The Distribution and Persistence of Genetic Markers of Fecal Pollution on Lake Superior Beaches

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DEDICATION

I dedicate this thesis to my husband, Erik Smith, and to my parents, Steven and Jacklyn Eichmiller, for never once questioning the purpose of 12 years of college.

ABSTRACT

Fecal contamination of surface waters is a widespread environmental problem and a public health concern. The presence and degree of fecal contamination in surface waters is based on the abundance of culturable fecal indicator bacteria (FIB), such as *Escherichia coli* or *Enterococcus* spp. Culture-based methods, however, require 18 to 48 hr to process and are unable to determine sources and assess risk in real time. Advances in molecular methods has led to the development of several promising "real time" detection assays that quantify the abundance of genetic markers for FIB, but their distribution and persistence in freshwater environments is not well-studied. This work explores the use of rapid, culture-independent methods for the identification of fecal pollution. In particular, the application of rapid tools is considered for freshwater beaches, particularly those of Lake Superior. In this thesis I characterized the short time scale variation in goose/duck and human sources of fecal contamination, *Salmonella* spp., and pathogenic *E. coli* at three Duluth area beaches, examined the distribution of genetic markers of fecal pollution in sand and sediment of a Duluth-Superior Harbor beach near a wastewater outfall, measured the effects of temperature and moisture on the persistence of genetic markers in water, sand, and sediment, and compared the decay rates of genetic markers of fecal pollution and bacterial pathogens. Goose/duck-borne *E. coli* consistently made up 12 to 29% of *E. coli* at Duluth-Superior Harbor sites and 5% of *E. coli* at a Lake Superior beach. In contrast, a human-specific *Bacteroides* genetic marker exhibited high temporal variability, and was detected at great frequency in the water column at a site located on the inner harbor. *Salmonella* spp. and potentially pathogenic *E. coli* were

infrequently detected at the study beaches. At a beach near a wastewater treatment plant outfall, effluent loading likely controlled the abundance of molecular indicators of fecal pollution in the water column. The concentration of enterococci and human-specific *Bacteroides* genetic markers in the water column was correlated to the abundance of genetic markers at some depths in sand and sediment. Sand and sediment contained more enterococci and total *Bacteroides* genetic markers on a per mass basis than water, whereas the concentration of human-specific *Bacteroides* was similar across sample types. In most instances, genetic markers were most abundant in the top 1 to 3 cm of sand and sediment. The decay of genetic markers of fecal pollution in sand and sediment was slow relative to the water column, and some genetic markers persisted or increased over time within sand and sediment. Molecular indicators decayed more rapidly at higher temperatures in all sample types and this decay was negatively correlated with sand moisture. The genetic marker for human-specific fecal contamination exhibited decay rates similar to markers for bacterial pathogens in sand, whereas non-source-specific markers decayed more slowly than bacterial pathogen markers under most conditions. Taken together, the relative sources of contamination, beach location, and other site-specific factors, such as the potential for resuspension of sand and sediment and pathogen abundance, should be considered in the choice of genetic markers for water quality monitoring on freshwater beaches.

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CHAPTER 1:

General Introduction

FECAL CONTAMINATION OF RECREATIONAL WATERS

Fecal contamination of surface waters is a widespread environmental problem and a public health concern. Contamination by fecal material results from deposition by wildlife, agricultural runoff, inadequate sewage treatment, or faulty septic systems (71, 84, 170). Waters contaminated by fecal matter can contain a wide range of infectious agents, exposure to which can lead to illness or death. Therefore, water bodies contaminated by fecal material are temporarily or permanently restricted for use by the public. The presence of fecal contamination is the second leading cause of beneficial use impairment in the United States in accordance with Section 303(d) of the Clean Water Act (169). Nationwide, lotic waters, bays, and estuaries are most affected, with 82,000 miles of impaired creeks, streams, and rivers and 2,300 square miles of impaired bays and estuaries (169). However, the scale of waters impaired by fecal pollution is undoubtedly much greater, as not all water bodies are subject to state monitoring efforts.

Nearly 14,000 incidences of waterborne illness were confirmed for a two year period in the most recent report from the Center for Disease Control and Prevention (CDCP) (37). However, this number represents only a small proportion of the total population affected by microbial disease from recreational water use, as illness often goes unreported. The CDCP also concluded that waterborne illness has been on the rise over the past several decades, and the 2007 to 2008 reporting period confirmed the highest number of waterborne disease outbreaks in the United States to date. This trend will likely continue due to the effects of climate change. Increased climate variability and

extreme precipitation events, both effects of climate change, are associated with increased presence of waterborne pathogens in marine and freshwaters (131, 141).

Fecal contamination resulting in beach closures can result in substantial economic losses. Beach closures can result in economic loss to the state or local community through decreased tourism, loss in consumer value of local resources, and health costs. On a Lake Michigan beach, closures were estimated to result in a net economic loss of $1,300 to $37,000 per day (138). On California beaches, individuals valued a day visit to beaches at an average of $28 (104). On marine beaches, the economic revenue can range from $5 to $3,500 per person per day (56). In addition, the cost associated with treatment of gastrointestinal illness from recreational water use was estimated at $37 per incidence, and total cost of illnesses acquired from beach use was estimated between $21 to $51 million per year (49). The cost of beach closures could increase, as the number of beach closures has increased over the past decade (Fig. 1.1) (120).

CULTURABLE INDICATORS OF FECAL POLLUTION

The presence and degree of fecal contamination in surface waters is determined based on the abundance of fecal indicator bacteria (FIB), such as *E. coli* or *Enterococcus* spp. Individual pathogens are rarely the subject of routine monitoring. A wide range of pathogens may be present at a single recreational beach due to the presence of multiple contamination sources (50, 183, 189). Monitoring pathogens is also logistically difficult. Detection of enteric viruses requires processing of large volumes of water, and monitoring for a suite of pathogens, rather than a single FIB, can be costly and laborious.

Moreover, pathogens are generally lower in abundance than FIB, and they can exhibit extremely high temporal variability (70).

Ambient water quality criteria were established in 1986 based on epidemiological studies on marine and freshwater beaches (35, 36, 48, 166). In freshwaters, the geometric mean of culturable *E. coli* and *Enterococcus* spp. should not exceed 126 colony forming units (CFU) 100 mL^{-1} or 33 CFU 100 mL^{-1}, respectively. In marine waters, the geometric mean of culturable *Enterococcus* spp. should not exceed 35 CFU 100 mL^{-1}. No single sample should exceed 235 CFU 100 mL^{-1} for *E. coli* and 61 CFU 100 mL^{-1} for *Enterococcus* spp. in freshwaters. In marine waters, the single sample limit for *Enterococcus* spp. is 104 CFU 100 mL^{-1}. These criteria limit incidence of gastroenteritis in freshwaters to 8 cases per 1,000 swimmers and 19 cases of gastrointestinal illness per 1,000 swimmers on marine beaches. The 1986 water quality monitoring criteria were not widely adopted until the passage of the Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 that mandated monitoring of fecal indicator bacteria (FIB). To aid in this monitoring, the fecal indicator bacterium concept was adopted.

A valid indicator of fecal contamination must satisfy several criteria (22, 28). The criteria for an ideal fecal indicator organism are:

1. It should be a member of the intestinal microflora of warm-blooded animals.
2. It should be present when pathogens are present, and absent in uncontaminated samples.
3. It should be present in greater numbers than the pathogen.

4. It should be at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants.
5. It should not multiply in the environment.
6. It should be detectable by means of easy, rapid, and inexpensive methods.
7. The indicator organism should be non-pathogenic.

Although *E. coli* and *Enterococcus* spp. appear to satisfy most of the criteria, there is conflicting evidence on the correlation to disease risk. The 1986 criteria are correlated with incidence of gastroenteritis in beachgoers, but there is a lack of information on the correlation between non-gastrointestinal illnesses, such as respiratory infections, skin rashes, or eye infections, and FIB (25, 35, 48). Traditional fecal indicators were not correlated with incidence of illness on a beach contaminated by non-point source pollution (38).

Moreover, some strains of *E. coli* are naturally occurring in sands and soils, with the potential to grow in this secondary habitat (34, 57, 81). *Enterococcus* spp. can persist and grow within moist marine sand microcosms (190). There is evidence that some *Enterococcus* strains can persist and grow in marine environments, especially within submerged aquatic vegetation (8, 9). Algae can also promote the persistence of *E. coli* and *Enterococcus* spp. in freshwater (33, 93).

The quantification of culturable FIB requires between 18 to 48 hours before results are obtained. Therefore, culturable FIB cannot assess health risk in real time, and the time lag between sample collection and beach posting can lead to exposure of

beachgoers to pathogens (138). In addition, beaches cannot be opened until bacterial counts fall below state and federal guidelines, potentially leading to false beach closures as well (138). As molecular techniques are now fairly well-established, there will be a necessary shift toward their implementation (158).

MOLECULAR INDICATORS OF FECAL POLLUTION

The National Epidemiological and Environmental Assessment of Recreational Water (NEEAR) Study was conducted on marine and Great Lakes beaches to evaluate rapid measures of water quality. The quantity of genetic markers for *Enterococcus* spp., total *Bacteroides*, human-specific *Bacteroides*, and *Clostridium* spp. were evaluated by quantitative PCR (qPCR) (38, 73, 173). In addition, the enumeration of F+ coliphage was conducted using a rapid assay (173). QPCR can produce results in as little as 2 to 3 hr, enabling same-day posting of beaches that exceed the water quality criteria. Among the rapid indicators tested, only qPCR for *Enterococcus* spp. and total *Bacteroides* were significantly associated with disease risk (172, 173). Importantly, qPCR for *Enterococcus* spp. was more strongly associated with gastrointestinal illness in children under 10 than culture-based enumeration of *Enterococcus* spp. (172). This is especially promising, as children are more susceptible to waterborne illnesses (25).

The abundance of *Enterococcus* spp. by culture-based methods and enumeration by qPCR are generally correlated. For 37 sites across the United States, encompassing marine and freshwater beaches, qPCR markers and CFU were significantly correlated (180). However, intense sampling at California beaches revealed that qPCR is biased

towards underestimation of culturable *Enterococcus* spp. abundance and could therefore lead to a failure to close beaches (122). Moreover, the relationship between *Enterococcus* spp. by culture-based methods and qPCR is temporally and spatially variable, and the correlation between culturable *Enterococcus* spp. and enterococci genetic markers is weaker at beaches that are primarily affected by non-point source pollution (39). Taken together, factors that affect the abundance of genetic markers for *Enterococcus* spp. should be reexamined before the wide-scale recommendation and implementation of qPCR for *Enterococcus* spp. for beach monitoring purposes.

The anaerobe *Bacteroides* spp. satisfies the major criteria as a marker of fecal pollution in environmental waters, and its potential as an indicator of human fecal pollution has been long recognized (3, 135). *Bacteroides* is abundant in human feces, constituting up to a third of the total microbial population (75, 116). Molecular methods of detecting human-specific *Bacteroides* are necessary because source-specific *Bacteroides* have not been isolated (43). On Lake Michigan beaches, total *Bacteroides* markers were proven to be more sensitive indicators of fecal pollution than *E. coli* (30). Boehm *et al.* (26) quantified human-specific *Bacteroides* to pinpoint beaches contaminated with sewage after identification of "hot spots" of pollution with traditional indicators. Additionally, *Bacteroides* has been positively correlated with pathogens such as *E. coli O157:H7*, *Salmonella*, and *Campylobacter* in environmental waters (150, 176).

A review by Scott *et al.* (152) concluded that the use of *Bacteroides* as a fecal indicator is promising, but too little is known about survival rates. In recent years, researchers have identified that the major factors controlling persistence in water are

temperature and predation (Table 1.1). Light exerts variable effect on survival, and its affect is likely negligible within sediment and sand relative to other factors. At ambient temperatures (15–20°C), *Bacteroides* persists for approximately one week (92, 175). In sterile environments, it can persist for up to 200 days (148).

There is some question, however, what presence of genetic markers for enterococci or *Bacteroides* spp. indicates, as DNA can persist for days or weeks in the environment following cell death (124). For example, Bae and Wuertz (11) found that live *Bacteroides* only survived for 28 hr in seawater, but the marker persisted at near detection levels for almost a month. Similarly, several studies have found that the decay of culturable enterococci is significantly faster than that of genetic markers for enterococci (12, 189). Both *Enterococcus* spp. and *Bacteroides* spp. can rapidly enter into a viable but not culturable (VBNC) state upon introduction to the natural environment (125–127). Consequently, studies of persistence must assess not only marker abundance, but in what form it persists.

MICROBIAL SOURCE TRACKING

The source of fecal contamination influences the degree of health risk associated with exposure. Exposure to feces from humans is presumed to carry a greater health risk, in part, due to the presence of human-specific enteric viruses (118, 134). Ruminants, such as cattle and sheep, are reservoirs for the bacterial pathogen *Salmonella* and pathogenic *Escherichia coli* (82, 87, 117). In addition, feces of wild birds can harbor a variety of viral, bacterial, and protozoan pathogens (78, 137, 142). Notably, wild birds can harbor

and transmit *Cryptosporidium*, a protozoan pathogen frequently associated with waterborne disease outbreaks (61, 118). However, humans, livestock, and wildlife can shed the same pathogens, so, in most instances, contamination source cannot be inferred from the presence of a particular pathogen (21).

Current water quality monitoring methods that quantify culturable enterococci, *E. coli*, or genetic markers for enterococci indicate the degree of fecal contamination (158, 192). However, in order to remediate or mitigate fecal pollution on beaches, the source or sources of contamination must be determined. There is no standard method for microbial source tracking (MST), but numerous molecular, biochemical, and chemical methods have been developed (112, 152). MST of fecal bacteria can be classified according to the need for a source library and whether culturing is required. Several common MST methodologies are listed in Table 1.2.

Library-dependent techniques typically require isolates of common fecal indicator bacteria, such as *E. coli*. Bacterial isolates from unknown sources are compared to thousands of known source isolates though ribotyping, rep-PCR, or other method. The need for a source library can be problematic. Libraries are well-suited to classify sources from the same geographic area in which the sources were collected; however, most libraries are not capable of classifying sources over wide geographic ranges (147). Moreover, the classification of source populations can change over time (67).

The amplification of source-specific genetic markers an increasingly preferred technique in MST studies (17, 18). This is likely due to the lack of need for a source library or bacterial culturing. Source-tracking markers have been developed for humans,

cattle, pigs, elk, dogs, horses, gulls, among other animals (143). However, the sensitivity (the ability to detect fecal pollution from a given source when it is present) and specificity (limited signal of the marker in non-target fecal material) of a marker must be measured and evaluated before its use in a MST study (143, 145).

SAND AND SEDIMENT AS A RESERVOIR OF FIB AND PATHOGENS

Although sand and sediment compartments are integral to understanding microbial load to recreational beaches, the dynamics of genetic markers in sand and sediments is not well-studied. Sand and sediment can substantially contribute to bacterial numbers through resuspension. Culturable *E. coli* and *Enterococcus* are found in large enough amounts in beach sand to significantly influence water column counts on several beaches (181, 188). A statistically significant effect of sediment *E. coli* counts upstream from sampling points has also been confirmed through a modeling approach (186). Higher bacterial abundance in sand and sediment may be due to the protection these environments offer from ultraviolet radiation, predation, temperature fluctuations, and other factors that negatively affect persistence in the water column (5, 80, 128, 181). Alternatively, great abundance may be a result of growth within sand or sediment, a has been previously observed for culturable *E. coli* and *Enterococcus* (5, 81, 190).

Extended persistence or growth of indicator bacteria in sand and sediment violate criterion 5 of an ideal fecal indicator, according to Bitton (22). However, few studies have examined the persistence or growth, as measured by genetic markers, of *Enterococcus* spp. or *Bacteroides* spp. in sand or sediment. In marine sands and manure

enterococci genetic markers decayed more slowly than culturable *Enterococcus* spp. (89, 189). Although the growth of enterococci in marine sand has been observed, the effect of growth on genetic marker abundance in sand or sediment has not been described.

No studies have documented evidence of the growth of *Bacteroides* genetic markers for fecal pollution within sand or sediment. However, *Bacteroides* is an obligate anaerobe, and could potentially persist for longer periods within anoxic sediments. In addition, *Bacteroides* is one of the most aerotolerant obligate anaerobes, surviving at atmospheric oxygen concentrations for up to three days (164). Baughn and Malamy (14) also found that *Bacteroides* can grow in nanomolar concentrations of oxygen. In a freshwater stream, human-specific *Bacteroides* was qualitatively more abundant in sediments than in overlying waters (171). In a freshwater microcosm, sediment exposure promoted faster decay of *Bacteroides* markers relative to microcosms without sediment. However, when the sediment was resuspended, the marker abundance nearly returned to initial values (44). The physiological requirements of *Bacteroides* spp. and distribution and persistence studies suggest that sand and sediment could be a potential reservoir for *Bacteroides* spp., although there is a lack of research in this regard.

Sand and sediment can also harbor pathogens. Pathogenic *E. coli*, *Pseudomonas aeruginosa*, *Salmonella newport*, and *Klebsiella pneumoniae* exhibit increased survival in sediments relative to water (31, 54). The effect was consistent across five sediment types, and supported the conclusion that sediment may serve as a reservoir for pathogenic bacteria, surviving for up to a month in this secondary habitat (31). Recent epidemiological studies show that contact with beach sand is associated with increased

risk of gastroenteritis in beachgoers (73, 74). Hand to mouth contact following hand to sand contact can facilitate transmission of bacteria and viruses (182). Therefore, the failure to account for bacterial populations within sand and sediments can lead to underestimation of health risk (47, 64).

FECAL CONTAMINATION ON LAKE SUPERIOR BEACHES

The Duluth-Superior Harbor is a freshwater estuary at the mouth of the St. Louis River. The St. Louis River system has been designated an Area of Concern (AOC) by the United States Environmental Protection Agency based on impairment of 9 out of 14 beneficial use criteria. Beach closings resulting from exceedances of bacterial water quality standards for recreational water contact have been an increasing problem for Duluth-Superior Harbor beaches (119). From 2003 to 2009, the Minnesota Pollution Control Agency (MPCA) monitored fecal coliform counts at 39 beaches, 27 of which were monitored on a weekly basis. Most beaches were seldom closed, but four beaches exhibit higher than average closure rates (Fig. 1.2) (Data from: http://www.lakesuperiorstreams.org). The four beaches with the highest closure rates are located in the Duluth-Superior Harbor (Fig. 1.3). The water flowing into the harbor from the St. Louis River and Lake Superior had relatively low *E. coli* counts over this period.

In the Duluth-Superior Harbor, possible sources of *E. coli* include sewage, wildlife, and naturalized *E. coli* populations. Two sewage treatment plants discharge effluent into the harbor, Western Lake Superior Sanitary District (WLSSD) and Superior Wastewater Treatment Plant (SWTP). WLSSD likely has a stronger effect on water

quality, as it is situated in the inner harbor before the first constriction. Contaminants discharged into the inner harbor, have a residence time of 30 to 40 d (163). In addition, seiche oscillations, which occur every two to seven hr, and range in amplitude from 3 to 25 cm, can be strong enough to transport contaminants 20 km up the St. Louis River (59, 85, 163). Sewage overflows were historically a problem in the Duluth-Superior Harbor. Overflows result from inflow and infiltration of stormwater into the sewer system, leading to high influent volumes and inefficient treatment of sewage. In 2008, WLSSD and the City of Duluth constructed storage basins with over 10 million gallon capacity to prevent untreated sewage releases during significant wet-weather events (J. Mayasich, personal communication). Monthly sampling of a site within the outer harbor revealed that of *E. coli* that could be classified as to its respective source, those from human sources were highest in spring, and decreased over the course of the summer (80).

In Duluth-Superior Harbor, *E. coli* from non-human sources was hypothesized to substantially contribute to area beach closures. The harbor provides habitat to many bird species, and the most abundant among them are *Larus delawarensis* (Ring-billed gulls), *Branta canadensis* (Canada goose), and *Anas platyrhynchos* (Mallard duck). Although *B. canadensis* is not the most abundant bird on harbor beaches, it contributes the highest proportion of *E. coli* that can be source-identified in water, sand, and sediment (68, 80). *E. coli* from *B. canadensis* is also more abundant than *E. coli* from human fecal sources during summer and fall (80). Some strains of *E. coli* in Duluth-Superior Harbor have been isolated multiple times over the sampling season and over multiple years, suggesting that they are naturalized to the environment (80, 93). Naturalized *E. coli*

strains have been found in soils of the Lake Superior watershed, with the potential to grow in the soil environment (81).

SUMMARY OF THESIS

This work explores the use of rapid, culture-independent methods for the identification of fecal pollution. In particular, the application of rapid tools for freshwater beaches is considered. Chapters 1 and 2 describe field studies in which the spatial and temporal dynamics of rapid indicators are examined. Chapter 2 examines the short timescale variation in goose/duck and human sources of fecal contamination of two Duluth-Superior Harbor beaches and a beach on Lake Superior. In contrast to previous studies, it employs culture-independent techniques of high-throughput colony hybridization with a goose/duck specific DNA probe and qPCR for human-specific *Bacteroides*. Chapter 3 explores the dynamics of genetic markers for enterococci, total *Bacteroides*, and human-specific *Bacteroides* on a Duluth-Superior Harbor beach near the WLSSD wastewater outfall. In particular, the role of sand and sediment as potential reservoirs for genetic marker of fecal contamination is explored. Chapters 3 and 4 are microcosm studies that examine the persistence of genetic markers in freshwater matrices. In chapter 4, the persistence of genetic markers for enterococci, total *Bacteroides*, and human-specific *Bacteroides* is determined in Duluth-Superior Harbor water, sand, and sediment. The effects of temperature and sand moisture on marker persistence were also examined. Lastly, chapter 5 delves deeper into the concept of genetic marker persistence by using propidium monoazide to distinguish between the

persistence of total genetic markers and those from live cells in sand microcosms. The decay rates of genetic markers for FIB are compared to bacterial pathogens in sand at two moisture levels.

Table 1.1

Summary of *Bacteroides* persistence experiments in water.

Reference	Light	Temp	Predation	Salinity
(92)		–	–	
(125)		–		+
(11)	–[a]			
(15)		–	–	
(148)			–	
(175)	0			

[a]A cow-specific marker exhibited more rapid decay in the presence of light, whereas a human-specific marker was not affected.

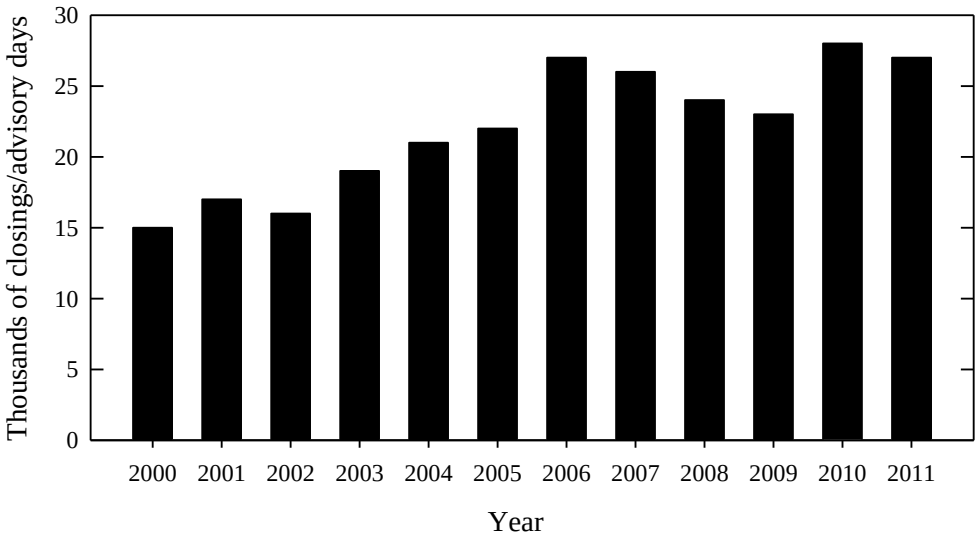
Table 1.2

Overview of several microbial source tracking methodologies.

Method	Description	Advantages	Disadvantages
Library-dependent, culture-dependent			
Ribotyping	Southern blot of genomic DNA cut with restriction enzymes and probed with ribosomal sequences	Reproducible; some methods useful for classifying isolates from multiple sources	Labor-intensive; reference database required; may be geographically specific; variations in methodology exist
Rep-PCR	PCR used to amplify palindromic DNA sequences coupled with electrophoretic analysis	Relatively simple	Reproducibility a concern; large database required; variability increases as database increases
Antibiotic resistance profile	Biochemical technique. Differentiates bacteria using antibiotics associated with human and animal therapy and animal feed.	Easy assay	Geographically specific; can be highly prone to false positives; genes on plasmids often lost with environmental conditions;
Library-independent, culture-dependent			
Bacteriophage or coliphage typing	Isolation and enumeration of bacteriophages of source-specific bacteria; serotyping or genotyping of F+ coliphages	High specificity	Variable distribution and survival rates; can be low in abundance
High-throughput colony hybridization	Isolation of bacteria, robotic arraying, and probing with a source-specific DNA sequence	Can rapidly screen large number of isolates	Need expensive equipment
Library-independent, culture-independent			
Amplification of host-specific bacteria or viruses	PCR or qPCR of source-specific marker; discriminates human, cattle, pigs, and other sources	Rapid; high-throughput	Technically demanding; need expensive equipment; survival rates of markers unknown

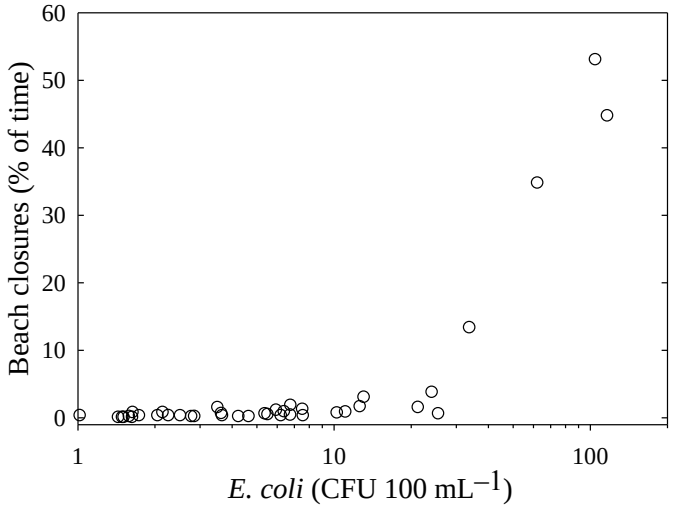
[a]From (112, 152, 158).

Figure 1.1



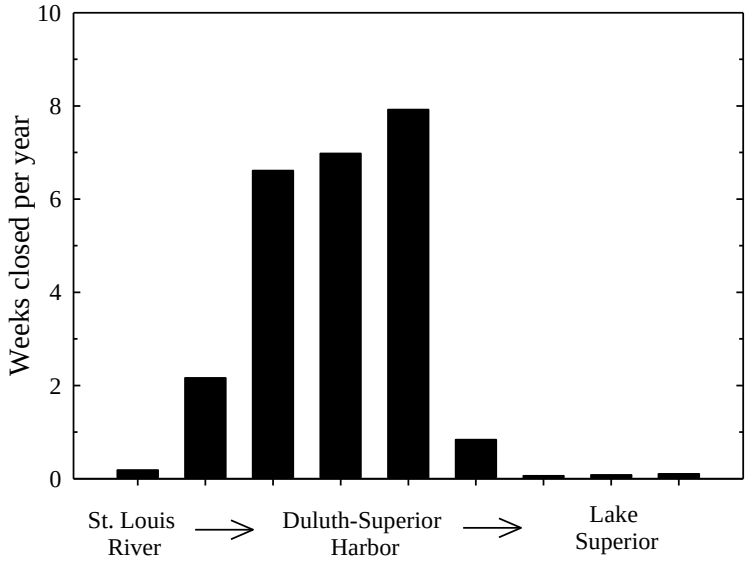
Total nationwide beach closings from 2000 to 2011. From NRDC (120).

Figure 1.2



The percentage of time Duluth area beaches were closed due to exceedances of *E. coli* water quality criteria from 2003 to 2009. The x-axis represents the geometric mean of *E. coli* counts for beaches from 2003 to 2009. Adaptive sampling, the daily sampling following beach closure, has not been included in the calculation of the geometric mean.

Figure 1.3



Average number of weeks of beach closings throughout the St. Louis River estuary from 2003 to 2009.

CHAPTER 2:

Short Term Dynamics of Fecal Bacterial Sources and Virulence Genes at a Lake Superior Boat Landing and Beaches

OVERVIEW

Beach closures due to elevated levels of fecal indicator bacteria (FIB) are common in the Duluth-Superior Harbor. The short-term dynamics of FIB, the relative contribution of goose/duck and human fecal contamination sources, and the presence of pathogen-related *Escherichia coli* genes and *Salmonella* spp. at a beach on Lake Superior and a beach and boat landing on Duluth-Superior Harbor were studied in the summers of 2007 and 2008 using culture and molecular techniques. Water, sand, and sediment samples were taken twice per week, and daily following a storm event. There was high spatial and temporal variability in concentrations of FIB and the sources of fecal contamination. Goose/duck-borne *E. coli* constituted 5 to 29% of the total *E. coli* population, and it did not substantially contribute to single-sample bacterial exceedances as previously hypothesized. Human fecal contamination was associated with higher densities of fecal indicator bacteria. While the site in the inner harbor, nearest to a wastewater treatment plant outfall had the greatest percentage of samples that tested positive for a human-specific *Bacteroides* marker (HF183) gene (39%), the incidence of *Salmonella* spp. and *E. coli* pathogen genes was low (~1% of total *E. coli*) and precludes the use of indicators to predict their occurrence. The *E. coli* pathogen gene *eae* was more frequently detected than *Salmonella* spp. and *E. coli* shiga-like toxin genes, which were rarely detected. However, there was no significant relationship between *eae* and traditional or source-specific indicators.

