MICROBIAL COMMUNITIES AND THE DIVERSE ECOLOGY OF FECAL INDICATORS AT LAKE MICHIGAN BEACHES

by

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ABSTRACT

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Fecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and enterococci are used to assess microbiological water quality in recreational waters. The use of FIB follows the assumption that their presence correlates with that of fecal-associated pathogens in recreational waters. The beach ecosystem is complex however and multiple factors can influence the concentration of *E. coli* and enterococci in the beach environment. Microbial communities within beach sand play a key role in nutrient cycling and are important to the nearshore ecosystem function. *E. coli* and enterococci, two common indicators of fecal pollution, have been shown to persist in the beach sand, but little is known about how microbial community assemblages are related to these fecal indicator bacteria (FIB) reservoirs. The first objective of this project was to harness the power of next-generation sequencing to profile the indigenous communities within beach sand and examine key environmental drivers of community structure. FIB were found at similar levels in sand at beaches adjacent to urban, forested, and agricultural land and in both the berm and backshore. However, there were striking differences in the berm and backshore microbial communities, even within the same beach, reflecting the very different environmental conditions in these beach zones in which FIB can survive. Results indicate that microbial community structure in beach sand is most associated with the concentrations of total

organic carbon (TOC) and total phosphorus (TP). Fine scale nucleotide differences in the V4V5 region of the 16S rRNA gene of abundant taxa were identified and sequence patterns suggest a biogeographic influence. This work demonstrates that microbial communities are reflective of environmental conditions at freshwater beaches and are able to provide useful information regarding long-term anthropogenic stress. The second objective of this project was to use host-specific alternative fecal indicator assays to identify the major pollution sources that are responsible for contributing high levels of *E. coli* in both beach sand and water. At the six beaches studied, berm sand contained the highest levels of *E. coli* versus to water ($P < 0.01$), using a weight-to-volume comparison. The gull-specific assay (Gull2) was detected more than any other host-specific alternative fecal indicator assay with 80% detection in water samples during water quality advisories. Human-specific *Bacteroides* (HB) and *Lachnospiraceae* (Lachno2) were detected in only 2.4% of water samples, however a large number of sand samples had an uncoupled occurrence of the two human-specific alternative indicators. Results from *in situ* microcosm experiments indicate that the HB and Lachno2 markers decay at different rates, helping explain their differential occurrence in environmental samples. I*n situ* microcosm experiments also revealed that signals from the alternative indicators decay approximately 20% faster than culturable *E. coli.* Overall, a significant amount of the *E. coli* burden in sand cannot be accounted for with the use of host-specific alternative indicators suggesting that *E. coli* concentrations in sand are uncoupled from fecal sources and that *E. coli* may be able to persist in beach sand post-deposition. The final objective of this project is to assess the survival of different *E. coli* isolates and to identify possible genomic characteristics that may support a persistence phenotype. *In situ* survival experiments revealed that the die-off of a non-environmental *E. coli* type strain was significantly faster than an environmental *E. coli* strain

isolated from beach sand. Comparative genomics suggested that biofilm formation and programmed cell death might be important mechanisms supporting the increased survival of the environmental *E. coli* strain. Overall, the findings presented in this dissertation provide new insights into the environmental ecology of enteric bacteria, highlighting the importance of nutrients, land-use, the indigenous microbial community, and genomic elements as determinants of the fate of FIB in the beach environment.

To my parents,

my husband,

and my late-grandmother Dr. Ann Robinson

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLD	Below the Limit of Detection
BLQ	Below the Limit of Quantification
CFU	Colony Forming Units
CN	Copy Number
CSO	Combined Sewer Overflow
E. coli	*Escherichia coli*
EDTA	Ethylenediamine Tetra-acetic Acid
EPS	Extracellular Polymeric Substances
FIB	Fecal Indicator Bacteria
LLQ	Lower Limit of Quantification
MLST	Multi Loci Sequence Typing
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorous
USEPA	United States Environmental Protection Agency

Chapter 1.

Background

Among developed nations, the United States is a top leader in water resource management providing 286 million Americans with some of the safest drinking water in the world (1). However despite advancements in water quality management, infrastructure, and federal regulations, some sanitation management issues persist. The failure to properly manage wastewater has critical implications for public health. Polluted runoff and untreated sewage released into recreational water expose swimmers to pathogenic bacteria and viruses. The most recent study from the US Centers for Disease Control and Prevention (CDC) reported 90 outbreaks of illness associated with recreational water contact in 2011-2012. The reported recreational illness outbreaks resulted in at least 1,788 individual cases, 95 hospitalizations, and one death (2).

General Fecal Indicator Bacteria

The link between contaminated water and human disease has led researchers to search for an organism that can help predict the presence of waterborne pathogens. Characteristics of ideal fecal indicator organisms include:

- Presence within the intestinal microbial population of warm-blooded animals.
- Co-occurrence with pathogens in the animal host and in the environment.
- High relative abundance compared to pathogens.
- Similar environmental fate compared to pathogens.
- Detectable and quantifiable by easy, rapid, and inexpensive methods.
- Limited pathogenic risk.

The U.S. Environmental Protection Agency (USEPA) recommends *E. coli* and enterococci as fecal indicator bacteria (FIB) for freshwater, and enterococci for marine water (3). Current enumeration methods rely on the ease of culturing these bacteria using growth media that is both

selective and differential. Culturing FIB requires 18-24 hr for incubation thus results are obtained the next day after sample collection (4, 5). Since beach fecal contamination has been shown to be highly variable, short lived, and episodic (6, 7), the use of culturable fecal indicators result in beach advisories that do not represent current water quality conditions. Also, not all sources of fecal contamination pose the same risk to human health, but all can contribute a significant amount of *E. coli* and enterococci to the beach environment. Thus, the use of these general fecal indicators can lead to beach managers posting beach closures when there is not a actual public health risk. Since Lake Michigan beach closures can cost local communities upwards of $37,000 per day (8), there has been a push for the development of same-day laboratory methods for the detection and quantification of fecal indicator bacteria.

Alternative Fecal Indicators

In recent years, research has been focused on the discovery of alternative, host-specific fecal indicator bacteria that can be detected using rapid molecular-based methods such as quantitative polymerase chain reaction (qPCR). Fecal anaerobes are commonly the target for alternative indicator assays, since they are dominant members in the intestinal tract of humans and other animals. Fecal pollution can originate from wastewater infrastructure issues, agricultural run-off, and from wild and domesticated animals. However, because untreated sewage poses the highest risk due to the transmission of human pathogenic bacteria and viruses, much attention has been given to the development of human-specific alternative indicators.

Previously developed qPCR assays for human-specific fecal pollution have targeted *Bacteroides*(9, 10) and *Lachnospiraceae* (11) among other groups. Fecal pollution originating from agricultural runoff, wildlife, and domesticated animals has also led to assays for bovine (12–14), swine (15, 16), chicken (17, 18), gull (17, 19, 20), dog (17, 21) fecal sources. Using

host-specific indicators in addition to general fecal indicators adds a layer of information to water quality testing that would allow beach managers to improve health risk estimates and devise remediation plans to remove fecal pollution sources. However, the environmental fate of alternative indicators relative to traditional fecal indicators after being deposited at the beach is unknown.

Bacteria in Beach Sand

Although beach monitoring has primarily focused on the detection of fecal indicators in water, beach sand and sediments have been shown to contain a large amount of culturable fecal indicator bacteria in freshwater (22–25) and marine beaches (26–28). Culturable *E. coli* and enterococci are found in high enough amounts to significantly influence water column counts on several beaches (22, 29, 30). Fecal contamination of beach sand is a major concern for beach managers and may be an important reservoir for not only fecal indicators, but pathogens as well. Studies have reported the detection of a wide variety of pathogens in sand including *Cryptosporidium (31, 32), Salmonella (27), Campylobacter (27),* and *enterovirus (31).* The presence of pathogens in sand is especially concerning considering children under the age of five have the highest direct contact with beach sand compared with any other beachgoer age group (33). In fact, a survey of beach visitors reported that 6.3% of people described experiencing GI illness in a 10-12 day follow up questionnaire. The same study reported that the highest incidence of GI illness, 9.5%, was experienced by children under the age of five (34).

The presence of microbial life in beach sand is not limited to fecal-borne bacteria and viruses. Beach sand appears to be a microhabitat for a diverse microbial community of indigenous bacteria, protozoa, and fungi. By direct microscopic counts, total bacteria in beach

sand can be on the order of 10^7 per gram (35). Compared to other natural environments, our understanding of the indigenous microbial community in sand is limited. A study that was conducted following the Deep Horizon gulf oil spill reported that sand microbial communities have a common community structure across large geographic regions, and changes in abundance of key members occur with environmental stressors (e.g. oil contamination) (36). In Lake Erie, sediment microbial communities in samples collected from polluted sites were less sensitive to treatments of heavy metals than communities from pristine locations (37). Anthropogenic influences, in addition to natural environmental stress, may play an important role in both community structuring and adaptation to the local environmental conditions.

The relationship between fecal indicators and the natural microbial community has been investigated. Higher levels of biofilm, assumed to be associated to the indigenous microbial community, correlated to lower levels of enterococci in marine sand (28). In laboratory microcosm experiments, unaltered sand microbial communities supported increased die-off of *E. coli* compared to autoclaved sand (killed microbial community) (38). The reported temporal and spatial stability of the microbial community structure may allow for the evaluation of how the community may be disrupted by different anthropogenic stressors, such as fecal pollution that introduces large loads of non-indigenous bacteria into beach sand.

Despite potential exclusion of fecal indicator bacteria by the sand community, the high levels of fecal indicators in sand have prompted the scientific community to call for the development of standard methods for fecal indicator enumeration in sand and also regular monitoring of bacteria in sand (39, 40). In order to establish limits for fecal indicator bacteria in sand, it would be important to determine if the presence of traditional and alternative fecal

indicators in sand reflects recent fecal pollution events rather than the persistence of these enteric organisms and/or the integration of the indicator organisms into the native community.

Naturalized *E. coli*

In addition to examining the different factors responsible for modulating *E. coli* survival in the environment, population genetics of *E. coli* suggest that some strains may be more adapted than others to stress experienced in the beach environment. Primary assumptions that support the use of *E. coli* as a general fecal indicator is that the gastrointestinal tract of warm-blooded animals is the primary habitat for strains and strains do not survive for extended periods of time outside a host. However as more isolates of *E. coli* have been recovered from the environment, it has become clear that the ecological diversity of *E. coli* has been underestimated. With the use of MLST (multilocus sequence typing), Walk et al. reported novel cryptic clades (CI – CV) within the genus *Escherichia* with isolates collected from freshwater surface waters and sediment (41, 42). A study by Lu et al. further investigated the genomes of the cryptic clade isolates confirming that they are most closely related to *E. coli* (compared to other *Escherichia* spp.) (43).

The identification of mechanisms by which *E. coli* is able to survive in the beach environment is important for understanding the ecology of this organism and also critical for evaluating its suitability as a fecal indicator. Outside the host GI system, enteric bacteria such as *E. coli* are subjected to harsh conditions including: UV radiation, large variations in temperature and pH, osmotic stress, low nutrient availability, and increased predation and competition from other microorganisms (44). Beach sand may provide a protective environment for *E. coli* buffering some of these environmental stressors. In addition, sand provides a means for bacterial attachment and for the formation of biofilm. Biofilms can be defined as complex, highly

organized communities of bacteria growing on a solid surface encapsulated in a self-produced extracellular polymeric matrix (45). A survival strategy for bacteria, biofilms have been shown to assist in the persistence of bacteria. In laboratory experiments, *E. coli* isolates recovered from the beach environment have been shown to be more tolerant to low temperatures with growth observed at temperatures as low as 5 ^{o}C (46).

Bacterial persistence is an important phenomenon in bacterial survival that results in the increased tolerance of a subpopulation (called "persister cells") to stressful conditions. Persister cell formation is a non-genetic, phenotypic switch to a dormant state (47). Persister cells have important clinical relevance, as they have been associated with the reemergence resistant bacterial infections after antibiotic treatments (47, 48). Much attention has been given to the clinical importance of bacterial persistence, however persister cells may be an important mechanism underlying the high concentrations of *E. coli* in the environment. A recent study by Hofsteenge et al. reported that two subpopulations of cells, normal and persisters, coexisted within cultures of the 11 environmental *E. coli* isolates studied (49). The same study noted large variations in the fraction of persister cells between different *E. coli* isolates and between different antibiotic treatments suggesting that different persister cells exist that might arise from a variety of different mechanisms. A better understanding of the population structure of *E. coli* is needed to determine if *E. coli* recovered from the environment represent truly environmentally adapted organisms compared to host-associated *E. coli* populations.

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Chapter 2

Influence of Landuse, Nutrients, and Geography on Microbial Communities and Fecal Indicator Abundance at Lake Michigan Beaches

Abstract

Microbial communities within beach sand play a key role in nutrient cycling and are important to the nearshore ecosystem function. *Escherichia coli* and enterococci, two common indicators of fecal pollution, have been shown to persist in the beach sand, but little is known about how microbial community assemblages are related to these fecal indicator bacteria (FIB) reservoirs. We examined eight beaches across a geographic gradient and range of land use types and characterized the indigenous community structure in the water and the backshore, berm, and submerged sands. FIB were found at similar levels in sand at beaches adjacent to urban, forested, and agricultural land and in both the berm and backshore. However, there were striking differences in the berm and backshore microbial communities, even within the same beach, reflecting the very different environmental conditions in these beach zones in which FIB can survive. In contrast, the microbial communities in a particular beach zone were similar among beaches, including at beaches on opposite shores of Lake Michigan. The differences in the microbial communities that did exist within a beach zone correlated to nutrient levels, which varied among geographic locations. Total organic carbon and total phosphorus were higher in Wisconsin beach sand than in beach sand from Michigan. Within predominate genera, fine-scale sequence differences could be found that distinguished the populations from the two states, suggesting a biogeographic effect. This work demonstrates that microbial communities are reflective of environmental conditions at freshwater beaches and are able to provide useful information regarding long-term anthropogenic stress.

Introduction

Since the creation of the Federal Beach Environmental Assessment and Coastal Health (BEACH) Act of 2000, coastal communities have been challenged with implementing programs for the monitoring of recreational waters. Water quality monitoring relies on fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, and the assumption that both are fecal in origin. In recent years, many Great Lakes coastal recreational waters were monitored for the first time and subsequently were found to have unacceptable levels of *E. coli*, indicating the presence of contamination from sources such as sewage, stormwater, or agricultural runoff. However, recent studies have detected high concentrations of FIB in recreational beach sands compared to the concentrations in the nearby monitored waters (1–4). Importantly, several studies have documented that sand reservoirs of FIB play a large role in beach water samples exceeding regulatory limits (1, 3, 5–7).

Persistent FIB in the beach environment is of great concern. The presence of FIB in either recreational waters or sand is assumed to indicate a recent pollution event. However, the persistence and possible proliferation of FIB in the beach environment can confound beach-monitoring efforts and poses additional challenges for beach managers. In addition, there is mounting evidence that *E. coli* isolated from the environment may comprise its own ecotype, capable of surviving in the environment (8, 9). Since sand FIB reservoirs have been shown to act as a source of FIB to the waters, studying the complex dynamics at the sand-water interface is an important area of study for beach research, particularly for research that aims at the reduction of beach closures and accurate assessment of associated public health risks.

The indigenous microbial community can exert antagonistic influence over the establishment of sand reservoirs of FIB. For example, *E. coli* has been shown to survive in

autoclaved sand (i.e., killed microbial community) at levels 100-fold higher than in natural sand (10–13). Piggot et al. reported decreasing levels of enterococci in marine sand with increased levels of biofilm, suggesting that competitive exclusion by microbial communities may influence the establishment of FIB reservoirs (14). Our previous work on the Deepwater Horizon oil spill in the Gulf of Mexico showed that sand microbial communities share a structure across large geographic boundaries and that changes in abundance of key community members occur along an environmental stress gradient (15). Marine sand microbial community assemblages have been reported to be significantly different between beaches that receive little fecal contamination and those that commonly have water quality exceedances (16). These previous works underscore the ecological relevance of microbial communities and the need to better understand beach microbial communities in freshwater systems as they relate to environmental stress.

To better elucidate the drivers of microbial community structure in Lake Michigan beaches in water and in different beach zones, including the backshore sand (dry sand), berm sand (wave-washed sand), and submerged sand, we hypothesized that each of these unique beach zones would show some level of consistency between Wisconsin and Michigan beaches. The commonalities in community structure in each beach zone would allow us to evaluate how the community may be disrupted by stressors, such as river inputs, which introduce large loads of nutrients and nonindigenous bacteria into beach sand. In addition to community analysis, we performed surveys for FIB within all sand and water samples and compared beaches to examine the discrete habitats that harbor FIB. We hypothesized that differences in community structure would exist along a stress gradient of increasing FIB densities and nutrient concentrations. We examined the relationships between urban impact, riverine influence, nutrients, geography, and

microbial community structure within beach zones and across beach sites in order to underscore the utility of microbial communities as indicators of environmental stress.

Materials and Methods

Study area and sample collection. The present study was conducted along the eastern and western coastlines of Lake Michigan at four Wisconsin beaches and four Michigan beaches (Fig. 1). Wisconsin beaches included Point Beach State Park (PB), Kohler-Andrae State Park (KA), Atwater Park Beach (ATW), and Bradford Beach (BB), which spanned 85 miles of shoreline. Michigan beaches included Pere Marquette Park Beach (PM), PJ Hoffmaster State Park (PJ), North Beach Park (NB), and Grand Haven City Beach (GH), which spanned 13 miles of shoreline. The land use for each for each beach was determined using National Land Cover Database (NLCD; ca. 2011) (17). For this study, a beach was defined as urban based on having at least 50% of the land cover within a 5 km radius categorized as "developed" by the NLDC land cover classification, an approach similar to previous studies examining urban beaches (18). Beaches with <50% land cover that was "developed" were defined as nonurban. For microbial community comparisons and statistics based on land cover, land cover was treated as a categorical variable.

At each of the eight beaches, sand was collected at the following distinct beach zones: submerged, berm, and backshore. Each beach was sampled along three transects from backshore to water, with the exception of ATW beach, which contained four transects. Submerged sand was defined as sand located beneath the surface of the water ~1 m from the shoreline. The berm or wash zone was defined as the lakeward portion of the beach within the range of wave action. The backshore was defined as the generally dry portion of the beach between the vegetation line

and the berm crest that is wetted from intermittent wave action (dry periods over a span of days to weeks).

Nearshore water was collected at a depth of 0.3 m adjacent to the beach. Samples were collected three times in the summer of 2013, and Wisconsin and Michigan sites were sampled within 48 h of each other. 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 laboratory 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.

Sand nutrient analysis. Nutrient concentrations, including total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP), were measured in all collected sand samples. Sand was dried completely prior to nutrient analysis. For the TN and TOC measurements, dried sand was washed with a 5% solution of HCl to remove the inorganic carbon fraction. Acid-washed sand was then dried prior to analysis. TN and TOC levels were determined simultaneously in all acidified sand samples using a Carlo-Erba NA-1500 CNS analyzer (Haak-Buchler Instruments, Saddlebrook, NJ) and an acetanilide standard. For TP measurements, 200 mg of sand was combusted in $Mg(NO_3)_2$ for 2 h, followed by a 16 h digestion in 1 N HCl. Sand extracts were then diluted and analysis was carried out using the ascorbic acid phosphomolybdate method originally outlined by Strickland and Parsons (19).

Culture-based bacterial enumeration. At each of three sample dates, nearshore water was collected along with sand from the backshore, berm, and submerged zones. Submerged beach zones were sampled on two of the sample days. A total of 72 water samples and 169 sand samples were analyzed for *E. coli* and enterococci densities. Submerged sand was not analyzed

for the presence of fecal indicators. *E. coli* and enterococci were eluted from sand samples using techniques adapted from those developed by Boehm et al. (20). To elute cells from sand, 45 g of either backshore or berm sand was shaken in sterile water for 2 min. There was no significant difference between eluting in phosphate-buffered water and/or shaking for longer durations. Water and sand extracts were filtered onto a 0.45 μm pore size nitrocellulose filter (Millipore, Billerica, MA) and transferred to modified mTEC (membrane-thermotolerant *E. coli*) and mEI (membrane *Enterococcus* indoxyl-d-glucoside) 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, and bacterial counts were reported as CFU/100 ml or CFU/100 g (dry weight) (21, 22).

DNA extraction, next-generation sequencing, and phylogenetic analysis. Water samples (400 ml) were also filtered onto 0.22 μm pore-size nitrocellulose filters (Millipore) for DNA collection. Filters were folded using sterile forceps and transferred to 2 ml screw-cap tubes. Beach sand was stored without further processing in 1 g aliquots. Both water filters and sand aliquots were stored at −80 °C until DNA extraction was performed.

To extract DNA from frozen nitrocellulose filters, screw-cap tubes were removed from −80 °C and crushed into small pieces, via manual force, with the use of sterile spatulas. Both crushed filter pieces and 1 g sand aliquots were extracted for DNA using the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. DNA concentrations were determined using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA). On each of the three sampling days, water, berm sand, and backshore sand samples were

extracted for each beach. Submerged sand samples were extracted only from the second and third sampling days.

All samples were sequenced using the MiSeq Illumina sequencing platform. Only two berm samples were sequenced from PJ Hoffmaster, rather than three, due to loss of the sample. The V4-V5 hypervariable regions of the 16S rRNA gene were amplified using fusion primer design according to protocols developed at the Josephine Bay Paul Center at the Marine Biological Laboratory, Woods Hole, MA (23). All sequencing data were processed, trimmed, quality controlled, and then stored as part of the VAMPS (Visualization and Analysis of Microbial Population Structures) database (24). The "Merge-Illumina-Pairs" script was used to analyze raw paired-end reads (25). Read-pairs with more than three mismatches within the ~80-nucleotide overlap were removed, and ≥ 66% of the nucleotides in the non-overlapping regions had to have a score of > Q30 (26). The program UCHIME (27) was used to remove chimera sequences using the methods outlined by Huse et al. (28).

Taxonomy was assigned through the Global Alignment for Sequence Taxonomy (GAST) using a 16S rRNA hypervariable region reference database (29). The oligotyping pipeline (v0.96) was used to determine ecological patterns of sequence similarities and differences in the eight most abundant genera in berm samples (30). The open source pipeline is available from http://oligotyping.org. This method has been previously used for numerous applications. For example, oligotyping has been used to identify host specificity of *Blautia*, to explore the ecology of *Arcobacter* in sewage, and to reveal habitat preferences of *Vibrio* (31–33). To assess the levels of *E. coli* and enterococci reads in our community sequences, we used the SRA BLAST tool. We performed a BLAST of the community sequences from the present study (SRP052297) against

16S sequences of enterococci (accession numbers KF250762 to KF250872) (34) and *E. coli* (X80724) obtained from GenBank.

Data analysis. Bioinformatic processes, data visualization, and statistical tests were performed in R (v0.98.501), using the packages "vegan," "permute," and "lattice." Pearson correlations and Student t-tests were deemed significant at P of ≤ 0.05 for FIB comparisons. Sequencing depth after processing ranged from 38,886 to 613,039 reads per sample, and samples were normalized to 124,361 reads, the median for the entire data set. Analysis of variance (ANOVA) followed by Tukey's post hoc test was carried out on nutrient concentrations within sand using a P of ≤ 0.01. Analysis of similarity (ANOSIM) calculations were used to test hypotheses relating to community structure differences within and between sample types. ANOSIM provides a way to statistically test whether there is a significant difference between two or more groups of samples based on a set grouping category. For example, ANOSIM was used to test whether communities grouped by beach zone and whether oligotypes grouped by state. All ANOSIM analyses used the Bray-Curtis index, permutations set at 999, and a P of ≤ 0.01. We identified genus-level taxa, with the greatest contribution to the dissimilarity observed between sand and water samples using similarity percentage analysis (SIMPER). The function "envfit" was used to calculate the regression statistic for individual environmental variables on ordination scores at a P-value of ≤ 0.01. Canonical analysis of principle coordinates (CAP), a form of distance-based redundancy analysis, was used to determine the contribution of measured environmental variables to the observed variation of bacterial community structure. Oligotype clustering and dendrogram generation were performed using minimum variance clustering (Ward method) from the Bray-

Curtis distances. Microbial community sequences have been deposited in NCBI's Sequence Read Archive (SRA) under project number SRP05229.

Results

Fecal indicator concentrations. Over the course of summer 2013, four Wisconsin and four Michigan beaches on Lake Michigan were monitored (Fig. 1 and Appendix A Figures 1-5). Wisconsin beach water had averages of 109 CFU of *E. coli*/100 ml and 67 CFU of enterococci/100 ml, while Michigan samples contained averages of 33 and 14 CFU/100 ml, respectively (Fig. 2). Overall, Wisconsin had significantly higher levels of *E. coli* and enterococci in beach water than Michigan beach water (Student t test, $P \leq 0.05$). We found that both *E. coli* and enterococci reservoirs were present in all sand samples with no significant difference in concentration between land use or state (Student t test, $P = 0.75$).

Although we cannot make a direct comparison between water and sand FIB levels, 84 and 71% of sand samples collected at the berm and 55 and 73% of samples collected from backshore sand were found to have higher levels of *E. coli* and enterococci, respectively, compared to water samples on a weight-to-volume basis; these were samples collected from the same transect on the same day. *E. coli* concentrations in the berm sand strongly correlated with *E. coli* concentrations in water (Pearson r = 0.90), while enterococci concentrations in sand and water had no significant correlation. Indicator concentrations in backshore sand were not correlated with levels of either indicator in water.

Riverine impacts. We examined the relationship of beach water quality to proximity of discharges from nearby rivers. Each beach included in the present study is located less than 10 miles from the mouth of a river that drains into Lake Michigan. For Michigan beaches, distance from river mouth was negatively correlated to beach water quality for *E. coli* and enterococci

(Pearson r = −0.39 and −0.35, respectively, with $P \leq 0.05$ and $P \leq 0.05$), meaning the greater the distance from the river mouth the lower the FIB levels. The Michigan site with the highest levels of FIB was Pere Marquette, which is located immediately south of the Muskegon River mouth. For Wisconsin beaches, the enterococci levels were negatively correlated to distance from river mouth (Pearson r = −0.37, $P \leq 0.05$), while the *E. coli* levels were not (Pearson r = 0.09).

Overall community composition. Illumina sequencing of 24 water and 63 sand samples revealed diverse microbial communities in all zones of all beaches sampled. Thirty-nine phyla were represented among the sequenced samples, with the greatest representation among *Bacteroidetes, Betaproteobacteria, Alphaproteobacteria, Actinobacteria, Acidobacteria, and Plantomycetes* (Fig. 3). Sequence reads corresponding to *E. coli*, *Enterococcus faecalis*, or *Enterococcus faecium* were not identified, which was expected since the levels were on average < 2 CFU FIB per g or ml of sample extracted. We utilized the SRA BLAST tool to compare our community sequences against enterococci and *E. coli* sequences obtained from GenBank to further explore the data set. Sequences matching the GenBank *E. coli* and *Enterococcus* species sequences appeared in low relative abundance (< 20 reads per sample) using this method, but these reads were not resolved to the species level in the community data set and were annotated as *Enterococcaceae* or *Enterobacteriaceae*. The relative proportion of sequences matching GenBank sequences was consistent with culture results when assuming 10^4 to 10^5 bacteria per g of sand.

A nonmetric multidimensional scaling (NMDS) plot based on taxa in the microbial communities from the water and backshore, berm, and submerged sands was created using the Bray-Curtis distance matrices (Fig. 4). NMDS analysis partitioned sequenced microbial communities into three distinct clusters, which were related to the beach zones from which the

samples were collected. Community comparisons revealed that the berm and submerged sand zones housed very similar communities that were not statistically different in overall community structure (ANOSIM R = 0.042, $P = 0.146$), while the backshore sand had a distinct community (ANOSIM R = 0.924, $P \leq 0.01$). Although backshore sand samples clustered away from other sample types, the grouping was more diffuse than those of other beach zones. Taken together, beach samples clustered by zone regardless of individual sites or state, indicating good temporal consistency in samples taken across three dates. In general, beach sand was found to have significantly different community structures compared to water (ANOSIM R = 0.816). Sand and water communities were only 22% similar, as revealed by SIMPER. Taxa contributing the greatest level of dissimilarity between sand and water samples included unclassified *Sporichthyaceae, Flavobacterium, Rhodoferax, and Fluviicola*, with higher average levels within water samples.

Diversity. The Shannon-Weaver diversity index was calculated for each sample separately and averaged across all samples for Michigan and Wisconsin samples (Appendix A Figure 6). The average diversity measures were 4.41 ± 0.36, 4.20 ± 0.08, 4.19 ± 0.11, and 3.51 ± 0.46 for the Michigan backshore, berm, submerged, and water samples, respectively. For the Wisconsin samples, the average diversity measures were 4.59 ± 0.21, 4.29 ± 0.11, 4.26 ± 0.11, and 3.82 ± 0.41 for the backshore, berm, submerged, and water samples, respectively. The Shannon-Weaver index revealed that sand is more diverse than water overall and that the sand environment housed increasingly diverse communities moving away from the water line. Water samples had significantly lower levels of diversity than all other beach zones ($P \leq 0.05$), as revealed by

Student t test comparisons. We did not find significant differences in sample diversity across states or between beaches within the same state.

Nutrient levels in beach sand. Concentrations of the TOC, TP, and TN were measured in all sand samples collected for the present study (Fig. 5). The levels of nutrients measured ranged from 122 to 3.56 × 104 μg/g, 18.4 to 415 μg/g, and 2.98 to 166 μg/g for TOC, TP, and TN, respectively. Wisconsin beaches had significantly higher levels of TOC and TP for submerged, berm, and backshore sands than Michigan beaches. We did not observe significant differences in TN across the states. For further site comparisons, beaches were grouped together by land use (urban versus nonurban). On the Michigan shoreline, pairwise Student t test comparisons of nutrients by land use (urban, PM-GH; nonurban, PJ-NB) revealed significantly higher levels of TOC in the backshore and TP in the berm within urban beaches, whereas on the Wisconsin shoreline, the same comparisons (urban, ATW-BB; nonurban, PB-KA) showed that urban beaches had significantly higher levels of TOC, TP, and TN among all beach zones.

Linking community structure to nutrients and FIB. A discriminant analysis, CAP, was used to examine relationships between measured environmental parameters and observed variation in the taxonomic structure and composition of a bacterial community. The geographic location of these beaches was autocorrelated with nutrient levels, meaning beaches in Michigan had lower levels of nutrients than beaches in Wisconsin. We used CAP to examine the major nutrient drivers of community structure, while constraining the geospatial gradient. Of all the parameters measured, the strongest determinants of community composition were TOC (envfit, $R^2 = 0.64$) and TP (envfit, $R^2 = 0.63$). When we controlled for TOC and TP, the geospatial effect (i.e., state) was

only able to account for 4.7% of the variation observed. Neither TN nor FIB densities were found to exhibit a significant correlation to the structure of these microbial communities. The factors of TOC and TP together provided 42.85% ($P \leq 0.01$) of the total explanatory power for the constrained axes. Although TN and FIB could not explain a significant amount of the community structure of these samples when analyzed independently, the combination of all environmental parameters was able to contribute an additional 15.15% to the total explanatory power for the constrained axes.

