General and Host-Associated Fecal Indicator Bacteria at Chicago Beaches

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THESIS

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This dissertation is dedicated to my parents, Giri Dass Shrestha and Neeru Shrestha, and my siblings, Gunjan Das Shrestha and Santwona Shrestha, for their unconditional love and support throughout my academic years in the United States.

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Contribution of Authors

Chapter I is a background chapter that places my dissertation questions in the context of beach monitoring as the larger field and highlights the significance of my research questions. Chapter II represents a published manuscript (Shrestha, A., & Dorevitch, S. (2019). Evaluation of rapid qPCR method for quantification of *E. coli* at non-point source impacted Lake Michigan beaches. *Water Research, 156*, 395–403. https://doi.org/10.1016/j.watres.2019.03.034) for which I was the primary author and major driver of the research. My committee chair and faculty advisor, Dr. Samuel Dorevitch, contributed to the editing of the manuscript. Chapter III represents an unpublished manuscript for which I am the first author. Catherine Kelty from the United States Environmental Protection Agency (USEPA) Office of Research and Development, Cincinnati, OH assisted me with the experiments and analysis of the water samples in the laboratory. Mano Sivaganesan, from the USEPA, helped with the data analysis and the fecal score calculations. Drs. Orin Shanks and Samuel Dorevitch contributed to the editing of the manuscript. Chapter IV represents a series of my own unpublished analysis directed at answering the question about the effects of precipitation on microbial water quality at Chicago beaches. The research also used data from Chapter III. I anticipate that this line of research will ultimately be published as part of a co-authored manuscript. Chapter V represents my synthesis of the research presented in this dissertation and my overarching conclusions. The future directions of this field and this research question are discussed.

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LIST OF ABBREVIATIONS

ΔΔCt	Comparative Cycle Threshold
AUC	Area Under the Curve
BAV	Beach Action Value
BCI	Bayesian Confidence Interval
BEACH	Beaches Environmental Assessment and Coastal Health
BSA	Bovine Serum Albumin
CCE	Calibrator Cell Equivalents
cEC	*E. coli* Culture
CI	Confidence Interval
CPD	Chicago Park District
Ct	Cycle Threshold
CV	Coefficient of Variation
CWA	Clean Water Act
DNA	Deoxyribonucleic Acid
DNQ	Detect but Non-quantifiable
EC	*E. coli*
ENT	Enterococci
FIB	Fecal Indicator Bacteria
IA	Index of Agreement
IAC	Internal Amplification Control
LLOSCQ	Lower Limit of Standard Curve Quantification
LQ	Lower Quartile
MeB	Method Blank
MPN	Most Probable Number
MST	Microbial Sources Tracking
NEEAR	National Epidemiological and Environmental Assessment of Recreational Waters
ND	Non-detect
NTC	No Template Control
OR	Odds Ratio
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
Q	Quantifiable
QC	Quality Control
qENT	Enterococci qPCR
QMRAs	Quantitative Microbial Risk Assessments
qPCR	Quantitative Polymerase Chain Reaction
R^2	Pearson's Correlation R-squared
ROC	Receiver Operating Characteristics
ROQ	Range of Quantification
RWI	Recreational Waterborne Illness
RWQC	Recreational Water Quality Criteria
SSDNA	Single Stranded Salmon Testes DNA
SPC	Sample Processing Control
TSC	Target Sequence Copies

LIST OF ABBREVIATIONS (continued)

TSM	Technical Support Materials
UIC-SPH	University of Illinois at Chicago School of Public Health
uLOQ	Upper Limit of Quantification
UQ	Upper Quartiles
USEPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant

SUMMARY

Recreational waterborne illness is a significant public health problem. Exposure to enteric bacteria, viruses, and protozoa are the main causes of swimming-related outbreaks of gastrointestinal illnesses. For decades, *E. coli* and enterococci have been measured at Great Lakes beaches to indicate fecal contamination. Although these "indicator bacteria" rarely cause disease in humans following recreational exposure, several epidemiological studies have established a direct relationship between the concentration of the indicator bacteria in beach water and the incidence of gastrointestinal illness among swimmers.

Aim 1: Evaluation of rapid qPCR method for quantification of *E. coli* at non-point source impacted Lake Michigan beaches

Most Great Lakes communities rely on culture-based *E. coli* methods for monitoring fecal indicator bacteria (FIB) at recreational beaches. These cultivation methods require 18 or more hours to generate results. As a consequence, public notifications about beach action value (BAV) exceedance are based on prior-day water quality. Rapid qPCR monitoring of bacteria in beach water solves the 24-hour delay problem, though the USEPA-approved qPCR method targets enterococci bacteria, while Great Lakes communities are familiar with *E. coli* monitoring. For an *E. coli* qPCR method to be useful for water quality management, it is important to systematically characterize method performance, and establish BAVs for public notification purposes. In this study, we 1) evaluated a draft USEPA *E. coli* qPCR method, 2) compared *E. coli* qPCR measurements with two established FIB (*E. coli* culture and enterococci qPCR) results, and explored potential strategies to establish *E. coli* qPCR BAV criteria in the absence of an epidemiological study. Based on analyses of 288 water samples collected from eight of Chicago's Lake Michigan beaches, the *E. coli* qPCR method demonstrates acceptable

performance characteristics. The method is prone to low level DNA contamination, possibly originating from assay reagents derived from *E. coli* bacteria. Both *E. coli* and enterococci BAVs were exceeded in approximately 18% of the samples. *E. coli* qPCR values were correlated with both *E. coli* culture ($r = 0.83$; $p < 0.0001$) and enterococci qPCR ($r = 0.67$; $p < 0.0001$) values. The approach recommended by the USEPA in its Technical Support Material (TSM) was used to generate candidate *E. coli* qPCR BAVs, as was receiver operating characteristic (ROC) analysis. Potential BAV thresholds differed substantially, ranging from 200.9 calibrator cell equivalents (CCE) / 100 mL (ROC analysis, enterococci qPCR BAV as the reference) to 1000 CCE/ 100 mL (TSM analysis, enterococci qPCR BAV as the reference). Because we found that different approaches to establishing potential BAVs generate quite different values, guidance from USEPA about approaches to defining comparable BAVs would be useful.

Aim 2: Characterizing fecal non-point pollution sources at Lake Michigan beaches in Chicago

A system of channels directs wastewater and stormwater away from Lake Michigan and towards the Mississippi River with the goal of protecting Lake Michigan from urban discharges. As a result, Chicago's beaches on Lake Michigan should not be impacted by point sources of fecal contamination. However, despite the absence of known point sources of human fecal pollution, USEPA recommended Beach Action Values (BAVs) are exceeded with some regularity at several of Chicago's Lake Michigan beaches. The goals of this study were 1) to evaluate the presence and concentration of host-associated genetic markers for human, dog, and bird fecal pollution sources at select Chicago beaches, 2) characterize their association with enterococci (EPA Method 1609.1) and *E. coli* (Colilert®) general fecal indicator bacteria (FIB) used for routine monitoring, and 3) explore microbial source tracking as a potential complement

to routine beach monitoring. During the summer of 2016, water was sampled five days per week at nine beaches and analyzed for *E. coli* by culture and enterococci by quantitative polymerase chain reaction (qPCR) for beach monitoring and public notification. Additional filters of water samples used for analyzing general FIB were also archived at -80°C for future analyses. We selected 195 of those archived filters for human (HF183/BacR287, HumM2), canine (DG3, DG37), and avian (GFD) fecal source characterization. Host-associated genetic markers were quantifiable in 4% (n=8) for HF183/BacR287, 1% (n=3) for HumM2, 6% (n=12) for DG3, 2% (n=4) for DG37 and 23% (n=45) for GFD. The most frequently detected host-associated marker at all beaches was GFD, which was detected in 40% of the samples tested. Human marker, HumM2, was detected least frequently (4%). Logistic regression results of overall samples showed strong, positive and statistically significant associations between the general FIB concentrations (on a $\log_{10}$ scale) and the presence of the GFD and DG3 markers [odds ratio (OR) range: 1.98-2.3]. Using a weighted-average fecal score approach, we observed that at all sites, host-associated concentrations of $\log_{10}$ copies per 100 mL sample for GFD (avian) was 8.4 times higher and DG3 (canine) was 4.2 times higher in samples that exceeded the enterococci qPCR BAV (1000 CCE/100mL) compared to samples that were less than 100 CCE/100mL. A similar pattern was observed in samples that exceeded the *E. coli* culture-based BAV (235 MPN/100mL) compared to samples less than 23.5 MPN/100mL for GFD (9 times higher) and DG3 (3.5 times higher). In contrast, human-associated marker average concentrations were not significantly different, regardless of general FIB BAV sample groupings. The findings that bird markers were widely distributed at beaches, that dog markers were limited to the beach that has an area for dogs, and that human markers were rarely detected were consistent with expectations.

Aim 3: Evaluating effects of precipitation on microbial water quality at Chicago beaches

Fecal bacteria concentrations have been reported to increase in surface waters after heavy rainfall mostly due to local municipal stormwater and sewer conveyance discharges. Chicago's Lake Michigan beaches present a unique setting in that stormwater and wastewater flows are engineered to discharge into the nearby Chicago River, which flows away from Lake Michigan, diverting pollution away from beaches. Therefore, under dry and most wet weather conditions, these beaches should not be impacted by these point sources of fecal contamination. However, several Chicago beaches often exceed the U.S. Environmental Protection Agency recommended Beach Action Values (BAVs). Here we investigate the potential influence of rainfall on general fecal indicator bacteria (FIB) and host-specific genetic markers at Chicago beaches.