INTRODUCTION

Bacterial contamination remains the leading cause of loss of beneficial use of recreational waters in the United States (169). The safety of recreational water contact is determined by the quantity of fecal indicator bacteria (FIB) in the water column, as they have been correlated with increased risk of gastrointestinal illness in beachgoers (136). Water quality on Great Lakes beaches is of particular concern. Great Lakes beaches are popular recreational destinations, and the number of yearly beach closures is well above the national average (119). Beach closings resulting from exceedances of *E. coli* standards (geometric mean of 126 CFU 100 mL^{-1} or single sample maximum of 235 CFU 100 mL^{-1}) are a consistent problem and a heavily debated public health issue. Incidences of poor water quality in Duluth-Superior Harbor are frequently blamed on sewage effluent discharge or overflows resulting from inflow and infiltration of storm water into the sanitary sewer system. Contact with human sewage is generally assumed to pose a greater health risk than contact with waste from non-human animals, so changes in sources of FIB can have important public health implications (118).

There are many potential sources of FIB on recreational beaches. Fecal matter from humans, birds, and other wildlife, as well as sands and soils, can contribute to elevated levels of FIB (34, 57, 81). Previous studies on Duluth-Superior Harbor beaches found that treated sewage effluent and geese were the most frequently detected sources of *E. coli* (68, 80). Monthly sampling indicated that the relative contribution of sources changed seasonally (80). However, more frequent beach sampling is necessary to elucidate short-term bacterial dynamics that may be occurring during FIB exceedances.

Bacterial populations within the beach environment exhibit extreme temporal variation, and bacterial exceedances are often single-day occurrences, even at frequently closed beaches (24, 100).

Few studies have characterized the short-term concentration changes and dynamics of fecal indicator bacteria and their respective sources. The specific aims of this study were to: 1) examine the short-term temporal variability of FIB originating from goose/duck and human sources across a range of impacted sites on Lake Superior and in the Duluth-Superior Harbor, and 2) assess the abundance of potentially pathogenic *E. coli* and *Salmonella* at the study sites and determine if their numbers correlate with sources of fecal contamination and environmental factors. While previous research studies used labor-intensive methods, which inherently limited sampling frequency, in this study, high-throughput robot-assisted colony hybridization and quantitative PCR (qPCR) methods were used to enable more frequent sampling and efficient processing of environmental samples and isolates.

METHODS

Study sites and sample preprocessing. The Blatnik Bridge Boat Landing (BB) and Southworth Marsh (SW) sampling sites are located within the Duluth-Superior Harbor, a freshwater estuary that comprises Superior and St. Louis Bays. The Beach House (BH) site was located on Lake Superior near Duluth, MN (Figs. 2.1 and 2.2). Water, sand, and sediment samples were taken two times per week in the summers of 2007 and 2008. One, week-long, storm event sampling occurred in 2007. Three replicate

samples were taken five m apart parallel to the shoreline of the site, and sand and sediment samples were obtained as a composite sample of 1 to ten cm depths. Water samples were collected in sterile Nalgene HDPE bottles (Rochester, NY), and sand and sediment samples were collected in sterile Whirpak bags (Nasco, Fort Atkinson, WI). All samples were transported to the lab on ice and processed within four h of sampling. Sewage influent and effluent samples were collected from the Western Lake Superior Sanitary District (WLSSD) wastewater treatment facility on 16 June 2009. Sewage influent was sampled prior to initial screening, and treated effluent was sampled after tertiary treatment by sand filtration at an onsite sampling station.

The concentration of *E. coli* and total fecal coliforms was obtained for each sample. Bacteria were elutriated from ten g subsamples of sand and sediment using 95 mL sterile ammonium phosphate solution with 0.01% gelatin and a wrist action shaker as previously described (81). Following shaking, solutions were allowed to sit for 15 min to allow large particles to settle before further processing. Water and supernatants from sand and sediment samples were filtered onto nitrocellulose filters (0.45 µm pore size, 47 mm diameter; Millipore, Billerica, MA), placed on mFC agar medium (6) (Difco, Detroit, MI) and incubated at 44 °C overnight to enumerate total fecal coliforms. *E. coli* were collected by filtering water and supernatants through polycarbonate filters (0.22 µm pore size, 47 mm diameter; Whatman, Piscataway, NJ). Filters were placed in five mL sterile phosphate buffered saline containing 0.01% gelatin and shaken for 30 min with three g, one mm sterile glass beads to suspend attached cells. Glycerol was added to a final concentration of 25%, and the cells were frozen at –80°C. *E. coli* cells were enumerated

by spread plating one to five mL of the frozen cell stocks onto modified membrane thermotolerant *E. coli* (mTEC) medium, prepared with 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc), in 22 x 22 cm Q-tray bioassay plates (Genetix, Boston, MA) (168). Plates were incubated at 35°C for 2 h, then at 44°C for 22 h, and finally stored overnight at 4°C overnight to develop colony pigment before counting. Plates were then used for colony picking and subsequent hybridization.

***E. coli* colony hybridizations.** *E. coli* colonies were picked from Q-tray plates using a Genetix Q-Bot robot as previously described (66). Up to 300 colonies were picked per sample, and nearly 25,000 *E. coli* were isolated overall. Colonies were placed in 384-well microplates in Hogness modified freezing medium (191) and stored at –80°C until further processing. Isolates were spot-inoculated onto Performa nylon membranes (Genetix, Sunnyvale, CA) using the Q-Bot robotic platform as previously described (66, 191). Each membrane contained triplicate colonies of 2,300 isolates and hybridization controls. After arraying, each membrane was placed onto LB medium and colonies were allowed to grow until 1 mm in diameter.

Colony hybridizations were done as previously described using ^{32}P-labeled probes for shiga-like toxins 1 and 2 (*Stx*2, *Stx*2), the attaching and effacing (A/E) protein intimin (*eae*), and a goose and duck specific gene sequence (66, 130) (Table 2.1). Images were captured using a Storm 860 Phosphorimager (GE Healthcare, Chalfont St. Giles, UK) and analyzed with Array-Pro Analyzer software (MediaCybernetics, Bethesda, MD). The spot intensity was corrected for background noise, and the signal was averaged across

triplicate spots within each membrane. The determination of cutoff value for positive hybridizations was done as previously described (66, 191).

Intimin subtyping. Subtypes of intimin, encoded by *eae*, were determined as previously described (139). DNA was directly extracted from *E. coli* isolates grown in 100 µL of Lysogeny agar medium. Cells were pelleted by centrifugation at 3,000 g for five min, the supernant was discarded, and the pellet was suspended in 90 µl of 0.05 M NaOH. The cells were heated for 15 min at 95 °C, centrifuged at 3,000 g for five min, and two µl of the supernatant was used as DNA template for intimin subtyping, as previously described (139).

Quantitative PCR (qPCR) analyses. The qPCR analyses were done from template DNA isolated from one g subsamples of sand and sediment, and water samples that were filtered, until clogged, through Durapore membrane filters (0.22 µm pore size, 47 mm diameter; Millipore, Billerica, MA). Samples were stored at –80°C until further processing. DNA was directly extracted from one g of sand or sediment, whereas filters were cut into 1 x 4 mm pieces before extraction using PowerSoil® DNA Isolation kits (MoBio, Carlsbad, CA). DNA was quantified by using a Qubit 1.0 fluorometer (Invitrogen, Grand Island, NY).

Plasmid standards were created by cloning the target gene from PCR product using the StrataClone PCR kit (Stratagene, Santa Clara, CA). PCR product was produced using human fecal DNA as template for HF183 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028 for the *ttr* locus. Purified plasmid DNA was quantified by using the Qubit 1.0 fluorometer (Invitrogen, Grand Island, NY) before

preparation of 5, 10-fold dilutions for qPCR standards. Each run contained triplicate reactions of standards, non-transcript controls, and environmental samples. Amplification was performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA), and cycle threshold (Ct) values were automatically determined using the system software. Sample marker concentration was calculated on a per-run basis.

Quantitative PCR analysis of the human-specific *Bacteroides* marker gene HF183 and the *Salmonella ttr* gene were done using previously published primers and optimized protocols (Table 2.1). If the target marker was detected in a particular sample, then samples obtained from dates surrounding that sampling time point showing gene presence were analyzed until the marker gene was below the limit of detection. Due to the high prevalence of the HF183 at the Blatnik Bridge site, all samples from that site were analyzed.

The qPCR reaction mixture for HF183 contained GREEN Real-Time PCR MasterMix (Epicentre, Madison, WI), primers at a concentration of 300 nM, and ten ng of template DNA, in a 25 uL reaction volume. Reaction conditions consisted of an initial denaturation at 95°C for five min, and 40 cycles of 95°C for 30 sec, annealing at 56°C for one min, and extension at 72°C for one min. Melt curves were used to verify specific product amplification. The assay detection limit was 50 copies per reaction.

The reaction mixture used for qPCR of the *Salmonella ttr* locus contained 1x Taqman Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 0.2 µg/µl bovine serum albumin, primers at a concentration of 200 nM, and ten ng of template

DNA, in 25 uL reaction volume. The reaction conditions were 50ºC for two min, 95ºC for ten min, and 50 cycles of 95ºC for 15 s, and 60ºC for one min. The assay detection limit was 30 copies per reaction.

Statistical analyses. All statistical analyses were conducted using JMP Pro 9.0.2 (SAS Institute Inc., Cary, NC). Pearson product-moment correlation was used to determine the relationship between environmental parameters and FIB, HF183 and wastewater treatment plant flow and turbidity, and the correlation between HF183, fecal coliforms, and total *E. coli* with *eae* containing *E. coli* isolates. ANOVA with Tukey HSD post-hoc comparisons were used to determine significant differences in FIB counts among sample sites and proportion of goose/duck-borne *E. coli* across sampling months. Fisher's exact test was used to compare the: 1) statistical differences in presence/absence of the HF183 across sites and sample types, 2) detection frequency of HF183 across sampling month in the water column at BB, and 3) detection frequency of potential EPEC across sampling months. The Student's t-test was used to compare FIB abundance when HF183 was present and absent. Chi-square analysis was used to compare the proportion of *E. coli* from goose/duck and unknown sources across test sites.

RESULTS

Total fecal coliform and *E. coli* counts. Over the course of this study, both harbor sites exceeded the fecal coliform single sample water quality standard of 400 CFU 100 mL^{-1}, on 40% of sampling dates. Total exceedances for 2007 and 2008 at the BB and SW harbor sites steadily increased from 23% in June to 32% and 55% in July and

August, respectively. The BH site on Lake Superior never exceeded the fecal coliform standard. Fecal coliform bacteria were more abundant in water and sand from the Duluth-Superior Harbor sites compared to the lakeside beach (Fig. 2.3A). On a per mass basis, fecal coliforms counts were, on average, 34-fold greater in sand compared to water across all sites. The sediment samples from the BB and BH sites contained 7- and 5-times greater density of fecal coliforms than did the overlying water, respectively. At the SW site, however, the abundance of fecal coliforms in the sediment was two-thirds lower than the overlying water. Variables such as wave height, precipitation, and sand and sediment moisture content, did not correlated with fecal coliform counts ($p < 0.05$). In contrast, however, water temperature was positively correlated with fecal coliforms in water, sand, and sediment ($R^2 = 0.15, 0.37, 0.22$, respectively; $p < 0.001$). As expected, water and sand temperatures characteristically increased over the course of the summer months, and fecal coliform counts showed concomitant increases across these sample types.

E. coli concentration in the water column exhibited similar dynamics to those of total fecal coliforms across all sampling sites. The *E. coli* counts were least abundant at the BH site, and had the greatest concentration at the SW site (Table 2.2). The *E. coli* counts at the SW site exceeded the single sample water quality standard of 235 CFU 100 mL^{-1} on 21% of the dates sampled, BB exceeded the standard on 1 day (4% of sampling dates), and the BH site never exceeded the state standard. While the abundances of *E. coli* at the BB and SW sites were significantly different, *E. coli* abundance at the BH site was not significantly different from either harbor site, likely due to the limited number of

samples obtained. While the *E. coli* and fecal coliform counts were positively correlated, the explained variance was low ($R^2 = 0.16$, $p = 0.001$).

Human fecal contamination. Samples were selected for qPCR analysis based on peaks in total fecal coliform bacterial counts (Fig. 2.4). The abundance of HF183 varied by site and sample type. The genetic marker was most frequently detected in the water column at the BB site, with a detection frequency of 39% (Fig. 2.3B). There were no significant differences among the detection frequency of HF183 in BB sand and sediment and water, sand, and sediment at SW and BH (Fig. 2.3B). The detection frequency indicated that the water column of BB was likely impacted by human fecal material during ~ 1.5 months of the summer, in contrast to an average of 0.5 months at the SW and BH sites. The concentration of HF183 in the water column at the BB site was extremely variable, with no significant seasonal trend (Fig. 2.5). Detection (contamination) events at the BB site lasted approximately 6.5 d, and the geometric mean of the genetic marker concentration in the water column when contamination was present was 6,800 copies 100 mL^{-1}, and reached a maximum of 18,800 copies 100 mL^{-1} in early September 2007. While the BB site is in relative close proximity to the WLSSD wastewater treatment plant outfall, the presence of HF183 was not significantly correlated with effluent flow or turbidity. However, the HF183 genetic marker was abundant in plant effluent (2.2×10^5 copies 100 mL^{-1}), a 2.5 log reduction in marker gene concentration relative to plant influent (6.2×10^7 copies 100 mL^{-1}).

The concentration of fecal indicator bacteria was greatest on dates when HF183 was present than when it was absent. The average concentration of fecal coliform bacteria

at the BB site, when HF183 was present, was 443 CFU 100 mL^{-1}, but only 175 CFU 100 mL^{-1} when absent (t-test, $p < 0.001$), whereas there was no significant difference in *E. coli* concentration. The average concentration of *E. coli* at the SW site was 200 and 78 CFU 100 mL^{-1} when HF183 was present and absent, respectively ($p = 0.044$). There was no significant difference in fecal coliform counts.

According to the monitoring guidelines of the State of Minnesota, which currently uses *E. coli* to assess water quality, on SW beach 60% of sampling dates that the standard (235 CFU 100 mL^{-1}) was exceeded, HF183 was not detected. In contrast, on 30% of sampling dates when the water quality standard was not exceeded, HF183 was present (Fig. 2.5).

Goose/duck-borne *E. coli.* DNA hybridization studies indicated that goose/duck-borne *E. coli* was at all sites and was detected on every sampling date. The proportion of goose/duck-borne *E. coli* was significantly different among all three sites, and the harbor sites had a significantly greater proportion (Chi-square, $p < 0.0001$) of goose/duck-borne *E. coli* relative to that found on the lake-side beach (Table 2.2). Goose and duck sources contributed, on average, 62 CFU 100 mL^{-1} and were the source of 29% of total isolates at the SW site, whereas goose and duck *E. coli* abundance averaged 14 CFU 100 mL^{-1} and was the source of 12% of total isolates at the BB site. Although goose/duck-borne *E. coli* counts were variable at the BB (CV = 120%) and SW (CV = 140%) sites, the coefficient of variation in percentage of goose/duck-borne *E. coli* was lower, 67% and 62% at the BB and SW sites, respectively.

Over the course of the study period, seven total exceedances were observed, one at BB and six at the SW sites (Fig. 2.7A, B). Absent goose/duck-borne *E. coli*, the number of exceedances would be reduced by 1 at each site. The highest monthly average percentage of goose/duck-borne *E. coli* at the SW site was in August 2007 and June 2008 (Fig. 2.7). There were no significant differences in the proportion of goose/duck-borne *E. coli* at the BB site across sampling months.

Pathogen abundance. A total of 21,096 *E. coli* isolates obtained from water samples at the three sites were screened for the presence pathogen-related genes by high density macroarray hybridization. A small proportion (2.8%) of all tested isolates contained the intimin virulence factor (*eae*), and only three isolates were found to contain the stx1 or stx2 genes, encoding for shiga-like toxin. The BB site had the greatest proportion of *E coli* isolates containing the *eae* gene (1.5%) (Table 2.2).

The distribution of intimin subtypes varied among sampling locations (Fig. 2.8). There was no seasonal trend in intimin-containing isolates, and no goose/duck-borne *E. coli* harbored pathogen genes. Moreover, there was no correlation between counts of goose/duck-borne *E. coli* and fecal coliforms, and the prevalence of the *eae* gene (p=0.83). Similarly, there was no correlation between the prevalence of the *eae* gene and HF183 (p = 0.35). Lastly, only three of 241 (1.2 %) samples from water, sand, and sediment were positive for the presence of *Salmonella* by qPCR.

DISCUSSION

While it has previously been shown that FIB exhibit high temporal variability (23, 24, 100), the temporal variability of sources of fecal contamination and pathogens is less well known. The aim of this study was to characterize the short-term variability in fecal contamination from goose/duck and human sources at a boat landing and Lake Superior beaches and to identify potentially pathogenic *E. coli* and *Salmonella* in waterways. The results showed that although goose/duck-borne *E. coli* were consistently present at Duluth-Superior Harbor sites, they did not substantially contribute to single sample water quality exceedances. In contrast, human fecal contamination exhibited high temporal variability, and was detected at a significantly great frequency in the water column at the BB harbor site. The incidence of *Salmonella* and potentially pathogenic *E. coli* was low across all study sites, and was likely not a significant public health concern.

Human-fecal contamination, as assessed by detecting the human-specific *Bacteroides* genetic marker HF183, had extremely high spatial and temporal variability at the study beaches. Previously, Ishii *et al.* (80) and Hansen *et al.* (68) found *E. coli* from human sources on 80 and 50% of the sampling dates, respectively, within the Duluth-Superior Harbor using HFERP of cultured *E. coli*. In addition, Ishii *et al.* (80) found more *E. coli* from human sources during spring months. In this study, however, there was no seasonal trend in the detection frequency of HF183, and the discrepancy to previous studies is likely due to differences in methodology. The presence or absence of HF183 has been shown to vary between storm events at a single sampling site (129), or over the course of several hours in a river (162). Although the persistence of human sources of *E. coli* in the environment is unknown, *E. coli* has been shown to persist in water, sands, and

soils, in extraintestinal environments (34, 81). Bacteria in the genus *Bacteroides*, in contrast, are presumed to decay quickly in the environment, as they are obligate anaerobes.

The high variation of HF183 at the BB site was probably due to the interaction between effluent outflow rate, marker persistence, and water movement patterns. Accounting for water movement patterns within the Duluth-Superior Harbor is complicated due to the interaction between the harbor's internal seiche and the lake seiche occillations coupled with the influence of wind speed, wind direction, and St. Louis River flow (85, 163). Measuring these parameters was beyond the scope of this study. In addition, there may be alternative sources of the HF183 genetic marker within the harbor besides WLSSD, such as faulty sewer lines. However, the volume of effluent, the high concentration of source-specific markers in effluent, and the location of the effluent outfall pipe within the inner harbor, makes it a potential point source of HF183 to the Blatnik Bridge site. Wastewater treatment systems, even those equipped with tertiary treatment of effluent such as filtration or chlorination (used at WLSSD), are not capable of completely removing all human fecal marker genes from effluent water (94, 162). LaPara *et al.* (94) found elevated quantities of human fecal markers near the WLSSD and SWTF outflow pipes in the Duluth-Superior Harbor. Similarly, we found a high abundance of HF183 in WLSSD effluent outflow.

The HF183 genetic marker was infrequently detected in sand and sediment. Our results are in contrast to those of Lamendella *et al.* (95) who found that human-specific markers were qualitatively more abundant in sediments than in overlying waters. Since

Bacteroides is an obligate anaerobe, this bacterium may potentially persist for longer periods within anoxic sediments than in overlying water. However, predation by the existing microbial community has a significant negative effect on *Bacteroides* persistence and could play an important role in loss of this bacterium in sand and sediment (92). Further research is needed to determine the persistence of the HF183 and the factors which affect persistence in sand and sediment.

We hypothesized was that goose/duck *E. coli* were responsible for the majority of water quality exceedances at the sampling sites examined in this study. In previous studies, Hansen *et al.* (68) and Ishii *et al.* (80) reported that waterfowl-borne *E.coli* comprised up to 100% of identified *E. coli* strains, and a maximum of approximately 35% of the total *E. coli* examined. Our results are consistent with these values, and we frequently observed Canada geese and goose droppings at study sites within the harbor, particularly at the SW site. However, we did not observe a mid to late summer increase in waterfowl-borne *E. coli* as was previously reported by Hansen *et al.* (68) and Ishii *et al.* (80). Instead, we found no seasonal trend in goose/duck-borne *E. coli* at the BB site, and goose/duck-borne *E. coli* peaked at the SW site in August 2007 and June 2008. This difference may be due to more frequent sampling that captured the variability within each month or the difference methodology used in this present of this study. The previous studies used HFERP DNA fingerprinting, coupled with a source library, whereas we used colony-hybridization with a source-specific DNA probe, a library-independent method.

Goose/duck-borne *E. coli* exceeded the single-sample water quality standard in 2 of 7 single-sample *E. coli* exceedances observed during the study period. Geese and

ducks contributed 8 and 24% of total *E. coli* on those dates; however, in both cases *E. coli* from unknown sources also exceeded the standard. Management of the geese population at harbor sites may reduce total *E. coli* abundance. Geese, in particular, produce large amounts of fecal matter that contains a high abundance of FIB (2, 79). Consequently, geese have been identified as the major source of fecal contamination in a variety of water bodies, including agricultural watersheds (160). Management to reduce geese populations has been shown to decrease fecal coliform counts by 50 and 75% in two ponds relative to an unmanaged pond (165).

In the present study we found potentially pathogenic *E. coli* at all three study sites. The most frequently detected pathogen was enteropathogenic *E. coli* (EPEC). These bacteria contain the *eae* gene that encodes for the adhesin intimin. Potential EPEC made up a small (~1%) proportion of the total *E. coli* population; however, they were consistently detected at the BB site. Few studies have quantified the presence of potential EPEC on recreational beaches. Our results were consistent with previously reported incidences of EPEC on a California beach (3.6%) and on a swimming beach on Lake Erie (0.08%) (65, 96). Since the infectious dose for EPEC is approximately 10^7 organisms for healthy adults (103), it follows that the health risk posed by water contact at the study sites is low, as the concentration of potential EPEC in the water column was <4 CFU 100 mL^{-1}.

The distribution of intimin subtypes was distinct among the study beaches, suggesting that the sources of these potential pathogens are also distinct. Over 30% of potential EPEC isolated from the BB site were of the β intimin subtype. The β intimin

subtype is the most common subtype among clinical isolates (139); consequently, human fecal contamination may be a source of pathogenic *E. coli* at the BB site. However, since we were unable to amplify the *eae* gene from DNA extracted from WLSSD sewage effluent, there is no evidence to support the contention that effluent is the source of potential EPEC to the BB site. It is important to note, however, that the detection of *eae* from the study sites relied on isolation and hybridization rather than PCR amplification from DNA extracts. Intimin subtypes ν, ξ, κ, and μ, have been isolated from birds (82, 90). As there are few studies that address the relationship between intimin subtypes and contamination source, it is difficult to speculate on the sources of potential EPEC at the SW and BH study sites.

The inability of traditional and source-specific indicators to predict pathogen abundance or presence is further evidence to the limitations of FIB for assessment of health risk to beachgoers. The lack of correlation may be due in part to the low abundance of pathogens. A review by Wu *et al*. (185) found that the likelihood of significant pathogen/indicator correlations increases with pathogen abundance. None of the isolates sourced to geese and ducks contained *E. coli* pathogen genes; however, we cannot speculate on the health risk of exposure to goose/duck fecal contamination. Geese and other waterfowl can harbor viral, bacterial, and protozoan pathogens (16). *Bacteroides* has been positively correlated with bacterial pathogens in environmental waters (150, 176); though, recent epidemiological studies have found inconsistent relationships between *Bacteroides* genetic markers and disease risk (173).

Similar to that reported by Santoro and Boehm (146), we found that HF183 was associated with high abundances of traditional fecal indicator bacteria. Under the current standards, markers of human fecal contamination would be present approximately 30% of the time when harbor sites were open; however, the presence of human fecal markers may not indicate a health risk, as DNA can persist for days or weeks in the environment following cell death (124). Interpretation of the presence of fecal genetic markers is problematic for sites with high rates of water recirculation and retention times, such as that present in the Duluth-Superior Harbor.

High spatial and temporal variability of fecal indicator bacteria and sources of fecal contamination were observed on a Lake Superior beach and at a boat landing and beach site within Duluth-Superior Harbor. Goose/duck-borne *E. coli* were always present at the study sites, but they did not substantially contribute to bacterial exceedances as previously hypothesized. Therefore, management of geese and duck populations may reduce long term geometric mean FIB abundance, but may not result in fewer beach closures. Human fecal contamination is present within the Duluth-Superior Harbor, and it was associated with higher densities of fecal indicator bacteria. However, the relationship between the abundance of *E. coli* and the genetic marker indicative of human fecal contamination, HF183, was site dependent. There was no significant relationship between traditional or source-specific indicators and potentially pathogenic *E. coli*. The incidence of bacterial pathogens on the study beaches was low, and may preclude the use of indicator bacterial numbers to predict their occurrence. The Duluth-Superior Harbor environment poses inherently challenging problems for water quality managers. The

complexity of water movement within the harbor almost certainly contributes to the high short-term variability in abundance and sources fecal indicator bacteria.

Table 2.1

Primers and probe used in this study.

Target	Primer/Probe	Sequence (5'-3')[a]	Reference
Human-specific *Bacteroides* 16S rRNA gene	HF183	ATCATGAGTTCACATGTCCG	(17, 153)
	HF183R	CCATCGGAGTTCTTCGTG	
Salmonella ttr	ttr-6	CTCACCAGGAGATTACAACATGG	(109)
	ttr-4	AGCTCAGACCAAAAGTGACCATC	
	ttr-5	FAM-CACCGACGGCGAGACCG ACTTT-BHQ1	
Shiga-like toxin 1	stx1F	ATAAATCGCCATTCGTTGACTAC	(130)
	stx1R	AGAACGCCCACTGAGATCATC	
Shiga-like toxin 2	stx2F	GGCACTGTCTGAAACTGCTCC	(130)
	stx2R	TCGCCAGTTATCTGACATTCTG	
Attaching and effacing protein intimin (*eae*)	eaeAF	GACCCGGCACAAGCATAAGC	(130)
	eaeAR	CCACCTGCAGCAACAAGAGG	
Goose/duck-specific sequence	1	TCGAGCGGCCGCCCGGGCAGGT	(66)
	2R	AGCGTGGTCGCGGCCGAGGT	
Intimin subtyping	eaeVF	AGYATTACTGAGATTAAG	(139)
	eaeVR	AAATTATTYTACACARAY	
	eaeZetaVR	AGTTTATTTTACGCAAGT	
	eaeIotaVR	TTAAATTATTTTATGCAAAC	

[a] BHQ1, black hole quencher-1; 6FAM, 6-carboxyfluorescein

Table 2.2.

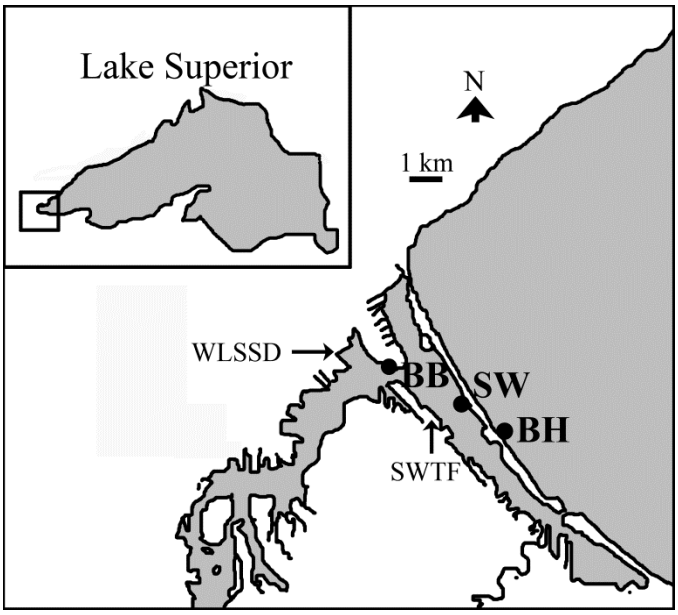
Identification of pathogen-related genes among *E. coli* water isolates.

Site	No. isolates analyzed	Concn. (CFU 100 mL^{-1})	Probe Hybridization (%)[a]		
			Goose/ duck	*eae*	*stx 1 or 2*
Blatnik Bridge	7849	74	12	1.5 (118)	<0.01 (2)
Southworth Marsh	11875	107	29	0.5 (42)	<0.01 (1)
Beach House	1372	35	4.6	0.8 (11)	ND[b]

[a]The number of isolates hybridizing to the pathogen gene probes *eae* and *stx* are shown in parentheses.