We also used SIMPER to examine the relationship between nutrient concentrations and the relative abundance of individual taxa within berm samples. In Wisconsin beaches, unclassified *Sphingobacteriales*, *Chlorobiales*, and *Ferribacterium* correlated to higher concentrations of TP and TOC. We then analyzed Wisconsin and Michigan beaches together and found that *Flavobacterium*, *Rhodoferax*, and unclassified *Sporichthyaceae* were most associated with higher levels of nutrients. When taxa were sorted based on the highest correlation with nutrients, we found that as few as 50 taxa were able to explain 65% of the community variation associated with TOC and TP. Oligotyping was then used to further examine nucleotide variation that existed within shared taxa in all berm samples.

Sequence level variation in major genera at beaches. To further explore our community data set at the sequence level, we performed oligotyping on the eight most abundant genera found in berm samples, which together comprised 60.0% of the berm communities on average. Oligotyping exploits the small base pair variations that exist within the hypervariable regions (in this case V4-V5) of sequences that are classified to a particular taxonomic group, in order to describe the fine-scale diversity that exists within a genus. We utilized oligotyping to describe

the within-genus population structure that is distinct across geographic boundaries. Genera examined included *Terrimonas* (Fig. 6), *Ferruginibacter* (Fig. 7), *Haliscomenobacter, Chloroacidobacterium, Pirellula, Methylibium, Rhodoferax, and Flavobacterium* (Appendix A Figures 7-11). Berm samples contained a similar number of reads for the groups selected, making these genera good candidates for oligotyping since large differences in abundance could influence results from comparisons. Overall, the berm samples contained the same oligotypes within most of the taxa examined, but in some cases there were marked differences in the relative abundances and distributions of oligotypes that distinguished samples by state and also by beach (for Wisconsin beaches). ANOSIM indicated that differences in both *Flavobacterium* oligotype ($R = 0.937$) and *Chloroacidobacterium* oligotype ($R = 0.938$) distribution were highly associated with the state. The oligotype profiles within the other taxa indicated a moderate level of association to state ($R = 0.408$ to 0.533). The same analysis was performed on the entirety of the community, resulting in a much lower, yet significant, association with state ($R = 0.348$). *Terrimonas* oligotypes were correlated even more specifically to the particular beach ($R = 0.795$). Further analysis revealed that distribution and abundance patterns of *Terrimonas* (Fig. 6) and *Ferruginibacter* (Fig. 7) oligotypes were highly correlated with land use (envfit, $R^2 = 0.82$ and $R^2 = 0.84$, respectively), with several oligotypes present only in urban-impacted beaches.

Discussion

We examined microbial communities from eight freshwater beaches along the eastern and western coastlines of Lake Michigan to evaluate riverine and urban impacts, as manifested by increased nutrients and/or fecal pollution loading, and the relationship of these impacts to fecal indicator reservoirs in the sand. We were most interested in the microbial community that exists

within beach sands and how it might correspond to FIB reservoirs. Recent studies have shown that bacterial community structure often varies along nutrient and land use gradients (35–39). Our previous work on the Deepwater Horizon oil spill in the Gulf of Mexico demonstrated that environmental perturbation, in that case petroleum contamination, can alter the structure and function of microbial communities in beach sand, which we characterized using next-generation sequencing technologies (15). The relationship between water quality and land use types has also been well documented (40–44). Likewise, perturbations resulting from urbanization may also be reflected in sand communities. Boehm et al. showed that beach sand microbial communities within a marine coastline were more similar when under a similar level of anthropogenic stress, indicated by developed land use and FIB concentration (45). In addition, urban-impacted coastal and river waters generally have elevated nutrient levels which can persist over large distances, even over several hundred kilometers (46).

Since beach sands can be expected to capture some of the nutrient composition of the overlying waters, we hypothesized that the impact of urbanization would be observed in differences in nutrient concentrations in sand and at the microbial community level within beaches located in urban environments compared to beaches that are surrounded by other land uses. Interestingly, although beach water and sand exist in close spatial proximity, several studies have demonstrated a reasonably small amount of shared microbial community members and have observed significant differences in community evenness and diversity in these zones (15, 16, 42, 47). Sand may be a good indicator of long-term impact, whereas water samples are more rapidly influenced by currents and subsequent fluxes of nutrients and/or allochthonous bacterial inputs.

Water and sand FIB. Overall, the levels of fecal indicators were found to be higher in Wisconsin than in Michigan beaches. Water quality was correlated to proximity to a river mouth, a result that has been documented by others at freshwater beaches (43, 48–51). Sand samples collected within the berm zone were consistently found to have high levels of FIB compared to concentrations within water grab samples collected in the same transect. High levels of fecal indicators in sand have been reported in Lake Michigan beaches; Alm et al. (3) reported up to 38 and 17 times higher levels of *E. coli* and enterococci, respectively, in beach sand than in the nearby bathing waters. Some researchers have also suggested that a sand environment has the potential to accumulate and resuspend bacteria in the overlying water (52, 53). In the present study, the levels of *E. coli* found in berm sand and water samples were correlated, indicating that the two reservoirs may be linked.

Since berm sand and nearshore water are constantly in close contact and are likely exchanging both nutrients and bacteria, we hypothesized that the berm microbial community could be used as a sentinel for a chronically contaminated beach. However, we did not find that community structure corresponded directly with FIB reservoirs. This finding is supported by Piggot et al. (14), who reported no difference in microbial community structure with differing levels of enterococci. We found that community changes correlated with nutrient concentrations in berm sand. Nutrients are relatively more conserved than *E. coli* and enterococci, which can die off in short periods of time and be highly transient (50, 54–57). These findings support that nutrient concentrations and microbial communities are relatively stable over time and reflect longer-term conditions at a beach better than densities of fecal indicators.

Microbial communities across beach zone and geography. In the eight beaches in the present study, the backshore, berm, and water had distinct microbial communities, and each beach zone assemblage was highly similar across all beaches. The berm and submerged sand microbial communities could not be distinguished. Overall, microbial communities were strikingly different across short distances (10 to 20 m from berm to backshore at the same beach), and these differences were much greater than what was found in communities from the same beach zone, regardless of geographic location.

However, within a beach zone, we found that the most striking differences in the taxonomic composition of the microbial community were between the two states, across the lake from each other. There are several factors that may account for this effect. One possible explanation is that local (i.e., within a state) similarities in community composition may reflect bacterial biogeography, making the argument that microbial community assemblages at a site have been conserved in space and time. Another explanation is that within-state similarities in community structure could be explained by local environmental or physical conditions that correspond to each respective state. Both explanations are plausible; however, local environmental conditions are more likely to be the predominate driver of community structuring. Of the nutrients we measured, the primary drivers of community structure in freshwater berm sands were TOC and TP; when these were controlled for, geographic separation (i.e., Wisconsin or Michigan) could only account for a minute percentage of community variation (4.7%) in berm samples. The geospatial effect may be due to environmental conditions not measured in the current study, such as turbidity, salinity, micronutrients, other macronutrients, or differences in sand substrate.

We used SIMPER to examine the relationship of individual taxa to TOC and TP. We did not find that any single taxon showed a striking difference between beaches with high or low

nutrients but rather found a cumulative effect of 50 taxa accounted for the majority of the variation attributed to TOC and TP. No single taxon contributed > 2% to the variation. With such a large number of taxa showing a similar response to nutrients, it is possible that differences are not driven solely by these major nutrients but by a multitude of factors that influence differences in community structure. Some taxa that correlated with higher levels of TP and TOC in berm sand samples. *Flavobacterium*, *Rhodoferax*, and *Ferribacterium* were among the top eight most abundant taxa and were further analyzed for fine-scale differences at the nucleotide level.

Fine-scale population structure among beaches. The distributions of taxa in berm sand samples at all beaches were quite similar, suggesting that despite the different locales, the berm environment retains some level of community continuity across beaches. We used oligotyping to assess the diversity within berm samples that was not initially apparent via examination at the taxon level. The genera selected for oligotyping analysis (*Terrimonas, Ferruginibacter*, *Haliscomenobacter, Chloroacidobacterium, Pirellula, Methylibium, Rhodoferax, and Flavobacterium*) were highly abundant and were consistently present within berm samples collected at the eight beaches. Interestingly, Michigan oligotypes were fairly consistent across all beaches, which were located more closely to one another, while in Wisconsin, the two urban beaches and the two beaches to the north were separated by greater distances and demonstrated greater variation. We would expect differences in the relative abundances of specific genera in response to nutrients, as was noted in the association of taxonomic composition and nutrient levels at beaches, but genetic variants (represented by oligotypes) within a genus may not always respond differentially to nutrients. We observed state-specific oligotype patterns in all eight of the genera examined. Although nutrient levels could account for these results, it was noteworthy

that the effect was seen across all eight genera examined, which might suggest a biogeographic effect.

Alternatively, the genetic variants tracked by oligotypes may be a fine-scale reflection of a combination of environmental conditions at beaches. Oligotypes of the group *Terrimonas* (Fig. 6) revealed distinct patterns that were associated with the particular beach, compared to other analyzed groups. *Terrimonas* is a common freshwater bacterium and its presence has been reported in Lake Michigan sediment communities (58). The common occurrence of a similar collection of these oligotypes is not surprising, as freshwater beaches on Lake Michigan are subjected to similar large-scale influences (e.g., pH, temperature, etc.) that influence communities as a whole. It is striking, however, that such a small number of nucleotide differences (represented by unique oligotypes) in a single taxon (*Terrimonas*) can distinguish samples not only by state but by beach. Further, the distinctive oligotype patterns were consistent across the three sample days over summer. Because it would be difficult to identify beaches with identical environmental conditions that span geographic distances, we cannot disentangle biogeographic effects from environmental drivers of structure. Within Wisconsin, however, our sites spanned 85 miles of Lake Michigan coastline encompassing both urban and nonurban land use types, which allowed us to make inferences regarding the effect of urban impact. When we examined the oligotypes generated from the genera *Terrimonas* and *Ferruginibacter* in Wisconsin samples, we found strong correlations to urban-impacted sites. These differences in oligotype patterns indicate that surrounding land use may play a role in shaping the genetic makeup of certain microbial community members in freshwater sand. Since land use is associated with gradients of anthropogenic stress, our results suggest that oligotyping of key taxa within the microbial community could be used to monitor the effect of land use on freshwater

sediment ecosystems, such as the influence of increasing urban development on the health of beach ecosystems. Further work should therefore include the investigation of chemical and anthropogenic variability accompanying differences in oligotype distribution to better understand the effect of surrounding land use.

The present study highlights the complexity of environmental and anthropogenic factors that influence microbial community structure at freshwater beaches on both local and regional scales. Previous studies have shown that microbial communities respond to environmental stressors (15, 16, 36, 37, 59). We conducted a comprehensive study of freshwater beach microbial communities and provide insight into changes related to nutrients, land use, and evidence of fecal pollution. In general, beaches within urbanized areas had higher concentrations of nutrients in berm samples than beaches in agricultural or residential areas. In addition, beach proximity to a river mouth was correlated with increased fecal indicator concentrations in beach water. Oligotyping allowed us to examine fine-scale changes within major taxa and revealed site- and region-specific patterns. Taken together, the data suggest that microbial communities in freshwater beaches respond to both local and regional influences and that examining microbial community structure could provide insights into ecosystem disturbances and function.

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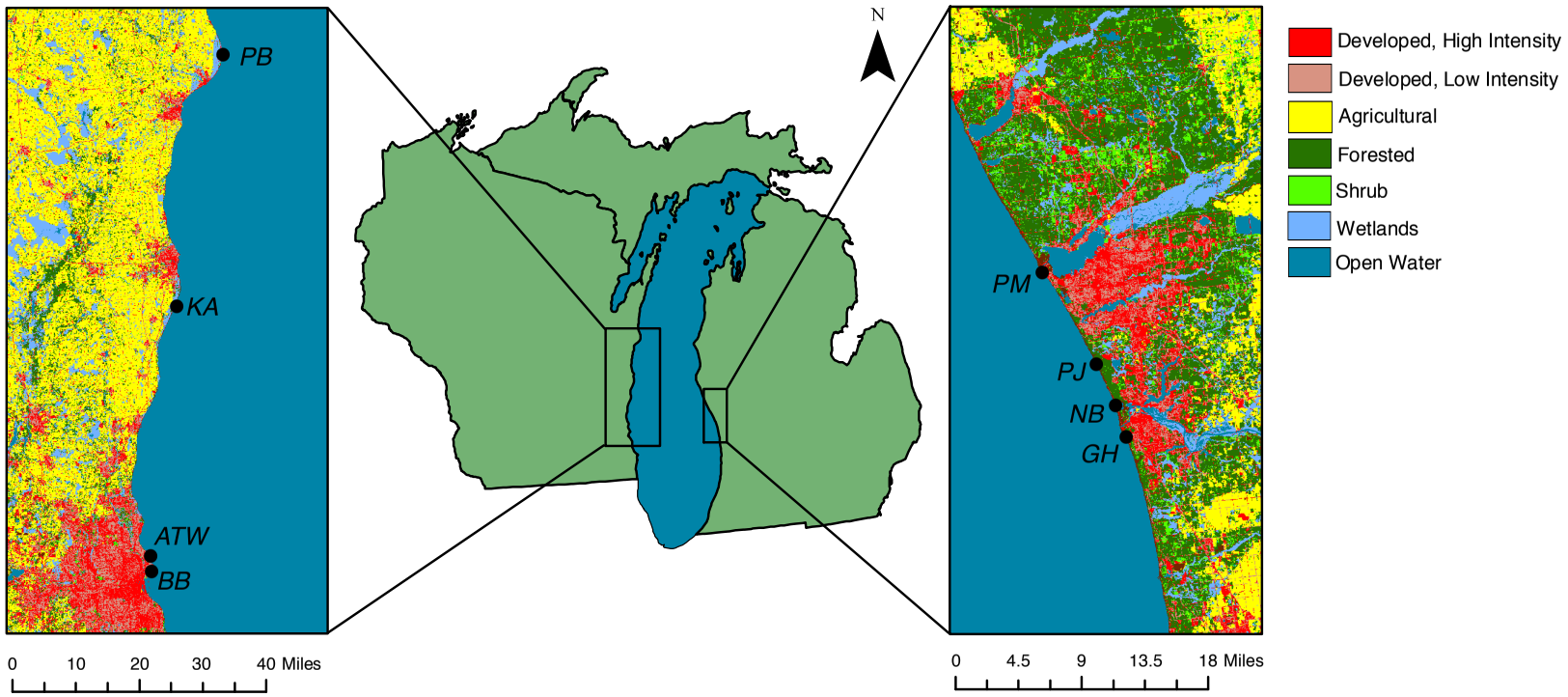

Figure 2.1. Map of sampling sites and surrounding land use. Wisconsin sites: Point Beach State Park (PB), Kohler-Andrae State Park (KA), Atwater Park Beach (ATW), and Bradford Beach (BB). Michigan sites: Pere Marquette Park Beach (PM), PJ Hoffmaster State Park (PJ), North Beach Park (NB), and Grand Haven City Beach (GH). (Map created using ArcMap 10.2.).

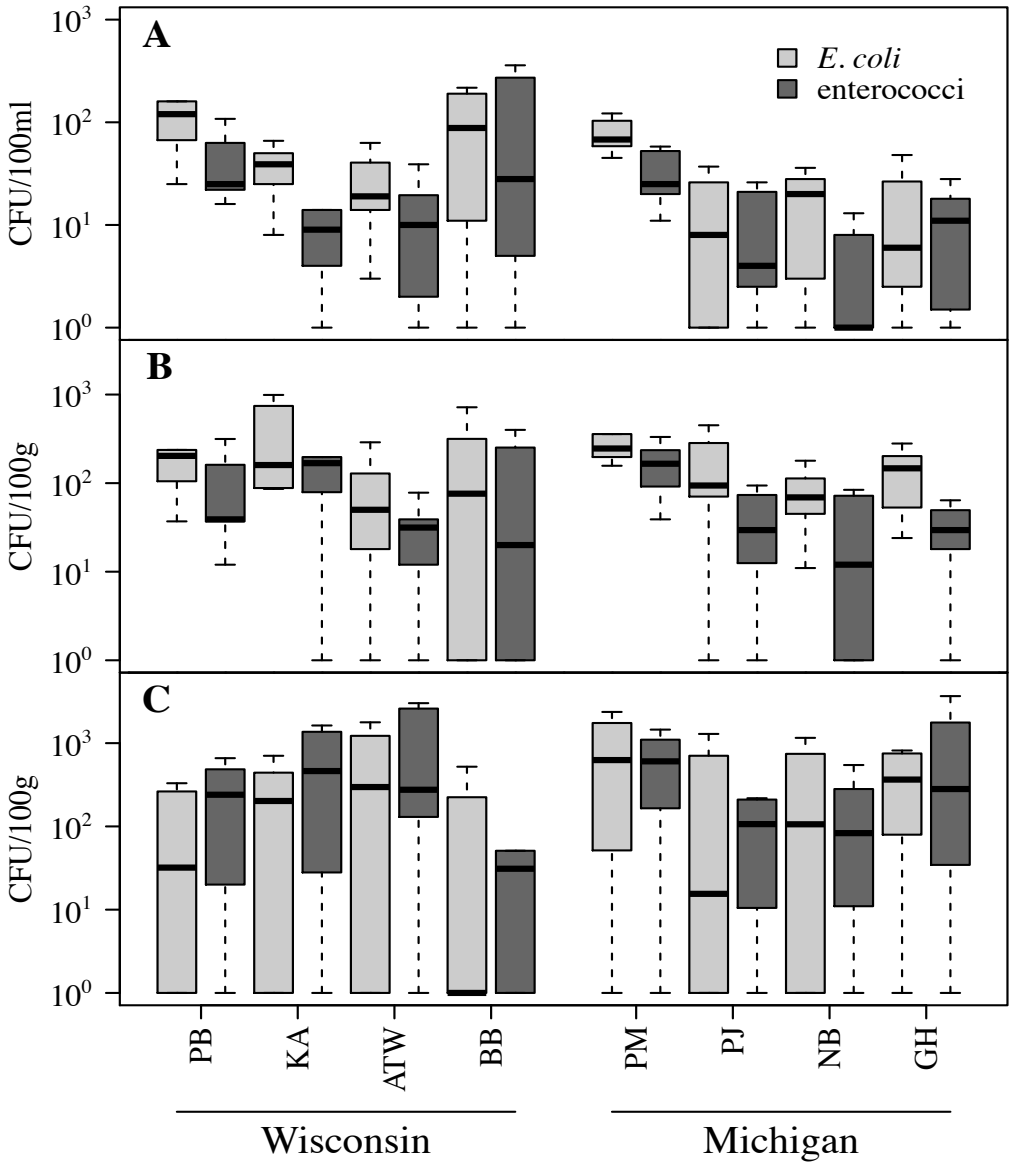

Figure 2.2. *E. coli* (shown in light gray) and enterococci (shown in dark gray) densities in water (A), berm sand (B), and backshore sand (C) samples collected throughout the summer of 2013. Both Wisconsin and Michigan beaches are ordered from north to south.

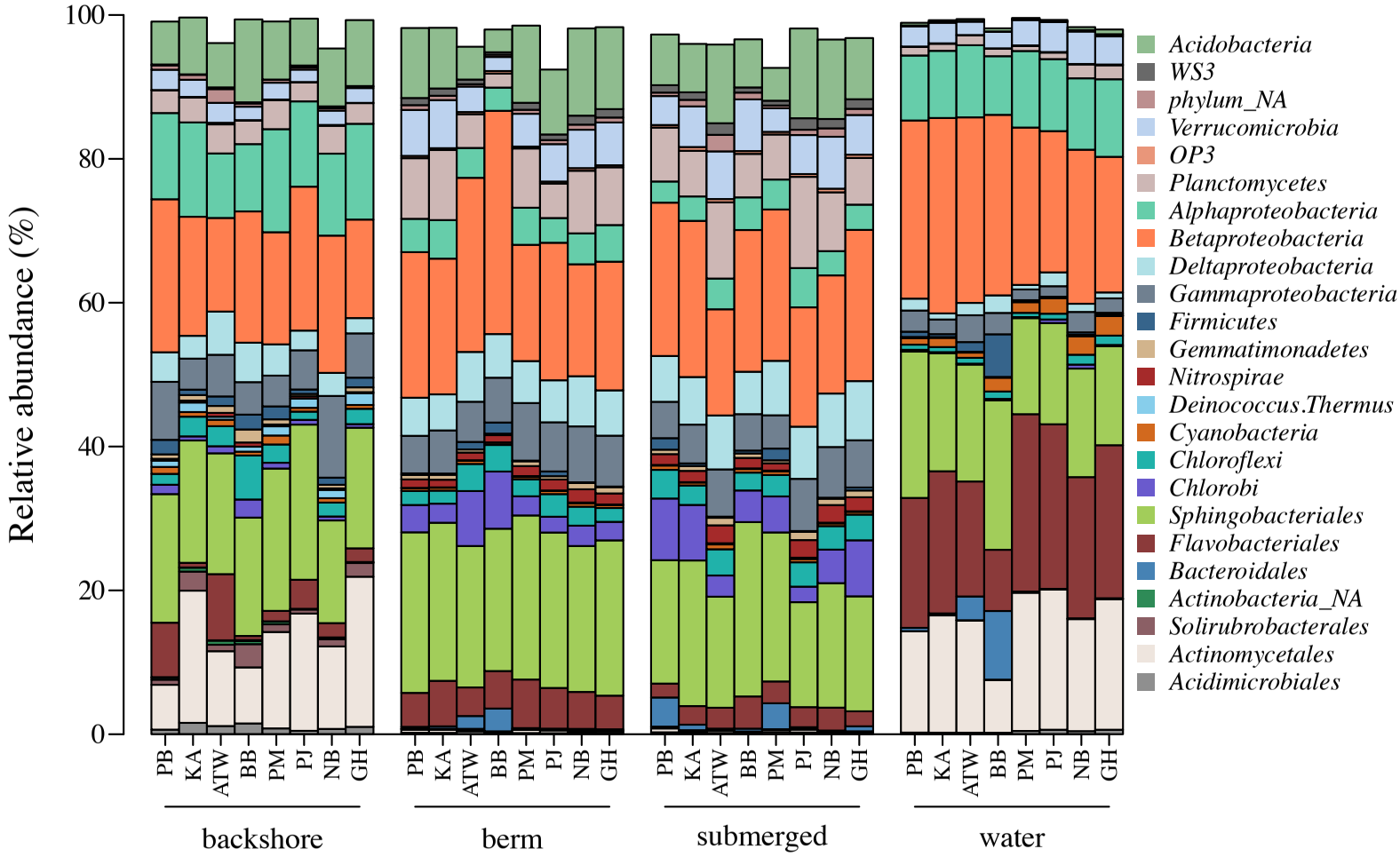

Figure 2.3. Relative abundances of dominant taxa in backshore sand, berm sand, submerged sand, and water samples. Bars represent the averaged relative abundances of taxa for three samples from each beach zone.

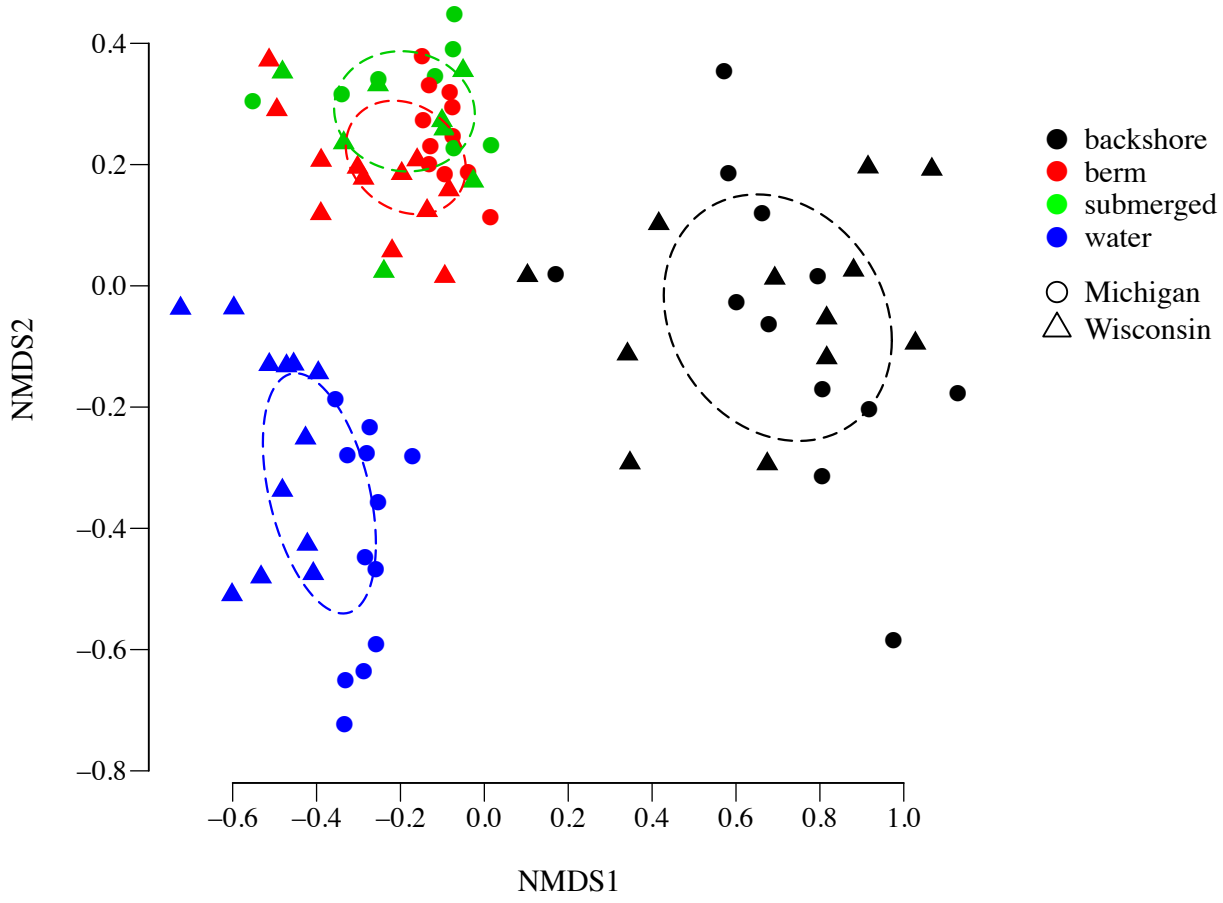

Figure 2.4. Nonmetric multidimensional scaling (NMDS) plot based on the Bray-Curtis distances of microbial communities from backshore sand, berm sand, submerged sand, and water. The relative abundances of all taxa were used for comparisons. The dashed colored lines surrounding each sample type represent covariance ellipsoids.

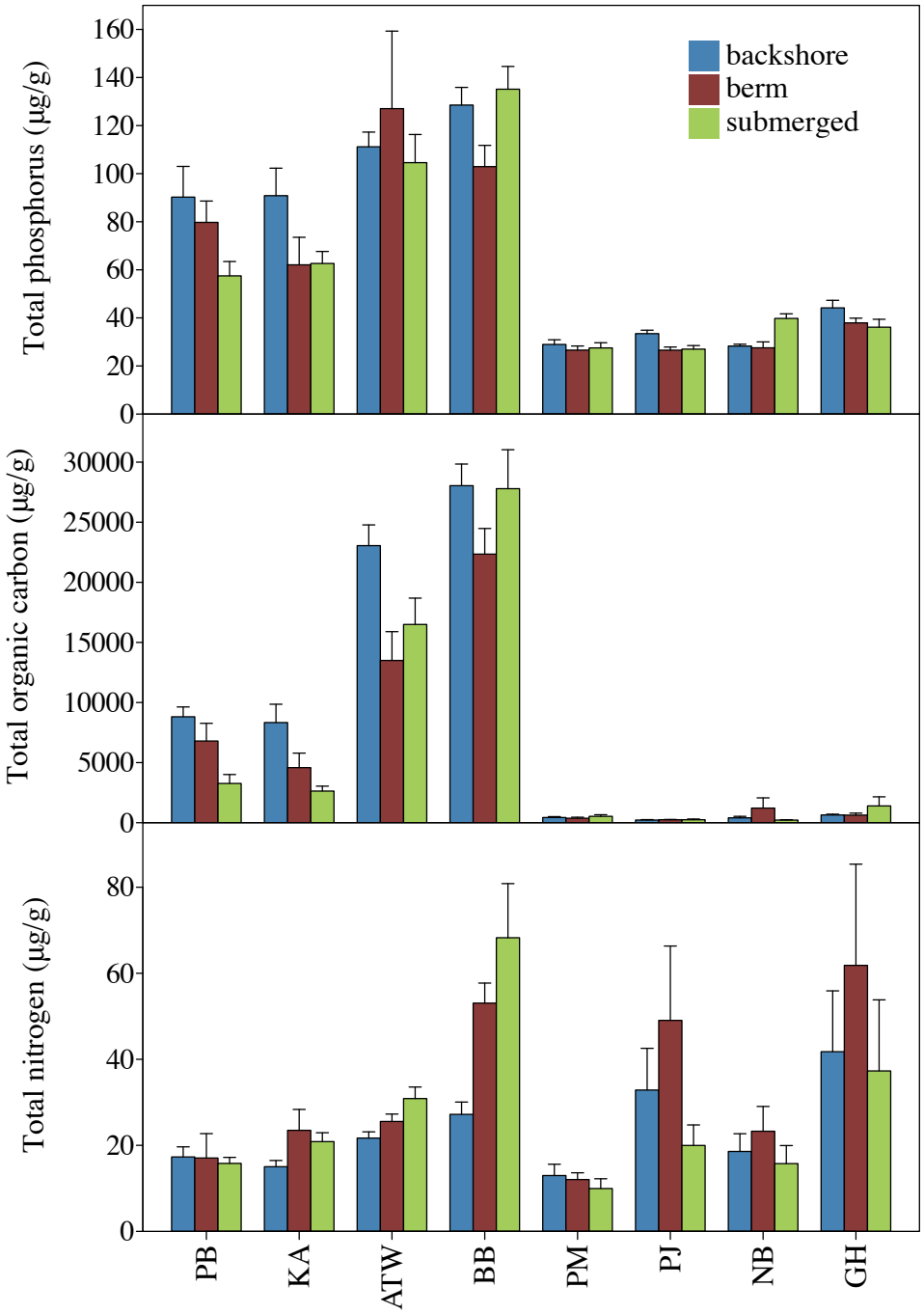

Figure 2.5. Total phosphorus, total organic carbon, and total nitrogen concentrations in backshore, berm, and submerged sands. The average concentrations from three samples taken at the same location at different time points are shown, and error bars represent the standard errors of measurements among all samples for a particular site.