During the summer of 2016, water samples were collected at nine Chicago beaches and analyzed for *E. coli* by culture and enterococci by qPCR. Select samples (n = 195) were tested for human (HF183/BacR287, HumM2), canine (DG3, DG37), and avian (GFD) fecal source characterization. We then examined the occurrence and concentrations of FIB and MST paired measurements under wet and dry conditions. Precipitation tended to increase concentrations of *E. coli* but not enterococci. Following rainfall, the odds of *E. coli* BAV exceedance increased, though again this was not true for enterococci BAV exceedance. Point estimate of the odds of detecting the MST markers were higher following wet weather (vs. dry weather) for all five host-associated markers, though none of these associations reached statistical significance. Using a weighted-average fecal score approach, we observed that the host-specific marker concentration (log10 copies per 100 mL) for DG3 was 2.4 times higher, DG37 was 2.1 times higher and GFD was 1.6 times higher during wet compared to dry weather conditions. In contrast, HF183/BacR287 average concentrations were not significantly different ($p > 0.05$), regardless of

weather conditions, though this marker was detected less frequently than the others. The impact of rainfall on general FIB and host-associated marker concentrations varied by beach. MST findings coupled with precipitation information can provide a better picture of different sources of fecal pollution and their pathways and can inform the development of remediation plans for beach water quality management to better heath protection of beachgoers.

I. INTRODUCTION

A. Background

1. Protecting water quality at recreational beaches in the United States

Recreational waterborne illness is a significant public health problem leading to dozens of outbreaks and thousands of cases of illness per year (Centers for Disease Control and Prevention, 2015). Such outbreaks of gastrointestinal illness are caused by the ingestion of water contaminated by bacterial, viral, and protozoan pathogens found in fecal matter from humans and other animals. Data from epidemiological studies show that about 15-25 swimmers, kayakers, fishermen and boaters per 1000 develop acute gastrointestinal illness attributable to their activities in recreational waters (Dorevitch et al., 2012; Wade et al., 2006, 2010). Under provisions of the Clean Water Act (CWA), the prevention of recreational waterborne illness centers around water quality monitoring at beaches, followed by public notification of elevated concentrations of bacteria in beach water. Beach managers can use data about elevated bacteria levels to identify potential sources of fecal pollution and attempt to control those sources.

To protect the health of beachgoers and prevent exposure to contaminated water, local and state environmental agencies in the United States routinely monitor recreational water quality, and have done so for decades by measuring *E. coli* and enterococci, often referred to as fecal indicator bacteria (FIB). These bacteria are not pathogenic themselves, but they may indicate the presence of pathogenic organisms, fecal contamination, and/or health risk. Although FIB are normal inhabitants of the human gut, they can originate from multiple sources and can persist in the environment. They are used to indicate the presence of fecal contamination due to their abundance and because

they are relatively easy and inexpensive to measure. Further, epidemiological studies have also shown a direct relationship between adverse human health risks among water recreators and the presence of *E. coli* and enterococci in recreational waters. However, we typically refer to the FIB *E. coli* and enterococci as "non-specific FIB" or "general FIB" because they do not give us information on the specific sources of fecal pollution (humans, dogs, ruminants, birds or other wildlife).

2. Recreational Water Quality Criteria (RWQC)

Under the authority of the CWA, the United States Environmental Protection Agency (USEPA) issues national recommendations for monitoring recreational water quality (United States Environmental Protection Agency, 2012). Current recreational water monitoring and notification efforts measure general FIB, and apply criteria termed "beach action values" that were developed from epidemiological studies predominantly conducted at beaches impacted by human sewage from treatment facility effluent ("point source impacted"). The National Epidemiological and Environmental Assessment of Recreational Waters (NEEAR) study was an important part of the USEPA's Beaches Environmental Assessment and Coastal Health (BEACH) program that evaluated the association between human health effects and novel rapid indicators of recreational water quality, as well as the traditional measures of FIB. That study described the use of real-time quantitative polymerase chain reaction (qPCR) methods for FIB measurements to provide same-day water quality results. The rapid qPCR method is a significant improvement over the culture method, which provides results only the following day. NEEAR study results indicate that the qPCR method using enumeration of enterococci DNA was at least as good as culture measurements in predicting the occurrence of acute

gastrointestinal illness among beachgoers (Wade et al., 2008, 2006, 2010). Based on the findings from the NEEAR studies, the USEPA derived specific beach action values (BAV) in the 2012 Recreational Water Quality Criteria (RWQC), for monitoring water quality measured by both culture and qPCR (United States Environmental Protection Agency, 2012). The mean illness rates associated with the *E. coli* culture BAV of 235 CFU/100 mL and enterococci qPCR BAV of 1,000 CCE/100 mL are approximately 36 cases of NEEAR-gastrointestinal illness (NGI) per 1,000 primary contact recreators based on the NEEAR studies (United States Environmental Protection Agency, 2012). Both the culture and qPCR BAVs are intended to provide a comparable degree of public health protection. However, since the NEEAR studies were restricted to beaches affected by point source pollution (wastewater treatment plant discharges), their associated epidemiological findings and derived BAV criteria may not contain the same health risk information at beaches that are not impacted by point sources of pollution.

3. Limitations of current monitoring frameworks

Despite the widespread implementation of FIB monitoring at beaches, this approach suffers from several limitations. First, monitoring that uses culture-based methods for measuring FIB requires 18-24 hours of analysis time to obtain results. Consequently, beach managers have made decisions about issuing swim bans or public advisories at beaches based on FIB levels from water samples collected the previous day. This lag time can lead to delays in issuing beach advisories and beachgoers may unknowingly be exposed to contaminated water. Dorevitch et al., (2017) found the results of prior-day *E. coli* levels are no better than chance at predicting current-day water quality.

Second, over the years, it has also become clear that high concentrations of FIB in beach water samples do not necessarily indicate that sewage contamination of beaches has occurred, as was once believed. Many studies have shown FIB can persist and multiply in sediments and sands of marine and freshwater beaches (M. Byappanahalli & Fujioka, 2004; M. N. Byappanahalli et al., 2006; Kinzelman, Whitman, Byappanahalli, Jackson, & Bagley, 2003).

A third limitation of the current framework is that the FIB monitored do not differentiate between sources of fecal pollution (human, bird, dog, wildlife, agricultural animals). Studies have shown that fecal matter from multiple non-human animals, such as dogs, birds, and other wildlife can contribute to high levels of FIB in recreational waters (Harwood, Staley, Badgley, Borges, & Korajkic, 2014; Wright, Solo-Gabriele, Elmir, & Fleming, 2009). This is important because quantitative microbial risk assessments (QMRAs) have suggested that recreational water impacted by human fecal contamination poses higher human health risk in beachgoers than water impacted by non-human sources of fecal pollution (J. A. Soller, Bartrand, Ashbolt, Ravenscroft, & Wade, 2010; J. A. Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010; J. Soller et al., 2015). In fact, many viruses that cause infectious gastroenteritis outbreaks, such as norovirus, are human-specific enteric pathogens and only present in human fecal matter (Centers for Disease Control and Prevention, 2015; Mawatari & Kato, 2014). While current beach-monitoring programs make use of only general fecal indicators, developing pollution control initiatives may be more effective if human and animal sources of the pollution could be distinguished. Towards that end, beach management, particularly at locations that often exceed USEPA BAVs, could be improved by using indicators that not only

provide information about the degree of fecal contamination but also about the source(s) of fecal pollution. Given Chicago's numerous beaches, prioritizing them for further analysis and remediation could take into account information about the presence of human fecal pollution at some beaches (but not others). Over the last several years, methods for source identification of FIB have been developed and refined, but routine use of host-associated markers has not been incorporated into beach monitoring and notification. Incorporation of such measures could address the inherent drawbacks associated with using general FIB.

4. Chicago beach monitoring and management

Chicago has 26 miles of lakeshore front with 27 public beaches that attract an estimated 20 million visitors annually. The Chicago Park District (CPD) has one of the most comprehensive beach monitoring programs in the United States. Until 2017, the CPD tested water samples from all 27 beaches at least 5 days per week during the beach season (between Memorial Day and Labor Day). Prior to 2015, like many beach monitoring programs across the country, CPD relied on culture-based methods such as Colilert® for monitoring FIB at Lake Michigan beaches. In the summer of 2015, the CPD enhanced its beach monitoring and notification through a pilot program of rapid molecular testing of beach water from five Chicago beaches using qPCR, performed by researchers at the University of Illinois at Chicago School of Public Health (UIC-SPH). This method utilizes a real-time polymerase chain reaction (PCR) amplification of a particular segment of deoxyribonucleic acid (DNA) unique to enterococci. Thus, monitoring targeted a rapidly measured but non-host-associated fecal indicator bacterium. The qPCR-based beach notification program was expanded to nine beaches, five days per

week in summer 2016. Beginning in 2017, the CPD discontinued Colilert® monitoring and extended the UIC-SPH testing to include 20 sampling sites (representing 27 beaches, some of which are small and adjacent to one another) every day in summer 2017.