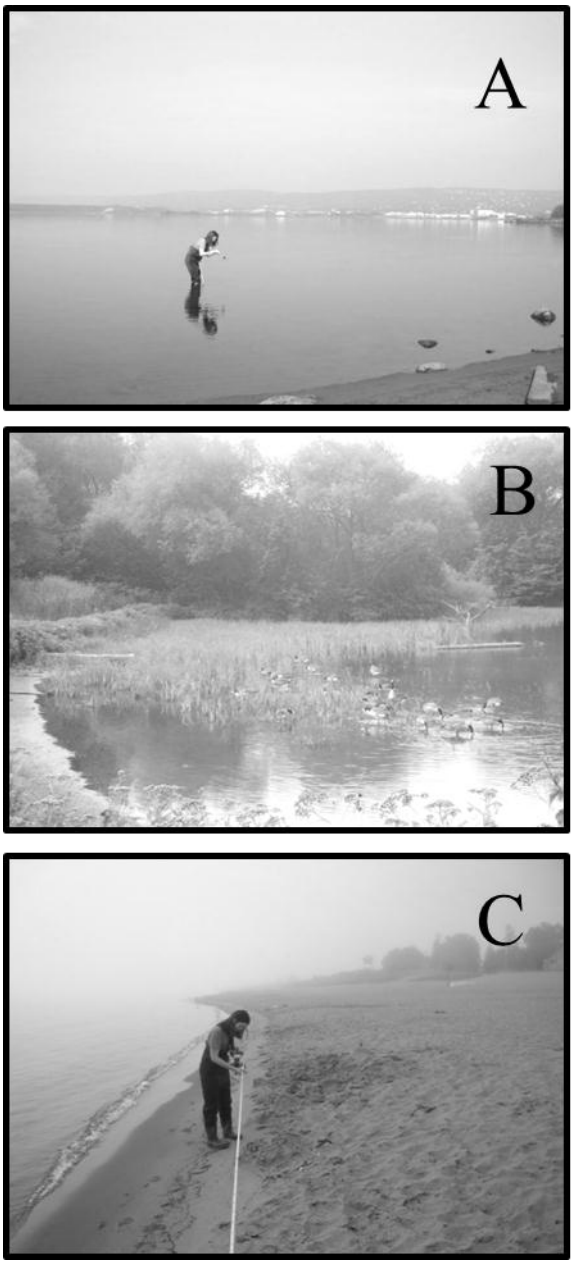
[b]ND=none detected.

Figure 2.1



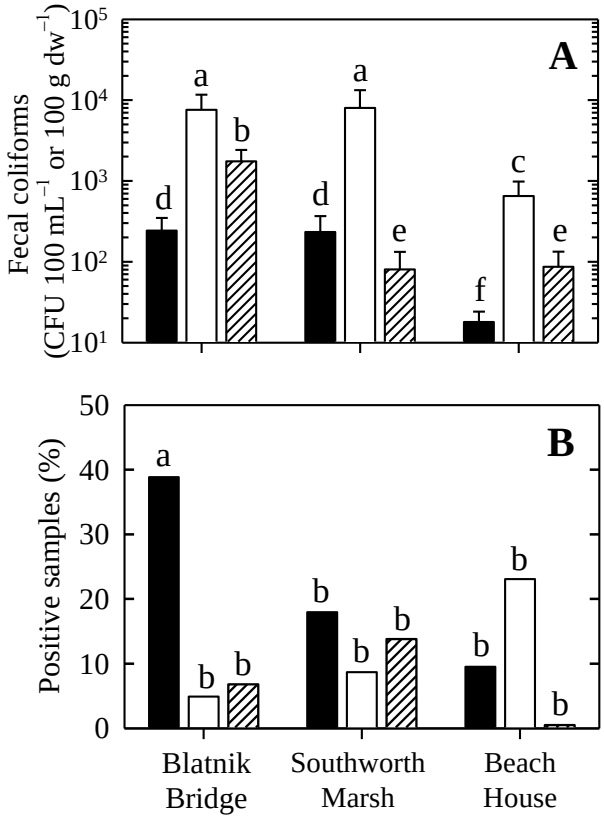
Map of study sites in The Duluth-Superior Harbor. The Blatnik Bridge (BB), Southworth Marsh (SW), and Beach House (BH) sites are indicated by black circles. Arrows indicate the location of wastewater treatment plants; the Western Lake Superior Sanitary District (WLSSD) and the Superior Wastewater Treatment Facility (SWTF).

Figure 2.2



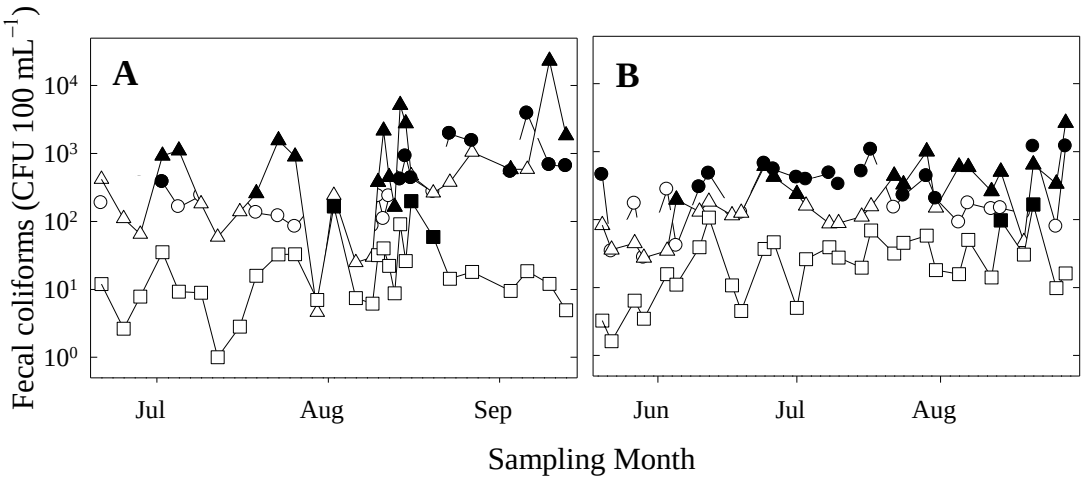
Photographs of locations of study reaches at Blatnik Bridge (A), Southworth Marsh (B), and Beach House (C) sites.

Figure 2.3



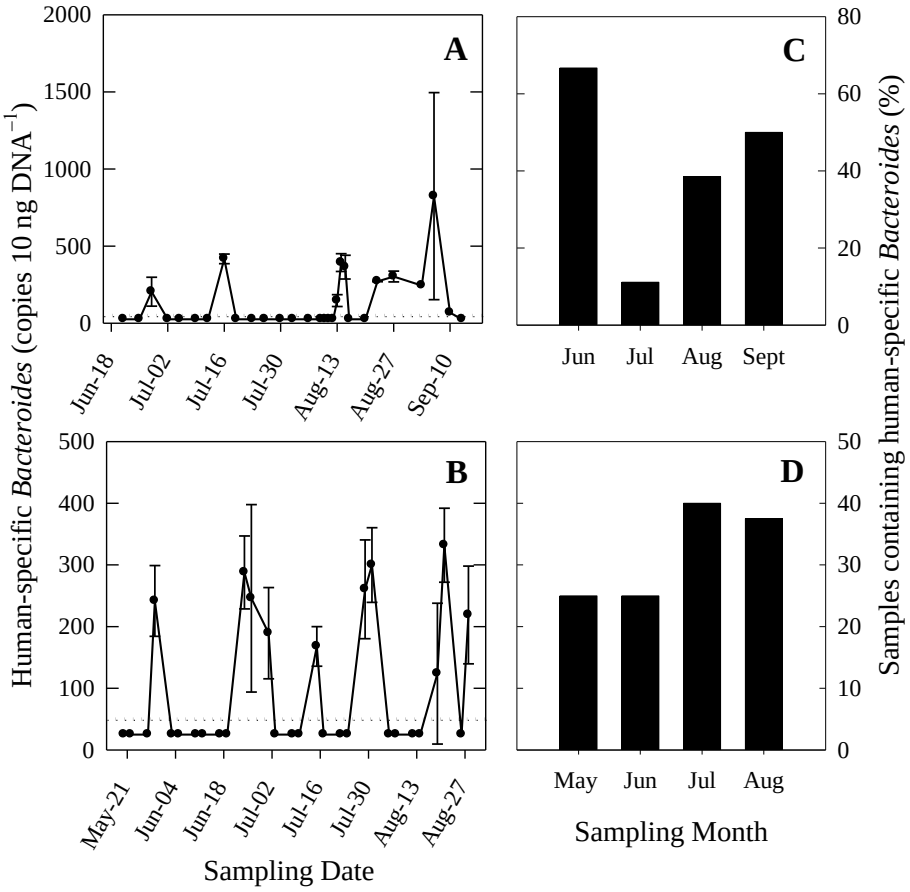
(A) Abundance of fecal coliforms in water (solid bars), sand (open bars), and sediment (striped bars) across sampling sites. Letters indicate significant differences based on ANOVA and Tukey HSD post-hoc comparisons. Error bars show 95% confidence intervals of the mean. (B) Percentage of samples that contained HF183 across sampling sites and substrate types. Bars with the same letters indicate no significant difference at $\alpha = 0.05$, based on Chi-square analysis.

Figure 2.4



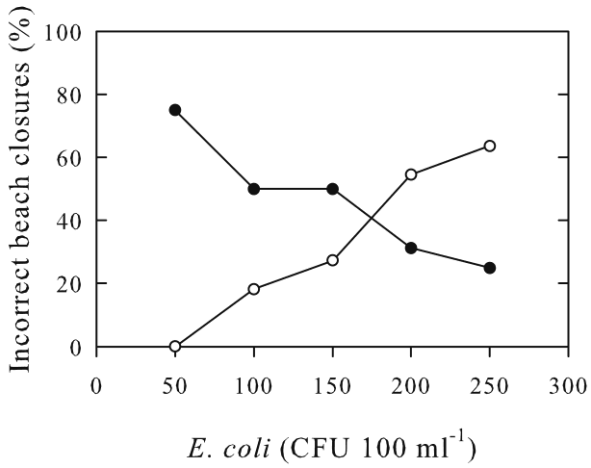
Fecal coliform counts in the water column at the Blatnik Bridge (○), Southworth Marsh (Δ), and Beach House (□) sites in 2007 (A) and 2008 (B). Solid symbols indicate the subset of sampling dates that were chosen for microbial source tracking and pathogen analysis.

Figure 2.5



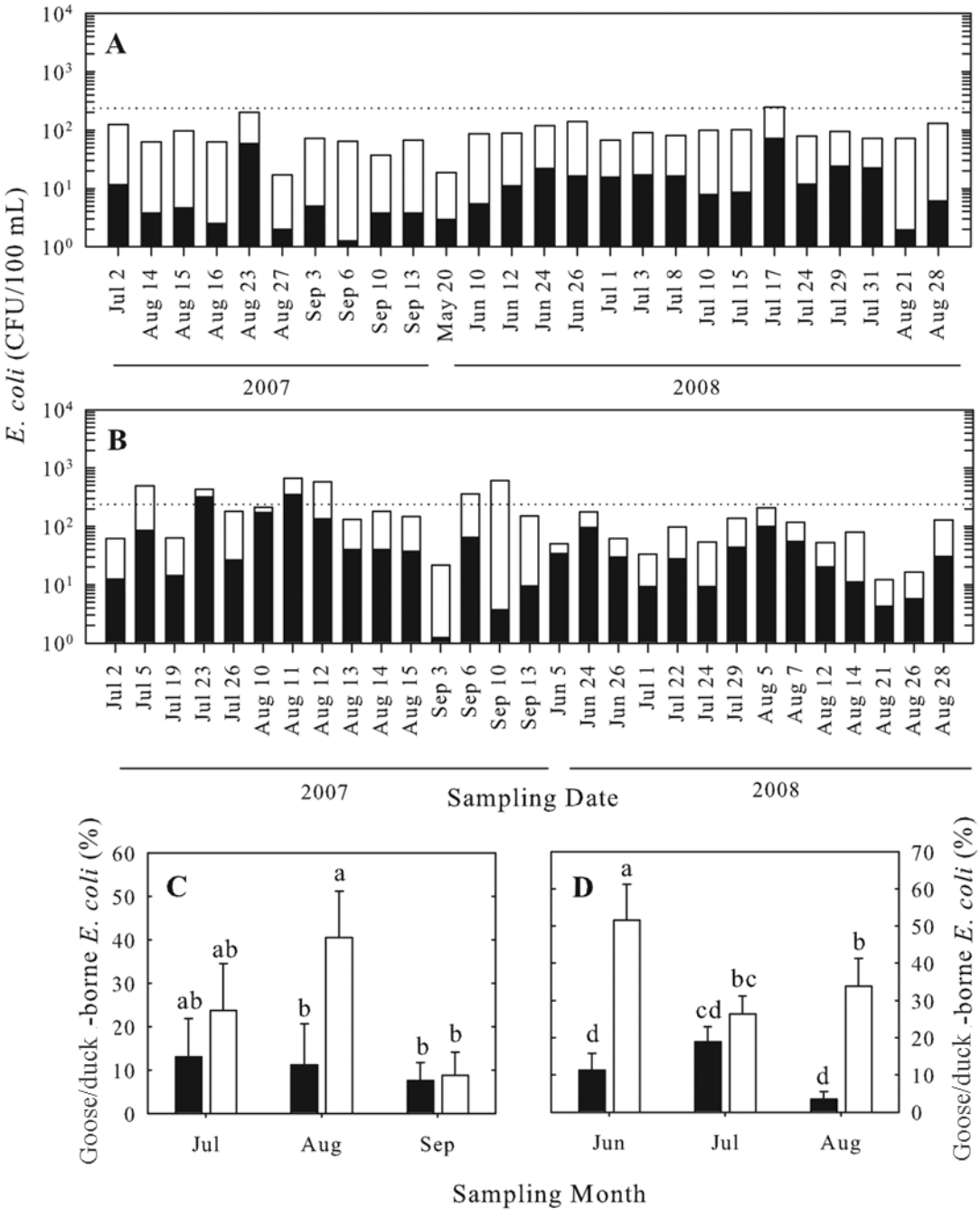
Concentration of HF183 in the water column at the Blatnik Bridge site. The concentration of HF183 on individual sampling dates is shown for 2007 (A) and 2008 (B). Error bars show the 95% confidence interval of the mean. The dotted line indicates detection limit of 50 copies 10 ng DNA^{-1}. Samples below the detection limit of 50 markers 10 ng DNA^{-1} are shown as one-half the detection limit. The proportion of water samples positive for HF183 by month is shown for 2007 (C) and 2008 (D). There was no significant difference in HF183 among sampling months.

Figure 2.6



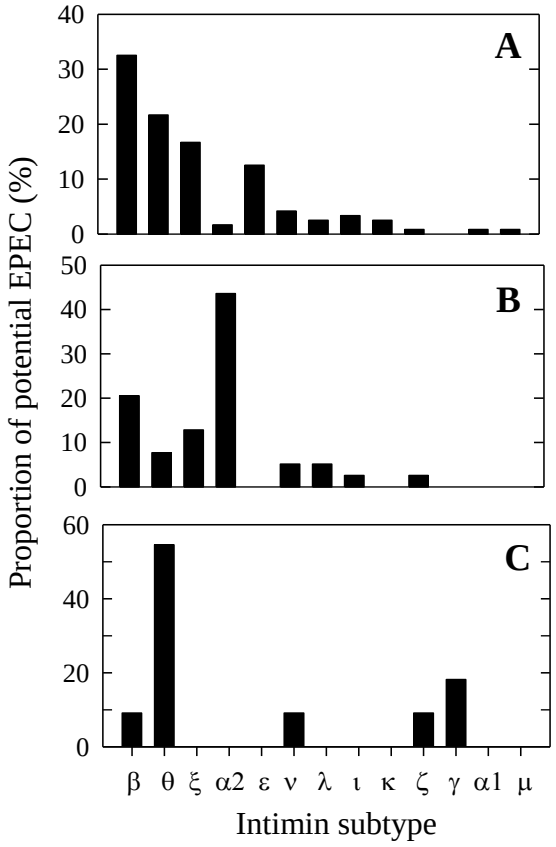
Type I (incorrect beach closure, ○) and Type II (failure to close beach, ●) error rates at hypothetical *E. coli* water quality standards ranging from 50 to 250 CFU 100 mL^{-1} when the presence of HF183 was considered the determinant of safety for recreational water contact at the Southworth Marsh beach site based on 2007 and 2008 data. The type I error rate results when a beach is closed when no HF183 is detected, whereas the type II error rate results when a beach remains open when HF183 is detected.

Figure 2.7


A
B
C
D
E. coli (CFU/100 mL)
Jul 2
Aug 14
Aug 15
Aug 16
Aug 23
Aug 27
Sep 3
Sep 6
Sep 10
Sep 13
May 20
Jun 10
Jun 12
Jun 24
Jun 26
Jul 1
Jul 3
Jul 8
Jul 10
Jul 15
Jul 17
Jul 24
Jul 29
Jul 31
Aug 21
Aug 28
2007
2008
Jul 2
Jul 5
Jul 19
Jul 23
Jul 26
Aug 10
Aug 11
Aug 12
Aug 13
Aug 14
Aug 15
Sep 3
Sep 6
Sep 10
Sep 13
Jun 5
Jun 24
Jun 26
Jul 1
Jul 22
Jul 24
Jul 29
Aug 5
Aug 7
Aug 12
Aug 14
Aug 21
Aug 26
Aug 28
2007
Sampling Date
2008
Goose/duck -borne E. coli (%)
Jul
Aug
Sep
Jun
Jul
Aug
Sampling Month
Goose/duck -borne E. coli (%)

E. coli from goose/duck (solid bars) as a proportion of total *E. coli* at Blatnik Bridge (A) and Southworth Marsh (B). The dotted line indicates the single sample water quality standard of 235 CFU 100 mL^{-1}. Bottom panel shows the proportion of goose/duck-borne *E. coli* per sampling month in 2007 (C) and 2008 (D) at BB (solid bars) and SW (open bars). Bars with the same letters indicate no significant difference at $\alpha = 0.05$ based on ANOVA and Tukey HSD post-hoc comparisons. Error bars show 95% confidence intervals of the mean.

Figure 2.8



Distribution of intimin subtypes from potential EPEC isolated from the water column at the Blatnik Bridge (A), Southworth Marsh (B), and Beach House (C) study sites. Subtypes are arranged along the x-axis in order of decreasing overall prevalence.

CHAPTER 3:

The distribution of genetic markers of fecal pollution on a freshwater sandy shoreline

OVERVIEW

Sand and sediment are important sources and sinks of culturable fecal indicator bacteria in the water column. However, the distribution and persistence of molecular markers of fecal pollution in sand and sediments are not known. Water, sand, and sediment were sampled monthly from a freshwater shoreline site receiving consistent input of wastewater effluent from June to October in 2010 and from May to September in 2011. Genetic markers for enterococci (Entero1), total *Bacteroides* (AllBac), and human-specific *Bacteroides* (HF183) were quantified by qPCR. Sand and sediment concentrations were 5.6 ± 0.2 $\log_{10}$ copies g dw^{-1} (mean ± standard deviation) and 5.3 ± 0.6 for Entero1, 5.5 ± 0.2 and 5.4 ±0.8 for AllBac, and 2.1 ± 0.1 (sand and sediment) for HF183. AllBac and HF183 in the water column were positively correlated with effluent concentrations (R^2 = 0.46 and 0.77). Effluent turbidity was positively correlated with AllBac and HF183 in effluent but was only correlated with AllBac in the water column (R^2 = 0.40). Entero1 and AllBac were most abundant in the upper 1 to 3 cm of sand and sediment, whereas HF183 was most abundant in the top 1 cm of sand and at 7 cm depth in sediment. The persistence of genetic markers over the course of one month was examined by installing capped, transparent cores into sand and sediment, allowing for no new inputs of fecal contamination. Three persistence experiments were conducted: June, July, and August 2011. In all three months, Entero1 and AllBac in the water column of the core did not change, but HF183 dropped below the detection level (3 copies mL^{-1}). In sand and sediment, experimental month significantly affected persistence. In July 2011, all genetic markers increased in sediment, indicating possible growth. Our results

show that sand and sediment may act as a reservoir for molecular markers of fecal pollution at some Great Lakes beaches.

INTRODUCTION

The degree of fecal contamination in surface waters is inferred by the presence and abundance of fecal indicator organisms. Fecal indicator bacteria can be enumerated by culture-based or molecular methods that target and quantify genus, species, or strain specific DNA sequences. Molecular methods such as quantitative PCR (qPCR) yield results in several hours, as opposed to 18 to 48 hrs for culture-based methods. In addition, the development of source-specific primers allows for identification of fecal pollution sources (112, 158). Consequently, there is a shift in the use of culture-based methods to quantitative PCR (qPCR) for molecular markers for water quality monitoring. The qPCR technique for bacteria within the genus *Enterococcus* is a promising method for identifying fecal contamination, as the abundance of the enterococci marker has been correlated to gastroenteritis disease risk in marine and freshwaters (172, 173). Similar to enterococci qPCR, qPCR for bacteria within the genus *Bacteroides* indicates the presence of fecal contamination, as it is abundant in fecal matter of both humans and animals (75, 179). *Bacteroides* is an obligate anaerobe, so its presence may indicate recent contamination. Additionally, *Bacteroides* markers have been developed that target source-specific strains, such as those that identify contamination from human sources (17).

Concentration of fecal bacteria in the water column can be influenced by sand and sediment bacterial concentrations. Sand and sediment can serve as reservoirs of fecal indicators in lake, river and ocean environments (80, 83, 188). Bacteria from the water column can be deposited in sand from wave action or settle out from the water column to

the sediment (128). As *E. coli* and enterococci are more abundant in shallow sand and sediment, resuspension of shallow sand and sediment may lead to water quality exceedances (63, 98, 128). Sands and sediments also offer protection from light, and nutrients may be more abundant in sand and sediment, potentially leading to indicator growth (20, 190). Taken together, there is high potential for indicators to accumulate in sand and sediment, so understanding indicator dynamics in these matrices is essential to accurately characterize the level of fecal contamination and associated health risk.

The role of sand and sediment as a reservoir for molecular indicators is not well understood. Molecular indicators for enterococci and *Bacteroides* markers have been detected in sand and sediment, but their vertical distribution has not been well-characterized. The presence of *Bacteroides* markers in streambed sediments and enterococci markers in sand were found to vary with depth (58, 171), but the quantitative distribution has not been examined. In addition, the persistence of molecular indicators in sand and sediment is not known. The qPCR technique can amplify DNA from live, dead, or dying cells and free DNA (86, 184); therefore, potentially contamination may be detected long after it first occurred. Before widespread implementation of qPCR methods for water quality monitoring, the distribution and persistence of molecular markers in sand and sediment should be examined.

In the present study, culturable and molecular indicators for enterococci, total *Bacteroides,* and human-associated *Bacteroides* were measured on a shoreline site of a freshwater harbor of Lake Superior. The site was located near a treated effluent outfall that served as point source of culturable and molecular indicators. The first aim was to

examine the effect of effluent inputs on culturable and molecular indicator abundance in the water column. Second, the vertical distribution of molecular markers in sand and sediment was characterized. Finally, the persistence of molecular indicators in water, sand, and sediment over a period of one month was determined through the use of field microcosms. Results confirmed the hypothesis that sand and sediment may serve as a reservoir of molecular markers of fecal contamination, especially for enterococci and total *Bacteroides* in the Great Lakes system examined.

METHODS

Study site and sample collection. The study site is non-recreational beach located on Duluth-Superior Harbor in Duluth, MN (47°13’37”N, 91°54’2”W) on the property of Western Lake Superior Sanitary District (WLSSD). The sampling site is located approximately 100 m from an outflow pipe that discharges treated effluent below the water’s surface (Figs. 3.1 and 3.2A). A 10–20 cm berm was located approximately 30 cm from the shoreline, beyond which the area was heavily vegetated by grass.

Samples were collected monthly from June to October in 2010 and from May to September in 2011. Three sand and sediment cores were sampled on 1 October 2012 for texture analysis, oxidation-reduction potential, and dissolved oxygen. WLSSD treated effluent received high doses of hypochlorite periodically over the study period. Hypochlorite doses other effluent characteristics are detailed in Table 3.1. Three replicate water samples were collected 2 m from the shoreline below the water’s surface. Three replicate cores of sand and sediment were taken one meter apart parallel to the shoreline.

Sand was sampled adjacent to the berm, and sediment was sampled 2 m from shore parallel to the shoreline at approximately 30–40 cm water depth. Cores were taken by manually inserting an AMS, Inc., 2.5 cm × 61 cm butyrate plastic core liner (American Falls, ID) into sand or sediment to a depth of approximately 15 cm. Three replicate treated effluent samples were taken from the effluent sampling station at WLSSD. Three times over the two-year study period, three replicate samples of raw influent were taken with the assistance from WLSSD staff.

The persistence of indicator bacteria was evaluated with three, one-month field experiments in June, July, and August of 2011. Three replicate transparent core tubes (same as those used for sampling) were installed to a depth of 15 to 20 cm in sand and sediment (Fig. 3.2B). The top of the cores were capped to prevent external inputs of contamination. Cores were positioned similarly to the sampling regime. Upon sampling, water from the upper portion of sediment cores was collected by decanting.

Sample processing and analyses. Cores were carefully removed from core tubes and sliced into 1 cm fractions. The top cm, 3, 5, 7, and 9 cm core depths were used for analyses in 2010, and 2011, and an 11 cm depth was also analyzed in 2011.

Oxidation-reduction potential and dissolved oxygen were measured for three replicate cores taken on 1 October 2012. Oxidation-reduction potential (mV) was measured with a pH58 (Milwaukee Instruments, Inc., Rocky Mount, NC) calibrated according to manufacturer instructions. Oxygen content (mg/L) of sand and sediment was measured by placing a calibrated MW600 standard portable dissolved oxygen meter (Milwaukee Instruments, Inc., Rocky Mount, NC) into sand or sediment. The probe was

allowed to equilibrate for 10 min before the reading was taken. Results are shown in Table 3.2.

For textural analysis, the approximate proportion of clay (< 2.0 μm), silt (2.0 – 50 μm), and sand (50 – 2000 μm) particles were determined from composite samples of three replicate cores taken on 1 October 2012. Forty grams of air dried (crushed to < 2 mm) sample was shaken for 16 h with 100 mL of 5% sodium hexametaphosphate. The suspension was quantitatively transferred to a sedimentation cylinder and brought to a total volume of 1L with deionized water. After a 2 h temperature equilibration, the suspension was stirred vigorously for one minute to re-suspend the particles. An ASTM No. 152H hydrometer was carefully placed in the suspension and used to take two readings, one at 40 s and another at 6–8 h (depending on the temperature of the suspension). The percentage of sand, silt and clay in the soil was calculated from the resulting hydrometer readings. Results are shown in Table 3.3.

DNA for qPCR analysis was extracted from a 1 g subsample of homogenized core material by using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA). Water, effluent, and influent samples were filtered through 0.45 μm nitrocellulose filters (Millipore, Billerica, MA) and sliced into 1 × 4 mm fragments with a sterile razor blade before DNA extraction with the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA). Sand and sediment was analyzed for percent moisture content. A 10 g subsample was weighed and then dried at 100°C for 24 h. The percent moisture was calculated as the difference between the sample wet and dry weights, divided by the dry weight of sample. All sand and sediment values are reported per dry g of sand or sediment.

In 2011, the concentration of *E. coli* and enterococci was determined by agitating 10 g subsamples of sand and sediment in 100 mL sterile ammonium phosphate solution with 0.01% gelatin in sterile milk dilution bottles. This was done using a wrist action shaker as previously described (81). Following shaking, solutions were allowed to sit for 15 min to allow large particles to settle before further processing. Supernatants from sand and sediment samples and water, effluent, and influent samples were filtered onto 0.45 µm nitrocellulose filters (Millipore, Billerica, MA) and placed on Modified mTEC (168) or mEI media (167) to enumerate *E. coli* and enterococci, respectively.

Genetic markers for total enterococci (Entero1), total *Bacteroides* spp. (AllBac) and human-specific *Bacteroides* (HF183) were quantified by quantitative PCR (qPCR) (Table 3.4). Reactions consisted of template DNA (0.5 uL – 5 uL of DNA extract), primers, probe, and iTaq Supermix or iTaq Supermix with ROX (Bio-Rad, Hercules, CA). Concentration of primers and probe were optimized for each assay (Table 3.4). Amplification of Entero1 and AllBac consisted of an initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Amplification of HF183 consisted of an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 30 s, extension at 60°C for 1 min, and a final melt curve analysis to verify specific product amplification.

Plasmid standards were created by cloning the target gene from PCR product amplified from *E. faecalis* strain ATCC 29212 for Entero1 and from sewage influent for AllBac and HF183 using the StrataClone PCR kit (Stratagene, Santa Clara, CA). Purified plasmid DNA was quantified by the Qubit 1.0 fluorometer (Invitrogen, Grand Island,

NY) before preparation of six 10-fold dilutions for qPCR standards ranging from 3 to 300,000 target markers 5 μL^{-1}. Each run contained triplicate reactions of standards, triplicate reactions of non-transcript controls, and triplicate reactions of samples. Amplification was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA), and quantification cycle (C*q*) values were automatically determined using the system software. Sample marker concentration was calculated on a per-run basis.

Statistical analyses. The detection limit of genetic markers in water samples was 300 copies per 100 mL, and in sand and sediment the detection limit was 30 copies g^{-1}. For culturable indicators, the detection limit was 2 CFU 100 mL^{-1} in water samples and 1 CFU one-third g^{-1} in sand and sediment. For statistical analyses, samples below the detection limit were assigned a value of one-half the limit of detection, and concentrations of indicators were log-transformed to approximate a normal distribution.

Paired t-tests were used to determine differences between site water column and effluent over the entire sampling period, between pre and post marker concentrations in water column persistence experiments, and between $\log_{10}$ change in enterococci and *E. coli* counts in sand and sediment. Chi-square was used to determine differences in the proportion of samples below the limit of detection for HF183 in sand and sediment. Pearson product-moment correlation was used to determine significant relationships between indicators in water, sand and sediment and correlation between effluent characteristics and water and effluent molecular indicator abundance. The relationship between depth of sand or sediment sample and concentration of indicators was examined

through linear regression. Two-way ANOVA was used to determine the effect of treatment month and genetic marker type on marker reduction from sewage treatment and to determine differences in indicator abundance between site water column and effluent by sampling month. Post-hoc comparisons were conducted with Tukey HSD at $\alpha = 0.05$.

Effluent flow and turbidity data were accessed online at <http://www.lakesuperiorstreams.org/streams/stream_data.html> through the data visualization tool for WLSSD on 10 October 2011.