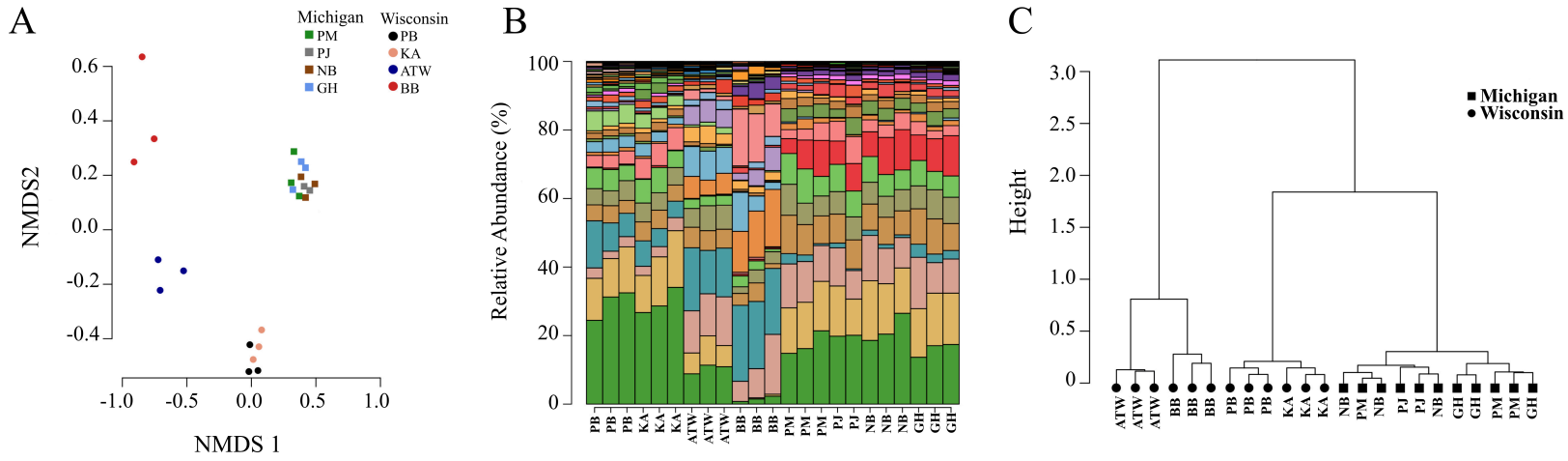

Figure 2.6. Oligotypes generated from the genus *Terrimonas* in all berm samples. (A) NMDS based on Bray-Curtis distance for *Terrimonas* oligotypes. (B) Oligotype relative abundance. Each color represents a unique oligotype. (C) Oligotype dendrogram, produced using a Bray-Curtis distance and Ward's method of linkage.

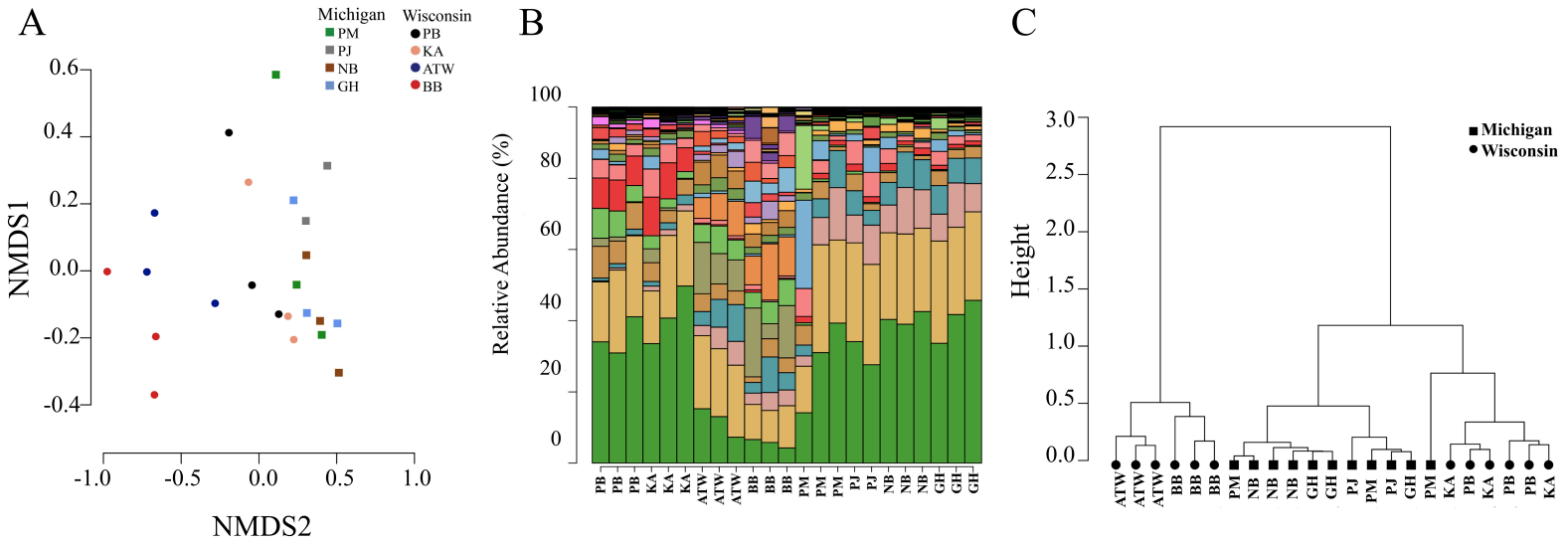

Figure 2.7. Oligotypes generated from the genus *Ferruginibacter* in all berm samples. (A) NMDS based on Bray-Curtis distance for *Ferruginibacter* oligotypes. (B) Oligotype relative abundances. Each color represents a unique oligotype. (C) Oligotype dendrogram, produced using Bray-Curtis distance and Ward's method of linkage.

Chapter 3

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

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.

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.

Introduction

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 enterococci, 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, in recent years it has been shown that enterococci and *E. coli* are less correlated with the presence of human pathogens in

environmental waters than previously (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 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.

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. 1). The beach site map was created using QGIS version 2.10.1 (39). 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. (40). 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 (41, 42). 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. (43).

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 (44).

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 Appendix B Table 2. The assays employed in this study targeted *E. coli* (45), *Enterococcus* spp. (Entero) (46), human-associated *Lachnospiraceae* (Lachno2) (26), human-associated *Bacteroides* (HB) (19, 44, 47), gull-associated *Catellicoccus marimammalium* (Gull2) (28, 48), and ruminant-associated *Bacteroidetes* (BacR) (22). Standard curves were created using six serial 1:10 dilutions from 1.5 X 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 Appendix B Table 3. All samples were run in duplicate 25 µl reaction mixtures containing 1X 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 sec, 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 Appendix B. 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. (49). 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 modified 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 (50). 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 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 mean 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.

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. 2). 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 X 10^2 to 3.2 X 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 X 10^3 CFU/100 g for berm sand, and 47 to 1.3 X 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 mean *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. 3. 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 to 6.6×10^5

copy number (CN)/100 ml and 1.0 X 10^4 to 4.3 X 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 X 10^2 to 9.2 X 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 mean concentration was 1.8 X 10^3 CFU/100 ml in water and 1.5 X 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 (Appendix B, Fig. 1). 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 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 Appendix B Fig. 2-5. 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 environ- mental 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 com- pared 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 mean concentrations (t = 0) of culturable *E. coli* and enterococci were 5.0 X 10^5 and 8.9 X 10^4 CFU/g, respectively. The initial mean concentrations of *E. coli*, enterococci, and Gull2 markers detected by qPCR were 2.4 X 10^4, 1.0 X 10^6, and 4.1 X 10^6 CN/g, respectively. The concentrations of Gull2, *E. coli*, and enterococci detected in the microcosms over time are shown in Fig. 4 and Appendix B Fig. 6. After 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 mean concentrations of *E. coli* and enterococci were 2.5 X 10^3 and 2.1 X 10^3 CFU/g, respectively, as measured by culture methods. The initial mean concentrations of *E. coli*, enterococci, HB, and Lachno2 were 5.3 X 10^3 CN/g, 1.5 X 10^5 CN/g, 4.7 X 10^4 CN/g, and 6.4 X 10^4 CN/g, respectively, as measured by qPCR. The concentrations of indicators measured over time are shown in Fig. 5 and Appendix B Fig. 7. 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 after inoculation. 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.

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 (51). 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, 52), 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 (53) and between 10^2 and 10^{10} CFU/g (54–56), 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 (Appendix B Fig. 1). The large range of *E. coli* and enterococci in gull feces has not been shown to vary by season, geography, or age of gull (54, 57); 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 (55). The same study showed that *Enterococcus* spp. and *Escherichia* spp. represent a smaller fraction of the population, at ~10% (55). Gull feces can also contain some human pathogens, such as *Campylobacter* and *Salmonella* (58). 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).

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. Storm- water outfalls have been reported to be frequently contaminated with sanitary sewage (59, 60) 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 *E. coli* and enterococci levels 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, 61). 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. 1), 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, 40, 62, 63). 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, 64); 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. (65). 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. 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, 45, 66), 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 mean 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 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 alter- native indicator assays, targeting *Bacteroides* (67, 68), 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 (69). 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 (70); however, testing with an established canine marker (71) 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 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 (72). 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.

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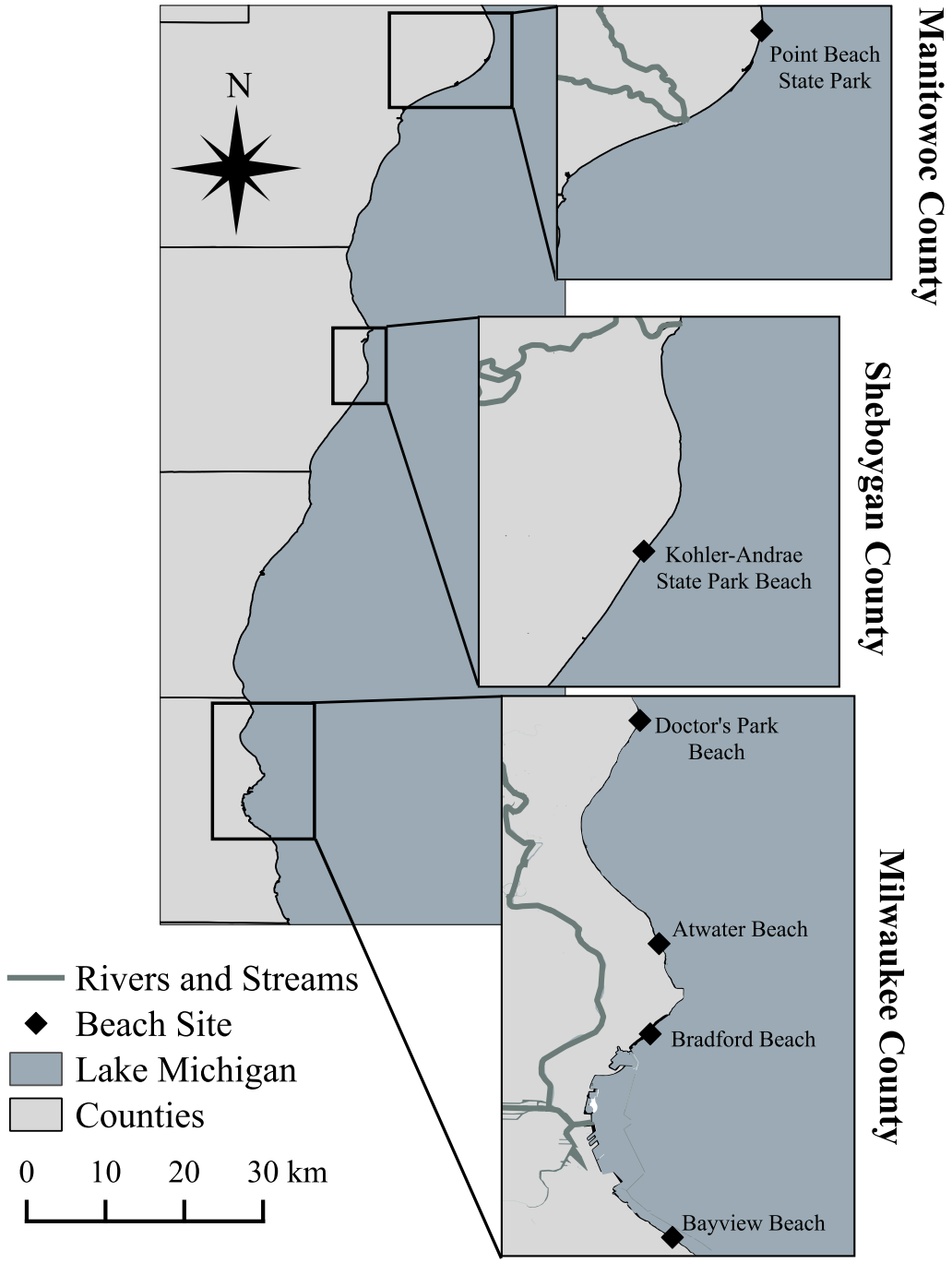

Figure 3.1. Beach sites. Manitowoc County: Point Beach State Park; Sheboygan County: Kohler-Andrea State Park Beach; Milwaukee County: Doctor's Park Beach, Atwater Beach, Bradford Beach, and Bayview Beach. (Map created using QGIS version 2.10.1-Pisa).

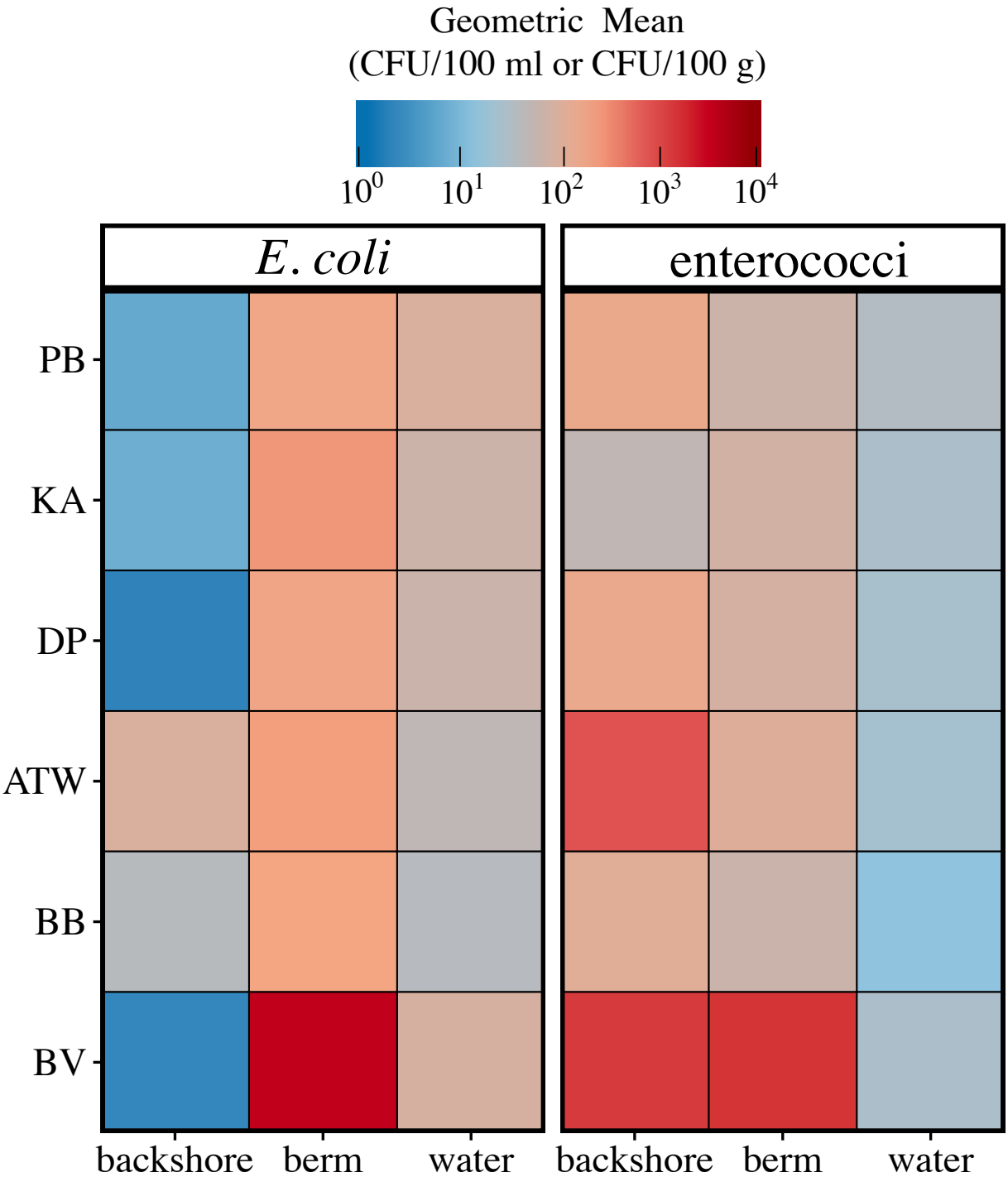

Figure 3.2. Heat map illustrating the geometric mean concentrations of *E. coli* and enterococci, as measured by membrane filtration, in sand and water samples collected at beaches during 2012 – 2013. Sand samples were compared to water samples on a weight to volume basis.

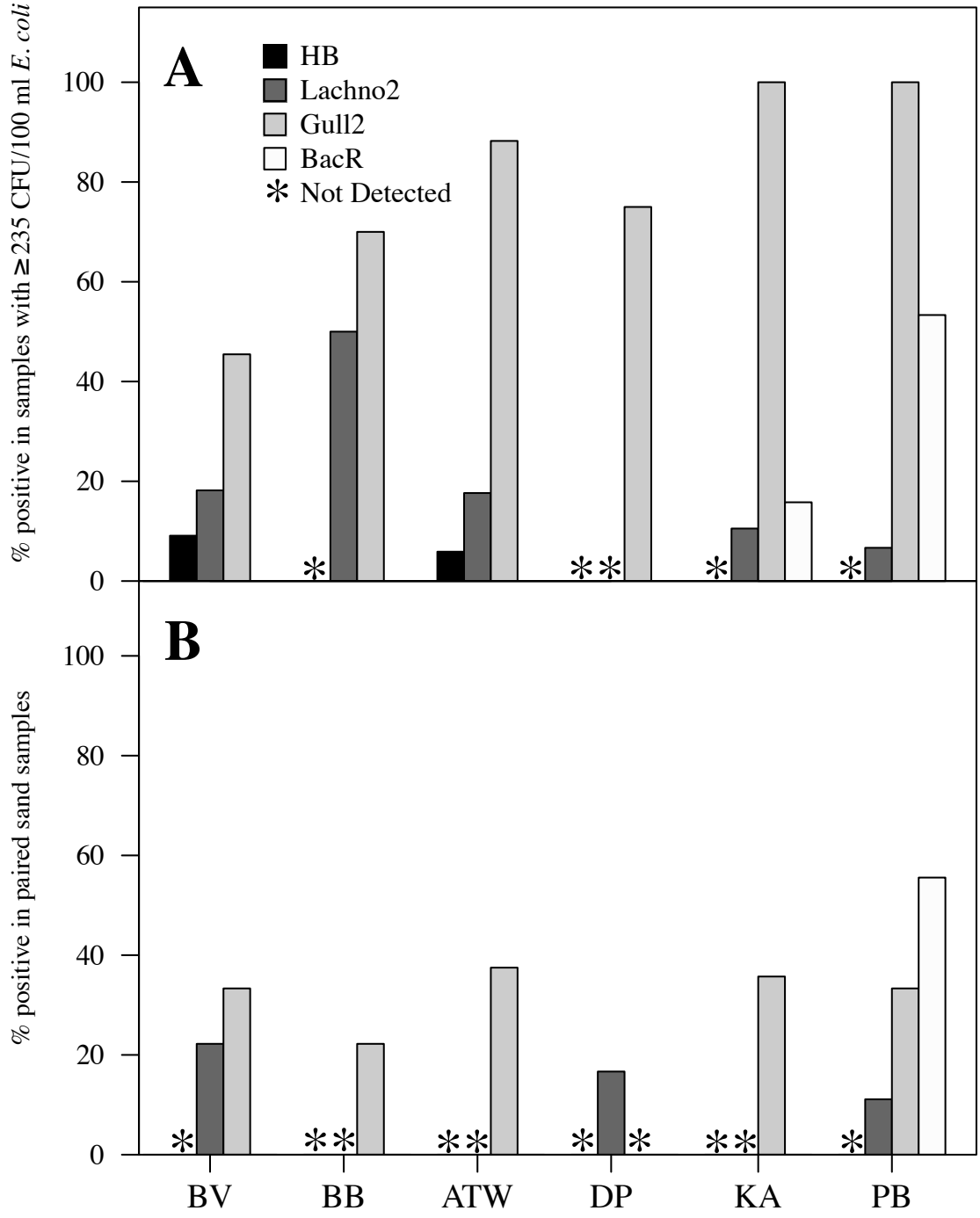

Figure 3.3. Alternative indicator detection frequencies measured during advisory conditions for (A) water samples ≥ 235 *E. coli*/ 100 ml (n = 84) and (B) sand samples paired by date and transect (n = 69) collected during 2012 - 2013. Water samples collected during CSO conditions were not included in this figure.

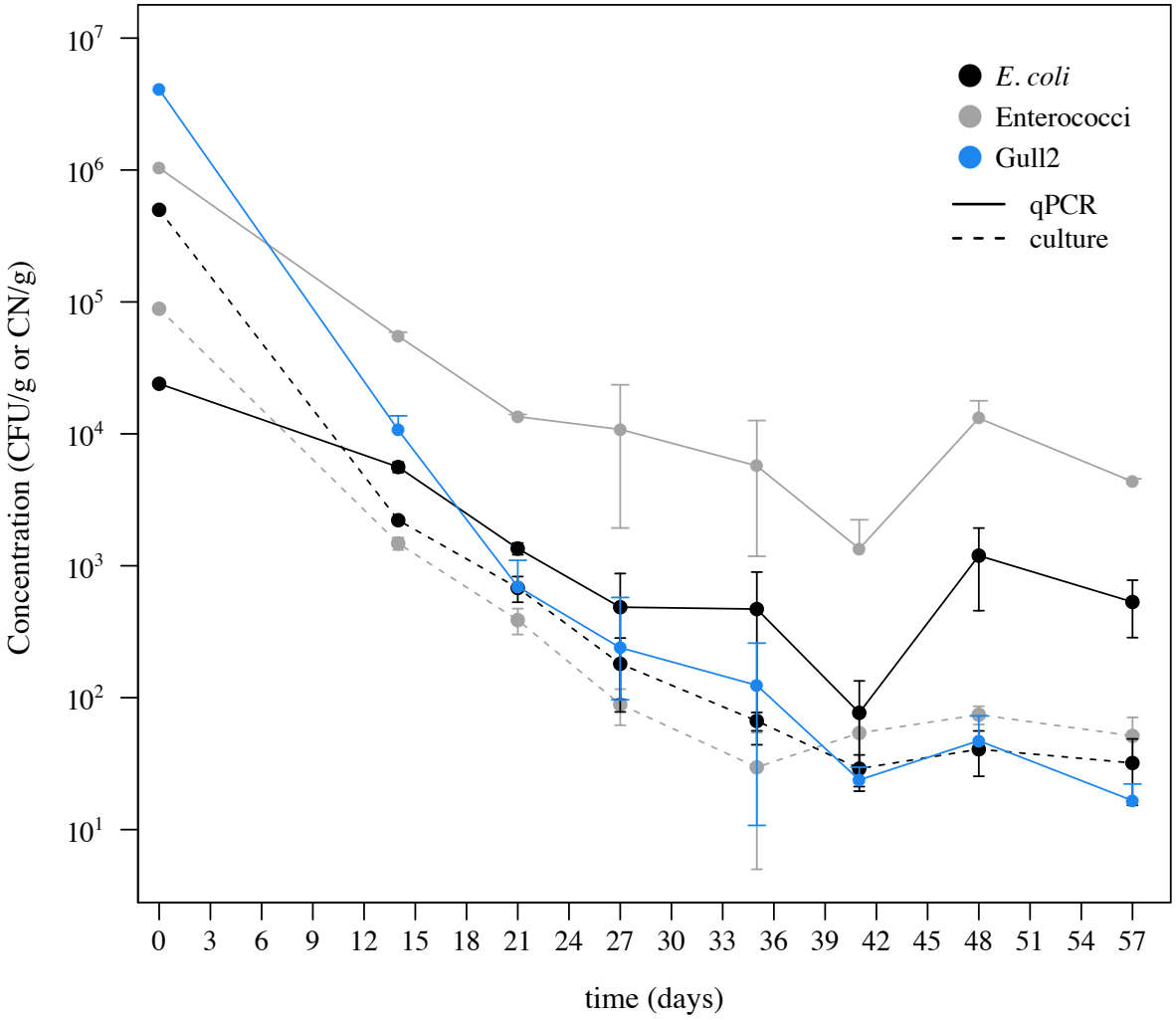

Figure 3.4. Concentrations of markers and fecal indicators measured over-time during the gull microcosm experiment. Circles represent mean concentrations for triplicate microcosms. Error bars indicate the standard deviation about the mean concentrations.

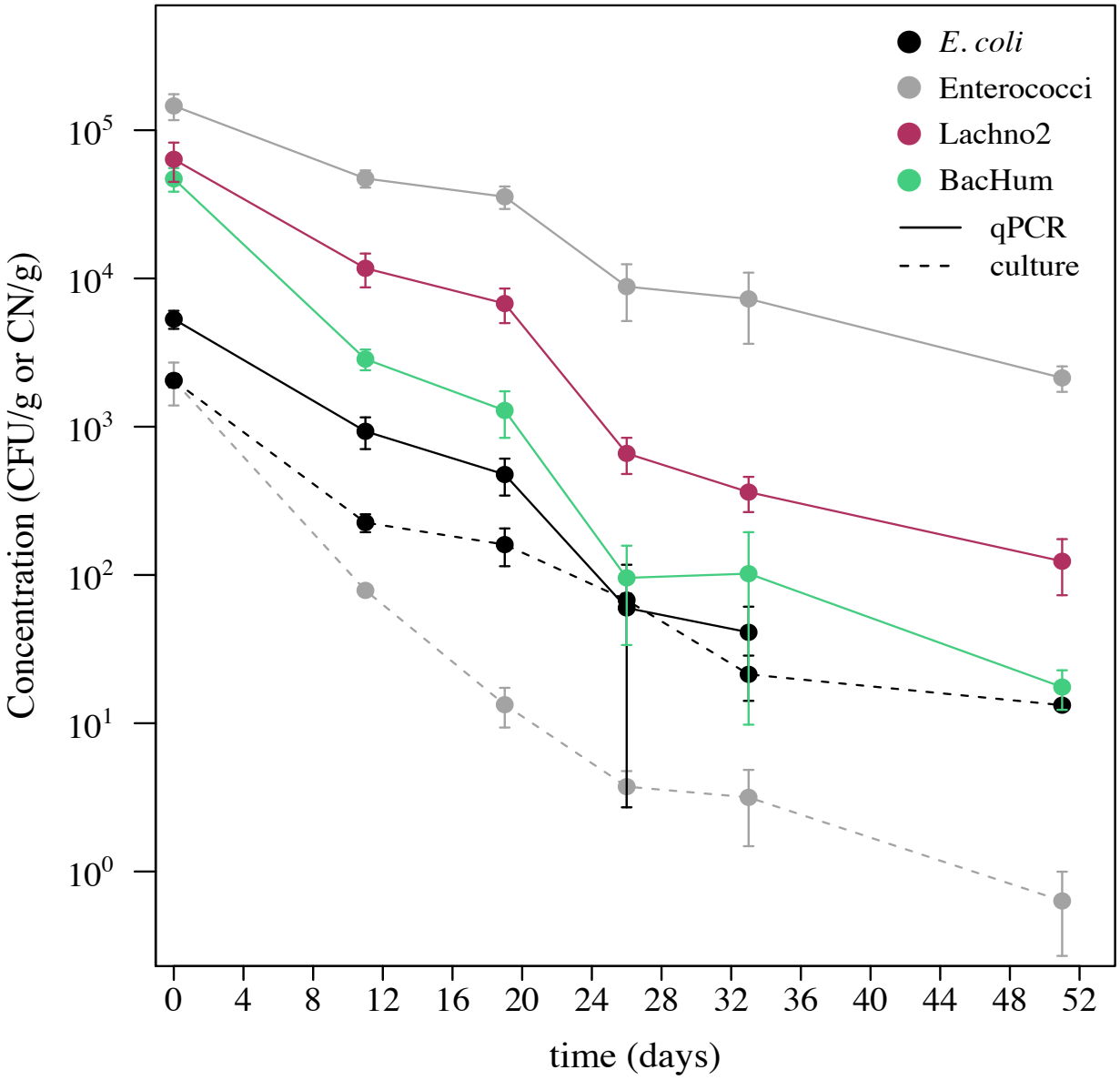

Figure 3.5. Concentrations of markers and fecal indicators measured over-time during the sewage microcosm experiment. Circles represent mean concentrations for triplicate microcosms. Error bars indicate the standard deviation about the mean concentrations. *E. coli*, as measured by qPCR, was analyzed at every time point and was detected but not quantified after t = 33 days post-inoculation.

Table 3.1. Mean concentrations and standard deviations of host-associated marker sequences detected in source material and environmental samples, and comparison to fecal indicator abundance.

				qPCR marker ratios (avg. ± SD)[c]	
Host-specific assay	**Sample Type[a]**	**No.[b]**	**Host marker concn (CN/g or 100 ml) (avg. ± SD)**	**Host marker per *E. coli***	**Host marker per *Enterococcus* spp.**
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* (≥ 235 CFU/100 ml) and low *E. coli* (< 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.

Table 3.2. First order day 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.

Chapter 4

Pangenome Comparisons of *E. coli* Isolates From Diverse Habitats

Introduction

Escherichia coli (*E. coli*) has been the preferred fecal indicator used for water quality monitoring and has been used in Wisconsin water for this purpose for 30 years (1). Recent studies, in addition to the work presented in this dissertation, have shown that *E. coli* can also survive naturally in the environment (2–6) and may be present in the absence of a known fecal source. The mechanisms by which *E. coli* are able to persist in beach sand remain elusive. Traditionally, *E. coli* has been thought to mainly inhabit the intestinal tract of humans and other animals. We now know that *E. coli* is a much more robust generalist capable of surviving in many environments. With the use of multilocus sequence typing (MLST) and whole genome sequencing, researchers have shown that many *E. coli* isolates recovered from the environment are related to but genetically distinct from their host-associated counterparts (7, 8).

Once outside the host, *E. coli* and other enteric bacteria are challenged with stressful environmental conditions such as large variations in temperature, pH, salinity, UV-radiation and nutrient levels (9, 10). Whole genome comparisons, together with physiological experiments, support the notion that environmental strains of *E. coli* may indeed survive in the external environment better than enteric *E. coli* (7). Although environmental *E. coli* strains are considered little or no threat to public health, it is essential to understand the population structure of these strains and their ability to resist environmental stressors due to their potential to confound water quality measurements. The complexity and fluctuations of conditions in the environment make it difficult to predict the survivability of *E. coli* strains in the natural environment. Although the relationship between *E. coli* genotypes and resulting persistence phenotypes is unclear, the present study aims to begin to shed light on this phenomenon.

