwater Park Beach (ATW), Bradford Beach (BB), and Bayview Beach (BV) (Fig. 5). The beach site map was created using QGIS version 2.10.1 (63). At each of the six beaches, in addition to water samples, sand was collected at the berm (wash zone wetted by wave action) and backshore beach zones (dry sand). Backshore sand was only collected during 2013 sampling dates. Sampling was conducted along three sites, spaced 50 to 100 m apart, parallel to the shoreline for PB, KA, BB, and BV, while ATW and DP were sampled at four sites. At each beach, the berm zone was considered the lakeward portion of the beach within the range of wet sand subjected to wave action. The backshore was defined as the generally dry portion of the beach between the vegetation line and the berm, which is wetted from intermittent wave action with dry periods over a span of days to weeks. Nearshore water was collected at a depth of 0.3 m approximately 3 to 5 m from shore. Water was collected in sterile 1-liter Nalgene bottles, and all sand samples were collected in sterile Whirl-Pak bags. Sand and water samples were transported to the lab on ice and processed within 24 h of collection. Sand moisture content was determined based on the mass difference before and after a 24-h drying period at 45°C.

Culture-based fecal indicator enumeration. *E. coli* and enterococci were isolated from sand samples using techniques adapted from those developed by Boehm et al. (50). To isolate cells from sand, 45 g of either backshore or berm sand was shaken in 450 ml of sterile water for 2 min by hand. Water and sand extracts were filtered through a 0.45-μm-pore-size nitrocellulose filter (Millipore, Billerica, MA) and transferred to modified membrane-thermotolerant *E. coli* (mTEC) and membrane *Enterococcus* indoxyl-D-glucoside (mEI) agar plates (Becton Dickinson, Franklin Lakes, NJ). Incubation and enumeration were performed according to USEPA methods 1603 and 1600 for *E. coli* and enterococci, respectively (64, 65). FIB concentrations were reported as CFU/100 ml or CFU/100 g (dry weight). Various filter volumes or sample dilutions were used to attain colony counts within a target countable range of 10 to 300 CFU. For samples filtered at 100 ml having colony counts fewer than 10, whole numbers were reported; otherwise, concentrations were reported to two significant figures, per Myers et al. (66).

Sample processing and selection. Water samples (400 ml) and sand eluent (100 ml) were also filtered through 0.22-μm-pore-size nitrocellulose filters (Millipore, Billerica, MA) for DNA extraction procedures. Filters were folded using ethanol-sterilized forceps and transferred to 2-ml sterile screw-cap tubes. Both water and sand DNA filters were stored at −80°C until DNA extraction was performed. Water samples were chosen for qPCR analysis if the culture counts exceeded 235 CFU/100 ml. A total of 84/422 samples were found to exceed the *E. coli* water criteria. We also selected a subset of water (n = 60) samples that had low levels of *E. coli*, which were collected at the same beach on the same day or within 1 day of the exceedance water samples. Paired sand samples, collected at the same transect and day as the selected water samples, were also analyzed by qPCR. Of the 84 water samples with ≥235 CFU *E. coli*/100 ml, 69 paired berm sand samples were analyzed by qPCR. Of the 60 water samples with ≤235 CFU *E. coli* /100 ml, 41 paired berm sand samples were analyzed. Sand was not collected on some of the sample dates. Additional water samples from Bradford (n = 6), Atwater (n = 8), and Bayview Beaches (n = 6), collected 1 day and 3 days after a combined sewer overflow (CSO) event in the Milwaukee estuary, were also analyzed using the same qPCR assays. We did not collect sand samples under CSO conditions. A summary of beach locations, number of days sampled, total samples collected, and samples analyzed using qPCR by beach can found in Table S1.

DNA extraction and qPCR analysis. To extract DNA from the selected samples, frozen nitrocellulose filters were removed from −80°C storage and placed on dry ice. Filters were then manually crushed into small pieces using sterile steel spatulas. DNA was extracted from the crushed filter pieces using the Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH), according to the manufacturer's instructions. The concentration and purity of DNA were determined using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA). Extraction efficiencies have consistently been ~20%, as previously reported (67).

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The qPCR assays were carried out using an ABI StepOne Plus real-time PCR system with TaqMan (Applied Biosystems, Foster City, CA) hydrolysis probe chemistry. All qPCR assays included in this study were previously published, and the primer-probe sequences can be found in Table S2. The assays employed in this study targeted *E. coli* (55), *Enterococcus* spp. (Entero) (68), human-associated *Lachnospiraceae* (Lachno2) (26), human-associated *Bacteroides* (HB) (19, 67, 69), gull-associated *Catellicoccus marimammalium* (Gull2) (28, 70), and ruminant-associated *Bacteroidetes* (BacR) (22). Standard curves were created using six serial 1:10 dilutions from 1.5×10^6 to 15 copies per reaction of a linearized plasmid containing the target sequence. Standard curves were run in triplicate and were included on each run. The slope, *y*-intercept, and assay efficiencies can be found in Table S3. All samples were run in duplicate 25-μl reaction mixtures containing 1× TaqMan gene expression master mix (Applied Biosystems), primers and probes at final concentrations of 1 μM and 80 nM, respectively, and 25 to 100 ng of extracted DNA. The PCR cycling conditions were performed as follows: 2 min at 50°C to activate the uracil-*N*-glycosylase (UNG), 10 min at 95°C to inactivate UNG and activate the *Taq* polymerase, 40 cycles of 95°C for 15 s, and 60°C for 1 min.