RESULTS

Indicators in sewage influent, treated effluent, and the water column. In untreated sewage influent, Entero1, AllBac, and HF183 markers were found at an average concentration of 7.6 ± 0.7 (mean ± standard deviation), 8.8 ± 0.6, and 7.0 ± 0.4 $\log_{10}$ copies 100 mL^{-1}, respectively. Treatment of sewage resulted in a 2.5 ± 0.7 $\log_{10}$ average reduction in genetic marker concentrations, although efficacy varied with sampling date and marker type. Reduction of markers followed the trends: HF183 > AllBac > Entero1 and June 2011 > August 2010 > June 2010, although differences were not statistically significant (Two-way ANOVA, $p > 0.5$). Treated effluent contained Entero1, AllBac, and HF183 at average concentrations of 5.8 ± 0.4, 6.4 ± 0.5, 4.4 ± 0.6 $\log_{10}$ copies 100 mL^{-1}, respectively. The concentration of culturable enterococci and *E. coli* in sewage was measured on June 2011. Treatment of sewage resulted in a 5.8 $\log_{10}$ reduction in enterococci and a 4.7 $\log_{10}$ reduction in *E. coli*. For treated effluent in 2011,

the geometric mean enterococci concentration was 12 CFU 100 mL^{-1}, 95% CI [1.2, 137], and *E. coli* was 272 CFU 100 mL^{-1}, 95% CI [52, 1427].

Entero1, AllBac, and HF183 markers were detected in the water column at all sampling times. On average, AllBac markers were most abundant at 6.3 ± 0.5 (mean ± standard deviation) $\log_{10}$ copies 100 mL^{-1}. The concentration of Entero1 and HF183 markers was 5.4 ± 0.4 and 4.1 ± 0.8 $\log_{10}$ copies 100 mL^{-1}, respectively. The concentration of culturable enterococci and *E. coli* measured in 2011 was 62 CFU 100 mL^{-1}, 95% CI [32, 121], and *E. coli* averaged 368 CFU 100 mL^{-1}, 95% CI [187, 722]. The concentration of Entero1 and HF183 genetic markers was lower in the water column compared to effluent (paired t-test, Entero1, $p = 0.006$; HF183, $p = 0.03$), but there was no difference between the site water column and treated effluent in AllBac markers, culturable enterococci, or *E. coli* (paired t-test, $p > 0.05$). On individual sampling dates, few differences between effluent and water column indicator concentrations were observed (Figs. 3.3 & 3.4). In eight of nine significant differences observed between effluent and water column genetic marker concentrations, the genetic marker concentration in effluent was higher than the site water column. For culturable indicators, enterococci and *E. coli*, three of four significant differences occurred when the water column had higher culturable indicators than effluent.

Genetic markers were significantly correlated within and between water column and effluent samples (Table 3.5). In the water column, Entero1 and AllBac were not correlated, but they were both positively correlated to HF183, with Entero1 having a slightly stronger correlation. Within effluent, markers were all positively cross-correlated,

with similar R^2 values. The positive correlation between water column and effluent HF183 concentrations explained more variation in marker concentrations than AllBac with respect to R^2 values. There was no significant relationship between water column and effluent Entero1 concentrations. Effluent turbidity was strongly correlated to effluent AllBac concentrations. Site water column AllBac and effluent turbidity were also correlated, but the R^2 was half that observed for effluent. Effluent turbidity was correlated to effluent HF183, but there was no similar correlation in the water column. There was no relationship between effluent turbidity and Entero1 markers.

Wastewater treatment plant effluent flow rates and hypochlorite treatment level were not significantly correlated to marker concentrations ($p < 0.05$). Effluent flow and effluent turbidity were not correlated ($p > 0.05$), likely due an observed time lag of up to 10 hrs between peaks in effluent flow and peaks in turbidity. There was no significant correlation between culturable indicators and genetic markers, or between culturable indicators and effluent turbidity, flow, or chlorination treatment in water samples.

Distribution of indicators in sand and sediment. The Entero1, AllBac, and HF183 genetic markers were more abundant in upper portions of sand and sediment. On average, there was no significant difference between the concentration of molecular indicators in sand and sediment. Sand and sediment marker concentrations were 5.6 ± 0.2 and 5.3 ± 0.6 for Entero1, 5.5 ± 0.2 and 5.4 ±0.8 for AllBac, and 2.1 ± 0.1 for HF183 in both sand and sediment. HF183 fell below the limit of detection in 19% of sand samples, and 17% of sediment samples. In 2010, the proportion of sediment samples below the detection limit was 9.6%, and was significantly different than that in sand in 2010 (χ^2 =

7.6, $p = 0.006$), and sediment in 2011 ($\chi^2 = 9.8$, $p = 0.002$). In sand, all markers were most abundant in the upper 1 to 3 cm (Fig. 3.5). In sediment, Entero1 and AllBac were abundant in the upper cm, similar to the distribution in sand. In contrast, HF183 was most abundant in 5 and 7 cm portions of sediment. The Entero1 and AllBac concentrations in sand and sediment were negatively correlated with depth (Entero1, sand $p = 0.04$, sediment $p < 0.0001$; AllBac, sand $p = 0.002$, sediment $p < 0.0001$). Markers decreased less with increasing depth in sand (regression slope, Entero1 = –0.06; AllBac = –0.08) compared sediment (regression slope, Entero1 = –0.14; AllBac = –0.20) for both markers. Although HF183 markers decreased with depth, the correlation was not significant ($p = 0.23$).

The culturable indicator bacteria enterococci and *E. coli* were most abundant in the upper centimeters of sand and sediment, similar to the distribution of molecular indicators (Fig 3.6). The concentration of culturable indicators was slightly higher in sand relative to sediment. The concentration of enterococci was 3.9 CFU g^{-1}, 95% CI [0.8, 19] in sand and 1.7 CFU g^{-1}, 95% CI [0.8, 3.8] in sediment. The concentration of *E. coli* was 4.0 CFU g^{-1}, 95% CI [1.2, 13] in sand and 3.9 CFU g^{-1}, 95% CI [2.5, 6.1] in sediment. Counts of enterococci in sediment and *E. coli* in sand and sediments were significantly negatively correlated with depth (enterococci, sand $p = 0.09$, sediment $p < 0.0001$; *E. coli*, sand $p = 0.003$, sediment $p = 0.0009$).

There were significant cross-correlations between culturable and molecular indicators in sand and sediment (Table 3.6). Indicator correlations in sand had a higher R^2 compared to sediment. There was a strong correlation between AllBac and Entero1 in

both sand and sediment, but the correlation of AllBac and Entero1 to HF183 explained little variance in sand and was insignificant in sediment. Culturable indicators enterococci and *E. coli* were significantly correlated to Entero1 and AllBac in sand and sediment, but the explained variance for Entero1 was low in sediment. Culturable indicators were not significantly correlated or did not explain much variance in HF183 marker abundance in sand and sediment. Similarly to AllBac and Entero1, culturable enterococci and *E. coli* were strongly correlated in both sand and sediment. In correlations of identical indicators between sand and sediment, all molecular and culturable indicators were significantly correlated. However, the correlations between identical molecular indicators explained less variation than the correlations between culturable indicators.

Correlations between water column and sand or sediment indicator abundances were dependent on indicator and depth. Entero1 abundance in sand and water was negatively correlated at 3 cm ($R^2 = 0.53$, $p = 0.02$), 5 cm ($R^2 = 0.43$, $p = 0.05$), 7 cm ($R^2 = 0.39$, $p = 0.05$), and 9 cm ($R^2 = 0.44$, $p = 0.04$) depth. Entero1 in water was also negatively correlated to sediment concentration at 1 cm depth ($R^2 = 0.41$, $p = 0.04$). However, Entero1 in water was not correlated with sand at 1 cm and 11 cm, or in sediment at depths greater than 1 cm ($p > 0.05$). The concentration of AllBac in sediment was not correlated with site water column concentrations at any depth in sand or sediment ($p > 0.05$). HF183 in water and sand were not correlated, but, in sediment, they were positively correlated at 1 cm ($R^2 = 0.48$, $p = 0.03$) and 7 cm ($R^2 = 0.40$, $p = 0.05$) depth. Culturable indicator concentrations of enterococci and *E. coli* in the water column were not correlated to sand or sediment concentrations at any depth ($p > 0.05$).

Comparison of calibrator cell equivalents (CCE) and CFU. Assuming four copies of the Entero1 marker per *E. faecalis* genome (72), comparisons were made between the number of enterococci as measured by qPCR and culture methods in 2011 (Fig. 3.7). There was a significant interaction between sample type and analysis method on enterococci abundance ($p = 0.04$). Overall, enterococci CCE were 2.9 to 4.8 $\log_{10}$ greater than enterococci CFU. The magnitude of the difference in CCE and CE of enterococci followed the trend: sand > sediment > effluent > sewage > water column.

Field persistence of indicators. The persistence of culturable and molecular indicators of fecal pollution was determined in field microcosms for a period of one month. In the water column, there was no significant change in Entero1 or AllBac markers (paired t-test, $p = 0.24$ and $p = 0.63$), but HF183 decreased from 1,800, 12,000, and 26,000 markers 100 mL^{-1} to below the detection limit of 300 markers 100 mL^{-1}. Culturable enterococci fell from 22, 40, and 116 CFU 100 mL^{-1} to below the detection limit of 2 CFU 100 mL^{-1}. *E. coli* decreased from 137 CFU 100 mL^{-1} to 13 CFU 100 mL^{-1}, 95% CI [1.0, 152] in the June field incubation. In the July and August 2011 field incubations, *E. coli* decreased from 233 and 680 CFU 100 mL^{-1} to below the detection limit.

Incubation month had a strong effect on the persistence of indicators in sand and sediment (Fig 3.8). Few significant changes in genetic marker concentration were observed in June. In July, sand concentrations of Entero1 and AllBac did not change, but HF183 decreased at 1 and 3 cm sand depths. Also, Entero1, AllBac, and HF183 increased at several depths in sediment. In August, Entero1, AllBac and HF183 decreased in sand

at several depths. Entero1 and AllBac exhibited decreases in sediment concentrations in August as well, whereas HF183 increased at most depths. The $\log_{10}$-fold change in markers ranged from –2.7 to +1.8, and, on average, all markers decreased in sand and increased in sediment. The average change in enterococci and *E. coli* was –1.0 $\log_{10}$ and ranged from –3.3 to 0.1 $\log_{10}$. There was no significant difference in $\log_{10}$ fold change in enterococci and *E. coli* counts (paired t-test, $p = 0.21$). Bacterial counts in sand and sediment decreased over the course of the summer, leading to an increase in samples below the detection limit of 1 CFU one-third g^{-1} of sand or sediment. After one month incubation, there were increases in the number of samples below the detection limit (Fig. 3.9). Sediment exhibited a greater increase in the number of samples that fell below the detection limit for both enterococci and *E. coli*. Overall, the increase in percentage of samples below the detection limit was 26% for enterococci and 35% for *E. coli* in sand, whereas in sediment the increase was 57% for enterococci and 61% for *E. coli*.

DISCUSSION

Sand and sediment can be an important reservoir of indicator bacteria; however, the distribution and persistence of molecular indicators in sand and sediment has not been thoroughly assessed. The first aim of this study was to examine the effect of effluent inputs on genetic markers for enterococci (Entero1), total *Bacteroides* (AllBac), and human-specific *Bacteroides* (HF183) in the water column of a nearby shoreline. Results indicate that effluent likely controls the abundance of molecular indicators of fecal pollution in water, due to the correlation between effluent and water column genetic

marker concentrations and the positive relationship between effluent turbidity and water column AllBac abundance. Second, the vertical distribution of molecular indicators in sand and sediment was determined. Sand and sediment contained more Entero1 and AllBac on a per mass basis than water, whereas the concentration of HF183 was similar among sand, sediment and water. In most instances, markers were most abundant in the top 1 to 3 cm; therefore, resuspension of shallow sediments could significantly raise indicator levels in water. Finally, the persistence of genetic markers in water, sand, and sediment over a period of one month was examined. Over the course of one month, Entero1 and AllBac markers in the water column persisted with no significant change in concentration, whereas HF183 decreased to below detection levels. In sand and sediment, experimental month had a strong effect on persistence, but all markers exhibited significant increases in sediment in July. Taken together, sand and sediment play an important role as a reservoir of molecular indicators at the study site, which has significant loading of indicators into the water column through discharge of treated effluent.

Effluent inputs largely control indicator abundance in the water column of the study site. Treatment of sewage influent resulted in a 2.5 $\log_{10}$ reduction in genetic markers, which is within the range observed in previous studies that reported reductions of 2.5 to 3.5 $\log_{10}$ (10, 155, 161). Even with a significant reduction in markers, in Duluth-Superior Harbor, the discharge of treated effluent resulted in a spike of genetic markers for total *Bacteroides* and human-specific *Bacteroides* within the water column (94). In the present study, on most sampling dates, there was no difference in effluent and site

water column molecular indicator concentrations. Moreover, effluent turbidity was significantly positively correlated to AllBac and HF183 in effluent and AllBac in the water column. Increases in effluent turbidity at WLSSD are the result of a chain reaction following a large precipitation event. Precipitation can result in inflow and infiltration, which, in turn, increases the input volume to the wastewater treatment plant thereby decreasing its efficiency (T. Tuominen, personal communication). The increase in turbidity following peak plant flows may indicate less efficient processing of wastewater, as bacteria are often associated with suspended particles. Similarly, *Salmonella* abundance in treated effluent was positively correlated with turbidity across four wastewater treatment plants of varying efficiencies (91). Surprisingly, effluent turbidity was not correlated to water column Entero1 and HF183. On average, water column concentrations of Entero1 and HF183 were lower than effluent, so processes such as settling or decay may have decoupled any existing relationship between markers and turbidity.

Effluent outflows can increase sediment concentrations of indicator bacteria. Proximity to wastewater outfalls was found to affect the spatial distribution of culturable *E. coli* and enterococci in sediments of a freshwater lake (63) and *Clostridium perfringens* in ocean sediments near a research station in Antarctica (51). In this study, sand and sediment contained approximately 4.9 $\log_{10}$ calibrator cell equivalents (CCE) g^{-1}, assuming four copies of the Entero1 marker per *E. faecalis* genome (72). The concentration of enterococci CCE in sand and sediment at the study site is well above the range of 2.2 to 3.2 $\log_{10}$ CCE g^{-1} in marine beach sand (73, 190). There are few studies

that quantify *Bacteroides* markers in sand and sediment. In Duluth-Superior Harbor, the concentration of total *Bacteroides* in sediment was elevated near a wastewater outfall, but human-specific *Bacteroides* was not detected in sediment (94). On the study shoreline, HF183 was 1 and 2 $\log_{10}$ less abundant than Entero1 and AllBac, respectively, in the water column, whereas HF183 was 3.4 $\log_{10}$ less abundant than Entero1 and AllBac in sand and sediment. Potentially, HF183 decays in the water column before it can be deposited in sand and sediment, or HF183 decays more rapidly than Entero1 and AllBac following deposition. However, HF183 was detected in every core sample, so at sites with consistent human fecal inputs, HF183 is likely to occur.

Molecular indicators of fecal pollution have a similar depth distribution profile to culturable indicator bacteria. Molecular indicators were, in most instances, most abundant in the upper one to three cm of sand and sediment. Similarly, *E. coli* and enterococci were found to be most abundant in the upper layers of sediment in freshwater streams, rivers, lakes, and estuarine bays (8, 42, 63). In freshwater sand, *E. coli* was most abundant from 0 to 5 cm, and enterococci were most abundant from 5 to 10 cm depth (4). A presence/absence study of a total *Bacteroides* and a source-specific marker for humans found no decrease in the detection frequency of total *Bacteroides* with sediment depth in a freshwater stream, but human markers were most frequently detected from 10 to 20 cm depth (171). HF183 was found at near detection limits at 11 cm depth in sand and sediment. The decrease in HF183 with depth in sand and the peak in HF183 at 7 cm depth in sediment, and subsequent decrease with depth, makes it unlikely that this marker is most abundant from 10 to 20 cm depth.

Resuspension of shallow sediments high in fecal indicators can lead to spikes in water column concentrations (128). Resuspension of sand and sediment at the study site could occur through surface runoff, a dramatic increase in the flow of the St. Louis River, or wave action from strong winds. However, wave height at the study site was never observed to exceed 5 cm, possibly, in part, due to the sheltered environment of the study beach. Therefore, it is unlikely that wave action would lead to resuspension of sediment greater than 1 cm depth. Nevertheless, resuspension of less than 0.5 cm depth of sediment can lead to water quality exceedances if sediment concentrations are high (8). It follows that factors that may lead to resuspension of sand or sediment must be taken into consideration when using molecular indicators for water quality monitoring or microbial source tracking. Similar to observations of culturable indicators, it appears that sand and sediment may act as a reservoir of molecular indicators, especially for total *Bacteroides* and enterococci.

Unexpectedly, Entero1 and AllBac markers persisted in the water column over the course of one month, with no change in concentration. Previous literature reported decay rates of qPCR markers for enterococci in the water column ranging from –0.24 to –2.2 $\log_{10}$ copies day^{-1} (12, 177) and from –0.6 to 2.2 $\log_{10}$ copies day^{-1} for *Bacteroides* (11, 44, 125, 151, 175). Assuming these decay rates, all markers should have fell below detection limits within one month; however, only HF183 fell below detection. Initial concentrations of enterococci CCE 100 mL^{-1} were 3.1 $\log_{10}$ greater than culturable enterococci, and enterococci CCE did not change when culturable cells fell below 0.3 $\log_{10}$ CFU 100 mL^{-1}. Culture methods may underestimate the actual number of viable

fecal indicators as exposure to environmental stressors not present in the host habitat may trigger bacteria to enter a viable but non-culturable (VBNC) state (52, 127). Attachment of *E. faecalis* in a VBNC state to zooplankton was found to facilitate persistence in freshwater and marine water (157). The difference between cell counts and qPCR markers may also differ as qPCR can amplify markers from dead or dying cells and free DNA (10, 86).

The persistence of molecular markers in sand and sediment exhibited variability with respect to month and depth. Differences in persistence among months suggest that differences in temperature played a role in persistence. Higher temperatures negatively affect persistence of fecal indicators (77, 110, 113); however, the average monthly temperature was highest in July, when increases in sediment abundance were observed for all genetic markers (108). Increases in marker concentrations suggest that enterococci and *Bacteroides* can grow within the sediment environment, if conditions are favorable. Growth of *Bacteroides* in experimental microcosms has not been reported, but enterococci have been shown to grow in wetted beach sand and streambed sediments (105, 190). In some instances, the average $\log_{10}$ change in marker concentration in sand or sediment was 1 or 2 $\log_{10}$, but was not significant. Due to the highly heterogeneous nature of sand and sediment environments (76), the accurate measurement of persistence of fecal indicators in sand and sediment, that is non-homogenized and non-inoculated, will remain a difficult question.

The distribution and persistence of molecular indicators at a site that is continuously impacted by wastewater outflows may differ from pristine systems. The

sheer number of strains that are introduced into the system on a daily basis favors selection for persistent strains. In addition, the high concentration of markers in the water column, coupled with the sheltered environment of the study site, may lead to higher settling rates than in other lotic systems. The complexity of factors that control persistence of fecal indicators necessitates the use of methods to limit the effects of environmental variability. Microcosms were used to address persistence of fecal indicators in this study in order to more accurately reflect persistence under environmental conditions, such as diurnal fluctuation in temperature and light. Still, it is possible that there were effects of the micrcosm itself on persistence, and the reductions in this study should be considered as an approximation of actual persistence.

Fecal pollution of surface waters is a public health concern worldwide. Demand for fast and accurate methods for water quality monitoring will lead towards a shift from the use of culture-based methods to quantification of molecular markers of fecal pollution. Recent studies have focused on the dynamics of molecular indicators of fecal pollution in the water column, even though sand and sediment can contain a greater proportion of indicators within the beach environment (8, 128). In addition, exposure to beach sand is associated with risk of gastroenteritis, and illness is associated with increased abundance of genetic markers for enterococci and *Bacteroides* (73, 74). Results from this study show that sand and sediment can act as a reservoir of molecular fecal indicators. Furthermore, the accumulation of markers in sediment may be due to growth, and should be examined further. The use of qPCR for molecular markers of fecal

pollution for beach monitoring and assessment should take into account the role of sand and sediment before making a management decision is made.

Table 3.1

Wastewater treatment plant characteristics on sampling dates.

Sampling date	Plant flow[a] (MGD)	Effluent turbidity[a] (NTU)	Chlorine[b] (lbs/day)
6/25/2010	64.5	5.8	284
7/28/2010	51.9	18.8	861
8/23/2010	46.5	1.5	1153
9/30/2010	37.5	7.1	3101
10/28/2010	36.0	30.8	297
5/23/2011	36.9	3.5	157
6/20/2011	34.4	5.1	1718
7/25/2011	35.9	7.0	150
8/24/2011	35.2	11.3	2196
9/20/2011	30.8	18.0	244

[a]Data from http://www.lakesuperiorstreams.org/streams/stream_data.html.

[b]Data from J.Mayasich (personal communication).

Table 3.2

Chemical characteristics of sand and sediment cores. [a]

Depth (cm)	Redox (mV)		Dissolved oxygen (mg/L)	
	Sand	Sediment	Sand	Sediment
1	96.6	–166	2.5	0.0
3	43.7	–156	2.0	0.0
5	22.0	–118	2.3	0.0
7	–29.3	–89.7	1.6	0.0
9	–85.7	–81.7	0.3	0.0
11	–69.3	–81.7	0.0	0.0

[a]Redox and dissolved oxygen data are averages from three replicate cores sampled on 1 October 2012.

Table 3.3

Texture analysis of sand and sediment cores.[a]

Depth (cm)	Sand cores			Sediment cores		
	Sand (%)	Silt (%)	Clay (%)	Sand (%)	Silt (%)	Clay (%)
1	73.8	7.5	18.7	72.5	6.3	21.3
3	65.0	11.3	23.8	62.5	15.0	22.5
5	67.5	12.5	20.0	67.5	13.8	18.8
7	66.2	13.8	20.0	70.0	11.3	18.8
9	72.5	13.8	13.8	77.5	10.0	12.5
11	65.0	12.5	22.5	72.5	8.8	18.8

[a]Values from composite samples of three replicate cores sampled on 1 October 2012.

Table 3.4

Oligonucleotide sequences and final concentrations for qPCR assays.

Assay primer and probe	Sequences (5'→ 3')[a]	Conc. (nM)	Target	Reference
Entero1				
ECST748F	AGAAATTCCAAACGAACTTG	400	*Enterococcus* spp.	(72)
ENC854R	AATGATGGAGGTAGAGCACTGA	400		
GPL813TQ	(FAM)TGGTTCTCTCCGAAATAGCTTTAGGGCTA(TAMRA)	200		
AllBac				
AllBac296F	GAGAGGAAGGTCCCCCAC	200	All *Bacteroidales*	(97)
AllBac467R	CGCTACTTGGCTGGTTCAG	200		
AllBac375Bhqr	(FAM)CCATTGACCAATATTCCTCACTGCTGCT(BHQ-1)	300		
HF183				
HF183	ATCATGAGTTCACATGTCCG	300	Human-associated	(18, 153)
HF183R	CCATCGGAGTTCTTCGTG	300	*Bacteroides*	

[a] FAM, 6-carboxyfluorescein; TAMRA, Carboxytetramethylrhodamine; BHQ-1, black hole quencher 1.

Table 3.5

Pearson product moment correlation R^2 values among beach water column marker concentrations, effluent marker concentrations, and effluent turbidity.

Variable	Beach[a,b]			Effluent		
	Entero1	AllBac	HF183	Entero1	AllBac	HF183
Beach						
Entero1	–			0.32	0.25	**0.51**
AllBac	0.31	–		0.07	**0.46**	0.35
HF183	***0.62***	**0.46**	–	0.12	0.21	***0.77****
Effluent						
Entero1				–		
AllBac				**0.46**	–	
HF183				**0.41**	**0.50**	–
Effluent Turbidity[c]	0.21	**0.40**	0.36	0.22	***0.80****	**0.49**

[a]All correlations are positive.

[b]Bold, $p \leq 0.05$; Bold and italics, $p \leq 0.01$; Bold, italics, and asterisk, $p \leq 0.001$.

[c]NTU

Table 3.6

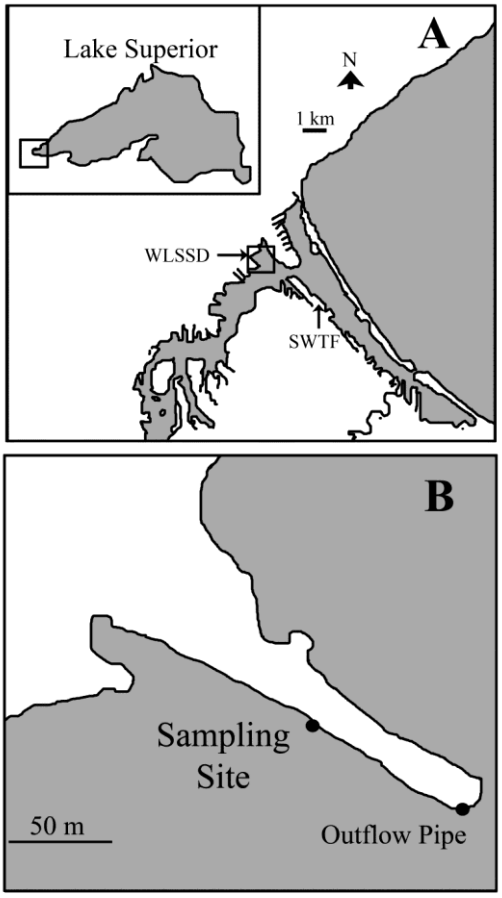
Pearson product moment correlation R^2 values among sand and sediment marker concentrations, *E. coli*, and enterococci concentrations.

Variable	Sand[a,b]					Sediment				
	Entero1	AllBac	HF183	*E. coli*	Enterococci	Entero1	AllBac	HF183	*E. coli*	Enterococci
Sand										
Entero1	–					***0.24****	***0.32****	0.01	***0.42****	***0.50****
AllBac	***0.85****	–				***0.26****	***0.39****	0.02	***0.58****	***0.65****
HF183	***0.13***	***0.17****	–			**0.12**	***0.21****	***0.27****	0.00	0.00
E. coli	***0.65****	***0.71****	0.02	–		**0.16**	***0.37****	***0.27***	***0.53****	***0.82****
Enterococci	***0.49****	***0.47****	0.00	***0.89****	–	0.05	**0.17**	***0.22***	***0.37****	***0.77****
Sediment										
Entero1						–				
AllBac						***0.68****	–			
HF183						0.00	0.04	–		
E. coli						**0.19**	***0.37****	0.09	–	
Enterococci						**0.13**	***0.24***	**0.20**	***0.59****	–

[a] All correlations are positive

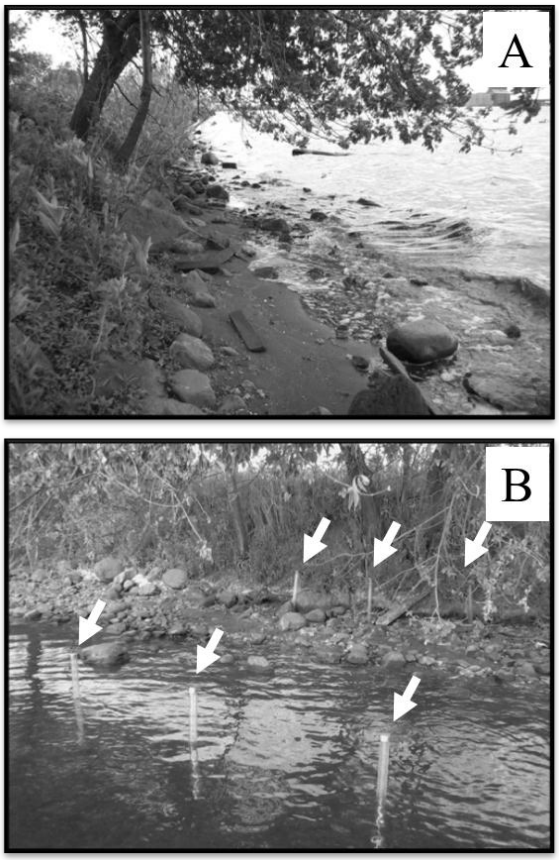
[b] Bold, $p \leq 0.05$; Bold and italics, $p \leq 0.01$; Bold, italics, and asterisk, $p \leq 0.001$.

Figure 3.1



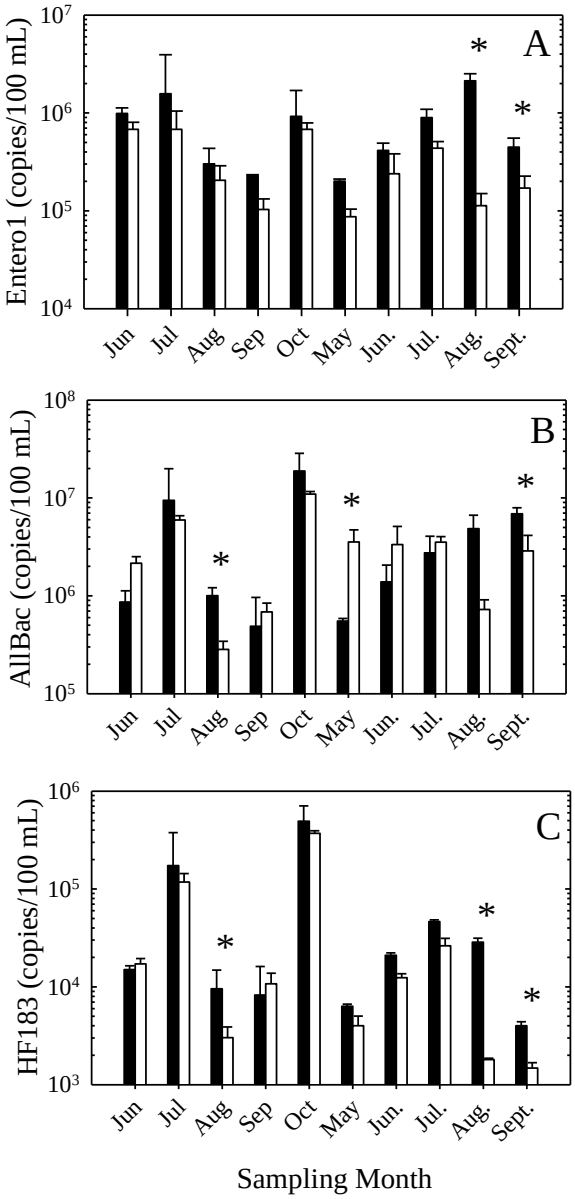
(A) Inset shows the location of Duluth-Superior Harbor on Lake Superior. Arrows indicate wastewater treatment plant outflow locations for Western Lake Superior Sanitary District (WLSSD) and Superior Wastewater Treatment Facility (SWTF). (B) Detail of shoreline near WLSSD, and location of the sampling site relative to the treated effluent outflow pipe.