Materials and Methods

Microcosm experiments. *E. coli* BB_Berm1 was isolated from Bradford Beach berm sand on 06-17-2013 using modified mTEC (membrane-thermotolerant *E. coli*) media. Incubation was performed according to USEPA Methods 1603 (11). A single well-isolated colony of BB_Berm1 was transferred to 50 ml of lysogeny broth and incubated at 37 °C with continuous shaking (200 rpm) overnight. The *E. coli* type strain ATCC® 11775™ (ATCC, Manassas, Virginia) that was originally isolated from human urine in 1941 but has been maintained in the laboratory for many decades was cultured similarly (12). Each culture was harvested at stationary phase (OD_{600} > 1.2). Harvested cells were pelleted via centrifugation at 10,000 g for 30 sec and washed two times with sterile PBS. Working cell suspensions were prepared using serial dilutions in sterile MilliQ water and were used as the inoculum for microcosm experiments.

Microcosms were constructed using PVC pipe material cut into 5 X 9 cm pieces. End caps for 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. Microcosm design was adapted from Alm et al. (3). Prior to microcosm use, the interior surfaces of the PVC pipes were sterilized using a 70% ethanol wash. Beach sand was collected at the berm from Bradford Beach. Inoculated sand was divided into prepared microcosms. The remaining inoculated sand was used for the triplicate t = 0 measurement. We conducted two separate yet identical microcosm experiments to assure reproducibility. The first microcosm experiment was conducted in August 2013 and microcosms were buried at a private beach north of Atwater Beach in Shorewood, WI. The second

microcosm experiment was conducted in July 2014 and microcosms were buried at Bayview Beach in St. Francis, WI.

For both experiments, three microcosms were sacrificed per sampling time point and the three microcosms were treated as triplicate measurements. At the time of sampling, the total content of each microcosm was transferred to a sterile Whirl Pak bag and fully homogenized. *E. coli* was isolated from sand microcosm samples using techniques adapted from those developed by Boehm et al. (13). To isolate *E. coli* cells from sand, 45 g microcosm sand was shaken in 450 ml sterile water for 2 min by hand. Sand extracts were filtered onto a 0.45 μm pore size nitrocellulose filter (Millipore®, Billerica, MA) and transferred to modified mTEC. Variable filter volumes or sample dilutions were used to attain colony counts within a target countable range of 10-300 CFU. For colony count data reporting, whole numbers were reported, otherwise concentrations were reported to two significant figures per Meyers and Sylvester 1997 (14). Sand moisture content was determined based on the mass difference before and after a 24 h drying period at 45° C. The arithmetic mean and standard deviation was calculated for each replicate time point in both microcosm experiments. Linear regressions were propagated using the first order exponential decay equation $\mathrm{Ln}(C / C_0) = kt$, 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.

Genomic sequencing, assembly, and annotation. A single well-isolated colony from *E. coli* BB_Berm1 was grown overnight in 10 ml lysogeny broth at 37 °C with shaking. Genomic DNA (gDNA) from *E. coli* BB_Berm1 was isolated following the standard cetryltrim-ethylammonium bromide (CTAB) isolation protocol for bacterial genomic DNA (15). MPure bead size-selected 20-kb libraries were constructed according to the Pacific Biosciences RSII protocol. PacBio

single-molecule real-time (SMRT) cells were loaded with Pacific Biosciences sequencing reagent 4.0, C4 chemistry, and P6 version 2 polymerase. Sequencing yielded a total of 18,459 reads with mean read length of 9.9 kbps, totaling 182,353,893 bps (≈ 400x coverage). Genome assembly was done using the PacBio PBcR HGAP 2.3.0 pipeline, with default settings (16). The final assembly consisted of a single contig 4,752,236 bp. The complete genome of *E. coli* ATCC® 11775™ is available through Genbank accession number AGSE00000000. *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™ genome fasta files were uploaded to the RAST server and de novo annotation was carried out using default RAST server settings (17). A total of 4,491 candidate protein-coding genes were predicted using RAST with a total G+C content of 50.7% for *E. coli* BB_Berm1.

Pangenome analysis. Pangenome analysis was carried out on 23 genomes, which included *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™. With the exception of *E. coli* BB_Berm1, all genomes were publically available on NCBI. Genomes included in pangenome analyses represent diverse habitats, including clinical, extra-intestinal (environmental), and laboratory environments. Detailed information and genome accession numbers can be found in Table 1. All genomes were annotated as described previously. The "get_homologues" program (18) was applied using the annotated Genbank files that were created via the RAST annotation. Get_homologues was used to identify clusters of orthologous sequences using the OrthoMCL clustering algorithm and default settings.

Genome phylotyping. *E. coli* phylotyping followed an *in silico* adaptation of the improved Clermont method for *E. coli* phylotype identification described in Clermont et al. (19). An *in*

silico PCR was performed on all genomes to determine phylotype designations, with the exception of genomes located within cryptic clades (CI, CIII, CIV, and CV) which had previously been reported (7). Primer pairs for *in silico* phylotyping can be found in Table 2. In short, a quadruplex PCR was initially performed that identified phylotypes A, B1, and B2. An additional two primer pairs were then used to differentiate between phylotype C, D, and E.

Results

Microcosm fitness experiments. As a preliminary assessment of environmental *E. coli* differential survival, *in situ* microcosms were used to compare the survival of *E. coli* BB_Berm1, an environmental *E. coli* isolate collected from Bradford Beach in Milwaukee, WI, to the *E. coli* type strain ATCC® 11775™. *E. coli* ATCC® 11775™ was originally isolated from human urine but has been maintained in the laboratory for decades. Two separate microcosm experiments were conducted to test the validity of findings and also to determine whether local forces affect observed survival dynamics of the two strains. Data from the first microcosm experiment, conducted in summer of 2013 at a private beach north of Atwater Beach, is shown in Figure 1. Data from the second microcosm experiment, conducted in Summer 2014 at Bayview Beach, is shown in Figure 2. Linear regression analysis was carried out using the first-order model of decay for *E. coli* culturable counts over the duration of the experiments (Table 3).

Both isolates remained detectable throughout the experiments. In both experiments, *E. coli* BB_Berm1 showed an increased survival compared to *E. coli* ATCC® 11775™. The decay constants for *E. coli* BB_Berm1 were significantly smaller than for *E. coli* ATCC® 11775™ ($P < 0.01$), indicating that *E. coli* BB_Berm1 die-off was lower than for *E. coli* ATCC® 11775™. Although the experiments were not carried out to *E. coli* extinction, the findings from these two experiments shed light on the survival of *E. coli* outside its "primary habitat" (i.e. animal GI

tract).

Lab and environmental strain comparative genomic analyses. To explore possible genotypic traits that support the survival phenotype of *E. coli* BB_Berm1 observed in the two microcosm experiments, we harnessed the power of Pacific Biosciences RSII to sequence the whole genome of *E. coli* BB_Berm1. After sequencing and assembly, the RAST NMPDR, SEED-based, prokaryotic annotation tool was used for ORF calling and annotation. We also used RAST NMPDR to annotate the previously sequenced genome of *E. coli* ATCC® 11775™.

A total of 4491 coding and 110 RNA sequences were found in *E. coli* BB_Berm1, while 4929 and 105 were harbored in *E. coli* ATCC® 11775™. Nucleotide and amino acid sequences were used as the get_homologues input for whole genome comparisons between *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™. Genomic comparisons were carried out using the OrthoMCL algorithm available in the get_homologues software package, imposing minimum alignment coverage of 75% and a maximum e-value of 1e−05. A total of 5433 unique orthologous clusters were detected using get_homologues, with 3689 (68%) found in both genomes, which corresponds to the core genome of the *E. coli* strains analyzed in this study.

The RAST NMPDR, SEED-based, prokaryotic annotation tool was used to assign protein-encoding sequences to functional categories called "*subsystems*". RAST annotation technology involves an expert curator defined "*subsystem"* that is comprised of proteins required to perform the function of the subsystem (e.g. peptidoglycan biosynthesis). RAST NMPDR calculated a total of 617 subsystems between the two genomes and found 129 subsystems unique to *E. coli* BB_Berm1 and 196 to *E. coli* ATCC® 11775™. The shared subsystems (n = 3919) included genes related to essential metabolic functions, such as energy metabolism, cellular division,

DNA replication, transcription, and protein synthesis. The 129 unique gene functions (Table 4) found only in *E. coli* BB_Berm1 comprised 60 different subsystems (Figure 3), while *E. coli* ATCC® 11775™ had 196 unique gene functions comprising 70 subsystems.

E. coli pangenome structure. *E. coli* genomes used in the comparative pangenome analyses are found in Table 1. Of the 23 genomes included in these analyses, 12 are considered "environmental" *E. coli* strains representing natural, extra-intestinal habitats including lake freshwater lake, beach sand, creek water, marine water, and soil. The remaining 11 genomes are considered "host" *E. coli* strains and the majority of the host-associated *E. coli* were isolated directly from fecal samples. Host-associated *E. coli* cover a diversity of animal sources including feline, canine, human, cattle, raccoon, and swine.

Pangenome analysis of the 23 genomes was carried out using the open-source program get_homologues. To determine the global gene repertoire of the 23 *E. coli* genomes (pangenome) the number of new genes added by each genomic sequence is estimated by get_homologues. The pangenome curve produced by get_homologues (not shown) suggests an open nature of the *E. coli* pangenome because the curve does not reach a plateau. The pangenome is broken down into cloud (genes shared by ≤ 2 genomes), shell-genome (genes shared by 3-20 genomes), soft-core (genes shared by ≥ 21 genomes), and core-genome (genes shared by all 23 genomes). The pangenome structure of the 23 genomes is shown in Figure 4 and Figure 5.

Ecotype pangenome structure was assessed by separating the cloud, shell-genome, soft core, and core genomes by "environmental" genomes and "host" *E. coli*. The environmental genomes were found to have the following pangenome classification: 5959 clusters (cloud, ≤ 2 genomes), 1904 clusters (shell-genome, 3-9 genomes), 3300 clusters (soft-core, ≥ 11 genomes),

and 2601 clusters (core-genome, 12 genomes). The host-associated genomes were found to have the following pangenome classification: 5723 clusters (cloud, ≤ 2 genomes), 1814 clusters (shell-genome, 3-9 genomes), 3586 clusters (soft-core, ≥ 10 genomes), and 2972 clusters (core-genome, 11 genomes). The *uidA* gene, which encodes beta-glucuronidase, was used to create the phylogenetic reconstruction shown in Figure 6. Also shown in Figure 6 are the phylotype results from the *in silico* Clermont analyses designated by the alphabetic letter-code (19). From the phylogenetic tree reconstruction alone, there does not appear to be a clear distinction among *E. coli* genomes from similar habitats (e.g. host genomes). Although there does not appear to be a strong correlation between phylogeny and ecotype within the 23 *E. coli* genomes, the *in silico* Clermont results are consistent with the *E. coli* lineages shown in the *uidA* phylogenetic tree.

Discussion

The comparative survival microcosm experiments of *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™ shed light on the increased survival phenotype of environmentally isolated strains of *E. coli* alluded to in the literature. The comparative survival and genomic analyses conducted on *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™, although preliminary, represent novel research and present insight into genotypic traits that may support the increased survival of *E. coli* BB_Berm1. The RAST annotation comparison of *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™ resulted in a total of 129 unique gene functions found in *E. coli* BB_Berm1 but absent in *E. coli* ATCC® 11775™ (Table 4). While not all genes listed in Table 4 may be related to the survival phenotype, there is a cohort of genes, namely those related to cellular regulation and signaling pathways, that should be explored in future studies due to their known association with survival and persistence.

The first cohort of genes specific to *E. coli* BB_Berm1 are genes involved with autoinducer 2 (AI-2) transport/processing. The operon responsible for AI-2 transport/processing is the *lsrACDBFGE* operon. *E. coli* BB_Berm1 harbors all genes within the *lsrACDBFGE* operon as well as genes required for operon regulation. The RAST annotation portal has also indicated that the *lsrACDBFGE* operon is putatively functional in *E. coli* BB_Berm1. *E. coli* BB_Berm1 also harbors the two genes required for regulating AI-2 uptake; *lsrR*, which encodes the transcriptional repressor of *lsr* operon, and *lsrK*, the gene encoding the AI-2 kinase. AI-2, produced by LuxS, also found in *E. coli* BB_Berm1, is a signaling molecule used in *E. coli* quorum sensing and thought to also be involved in interspecies communication (Appendix C, Figure 1). *E. coli* populations use quorum sensing as a means of population-density-sensing via the production and uptake of small signaling compounds, such as AI-2, that are secreted into the environment (20).

AI-2-mediated quorum sensing is widely distributed in both gram negative and positive bacterial species. Bioluminescence of *Vibrio harveyi* was the first reported bacterial function controlled by AI-2-mediated quorum sensing (21). Facilitated by quorum sensing, the formation of bacterial biofilms has been shown to support the persistence of microorganisms. Biofilms protect their inhabitants from inhospitable environmental conditions including oxidative stress, desiccation, nutrient starvation, and grazing by other organisms. Biofilm formation is complex and involves the production and reception of quorum sensing signal chemicals, such as AI-2 (22). Increasing concentrations of AI-2 have been shown experimentally to stimulate biofilm formation in *E. coli* and other species (23).

The presence of the *lsrACDBFGE* operon and regulatory genes in *E. coli* BB_Berm1 is notable as these genes function to promote environmental survival via biofilm formation.

Although the presence of biofilm was not tested in the present study, the ATCC® 11775™ type strain has been shown to produce biofilm in controlled laboratory experiments using a CDC Biofilm Reactor® (24, 25). The absence of these genes in *E. coli* ATCC® 11775™ could suggest a reduced ability to produce biofilm by this strain in non-laboratory settings. Identification of the specific pathway responsible for a biofilm forming phenotype may be difficult, because *E. coli* can have several different quorum-sensing pathways (26). Nevertheless, future research should assess the *in situ* production of biofilm, because differential biofilm formation could be an important factor in the differential survival of *E. coli* BB_Berm1 and *E. coli* ATCC® 11775™.

The second group of genes harbored by *E. coli* BB_Berm1 that merit further investigation are genes involved in toxin-antitoxin (TA) systems and programmed cell death. TA systems encode for a toxic protein that will attack an essential cellular process unless its antitoxin protein is also present; thus, TA systems are responsible for self-modulating cellular growth and metabolism. As many as 33 TA systems have been identified in *E. coli* K12 (27). Both *E. coli* BB_Berm1 and ATCC® 11775™ have TA genes that represent complete or partially complete TA systems. A *mazEF* family TA system, found only in *E. coli* BB_Berm1, is represented by toxin-ChpB and downstream antitoxin-ChpS. In laboratory studies, over-expression of *chpB*, as well as other *mazEF* family toxin genes, inhibits translation globally, but has no effect on DNA or RNA synthesis (28).

The regulation of cellular processes is essential to the survival of microorganisms under stressful environmental conditions. Research has suggested that TA systems mediating programmed cell death, biofilm formation, and environmental persistence are interrelated. Within a bacterial biofilm, programmed cell death can support the survival of bacterial subpopulations by the releasing cellular components of killed cells, thereby providing nutrients

to the surviving population. In *E. coli*, the TA family with the most experimental evidence of a relationship linked to programmed cell death is the *mazEF* family of TA systems (29).

Another compelling future direction of this work is the study of TA systems and their modulation of persister cell formation. Bacterial cells that survive otherwise death-inducing conditions because of dormancy rather than resistance are called persisters (30). Functionally active TA systems cause growth stasis and thus their relationship to bacterial persistence has been considered. Maisonneuve et al. showed that the over-expression of 5 different mRNAase TA systems, including *mazEF* family toxins, resulted in higher fractions of persisters to both ciprofloxacin and ampicillin antibiotic treatments (31). Due to the large variation in TA system genes within different *E. coli* genomes, it has been suggested that TA system loci are lost/gained over a short time scale within the species (32). Overall, TA system regulation of *E. coli* survival in the environment is an interesting phenomenon that should be explored further. However, careful attention should be exercised when attempting to isolate the effect of any one TA system due to the functional redundancy of chromosomal TA loci in *E. coli.*

Our understanding of *E. coli* comparative genomics has been biased due to the over representation of pathogenic and commensal genomes reported in the literature and available in NCBI. There have been a limited number of genomic studies performed on environmentally isolated *E. coli* strains. The majority of environmental *E. coli* genomic research has focused on studying environmental members of cryptic clades (7, 8, 33). Although cryptic lineages of *E. coli* have been shown to predominately harbor environmental organisms, cryptic clade *E. coli* do not appear to be common in aquatic habitats (34).

The phylotype analyses in the present study identified 5 Clermont-defined *E. coli* phylogroups, in addition to 4 cryptic clades previously reported (7). Environmental and host-

associated *E. coli* genomes were distributed across the 5 *E. coli* phylogroups (Figure 6) and there did not appear to be a distinct ecotype-specific phylogenetic pattern. Although the *uidA* phylogentic tree appears to be consistent with the Clermont phylotype profiles of the 23 genomes, further comparative genomic analyses should be focused on identifying ecotype-specific gene patterns (i.e. gene presence/absence).

The driving hypothesis of this study was that *E. coli* isolated from similar habitats would be phylogenetically distinct from *E. coli* isolated from fundamentally different habitats (host-associated versus environmental), however this did not appear to be supported by the genomes included in the study. Although the homogeneity of *E. coli* isolates among the *uidA* phylogenetic lineages was not expected, the phylogroup association may be a promising direction to explore in the future. Phylogroup B1 has been shown to be the dominant lineage for *E. coli* isolated from environmental waters (35, 36). Although the membership of the B1 phylogroup is dominated by isolates from the environment, host-associated isolates have been found within the B1 phylogroup (Figure 6). It stands to reason that members of B1 may be environmentally adapted organisms capable of increased survival under harsh environmental conditions. *E. coli* BB_Berm1 (B1 phylogroup) survived longer than *E. coli* ATCC® 11775™ (B2 phylogroup) in the *in situ* microcosm experiments, which may suggest a phylogroup-associated survival phenotype warranting further investigation.

In conclusion, the ability for certain strains of *E. coli* to persist in the environment is concerning due to their potential to confound water-monitoring results. The present study provided evidence for the heightened survival of an environmental *E. coli* isolate compared to a non-environmental strain. Whole genome sequencing of the two *E. coli* genomes provided

insight into genotypic differences that may support the *in situ* differential survival observed experimentally.

Future Directions

The work presented in this Chapter represents a preliminary assessment of environmental *E. coli* survival and provides insight into potentially important survival mechanisms. It is unknown if enhanced survival/persistence is a general characteristic of environmental *E. coli* strains, since the survival experiments included only one environmental *E. coli* strain (BB_Berm1)**.** Future research directions and ideas about specific experiments are highlighted in this section; however**,** a major focus of future research endeavors should expand these analyses to other ecologically similar strains (Table 1).

Based on previous studies highlighting the importance of biofilm formation in bacterial persistence coupled with the genomic identification of the AI-2 genes, it stands to reason that biofilm formation maybe an important mechanism for enhanced survival of *E. coli* BB_Berm1 compared to *E. coli* ATCC® 11775™. It would be inappropriate to assume that the absence of the AI-2 genes in *E. coli* ATCC® 11775™ indicates that the strain is unable to produce biofilm; in fact, ATCC® 11775™ has actually been shown to produce biofilm in laboratory bioreactor studies (24). The quorum sensing pathway used by *E. coli* ATCC® 11775™ for biofilm formation may be less suited for environmental conditions compared to *E. coli* BB_Berm1; thus it is possible that the *in situ* production of biofilm differs between these two strains.

To test for differential biofilm production between the two strains, the *in situ* microcosm experiment should be repeated while including an additional analysis for the quantification of EPS (Extracellular Polymeric Substances) in microcosm sand. The outlined EPS protocol has been adapted from previously published studies (37, 38), with a similar method adaptation

reported by Piggot et al. (39). However, the method has not yet been evaluated for suitability for EPS detection in microcosm experiments. All glassware used in the outlined analysis should be acid-washed (10% HCl), then rinsed five times in deionized water and air-dried.

1. Retrieve *in situ* microcosms and place immediately on ice during transport to the laboratory.
2. Empty microcosm contents into sterile Whirl Pak bags. Process microcosm sand for microbiology and DNA (as previously described) and place remaining sand on ice.
3. Using a sterile spatula, weigh 3 g sand into a sterile weigh boat. Record the exact mass to the hundredths place. Dispense weighed sand into a 15 ml glass conical vial. Repeat this step subsampling each microcosm 3x.
4. Add 3 ml 0.5 mM ethylenediamine tetra-acetic acid (EDTA) to the sand and invert vials to mix.
5. Place vials in a tube rack and transfer to a 40 °C water bath for a 15 min incubation. Remove and invert rack every 5 min to mix. This step will solubilize the EPS. If possible perform this step in a dark or dimly lit room.
6. Centrifuge vials at 8,000 g for 6 min. Using Pasteur pipettes, transfer supernatants to a larger glass conical vial. Pool supernatants from the 3 subsamples. Do not dispose of remaining sand pellets.
7. Add cold (-20 °C) ethanol to the pooled supernatant to a final concentration of 70% ethanol and invert vials to mix. Store vials overnight at -20 °C to precipitate the EPS.

*Repeat steps 4-7 for a total of 3 extractions/subsample. Previous studies have shown that performing a total of 3 extractions is sufficient for collecting residual EPS

8. Pool subsample fractions. Pellet the precipitated EPS via centrifugation (8,000 g for 6 min) and resuspend in sterile deionized water (record volume).

9. Perform EPS quantification via the phenol-sulfuric acid method (39).

In addition to comparing the concentrations of EPS produced between BB_Berm1 and *E. coli* ATCC® 11775™, it would also be interesting to compare the concentrations of EPS across beach sand samples with varying concentrations of *E. coli*.

The importance of toxin-antitoxin systems in programmed cell death has been documented. Overall, toxin-antitoxin systems have diverse mechanisms and cellular targets to modulate bacterial physiology under stressful environmental conditions. As mentioned previously, both *E. coli* BB_Berm1 and ATCC® 11775™ harbor toxin-antitoxin systems (both partial and complete). Studying the importance of the partial toxin-antitoxin systems may be challenging because neutralization of toxins by non-cognate antitoxins (i.e. antitoxin from of a different toxin-antitoxin system) has been reported (40, 41). It would however be interesting to determine whether the ChpB/ChpS toxin-antitoxin system, found only in BB_Berm1, is an important mechanism supporting the differential survival observed in the *in situ* microcosm experiments.

Many possible methods could be harnessed to assess the *in situ* importance of the ChpB/ChpS system for the survival of *E. coli* at the beach. One possible way to explore the link between ChpB/ChpS and environmental *E. coli* survival would be with the use of wildtype *E. coli* MG1655 and isogenic Δ*chpB* (*E. coli* MG1655 harboring a mutated *chpB* gene). A collection of 3985 single-gene knockout mutants, including an *E. coli* MG1655 Δ*chpB* mutant, were previously been developed as part of the "Keio collection" of *E. coli* chromosomal mutants (42). Members of the Keio collection project were unable to produce an *E. coli* MG1655 Δ*chpS* mutant because in the absence of *chpS, chpB* results in *E. coli* death (42). Comparative survival experiments of the wildtype *E. coli* MG1655 and Δ*chpB* mutant may provide preliminary evidence of the importance of the ChpB/ChpS module in *E. coli* survival in beach sand.

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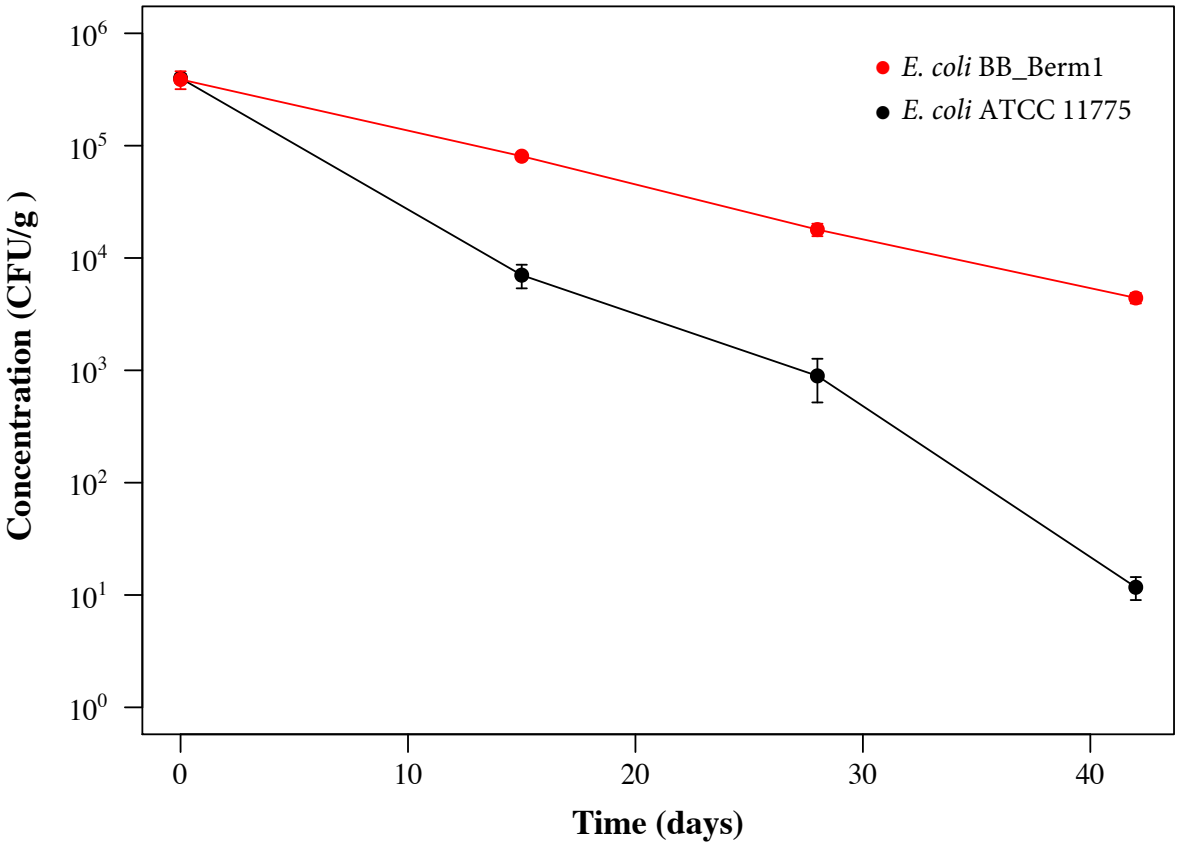

Figure 4.1. Concentrations of *E. coli* BB_Berm1 and *E. coli* ATCC 11775™ measured over-time during the first microcosm experiment conducted during August 2013 in Shorewood, WI. Circles represent mean concentrations for triplicate microcosms. Error bars indicate the standard deviation about the mean concentrations.

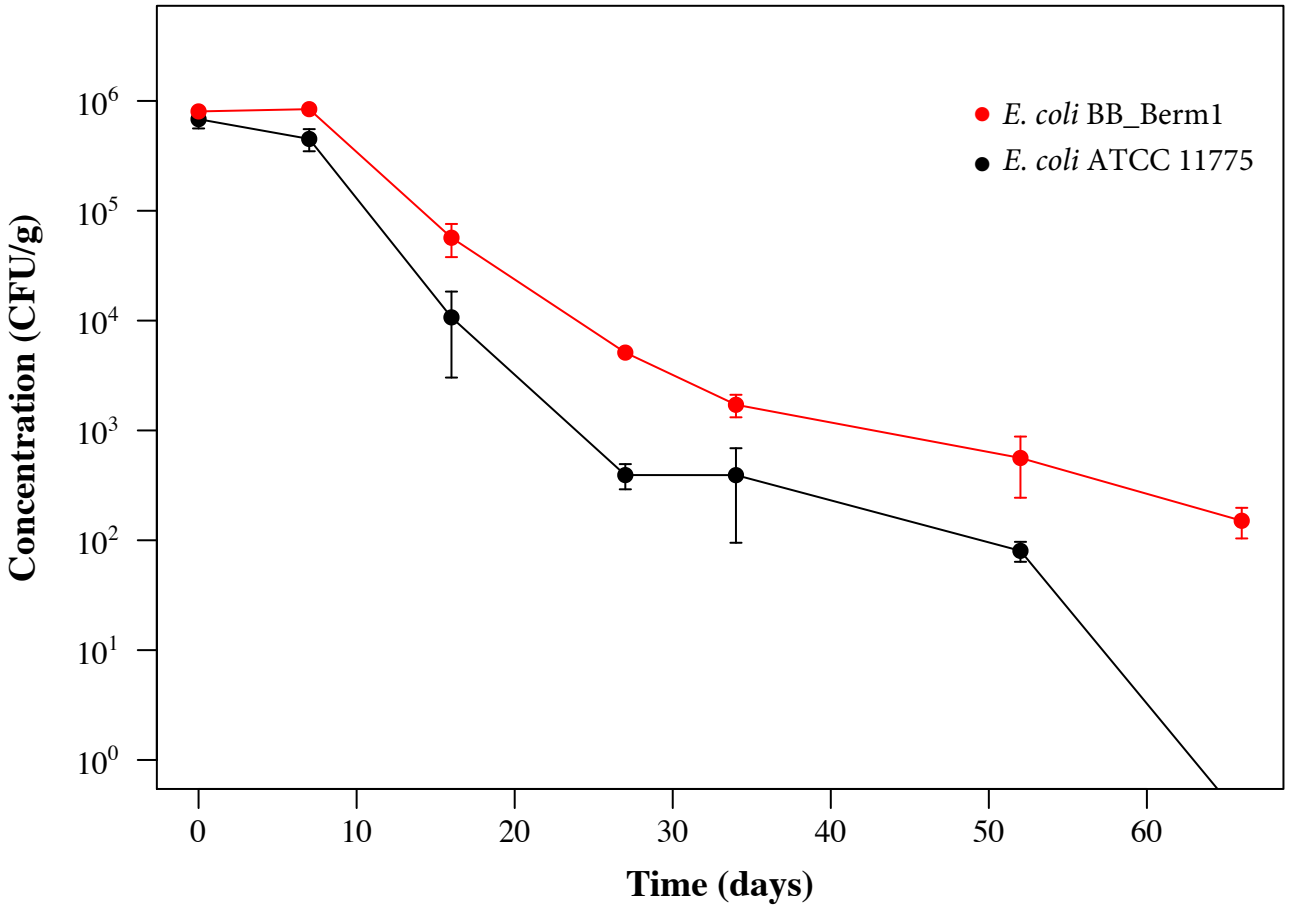

Figure 4.2. Concentrations of *E. coli* BB_Berm1 and *E. coli* ATCC 11775 measured over-time during the first microcosm experiment conducted during July 2014 at Bayview Beach in St. Francis, WI. Circles represent mean concentrations for triplicate microcosms. Error bars indicate the standard deviation about the mean concentrations.