5. *E. coli* qPCR (USEPA Draft Method C)

E. coli is the most common general FIB used for recreational water monitoring at freshwater beaches. *E. coli* are fecal coliform bacteria found in the digestive tracts and associated fecal matter of warm-blooded animals including humans (National Research Council, 2004). Historically, managers of Great Lakes beaches have relied on culture-based methods of *E. coli* (such as membrane filtration or Colilert®) rather than enterococci for monitoring FIB at recreational beaches. There is continued interest in the development of a qPCR method to rapidly measure *E. coli* in beach water, as beach managers as well as the public in the Great Lakes basin are more familiar with *E. coli* than enterococci for beach monitoring and notification. While enumeration of *E. coli* culture has been well established, methods for quantifying *E. coli* using qPCR is still under development. The performance of the draft *E. coli* qPCR method published by the USEPA is not well characterized and requires further evaluation before it can be implemented on a routine basis for beach water monitoring.

The association between *E. coli* concentration and risk of gastrointestinal illness among water recreators in coastal, Great Lakes and inland waters has been well established through large epidemiologic studies (Dufour, 1984; Marion, Lee, Lemeshow, & Buckley, 2010; United States Environmental Protection Agency, 1986). Even though several studies in the past have used qPCR for detection and quantification of *E. coli* DNA targets, the USEPA only approves culture-based methods, not qPCR methods, for

assessing *E. coli* for water quality monitoring. A major challenge to quantifying low levels of *E. coli* using qPCR is that the polymerase in the PCR reagents (e.g. Environmental MasterMix 2.0) is cloned in *E. coli* and thus contains *E. coli* DNA, resulting in some low-level DNA amplification from the reagents. The reagent contamination makes it difficult to analyze samples with similar amplification curves, and increases the likelihood of false positive results. Nonetheless, the USEPA is evaluating a qPCR protocol targeting *E. coli* as an option for monitoring beach water quality.

New epidemiological data are not concurrently being collected with the development of the *E. coli* qPCR method makes it difficult to determine a criterion value or BAV for beach management and notification directly. Instead, the BAV must be extrapolated to *E. coli* qPCR units based on the relationships between *E. coli* measured by qPCR and other FIB metrics for which BAVs have been established. Although several recent studies have assessed the relationship between the *E. coli* culture and enterococci qPCR results (Francy, Bushon, Brady, & Kephart, 2013; Haugland, Siefring, Wymer, Brenner, & Dufour, 2005; Lavender & Kinzelman, 2009; Noble, Blackwood, Griffith, McGee, & Weisberg, 2010; Raith, Ebentier, Cao, Griffith, & Weisberg, 2014; Schang et al., 2016), few have compared *E. coli* culture results with qPCR results for *E. coli* on the same water samples (Lam et al., 2014; Noble et al., 2010). Both Noble et al. (2010) and Lam et al. (2014) reported significant positive linear relationships between *E. coli* quantified by culture and real-time PCR methods in surface water. The water sampling sites in the Lam et al. study, and all but one of the Noble et al. study sites, were marine water locations. At several Racine, WI beaches, Lavender & Kinzelman (2009) found

only moderately strong associations between *E. coli* culture and *E. coli* qPCR ($0.47 \leq R^2 \leq 0.56$). It is not clear from the above studies how the new *E. coli* qPCR method will translate to current BAV thresholds for posting beach advisories at freshwater beaches not impacted by point-source of pollution. Further research is needed to define how its threshold values will correspond with existing culture and qPCR threshold values.

6. Use of microbial sources tracking (MST) markers in recreational waters

In the past decade, microbial source tracking (MST) methods have been developed to distinguish sources of fecal waste by targeting DNA sequences or gene fragments of FIB that vary among different animal hosts (at the genus or species level). MST technologies targeting human, canine, avian and other agricultural and wildlife wastes have been used to identify sources of fecal contamination within various water systems, including fresh and marine recreational water around the United States (Harwood et al., 2014). The use of these MST markers might lead to increased accuracy in estimating health risks to beachgoers and aid in the implementation of effective mitigation of fecal pollution sources.

Bacteroides species bacteria are found in high concentrations in fecal matter of warm-blooded animals. Because segments of DNA are different in of *Bacteroides* spp. in the intestines of humans than in *Bacteroides* spp. in the intestines of dogs or cows, MST methods have been developed using *Bacteroides* spp. (Wexler, 2007). In addition, the inability of *Bacteroides* spp. to proliferate in the environment and short survival duration outside hosts also makes them good for MST (Pepper, Gerba, & Gentry, 2014), as their presence in water reflects recent pollution by fecal matter.

Unlike for general FIB, standard methods for MST markers have not been published by the USEPA. However, several molecular MST markers in *Bacteroides* spp. for humans, dogs, birds, ruminants and other animals have been developed and tested (Harwood et al., 2014; Krentz, Prystajecky, & Isaac-renton, 2013; Roslev & Bukh, 2011). The host specificity of human-host *Bacteroides* gene markers, HF183 and HumM2, is well characterized in the literature (Ahmed, Hughes, & Harwood, 2016; Boehm, Soller, & Shanks, 2015). Other commonly used MST markers shown to be robust in different environments identify *Bacteroides* spp. specific to humans (general *Bacteriodales*, *Methanobrevibacter smithii*, & *Enterococcus faecium*), ruminants (BacR & CowM2), pigs (Pig-2-Bac), dogs (*Bacteriodales*-dog), and gulls (*Catellicoccus*-gull & Gull2) (Harwood et al., 2014).

Although host-associated MST markers have been used in research studies of fecal contamination in recreational waters, the relatively limited number of studies that have evaluated the relationship between the host-associated MST markers and measures of general (non-host-associated) FIB have had inconsistent results. While many studies have found no significant correlation between the general FIB tested and the MST markers (Flood et al., 2011; McQuaig, Griffith, & Harwood, 2012; Mika, Ginsburg, Lee, Thulsiraj, & Jay, 2014; Santoro & Boehm, 2007; Shibata et al., 2010; Zachery R. Staley, Vogel, Robinson, & Edge, 2015), others have found host-specific MST markers in surface waters to be significantly associated with higher enterococci levels by culture and by qPCR (Eichmiller, Hicks, & Sadowsky, 2013; Gordon et al., 2013; Hughes, Beale, Dennis, Cook, & Ahmed, 2017). Conversely, Boehm et al., (2009), reported that the presence and concentration of the human-associated HF183 marker was inversely

associated with the concentration of *E. coli* and enterococci in marine water (Boehm et al., 2009). Some of the disparities among the study findings may be due in part to different sources of pollution, hydrology and climate of the settings evaluated, and MST measurement methods used in the various studies, but overall it suggests that further research is needed to explore the value of MST methods for freshwater beach management.

7. Precipitation and water quality

Natural and anthropogenic factors influence the concentration and distribution of FIB in recreational surface waters. Numerous weather-related variables such as precipitation, temperature, and solar radiation, and their association with FIB concentrations in surface water have been studied previously (Jones, Liu, & Dorevitch, 2013). Statistical modeling has also been used in the past to predict *E. coli* concentrations at various sites utilizing water quality monitoring data and meteorological conditions (Nevers & Whitman, 2008).

Although many previous studies have shown a strong positive correlation of precipitation with FIB, especially *E. coli* concentrations, in surface waters (Ackerman & Weisberg, 2003; Dwight et al., 2011; Kirs et al., 2017; Kleinheinz, McDermott, Hughes, & Brown, 2009; McLellan et al., 2007), a study conducted by Sampson et al. (2006) did not show any significant relationship between amount of rainfall and bacterial concentrations at 15 beaches along Lake Superior. Then again, many studies in the last decade support that after heavy rainfall events, concentrations of bacteria typically increase in surface waters. Such spikes in bacterial concentration could occur through multiple mechanisms, including but not limited to: wastewater discharge, storm water or

wastewater infrastructure failure, a combined sewer overflow event, or simply resuspension of bacteria already in the water due to the stirring up of sand and sediment by high waves (Kleinheinz et al., 2009; McLellan et al., 2007; Whitman, Nevers, & Byappanahalli, 2006). In Chicago, wastewater is not discharged into Lake Michigan except following extreme rain events. Thus, elevations of FIB following rainfall at Lake Michigan beaches in Chicago could be due to surface flow of fecal matter (or bacteria from fecal matter) into beach water. Data from a multi-year study data revealed that extreme precipitation occurring the previous day to be significantly associated with beach closures at several Great Lake region beaches (Bush et al., 2014). In certain situations, higher levels of FIB after heavy rainfall were also specific to the beach or its location.

Currently, information is scant regarding the effect of rainfall on beach closures and the role of heavy precipitation on FIB concentrations at non-point source impacted beaches. There is also limited data available on the relationship between heavy rainfall and water quality monitoring with respect to the non-specific enterococci qPCR method, and the association between precipitation and FIB from specific sources of fecal pollution (using MST methods).