The copy number (CN) was converted to CN/100 ml of water or CN/100 g of sand (original sample) using the DNA elution volume (150 μl), sample filtration volume, total sample mass (for sand), and wet/dry mass (for sand). The lower limit of quantification (LLQ) was determined for each assay using a cycle threshold (C_T) corresponding to the standard curve dilution that was within the linear range. The limit of reliable quantification or LLQ was 15 copies per reaction, which is equivalent to 112 CN/100 ml for water samples filtered at 400 ml. The LLQ occurred at a C_T of 35, with the exception of the Gull2 assay, which had a C_T of 38. Any amplification that occurred below the LLQ but above background was recorded as detected but not quantifiable (DNQ). All beach water samples were analyzed using the *E. coli*, Entero, Gull2, Lachno2, and HB assays. Beach sand samples were analyzed using the Gull2, Lachno2, and HB assays. Selected water and sand samples that were collected from either Point Beach State Park or Kohler-Andrae State Park were also analyzed using the ruminant-specific BacR assay due to the proximal agricultural land use practices (14).

Gull, ruminant, and untreated sewage sample analysis. To examine the variation of host-associated genetic markers (Gull2, Lachno2, HB, and BacR) and their relationship to traditional indicators in source material, we performed qPCR on gull fecal pellets ($n = 22$), sewage influent samples ($n = 43$), and bovine fecal samples ($n = 6$). Fecal and sewage influent samples were processed using the Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH), according to the manufacturer's protocol. Additional information about the collection of these samples can be found in the supplemental material. We also analyzed these samples using the *E. coli* and Entero qPCR assays and compared the relative concentrations of host markers to those of *E. coli* and Entero.

***In situ* beach microcosms.** Microcosms were constructed using polyvinyl chloride (PVC) pipe material cut into 5 by 9-cm pieces. End caps for the microcosms were prepared by drilling 20 to 30 1-mm-diameter holes into PVC knockout test caps (Oatey, Cleveland, OH) and affixing a sterile 0.22-μm-pore filter to the interior surface using standard silicone sealant. The microcosm design was adapted from that of Alm et al. (71). Prior to microcosm use, the interior surfaces of the PVC pipe were sterilized using a 70% ethanol wash.

For gull fecal microcosm experiments, 13 fecal pellets were collected from metropolitan Milwaukee locations. A 1-ml aliquot of sterile phosphate-buffered saline (PBS) was added to each pellet and vortexed for 30 s to create a pooled fecal slurry. All fecal slurries were combined for a single pooled sample and used within 24 h of collection. Initial concentrations of *E. coli* and enterococci were determined for the pooled sample via membrane filtration and incubation on mod-mTEC and mEI media, as described previously. Beach sand was collected at the berm from Bradford Beach and tested for levels of *E. coli* and enterococci. A 5-ml subsample of the fecal slurry was inoculated into 5,000 g of sand and homogenized manually. Inoculated sand was divided into 21 prepared microcosms, which were used for seven triplicate time points. The remaining inoculated sand was used for the triplicate measurement at time 0. With permission from Milwaukee County Parks, inoculated microcosms were transferred to Bayview Beach on ice and buried within the berm zone in a single layer 7 to 10 cm below surface level, spaced 5 cm apart. After burial, a random selection of three microcosms were removed every 7 to 10 days and transferred to the lab for analysis. All samples were analyzed using previously described culture-based methods. Sand extracts were also filtered for DNA, extracted, and analyzed using the *E. coli*, Entero, and Gull2 qPCR assays. The experiment was run for 57 days.

Sewage microcosms used the same setup protocol as outlined above. The inoculum for the sewage microcosm consisted of untreated sewage influent collected at the South Shore Water Reclamation facility supplied by Milwaukee Metropolitan Sewerage District (MMSD). Sewage influent was transferred to the laboratory at 4°C and used within 4 h of collection. A 100-ml aliquot of sewage was inoculated into 3,000 g of sand, homogenized manually, and divided into 15 microcosms. Time points were 7 to 10 days apart, with the final triplicate microcosms sacrificed at 51 days after inoculation, for a total of six time points. Sand extracts were processed as described in the previous experiment and analyzed using the *E. coli*, Entero, HB, and Lachno2 qPCR assays.

Data analysis. Data analysis and statistical procedures were performed in R (version 3.1.1) using R Core packages. Data visualization and figure generation were carried out with the lattice and ggplot2 packages and package dependencies (72). Culture counts were log transformed (those with no detectable CFU were given a value of 1). Counts of *E. coli* and enterococci were compared using the 2-tailed Student's *t* test, assuming equal variance and significance at a P value of ≤ 0.01. The geometric mean was determined independently for each beach in backshore sand, berm sand, and water samples. Spearman's rank order coefficient was used to assess the correlation between *E. coli* levels in water and berm sand

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samples along the same transect, and to assess the correlation between human-associated marker concentrations in samples collected under CSO conditions. The geometric mean values were used as the input for the heatmap generation. Ratios of alternative indicators to *E. coli* and enterococci were calculated for each sample individually. The average and standard deviation of sample ratios were reported.

The arithmetic mean and standard deviation were calculated for each replicate time point in both microcosm experiments. Linear regressions were propagated using the first-order exponential decay equation $\ln(C/C_0) = kt$, where C_0 is the initial concentration, k is the decay rate constant in days^{-1}, and t is equal to elapsed time in days. Analysis of variance (ANOVA) was used to evaluate if significant differences exist between the regression coefficients for indicator decay in microcosm experiments. Differences in decay rate coefficients were deemed significant at a P value of ≤ 0.01. In discussing differences in decay constants among fecal indicators and qPCR markers, the absolute value of the decay constant was used.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02881-16.

TEXT S1, PDF file, 0.8 MB.

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