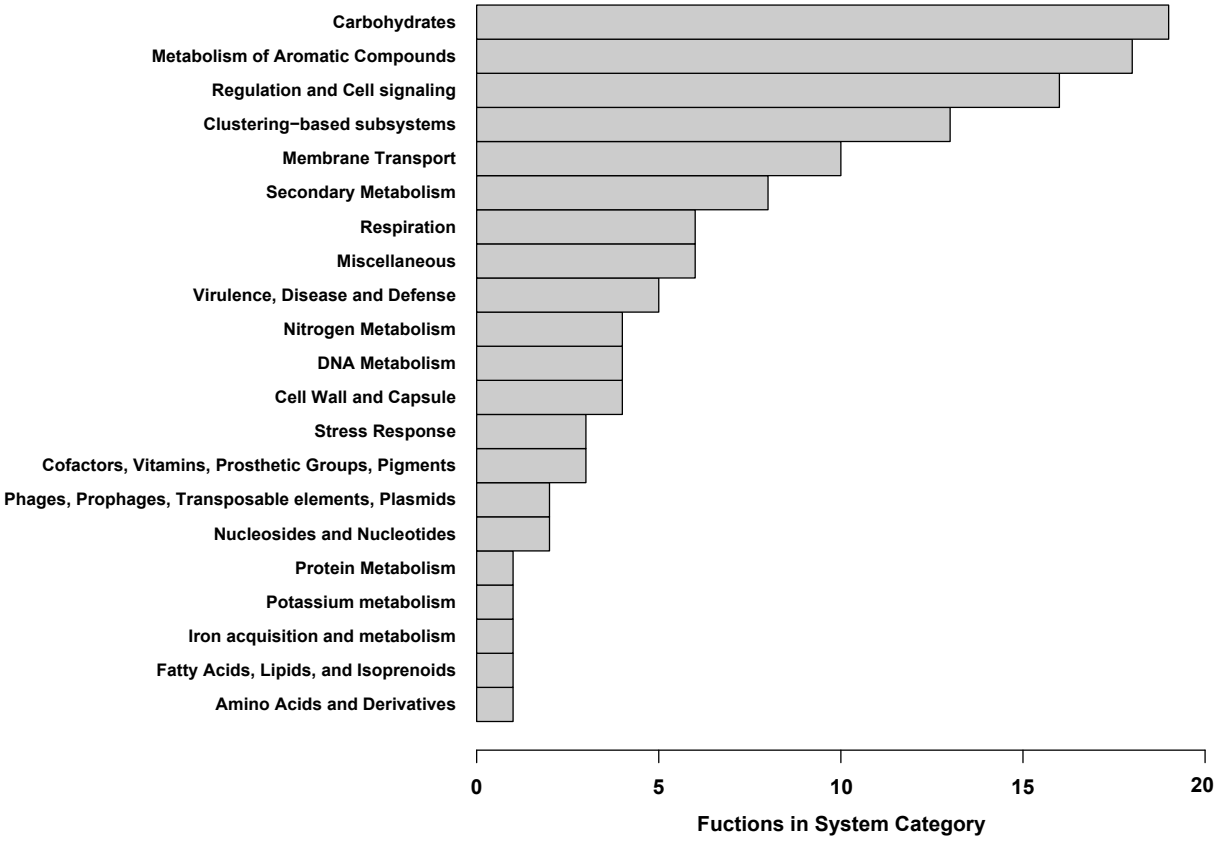

Figure 4.3. RAST annotated gene functional categories found in *E. coli* BB_Berm1 and absent in *E. coli* ATCC 11775.

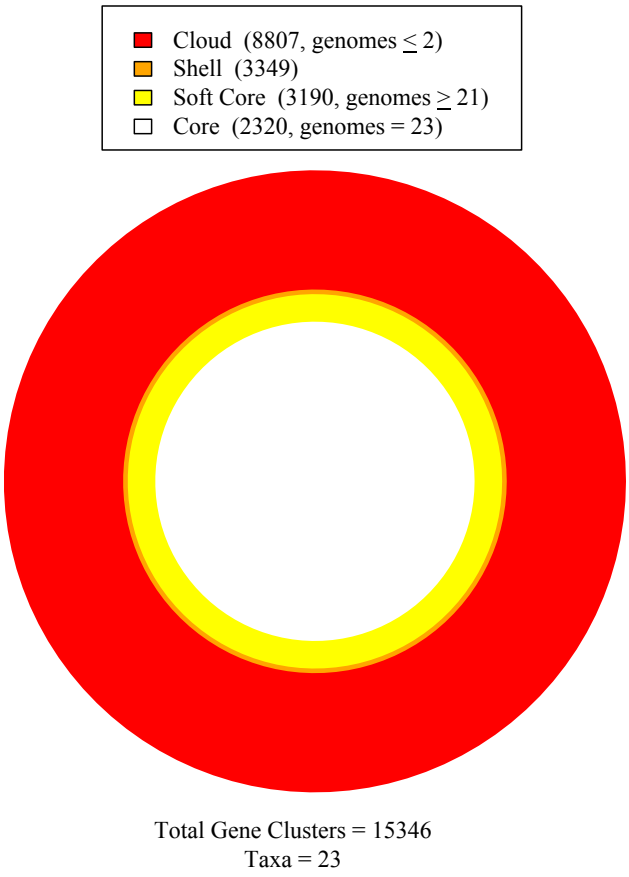

Figure 4.4. Partition of the pangenomic matrix into shell, cloud, soft-core, and core compartments.

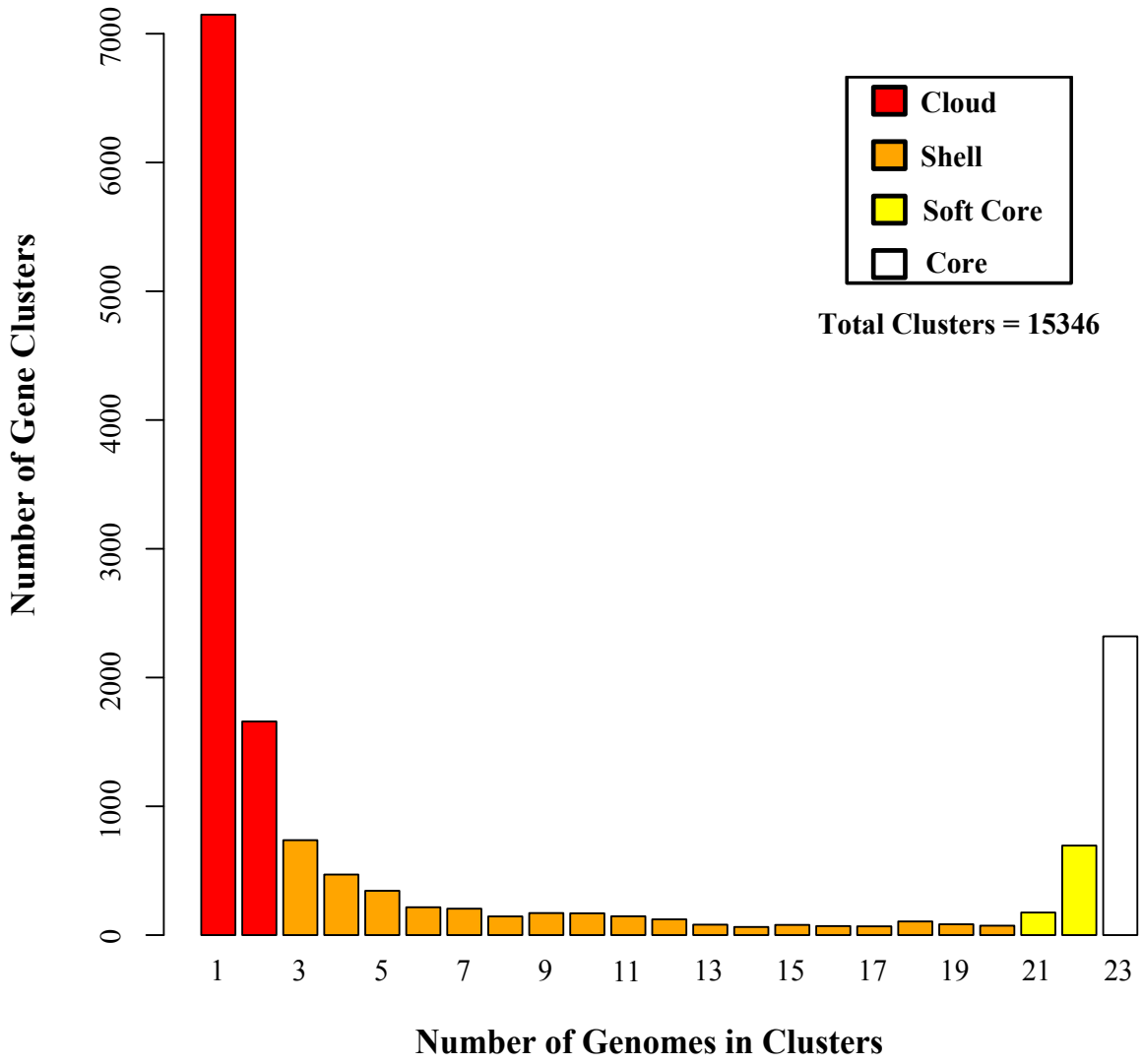

Figure 4.5. Shell, cloud, soft-core, and core components of the pangenome showing the total number of gene clusters in each pangenome component.

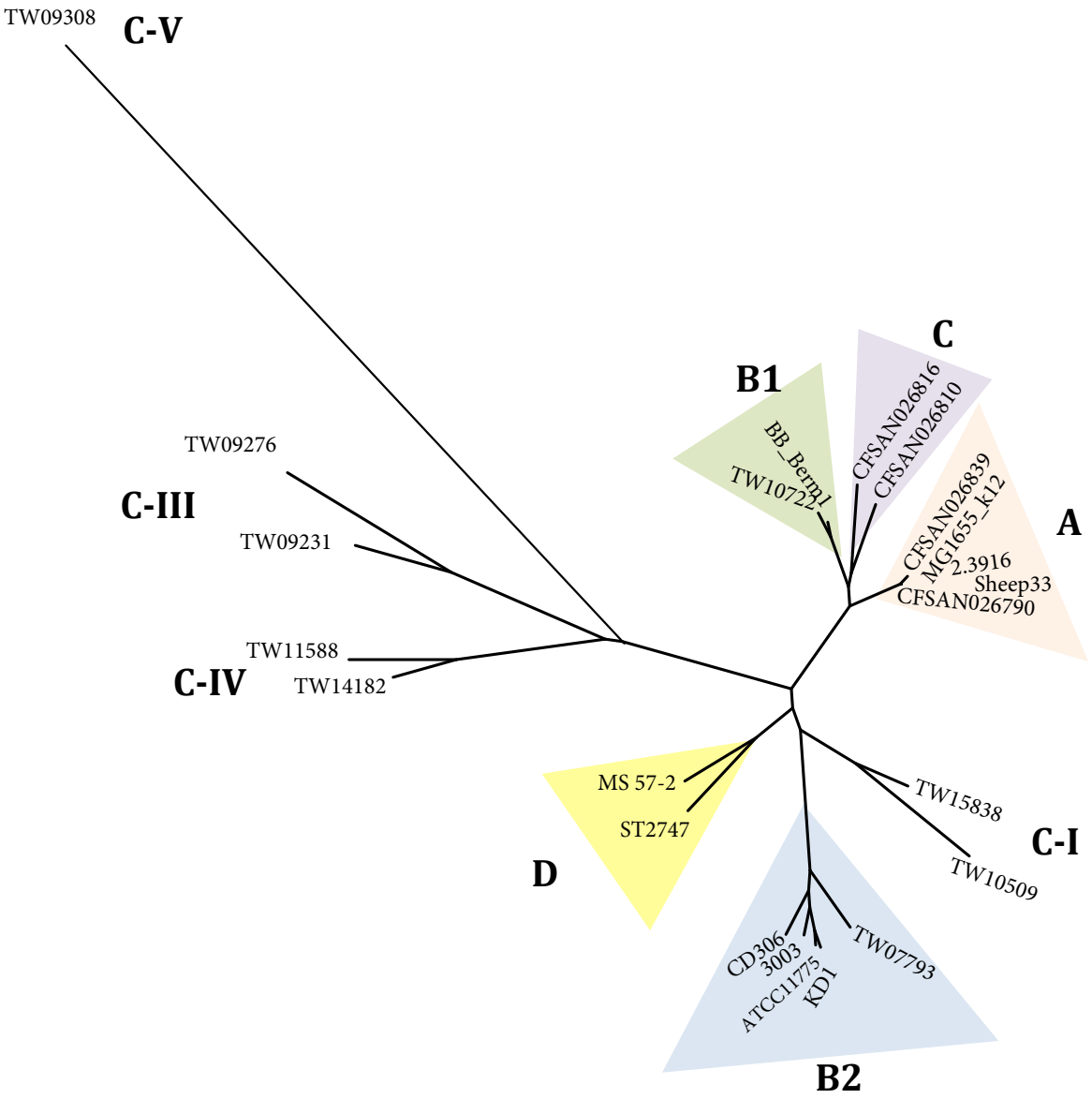

Figure 4.6. *E. coli* species phylogeny of the 23 genomes used in this study. The phylogenetic network was constructed with MegAlign Pro software, using an alignment of *uidA* (B-glucuronidase) gene sequences in all genomes. Bold letter and number labels signify the clade or phylotype designations. Clades I, III, IV, V and their respective genomes were reported in Walk et al. Phylotypes A, B1, B2, C, and D were determined based on *in silico* adaptation of the identification procedure (i.e. Clermont Method) using primer sequences reported in Clermont et al.

Table 1. Genomes included in this study

Genome	Ecotype	Geo. Origin	Isolation	Source	GenBank Accession
2.3916	host		pig	feces	AFAB00000000
ATCC® 11775™	host	New York	human	urine	AGSE00000000
CD306	host		cat	feces	CP013831
CFSAN026839	host	Kansas	racoon	feces	LDDM01000000
BB_Berm1	environmental	Wisconsin	environment	beach sand	
KD1	host	USA	dog	feces	AJWO00000000
MG1655_k12	host		human	feces	AYEK01000000
MS57-2	host		human	feces	ADUG00000000
ST2747	environmental	Belgium	water	water	CP007394
TW07793	environmental		water	water	AFAG00000000
TW09231	environmental	Michigan	environment	beach water	AEJW00000000
TW09276	environmental	Michigan	environment	beach water	AEJV00000000
TW09308	environmental	Michigan	environment	beach water	AEME00000000
TW10509	host	India	human	feces	AEHW00000000
TW10722	host	Guinea-Bissau	human	feces	AELB00000000
TW11588	environmental	Puerto Rico	environment	soil	AEMF00000000
TW14182	environmental	Michigan	environment	beach water	AEJZ00000000
TW15838	environmental	Australia	environment	beach sand	AEJX00000000
3003	environmental		environment	water	AFAF00000000
sheep33	host	China	sheep	marine water	LVQC00000000
CFSAN026816	host	USA	cow	feces	LDCS01000000
CFSAN026790	environmental	USA	environment	creek water	LDDP01000000
CFSAN026810	environmental	USA	environment	pond warer	LDDG01000000

Table 2. Primers pairs and PCR profiles used for *E. coli* phylotype identification.

			***E. coli* phylogroup**					
Primer Set	**Target**	**Primer pairs (5' → 3')**	**A**	**B1**	**B2**	**C**	**D**	**E**
Quadruplex	*arpA*	AACGCTATTCGCCAGCTTGC						
		TCTCCCCATACCGTACGCTA	+	+	-	+	+	+
		ATGGTACCGGACGAACCAAC						
	chuA	TGCCGCCAGTACCAAAGACA	-	-	+	-	+	+
	yjaA	CAAACGTGAAGTGTCAGGAG						
		AATGCGTTCCTCAACCTGTG	-/+	-	-/+	+	-	-
	TspE4.C2	CACTATTCGTAAGGTCATCC						
		AGTTTATCGCTGCGGGTCGC	-	+	-/+	-	-/+	-/+
Phylogroup E		GATTCCATCTTGTCAAAATATGCC						
	arpA	GAAAAGAAAAAGAATTCCCAAGAG	NA	NA	NA	NA	-	+
Phylogroup C		AGTTTTATGCCCAGTGCGAG						
	trpA	TCTGCGCCGGTCACGCCC	-	NA	NA	+	NA	NA

Table 3. First-order decay coefficients and standard deviations calculated for August 2013 and July 2014 microcosm experiments.

Experiment	Isolate	Decay rate constant *k* ± SD (days^{-1})
1	*E. coli* BB_Berm1	-0.114 ± 0.004
	E. coli ATCC® 11775™	-0.244 ± 0.004
2	*E. coli* BB_Berm1	-0.146 ± 0.006
	E. coli ATCC® 11775™	-0.219 ± 0.008

Table 4. List of RAST annotated gene categories and functions detected in *E. coli* BB_1 and absent in *E. coli* ATCC 11775.

Category	Subcategory	Subsystem	Role	FIGFAM
Amino Acids and Derivatives	Arginine polyamines	Putrescine utilization pathways	Gamma-glutamyl-GABA	FIG00007600
Carbohydrates	Aminosugars	Chitin and N-acetylglucosamine utilization	Acetylglucosamine transcriptional regulator	
Carbohydrates	Aminosugars	Fructoselysine utilization pathway	Fructoselysine transporter	FIG00008615
Carbohydrates	Aminosugars	Fructoselysine utilization pathway	Fructoselysine 6-phosphate deglycase	FIG00142855
Carbohydrates	Aminosugars	Fructoselysine utilization pathway	Fructoselysine 3-epimerase	FIG00010697
Carbohydrates	Aminosugars	Fructoselysine utilization pathway	Fructoselysine kinase	FIG00008709
Carbohydrates	Aminosugars	Fructoselysine utilization pathway	Fructoselysine transcriptional regulator	FIG00008107
Carbohydrates	Di & oligosaccharides	Beta-Glucoside Metabolism	PTS system, cellobiose-specific component	
Carbohydrates	Di & oligosaccharides	Maltose and Maltodextrin Utilization	Maltose-6'-phosphate glucosidase	FIG00003756
Carbohydrates	Di & oligosaccharides	Maltose and Maltodextrin Utilization	Sugar-P isomerases/epimerases protein	
Carbohydrates	Di & oligosaccharides	Maltose and Maltodextrin Utilization	Neopullulanase	FIG00066643
Carbohydrates	Di & oligosaccharides	Melibiose Utilization	Melibiose/NA+ carrier symportor	FIG00088522
Carbohydrates	Di & oligosaccharides	Sucrose utilization	Sucrose permease	
Carbohydrates	Di & oligosaccharides	Sucrose utilization	Sucrose-6-phosphate hydrolase	
Carbohydrates	Di & oligosaccharides	Sucrose utilization	Sucrose specific transcriptional regulator	FIG00023779
Carbohydrates	Monosaccharides	Galacturonate/Glucuronate Utilization	Altronate hydrolase	
Carbohydrates	Monosaccharides	D-ribose utilization	Ribose/xylose/arabinose/galactoside ABC transport, permease	FIG00022724
Carbohydrates	Monosaccharides	Mannose Metabolism	Alpha-mannosidase	FIG00009194
Carbohydrates	Monosaccharides	Xylose utilization	D-xylose proton-symporter XylE	FIG00106273
Carbohydrates	Polysaccharides	Alpha-Amylase locus	putative esterase	FIG00016132
Cell Wall and Capsule	Extracellular polysacchrides	Capsule Biosynthesis and Assembly	Putative polysaccharide export protein	FIG00146546
Cell Wall and Capsule	Extracellular polysacchrides	Capsule Biosynthesis and Assembly	Putative outer membrane lipoprotein YmcA	FIG00146759
Cell Wall and Capsule	Extracellular polysacchrides	Capsule Biosynthesis and Assembly	Uncharacterized lipoprotein YmcC precursor	FIG00007519
Cell Wall and Capsule	no subcategory	Peptidoglycan Crosslinking	L,D-transpeptidase ErfK	FIG01304270
Clustering-based subsystems	CRISPRs and associated hypotheticals	CBSS-216592.1.peg.3534	CRISPR-associated protein, Cas2	FIG00054590
Clustering-based subsystems	CRISPRs and associated hypotheticals	CBSS-216592.1.peg.3534	CRISPR-associated protein, Cse3 family	FIG00005983
Clustering-based subsystems	CRISPRs and associated hypotheticals	CBSS-216592.1.peg.3534	CRISPR-associated protein, Cas5e family	FIG00008770
Clustering-based subsystems	CRISPRs and associated hypotheticals	CBSS-216592.1.peg.3534	CRISPR-associated protein, Cse4 family	FIG00002459
Clustering-based subsystems	no subcategory	CBSS-374931.9.peg.1048	FIG001353: Acetyltransferase	FIG00001353
Clustering-based subsystems	no subcategory	CBSS-374931.9.peg.1048	FIG032766: hypothetical protein	FIG00021257
Clustering-based subsystems	no subcategory	Conserved cluster in Enterobacteriaceae	Inner membrane protein YhaI	FIG01304266
Clustering-based subsystems	no subcategory	Long-chain-fatty-acid-CoA ligase	Phosphopantetheine-binding acyl carrier protein	
Clustering-based subsystems	no subcategory	Putative hemin transporter	Putative hemine transporter ATP-binding subunit	FIG00008609
Clustering-based subsystems	Putrescine/GABA utilization cluster	GABA and putrescine metabolism from cluters	Gamma-glutamyl-putrescine synthetase	FIG00114297
Clustering-based subsystems	Putrescine/GABA utilization cluster	GABA and putrescine metabolism from cluters	Putrescine utilization regulator	FIG00137548
Clustering-based subsystems	Putrescine/GABA utilization cluster	GABA and putrescine metabolism from cluters	Gamma-glutamyl-aminobutyraldehyde dehydrogenase	FIG00082709
Clustering-based subsystems	Putrescine/GABA utilization cluster	GABA and putrescine metabolism from cluters	Gamma-glutamyl-putrescine oxidase	FIG00002047
Cofactors, Vitamins, Pigments	Folate and pterines	Folate Biosynthesis	Dihydroneopterin triphosphate pyrophosphohydrolase type 2	
Cofactors, Vitamins, Pigments	Folate and pterines	p-Aminobenzoyl-Glutamate Utilization	Catalyzes the cleavage of p-aminobenzoyl-glutamate to p-aminobenzoate and glutamate, subunit B	FIG00139150
Cofactors, Vitamins, Pigments	no subcategory	Thiamin biosynthesis	Thiazole biosynthesis protein ThiH	

Table 4. List of RAST annotated gene categories and functions detected in *E. coli* BB_1 and absent in *E. coli* ATCC 11775.

Category	Subcategory	Subsystem	Role	FIGFAM
DNA Metabolism	CRISPs	CRISPRs	CRISPR-associated protein, Cse2 family	FIG00010563
DNA Metabolism	CRISPs	CRISPRs	CRISPR-associated protein, Cse1 family	FIG00006009
DNA Metabolism	CRISPs	CRISPRs	CRISPR-associated helicase Cas3	FIG00002034
DNA Metabolism	DNA repair	DNA repair, bacterial	DNA-damage-inducible protein J	FIG00002504
FattyAcids, Lipids, and Isoprenoids	no subcategory	Polyhydroxybutyrate metabolism	D-beta-hydroxybutyrate permease	FIG00015338
Iron acquisition and metabolism	Siderophores	Siderophore Enterobactin	Ferric enterobactin transport system permease protein FepG	
Membrane Transport	Protein secretion system, Type VII	alpha-Fimbriae	Alpha-fimbriae tip adhesin	FIG00017309
Membrane Transport	Protein secretion system, Type VII	alpha-Fimbriae	Alpha-fimbriae usher protein	FIG00018400
Membrane Transport	Protein secretion system, Type VII	alpha-Fimbriae	Alpha-fimbriae major subunit	FIG00018939
Membrane Transport	Protein secretion system, Type VII	alpha-Fimbriae	Alpha-fimbriae chaperone protein	FIG00019370
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Fimbriae-like adhesin SfmA	FIG00009730
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Fimbrial periplasmic chaperone SfmC	FIG01305019
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Outer membrane usher protein SfmD	FIG00007547
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Fimbriae-like periplasmic protein SfmF	FIG01070680
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Transcriptional regulator of fimbriae expression (LuxR/UhpA)	FIG00011243
Membrane Transport	Protein secretion system, Type VII	The fimbrial Sfm cluster	Fimbriae-like adhesin SfmH	FIG00006488
Aromatic Compound Metabolism	Aromatics, anaerobic degradation	Hydroxyaromatic decarboxylase family	Hydroxyaromatic non-oxidative decarboxylase protein D	FIG00006766
Aromatic Compound Metabolism	Aromatics, anaerobic degradation	Hydroxyaromatic decarboxylase family	Hydroxyaromatic non-oxidative decarboxylase protein C	FIG00133738
Aromatic Compound Metabolism	Aromatics, anaerobic degradation	Hydroxyaromatic decarboxylase family	Hydroxyaromatic non-oxidative decarboxylase protein B	FIG01970554
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	4-hydroxyphenylacetate 3-monooxygenase, reductase	FIG00001471
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	4-hydroxyphenylacetate 3-monooxygenase	FIG00138613
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	Transcriptional activator of 4-hydroxyphenylacetate 3-monooxygenase operon	FIG00005741
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	4-hydroxyphenylacetate symporter	FIG00007527
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	2-oxo-hepta-3-ene-1,7-dioic acid hydratase	FIG00001799
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-hydroxymuconate delta-isomerase	FIG00137536
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	3,4-dihydroxyphenylacetate 2,3-dioxygenase	FIG00001722
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase	FIG00133544
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-oxo-hex-3- ene-1,7-dioate decarboxylase	
Aromatic Compound Metabolism	Central aromatic metabolism	4-Hydroxyphenylacetic acid catabolic pathway	Homoprotocatechuate degradative operon repressor	
Aromatic Compound Metabolism	Central aromatic metabolism	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B	FIG00133276
Aromatic Compound Metabolism	Central aromatic metabolism	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	FIG00133228
Aromatic Compound Metabolism	Central aromatic metabolism	Meta-cleavage pathway of aromatic degradation	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol	FIG00002495
Aromatic Compound Metabolism	no subcategory	Aromatic Amin Catabolism	Phenylacetaldehyde dehydrogenase	FIG00134532
Aromatic Compound Metabolism	no subcategory	Aromatic Amin Catabolism	Monoamine oxidase	FIG00116538
Aromatic Compound Metabolism	Central aromatic metabolism	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B	FIG00133276
Aromatic Compound Metabolism	Central aromatic metabolism	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	FIG00133228

Table 4. List of RAST annotated gene categories and functions detected in *E. coli* BB_1 and absent in *E. coli* ATCC 11775.

Category	Subcategory	Subsystem	Role	FIGFAM
Aromatic Compound Metabolism	Central aromatic metabolism	Meta-cleavage pathway of aromatic degradation	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol	FIG00002495
Aromatic Compound Metabolism	no subcategory	Aromatic Amin Catabolism	Phenylacetaldehyde dehydrogenase	FIG00134532
Aromatic Compound Metabolism	no subcategory	Aromatic Amin Catabolism	Monoamine oxidase	FIG00116538
Miscellaneous	no subcategory	Aromatic dioxygenase mess	3-phenylpropionate dioxygenase, alpha subunit (EC 1.14.12.19)	FIG00137602
Miscellaneous	no subcategory	Aromatic dioxygenase mess	3-phenylpropionate dioxygenase, beta subunit (EC 1.14.12.19)	FIG00138944
Miscellaneous	no subcategory	Aromatic dioxygenase mess	3-phenylpropionate dioxygenase ferredoxin subunit	FIG00007834
Miscellaneous	no subcategory	Aromatic dioxygenase mess	3-phenylpropionate dioxygenase ferredoxin--NAD(+) reductase component (EC 1.18.1.3)	FIG00008663
Miscellaneous	no subcategory	Aromatic dioxygenase mess	3-carboxyethylcatechol 2,3-dioxygenase (EC 1.13.11.16)	
Miscellaneous	no subcategory	Broadly distributed proteins not in subsystems	Putative oxidoreductase YncB	FIG00005027
Nitrogen Metabolism	no subcategory	Ammonia assimilation	Nitrogen regulation protein NR(I)	
Nitrogen Metabolism	no subcategory	Cyanate hydrolysis	Cyn operon transcriptional activator	FIG00011094
Nitrogen Metabolism	no subcategory	Cyanate hydrolysis	Cyanate hydratase (EC 4.2.1.104)	
Nitrogen Metabolism	no subcategory	Cyanate hydrolysis	Cyanate transport protein CynX	FIG00101140
Nucleosides and Nucleotides	no subcategory	Pseudouridine catabolism	Pyrimidine nucleoside transport protein	FIG00006658
Nucleosides and Nucleotides	Purines	Purine Utilization	Periplasmic aromatic aldehyde oxidoreductase	FIG00060371
Phages, Prophages, Plasmids	Phages, Prophages	Phage capsid proteins	Phage head-tail joining protein	
Phages, Prophages, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail sheath monomer	FIG00001843
Potassium metabolism	no subcategory	Potassium homeostasis	Trk system potassium uptake protein	FIG00229234
Protein Metabolism	Protein processing and modification	G3E family of P-loop GTPases	Periplasmic protein kinase ArgK and related GTPases of G3E	
Regulation and Cell signaling	no subcategory	cAMP signaling in bacteria	Prophage Clp protease-like protein	
Regulation and Cell signaling	no subcategory	DNA-binding regulatory proteins, strays	LysR family transcriptional regulator near succinyl-CoA:3-ketoacid-coenzyme A transferase	
Regulation and Cell signaling	no subcategory	LysR-family proteins in Escherichia coli	Regulatory protein (induces abgABT, used to catabolize p-aminobenzoyl-glutamate)	FIG00008656
Regulation and Cell signaling	no subcategory	LysR-family proteins in Escherichia coli	LysR family transcriptional regulator STM2281	FIG00011906
Regulation and Cell signaling	Programmed Cell Death	MazEF toxin-antitoxin system	Programmed cell death antitoxin ChpS	FIG00019079
Regulation and Cell signaling	Programmed Cell Death	MazEF toxin-antitoxin system	Programmed cell death toxin ChpB	FIG00020038
Regulation and Cell signaling	Programmed Cell Death	Toxin-antitoxin replicon stabilization systems	VapB protein (antitoxin to VapC)	FIG00004470
Regulation and Cell signaling	Programmed Cell Death	Toxin-antitoxin replicon stabilization systems	YafQ toxin protein	FIG00008080
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	Autoinducer 2 (AI-2) kinase LsrK	FIG00040421
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	LsrR, transcriptional repressor of lsr operon	FIG00012871
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	AI-2 ABC transport system,AI2 transporter subunits	FIG00137917
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	AI-2 ABC transport system, membrane channel protein LsrC	FIG00003820
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	AI-2 ABC transport system, membrane channel protein LsrD	FIG00138020
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	AI-2 ABC transport system, periplasmic binding protein LsrB	FIG00002057
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	AI-2 aldolase LsrF	FIG00003369
Regulation and Cell signaling	Quorum sensing and biofilm formation	AI-2 transport/processing (lsrACDBFGE operon)	Autoinducer 2 (AI-2) modifying protein LsrG	FIG00003614

Table 4. List of RAST annotated gene categories and functions detected in *E. coli* BB_1 and absent in *E. coli* ATCC 11775.