B. Objectives

The first study in this dissertation responds to the lack of information about the performance of the USEPA draft *E. coli* qPCR method and relevance of *E. coli* measured by qPCR to existing beach action values (BAVs). Using water samples collected from eight of Chicago's Lake Michigan beaches during the summer of 2017, the study has the following specific objectives:

i. To characterize the performance of the *E. coli* qPCR draft USEPA Method with specific attention to standard curve linearity (R^2), amplification efficiency (*E*), lower limit of quantification, and false positive rate;
ii. To compare *E. coli* qPCR measurements with two established FIB measurement methods (*E. coli* culture and enterococci qPCR);
iii. To evaluate approaches to establish health-based candidate *E. coli* qPCR BAV criteria in the absence of an epidemiological study; and
iv. To assess the degree to which beach-specific BAVs, developed for *E. coli* measured by qPCR, differ from an optimized BAV value for the set of the eight beaches.

The second study in this dissertation combines a sample intensive strategy with routine general FIB monitoring and molecular fecal source identification tools to characterize fecal pollution trends at Chicago beaches. Using archived filters of water samples collected from eight of Chicago's Lake Michigan beaches during the summer of 2016, the study has the following specific objectives:

i. To evaluate the presence and concentration of host-associated genetic markers for human (HF183/BacR287 and HumM2), dog (DG3 and DG37), and bird (GFD) fecal pollution sources at select Chicago beaches;
ii. To characterize association between MST results and general FIB measurements generated for beach monitoring and notification; and
iii. To explore whether MST testing results might complement the information obtained in routine beach monitoring.

The third study in this dissertation investigates the potential influence of rainfall on general FIB and source tracking genetic markers at non-point source impacted Lake Michigan beaches in Chicago. The study has the following specific objectives:

i. To assess the effect of rainfall on general FIB concentrations at non-point source impacted Chicago beaches;
ii. To examine the association between precipitation and detection of MST markers at these beaches; and
iii. To evaluate whether the observed associations vary by beach. We hypothesized that heavy rainfall is associated with higher general FIB concentrations and detection of MST markers leading to more frequent posting of beach advisories due to general FIB BAV exceedances.

Chapter II is an Accepted Manuscript of an article published by Elsevier in Water Research on 21 March 2019 online: Shrestha, A., & Dorevitch, S. (2019). Evaluation of rapid qPCR method for quantification of *E. coli* at non-point source impacted Lake Michigan beaches. Water Research, 156, 395–403. See Appendix A.

II. EVALUATION OF RAPID QPCR METHOD FOR QUANTIFICATION OF *E. COLI* AT NON-POINT SOURCE IMPACTED LAKE MICHIGAN BEACHES

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A. Introduction

Escherichia. coli (E. coli) are fecal indicator bacteria (FIB) commonly used for recreational water quality monitoring at freshwater beaches. Historically, beach managers in the Great Lakes basin have employed culture-based *E. coli* methods rather than enterococci even though the U.S. Environmental Protection Agency (USEPA) has established water quality criteria for freshwater using both bacteria (United States Environmental Protection Agency, 1986, 2012). Although culture-based chromogenic substrate methods such as Colilert® are easy to use and relatively inexpensive, they require 18-24 hours of incubation. Since FIB concentrations in water can change from day to day, this turnaround time can often lead to incorrect assessment of beach water quality resulting in erroneous public health information (Kim & Grant, 2004). A recent study revealed that lag *E. coli* culture results are no better than chance alone at predicting current-day water quality at Chicago beaches (Dorevitch et al., 2017). In 2012, the USEPA released new Recreational Water Quality Criteria (RWQC) that included a rapid quantitative polymerase chain reaction (qPCR) method for enterococci, which unlike culture methods, can generate results within 3-4 hours for same-day water quality notifications (United States Environmental Protection Agency, 2012). Not only are enterococci qPCR results rapid, they appear to be better predictors of children's health

risk than culture measures of enterococci, despite the fact that qPCR may detect dead and non-viable cells (Wade et al., 2008). Because several states including those in the Great Lakes basin have adopted *E. coli* standards rather than enterococci standards for monitoring their freshwater and inland beaches (United States Environmental Protection Agency, 2003), there is interest in developing a qPCR method to rapidly measure *E. coli* in beach waters. An *E. coli* qPCR method has been described (Chern, Siefring, Paar, Doolittle, & Haugland, 2011). However, the evaluation of that method focused on the affinity of different primer/probe sets for *E. coli* and a variety of related bacterial species. Thus, a systematic characterization of *E. coli* qPCR method performance is lacking. Likewise, paired comparisons of *E. coli* qPCR test results to currently approved FIB test methods (i.e. *E. coli* culture and enterococci qPCR) are also lacking. Assuming that an *E. coli* qPCR testing method demonstrates acceptable performance, beach mangers will need a Beach Action Value (BAV) for this method in order to apply monitoring results for public notification.

Epidemiologic data used to determine recreational water criteria values have shown that *E. coli* measured by culture is predictive of the occurrence of gastrointestinal illness at freshwater recreation sites impacted by wastewater treatment plants (Dufour, 1984; United States Environmental Protection Agency, 1986). Although some studies of water quality using qPCR methods for *E. coli* have been conducted, none evaluated *E. coli* qPCR results as a predictor of illness (Francy, Stelzer, et al., 2013; Haugland et al., 2005; Lavender & Kinzelman, 2009; Noble et al., 2010; Raith et al., 2014; Schang et al., 2016). While multiple studies have assessed the relationship between the *E. coli* culture and enterococci qPCR results, few focus on comparisons between *E. coli* culture and

qPCR results on paired water samples (Lam et al., 2014; Noble et al., 2010). Both Noble et al. (2010) and Lam et al. (2014) reported significant linear relationships between *E. coli* culture and qPCR concentrations, however all but one of the test samples were marine waters. At several Racine, WI beaches, Lavender & Kinzelman (2009) found only moderately strong associations between *E. coli* culture and *E. coli* qPCR (R^2 0.47 to 0.56). To our knowledge, there are currently no peer reviewed publications that have evaluated the *E. coli* qPCR draft USEPA Method for routine beach water monitoring and notification purposes.

Since establishing the 1986 water quality criteria based on epidemiologic studies, the USEPA more recently established the 2012 RWQC for enterococci measured using qPCR methods, again, using data from epidemiological studies (United States Environmental Protection Agency, 2012). Thus, although new *E. coli* qPCR laboratory methods have been developed, strategies are needed to establish health-based *E. coli* qPCR BAV that are tied to health risk. Technical support materials (TSM) linked to the 2012 RWQC were developed by USEPA to provide guidance for establishing site-specific recreational water quality criteria for newly developed indicators and methods under consideration for recreational water quality monitoring. Another analytic approach suitable for generating BAVs for new testing methods is receiver operating characteristics (ROC). Receiver operating characteristics or ROC is a data analysis method used to generate a threshold value from a continuous variable data set, such as *E. coli* qPCR results, that optimally differentiates between dichotomous conditions of second variable (i.e. *E. coli* culture or enterococci qPCR BAV exceedance). This method has been previously applied to other water quality analyses to predict enterococci criteria

value exceedance in relation to rainfall (Morrison et al., 2003) and the presence of protozoan pathogens (Yavuz et al., 2014).

Study objectives include 1) performance characterization of the *E. coli* qPCR draft USEPA method based on standard curve linearity (R^2), amplification efficiency (*E*), lower limit of quantification, and false positive rate; 2) comparison of *E. coli* qPCR measurements with two established FIB (*E. coli* culture and enterococci qPCR) results using water samples collected from eight of Chicago's Lake Michigan beaches; 3) evaluation of approaches to establish health-based candidate *E. coli* qPCR BAV criteria in the absence of an epidemiological study; and 4) to assess the degree to which beach-specific BAVs differ from an optimized BAV value for the set of the eight beaches.

B. Material and methods

1. Site description and sample collection

Chicago has 26 miles of lakeshore front with 27 public beaches that attract an estimated 20 million visitors each summer (Chicago Park District; USEPA, 2011). Local wastewater and stormwater flow in the Chicago area is intentionally diverted away from Lake Michigan towards the Mississippi River to prevent beach contamination. Seven days per week, qPCR beach monitoring using enterococci took place at 20 locations in the summer of 2017. Eight of these beaches were selected to evaluate the *E. coli* qPCR method to include at least two beaches from the north, central, and south sections of Chicago's beachfront. Within those spatial constraints, the two beaches that in the previous year exceeded the enterococci qPCR BAV most frequently, the two that exceeded the enterococci qPCR BAV least frequently, and the two that exceeded the 2016 *E. coli* culture criteria most frequently were selected

(**Table XI**, Appendix A and **Figure 7**, Appendix A). A total of 288 samples were collected from these eight beaches and tested for *E. coli* culture and qPCR four days per week (Monday-Thursday) between July 3 and August 31, 2017. Chicago Park District personnel collected 1-liter water samples in clean Nalgene bottles (Thermo Fisher Scientific, Waltham, MA), stored them in coolers with ice packs and transported the samples to UIC for analysis within approximately 1.5 hours of collection.

2. *E. coli* culture

E. coli were cultivated without dilution using Colilert® (IDEXX Laboratories, Westbrook, ME) assay following the manufacturer's recommendations and reported as most probable number (MPN) per 100 mL. The upper limit of quantification (uLOQ) for this method is 2,419 MPN/100 mL. Results above the uLOQ were assigned the value of 2,420 MPN/ 100 mL.