Figure 3.2



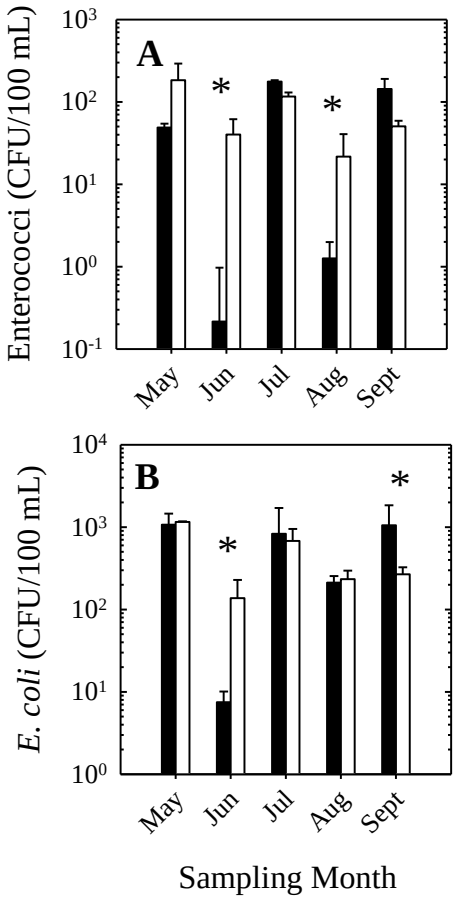
Photographs of the study reach (A) and installed, capped cores during persistence experiments (B). White arrows indicate core tubes.

Figure 3.3



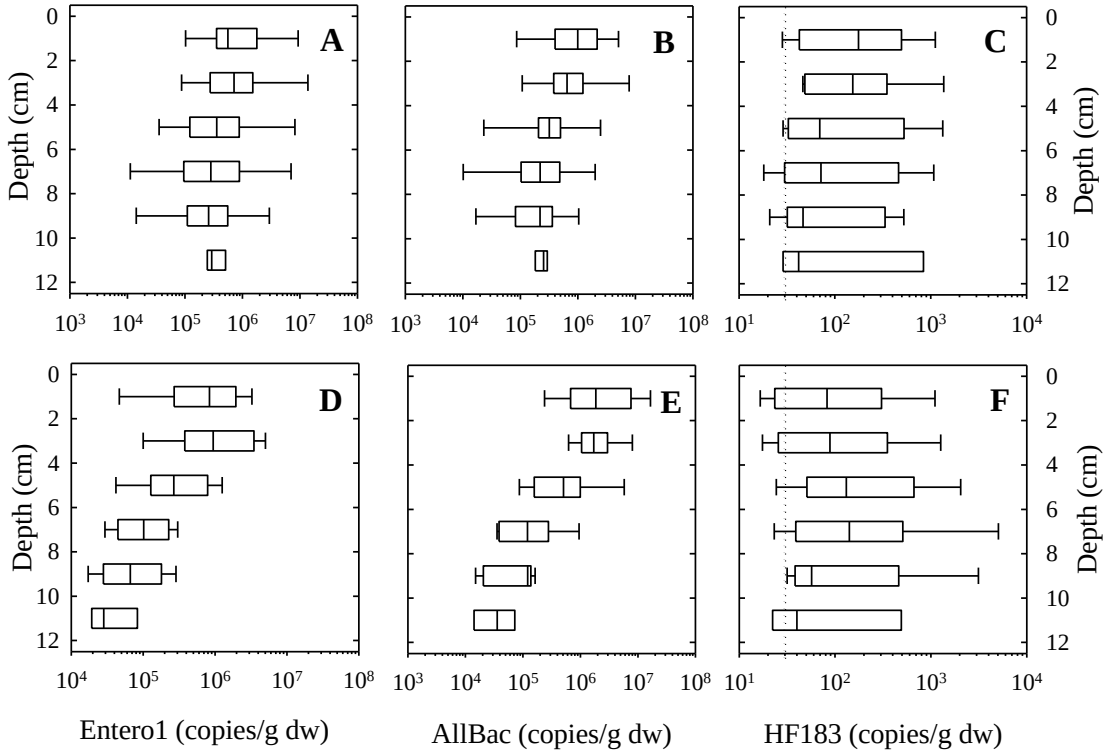
The concentration of Entero1 (A), AllBac (B), and HF183 (C) molecular markers of fecal contamination in treated effluent (solid bars) and beach water (open bars). Asterisks indicate significant differences between the concentration of markers on a sampling date based on Tukey's HSD ($\alpha = 0.05$).

Figure 3.4



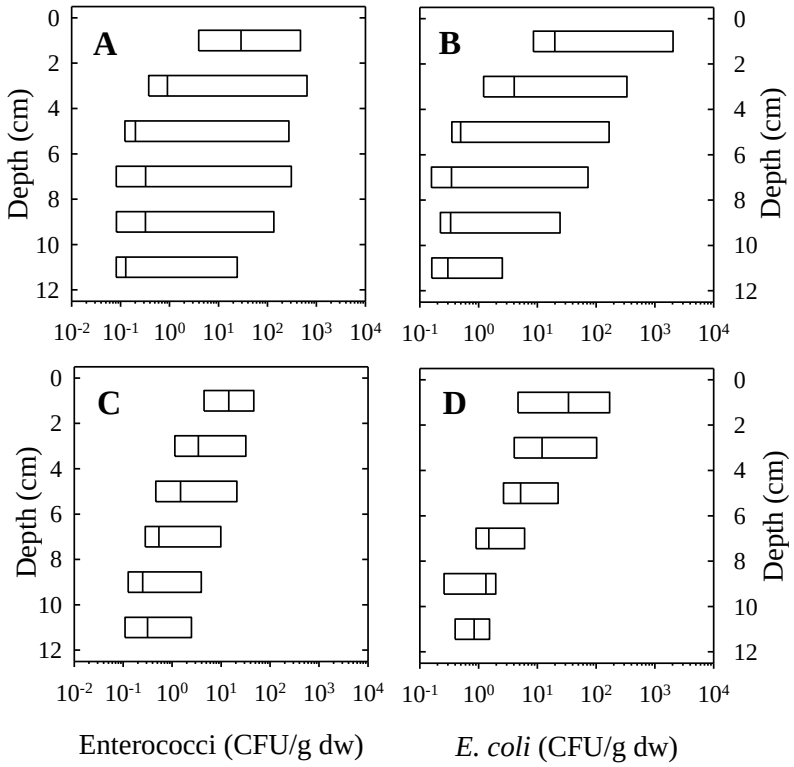
The concentration of culturable enterococci (A) and culturable *E. coli* (B) in treated effluent (solid bars) and beach water (open bars) in 2011. Asterisks indicate significant differences between the concentration of markers on a sampling date based on Tukey's HSD ($\alpha = 0.05$).

Figure 3.5



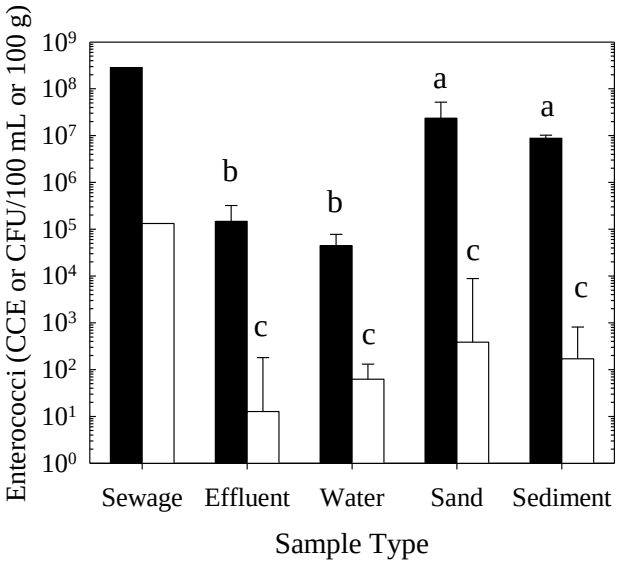
Box plots of Entero1 (A, D), AllBac (B, E), and HF183 (C, D) with depth in sand (A, B, C) and sediment (D, E, F) across all sampling dates in 2010 and 2011. The left boundary of the box indicates the lower quartile of the data, the right boundary indicates the upper quartile, and the line within the box indicates the median. Whiskers show the 90th and 10th percentiles. The dotted line indicates the limit of detection for the HF183 assay.

Figure 3.6



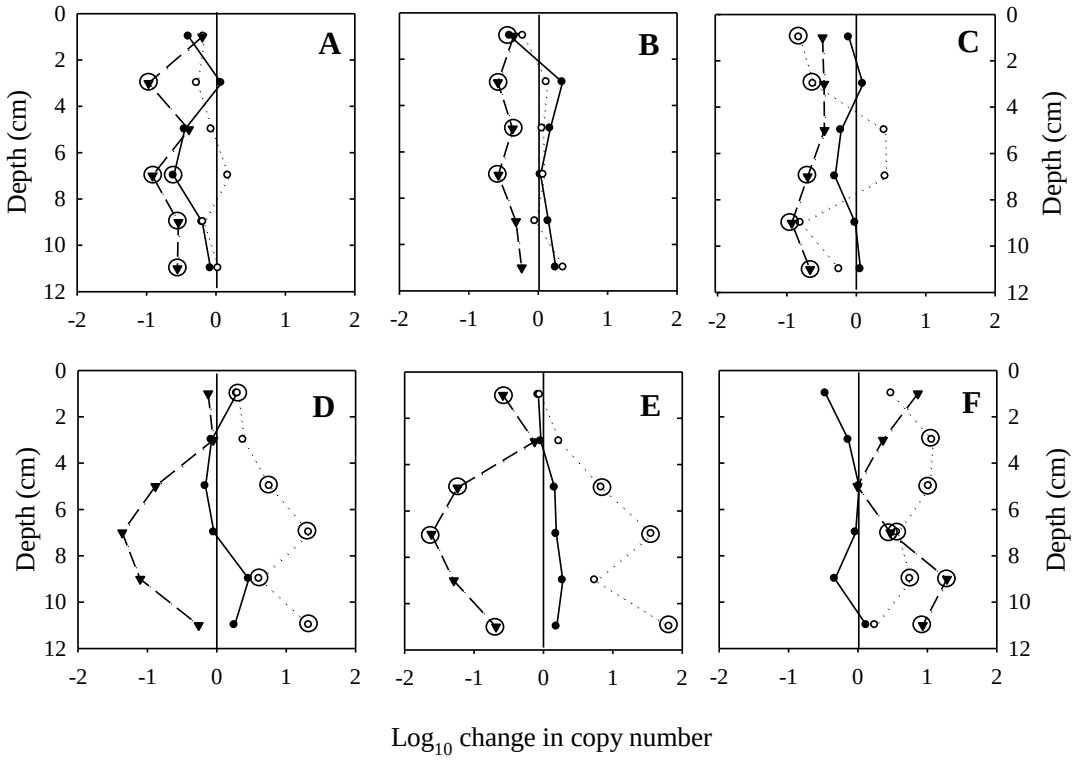
Box plots of culturable enterococci (A, C) and *E. coli* (B, D) with depth in sand (A, B) and sediment (C, D) across all sampling dates in 2010 and 2011. The left boundary of the box indicates the lower quartile of the data, the right boundary indicates the upper quartile, and the line within the box indicates the median.

Figure 3.7



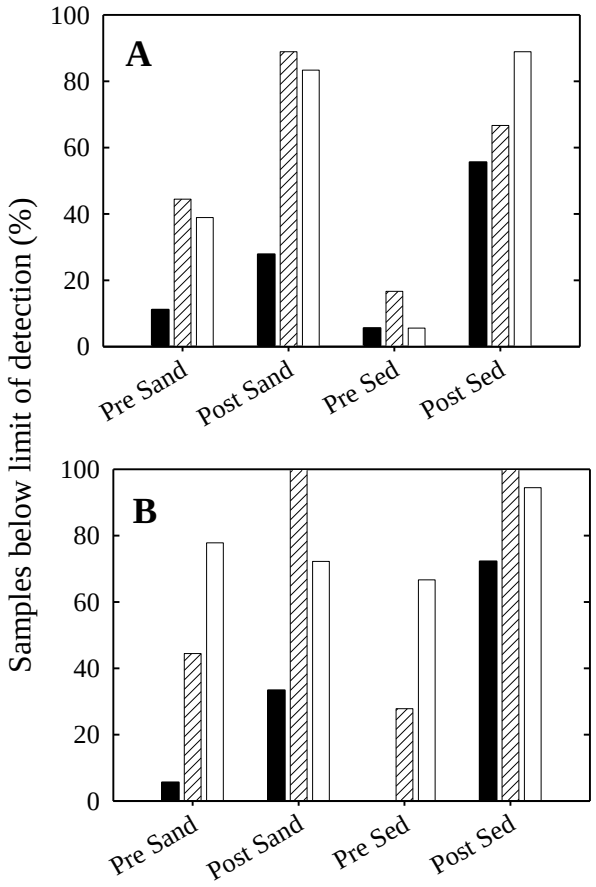
Enterococci colony forming units (CFU) and calibrator cell equivalents (CCE) in 2011 samples. Sewage values are from June 2011. Error bars indicate the 95% confident interval of the mean. Bars with the same letter are not significantly different based on Tukey's HSD at $\alpha = 0.05$. Sewage was excluded from the analysis.

Figure 3.8



Log_{10}-fold change in abundance of Entero1 (A, D), AllBac (B, E), and HF183 (C, F) genetic markers in sand (A, B, C) and sediment (D, E, F). The results from June (solid circles), July (open circles), and August (inverted triangles) one month long field incubations are shown. Samples that exhibited a significant change ($\alpha = 0.05$) in genetic marker concentration are circled.

Figure 3.9



Percentage of sand and sediment samples (n = 18) below the limit of detection of 1 CFU per one-third g dw for enterococci (A) and *E. coli* (B) in June (solid bars), July (hatched bars), and August (open bars).

CHAPTER 4:

Influence of Moisture and Temperature on the Persistence of Genetic Markers for Enterococci, total *Bacteroides*, and human-specific *Bacteroides* in Lake Superior Water, Sand, and Sediment

OVERVIEW

The persistence of genetic markers for enterococci (Entero1), total *Bacteroides* (AllBac), and human-associated *Bacteroides* (HF183) was examined in microcosms containing water and sediment or sand from Duluth-Superior Harbor inoculated with raw sewage influent. The influence of temperature on persistence was determined at 6, 13, 21, 30, and 37°C, and the effect of moisture on persistence in sand was examined at 10, 20, and 30% moisture. Marker concentrations were measured by quantitative PCR. The decay rates of Entero1, AllBac, and HF183 markers were negatively correlated with temperature for most treatments. The Entero1 and AllBac markers decayed more slowly in sand at 30% moisture, relative to that seen for 10 and 20% moisture, except for Entero1 at 6°C. The effect of moisture on the decay of HF183 in sand varied with temperature and did not show a consistent trend. The AllBac marker had a positive decay rate at 6 and 13°C in sand at 30% moisture, indicating possible growth within the microcosm. Markers decayed faster in water than in sand and sediment. The decay rates of the Entero1 and AllBac markers were 92% similar, whereas the AllBac and HF183 marker decay rates were only 32% similar. In instances when HF183 and AllBac decay rates were dissimilar, the HF183 marker exhibited a faster decay. Results of these studies indicate that environmental conditions that influence temperature and moisture, as well as sample matrix, must be taken into account when evaluating fecal contamination using molecular markers.

INTRODUCTION

Fecal pollution of land and surface waters is a widespread environment problem and of public health concern. In order to detect fecal pollution, bacterial species that are abundant in fecal matter, typically *Escherichia coli* or *Enterococcus* spp., are quantified by using culturing methods. The presence of these fecal indicator bacteria (FIB) has been used to imply the potential presence of fecal contamination and potential risks to human health. Since culture-based methods require 18 to 48 hr to process, however, they are unable to assess risk in real time. Advances in molecular methods and knowledge of the molecular biology of FIB has led to the development of several promising "near real time" detection assays, especially the development of quantitative PCR (qPCR) primers and probes for FIB. Once qPCR methods are appropriately validated there will be a necessary shift towards their implementation to assess water quality (158).

Several criteria must be satisfied in order to establish the validity of method to quantify FIB. The method must be correlated to the incidence of illness or presence of human pathogens, be sensitive to the presence of fecal contamination, be specific to its respective source or host, detect microbes that do not grow outside of the host environment, and have a consistent rate of decay outside of the host (25, 69). Recent studies have suggested that *E. coli* are unsuitable indicators due to their widespread presence and growth in sands, soils, and algae (34, 57, 80, 81, 93). Some enterococci are primarily environmental in origin, and enterococci have also been shown to exhibit extended persistence in secondary habitat, and increased survival in the presence of aquatic vegetation (7, 9, 52). However, epidemiological studies have demonstrated that

Enterococcus qPCR is among one of the most reliable methods to predict the incidence of disease in beachgoers at both marine and freshwater beaches (172, 173). The persistence of *Enterococcus* qPCR markers in the environment needs to be adequately assessed in order to accurately interpret contamination events. In addition, the use of *Bacteroides* spp. as indicators of fecal pollution has been long recognized, as this bacterium is abundant in feces, constituting up to 30% of the total microbial population (3, 75, 116, 135). *Bacteroides* qPCR is particularly applicable to microbial source-tracking studies due to the development of source-specific markers for bovine, porcine, human, and other fecal sources (53, 143).

Although sand and sediment are integral to understanding the microbial loading of recreational beaches, the survival of genetic markers of FIB in sand and sediment is not well-studied (64). Culture-dependent methods have shown that sand and sediment contribute substantially to bacterial numbers through resuspension. Both *E. coli* and enterococci have been found to be in large enough quantities in beach sand to significantly influence water column FIB counts at several beaches (181, 188). It has been postulated that these matrices may offer protective benefits for microorganisms from predation, temperature fluctuations, or death due to desiccation (156, 190). Some pathogenic bacteria have been shown to exhibit prolonged survival in sediments relative to water (31, 54). These effects were consistent across five sediment types, and supported the conclusion that sediments may serve as a reservoir for pathogenic bacteria, surviving for up to a month in this secondary habitat. Furthermore, exposure to contaminated beach sands can raise the risk of gastrointestinal illness (73).

The persistence of genetic markers of FIB in sand and sediment cannot be directly inferred from the decay rate of culturable FIB, as the presence of viable but not culturable (VBNC) cells and extracellular DNA may result in slower decay of genetic markers of FIB relative to culturable cells (124, 125). For example, Yamahara *et al*. (189) and Klein *et al*. (89) found that enterococci measured by qPCR decayed more slowly than cultured enterococci in marine beach sand and manure. However, the factors that decrease FIB persistence in water (increases in temperature, light, and predation) and sand and sediment (increased temperature and decreased moisture) are consistent for culturable FIB and molecular markers (11, 77, 111, 177). In manure and soil, increased temperature hastened the decay of enterococci and total *Bacteroides* markers (89, 140). Studies on culturable FIB have also shown that low moisture or drying stress can greatly increase bacterial decay, whereas increased moisture can stimulate growth (20, 32, 114). Similarly, increased moisture in soil and wetting of marine beach sand slows decay of molecular markers of fecal pollution (140, 189). Although several studies have examined the persistence of molecular markers in freshwater (12, 44, 62, 153), there are no studies that have examined the persistence of molecular markers in freshwater sand or sediment.

The objective of this study was to determine the persistence of genetic markers of fecal pollution in water, sand, and sediment from a freshwater beach. Sand and sediment laboratory microcosms inoculated with raw sewage influent were used to examine the effects of temperature, sand moisture, and matrix type on decay rate. The concentration of non source-specific markers, the *Enterococcus* qPCR marker Entero1, the *Bacteroides* genus-specific marker AllBac, and the source-specific *Bacteroides* marker for human

feces, HF183, were monitored up to four months. Results of this study have implications for the use of qPCR markers for the detection of fecal contamination and source-tracking of human fecal pollution on freshwater beaches, provide a framework for understanding the dynamics of genetic markers in freshwater environments, and enhance our understanding of the ecology of these fecal bacteria.

METHODS

Microcosms. Experiments could not be conducted in the field because some *Bacteroides* spp. are opportunistic pathogens which necessitated the use of experimental microcosms. Two types of microcosms were used: (1) sand only; and (2) water with submerged sediment (Fig. 4.1). Water, sand, and sediment for the experimental microcosms were collected from Duluth Boat Club Beach (DBC) in Duluth, MN, (46°46'10"N, 92°05'23"W) on June 20, 2011. Raw sewage inoculum was obtained from Western Lake Superior Sanitary District (WLSSD) treatment plant on June 20, 2011. Sand microcosms consisted of sterile 55 mL glass screw top test tubes filled with 40 g sand and 4 mL of inoculum. Water and submerged sediment microcosms consisted of 40 g of sediment overlaid with 112.5 mL of DBC beach water and 12.5 mL of inoculum, in 160 mL sterile glass milk dilution bottles.

Prior to sterilization, glass milk dilution bottles were pretreated with dimethyldichlorosilane (DCDMS) to reduce bacterial adhesion to glass surfaces. Bottles were filled with a 10% solution of DCDMS in toluene and allowed to sit for 10 min. The bottles were rinsed with toluene and then rinsed with methanol.

Treatments and sampling. Three replicate uninoculated samples of sand and sediment were frozen for DNA extraction and subsequent analysis by qPCR of the initial marker concentration. Three replicate samples of DBC beach water, prior to inoculation and the addition of wastewater influent, were filtered through nitrocellulose filters (0.45 μm pore size, 47 mm diameter; Millipore, Bedford, MA) to collect bacteria for DNA extraction.

The effect of temperature on marker persistence in sand and sediment microcosms was tested at five temperatures. Four temperatures spanned the range of those observed in Duluth, MN, from the spring to fall open water season (6, 13, 21, and 30°C), and the fifth temperature, 37°C, was included to select for potential preferential growth of gut-associated bacteria. Incubator temperatures were monitored at each sampling, and they did not change over the course of the experiment. Microcosms were incubated in the dark for 120 days.

The effect of sand moisture on persistence was tested at three moisture levels (10, 20, and 30%) encompassing values observed on Duluth, MN, area beaches (J. Eichmiller, unpublished data). For moisture analysis, samples were weighed and then dried at 100°C for 24 h. The percent moisture was calculated as the difference between the sample wet and dry weights, divided by the dry weight of sample. All sand and sediment values are reported per dry g of sand or sediment. Filter sterilized DBC water was added to sand microcosms in order to adjust the sand moisture level from a starting moisture of 1% to the desired level. After four months of incubation, the sand moisture slightly decreased from 10, 20, and 30% to 9, 19 and 28%, respectively. The initial moisture of the

sediment was 26%, and the moisture increased to 35% after the microcosms were constructed. After this time, sediment moisture did not change over the course of the experiment.

The approximate proportion of clay (< 2.0 μm), silt (2.0 – 50 μm), and sand (50 – 2000 μm) particles in DBC sand and sediment were determined. Forty grams of air dried (crushed to < 2 mm) sample was shaken for 16 h with 100 mL of 5% sodium hexametaphosphate. The suspension was quantitatively transferred to a sedimentation cylinder and brought to a total volume of 1 L with deionized water. After a 2 h temperature equilibration, the suspension was stirred vigorously for one minute to re-suspend the particles. An ASTM No. 152H hydrometer was carefully placed in the suspension and used to take two readings, one at 40 s and another at 6–8 h (depending on the temperature of the suspension). The percentage of sand, silt and clay in the soil was calculated from the resulting hydrometer readings. Results of textural analysis are shown in Table 4.1.

Three sand and sediment microcosms of each treatment were sacrificed at each sampling time: 1, 3, 7, 14, 21, 28, and 120 d post-inoculation. Water from sediment microcosms (100 mL) was filtered through a nitrocellulose filter (0.45 μm pore size, 47 mm diameter; Millipore, Bedford MA) to collect material for DNA extraction. Sand and sediment was homogenized and assayed for moisture content before DNA was extracted from 1 g subsamples. Filters, sand, and sediment samples were stored at –20°C until used.

DNA extraction and qPCR assays. DNA from frozen filters, and sand and sediment samples was extracted using a PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA). Prior to DNA extraction, filters were sliced into 1 mm × 4 mm fragments using a sterile razor blade. The MoBio protocol was modified to include an additional DNA wash with buffer C5. DNA was eluted in a final volume of 50 µl.

Plasmid standards were created by cloning PCR products amplified from *Enterococcus faecalis* strain ATCC 29212 for the Entero1 genetic marker and from sewage influent for AllBac and HF183 genetic markers using previously published primers (Table 4.2) and the StrataClone PCR kit (Stratagene, Santa Clara, CA). Purified plasmid DNA was quantified by using a Qubit 1.0 fluorometer (Invitrogen, Grand Island, NY) before preparation of six 10-fold dilutions for qPCR. Standards ranged from 3 to 300,000 target copies per 5 µL.

The qPCR analyses were conducted using previously published protocols, but primer and probe concentrations were optimized for this study (Table 4.2). The HF183 assay was done using iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), and the Entero1 and AllBac assays used iTaq Supermix With Rox (Bio-Rad, Hercules, CA). Reactions contained 12.5 µL mastermix, primers and probe, and 5 µL of undiluted or a 10-fold dilution of template DNA in a final reaction volume of 25 µL. The HF183 reactions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C denaturation for 15 s, 56°C annealing for 30 s and an extension at 60°C for 45 s. A melt step was included to verify specific product amplification. For the Entero1 and AllBac reactions, an initial denaturation at 95°C for 5 min was used, followed by 40

cycles of a denaturation at 95°C for 15 s and an annealing and extension step at 60°C for 1 min.

Each qPCR run contained triplicate reactions of standards and non-transcript controls, and duplicate reactions of samples. Amplifications were performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA), and quantification cycle (C*q*) values were automatically determined using the system software. Sample marker concentrations were calculated on a per-run basis.

Analyses. Sand and sediment marker concentrations are reported as the value per g dry wt. A two-way ANOVA was used to determine the mains effects and interaction of temperature and sample type on marker gene concentration for the individual markers. Tukey's HSD was used to determine significant differences between means. First-order decay rates were calculated as the slope of the linear regression of ln-transformed genetic markers up to 28 d. In instances where marker concentrations fell below the limit of detection, only sampling dates for which markers could be determined for all three replicates were included in the calculation of the decay rate. First order decay is described by the following equation:

(1) $$C = C_0 e^{-kt}$$

where C is the concentration of genetic markers at time (t), C_0 is the initial concentration of markers, and k is the decay rate constant.

Linear regression and Pearson product-moment correlation were used to examine the relationship between decay rate and temperature, and the relationship between marker concentration at 120 d and temperature. Tukey's studentized range statistic multiple

comparisons test was used to determine the significant differences in decay rates between five treatments/sample types (Sand at 10, 20, and 30% moisture, water, and sediment) at each temperature level. Multiple comparisons tests were also used to determine the effect of temperature on the decay rate of marker genes in water. Test values were calculated by dividing the difference between two slopes by the pooled standard error.

(2) $$q = \frac{\left(\overline{X_i} - \overline{X_J}\right)}{\sqrt{\frac{MS_W}{n}}}$$

As the critical value for Tukey's range statistic (q) accounts for a factor of 2 (appears under the square root sign), q divided by the square root of 2 was used for the critical value for mean comparisons. Tukey's range test was done using Microsoft Office Excel 2007 (Microsoft, Redmond, WA) and critical values were determined using R (http://www.r-project.org/). All other statistical analyses were done using JMP®Pro version 9.0.2 (SAS Institute Inc., Cary, NC). All statistical analyses were done at a level of statistical significance of $\alpha = 0.05$.

RESULTS

Validity of qPCR runs and initial marker concentrations. The amplification efficiencies of all runs were between 93 and 110%, and the R^2 values were above 0.993 (Table 4.3). No amplification was observed in non-transcript controls for the HF183 and AllBac assays. The non-transcript controls for the Entero1 assay had a significantly lower Cq value than the lowest standard (t-test, $p < 0.0001$). The limit of detection (LOD) was consistent across all assays. The LOD for sand and sediment was 30 copies g^{-1}, and water

samples had a LOD of 3 copies mL^{-1}. The genetic marker concentrations of the DBC samples prior to inoculation and of the raw sewage inoculum are shown in Table 4.4.

Influence of temperature on genetic markers. Temperature had a significant effect ($p \leq 0.05$) on the concentration of the Entero1, AllBac, and HF183 genetic markers. At 1 and 3 d after inoculation, there was no clear effect of temperature treatments (Figs. 4.2 & 4.3). The 6°C microcosm treatment had the highest mean concentrations from day 7 to 28, with the exception of the Entero1 and AllBac markers in sand at 30% moisture. At 120 d, the copy numbers of Entero1 and AllBac were negatively correlated with temperature ($p \leq 0.01$), except for Entero1 in sand at 30% moisture (Table 4.5). At 120 d, the HF183 genetic marker in sand and sediment fell below the LOD at all temperatures greater than 6°C. In water, the copies of HF183 fell below the LOD for all temperatures at 120 d.