Category	Subcategory	Subsystem	Role	FIGFAM
Respiration	Electron accepting reactions	Anaerobic respiratory reductases	Vanillate O-demethylase oxidoreductase	FIG00001276
Respiration	no subcategory	Formate hydrogenase	Hydrogenase-4 component A	
Respiration	no subcategory	Formate hydrogenase	Hydrogenase-4 component B	
Respiration	no subcategory	Formate hydrogenase	Hydrogenase-4 component D	
Respiration	no subcategory	Formate hydrogenase	Hydrogenase-4 component E	
Respiration	no subcategory	Formate hydrogenase	Hydrogenase-4 component I	
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	3-phenylpropionic acid catabolism, transcriptional activator	FIG00137462
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	2,3-dihydroxy-2,3-dihydro-phenylpropionate dehydrogenase	FIG00006725
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	Mhp operon transcriptional activator	FIG00016600
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	3-(3-hydroxy-phenyl)propionate hydroxylase	FIG00138127
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase	
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	2-keto-4-pentenoate hydratase	FIG01304357
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	4-hydroxy-2-oxovalerate aldolase	
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	4-hydroxybenzoate transporter	FIG00015882
Stress Response	Cold shock	Cold shock, CspA family of proteins	Cold shock protein CspB	FIG00006252
Stress Response	Cold shock	Cold shock, CspA family of proteins	Cold shock protein CspF	FIG01304325
Stress Response	no subcategory	Commensurate regulon activation	Transcriptional regulator YkgA	FIG00014136
Virulence, Disease and Defense	Adhesion	Mediator of hyperadherence YidE	HTH-type transcriptional regulator YidP	FIG00149137
Virulence, Disease and Defense	Invasion and intracellular resistance	Internalin-like proteins	internalin, putative	FIG01287332
Virulence, Disease and Defense	Antibiotic/Toxin Resistance	Arsenic resistance	Arsenical resistance operon repressor	FIG01124301
Virulence, Disease and Defense	Antibiotic/Toxin Resistance	Arsenic resistance	Arsenic efflux pump protein	FIG00081580
Virulence, Disease and Defense	Antibiotic/Toxin Resistance	Cobalt-zinc-cadmium resistance	Copper-sensing two-component system response regulator CusR	

Chapter 5.

General Discussion and Conclusions

The central theme of this dissertation project was to better understand the ecology of fecal indicators in the natural beach environment. In freshwater systems, recreational water quality monitoring relies on established criteria indicating acceptable levels of *E. coli* in water. The use of *E. coli* as an indicator relies on the characteristics outlined in Chapter 1, with the relationship to fecal pathogens and limited environmental survival among the most fundamental characteristics. Following the established *E. coli* criteria, the motivation of mandated beach monitoring is to limit potential health risks associated with contaminated recreational water contact.

In line with previous studies, the present study has noted high levels of *E. coli* in beach sand. Physical forces, such as wave-action, can lead to the resuspension of *E. coli* from sand. It is also possible that some of the suspended *E. coli* in the water column may filter-out thus increasing levels of *E. coli* in sand. Researchers have used laboratory sand column experiments as well as modeling to study the detachment, mobilization, and fate of FIB within the beach wash-zone (1–3). Although it was not within the scope of this study to identify exchange dynamics of *E. coli* at the sand-water face, we have documented that *E. coli* concentrations in water are correlated to the high levels of *E. coli* in sand (Chapter 3). Since water quality measurements inform beach advisories, the potential for sand to affect nearshore water quality is a concern. A myriad of additional factors also likely influence the concentrations of *E. coli* in the beach environment such as continuous fecal deposition, competition from the indigenous microbial community, anthropogenic nutrients inputs, and other environmental factors (Figure 1).

Chapter 2 highlights that indigenous sand microbial communities vary between beaches at both local and regional geographic scales. Despite the characteristic differences among the

beaches studied, *E. coli* was detected universally and in high concentrations in sand. The results from Chapter 2 suggest that the sand *E. coli* reservoir is not highly influenced by beach conditions that vary along environmental and urban-impact gradients. Since the source and degree of fecal bacteria inputs also vary along environmental and urban-impact gradients, the origin of this universal *E. coli* sand reservoir cannot be explicitly identified. Furthermore, these data present the possibility that *E. coli* may not be linked to fecal contamination events, thus calling into question previous assumptions about its utility as a fecal indicator.

External environmental conditions cannot account for the majority of the sand *E. coli* burden, thus we set out to identify other possible mechanisms that can explain the abundance of *E. coli* in sand. It has yet to be determined if the concentrations of *E. coli* in the beach environment, particularly within sand, are associated with an innate ability of some *E. coli* strains to resist the stressful environmental conditions that can otherwise result in extinction. In comparative decay experiments, *E. coli* isolated from beach sand survived longer than an *E. coli* type-strain that has been maintained in a laboratory setting. Preliminary comparative genomic analyses identified several genes in the environmental *E. coli* isolate with known associations to stress resistance and survival. Additional experimentation should expand on the preliminary evidence presented in Chapter 4 to describe the importance of the genes in *in situ E. coli* survival. In the future, especially if *E. coli* continues to be the preferred fecal indicator, environmental *E. coli* genes could be targets of ecotype specific PCR or qPCR assays. The potential development of ecotype specific assays could be used to determine the relative amount of naturalized to host-associated *E. coli* within an environmental sample. From a health-risk management prospective, an environmental *E. coli* assay could help beach managers interpret *E. coli* measurements to more accurately predict potential water-borne illnesses.

In general, the use of traditional fecal indicators can be problematic and may lead to overestimations or underestimations of human health risk if not combined with more robust techniques. Rapid testing methods for alternative fecal indicators show promise for recreational beach monitoring. To date, the use of alternative indicator qPCR assays has been limited to investigative microbial source tracking and beach restoration studies. The current advisory and closure criteria for recreational beaches has been defined based on epidemiological studies establishing a relationship between *E. coli* and reported gastrointestinal illness of beachgoers. The comprehensive alternative indicator study (Chapter 3) reported that during water quality advisories (≥ 235 CFU/100 ml), with few exceptions, the majority of the *E. coli* burden could be attributed to fecal contamination from gulls. Although gull feces can harbor pathogenic bacteria, the detection of pathogens can be spotty and the overall human health risk is low when compared to untreated sewage contamination. Beach managers are tasked with developing routine beach monitoring programs that issue beach advisories via the established *E. coli* criteria. However, since gull feces can contribute a large amount of *E. coli*, from a beach monitoring compliance standpoint, gull contamination can be a concern. If the majority of beach advisories stem from gull contamination, then current standards are likely overestimating the associated human-health risk.

In the case of sewage contamination following a combined sewer overflow (CSO) event, the concentrations of culturable *E. coli* and enterococci were low or undetectable (0-23 CFU/100 ml) in nearshore water while the two human-specific alternative indicators produced a qPCR robust signal. Since the concentrations of *E. coli* were below the USEPA criteria, after the CSO event the use of traditional indicators alone would not have triggered a water quality advisory;

thus, in this case the use of culturable *E. coli* alone was found to underestimate the true human-health risk.

Among other concerns, fundamental questions about the fate of alternative indicators in the environment have impeded their use in routine monitoring programs. Using *in situ* microcosm experiments we were able to track the die-off of alternative fecal indicator markers compared to culturable traditional indicators. In general, the host-specific alternative indicator markers decayed faster than culturable indicators. However, due to the overabundance of alternative indicators compared to *E. coli* or enterococci in source material, the differential decay is not a major concern as alternative indicator concentrations would remain above culturable indicators up to ~30 days.

If the goal of recreational beach monitoring is to protect human health, it stands to reason that beach monitoring should begin transitioning to the adoption of alternative indicators. Alternative indicators can provide the sensitivity and specificity for sources that pose a high health risk, which traditional fecal indicators lack. It may be possible to create a multi-tiered monitoring approach that integrates both general and host-specific indicator criteria for beach advisory postings. For instance, the first-tier of beach monitoring could include culturable *E. coli* criteria. The alternative indicator assay used in the second-tier could then depend on the concentrations of *E. coli* (first-tier) and/or other observational data. If a beach were found to have low *E. coli* concentrations, then as long as the sample tests negative for human-specific indicators, an advisory would not be issued. If the beach tested positive for human-specific indicators or if there was a known sewage contamination event, a beach advisory would be issued. If a sample was instead found to have high *E. coli* concentrations, the sample would then be tested for both human and gull specific indicators. In this case, if the gull specific indicator

was detected then an informed public health decision could be made as to what limit could be for gull waste. Quantitative microbial risk assessment (QMRA) modeling indicates that the gastrointestinal illness risk associated with gull contaminated water is at least two orders of magnitude less than what is predicted by the USEPA recreational FIB criteria (4, 5). Alternatively, a beach advisory would be issued if the human specific indicators were detected when *E. coli* concentrations were high. A multi-tiered approach would also provide beach managers with the benefit of comparing historical water quality measurements that relied on the sole use of *E. coli* while integrating some of the host-specific rapid testing techniques.

In addition to host-specific alternative indicators, advances in technology and modeling have also provided beach managers with new tools for predicting water quality issues at beaches. The USEPA has developed Virtual Beach (6), which is a software package that can be used to develop site-specific statistical models for predicting fecal indicator levels at beaches. Virtual Beach models calculate expected *E. coli* levels by utilizing the predictive quality of climate data, physical beach characteristics, and other observational data. Individualized models are developed that incorporate data such as rainfall, water current, turbidity or water clarity, temperature, and wave-height. However, with the complexity of the beach environment and the diverse ecology of *E. coli,* in many cases Virtual Beach and other predictive models have not been able to produce reliable predictions of *E. coli* levels. Perhaps using alternative indicators in these models could improve prediction accuracy of conditions with high human health risk.

New technologies, such as autonomous *in situ* sensors, can also provide beach managers with real-time measurements of *E. coli.* Although autonomous *in situ* sensor technologies still remain at the research and development stage, they may prove highly useful for beach managers in the future - particularly for beaches where predictive *E. coli* models have not been successful.

Autonomous *in situ* technologies have used adaptations of PCR techniques (7), fluorescence *in situ* hybridization, and flow cytometry (8). However, even with the advancements in water quality prediction and testing technologies, real-time *E. coli* measurements may or may not be an indication of an actual health risk. Alternatively, host-specific indicator assays can provide same-day results that can be directly used in risk assessments. With the progression of water quality technologies and increased interest in rapid testing, real-time measurements of the host-specific alternative indicators may soon be possible.

In conclusion, the work presented in this dissertation highlights the complexity of the beach ecosystem and also calls into question the dogma of traditional methods of recreational water quality testing. The development of rapid technologies is an important step towards decreasing health risks for beachgoers. The ability for alternative fecal indicator assays to produce same-day results is critical for ensuring that beach advisories are posted only when appropriate. The field of beach research is evolving, as is our understanding of the ecology of fecal indicator organisms. With the goal of protecting human health and the economic stability of coastal communities that rely on beach recreation, recreational water quality testing methodologies must evolve as well.

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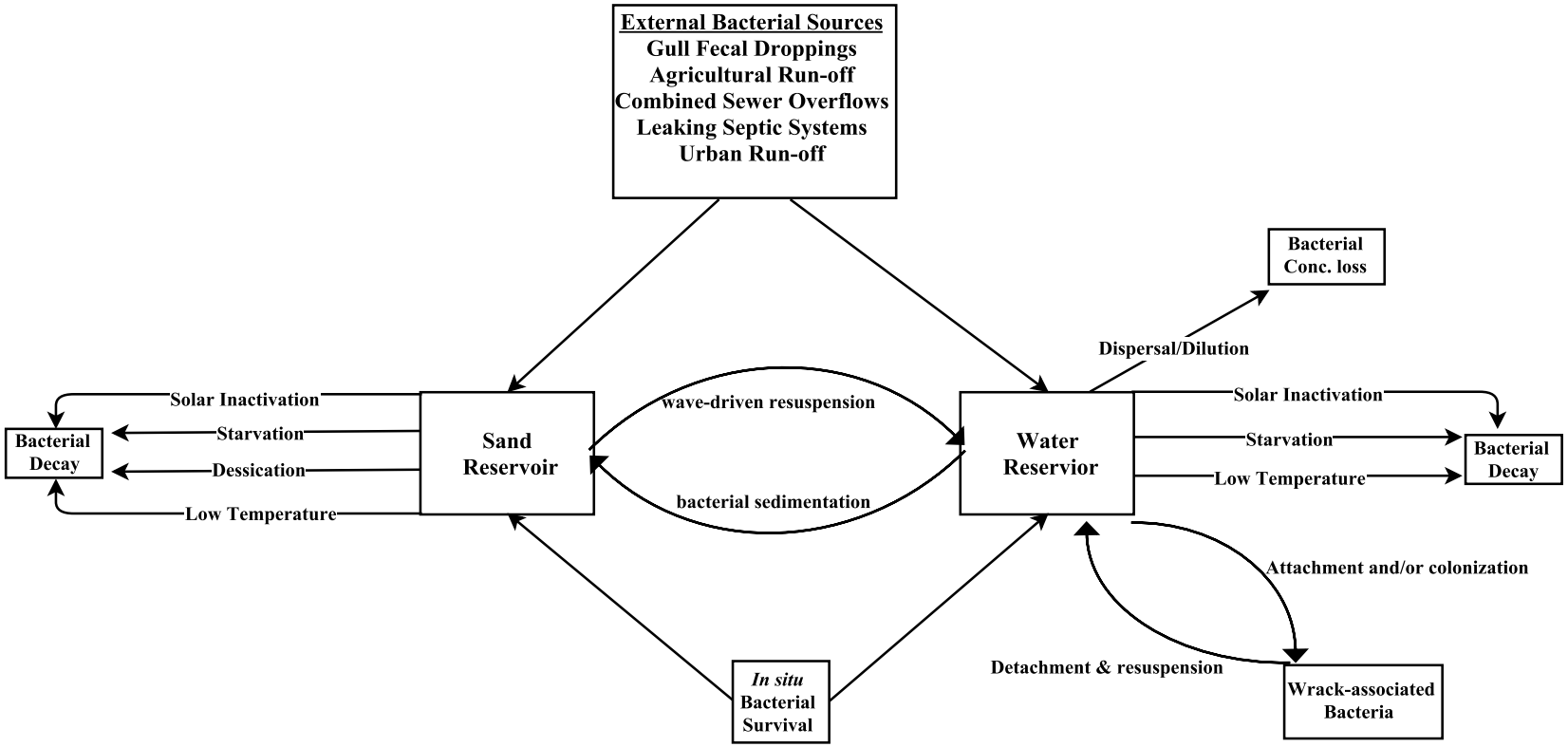

Figure 5.1. Conceptual model of the dynamics and fate of beach fecal pollution.

Appendix A. Supplemental Material for Chapter 2

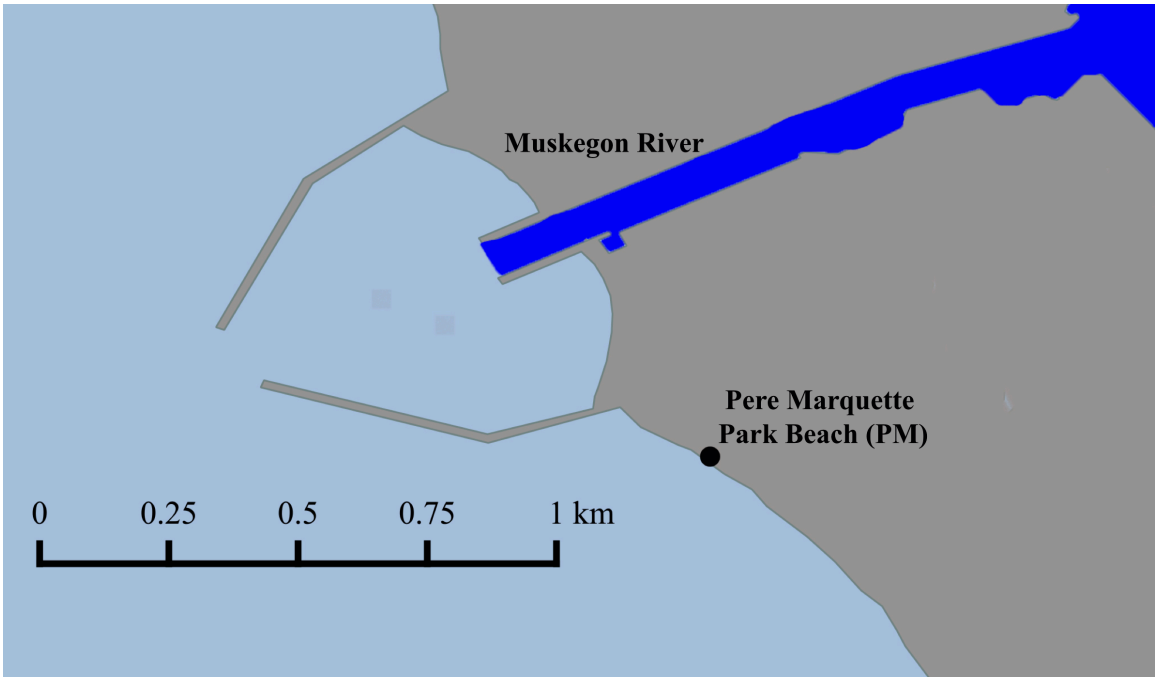

Figure 1. Map of Pere Marquette beach and Muskegon River. Background map ©OpenStreetMap contributors http://www.openstreetmap.org/copyright.

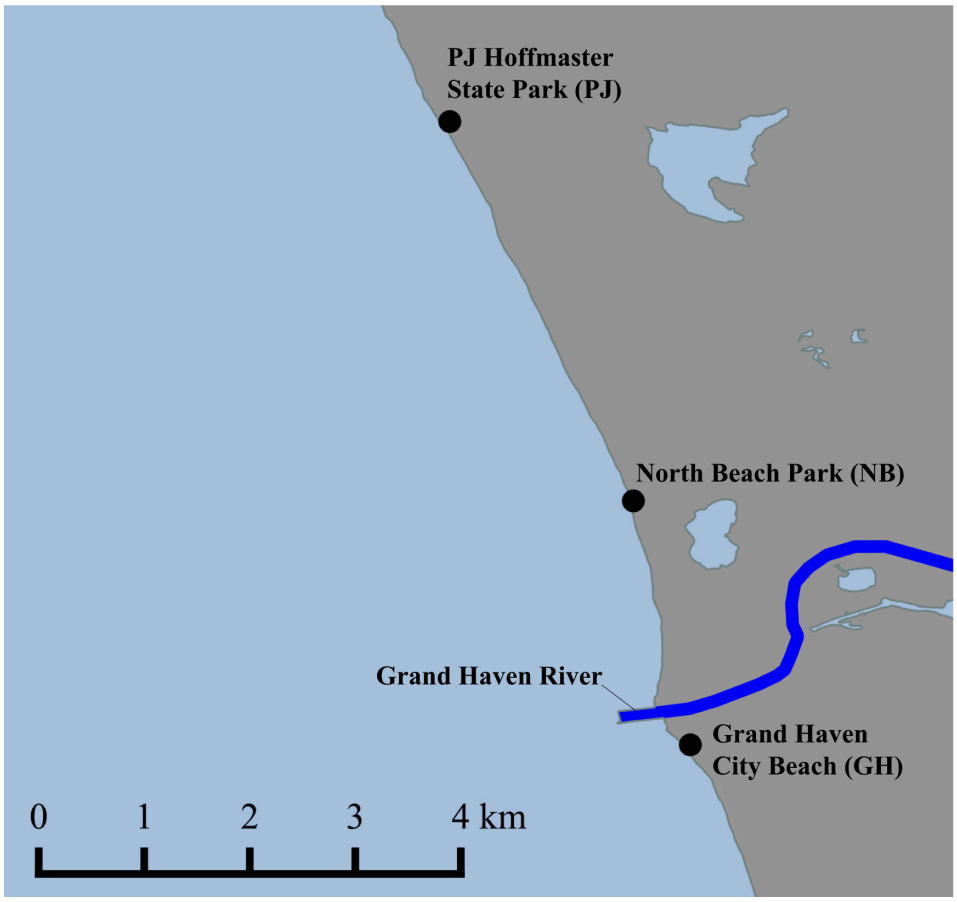

Figure 2. Map of PJ Hoffmaster, North Beach, and Grand Haven beaches in proximity to the Grand Haven River in Michigan. Background map ©OpenStreetMap contributors http://www.openstreetmap.org/copyright.

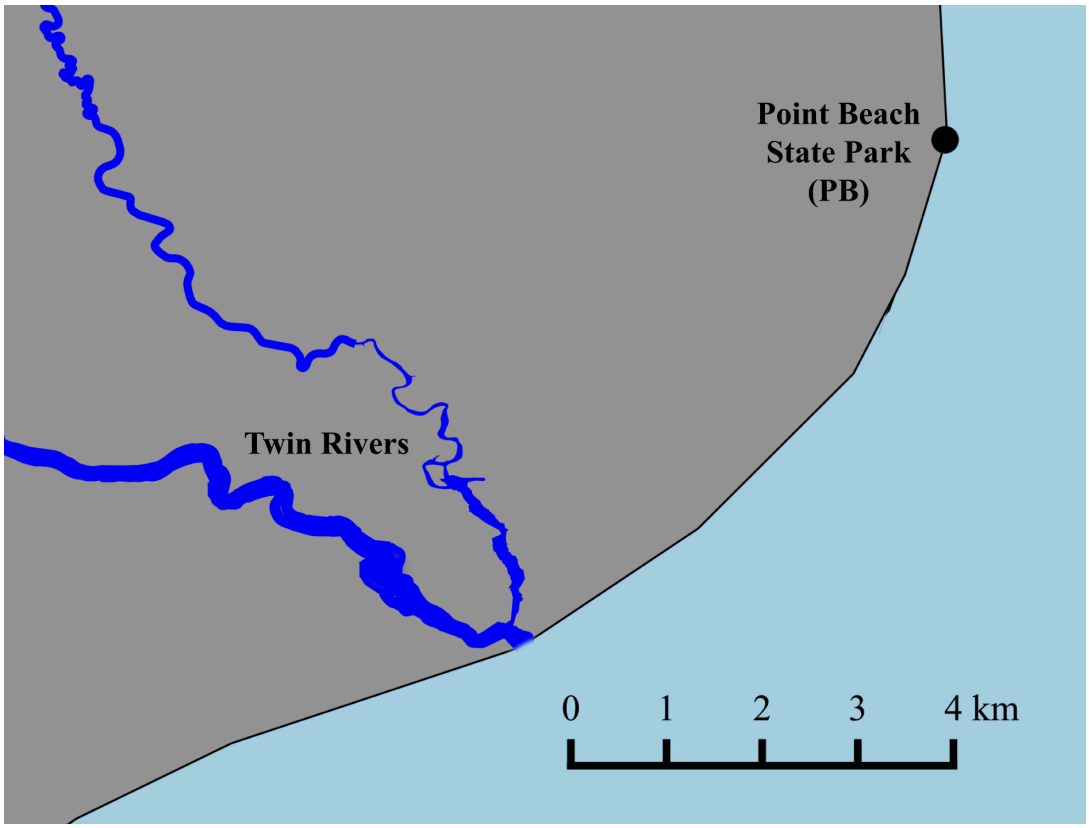

Figure 3. Map of Point Beach in proximity to the Twin Rivers in Wisconsin. Background map ©OpenStreetMap contributors http://www.openstreetmap.org/copyright

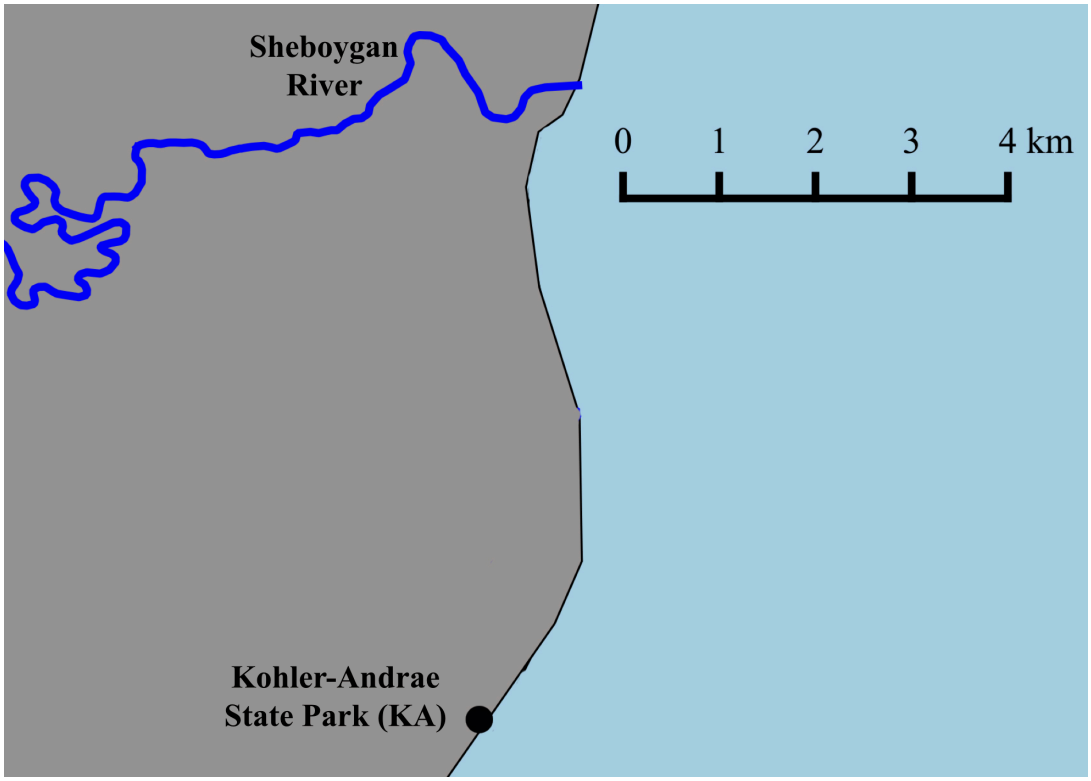

Figure 4. Map of Kohler-Andrae Beach adjacent to the Sheboygan River in Wisconsin. Background map ©OpenStreetMap contributors http://www.openstreetmap.org/copyright.

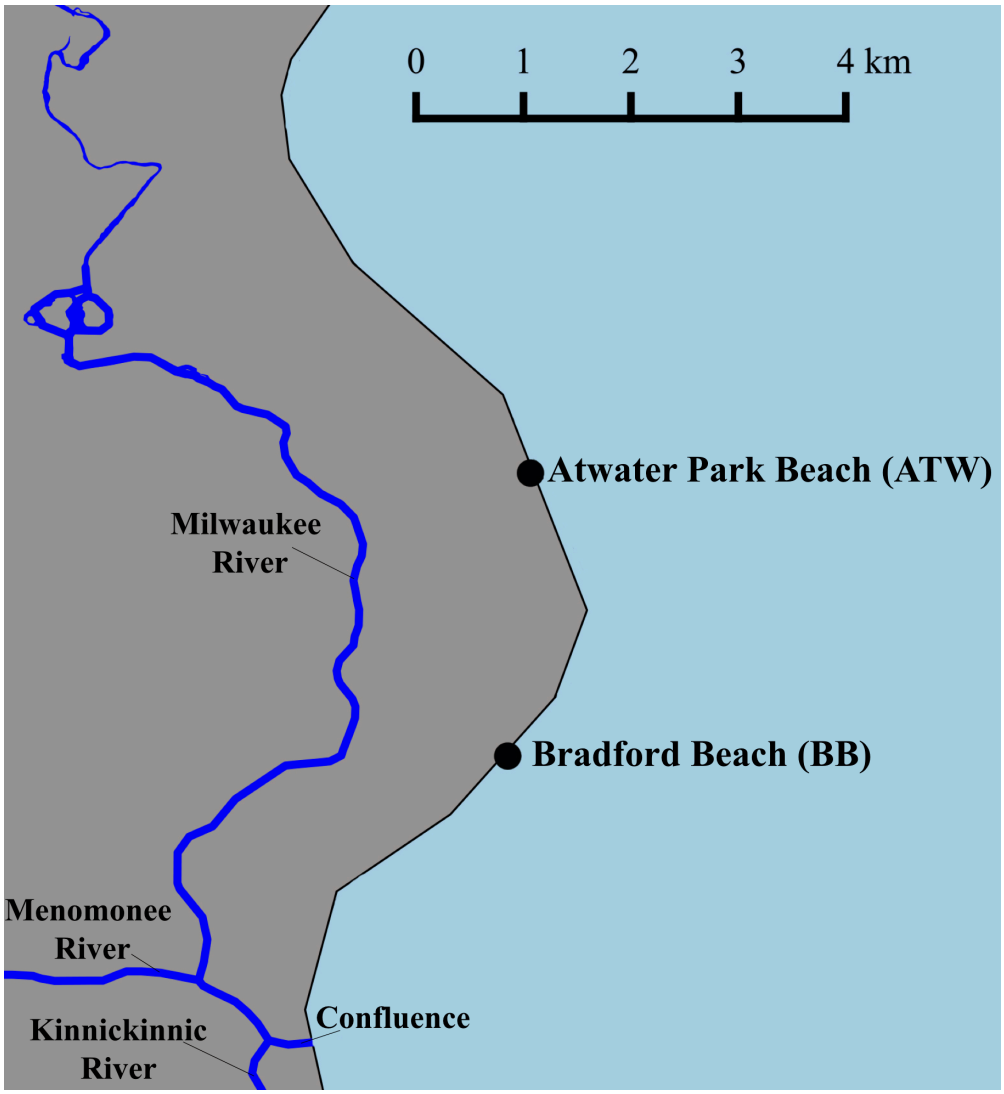

Figure 5. Map of Atwater and Bradford beaches in proximity to the Milwaukee, Menomonee, and Kinnickinnic River confluence in Wisconsin. Background map ©OpenStreetMap contributors http://www.openstreetmap.org/copyright.

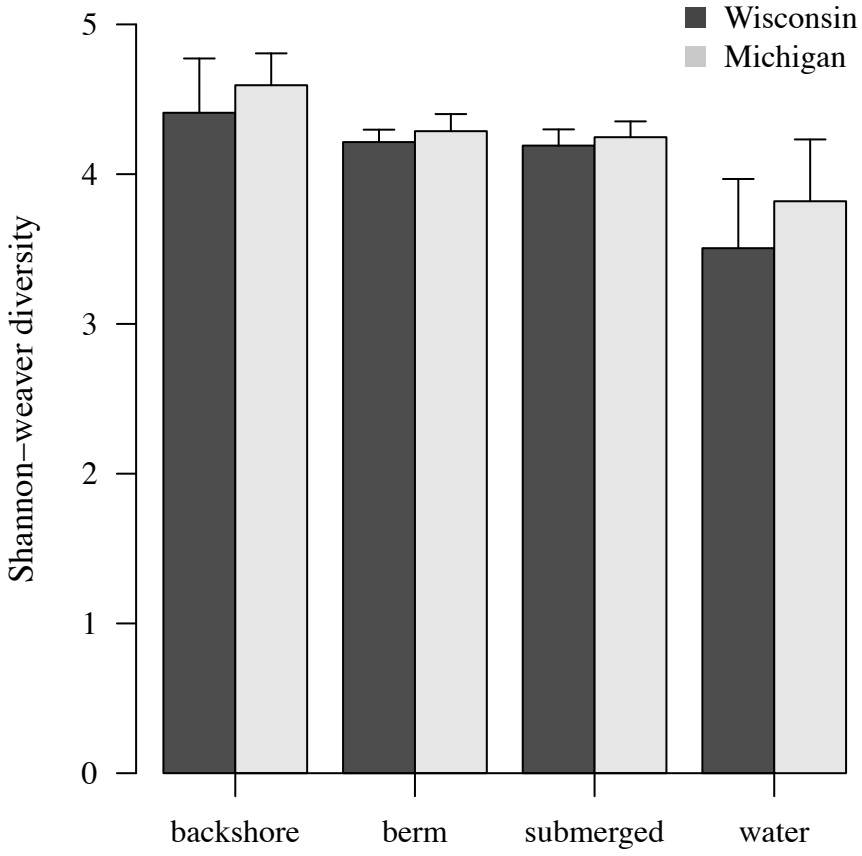

Figure 6. Averaged Shannon diversity measurements in backshore, berm, submerged and water samples. Error bars represent the standard deviation in diversity measured.