3. qPCR analysis

The procedures outlined in Method 1609.1: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay (United States Environmental Protection Agency, 2015) and USEPA draft Method C: *E. coli* by qPCR (received from USEPA, Office of Research and Development, Cincinnati, OH) were followed for the extraction, amplification, and quantification of enterococci and *E. coli* DNA respectively. The procedure is briefly described below.

a. DNA extraction

From each sample, 100 mL of water was filtered through 0.4µm pore size 47mm diameter polycarbonate filters (MilliporeSigma, Burlington, MA). Filters were folded and placed in a 2-mL extraction tube containing 0.3 g of acid-washed glass beads (Generite, LLC, North Brunswick, NJ). A total of 600 µL 0.2 mg/L single stranded salmon testes DNA (SSDNA) (Sigma-Aldrich, St. Louis, MO) was added to each extraction tube. Genomic DNA was extracted by bead beating for 60 seconds at 5000 rpm. Tubes were subsequently centrifuged at 12,000 × g for 1 minute. Supernatants were transferred to sterile 1.5 mL low-retention microcentrifuge tubes (Sarstedt, Inc., Newton, NC), which were centrifuged at 12,000 × g for 5 minutes. Genomic DNA (in the supernatant) was transferred to a sterile 1.5 mL low-retention microcentrifuge tube and analyzed immediately.

b. DNA amplification

Undiluted 5 µL of final genomic DNA extracts were added to 20 µL of reagents, in duplicate. The reagent mixture included 12.5 µL Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA), 3 µL forward and reverse primers and a TaqMan® probe (Integrated DNA Technologies, Coralville, IA), final concentration in reaction: 1 µM of each forward/reverse primer and 80 nM of probe, as presented in the Appendix (**Table XII**, Appendix A), 2 µL sterile H_2O and 2.5 µL of 2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), as described elsewhere (United States Environmental Protection

Agency, 2015). All reactions for *E. coli* were performed on the Applied Biosystems StepOnePlus Real-Time PCR platform (Applied Biosystems, Foster City, CA), while the reactions for enterococci were performed on the Applied Biosystems QuantStudio 3 Real-Time PCR system with amplification conditions specified in respective protocols (**Table XIII**, Appendix A).

4. Quantification of target DNA

The comparative cycle threshold (ΔΔCt) method as described in Method 1609.1 was used to calculate the ratio of target sequences in the samples and calibrators to generate calibrator cell equivalents (CCE) for both *E. coli* and enterococci. The results for the qPCR tests were reported in CCE per 100 mL water sample.

5. Data quality

Colilert® quality control procedures included analysis of duplicate samples and negative controls using sterile phosphate-buffered saline (PBS) analyzed once per week. Quality control for qPCR consisted of standard curves, replicate reactions for all samples, use of no template controls (NTC), method blanks (MeB), positive control calibrator samples, and SSDNA sample processing control (SPC). For the MeB, 30 mL of sterile PBS was filtered before the samples were filtered and the MeB filter was extracted in similar way as the sample and calibrator filters. Over the nine-week duration of the study, five standard curve assays were completed for *E. coli*. Each standard curve for *E. coli* included triplicate reactions of *E. coli* plasmid standards obtained from USEPA Office of Research and Development in estimated concentrations of 5.9, 11.78, 43.75, 234.96, 2404.36 and 22698.65 target sequence copies per 5 µL (TSC/5 µL). Ten standard curve assays were run for enterococci

during the 2017 summer beach season. Enterococci standard curves were constructed using triplicate reactions of enterococci plasmids in same concentrations as *E. coli* standards, but excluded the lowest concentration of 5.9 TSC/ 5 µL. Composite standard curves were generated and analyzed for both targets using linear regression (Sivaganesan, Haugland, Chern, & Shanks, 2010). Every qPCR 96-well plate contained two NTC reactions, two MeB replicates for enterococci, and six replicates of MeB for *E. coli*. Every plate also included replicate reactions for calibrator samples [known concentration spike of laboratory-cultured *E. faecalis* (ATCC® 29212™)] for enterococci and reconstituted *E. coli* Bioballs (BioMerieux #56146) for *E. coli,* as positive controls.

Substances found in beach water samples can cause qPCR amplification inhibition and/or poor DNA recovery. Moderate degrees of matrix interference can be corrected by using the SPC to adjust respective *E. coli* and enterococci qPCR cycle threshold (Ct) measurements on a sample basis. However, water sample results with a high degree of matrix interference (> 3Ct shift between SPC spikes in water samples and calibrator samples) were considered not usable (refer to Method 1609.1 for a complete description of method procedures).

6. Data analysis

Datasets were generated in Microsoft Excel 2010 and exported to SAS software for Windows, version 9.4 (SAS Institute, Cary, NC) for data analyses. Graphs were produced using SAS. Descriptive statistics were summarized as medians with the interquartile ranges for both enterococci (qPCR) and *E. coli* concentrations (culture and qPCR). Frequency of BAV exceedances were calculated for enterococci qPCR

and *E. coli* culture. The lower limit of standard curve quantification (LLOSCQ) for *E. coli* qPCR assay was calculated from the upper 95% bound of the composite standard curve generated from all individual Ct data points for each standard. The normality of distribution of culture and qPCR results were determined using Kolmogorov-Smirnov tests, using $p>0.05$ to indicate normality. Since neither *E. coli* nor enterococci results were normally distributed, data were $\log_{10}$ transformed and the log-transformed data approximated normality for overall results. Bivariate associations between continuous measures of culture and qPCR results were characterized by Pearson correlation coefficients for log-normally distributed data and Spearman correlation coefficient for beach-specific data that was not distributed normally or log-normally. Kruskal-Wallis ANOVA was used to determine if there were significant differences in concentrations (*E. coli* MPN, *E. coli* CCE and enterococci CCE) between beaches followed by Dunn's post-hoc pairwise multiple comparisons for determining which beaches were different from one another. All results and relationships were considered significant at alpha (α) <0.05.

The USEPA TSM recommends the calculation of an index of agreement (IA) and Pearson's correlation R-squared (R^2) for the association between paired measurements of a new and an established method. In our case, the new ("alternative") method (*E. coli* qPCR) was paired separately with the two established reference methods (*E. coli* culture and enterococci qPCR). Per the TSM, the alternative indicator is acceptable for establishing site-specific water quality criteria or BAV if the calculated IA is greater than 0.7 and/or R^2 is greater than 0.6 (U.S. Environmental Protection Agency, 2014). If the IA is greater than or equal to 0.7, the

new indicator BAV is the same numerical criteria value of the reference method BAV. If this was true in our comparison of *E. coli* qPCR to *E. coli* culture, the BAV of *E. coli* qPCR would be 235 CCE/ 100 mL derived from the 235 MPN/ 100 mL BAV of *E. coli* culture. However, if the IA is less than 0.7, but the calculated R-squared determines that the two methods are correlated (i.e., R^2 greater than 0.6), the new criteria or BAV is derived through linear regression.

We used ROC analysis to identify thresholds of *E. coli* qPCR concentrations that are predictive of exceedance of the enterococci qPCR BAV (1,000 CCE/100 mL) and *E. coli* culture BAV (235 MPN/ 100mL). Associated graphs were generated using the MedCalc® software (MedCalc Version 18 for Windows, MedCalc Software, Ostend, Belgium) to calculate the Area Under the Curve (AUC). An AUC of 1.0 indicates perfect sensitivity and specificity whereas an AUC of 0.5 suggests no discriminatory ability of the predictor (comparable to chance alone). The optimal *E. coli* CCE threshold value was defined as the point on the ROC curve with the highest true positive and lowest false positive rate for predicting a respective BAV exceedance.

C. Results

1. Data quality

a. Colilert® quality control results

All negative controls for Colilert® analyzed resulted in <1 MPN/ 100 mL after 22-24 hours of incubation (n = 9). The average coefficient of variation (CV) between replicate analyses of samples by Colilert was 16.9% (data not shown).

b. qPCR performance metrics

Accuracy of qPCR data was defined by the R^2 of standard curves. The accuracy of the standard curves for both targets was high, with an overall R^2 of 0.999 for *E. coli* and R^2 of 0.965 for enterococci. Slope and intercept information from the pooled five standard curves for *E. coli* and from pooled 10 enterococci standard curves are summarized in **Table XIV**, Appendix A**.**

c. qPCR precision

All together 72 sets of enterococci calibrator samples were analyzed throughout the study period. In the course of 36 days of *E. coli* monitoring, 36 sets of *E. coli* calibrator samples were analyzed (six replicates of one calibrator sample per day). We observed high precision, with very little variability of Ct values in measurement on different days' cells. The coefficient of variation (CV) for the *E. coli* and enterococci SPC and for both the *E. coli* and enterococci calibrator cells were low. Descriptive statistics for the calibrator samples and SPC for both *E. coli* and enterococci are summarized in **Table XV**, Appendix A.

2. qPCR lower limit of standard curve quantification (LLOSCQ)

Based on the LLOSCQ calculation, the calculated LLOSCQ for *E. coli* qPCR was a Ct value of 37.27, indicating that the method should be able to reliably measure concentrations below 5.90 TSC/ 5 µL. A method for calculating the lower limit of quantification is not specified in the enterococci qPCR method (Method 1609.1). The lowest concentration used in constructing the enterococci standard curve was 11.78 TSC/5 µL. The mean Ct (sd) for detecting 11.78 TSC/5 µL of *E. coli* and enterococci targets were similar: 34.5 (0.45) and 33.2 (0.56) respectively.