The decay rates of the Entero1 genetic marker for sand at the 10 and 20% moisture levels had a negative, linear relationship with temperature ($p = 0.01$ and 0.001, respectively) (Table 4.5). When sand was at 30% moisture, there was no significant relationship between the decay rate and temperature ($p = 0.56$), and the decay rate was fastest at 21 and 30°C compared to other temperatures. In sediment, there was a significant relationship between decay rate and temperature ($p = 0.05$). Although the decay rate of Entero1 in water was not linearly related to temperature, at 6°C the decay rate was significantly slower ($p \leq 0.05$) than at 13, 21, and 37°C. The decay rates of the AllBac genetic marker in sand had a negatively linear relationship with temperature ($p =$ 0.005 to 0.02) for all sand moisture levels (Table 4.5). In sediment, however, the

regression was not significant ($p = 0.53$). Similar to Entero1, the decay rate of AllBac in water was not correlated to temperature, but the decay was significantly slower at 6°C ($p \leq 0.05$) compared to higher temperatures. The decay rate of the HF183 genetic marker was negative correlated with temperature ($p = 0.01$ to 0.02) for all sample types (Table 4.5). The increase in the slope of the regression between decay rate and temperature with increasing sand moisture content indicated that greater moisture promoted a faster decay rate at elevated temperatures (Table 4.5). The average slope of temperature and decay rate in sand for Entero1 and AllBac was –0.0018 ± 0.0002 (mean ± standard deviation) day^{-1} $°C^{-1}$, whereas the average slope of temperature and decay rate for HF183 in sand was –0.0077 ± 0.0012 day^{-1} $°C^{-1}$, indicating that increases in temperature more strongly affected the decay of the HF183 genetic marker compared to the other genetic markers.

Sand moisture effects. Sand at 30% moisture had a significantly higher concentration of the Entero1 and AllBac genetic markers ($p \leq 0.05$) than at 20 and 10% moisture a majority of the times samples were taken (Fig. 4.2). Temperature affected the initial time point at which significant differences were observed. Copies of Entero1 in sand became significantly greater ($p \leq 0.05$) at 30% moisture relative to the values seen at 20 and 10% moisture in 120 d at 6°C, 21 d at 13°C, 14 d at 30 and 37°C, and from the first day at 21°C. The copy number of AllBac became significantly greater ($p \leq 0.05$) at 30% moisture relative to the concentration seen at 20 and 10% moisture at 14 d at 6°C, 3 d at 13°C, and from the first day at 21°C, 30°C and 37°C. There was no significant effect ($p > 0.05$) of moisture on the copy number of HF183 at specific time points.

Moisture also had a significant effect ($\alpha = 0.05$) on the decay rate of the Entero1 and AllBac genetic markers. The decay rate of the Entero1 was not affected by moisture at 6°C (Fig. 4.4). However, Entero1 in sand at 30% moisture and incubated at 21 and 30°C had a slower decay rate than sand at 20% moisture. At 13 and 37°C, the Entero1 genetic markers in sand at 30% moisture had a slower decay rate than sand at both 10 and 20% moisture. The decay rate of AllBac in sand at 30% moisture was slower than the decay rate in sand at 10 and 20% moisture for all temperatures. Moreover, at 6 and 13°C, the decay rate of AllBac was greater than zero, suggesting that there was likely growth of *Bacteroides* within the microcosm. There was no consistent pattern in the effect of moisture on the decay rate of HF183 genetic markers. For example, HF183 in sand at 30% moisture decayed more slowly than in sand at 20% moisture at 6°C, but HF183 decayed faster at 30% moisture relative to sand at 10% moisture at 30°C. Although moisture appeared to exert a strong effect on the decay of Entero1 and AllBac, moisture did not seem to strongly affect HF183 decay rates in sand.

Matrix effects. Matrix type influenced the decay rate of all the tested genetic markers (Fig. 4.4). The decay rate of Entero1 in water was faster than in sand and sediment at 6, 13, and 21°C. At 30 and 37°C, the decay rate of Entero1 in water was significantly faster ($\alpha = 0.05$) than in sand at 30% moisture and in sediment, but was similar to sand at 10 and 20% moisture levels. The decay rate of AllBac in water was faster than that found in sand and sediment at 13 and 21°C. Moreover, at 6, 30, and 37°C, the decay rate of AllBac was faster in water than in sand at 30% moisture and in sediment, but was not different than in sand at 10 and 20% moisture.

The decay rate of HF183 in water was faster than in sand and sediment at 6 and 13°C. However, at 21°C, the decay rate of HF183 in water was slower than in sand, but not sediment. The decay rate of HF183 in water at 30°C was slower than in sand at 10% moisture, but not in the other matrices. At 37°C, the decay rate of HF183 in water was not different ($p \geq 0.05$) from sand or sediment. The decay rate of the Entero1, AllBac, and HF183 genetic markers in sand (at 30% moisture) was not different that that found in sediment at 35% moisture, except for Entero1 at 37°C, AllBac at 13°C, and HF183 at 6°C (Fig. 4.4). This suggests that other factors that affect decay of genetic markers were similar between sand and sediment, or that these other effects are negligible relative to the effect of moisture level.

Influence of genetic marker on decay rates. The decay rates for the Entero1 and AllBac genetic markers were 92% similar for all sample types based on Tukey's studentized range statistic (Fig. 4.4), and there was no significant difference between Entero1 and AllBac decay rates overall (paired t-test, $p = 0.95$). By comparison, the decay rates for the AllBac and HF183 marker were only 32% similar between sample types, and HF183 decay rates were significantly faster than AllBac overall (paired t-test, $p < 0.0001$).

Greater moisture was associated with increased differences between the AllBac and HF183 decay rates. At 10% moisture, the decay rate of AllBac genetic markers was slower than HF183, but only at 37°C. At 20% moisture, the decay rate of AllBac was slower than HF183 at 6, 30, and 37°C. At 30% moisture, the decay rate of AllBac was slower than HF183 at all temperatures. Similarly, AllBac decayed more slowly than

HF183 in sediment at all temperatures, except for 6°C. The HF183 genetic marker decayed faster than AllBac in water at all temperatures, except for 13°C.

DISCUSSION

The objective of this study was to determine the persistence of the Entero1, AllBac, and HF183 genetic markers of fecal pollution in water, sand, and sediment from a freshwater beach. Increased temperature was associated with the increased decay rate for most marker and treatment combinations. The AllBac and Entero1 genetic markers at 30% sand moisture decayed slower than at 10% and 20% moisture, except for Entero1 at 6 °C. The decay rate of HF183, by comparison, was negligibly affected by moisture. Genetic markers decayed more quickly in water than in sand and sediment. Overall, the decay rates of the Entero1 and AllBac genetic markers were 92% similar in this study, whereas the decay rates of the AllBac and HF183 markers were only 32% similar. The HF183 marker decayed more rapidly than the AllBac marker in most microcosm treatments. The decay rates of Entero1 were comparable to that seen in other studies in freshwater and marine sand (9, 12, 189); however, the decay rates of *Bacteroides* in this study were lower than previously reported decay rates for fresh and marine waters (11, 44, 151, 175).

The effect of temperature on the persistence of enterococci and *Bacteroides* genetic markers in marine and freshwaters is well established. The present study and previous studies have shown that lower temperature slows the decay of culturable enterococci (7, 77, 113), enterococci genetic markers (11), total *Bacteroides*, and source-

specific *Bacteroides* genetic markers for pigs, cows, and humans in water (11, 15, 92, 110, 125). Numerous studies have also confirmed that lower temperatures slow the decay of cultured FIB, such as fecal coliforms, *E. coli*, and enterococci in sand and sediment (7, 34, 40, 114).

In contrast, the effect of temperature on persistence of genetic markers for enterococci, total *Bacteroides*, and human-specific *Bacteroides* in freshwater sand and sediment has not been studied. We found that lower temperatures slowed the decay of enterococci and *Bacteroides* genetic markers of fecal pollution in freshwater, sand, and sediment, much like has been observed by others in freshwater or seawater studies.

Interestingly, the effect of temperature varied with marker type, and increases in temperature more greatly accelerated decay of the HF183 marker relative to the Entero1 and AllBac genetic markers. Moreover, increases in moisture increased the effect of temperature by hastening decay of HF183. The interaction of moisture and temperature on the decay of genetic markers in sand has not been previously noted, and the underlying mechanisms are unclear, as moisture generally results in slower decay or has no effect on decay. However, a similar phenomenon may have been observed in manure-amended soils. Rogers *et al*. (140) determined the decay rate of eight genetic markers in soil amended with swine and beef manure at two temperatures and at two moisture levels. Although the interaction of temperature and moisture on decay rate was not discussed, manure source appears to play an important role. In soil amended with swine manure, first-stage decay rate was comparable or slowed at higher temperatures at 80% field capacity (FC) relative to 60% FC. In soil amended with beef manure, first-stage decay

rate was similar or increased at higher temperatures at 80% relative to 60% FC. These results suggest that the interaction between temperature and moisture on decay rate is dependent on the source of the matrix, inoculum, or both.

Greater sand moisture is hypothesized to increase the persistence of bacteria by preventing death from desiccation. In this study, there was little difference in the decay rate of the tested genetic marker at 10 and 20% moisture, but at 30% moisture, the decay rates of the Entero1 and AllBac genetic markers were slowed. There are conflicting reports on the effect of moisture on the persistence of enterococci in sand and soil. Increased moisture has been found to slow decay or trigger growth of culturable enterococci in marine sand (42, 189, 190); however, moisture had little to no effect on the decay of cultured enterococci in freshwater beach sand (114) and enterococci genetic markers in marine sand and manure-amended soil (140, 189). We did not observe an increase in Entero1 over the course of our experiments. Previous studies observed growth of enterococci that was naturally present in the sand, rather than bacteria from an added inoculum (42, 190). Fecal contamination sources have been shown to significantly influence persistence of bacteria in secondary habitats (7), and the bacteria from sewage influent used in this study may be less adapted to sand as a secondary habitat.

In the present study, the Entero1, AllBac, and HF183 marker genes decayed more slowly in sand and sediment than in water at all tested temperatures. This finding is not surprising, as fecal coliforms, *E. coli*, and enterococci exhibit increased persistence in sediments relative to that seen in the water column (7, 9, 88, 99). Sediment likely offers protection from nutrient limitation, temperature fluctuations, desiccation, and predators

compared to the open water column. The average decay rate constant for genetic markers in the water column was 0.23 day^{-1} for Entero1 and the AllBac genetic markers and 0.55 day^{-1} for the HF183 marker. The average decay rate constant in sand and sediment was 0.07 day^{-1} for Entero1 and AllBac and 0.32 day^{-1} for HF183. The decay rates in water were at the low end of the range previously reported for total *Bacteroides* genetic markers, 0.05 to 1.64 day^{-1} (11, 44, 125, 151, 175), and Entero1, 0.24 to 2.2 day^{-1} (12, 177), in marine and freshwater. Since the sediment and water microcosms in our studies were not aerated or shaken, decreased oxygen may have led to an increase in the persistence of *Bacteroides* species. Previous studies on the decay of markers in water rarely included sediment in the microcosm. Dick *et al*. (44), however, included 10 g L^{-1} sediment in a freshwater microcosm. Although sediment exposure promoted faster decay of *Bacteroides* markers relative to the control, the marker abundance nearly returned to initial values when the sediment was resuspended (44).

In this study, the decay rate of the Entero1 marker gene in freshwater sand and sediment averaged 0.07 day^{-1}, similar to the decay rate of Entero1 in manure, manure-amended soil, and marine sand (89, 140, 189) and slower than, but comparable to, the decay rate of culturable enterococci reported by Badgley *et al*. (9) and Howell *et al*. (77). However, our decay rates were 0.2 to 0.9 day^{-1} slower than those found in other culture-based studies in sand (7, 114). One possible explanation for the observed differences is that culture-based methods tend to underestimate the total number of bacteria as they can rapidly enter a viable but non-culturable (VBNC) state upon exposure to stressors, such as nutrient limitation (144, 187). Quantitative PCR can amplify DNA from culturable and

VBNC cells, but qPCR also amplifies extracellular DNA (free DNA and DNA from dead or dying cells) (86, 121). The presence of extracellular DNA complicates estimating bacterial decay rates using molecular markers. For example, transgenic corn DNA can persist for 21 d in freshwater and 40 d in sediment at 15°C (46). Moreover, experiments using propidium monoazide (PMA) pretreatment to bind free DNA prior to qPCR have shown that extracellular DNA is a significant proportion of the total DNA amplified from freshwater after 24 h in a microcosm (10–12). Furthermore, extracellular DNA persists longer at lower temperatures, in part, to the lower activity of degradation enzymes (121, 133). Therefore, the extended persistence of extracellular DNA possibly contributed to our observation of slower decay rates of genetic markers at lower temperatures and slower decay rates compared to those found in studies using cultured enterococci. Regardless, this phenomenon is likely to have an impact using all molecular marker genes in temperate, freshwater environments.

The AllBac genetic marker had a positive decay rate at 30% moisture at 6 and 13°C, indicating the possible growth of *Bacteroides* spp. within the microcosms. Growth of *Bacteroides* has been observed in anaerobic sewage influent (174). Lower dissolved oxygen has been shown to promote persistence of *Bacteroides* in river water microcosms (13, 110). While Marti (110) observed an increase in total and pig-specific *Bacteroides* marker genes in river water microcosms at 4°C under microaerophilic conditions, the AllBac genetic marker only increased in the first 4 d of the experiment, and it returned to below the initial concentration by day 45 (110). In this study, elevated moisture in sand likely contributed to anoxic conditions that supported growth of this anaerobic bacterium,

while lower temperatures promoted persistence. The observation of *Bacteroides* growth suggests that the presence of the AllBac genetic marker in the environment may not be indicate recent fecal contamination, especially in sand or sediment saturated with freshwater.

The genetic markers examined in this study exhibited clear differences in long-term persistence. While HF183 had a relatively high decay rate that was generally unaffected by moisture, the copy number fell below the LOD within the 4 month time frame of the experiment. Schulz and Childers (151) have shown that persistent *Bacteroides* populations are genetically diverse. However, it is possible that the human source-specific strains of *Bacteroides* which contain the HF183 genetic marker are not resilient outside of the host environment. Therefore, our results support the use of HF183 as an indicator of recent human fecal contamination. In contrast, the Entero1 and AllBac genetic markers persisted for the length of the experiment at all temperatures. Both genetic markers had a slower decay rate in sand at 30% moisture compared to 10 and 20% moisture at most temperatures. Moreover, at 30% moisture at 6°C, the abundance of Entero1 at 4 months did not change greatly from the initial concentration at the start of the experiment, and AllBac increased by one $\log_{10}$ over the course of the experiment at 6°C. Since culturable FIB have been shown to overwinter and survive for extended periods at temperatures below 0°C (81, 159), it is possible that the Entero1 and AllBac genetic markers could overwinter in sand and sediment as well, potentially leading to inaccurate genetic marker abundances in early spring or summer.

Although sand and sediment are potentially large reservoirs of FIB and pathogens in beach environments, they are sometimes overlooked. This study found that the decay rate and persistence of genetic markers of fecal pollution in water, sand, and sediment collected from a freshwater beach are distinct. In addition, temperature and moisture played an important role in the decay rates and persistence of molecular marker genes in the matrices obtained from a Laurentian Great Lake. Since there already is a push for a shift towards the use of molecular indicators of fecal contamination to monitor water quality, further research is needed to validate genetic markers of fecal pollution for assessment of health risk from exposure to beach sand and sediment, especially in freshwater environments. Additionally, the decay rate of molecular indicators in sand and sediment should be incorporated into models of the dynamics of FIB in the Great Lakes.

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Table 4.1

Texture analysis of microcosm sand and sediment.

Microcosm component	Sand	Silt	Clay
Sand	93.7	< 2%	6.3
Sediment	87.1	< 2%	12.9

Table 4.2

Oligonucleotide sequences and final concentrations of primers and probes for qPCR assays.

Assay primer and probe	Sequences (5'→ 3')[a]	Conc. (nM)	Target	Reference
Entero1				
ECST748F	AGAAATTCCAAACGAACTTG	400	*Enterococcus* spp.	(106)
ENC854R	AATGATGGAGGTAGAGCACTGA	400		
GPL813TQ	(FAM)TGGTTCTCTCCGAAATAGCTTTAGGGCTA(TAMRA)	200		
AllBac				
AllBac296F	GAGAGGAAGGTCCCCCAC	200	All *Bacteroidales*	(97)
AllBac467R	CGCTACTTGGCTGGTTCAG	200		
AllBac375Bhqr	(FAM)CCATTGACCAATATTCCTCACTGCTGCT(BHQ-1)	300		
HF183				
HF183	ATCATGAGTTCACATGTCCG	300	Human-associated	(17, 153)
HF183R	CCATCGGAGTTCTTCGTG	300	*Bacteroides*	

[a] FAM, 6-carboxyfluorescein; TAMRA, Carboxytetramethylrhodamine; BHQ-1, black hole quencher 1.

Table 4.3

Validity of qPCR calibration curves.

Assay	Calibration curve[a]	PCR efficiency range (%)[b]	R^2 range of calibration curve	C*q* of LOD[c]
Entero1	$y = -3.30x + 36.79$	93 – 110	0.993 – 0.997	35.20 ± 0.18
AllBac	$y = -3.28x + 37.88$	94 – 108	0.994 – 0.999	36.26 ± 0.25
HF183	$y = -3.33x + 34.76$	94 – 107	0.994 – 0.998	33.05 ± 0.15

[a] Mean value for slope and intercept for 15 runs.

[b] Amplification efficiency = $10^{-1/\text{slope}} - 1$.

[c] Quantification cycle (C*q*) of the limit of detection, mean ± standard error.

Table 4.4

Genetic marker concentration in microcosm components prior to inoculation.

Microcosm component	Entero1	AllBac	HF183
Sand[a]	2.5×10^2	4.1×10^3	blod[c]
Sediment[a]	1.4×10^4	3.6×10^5	blod
Water[b]	3.8×10^1	6.1×10^3	blod
Raw sewage inoculum[b]	3.1×10^6	1.2×10^7	6.5×10^5

[a] Concentration in copies g^{-1}.

[b] Concentration in copies mL^{-1}.

[c] Sample below limit of detection (blod). For aqueous samples the limit of detection was 3 copies mL^{-1}, and for sediment and sand samples the limit of detection was 30 copies g^{-1}

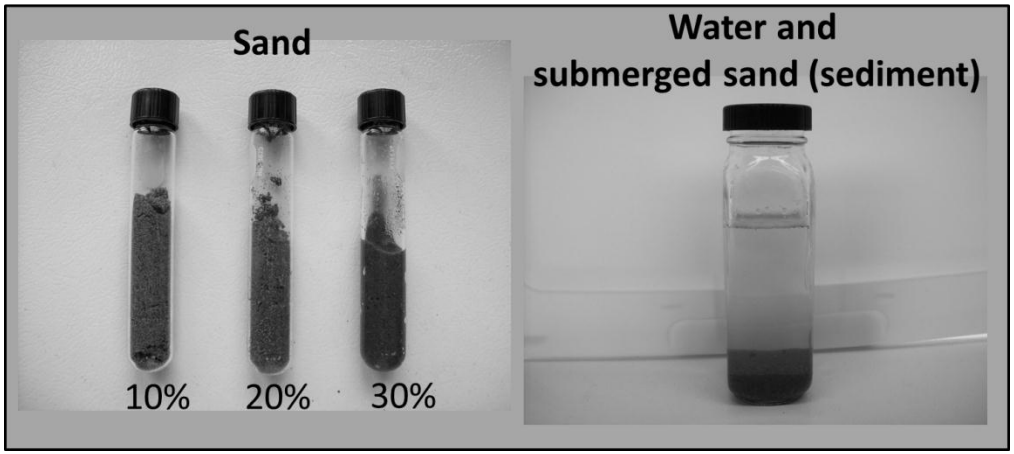
Table 4.5

Linear regression of temperature with genetic marker decay rate and copy number at 120 d.[a]

Sample type	Entero1				AllBac				HF183	
	Decay rate		120 d		Decay rate		120 d		Decay rate	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
Sand - 10%	–0.0016	0.91*	–0.0563	0.86**	–0.0019	0.94**	–0.0796	089**	–0.0066	0.89*
Sand - 20%	–0.0021	0.98**	–0.0307	0.54**	–0.0020	0.92*	–0.0529	0.77**	–0.0075	0.90*
Sand - 30%	–0.0004	0.12	–0.0152	0.09	–0.0015	0.87*	–0.0763	0.73**	–0.0090	0.87*
Sediment	–0.0016	0.77*	–0.0418	0.54**	–0.0008	0.14	–0.0673	0.79**	–0.0101	0.97**
Water	–0.0015	0.39	–0.0437	0.54**	–0.0022	0.42	–0.0509	0.56**	–0.0075	0.93**

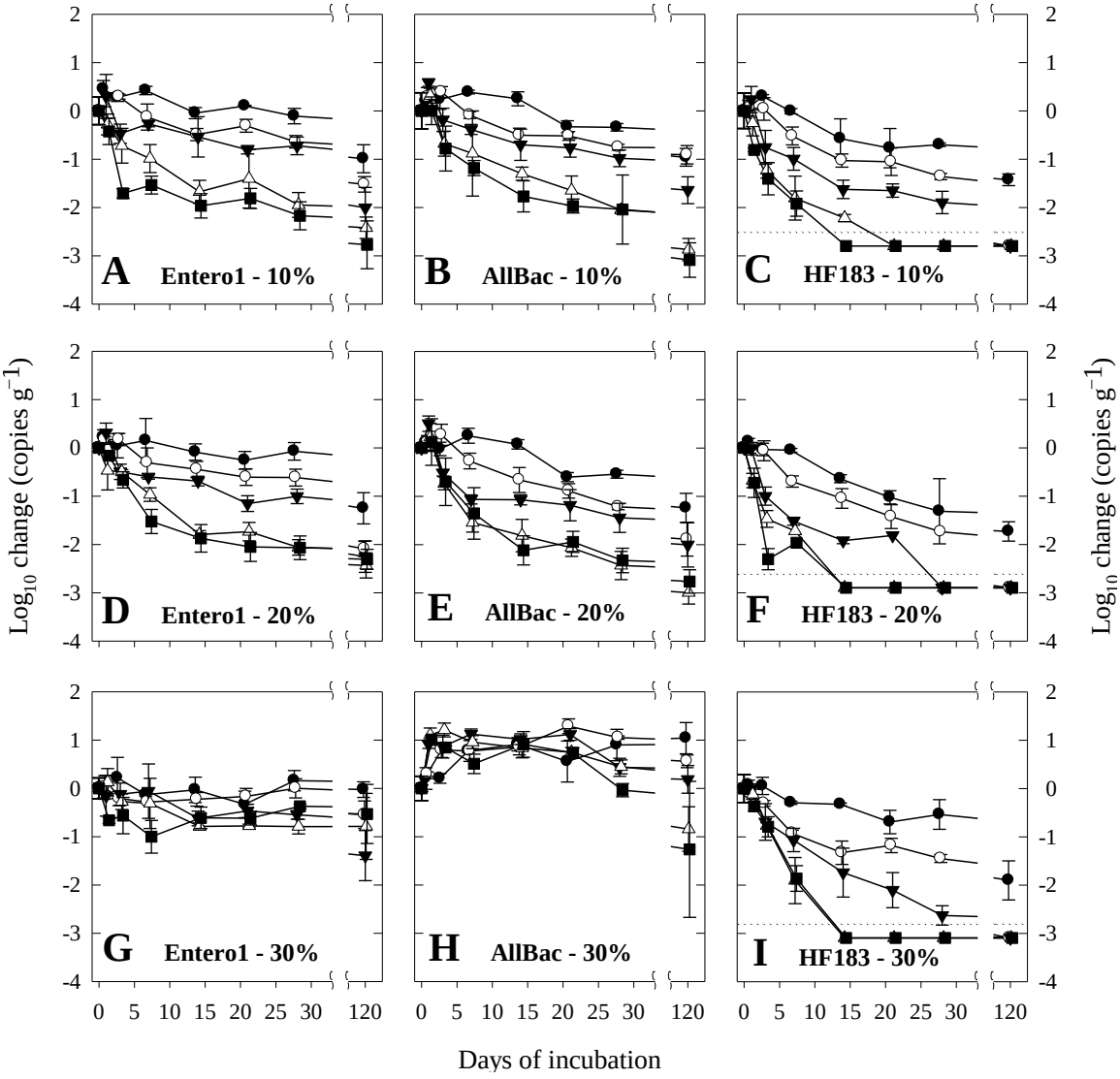
[a] *, $p \leq 0.05$; **, $p \leq 0.01$

Figure 4.1



Photgraphs of sand and sediment microcosm experimental setups.

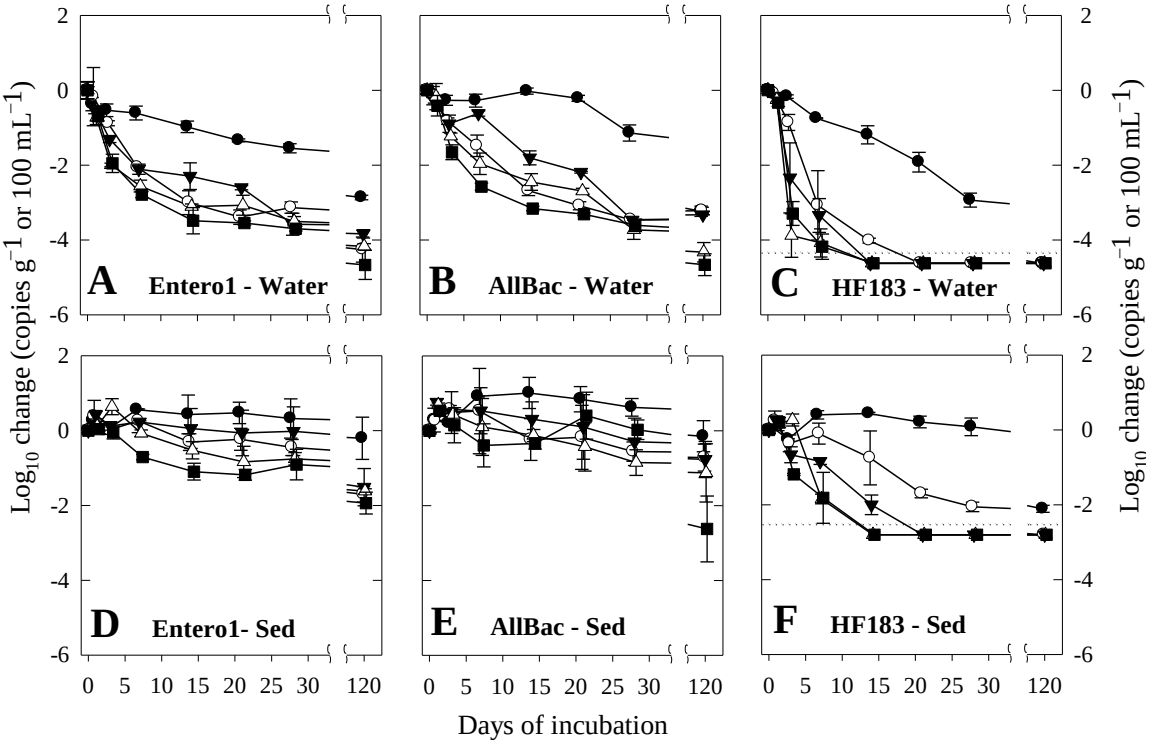
Figure 4.2



The log_{10}-fold change in abundance of genetic markers of fecal pollution in sand microcosms. Legend: Entero1 (A, D, G), AllBac (B, E, H), and HF183 (C, F, I) at 10% moisture (A, B, C), 20% moisture (D, E, F), and 30% moisture (G, H, I) at 6°C (filled circles), 13°C (open circles), 21°C (filled, inverted triangles), 30°C (open triangles), and

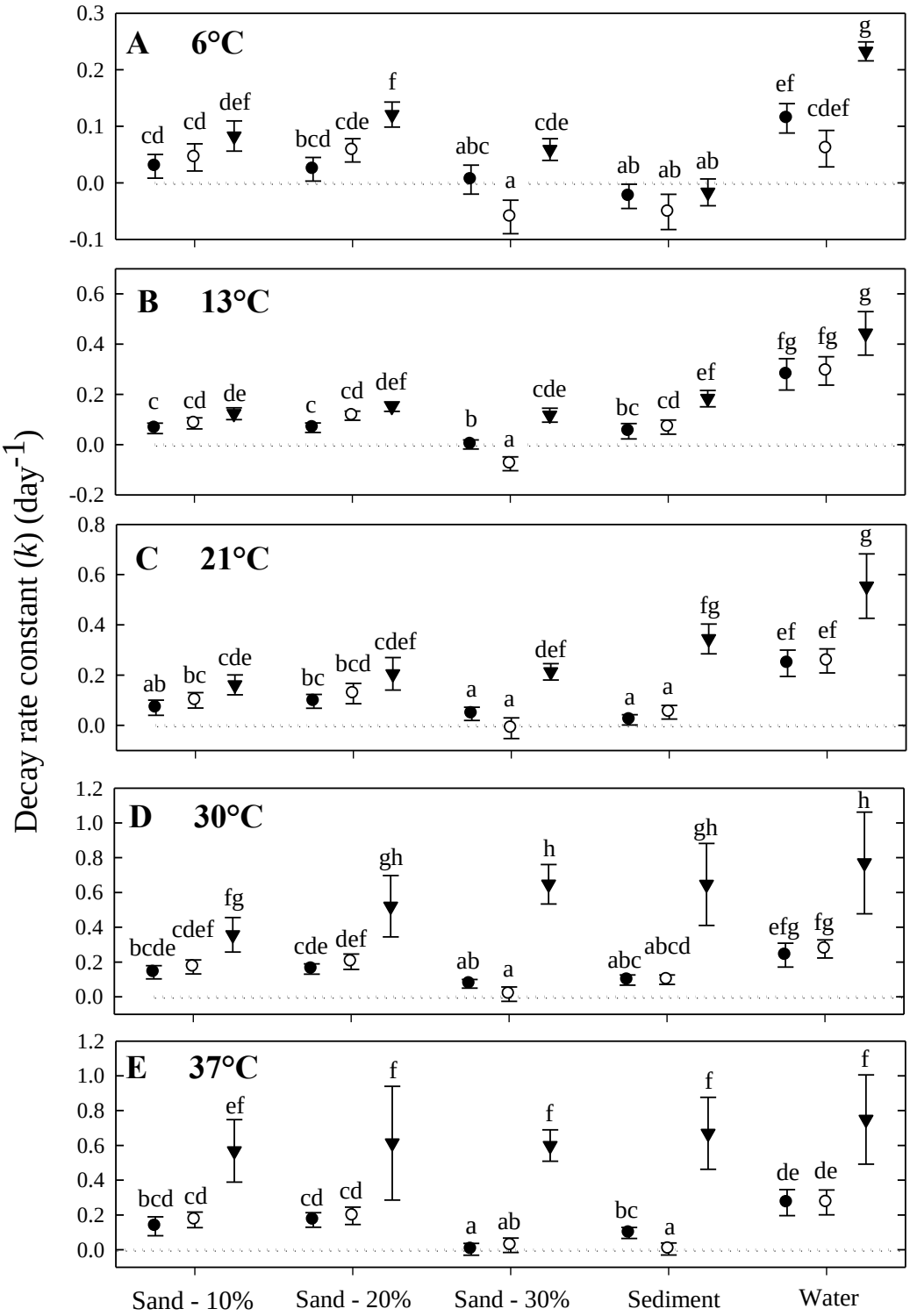
37°C (filled squares) over the course of the experiment. The dotted line indicates the limit of detection. Samples below the limit of detection are shown as one-half the value of the limit of detection. Error bars show the 95% CI of the mean.