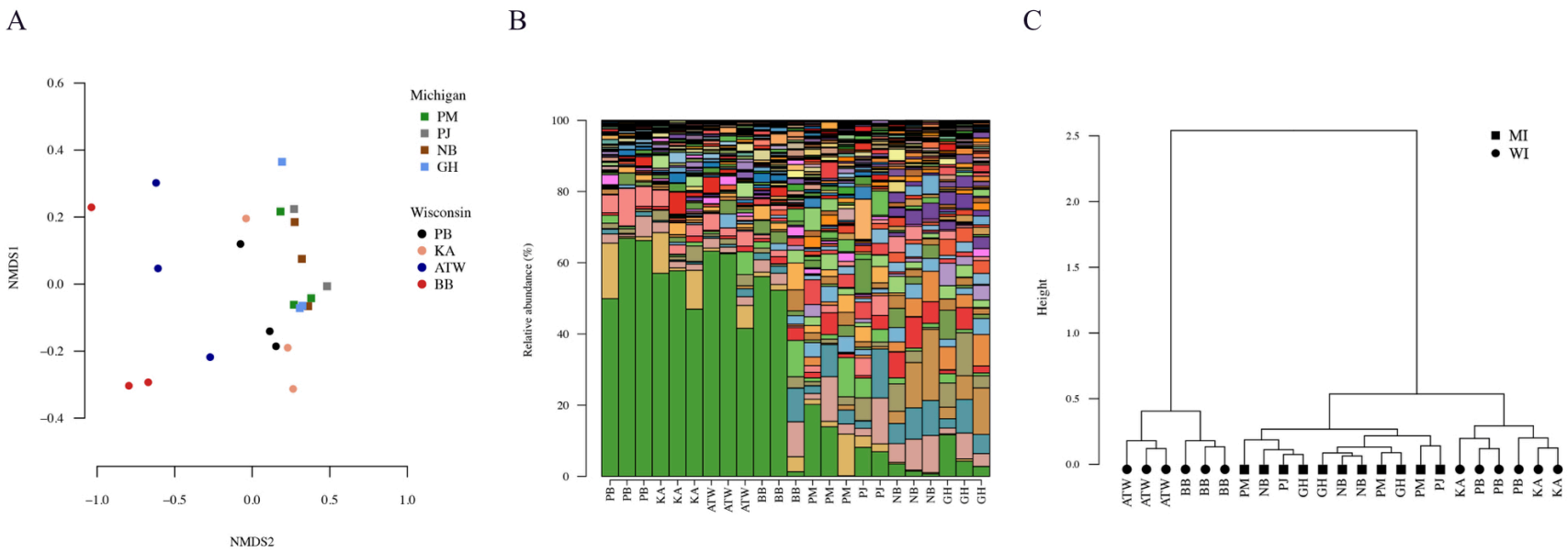

Figure 7. Oligotypes generated from the genus *Chloroacidobacterium* in all berm samples. NMDS based on Bray-Curtis distance matrix (A). Oligotype relative abundance (B). Oligotype dendrogram, produced using the ward-method and Bray-Curtis distance matrix (C).

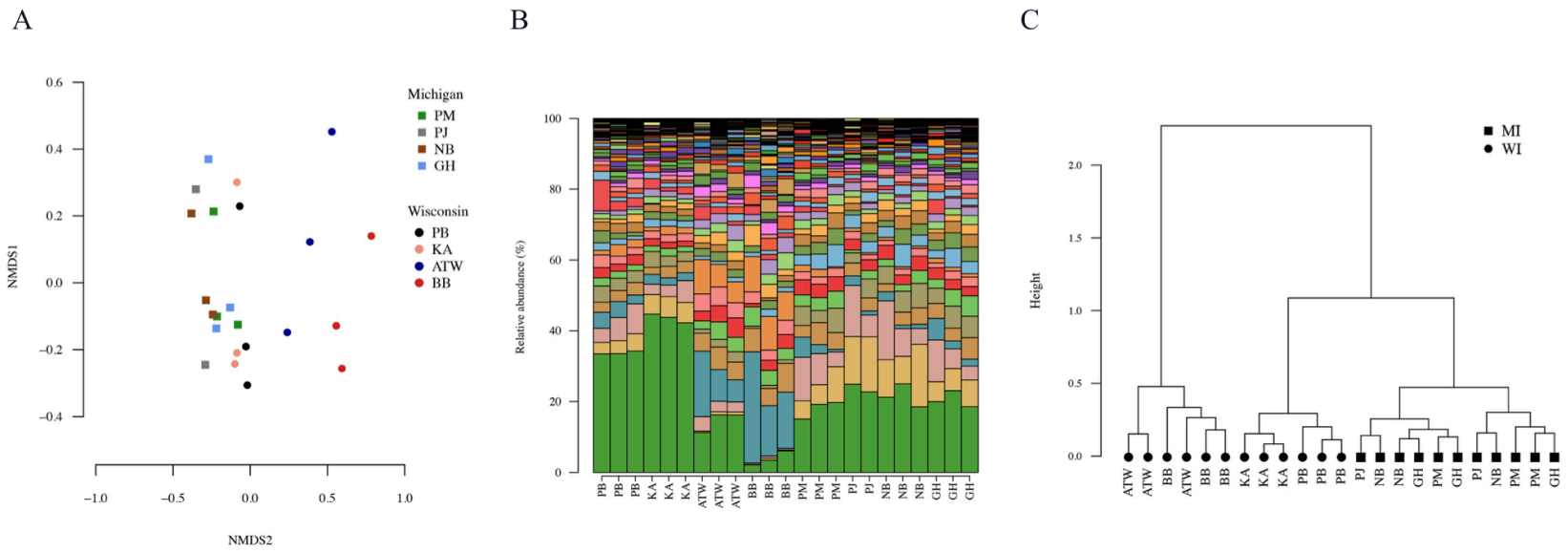

Figure 8. Oligotypes generated from the genus *Piruella* in all berm samples. NMDS based on Bray-Curtis distance matrix (A). Oligotype relative abundance (B). Oligotype dendrogram, produced using the ward-method and Bray-Curtis distance matrix (C).

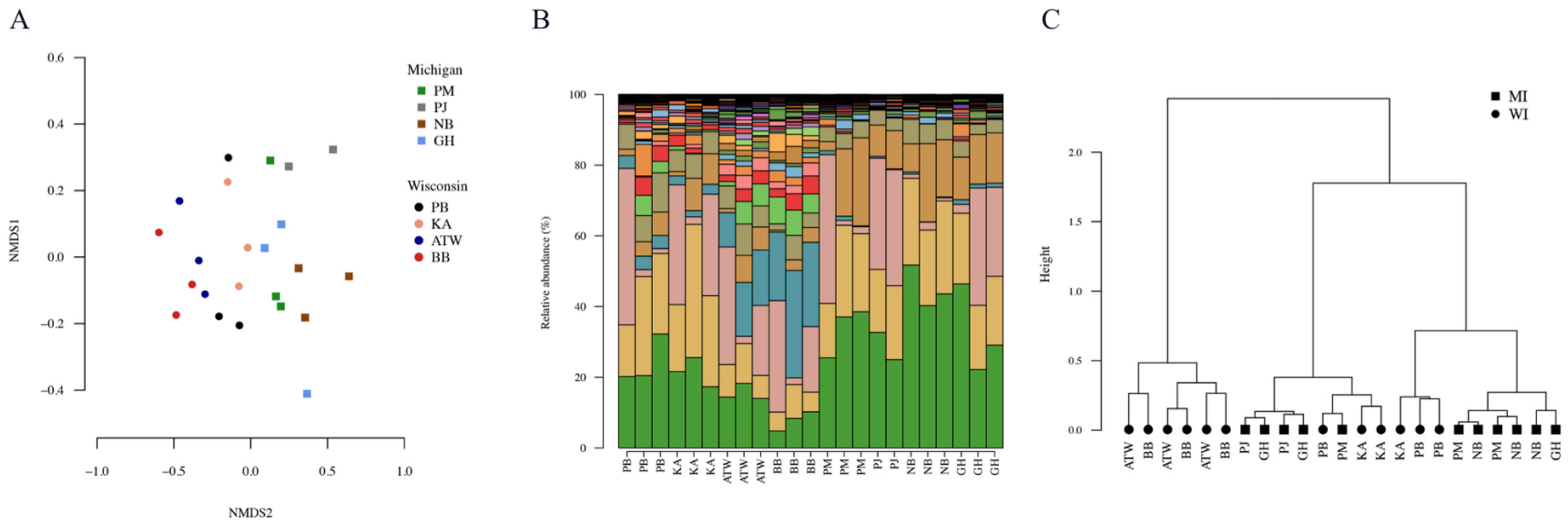

Figure 9. Oligotypes generated from the genus *Methylibium* in all berm samples. NMDS based on Bray-Curtis distance matrix (A). Oligotype relative abundance (B). Oligotype dendrogram, produced using the ward-method and Bray-Curtis distance matrix (C).

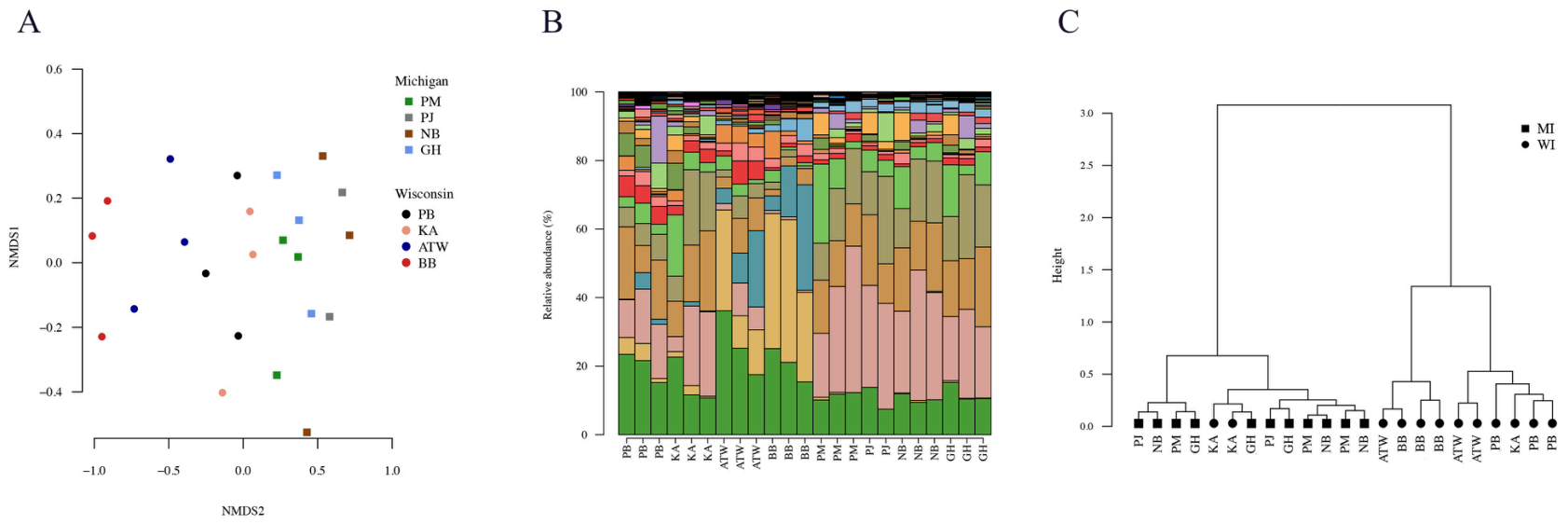

Figure 10. Oligotypes generated from the genus *Rhodoferax* in all berm samples. NMDS based on Bray-Curtis distance matrix (A). Oligotype relative abundance (B). Oligotype dendrogram, produced using the ward-method and Bray-Curtis distance matrix (C).

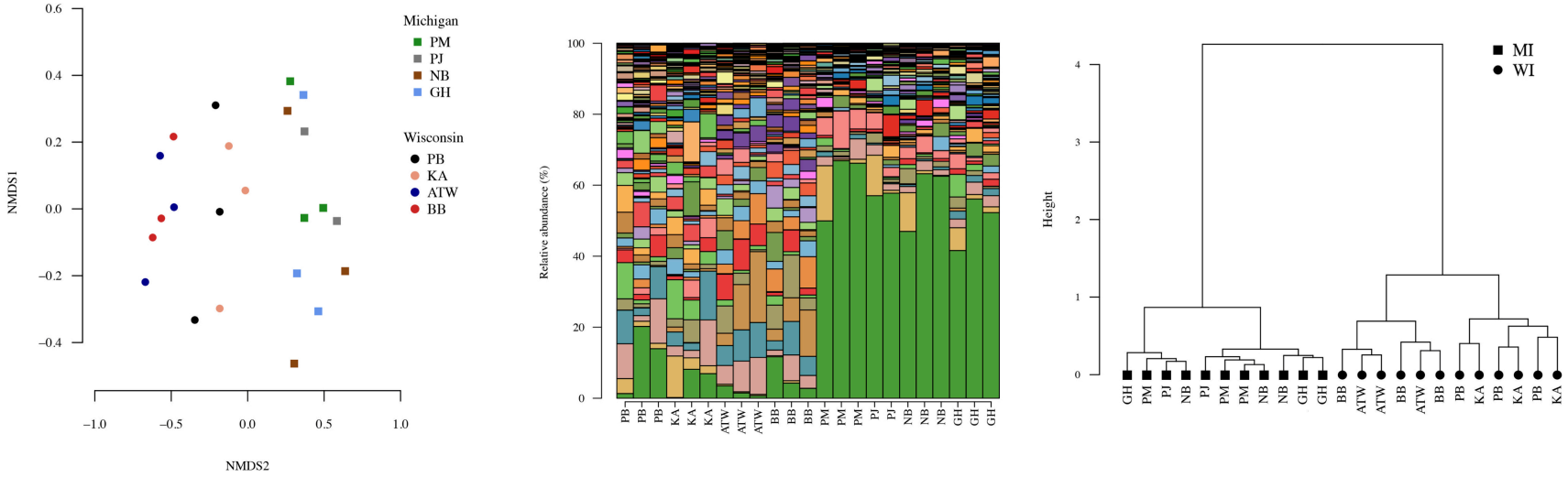

Figure 11. Oligotypes generated from the genus *Flavobacterium* in all berm samples. NMDS based on Bray-Curtis distance matrix (A). Oligotype relative abundance (B). Oligotype dendrogram, produced using the ward-method and Bray-Curtis distance matrix (C).

Table 1. Wisconsin and Michigan site descriptions.

State	Beach	Town	GPS Coordinates		Land-use	Closest River	Length (m)
Wisconsin	Point Beach State Park	Two Rivers	44.210381	-87.507676	Non-urban	Two Rivers	508
	Kohler-Andrae State Park	Sheboygan	43.667208	-87.715903	Non-urban	Sheboygan River	268
	Atwater Park Beach	Shorewood	43.09006	-87.872943	Urban	Milwaukee Confluence[a]	374
	Bradford Beach	Milwaukee	43.06176	-87.87473	Urban	Milwaukee Confluence[a]	984
Michigan	Pere Marquette Park Beach	Muskegon	43.2224	-86.336492	Urban	Muskegon River	933
	PJ Hoffmaster State Park	Muskegon	43.126692	-86.275615	Non-urban	Grand Haven	509
	North Beach Park	Ferrysburg	43.082651	-86.254274	Non-urban	Grand Haven	404
	Grand Haven City Beach	Grand Haven	43.054332	-86.247653	Urban	Grand Haven	494

[c]Confluence of the Milwaukee, Menomonee, and Kinnickinnic Rivers

Appendix B. Supplemental Material for Chapter 3

Gull, sewage influent, and ruminant fecal sample information

Fresh gull fecal samples (n = 22) were collected aseptically with sterile spatulas from parking lot surfaces at Bradford Beach (Milwaukee, WI), Grant Park (South Milwaukee, WI), and Point Beach State Park (Manitowoc, WI) during 2012-13. Samples were collected within 2 to 3 min after deposition to prevent overgrowth with non-fecal bacteria. The collected samples were stored in sterile 2 ml tubes, transported to the laboratory within 2 h, and stored at 80 °C. Forty-two raw influent samples were collected at the South Shore Water Reclamation Facility between 2008-2013. Six fecal samples were collected from dairy cows in Janesville and Franklin Wisconsin in February 2015.

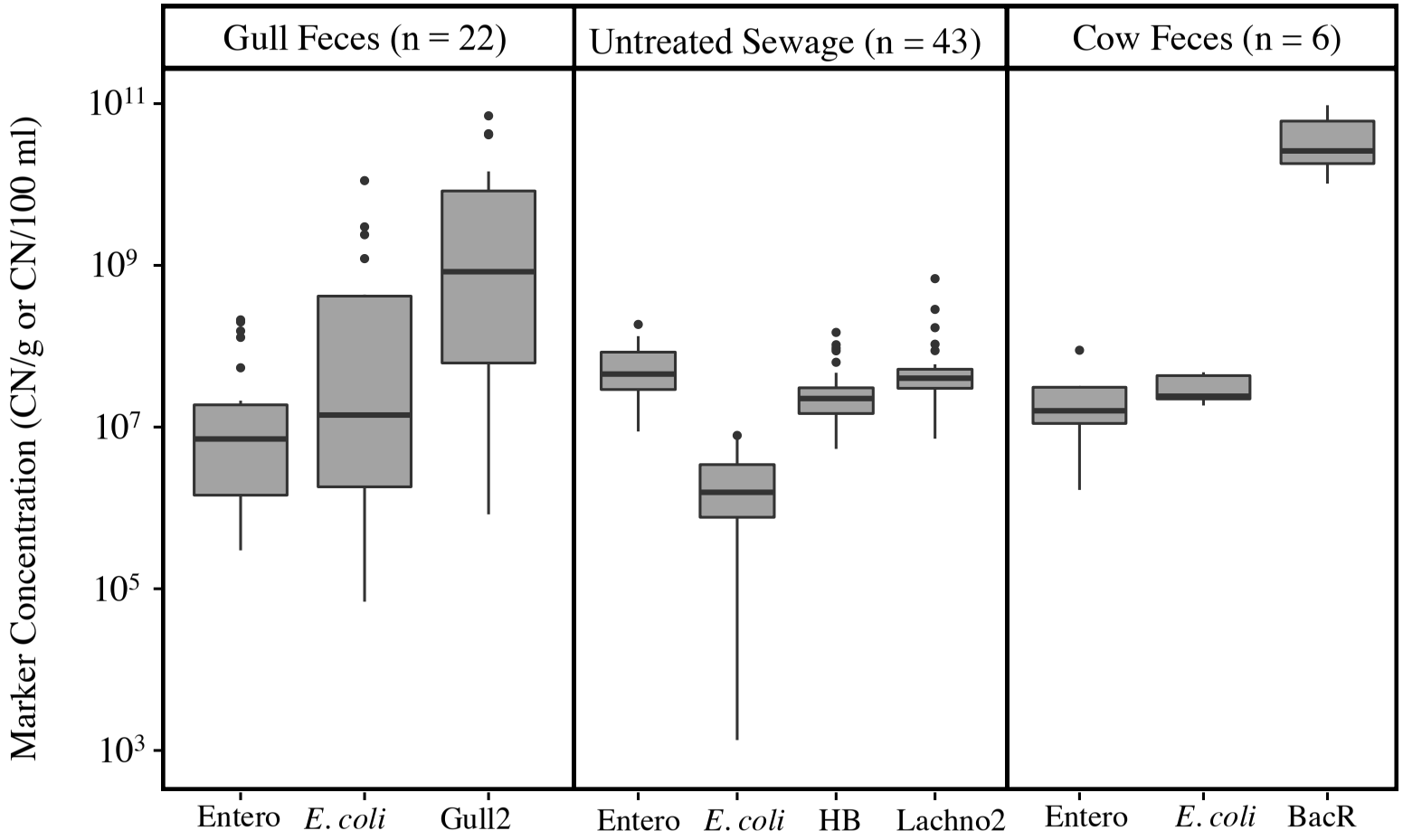

Figure 1. Alternative and traditional fecal indicator concentrations in gull, untreated sewage, and cow fecal samples.

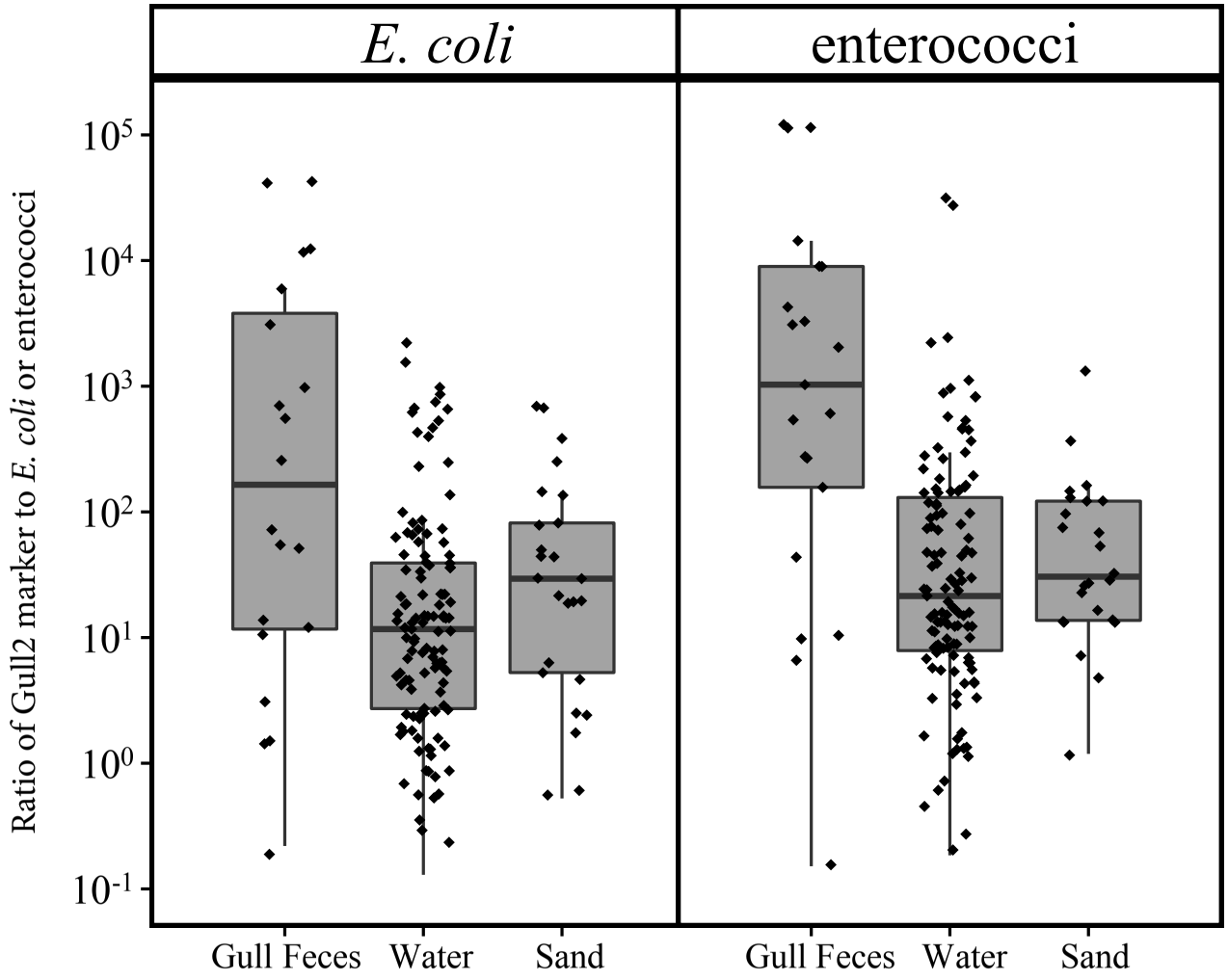

Figure 2. Ratios of the Gull2 marker to traditional fecal indicator bacteria in gull feces (n = 22), water (n = 119), and sand (n = 25) samples beach environmental samples

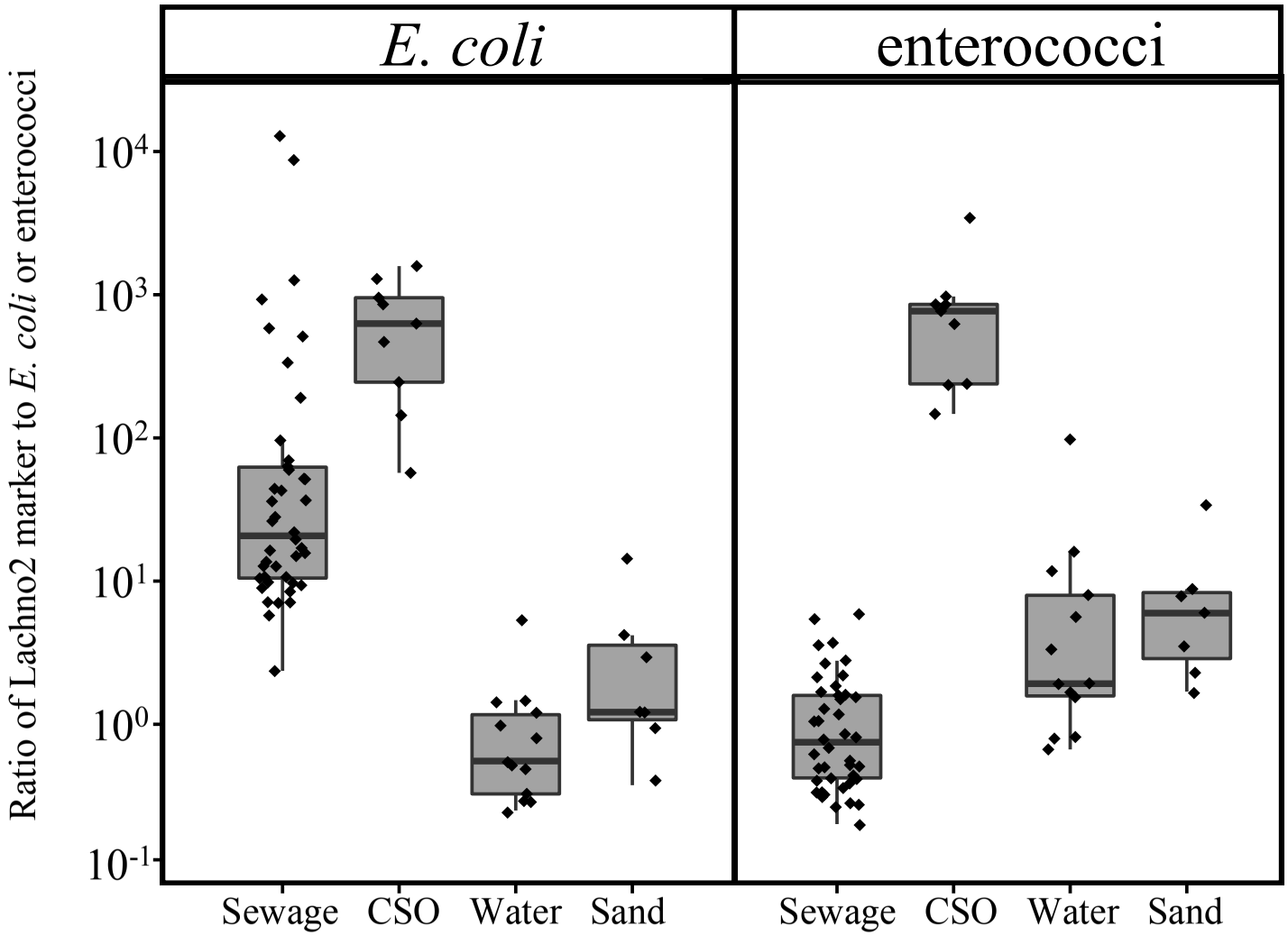

Figure 3. Ratios of the Lachno2 marker to traditional fecal indicator bacteria in untreated sewage (n = 43), CSO (n = 9), water (n = 13), sand (n = 7) samples.

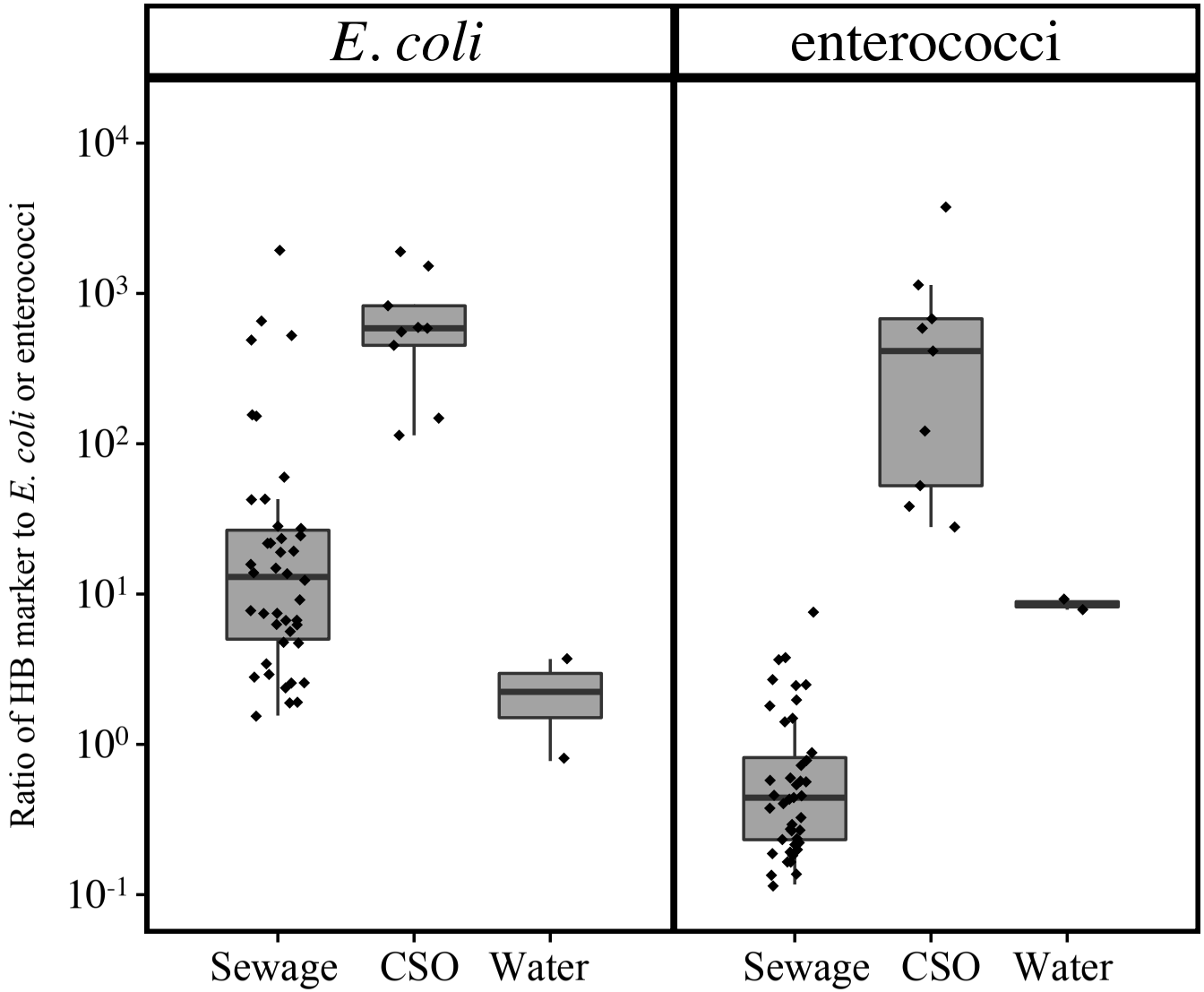

Figure 4. Ratios of the HB marker to traditional fecal indicator bacteria in untreated sewage (n = 43), CSO (n = 9), and water (n = 2) samples.