3. NTC and MeB contamination in *E. coli* qPCR and enterococci qPCR

The Ct values for the NTC and MeB for the *E. coli* qPCR runs were very similar with the average Ct of approximately 38 for the *E. coli* target (range 36- 40) as shown in **Table XVI,** Appendix A. Only 10 (13.89 %) out of 72 reactions of NTC Ct values for *E. coli* were below the LLOSCQ of 37.27. The number was much higher for MeB Ct values with 21.76 % (47 out of 216 reactions) below the LLOSCQ (**Table XVI,** Appendix A). This is consistent with the presence of extraneous *E. coli* DNA as a contaminant of the *Taq* polymerase found in the Environmental Master Mix. In comparison, the enterococci target was undetectable in 94% of the NTC (n = 144) and 91% of MeB (n = 144) during the 36 days of testing, with average Ct of 39.7 for both NTC and MeB (**Table XVI**, Appendix A).

4. qPCR inhibition

Three same samples (1.04%) out of the 288 samples analyzed exceeded the 3 Ct unit offset for SPC target for both *E. coli* and enterococci qPCR analyses. All three samples had relatively high turbidity (5.47 NTU-14.6NTU). For comparison, the mean (sd) turbidity on dates that no samples were inhibited was 3.73 NTU (5.63). All remaining samples had offsets <3 cycles for SPC target in both *E. coli* and enterococci qPCR assays. Thus, while Ct offsets can indicate either poor DNA recovery or amplification inhibition, the linkage with turbidity points to matrix interference due to qPCR inhibition.

5. Comparisons between *E. coli* culture, *E. coli* qPCR, and enterococci qPCR results

A total of 288 *E. coli* culture were generated with results ranging from 2 MPN/100 mL to >2419 MPN/100 mL with a median (interquartile range) of 59 MPN/ 100 mL (22-162 MPN/ 100 mL). As summarized in **Table I,** the median *E. coli* concentrations were highest at Montrose beach followed by Rainbow beach; the lowest median concentration was observed at Ohio Street beach. Kruskal-Wallis tests showed that the *E. coli* concentrations were significantly different between the 8 beaches tested ($p<0.0001$). Out of the total 288 samples, 52 samples (18.1%) exceeded the *E. coli* BAV of 235 MPN/ 100 mL, with considerable variability among beaches **(Table I**). The frequency of BAV exceedance was more common at the two beaches with the highest median *E. coli* concentrations (Montrose and Rainbow beaches), as well as North Avenue beach.

Similar to the culture results, the *E. coli* qPCR CCEs were significantly different between the beaches (Kruskal Wallis test, $p<0.0001$) with concentrations ranging from 9-39,431 CCE/ 100 mL. Out of the total 285 samples that did not have inhibition, 14 (4.9 %) had Ct values below the LLOSCQ (data not shown). Median *E. coli* qPCR concentrations was highest at Montrose beach followed by Rainbow and 63rd Street beach. Similar to *E. coli* culture results, Ohio Street beach had the lowest median *E. coli* concentration by qPCR.

Enterococci qPCR results from the eight beaches ranged from 3-47,484 CCE/ 100 mL. Median enterococci concentrations were lowest at Ohio Street beach and highest at South Shore and Montrose beaches. North Avenue beach, which frequently

exceeded the *E. coli* culture BAV, had the lowest frequency of exceeding the enterococci BAV of 1000 CCE/ 100 mL. Out of the total 285 samples that did not have inhibition, 52 samples (18.2%) exceeded the enterococci BAV of 1000 CCE/ 100 mL (**Table I**). Although the number of exceedances by *E. col*i culture BAV and enterococci qPCR BAV were similar (18%), the results were not always consistent among different beaches. Of the 52 samples that exceeded the enterococci qPCR BAV, only 25 (48%) also exceeded the *E. coli* culture BAV, while 208 (73%) samples that did not exceed the enterococci qPCR BAV also did not exceed the *E. coli* culture BAV.

6. <u>Association between qPCR and culture measures of water quality</u>

The associations between $\log_{10}$ transformed culture and qPCR results are presented in **Figure 1** along with Pearson's correlation coefficients (r). *E. coli* qPCR was most strongly associated with same-day Colilert® result ($r = 0.83$; $p<0.0001$). Moderately strong and statistically significant correlations were also observed between enterococci qPCR and *E. coli* qPCR ($r = 0.67$; $p<0.0001$) and between enterococci qPCR and same day culture results ($r = 0.67$; $p<0.0001$. Lag *E. coli* culture results (*E. coli* culture results from water samples collected, for example, on a Monday that became available on Tuesday) were not associated with the (Tuesday's) *E. coli* ($r = 0.12$; $p = 0.08$) or enterococci qPCR results ($r = 0.09$; $p = 0.17$). This indicates that *E. coli* culture results available to a beach manager on a given day (from samples cultured the prior day) is not predictive of current water quality. The relationship between qPCR and culture results varied by beach as shown in **Table XVII,** Appendix A.

7. Estimating potential *E. coli* qPCR BAV thresholds

a. Index of Agreement (IA) and R-squared (R^2)

Results for IA and R^2 were tabulated with paired measurements (all measurements above respective limit of quantification) using two different reference methods. The *E. coli* qPCR method met one of the acceptability criteria (IA = 0.81; n = 271), but failed the other (R^2 = 0.45; n = 271) using the enterococci qPCR method as a reference. In contrast, it met both acceptability criteria (IA = 0.70 and R^2 = 0.61; n = 262) while using the *E. coli* culture method as the reference. In accordance with USEPA guidance, the *E. coli* qPCR BAV threshold was assigned as 1000 CCE/100mL for the enterococci qPCR reference method and 235 CCE/100mL for the culture *E. coli* reference method [new indicator BAV = reference method BAV when IA > 0.7; (U.S. Environmental Protection Agency, 2014)].

b. ROC analysis

Potential *E. coli* qPCR BAV criteria were identified using ROC analysis. **Figure 2** shows ROC curves illustrating the relationship between sensitivity and specificity when comparing *E. coli* qPCR results with same day *E. coli* culture (Panel A), lag *E. coli* culture (Panel B), and enterococci qPCR (Panel C) BAV exceedances as predictor variables. The AUC values were over 0.8 and statistically significant ($p<0.001$) with *E. coli* culture and enterococci qPCR variables, but not with lag *E. coli* culture (AUC = 0.55; $p > 0.05$). **Table II** summarizes each *E. coli* qPCR BAV optimized criteria level to best predict *E. coli* culture BAV exceedance and enterococci qPCR BAV exceedance.

Beach-specific ROC analysis resulted in a range of criteria values from 84 CCE/ 100 mL to 1,102 CCE/ 100 mL as seen in **Table XVIII** and **Table XIX** resented in the Appendix A. The variability among beaches for location-specific BAVs was dependent upon the BAV reference value chosen as the reference value. If the same-day *E. coli* BAV exceedance is used as the reference value, the highest beach-specific *E. coli* qPCR BAV is approximately four times higher than the lowest beach-specific BAV. If the enterococci qPCR BAV exceedance was used as the reference values, the highest *E. coli* qPCR BAV is 10 times greater than the lowest beach-specific BAV. The sensitivity and specificity associated with potential criterion values of same-day culture *E. coli* BAV exceedance and qPCR enterococci BAV exceedance also varied widely between the eight beaches. **Table XVIII** in Appendix A demonstrates that *E. coli* qPCR threshold values for three of the beaches (North Avenue, South Shore and Calumet) can be developed that are relatively sensitive and specific for exceeding the same day *E. coli* culture BAV, with high AUCs. Similarly, **Table XIX,** Appendix A demonstrates that *E. coli* qPCR threshold values for same two of the three beaches (North Avenue and Calumet) can be developed that are highly sensitive and specific for exceeding the enterococci qPCR BAV, with high AUCs. However, the *E. coli* qPCR threshold criteria differed from one another depending on whether the *E. coli* culture or the enterococci qPCR BAV value was used as the 'reference value.'

c. Candidate *E. coli* qPCR BAV criteria thresholds

Four candidate *E. coli* qPCR BAVs (**Table III**) were generated using two analytical approaches (TSM and ROC). Two BAVs were used as references for each approach: that for the *E. coli* culture method (235 MPN/100mL) and for the enterococci qPCR method (1,000 CCE/100mL). The ability of the four candidate *E. coli* qPCR BAVs to correctly identify exceedance of established BAVs (*E. coli* culture and enterococci qPCR) were compared (**Table III**). Sensitivity ranged from 0.58 to 0.81 (**Table III**) indicating a failure to trigger beach advisories 19-42% of the time compared to the 'reference value' of *E. coli* qPCR BAV (1000 CCE/ 100 mL) criteria. No water sample exceeded the *E. coli* candidate BAVs when the enterococci qPCR results were below the enterococci qPCR BAV (perfect specificity).