Figure 4.3



The log_{10}-fold change in abundance of genetic markers of fecal pollution in water and sediment microcosms. Legend: for Entero1 (A, D), AllBac (B. E), and HF183 (C, F) in the water portion (A, B, C) and sediment (D, E, F) at 6°C (filled circles), 13°C (open circles), 21°C (filled, inverted triangles), 30°C (open triangles), and 37°C (filled squares) over the course of the experiment. The dotted line indicates the limit of detection. Samples below the limit of detection are shown as one-half the value of the limit of detection. Error bars show the 95% CI of the mean.

Figure 4.4


A 6°C
B 13°C
C 21°C
D 30°C
E 37°C
Decay rate constant (k) (day-1)
Sand - 10%
Sand - 20%
Sand - 30%
Sediment
Water

The decay rate constant (*k*) of Entero1 (filled circles), AllBac (open circles), and HF183 (filled, inverted triangles) at 6°C (A), 13°C (B), 21°C (C), 30°C (D), and 37°C (E). Error bars indicate 95% confidence interval of *k*. Samples that are not significantly different at $\alpha = 0.05$ based on Tukey's studentized range test share the same letter. Dotted line indicates *k* of zero. Negative *k* values indicate the accumulation rate of genetic markers.

CHAPTER 5:

Decay of culturable fecal indicators and genetic markers for indicators and pathogens in freshwater sand microcosms

OVERVIEW

In order for fecal indicator bacteria (FIB) to be a valid gauge of health risk associated with recreational water use, it must exhibit similar decay to the pathogens it is slated to predict. Beach sand poses both direct and indirect impacts on water quality and pathogen load; however, the comparative decay of FIB and pathogens in freshwater sand has not been examined. In this study, freshwater sand microcosms were inoculated with sewage and pure cultures of bacterial pathogens to compare relative decay rates. The abundance of culturable FIB *Enterococcus* spp. and *Escherichia coli,* genetic markers for FIB *Enterococcus* spp. (Entero1), total *Bacteroides* (AllBac), and human-specific *Bacteroides* (HF183), and genetic markers for the pathogens *Campylobacter jejuni,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Shigella flexneri* were monitored over the course of two weeks using conventional culture methods and quantitative PCR (qPCR). The effect of moisture on the persistence of culturable FIB, genetic markers for FIB, and genetic markers for bacterial pathogens was measured. In addition, propidium monoazide (PMA) treatment was used to examine differences in the persistence of total genetic markers and those from live cells. Moisture had a significant effect ($p \leq 0.05$) on the decay rates of culturable indicators, total AllBac markers, and genetic markers for FIB, *Salmonella*, and MRSA from live cells. At 14% sand moisture, the decay rate of total markers was slower than that of live cells for all qPCR assays, but at 28% moisture, there was no difference in the decay rates of total and live markers for any assay. AllBac and MRSA exhibited growth at 28% sand moisture. Overall, culturable FIB and HF183 had decay rates that

were most comparable to the bacterial pathogens examined in this study. Our results call into question the validity of Entero1 and AllBac as fecal indicators. The choice of FIB in freshwater sand should take into account the pathogen of concern and sand moisture conditions.

INTRODUCTION

Recreational use of beaches involves contact with water, sand, and sediment; however, consideration of the health risks from exposure to beach sand or sediment has received relatively little study. Heaney *et al.* (73, 74) reported the first epidemiological evidence that direct contact with beach sand was positively correlated to gastrointestinal illness. This is not surprising, as sand has been shown to serve as a reservoir of fecal indicator bacteria (FIB) (5, 20, 29, 80). FIB, such as *Enterococcus* spp. and *Escherichia coli*, are bacteria that indicate the presence of fecal contamination and potential presence of human pathogens in the beach environment. FIB have been detected at high concentrations in both marine and freshwater beach sand (132, 181, 188). Sand can harbor pathogenic bacteria as well. *Campylobacter* spp., *Salmonella* spp., *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and *Vibrio vulnificus* have been detected in marine beach sand (27, 154, 189). *S. aureus* and MRSA have also been detected in sand at freshwater beaches (102).

Beach sand can promote greater persistence of bacteria relative to the water column. Sand offers protection from ultraviolet radiation, predation, temperature fluctuations, among other factors, that negatively affect persistence in the water column (5, 80, 128, 181). Moreover, FIB have been shown to exhibit the potential for regrowth and long-term persistence in sand and sediment (5, 99, 105, 190), an effect that can be precipitated by rewetting or high sand moisture content (114, 190). Regrowth and persistence of FIB in beach sand may uncouple the predictive relationship between pathogens and indicators, as a valid fecal indicator should have a similar decay rate to the

pathogens it predicts (69). Several studies have indicated that FIB decay rates are slower than pathogens in sand and sediment (31, 189). In manure-amended soil, the similarity of decay rates between pathogens and indicators was dependent on the source of fecal material (140).

Although historically FIB were quantified by culture-based methods, the need for same-day water quality monitoring results has led to an increase in the recommendation for and use of comparable culture-independent methods, such as quantitative PCR (qPCR) for genetic markers (158). However, research suggests that the decay rate of genetic markers is slower than their culture-based counterparts (89, 125, 177, 189). DNA is able to persist for days or weeks in the environment after cell death, and PCR can readily amplify DNA from nonviable cells, calling into question whether presence of genetic markers act as reliable indicators of fecal pollution in the environment (86, 124). However, treatment of environmental samples with propidium monoazide (PMA) can be used to differentiate between total genetic markers and those from live cells (10, 123). PMA is a membrane-impermeable dsDNA binding dye that inhibits amplification of free DNA and DNA from dead or dying cells. Use of PMA prior to qPCR (PMA-PCR) has been used to differentiate indicator and pathogen total genetic markers and markers from live cells in marine and freshwater microcosms (10–12).

Beach sand is an important reservoir of FIB, and sand can act as an exposure route to pathogenic bacteria (64, 74). However, there have been few comparisons among the persistence of culturable FIB, genetic markers of FIB, and pathogens in sand. Moreover, the persistence of genetic markers of FIB and pathogens in freshwater sand

has not been studied. The objectives of this study were to: (i) measure the effect of moisture on the decay rate of culturable *Enterococcus* spp. and *E. coli*, (ii) measure the effects of sand moisture and PMA treatment on the persistence of genetic markers for the FIB *Enterococcus* spp. (Entero1), total *Bacteroides* (AllBac), and human-specific *Bacteroides* (HF183), and the pathogens *C. jejuni* (qCamp), MRSA (qMRSA), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (qSalm), and *Shigella flexneri* (qShig), and (iii) determine whether FIB have decay rates comparable to pathogens with respect to moisture and PMA treatment.

METHODS

Microcosm setup. Sand microcosms consisted of sterile 55 mL glass screw top test tubes filled with 40 g sand collected from the Duluth Boat Club Beach (DBC) in Duluth, MN, (46°46'10"N, 92°05'23"W) on May 30, 2012. Microcosms were inoculated with raw sewage and pure cultures of *Campylobacter jejuni* ATTC 33560, *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028, *Shigella flexneri* ATCC 20170, and methicillin-resistant *Staphylococcus aureus* COL strain. The *C. jejuni* was allowed to grow for 24 hr in Mueller Hinton broth at 42°C in a BBL GasPak 100 jar (Becton, Dickinson, and Company, Sparks, MD) with Pack-MicroAero Gas Generating system (Mitsubishi Gas Chemical Company, Inc., Japan). The remaining cultures were grown overnight in lysogeny broth (19) at 37°C. Liquid cultures of pathogens were centrifuged, supernatant was discarded, and the cell pellets were resuspended in raw sewage influent collected from the Western Lake Superior Sanitary District (WLSSD)

treatment plant on May 30, 2012. Each microcosm was inoculated with 4 mL sewage, the equivalent of 1.7 mL liquid culture of *C. jejuni*, and the equivalent of 1 mL liquid culture of the remaining pathogens. The amount of bacteria inoculated was equivalent to 1.03×10^8 *Campylobacter* cells, 8.32×10^8 *Salmonella* cells, 9.04×10^8 *Shigella* cells, 1.20×10^9 MRSA cells.

The effect of sand moisture was tested at two levels. The initial moisture of sand was 2%, and after addition of inoculum, sterile distilled water was added to adjust the moisture to 14 or 28%. The microcosms were homogenized by hand shaking and incubated in the dark at 25°C. The incubation temperature and sand moisture did not change over the course of the experiment.

The approximate proportion of clay (< 2.0 μm), silt (2.0 – 50 μm), and sand (50 – 2000 μm) particles in the sand microcosms was determined. Forty grams of air dried (crushed to < 2 mm) sample was shaken for 16 h with 100 mL of 5% sodium hexametaphosphate. The suspension was quantitatively transferred to a sedimentation cylinder and brought to a total volume of 1 L with deionized water. After a 2 h temperature equilibration, the suspension was stirred vigorously for one minute to re-suspend the particles. An ASTM No. 152H hydrometer was carefully placed in the suspension and used to take two readings, one at 40 s and another at 6–8 h (depending on the temperature of the suspension). The percentage of each fraction was calculated from the resulting hydrometer readings. For the material used for microcosms, the percentage of sand, silt and clay was 82.5%, 2.5%, and 15.0%, respectively.

Sampling and PMA treatment. Four replicate samples were taken of wastewater influent, sand prior to inoculation, and sand post-inoculation. Additionally, samples were taken at 1, 3, 5, 7, 9, 11, and 14 d after inoculation. At each sampling time point, four replicate microcosms were sacrificed for each moisture treatment. Test tube contents were homogenized, 10 g of sand were placed into a sterile 150 mL milk dilution bottles with 100 mL of 0.1 M ammonium phosphate solution with 0.01% gelatin, and the bottles were shaken on a wrist action shaker, as previously described (81). For culturable indicator counts, sand supernatant and wastewater influent were filtered onto nitrocellulose filters (0.45 µm, 47 mm; Millipore, Billerica, MA) and placed on the surface of modified mTEC (168) or mEI media (167) to enumerate *E. coli* and enterococci, respectively.

To distinguish between total genetic markers and markers from live cells, subsamples were treated with propidium monoazide (PMA) to bind free DNA (10). A 45 mL aliquot of supernatant or wastewater was centrifuged at 7,650 x g for 10 min, and the pellet was resuspended in 0.5 mL phosphate buffered saline and transferred to an ultra clear microcentrifuge tube (DOT Scientific, Inc., Burton, MI). For samples treated with PMA, 2.5 µL of 20 mM PMA dye in water (Biotium, Inc., Hayward, CA) was added to reach a final concentration of 100 nM PMA. Samples were incubated for 5 min in the dark. In order to inactivate remaining dye, samples were subjected to light treatment for 6 minutes 20 cm from a 1000W halogen light source (Osram, Germany) while on ice to limit heating of samples. While Bae and Wuertz (10) suggested a treatment time of 10 min for samples containing total suspended solids between 100 and 1,000 mg L^{-1} with a

600W halogen light source, the treatment time in this study was reduced proportional to the lumen intensity of the light source. Following light treatment, samples were centrifuged for 2 min at 16,000 x g, the supernatant was discarded, and samples were frozen at –20°C until DNA extraction.

DNA extraction and qPCR assays. DNA was extracted using a PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA). Frozen pellets were resuspended in extraction buffer and transferred to extraction tubes. Before extraction, 50 ng of UltraPure Salmon Sperm Solution (Life Technologies, Grand Island, NY) was added to adjust for differential recovery of DNA from individual replicates as previously described (72). The MoBio protocol was modified to include an additional DNA wash with buffer C5. DNA was eluted in a final volume of 50 µL.

Plasmid standards were created by cloning PCR product amplified from *Enterococcus faecalis* strain ATCC 29212 for the Entero1 genetic marker, from sewage influent for AllBac and HF183 markers, and from pathogens used in this study using previously published primers (Table 5.1) and the StrataClone PCR kit (Stratagene, Santa Clara, CA). Purified plasmid DNA was quantified by using a Qubit 1.0 fluorometer (Invitrogen, Grand Island, NY) before preparation of six 10-fold dilutions for qPCR standards, ranging from 3 to 300,000 target copies per 5 µL. For the recovery and internal amplification control (IAC), 10-fold dilutions of standards were created by diluting UltraPure Salmon Sperm Solution (Life Technologies, Grand Island, NY) to concentrations ranging from 1 to 10,000 pg DNA.

The qPCR analysis was done using previously published protocols; however, oligonucleotide concentrations were optimized for this study (Table 5.1). The HF183 assay was done using iTaq SYBR Green Supermix With ROX (Bio-Rad, Hercules, CA), and the remaining qPCR assays used iTaq Supermix With Rox (Bio-Rad, Hercules, CA). Reactions contained 12.5 µL mastermix, primers and probe, and 5 µL of 1:2 or 1:10 dilution of template DNA in a final reaction volume of 25 µL. The HF183 reactions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 56°C annealing for 30 s, and an extension at 60°C for 45 s. Finally, a melt step was included to verify specific product amplification. For probe-based assays, an initial denaturation at 95°C for 5 min was used, and was followed by 40 cycles of a denaturation at 95°C for 15 s and an annealing and extension step at 60°C for 1 min.

Each qPCR run contained triplicate reactions of standards and non-transcript controls, and duplicate reactions of samples. Amplifications were performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA), and quantification cycle (C*q*) values were automatically determined using the system software. Sample marker concentrations were calculated on a per-run basis.

Decay rate calculation. The % recovery was calculated by dividing the recovered quantity of salmon sperm DNA (S_r) relative to the amount added prior to DNA extraction (50 ng).

(1) $$\%\ \text{recovery} = \frac{S_r}{(50\ \text{ng})} \times 100$$

The concentration of genetic markers in each sample was determined by dividing the concentration of genetic markers, as determined by each assay, by the % recovery.

Finally, the concentration of markers was adjusted to the weight of sample extracted in dry g.

To determine the decay rates of culturable indicators and genetic markers, Aikaike information criterion was used to determine whether first-order decay or second-order decay model was preferred.

(2) $$C = C_0 e^{-kt}$$

(3) $$C = C_0 e^{-k_1 t'} e^{-k_2 t - t'}$$

C is the concentration of genetic markers at time (t), C_0 is the initial concentration of markers, and k is the decay rate. In second order decay, an initially rapid decay (k_1) occurs until a certain timepoint (t'), followed by a period of slower decay (k_2). For qMRSA, Aikaike information criterion was used to determine whether a first-order decay or plateau followed by first-order decay was preferred,

(4) $$C = C_0 e^{-k(t-t_0)}$$

where t_0 is the timepoint that decay begins after a lag period.

Decay rates were calculated up to 14 d or up to the point before replicates fell below the limit of detection. In addition, if genetic markers reached a plateau, then subsequent time points were excluded from decay rate calculations. Calculations were done using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA).

Analysis. A multiple comparisons test was used to determine significant differences in decay rates of indicators and pathogens, and among treatments for individual genetic markers. Test values were calculated by dividing the difference between two slopes by the pooled standard error.

(5) $$q = \frac{\left(\overline{X_i} - \overline{X_J}\right)}{\sqrt{\frac{MS_W}{n}}}$$

As the critical value for Tukey's range statistic (q) accounts for a factor of 2 (appears under the square root sign), q divided by the square root of 2 was used for the critical value for mean comparisons. Tukey's range test was done using Microsoft Office Excel 2007 (Microsoft, Redmond, WA) and critical values were determined using R (http://www.r-project.org/). Three-way ANOVA was used to compare the effect of moisture, PMA treatment, and time on concentration of Entero1. Tukey HSD was used for post-hoc mean comparisons. Student's t-test was done at each time point where the mean marker concentration was greater than the initial concentration to determine whether genetic marker concentration had significantly increased.

RESULTS

Percent recovery and initial concentrations of indicators. The percent recovery of salmon sperm (IAC) DNA was 3.3 ± 0.9 % (mean ± standard deviation) and ranged from 0.5 to 9%. The average calibration curve across 4 to 6 runs is shown in Table 5.2. The initial amount of DNA from all pathogenic bacteria in sand prior to inoculation and raw sewage was below the detection limit. The initial concentration of culturable *Enterococcus* spp. and *E. coli* in sand was also below the limit of detection (LOD) of 0.3 CFU per g. Additionally, there were no detectable HF183 markers in sand prior to inoculation. The initial concentration of indicators above the LOD in sand and sewage inoculum is shown in Table 5.3.

Decay curves. For culturable indicators (Fig. 5.1) and genetic markers for indicators (Fig. 5.2) and pathogens (Fig. 5.3), Aikaike information criterion confirmed that first-order decay was preferred over second-order decay. For qMRSA, there appeared to be a lag time, after which decay began. Aikaike information criterion confirmed that a model that had a plateau phase, followed by first-order decay was preferred over a simpler first-order decay model without a plateau phase. The length of the plateau phase for total qMRSA markers was 4.2 days at 14% moisture and 5 days at 28% moisture. For qMRSA in live cells, the plateau phase was reduced by 50% at both moisture levels.

Over the course of 14 d, the HF183 marker fell below the LOD at 14% moisture at 9 d for total markers and at 5 d for markers from live cells (PMA treated). HF183 in live cells also fell below the LOD at 11 d at 28% moisture. The concentration of qCamp in live cells fell below the LOD at 7 d at 14% moisture and at 9 d at 28% moisture. At 9 d, culturable *E. coli* in the 28% moisture microcosm reached a plateau, past which, no further degradation of the indicator bacterium took place, as seen by the relatively constant *E. coli* concentration from 9 to 14 days. A similar plateau occurred for total qCamp markers at 7 and 9 d for 14 and 28% moisture, respectively.

The first-order decay rates for culturable indicators and decay rates of genetic markers for indicators and pathogens are shown in Table 5.4. For all treatments, Entero1 had the lowest R^2 value, indicating a poor fit of the first-order decay model. The fluctuation in the average concentration of Entero1 over time did not indicate a constant

rate of decay. Moreover, replicate microcosms at individual times exhibited high variability for the concentration of Entero1 markers (Fig. 5.2).

Moisture and PMA treatment effects. *Enterococcus* spp. decayed 0.081 day^{-1} slower at 28% moisture than at 14% moisture, whereas *E. coli* decayed 0.099 day^{-1} slower at 14% moisture relative to 28% moisture. For total genetic markers, decay was significantly slower at 28% moisture for AllBac and HF183 markers relative to 14% moisture ($p \leq 0.05$), with decay rate decreasing by nearly half at higher moisture. For genetic markers from live cells only, Entero1, AllBac, HF183, qSalm, and qMRSA had slower decay rates at 28% moisture relative to 14% moisture. At 14% moisture, genetic markers from live cells decayed significantly faster than total genetic markers for all assays ($p \leq 0.05$). At 28% moisture, however, there was no significant difference in decay rates of total markers and markers from live cells only for any assay ($p > 0.05$).

As the first-order decay model did not explain much variance in Entero1 genetic marker concentrations, a three-way ANOVA was also used to examine the effects of moisture, PMA treatment, and sample date on Entero1 copy number. There was a significant interaction between sample date and moisture ($p = 0.0002$) and between sample date and PMA treatment ($p < 0.0001$) on copy number. At 14% moisture, copies of Entero1 wer higher at 0 and 1 d than at 5, 7, 9, and 14 d ($p \leq 0.05$), whereas there was no difference in Entero1 copy number over time at 28% moisture. Total copy number of Entero1 were relatively constant over the 14 day experiment. However, on day 1, total copies were significantly more abundant than on day 7 ($p \leq 0.05$), which had the lowest concentration of total copies over the course of the experiment. In contrast, Entero1

copies from live cells were more abundant ($p \leq 0.05$) on days 0 and 1 relative to 5 through 14 d for both moisture levels (Fig. 5.2).

The AllBac genetic marker increased over time at 28% moisture for both total and live markers (Fig. 5.2), indicating growth of cells within the microcosm. Moreover, the total copies of AllBac on day 1 and from days 5 to 14 and copies of AllBac from live cells on day 1 and from days 7 to 14 was significantly greater than the initial copy number at the start of the experiment ($p \leq 0.0009$). The qMRSA genetic marker also increased at several time points in the experiment relative to the initial conditions. Total copies of qMRSA at 14% moisture on day 3 was greater than the concentration at the start of the experiment ($p = 0.0006$). At 28% moisture, copies of total qMRSA on days 1 through 5 were higher than the initial concentration ($p \leq 0.05$), and copies of qMRSA from live cells was higher on day 1 relative to the initial concentration ($p = 0.03$).

Comparison of indicator and pathogen decay rates. Decay rates of indicators were compared to the decay rates of total and live pathogens at 14% and 28% moisture (Table 5.5). At 14% moisture, the decay rate of total qCamp was similar only to the decay rate of total HF183. Similarly, at 14% moisture, the decay of live qCamp was similar only to the decay rate of HF183 from live cells. There was no indicator that exhibited a similar decay rate to qCamp at high moisture. Culturable indicator *Enterococcus* spp. and *E. coli* and total HF183 and HF183 from live cells had decay rates similar to qSalm, qShig, and qMRSA under several conditions. In contrast, the decay rates of Entero1 total and live cells were similar only to the decay rate of total qMRSA at 28% moisture. AllBac markers from live cells had a similar decay rate to total qSalm,

qShig, and qMRSA at 14% moisture. The decay rate of total AllBac was not equivalent to the decay rate of any pathogen.

DISCUSSION

Understanding the dynamics of FIB and pathogens in sand is vital to understanding the microbial load of recreational beaches. Foreshore sand has been shown to significantly affect bacterial abundance in nearshore waters through swash zone movement and suspension (181, 188). In addition, recent studies have highlighted the health risks associated with exposure to beach sand (64, 74, 182). Despite this, there have been no studies on the persistence of FIB and pathogens in freshwater sand. In this study, the effect of moisture on the persistence of culturable FIB, and genetic markers for total and live FIB and bacterial pathogens was measured. Finally, decay rates of culturable indicator microorganisms and genetic markers for them were compared with genetic markers for bacterial pathogens to identify indicator microorganisms that had decay rates similar to the bacterial pathogens examined in this study.

Sand moisture affected the decay rates of culturable indicator bacteria, total *Bacteroides*, and genetic markers for indicator bacteria and *Salmonella* and MRSA from live cells. At 14% sand moisture, the decay rates of total genetic markers were slower than those from live cells in all qPCR assays, but at 28% moisture, there was no difference in the decay rates of total genetic markers and those from live cells for any assay. Overall, culturable FIB and the human-specific *Bacteroides* marker HF183 had decay rates that were most similar to the decay of *Campylobacter*, *Salmonella*, *Shigella*,

and MRSA genetic markers. Similar to other studies, our results call into question the use of genetic markers of *Enterococcus* spp., specifically Entero1, as a valid indicator of the presence of bacterial pathogens (12, 185, 189).

For culturable indicators and genetic markers of FIB and pathogens from live cells, higher sand moisture slowed decay rate, with the exception of culturable *E. coli* and genetic markers for *Campylobacter* and *Shigella*. The protective effect of high sand moisture on culturable FIB is well-established (20, 32, 42, 114, 190); however, the effect of moisture on the decay of genetic markers in sand is not well-known. Bolton *et al*. (27) found no relationship between *Campylobacter* and *Salmonella* abundance and sand moisture on marine beaches in the UK. In contrast, Yamahara *et al*. (189) found that greater sand moisture was associated with higher concentrations of FIB, *Campylobacter*, *S. aureus*, and *Salmonella* on beaches along the CA coast. In marine sand microcosms, moisture significantly slowed the decay of *Salmonella* and human-specific *Bacteroides* markers, but moisture did not significantly affect the decay rate of Entero1 and *Campylobacter* markers (189). On a subtropical beach, FIB and pathogens were negatively correlated with sand moisture (1, 154). In manure-amended soil microcosms, the effect of moisture on the decay rate of Entero1 and total *Bacteroides* markers was variable, depending on manure source, but higher moisture consistently slowed the decay of genetic markers for *Salmonella* and *Shigella* (140). Conflicting reports on the effect of moisture on pathogens and genetic markers of FIB in sand indicate that contamination source and/or environment-specific factors play a potentially large role in persistence.

Not surprisingly, the genetic marker for enterococci, Entero1, decayed much slower than culturable *Enterococcus* spp., likely due to the presence of extracellular DNA, DNA from dead or dying cells, and the presence of viable but non-culturable cells (VBNC) (10, 86, 123, 127, 149). The slower decay rate of Entero1 in PMA treated samples relative to culturable *Enterococcus* spp. indicated a significant proportion of VBNC that might be resuscitated, especially at 28% moisture where no difference was observed between the decay of Entero1 markers for total and live cells. The R^2 values of first-order decay models of culturable indicator bacteria were much greater than the Entero1 markers, ranging from 0.88 to 0.93 and 0.08 to 0.67, respectively. Davies *et al*. (41) suggested that the lack of decay model fit could be due, in part, to the complex relationship between growth and predation. Indeed, decay curves of enterococci in sand and sediment microcosms can exhibit considerable variation at different times (9, 189; also see results in Ch. 4).

AllBac was the only genetic marker to exhibit a positive decay rate over the course of the experiment. AllBac did not accumulate at 14% moisture, but AllBac markers for both total and live cells increased at similar rates in sand at 28% moisture. Shultz and Childers (151) compared the decay rates of genus and source-specific *Bacteroides* genetic markers in marine and freshwater microcosms, and no *Bacteroides* genetic marker increased over the course of the experiment. Although the decay of *Bacteroides* in sand has not been studied previously, in manure-amended soil, *Bacteroides* exhibited second-order decay kinetics (140). However, in river water microcosms under microaerophilic conditions, Marti *et al*. (110) observed an increase in

total and pig-specific *Bacteroides* markers at low temperatures, and total *Bacteroides* increased over the first four days of the experiment and gradually decreased to starting concentrations by day 45. Higher sand moisture may have reduced oxygen availability and created anoxic micro-niches in the sand microcosm that were favorable for the regrowth of *Bacteroides* cells, an obligate anaerobe. Regardless, our results definitively show that *Bacteroides* spp. are capable of growth within moist sand and the AllBac genetic marker for total *Bacteroides* spp. is an unsuitable indicator of recent fecal contamination at sites that could be influenced by sand washout or resuspension.

Upon introduction to sand, MRSA entered a stable phase for 2 to 5 d, after which it exhibited first-order decay. At 28% moisture, total qMRSA was more abundant than the initial concentration from days 1 through 5, and qMRSA from live cells on day 1 was elevated relative to the initial concentration, indicating growth occurred in the first 24 hr. Levin-Edens *et al*. (101) found that the decay of MRSA bacteria was dependent on salinity, temperature, and the MRSA strain, but did not observe a plateau followed by first-order decay or growth over the incubation period. However, Mohammed *et al*. (115) found that *S. aureus* proliferated 1 to 5 days in marine beach sand. Moreover, higher moisture slowed the decay of *S. aureus* (115). Recent beach surveys indicate that *S. aureus* is relatively common in beach water and sand, with detection frequencies of 14 to 59% in water and 1.9 to 14% in sand (60, 102, 154, 189). It is possible that the ability of MRSA to grow in the sandy environments, coupled with delayed onset of decay, may contribute to the high detection frequencies of this organism at some beaches.

In order to be useful, FIB must have decay rates in a secondary environment, such as sand, that are similar to the pathogen or pathogens they are used to indicate in order to be valid measures of health risk (69). Our results indicate that the genetic marker for enterococci, Entero1, and total *Bacteroides*, AllBac, rarely exhibited decay rates similar to genetic markers for the bacterial pathogens *Campylobacter*, *Salmonella*, *Shigella*, and MRSA. In contrast, culturable *Enterococcus* spp., *E. coli*, and the human-specific *Bacteroides* marker HF183 had decay rates similar to the markers for most bacterial pathogens examined in this study. Certain FIB may be more applicable to particular pathogens depending on sand moisture content. That the Entero1 genetic marker did not exhibit decay rates similar to the markers for pathogens in this study is surprising because Entero1 abundance in water and sand can be correlated with the incidence of gastroenteritis at recreational beaches (73, 173). However, the causative agents of disease were not determined in those studies, so the correlation of Entero1 and incidence of gastroenteritis of bacterial origin is unknown (73, 173). Similar to our results, several studies have shown that the decay of the Entero1 marker in seawater and freshwater is different than the decay of bacterial pathogens or genetic markers for them (12, 189). Only the human *Bacteriodes* HF183 marker had a decay rate similar to the genetic marker for *Campylobacter* cells, and no cultured FIB decayed as quickly as *Campylobacter*. Previous studies similarly found that *Campylobacter* decayed more quickly than FIB in marine sand and manure (89, 189).