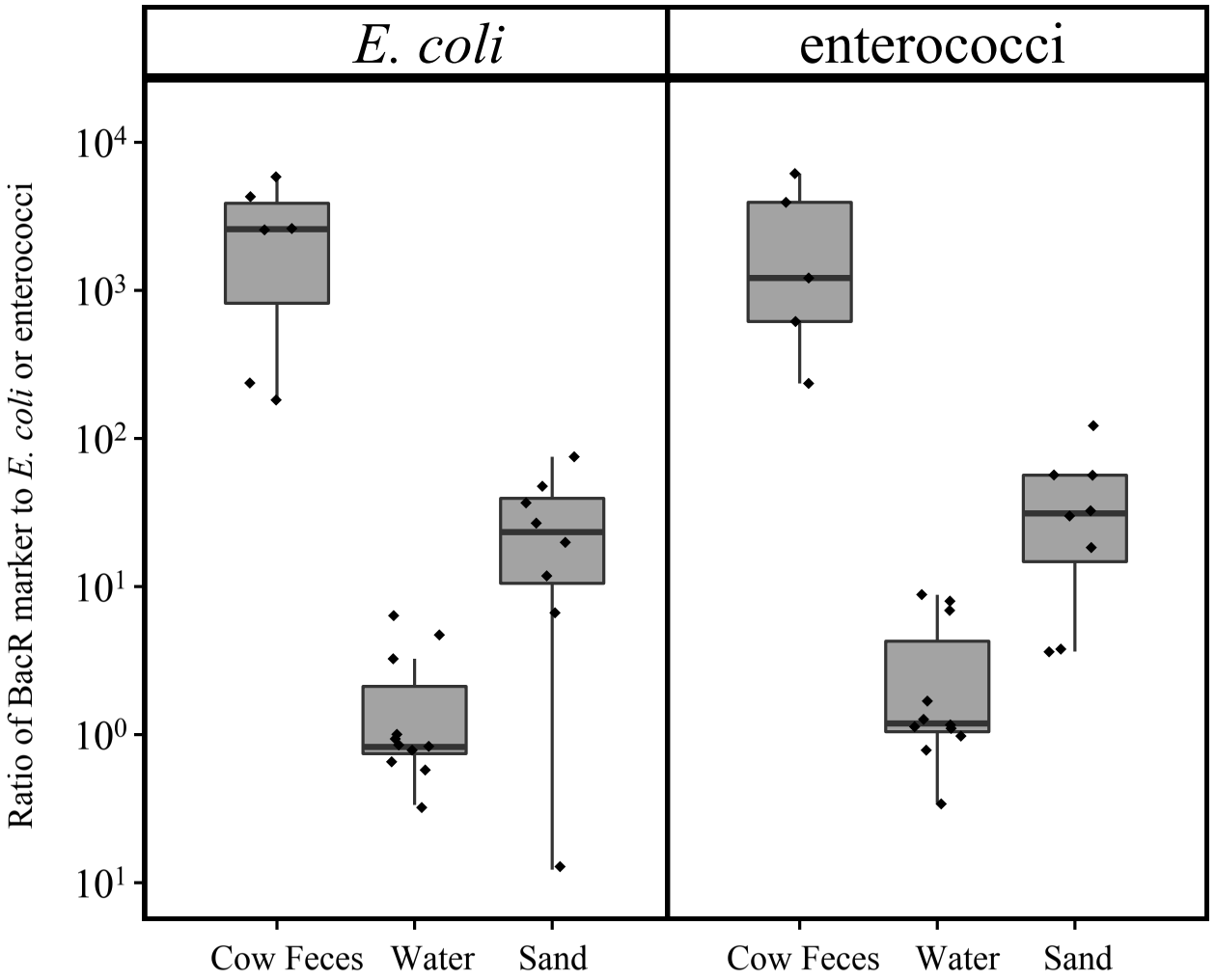

Figure 5. Ratios of the BacR marker to traditional fecal indicator bacteria in cow feces (n = 6), water (n = 11), and sand (n = 8) samples.

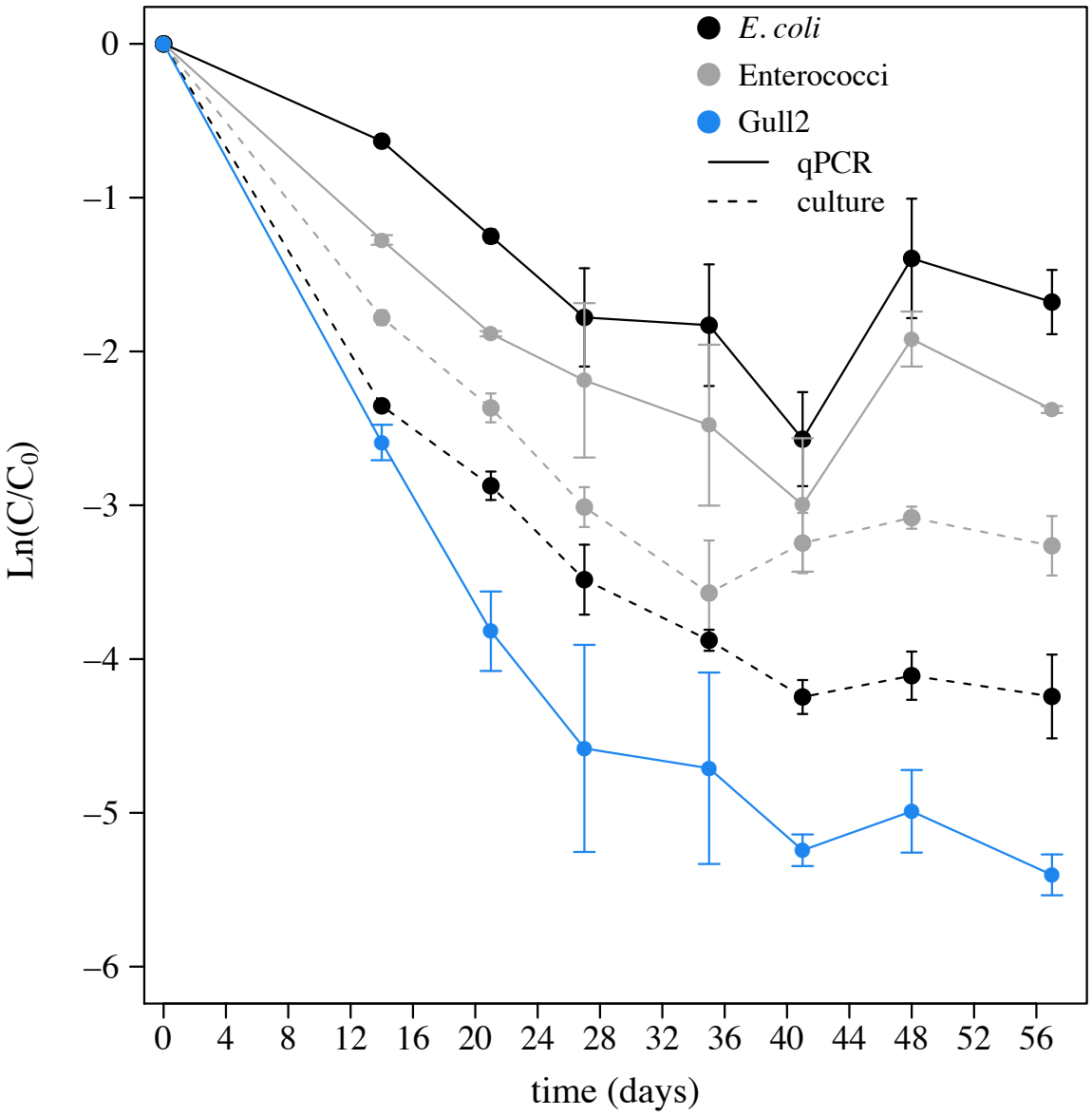

Figure 6. Decay of qPCR and culturable indicators during the gull microcosm experiment. Error bars indicate the standard deviation about the mean.

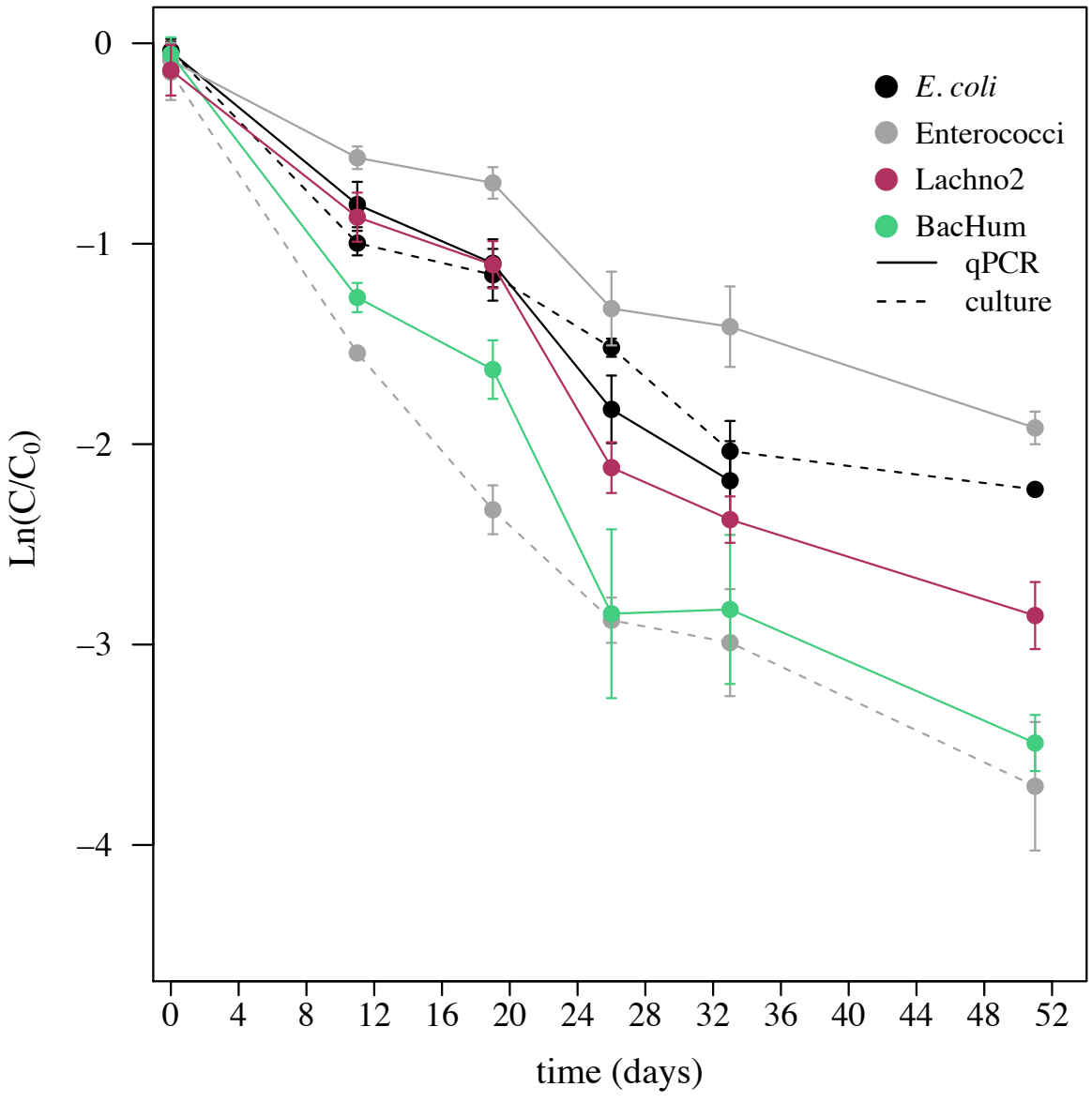

Figure 7. Decay of qPCR and culturable indicators during the gull microcosm experiment. Error bars indicate the standard deviation about the mean.

Table 1. GPS coordinates of beach sampling sites, sampling frequency, total samples collected and, samples analyzed with qPCR assays.

Beach	Coordinates	Days Sampled	Samples Collected			qPCR Samples (High *E. coli*)[a]		qPCR Samples (Low *E. coli*)[1]		qPCR Samples Post-CSO
			Water	Berm	Backshore	Water	Paired Sand	Water	Paired Sand	Water
BV	42 59.163, 87 52.044	14	39	36	12	11	9	1	1	6
BB	43 03.726, 87 52.332	23	68	58	33	10	9	5	5	6
ATW	43 05.455, 87 52.389	22	87	80	44	17	16	14	13	8
DP	43 10.224, 87 52.843	12	48	47	16	12	12	5	5	NA
KA	43 39.510, 87 43.258	11	89	93	39	19	14	17	10	NA
PB	44 13.117, 87 30.406	11	91	93	39	15	9	18	7	NA
Total		**30**	**422**	**407**	**183**	**84**	**69**	**60**	**41**	**20**

[a.] High *E. coli*: ≥ 235 in water; Low *E. coli:* < 235 *E. coli* in water.

Table 2. The nucleotide sequences of primers and probes that used for quantification polymerase chain reaction (qPCR) assays.

Assay	Target	Source	Primer/Probe (5'to 3')		Reference
E. coli	*E. coli*	General	uidA-1663F:	GCGACCTCGCAAGGCATA	Li 2006
			uidA-1790R:	GATTCATTGTTTGCCTCCCTGCTGCG	Li 2006
			uidA-1729p:	[6FAM] TGCAGCAGAAAAGCCGCCGACTTCGG [MGBNFQ]	Li 2006
Entero	*Enterococcus*	General	Entero23S-F:	AGAAATTCCAAACGAACTTG	Ludwig 2000
			Entero23S-R:	CAGTGCTCTACCTCCATCATT	Ludwig 2000
			Entero23S-p:	[6FAM] TGGTTCTCTCCGAAATAGCTTTAGGGCTA [MGBNFQ]	Ludwig 2000
Lachno2	*Lachnospiraceae*	Sewage	CcocR-F:	TTCGCAAGAATGAAACTCAAAG	Newton 2011
			Lachno2-R:	AAGGAAAGATCCGGTTAAGGATC	Newton 2011
			Lachno2-p:	[6FAM] ACCAAGTCTTGACATCCG [MGBNFQ]	Newton 2011
HB	*Bacteroides*	Sewage	HF-183F:	ATCATGAGTTCACATGTCCG	Bernhard 2000
			BacHum-241R:	CGTTACCCCGCCTACTATCTAATG	Kildare 2007
			BacHum-193p:	[6FAM] TCCGGTAGACGATGGGGATGCGTT [MGBNFQ]	Kildare 2007
Gull2	*C. marimammalium*	Gull	Gull2-F:	TGCATCGACCTAAAGTTTTGAG	Lu 2008
			Gull2-R:	GTCAAAGAGCGAGCAGTTACTA	Lu 2008
			Gull2-p:	[6FAM] CTGAGAGGGTGATCGGCCACATTGGGACT [MGBNFQ]	Sinigalliano 2013
BacR	*Bacteroidetes*	Ruminant	BacR_F:	GCGTATCCAACCTTCCCG	Reischer 2006
			BacR_R:	CATCCCCATCCGTTACCG	Reischer 2006
			BacR_p:	[6FAM] CTTCCGAAAGGGAGATT [MGBNFQ]	Reischer 2006

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2. **Ludwig W**, **Schleifer KH**. 2000. How quantitative is quantitative PCR with respect to cell counts? Systematic and Applied Microbiology **23**:556–562.
3. **Newton RJ**, **Vandewalle JL**, **Borchardt MA**, **Gorelick MH**, **McLellan SL**. 2011. Lachnospiraceae and Bacteroidales alternative fecal indicators reveal chronic human sewage contamination in an urban harbor. Applied and Environmental Microbiology **77**:6972–81.
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5. **Kildare BJ**, **Leutenegger CM**, **McSwain BS**, **Bambic DG**, **Rajal VB**, **Wuertz S**. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research **41**:3701–3715
6. **Lu J**, **Santo Domingo JW**, **Lamendella R**, **Edge T**, **Hill S**. 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Applied and Environmental Microbiology **74**:3969–3976.
7. **Sinigalliano CD**, **Ervin JS**, **Van De Werfhorst LC**, **Badgley BD**, **Ballesté E**, **Bartkowiak J**, **Boehm AB**, **Byappanahalli M**, **Goodwin KD**, **Gourmelon M**, **Griffith J**, **Holden PA**, **Jay J**, **Layton B**, **Lee C**, **Lee J**, **Meijer WG**, **Noble R**, **Raith M**, **Ryu H**, **Sadowsky MJ**, **Schriewer A**, **Wang D**, **Wanless D**, **Whitman R**, **Wuertz S**, **Santo Domingo JW**. 2013. Multi-laboratory evaluations of the performance of Catellicoccus marimammalium PCR assays developed to target gull fecal sources. Water Research **47**:6883–6896.
8. **Reischer GH**, **Kasper DC**, **Steinborn R**, **Mach RL**, **Farnleitner AH**. 2006. Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in alpine karstic regions. Applied and Environmental Microbiology **72**:5610–5614.

Table 3. Quantitative PCR (qPCR) assay slope, y-intercept, and efficiencies.

Assay	Slope	Intercept	R^2	Efficiency (%)
E. coli	-3.45	39.39	0.98	94.17
Entero	-3.43	39.32	0.99	95.76
Lachno2	-3.42	37.65	0.99	95.95
HB	-3.35	36.96	0.99	98.68
Gull2	-3.72	42.48	0.99	85.75
BacR	-3.46	40.15	0.99	94.52

Appendix C. Supplemental Material for Chapter 4

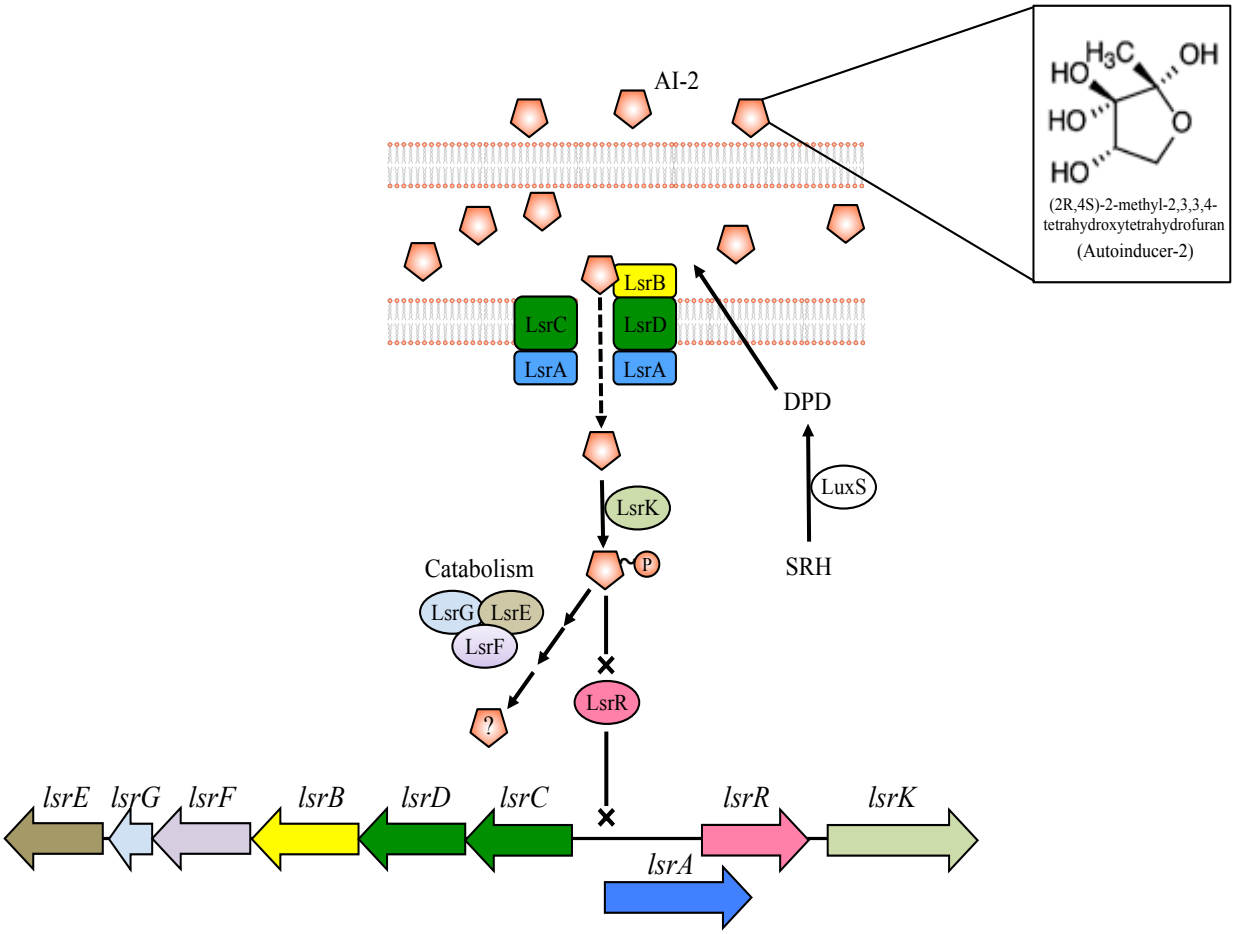

Figure 1. *lsrACDBFGE* operon in *E. coli* BB_Berm1. Figure was developed by Danielle Cloutier and was adapted from Vendeville et al. LsrE: Transaconitate 2-methyltransferase. LsrG: AI-2 modifying protein. LsrF: AI-2 aldolase. LsrB: AI-2 ABC transport system, periplasmic AI-2 binding protein. LsrD: AI-2 ABC transport system, membrane channel protein. LsrC: AI-2 ABC transport system, membrane channel protein. LsrA: AI-2 ABC transport system, fused AI-2 transporter subunits and ATP-binding component. LsrR: transcriptional repressor of *lsr* operon. LsrK: AI-2 kinase.

Curriculum Vitae

Danielle Cloutier

CURRICULUM VITAE

EDUCATION

University of Wisconsin-La Crosse Microbiology B.S., 2008-2012

DISSERTATION TITLE

"Microbial Communities and the Diverse Ecology of Fecal Indicators at Lake Michigan Beaches"

APPOINTMENTS

2016 Water Resource Specialist Intern, Wisconsin Department of Natural Resources
2015 Co-chair on Microbial Source Tracking, WI Coastal Beaches Workgroup
2015 Steering Committee, WI Coastal Beaches Workgroup
2013 Founder and President, American Society for Microbiology at UW-Milwaukee
2012-2016 Graduate Research Assistant, Dr. Sandra McLellan PI, UW-Milwaukee
2011-2012 Vice President, American Society of Microbiology at UW-La Crosse
2010-2011 Secretary, American Society of Microbiology at UW-La Crosse

HONORS & AWARDS

2017-2018 NOAA Sea Grant John A. Knauss Marine Policy Fellowship, Legislative fellow, Senator Brian Schatz (Hawaii)
2016 Graduate Student Excellence Fellowship, UW-Milwaukee
2016 Distinguished Dissertation Award Nomination, UW-Milwaukee
2012-2016 Graduate Research Assistantship, UW-Milwaukee
2015-2016 Student Chapter Support Award, American Society for Microbiology
2014-2015 Student Chapter Support Award, American Society for Microbiology
2013 Graduate Student Travel Award, UW-Milwaukee
2012-2013 Dean's Graduate Student Award, School of Freshwater Sciences, UW-Milwaukee
2012 Department Senior of the Year, Dept. of Microbiology, UW-La Crosse
2011 Laboratory Teaching Assistantship, Dept. of Microbiology, UW-La Crosse
2011 Department of Microbiology Scholarship, UW-La Crosse
2011 Office of Undergraduate Research Travel Award, UW-La Crosse

PEER-REVIEWED PUBLICATIONS

Cloutier DD and McLellan SL. 2017. Distribution and Differential Survival of Traditional and Alternative Indicators of Fecal Pollution at Freshwater Beaches. Applied and Environ Microbiol. 83: 1-16.

Cloutier DD, Alm EW, and McLellan SL. 2015. The Influence of Land-use, Nutrients, and Geography on Microbial Communities and Fecal Indicator Abundance at Lake Michigan Beaches. Applied and Environ Microbiol. 81:4904-4913.

LECTURES & CONFERENCE PRESENTATIONS

Cloutier DD and McLellan SL. 2015. Integrating Microbial Source Tracking into Beach Monitoring. WI Coastal Beaches Workgroup: Annual Meeting. Milwaukee, WI.

Cloutier DD and McLellan SL. 2015. Microbial Source Tracking and the Relationship of Sand to Degraded Water Quality. American Society for Microbiology (ASM) National Conference, New Orleans, LA.

Cloutier DD and McLellan SL. 2015. Investigating Sources of Fecal Pollution at Beaches With The Use Of Microbial Source Tracking. Wisconsin Beach Health: Past, Present and Future Meeting. Madison, WI.

Cloutier DD, Alm EW, and McLellan SL. 2014. Microbial Community Structure of Freshwater Beach Sand And Relationship to Fecal Indicator Densities. ASM National Conference, Boston, MA.

Cloutier DD and McLellan SL. 2013. The Effect of the Indigenous Microbial Community on the Persistence of Fecal Indicator Bacteria in Lake Michigan Beach Sand. International Association for Great Lakes Research Conference, West Lafayette, IN.

Cloutier DD, VandeWalle JL, and McLellan SL. 2013. Distribution Of *Bacteroidales* Relative To Traditional Markers Of Fecal Pollution From Water To Backshore Sand At Two Urban Lake Michigan Beaches. ASM National Conference, Denver, CO.

Cloutier DD and Bratina BJ. 2012. The Microflora of *A. fasciatus* Across Geographic Boundaries. ASM National Conference. San Francisco, CA.

Cloutier DD and Bratina BJ. 2012. The Microflora of *A. fasciatus* Across Geographic Boundaries. UW-La Crosse Undergraduate Research and Creativity Symposium, La Crosse, WI.

RESEARCH/PROJECT CONTRIBUTION ACKNOWLEDGEMENTS

- Templar HA et al. 2016. Quantification of Human-associated Fecal Indicators Reveal Sewage from Urban Watersheds as a Source of Pollution to Lake Michigan. Water research. 100:556-567.
- Fisher JC et al. 2015. Comparison of Sewage and Animal Fecal Microbiomes by Using Oligotyping Reveals Potential Human Fecal Indicators in Multiple Taxonomic Groups. . Applied and Environ Microbiol. 20:7023-7033.
- Newton RJ et al. 2015. Sewage Reflects the Microbiomes of Human Populations. MBio. 6.2:e02574-14.
- McLellan SL and Eren AM. 2014. Discovering New Indicators of Fecal Pollution. Trends in microbiology. 12:697-706.

PUBLISHED INTERVIEWS /MEDIA COVERAGE

- UW Sea Grant (YouTube). On-camera. 2016. “Wisconsin's 2017 Knauss Fellow: Danielle Cloutier.”
- Milwaukee Journal Sentinel. Print. 2016. “UWM Professor Taking a Deeper Dive on Beach Pollution Data.”
- ABC WISN12. On-camera. 2015. “Milwaukee Beach Closings Double, Advisories Up.”
- Urban Microbial Ecology Blog. 2015. “Effects of Environmental Factors On Sand Microbial Communities.”
- ABC WISN12. On-camera. 2014. “Growing Concerns Over Contamination Underground.”
- Milwaukee Journal Sentinel. 2014. Print. "Not as Bad as the '70s, But Alewives Are Piling Up on the Beaches Again.”

VOLUNTEER & ACADEMIC INVOLVEMENT

- Spring River Cleanup Volunteer (Milwaukee River), Milwaukee Riverkeeper, April 2016.
- Science Demonstrator/Guide for the Lake Sturgeon Bowl Competition, Feb 2016.
- UW-Milwaukee Science Bag Group Demonstrator, Jan 2016.
- Meal Preparation Volunteer, Milwaukee Hope House Shelter, Oct 2015.
- Society for Freshwater Science Conference Volunteer, May 2015.
- UW System Symposium for Undergraduate Research Poster Judge, Apr 2015.
- Sweet Water Clean Rivers, Clean Lake Conference Attendee, Apr 2015.
- Invited Science Speaker/Educator for the Girl Scouts of the USA, Feb 2015.
- Science/Math Tutor at Milwaukee Rescue Mission Homeless Shelter, 2014-2015.
- Expert Panelist at the Wisconsin Beach Health: Past, Present and Future Meeting, Nov 2015.
- Nominated Student Representative for the USEPA Administrator Visit at UW-Milwaukee, Oct 2014.
- Alliance for the Great Lakes Adopt-A-Beach Volunteer, Jun 2014.
- UN World Water Day Run-4 Water Milwaukee Volunteer, Apr 2014.
- Science Demonstrator/Guide for the Lake Sturgeon Bowl Competition, Feb 2014.
- Adopt-A-Family Program Volunteer, Journey House Shelter Milwaukee, Dec 2013.
- Great Lakes Beach Association Conference Attendee, Oct 2013.
- Science Demonstrator/Guide for the Lake Sturgeon Bowl Competition, Feb 2013.
- US Fish and Wildlife Service, Fish Health Center Laboratory Volunteer, 2011-2012.
- UW-La Crosse Undergraduate Researcher, 2010-2012.

ACADEMIC & PROFESSIONAL TRAINING

- Quantum GIS Data Curation and Manipulation, 2015, UW-Milwaukee held in Milwaukee.
- Microbial Whole Genome Analysis, 2015, Univ of Maryland Sch of Med held in New Orleans.
- Quantum GIS Geographic Information Systems, 2015, UW-Milwaukee held in Milwaukee.
- Statistics/Computing for Microbial Ecologists", 2014, UM Dept of Microbiol & Immunol held in Detroit.
- Analyses of Microbial Community Composition/Metagenomics, 2014, Univ. of Colorado held in Boston.
- Molecular Typing of Bacterial Pathogens, 2013, Univ. Hosp. Munster held in Denver.

ACADEMIC & PROFESSIONAL AFFILIATIONS

2016	American Geophysical Union
2015-2016	Wisconsin Coastal Beaches Workgroup
2013-2016	American Society for Microbiology Student Chapter at UW-Milwaukee
2013-2014	International Association for Great Lakes Research
2012-2016	Milwaukee Microbiology Society
2011-2016	American Society for Microbiology
2008-2012	American Society for Microbiology Student Chapter at UW-La Crosse