D. Discussion

1. *E. coli* qPCR method performance

Several metrics were used to assess the performance of the *E. coli* qPCR method using DNA standards and Chicago area beach water samples. Standard curves demonstrated exceptional linearity ($R^2 = 0.999$) and amplification efficiency ($E = 1.00$), well within the performance criteria recommended by qPCR experts [$R^2 \geq 0.980$ and $E = 0.90$ to 1.10; (Bustin & Nolan, 2006; ThermoFisher Scientific, 2016)]. In addition, the *E. coli* qPCR method was able to routinely measure DNA standard concentrations under 10 copies per reaction suggesting that this procedure is capable of measuring low levels of DNA target in environmental samples. In addition, only 1% of recreational water samples (n = 3) showed evidence of matrix interference,

indicating that this method can routinely recover and amplify *E. coli* DNA targets in Chicago area beach waters. Interestingly, matrix interference only occurred in samples with above average turbidity, suggesting that particulates suspended in the water column may influence method performance. Controls designed to identify contamination detected extraneous DNA in 65.3% and 79.2 % of *E. coli* qPCR test reactions in the NTC (n = 72) and MeB (n = 216) respectively, but were consistently at low concentrations (**Table XVI**, Appendix A**).** As a result, *E. coli* qPCR estimate CCE concentrations could be inflated, however contamination levels were extremely low (maximum observed contamination = 55 CCE/ 100 mL) and had minimal impact on water sample concentration estimates. It is possible that contamination in *E. coli* qPCR experiments originates from the reagents employed in this method, likely the DNA polymerase found in the Environmental Master Mix. It is common for *Taq* DNA polymerase to be manufactured via *E. coli* cloning (Frahm & Obst, 2003; Silkie et al., 2008) and it is possible that trace quantities of *E. coli* DNA may be present leading to low levels of contamination in controls and water samples. The addition of a qPCR reagent decontamination step or use of reagents with increased purification quality controls (less bacterial DNA) might be useful to reduce false-positives in *E. coli* qPCR and to ensure more accurate concentration estimates, albeit it might increase the time and cost required to analyze samples.

2. *E. coli* qPCR and FIB paired measurements

Comparing paired measurements of a new alternative indicator methodology (*E. coli* qPCR) with established FIB procedures can reveal important information on the ability of the novel approach to identify fecal pollution. *E. coli* qPCR results were

strongly associated with same-day *E. coli* culture and enterococci qPCR findings across beach sites. A similar trend was reported in studies conducted by Lam et al., 2014 and Noble et al., 2010 based on paired measurements of culture and qPCR methods for quantifying *E. coli* in freshwater. Interestingly, correlations between enterococci qPCR and *E. coli* qPCR ($r = 0.67$) and between enterococci qPCR and same day *E. coli* culture ($r = 0.67$) were weaker than the correlation between *E. coli* qPCR and same day *E. coli* culture ($r = 0.83$), suggesting that the target microbe (enterococci or *E. coli*), rather than the measurement method (culture or qPCR) may have a stronger influence on the level of agreement between two water quality fecal indicator approaches. This observation could have important ramifications when selecting a reference method to establish new BAV criteria thresholds for an alternative indicator in the absence of an epidemiological study. Additional research is warranted to confirm this observation across a broader range of site locations.

3. Comparison of potential candidate BAV criteria using different statistical approaches

Using the IA approach yielded reasonable agreement between results of *E. coli* qPCR analyses and with both *E. coli* culture and the enterococci qPCR reference methods at Chicago beaches. However, the candidate BAV for the *E. coli* qPCR method derived using the TSM calculation with enterococci qPCR as the reference was 4.25 times the BAV threshold obtained from IA approach using *E. coli* culture as the reference method. Since both *E. coli* culture and enterococci qPCR methods are recommended by USEPA for monitoring freshwater beaches, one might assume that they indicate a comparable degree of health risk. Our study suggest that this would

not be the case at non-point impacted beaches in the Chicago area. It is not known whether there would be closer agreement among the candidate *E. coli* qPCR BAVs had this study been conducted at beaches impacted by wastewater discharge.

4. Relationships between *E. coli* qPCR and established FIB vary by beach site

Furthermore, results from our study also indicated that *E. coli* qPCR ROC-derived BAVs differed from one another depending on whether the *E. coli* culture or the enterococci qPCR BAV value was used as the reference value. In our study we found that using the *E. coli* qPCR BAV derived from the enterococci qPCR BAV exceedance as the reference, beach advisories would be twice as frequent as using the *E. coli* qPCR BAV derived using ROC analysis with *E. coli* culture BAV exceedances as the reference. Since we do not have epidemiologic data linked to the water quality results, it is not possible to determine whether enterococci qPCR or the *E. coli* culture should be considered the 'gold standard' predictor of health risk at these sites. It is interesting to note that the *E. coli* qPCR threshold value derived using the ROC method with to enterococci BAV as the anchor is about the same as the current culture BAV of 235 MPN/ 100 mL. However, threshold values from beach-specific analysis varied substantially from each other, as did the sensitivity and specificity for BAV exceedance. A single criterion value for posting beach advisories might not be optimal for monitoring at similar, nearby beaches. Whether this truly reflects site-specific differences in health risk is not known.

Although the number of exceedances of *E. coli* culture BAV and enterococci qPCR BAV were similar (18%), the results were not always consistent among different beaches. For example, North Avenue beach had total nine (25%) *E. coli*

culture BAV exceedances whereas only 3 (8.3%) enterococci qPCR exceedances. On the other hand, at Ohio Street beach *E. coli* culture BAV exceedance was less common, 1 (2.8%), than enterococci qPCR BAV exceedances, total of 4 (11.1%). This discordance between the exceedance of BAV using *E. coli* culture and enterococci qPCR method suggest that these two methods may not provide comparable degree of public health protection at non-point source impacted freshwater beaches. Currently, BAVs for different microbes (*E. coli* measured by culture, enterococci measured by culture, enterococci measured by qPCR) are considered to be interchangeable at freshwater beaches. Several prior studies have also reported different levels of agreement between *E. coli* culture and enterococci qPCR BAV exceedances (Francy, Bushon, et al., 2013; Haugland et al., 2014; Sheth, McDermott, Busse, & Kleinheinz, 2016) . Recent results from a 2015-2016 study at most of the same beaches as in our study had far more BAV exceedances by *E. coli* culture than by enterococci qPCR (Dorevitch et al., 2017). Our results suggest that the USEPA should re-evaluate the assumption of equivalence of these different methods at non-point source impacted freshwater beaches and potentially revise guidance to state and local beach managers and others who ensure compliance with their water quality standards using the provisions listed in the 2012 RWQC.

5. Implications for water quality management

This study has several implications for water quality management. States and local government might face challenges in their attempts to develop alternative BAV criteria thresholds. The statistical approaches that we used resulted in different *E. coli* qPCR potential BAV criteria thresholds ranging from 200 CCE/100 mL to 1000

CCE/100mL using two USEPA recommended FIB protocols (*E. coli* culture and enterococci qPCR) as reference methods. It is not apparent which *E. coli* qPCR BAV would provide the same degree of health protection as those established for *E. coli* culture or for enterococci qPCR (which themselves do not appear to result in comparable beach management decisions in our setting).

Our findings provide opportunities for policy makers to address the challenges of developing a site-specific criterion or BAV for alternative indicator/ method. We found that the choice of the reference method used in TSM calculations influences the BAV for the alternative indicator. For that reason, it would be useful to know if they should be considered comparable, or whether the most conservative (lowest) BAV should be used. ROC analysis – which optimized sensitivity and specificity, a dichotomous cut point rather than relying on the linear relationship between the two sets of continuous variables, provided different BAVs than the TSM method. Consideration should be given to use of the ROC method in the establishment of alternative criteria.

6. Strengths and limitations

Chicago's diversion of treated wastewater and stormwater away from Lake Michigan makes its beaches relatively unique. Thus, our findings may not be applicable in other settings. The research took place over a single season, and the analyses were conducted in a single laboratory. It is not known whether the observed *E. coli* qPCR associations with *E. coli* culture and with enterococci qPCR results would be different in different settings or in the same setting at a different point in time. A strength of this study is that it is the first to compare *E. coli* culture results

with rapid qPCR methods quantifying both *E. coli* and enterococci using the same water samples on a daily basis. We used multiple pieces of information including lag *E. coli* culture, same-day *E. coli* culture, and same-day enterococci qPCR to derive potential BAV criteria thresholds for the draft USEPA *E. coli* qPCR. The qPCR analyses were conducted in real-time, rather than after defrosting archived filters, as would be the case if the methods were to be used for routine beach monitoring.

TABLE I

RESULTS OF *E. COLI* QPCR, ENTEROCOCCI QPCR, AND *E. COLI* CULTURE, BY BEACH (N = 288)

Beach	***E. coli* qPCR**	**Enterococci qPCR**		***E. coli* culture**	
	CCE/ 100mL Median (LQ-UP)	**CCE/ 100mL Median (LQ-UQ)**	**BAV Exceedance N (%)**	**MPN/ 100mL Median (LQ-UQ)**	**BAV Exceedance N (%)**
Montrose	408 (172-1073)	420 (193-927)	9 (25.0)	113 (57-297)	10 (27.8)
North Avenue	182 (109-389)	201 (93-620)	3 (8.3)	40 (21-224)	9 (25.0)
Ohio Street	77 (46-274)	107 (62-310)	4 (11.1)	20 (12-33)	1 (2.8)
Rainbow	312 (124-997)	324 (86-744)	7 (20.0)	93 (31-239)	11 (30.6)
South Shore	227 (92-433)	509 (220-941)	7 (19.4)	55 (29-120)	6 (16.7)
57th Street	147 (53-583)	334 (140-679)	7 (19.4)	32 (21-147)	6 (16.7)
63rd Street	278 (112-617)	264 (98-751)	8 (23.3)	73 (31-128)	5 (13.9)
Calumet	192 (60-456)	208 (59-530)	7 (19.4)	30 (12-97)	4 (11.1)
All beaches	203 (87-521)	281 (98-742)	52 (18.2)	59 (22-162)	52 (18.1)
p-value*	0.0001	0.0021	--	<.0001	--

The lower quartile (LQ) and upper quartiles (UQ) are included in the parentheses.