PMA-qPCR is a valuable new tool for assessing the abundance of genetic markers from live cells. The quantification of solely culturable bacteria underestimates abundance

by not accounting for VBNC bacteria, and the quantification of total genetic markers can overestimate abundance due to extracellular DNA or DNA from dead or dying cells (121, 123, 127). With respect to bacterial pathogens, precise quantification methods are extremely important in order to accurately gauge health risk. *Campylobacter*, *Salmonella*, and *Shigella* can readily enter into a VBNC state upon exposure to environmental stressors (126). Although the VBNC state has been reported for *S. aureus*, it is not well-established (193). Under low moisture conditions, there was a significant difference in the decay rates of total genetic markers and those from live cells for all FIB and pathogens in this study. Bae and Wuertz (12) also found that decay of *Campylobacter* genetic markers in live cells was more rapid than the decay of total genetic markers in freshwater, however, there was no difference between the decay rates for genetic markers of total or live *Salmonella* cells. Future studies are needed to examine the prevalence of the VBNC bacterial pathogens on recreational beaches in order to assess the validity of culture-based methods for assessment of health risk.

Beach sand is an important reservoir of FIB, but evidence suggests that sand may serve as a reservoir for pathogenic bacteria as well (64, 74). Similar to studies of seawater and freshwater, this study indicated that the bacterial pathogen of interest should be considered in choosing an appropriate indicator microorganism or molecular marker for freshwater sand habitats (185). Moreover, sand moisture also influences the applicability of a particular FIB or molecular indicator. There are clear limitations of our microcosm study, such as to whether the results are broadly applicable to the scale of entire beaches. Undoubtedly, site-specific features may also play a role in the persistence of culturable

FIB and genetic markers for FIB and pathogens. Future studies are needed to elucidate and examine factors, such as organic matter content and particle size, which may affect pathogen persistence in sand. In addition, the ability of MRSA to persist and proliferate in sand should be examined further.

Table 5.1 Quantitative PCR assays used in this study.

Assay	Target	Locus	Primer/ Probe	Sequence (5' to 3')[a]	Conc. (nM)	Ref.
Entero1	*Enterococcus* spp.	23S rRNA	ECST748F	AGAAATTCCAAACGAACTTG	400	(106)
			ENC854R	CAGTGCTCTACCTCCATCATT	400	
			GPL813TQ	(6FAM) TGGTTCTCTCCGAAATAGCTTTAGGGCTA (TAMRA)	200	
AllBac	*Bacteroides* spp.	16S rRNA	296F	GAGAGGAAGGTCCCCCAC	200	(97)
			467R	CGCTACTTGGCTGGTTCAG	200	
			375Bhqr	(6FAM) CCATTGACCAATATTCCTCACTGCTGCT (BHQ1)	300	
HF183	Human-specific *Bacteroides*	16S rRNA	HF183F	ATCATGAGTTCACATGTCCG	300	(17, 153)
			183R	TACCCCGCCTACTATCTAATG	300	
qCamp	*Campylobacter* spp.	16S rRNA	CampF2	CACGTGCTACAATGGCATAT	200	(107)
			CampR2	GGCTTCATGCTCTCGAGTT	200	
			CampP2	(6FAM) CAGAGAACAATCCGAACTGGGACA (BHQ1)	100	
qSalm	*Salmonella* spp.	*ttrRSBCA*	Ttr-6	CTCACCAGGAGATTACAACATGG	200	(109)
			Ttr-4	AGCTCAGACCAAAAGTGACCATC	200	
			Ttr-5	(6FAM) CACCGACGGCGAGACCGACTTT (BHQ1)	200	
qShig	*Shigella* spp.	*ipaH*	Shig1	CTTGACCGCCTTTCCGATA	400	(178)
			Shig2	AGCGAAAGACTGCTGTCGAAG	400	
			ShigP	(TET) AACAGGTCGCTGCATGGCTGAA (TAMRA)	200	
qMRSA	Methicillin-resistant *S. aureus*	*mecA*	FmecA	CATTGATCGCAACGTTCAATTT	200	(55)
			RmecA	TGGTCTTTCTGCATTCCTGGA	200	
			PmecA	(JOE) TGGAAGTTAGATTGGGATCATAGCGTCAT (TAMRA)	100	
IAC[b]	*Oncorhynchus keta*	ITS[c] region 2	SketaF2	GGTTTCCGCAGCTGGG	200	(45)
			SketaR3	CCGAGCCGTCCTGGTCTA	200	
			SketaP2	(6FAM) AGTCGCAGGCGGCCACCGT (TAMRA)	100	

[a] BHQ1, black hole quencher-1; 6FAM, 6-carboxyfluorescein; JOE, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; TET, tetrachlorofluorescein.

[b] Internal amplification control.

[c] ITS, internal transcribed spacer.

Table 5.2

Quantitative PCR calibration data.

Assay	Equation[a]	Range E[b]	Range R^2	LOD[c]
Entero1	$y = -3.32x + 36.6$	92 – 109	0.991 – 0.998	3
AllBac	$y = -3.38x + 38.2$	90 – 104	0.989 – 0.996	3
HF183	$y = -3.43x + 33.9$	89 – 100	0.996 – 0.999	3
qCamp	$y = -3.28x + 36.6$	100 – 113	0.985 – 0.999	30
qSalm	$y = -3.11x + 36.3$	97 – 121	0.993 – 0.999	150
qShig	$y = -3.23x + 35.6$	93 – 110	0.994 – 0.998	30
qMRSA	$y = -3.25x + 38.4$	92 – 112	0.979 – 0.999	30
IAC	$y = -3.25x + 31.2$	100 – 109	0.949 – 0.997	1

[a]Average calibration equation across 4 to 6 runs.

[b]Amplification efficiency = $(10^{(1/-\text{slope})} - 1)*100$.

[c]Limit of detection in genetic marker copies per assay. For IAC, LOD is in pg salmon sperm DNA.

Table 5.3

Initial concentrations of indicators in sand and sewage.

Microcosm component	*Enterococcus* spp.	*E. coli*	Entero1		AllBac		HF183	
			Total	Live[b]	Total	Live	Total	Live
Sand[a]	blod[c]	blod	47,700	24,800	128,000	42,600	blod	blod
Sewage inoculum	977	38,700	76,500	10,500	496,000	110,000	33,000	6,270

[a]Concentration of culturable cells or copies of genetic markers per g dry wt (sand) or per mL (sewage).

[b]PMA treated.

[c]Below the limit of detection.

Table 5.4

Decay rates and R^2 for culture-based and genetic marker-based indicators and pathogens.

Assay[a]	14% Moisture (wt/wt)				28% Moisture (wt/wt)			
	Control		PMA treated		Control		PMA treated	
	Decay rate[b] [std. err.]	R^2	Decay rate [std. err.]	R^2	Decay rate [std. err.]	R^2	Decay rate [std. err.]	R^2
Enterococcus spp.	0.361 [0.025]	0.88	–	–	0.280 [0.014]	0.93	–	–
E. coli	0.375 [0.025]	0.88	–	–	0.474 [0.026]	0.95	–	–
Entero1	0.062 [0.028]	0.14	0.178 [0.023]	0.67	0.035 [0.017]	0.12	0.050 [0.031]	0.08
AllBac	0.212 [0.020]	0.78	0.338 [0.034]	0.77	–0.112 [0.022]	0.47	–0.188 [0.038]	0.45
HF183	0.655[0.059]	0.87	1.52 [0.136]	0.93	0.342 [0.027]	0.85	0.473[0.059]	0.74
qCamp	0.798 [0.081]	0.84	1.21 [0.134]	0.85	0.767 [0.054]	0.90	1.01 [0.065]	0.93
qSalm	0.356 [0.020]	0.91	0.483 [0.025]	0.93	0.338 [0.015]	0.94	0.373 [0.028]	0.86
qShig	0.471 [0.029]	0.90	0.715 [0.041]	0.91	0.507 [0.046]	0.80	0.615 [0.054]	0.81
qMRSA[c]	0.369 [0.042]	0.81	0.596 [0.045]	0.89	0.208 [0.071]	0.38	0.356 [0.046]	0.73

[a]*Enterococcus* spp. and *E. coli* are culture-based assays, whereas remaining indicators and pathogens are genetic marker-based assays.

[b]Decay rate units are in $days^{-1}$. Negative decay rates indicate accumulation in the microcosm.

[c]qMRSA decay rate following plateau.

Table 5.5

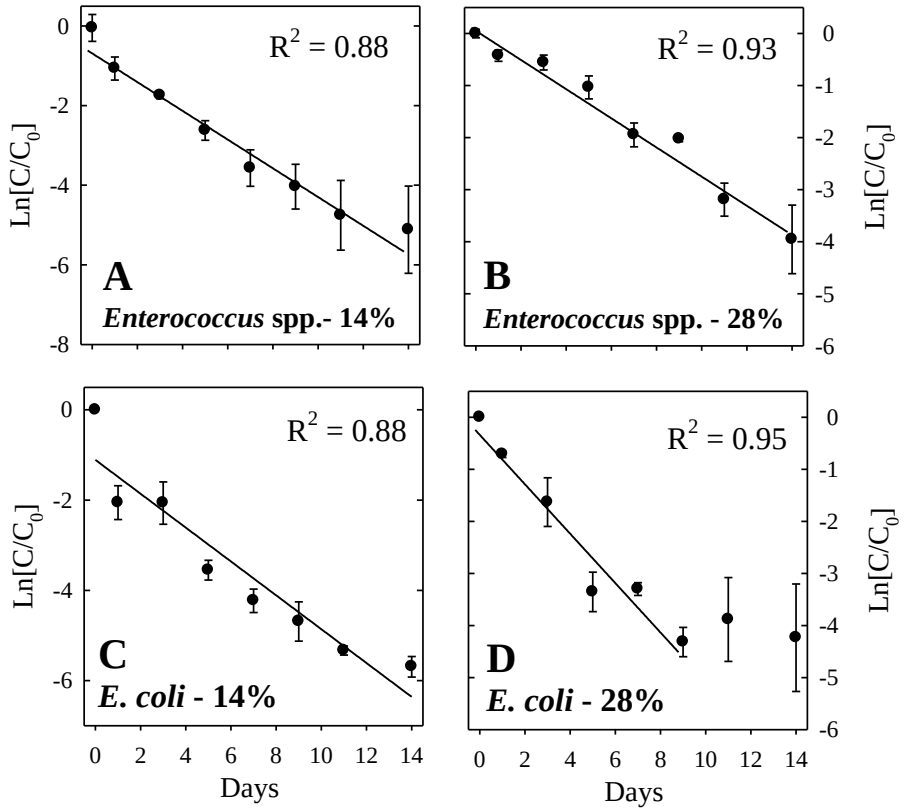
Similarity between indicator and pathogen decay rates.

Pathogen Assay	*Enterococcus* spp.	*E. coli*	Entero1		AllBac		HF183	
			Total	Live [a]	Total	Live	Total	Live
Low moisture								
qCamp	–[b]	–	–	–	–	–	T	L
qSalm	T	T, L	–	–	–	T	L	–
qShig	T	T	–	–	–	T	T, L	–
qMRSA	T	T	–	–	–	T	L	–
High moisture								
qCamp	–	–	–	–	–	–	–	–
qSalm	T, L	L	–	–	–	–	T, L	T, L
qShig	–	T, L	–	–	–	–	T	T, L
qMRSA	T, L	L	T	T	–	–	T, L	T, L

[a]PMA treated.

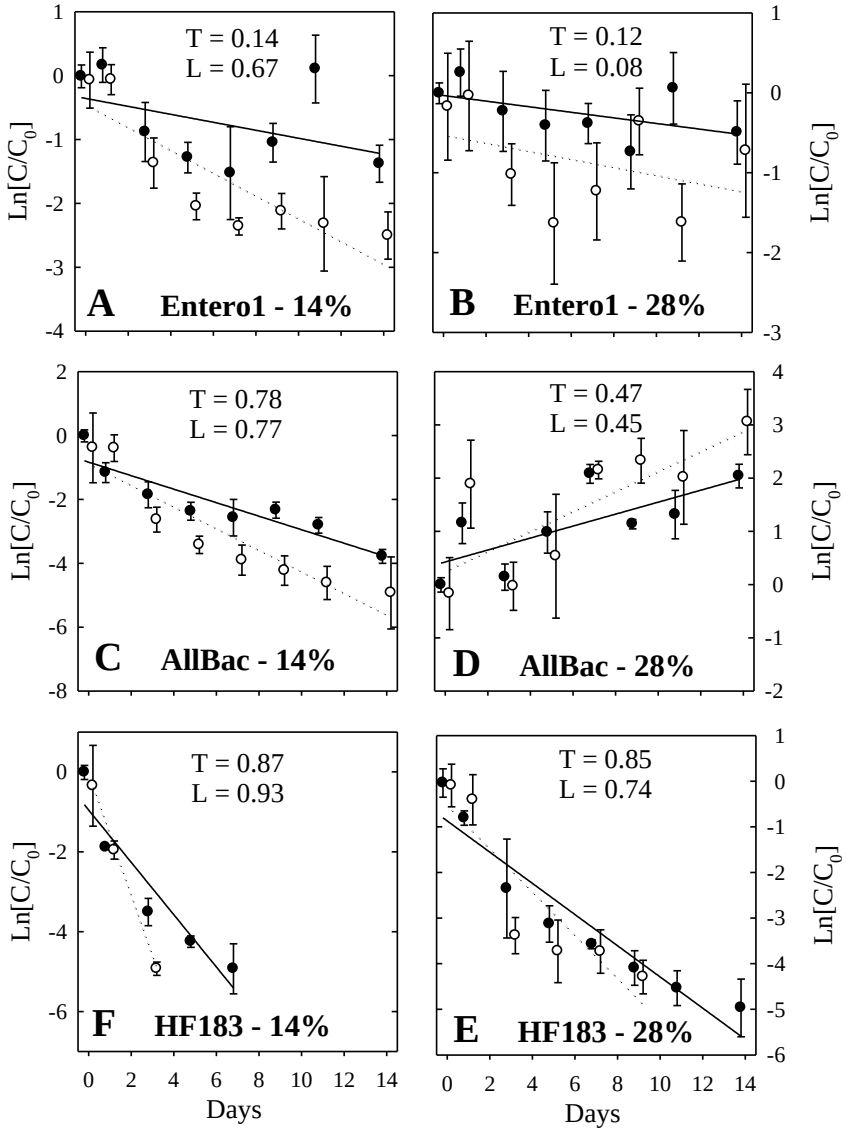
[b]Letters indicate decay rates that are not significantly different ($p \leq 0.05$) for total pathogen markers (T) or pathogen markers in live cells (L).

Figure 5.1



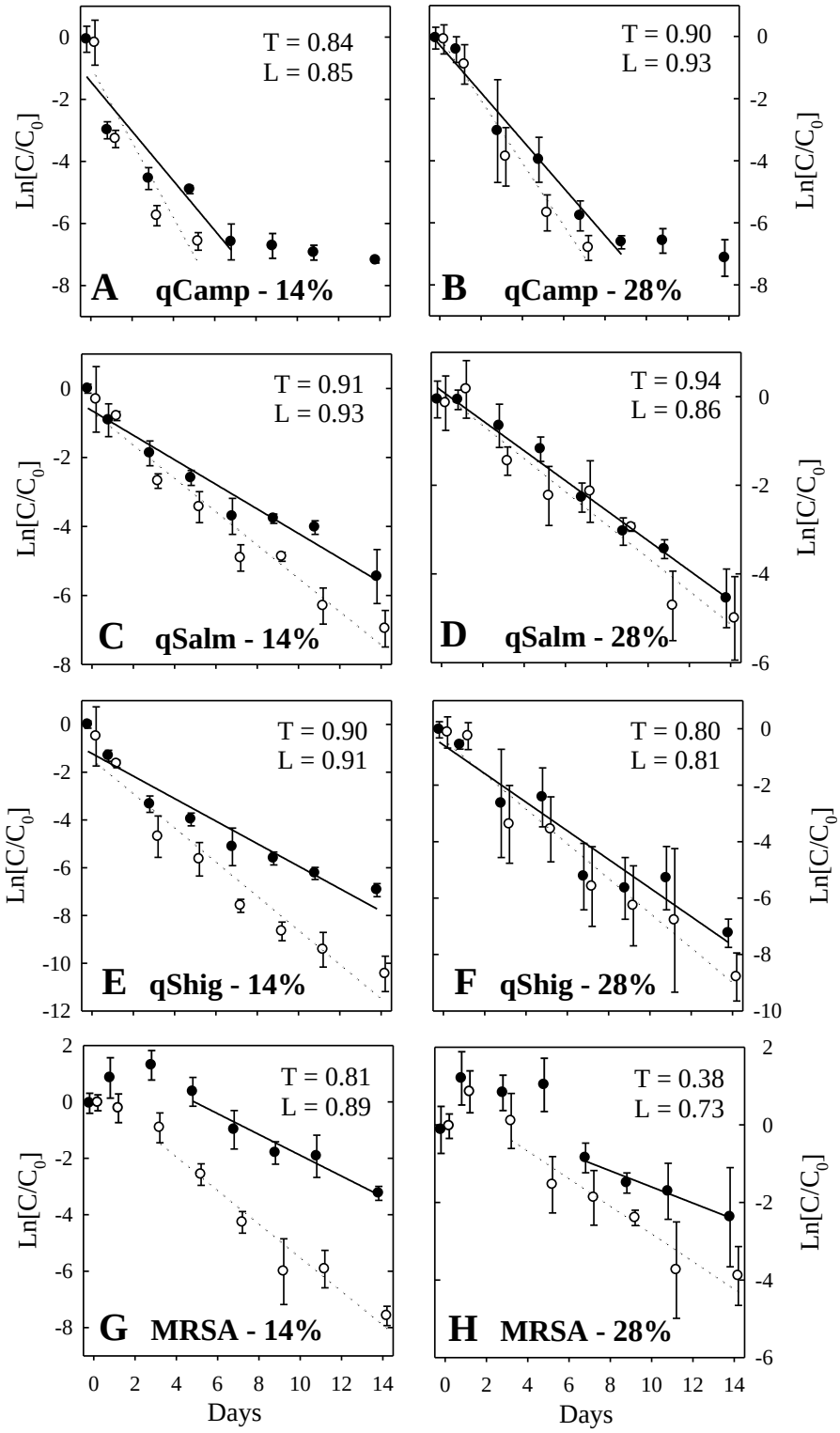
Decay of culturable indicators *Enterococcus* spp. (A, B) and *E. coli* (C, D) in sand at 14% (A, C) and 28% (B, D) moisture. Solid lines show the first-order decay curve. Error bars indicate 95% confidence intervals for the mean.

Figure 5.2



Decay of Entero1 (A, B), AllBac (C, D) and HF183 (E, F) genetic markers in sand at 14% (A, C, E) and 28% (B, D, F) moisture. The fitted first order-decay model for total markers (closed circles) and markers from live cells only (open circles) is indicated by solid and dotted line, respectively. R^2 values are shown for total markers (T) and markers from live cells (L). Error bars indicate 95% confidence intervals for the mean.

Figure 5.3


T = 0.84
L = 0.85
A qCamp - 14%
T = 0.90
L = 0.93
B qCamp - 28%
T = 0.91
L = 0.93
C qSalm - 14%
T = 0.94
L = 0.86
D qSalm - 28%
T = 0.90
L = 0.91
E qShig - 14%
T = 0.80
L = 0.81
F qShig - 28%
T = 0.81
L = 0.89
G MRSA - 14%
T = 0.38
L = 0.73
H MRSA - 28%
Ln[C/C0]
Days

Decay of qCamp (A, B), qSalm (C, D), qShig (E, F), and qMRSA (G, H) genetic markers in sand at 14% (A, C, E, G) and 28% (B, D, F, H) moisture. The fitted first-order decay model for total markers (closed circles) and markers from live cells only (open circles) is indicated by solid and dotted line, respectively. R^2 values are shown for total markers (T) and markers from live cells (L). Error bars indicate 95% confidence intervals for the mean.

CHAPTER 6:

Conclusions and Future Directions

CONCLUSIONS

The overarching goal of this thesis was to determine the utility of rapid, culture-independent methods for the identification and source-tracking of fecal pollution on freshwater beaches. In this thesis I examined the short time scale variation in goose/duck and human sources of fecal contamination on Duluth area beaches, measured the distribution of genetic markers of fecal contamination in water, sand, and sediment of a Duluth-Superior Harbor beach near a wastewater outfall, determined the decay rates of genetic markers in freshwater matrices, and compared the decay rates of genetic markers of fecal pollution and bacterial pathogens.

In Chapter 2, high-throughput colony hybridization of *E. coli* with a goose/duck-specific and pathogen probes and quantitative PCR (qPCR) for human-specific *Bacteroides* and *Salmonella* spp. was used to characterize the short-term variability in sources of fecal contamination and pathogenic E. coli and Salmonella at three Duluth area beaches. Goose/duck-borne *E. coli* made up 5 to 29% of *E. coli* at the study sites. Goose and duck *E. coli* did not substantially contribute to single-sample water quality exceedances, although few were observed over the study period. Therefore, management of goose populations on Duluth-Superior Harbor beach would likely lower total *E.coli*, but it may not reduce single-sample exceedances. In contrast, the human-specific *Bacteroides* marker gene exhibited high temporal variability, and was detected at a significantly great frequency in the water column in a site located in the inner harbor. Due to the site location, more frequent detection of human-specific markers is likely due to treated wastewater effluent. *Salmonella* spp. and potentially pathogenic *E. coli* were

low in abundance and were not correlated to indicator abundance or sources of fecal contamination. The Duluth-Superior Harbor environment poses inherently challenging problems for water quality managers, as water movement within the harbor almost certainly contributes to the high short-term variability and a lack of correlation between fecal indicator bacteria, their sources and pathogen genes, to environmental variables.

In Chapter 3, qPCR of genetic markers for the fecal indicator bacteria (FIB) enterococci, total *Bacteroides*, and human-specific *Bacteroides* was used to examine the distribution and relationship of genetic markers to effluent outflows in water, sand, and sediment of a Duluth-Superior Harbor beach near an effluent outfall. Results indicated that effluent loading likely controls the abundance of molecular indicators of fecal pollution in the water column of the study beach. Sand and sediment contained more enterococci and total *Bacteroides* markers on a per mass basis than water, whereas the concentration of human-specific *Bacteroides* was similar across matrices. In most instances, markers were most abundant in the top 1 to 3 cm of sand and sediment. Over the course of one month incubation in the field, enterococci and total *Bacteroides* markers in the water column did not significantly change, but human-specific *Bacteroides* decreased to below detection levels. In sand and sediment, experimental month had a strong effect on persistence, and all markers exhibited significant increases in sediment in July. In contrast with the sites studied in chapter 2, at which human-specific *Bacteroides* was infrequently detected in sand and sediment, the study site had similar abundance of human-specific *Bacteroides* in water, sand, and sediment. Taken together, sand and sediment can be reservoirs of genetic markers at freshwater beaches

with significant water column loading of molecular indicators. The observed accumulation of markers in sediment in July may be due to growth, and should be examined further. Beach monitoring and assessment with molecular markers of fecal pollution must account for the potential for sand and sediment to serve as sources of molecular indicators.

In Chapter 4, sand and water and sediment microcosms were used to determine the persistence of genetic markers for enterococci, total *Bacteroides*, and human-specific *Bacteroides* in freshwater matrices. Increased temperature was associated with increased decay rates for most marker and treatment combinations. The decay of enterococci and total *Bacteroides* markers was slowed at 30% sand moisture relative to 10 and 20% moisture, but moisture did not strongly affect the decay of human-specific *Bacteroides* in sand. Markers decayed more quickly in water than in sand and sediment. The human-specific *Bacteroides* marker decayed more quickly than enterococci and total *Bacteroides* at most temperatures and in most matrices; therefore, the human-specific *Bacteroides* marker may be well-suited as an indicator of recent contamination. All markers persisted for over 4 mo. at low temperatures, indicating there may be potential for overwintering of markers in sand and sediment. The increased persistence of enterococci and growth of total *Bacteroides* in saturated sands and sediments may preclude their use for detection of fecal contamination on some freshwater beaches.

In Chapter 5, sand microcosms were used to compare the decay rates of culturable FIB, genetic markers for FIB, and genetic markers for bacterial pathogens. PMA treatment was used to examine differences in the persistence of total genetic markers and those from live cells. At 14% sand moisture, the decay rate of total markers was slower than that of live cells for all qPCR assays, but at 28% moisture, there was no difference in

decay rates of total markers and those from live cells for any assay. This indicates that the presence of free DNA and DNA from dead or dying cells may overestimate pathogen abundance in low moisture sands. Prolific growth of total *Bacteroides* and limited growth of MRSA in microcosms was observed. Moisture affected the similarity between decay rates of indicators and pathogens. Overall, culturable FIB and the human-specific *Bacteroides* marker had decay rates that were most comparable to *Campylobacter*, *Salmonella*, *Shigella*, and MRSA genetic markers. Genetic markers for *Enterococcus* spp. and total *Bacteroides* may not be valid indicators of health risk on sandy, freshwater beaches impacted by bacterial pathogens. The moisture content, pathogen of interest should be considered in choosing an appropriate indicator of fecal contamination in freshwater sand.

Taken together, there are a multitude of factors that affect the distribution and persistence of FIB in the Duluth-Superior Harbor beach environment (Fig. 6.1).

FUTURE DIRECTIONS

The projects described in this thesis have opened up several avenues for future research on genetic markers of fecal pollution in freshwaters. Opportunities for future research include the construction of a predictive model for genetic markers in the Duluth-Superior Harbor, further study of the spatial distribution of genetic markers in sand and sediment, and examination of the effects of oxygen on *Bacteroides* survival in sediment.

High frequency sampling of Duluth-Superior Harbor beaches revealed high variability in human sources of fecal contamination. A typical predictive model that uses

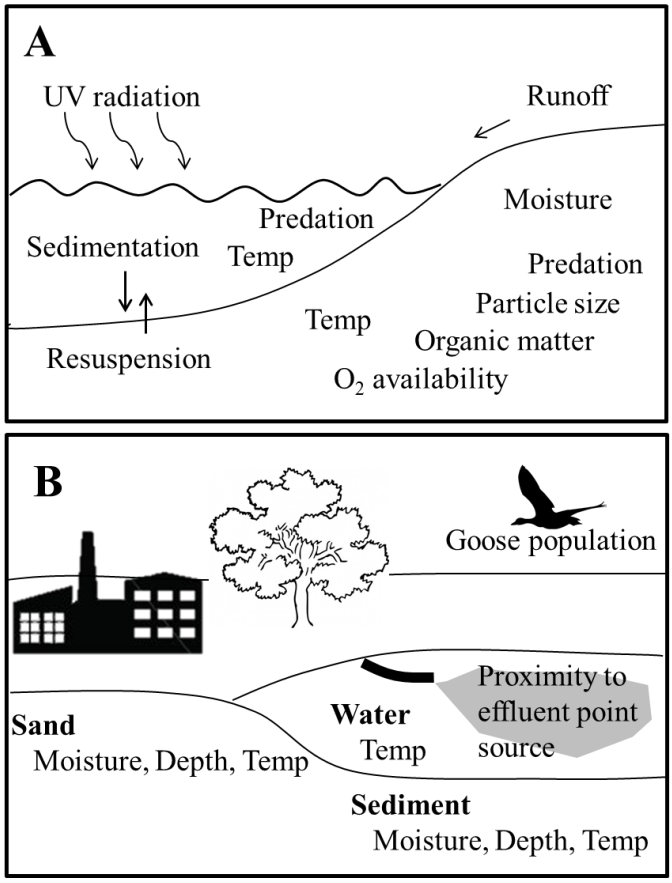
parameters such as wave height and temperature to predict FIB could not be constructed for human-specific *Bacteroides* presence or abundance. This is likely due to the complicated water movement patterns within the harbor, coupled with variability in genetic marker loading from effluent outflows. Dr. Jay Austin and Bruce Ludewig of the University of Minnesota-Duluth are currently developing a numerical model of the Duluth-Superior Harbor. Magnitude and location of contaminant loading are entered into the model, and wind speed, wind direction, and St. Louis River flow can be used to examine the movement of a contaminant plume (B. Ludewig, personal communication). With the concentration of genetic markers in effluent outflow, outflow volume, and marker decay rates determined in this thesis, it may be possible to model genetic marker concentrations on all Duluth-Superior Harbor beaches simultaneously. Moreover, a predictive model, coupled with sampling, could help identify and pinpoint sewage leaks in the harbor.

Although the vertical distribution of genetic markers of fecal pollution in sand and sediment was assessed, the horizontal spatial distribution of markers was not examined. Fine- and large-scale analysis of the spatial distribution of markers would improve sampling accuracy and efficiency. Current monitoring schemes do not include sand or sediment monitoring. However, the importance of sand and sediment as a reservoir of pathogens is increasingly recognized, and future monitoring efforts may include sand and sediment.

The current thesis has shown that the total *Bacteroides* genetic marker has the potential to grow in sand and sediment. Presumably, the low availability of oxygen in

sand at high moisture levels and in sediment facilitated growth. A future study could examine the vertical distribution of *Bacteroides* in experimental microcosms with various oxygen gradients. Furthermore, these results could be compared to the distribution of *Bacteroides* in stream upwelling (low oxygen) and downwelling (high oxygen) zones.

Figure 6.1



Factors that are hypothesized to affect the concentration and decay rate of fecal indicator bacteria in the beach environment (A). The factors which significantly affect the distribution and decay rate of culturable indicators and molecular indicators in the Duluth-Superior Harbor beach environment (B).

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