*Kruskal-Wallis. Differences were significant between beaches.

[a] Abbreviations: CCE = Calibrator Cell Equivalents; BAV = Beach Action Value; MPN = Most Probable Number.

TABLE II

OPTIMAL CRITERIA AND AREAS UNDER THE CURVE (AUC) FOR *E. COLI* QPCR USING ROC ANALYSIS

	N	**Associated criterion (CCE/100 mL)**	**Sensitivity**	**Specificity**	**AUC**	**AUC Standard Error [a]**	**AUC 95% CI [b]**
Same day *E. coli* culture BAV exceedance	285	410.4	82.00	80.43	0.88	0.03	0.84 - 0.92
Lag *E. coli* culture BAV exceedance	215	70.7	90.62	22.40	0.55	0.06	0.48 - 0.62
Enterococci qPCR BAV exceedance	285	200.9	92.31	58.80	0.84	0.03	0.79 - 0.88

[a] DeLong et al., 1988.

[b] Binomial exact method.

[c] Abbreviations: CCE = Calibrator Cell Equivalents; AUC = Area under the curve; CI = Confidence Interval; BAV = Beach Action Value.

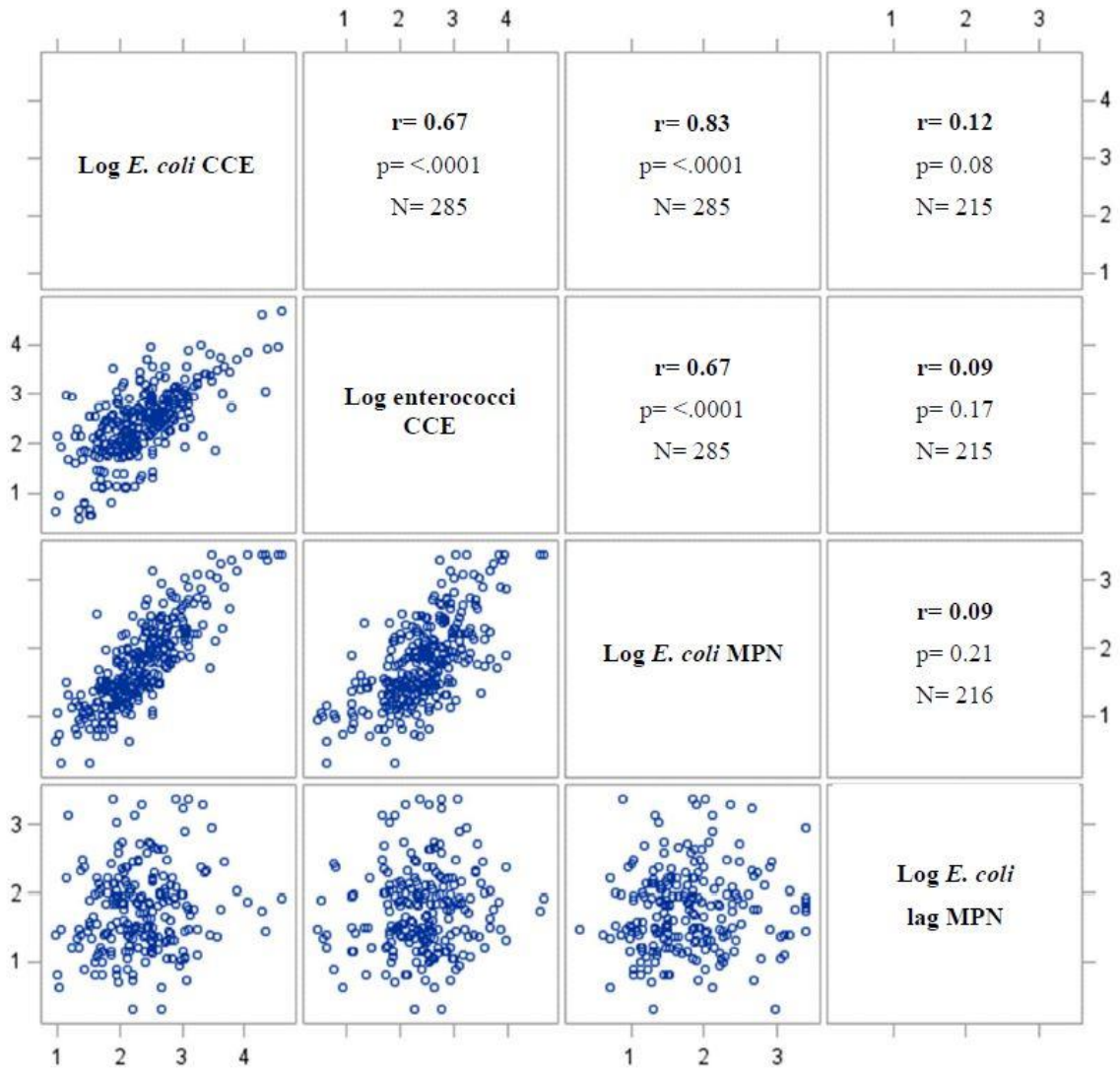

Figure 1: Scatterplot matrix with Pearson's correlation coefficients for all beaches combined
[a] Abbreviations: CCE = Calibrator Cell Equivalents; MPN = Most Probable Number.
[b] Units on the X-axis and Y-axis are either MPN/100 mL or CCE/100 mL on a log $_{10}$ scale.

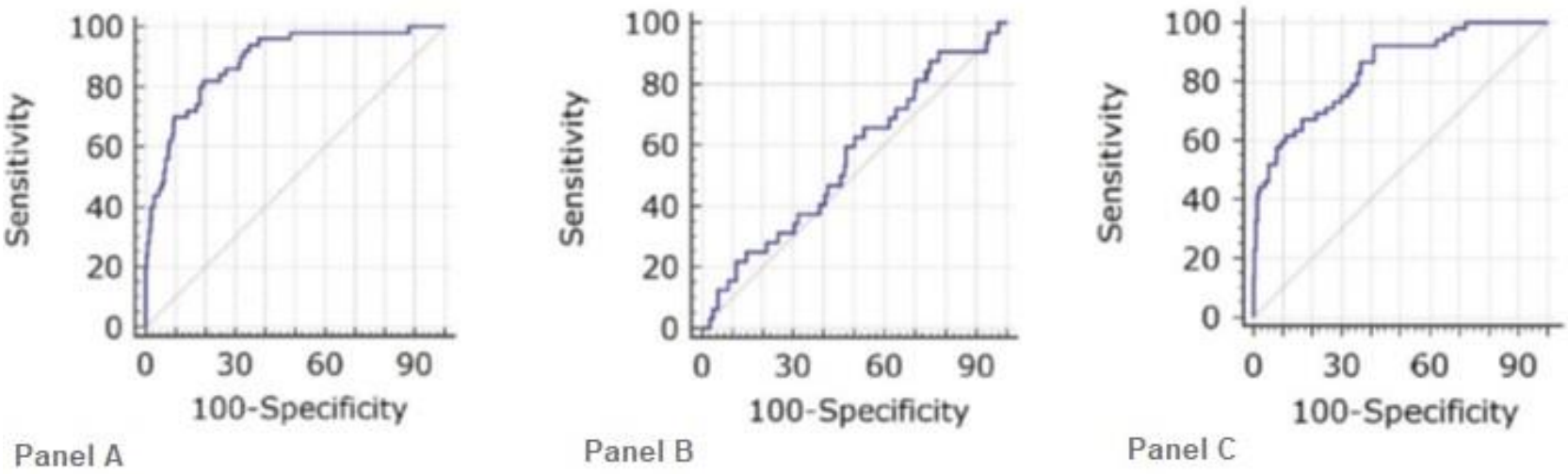

Figure 2: ROC curve for *E. coli* qPCR against (Panel A) Same day *E. coli* culture BAV exceedance (Panel B) Lag *E. coli* culture BAV exceedance (Panel C) Enterococci qPCR BAV exceedance

TABLE III

SENSITIVITY AND SPECIFICITY OF FOUR CANDIDATE *E. COLI* QPCR BAV VALUES

	Reference Value	**Analytic Approach**	**BAV Criterion (*E. coli* qPCR CCE/100mL)**	**Sensitivity**	**Specificity**
BAV_1	**Enterococci qPCR**	IA	1000	REF	REF
BAV_2		ROC	200.9	0.58	1.00
BAV_3	***E. coli* culture**	IA	235	0.62	1.00
BAV_4		ROC	410.4	0.81	1.00

[a] Abbreviations: BAV= Beach Action Value; CCE= Calibrator Cell Equivalents.

E. Conclusions

- The draft USEPA qPCR method targeting *E. coli* intended to be used for monitoring freshwater beaches demonstrated good technical performance.
- Based on the high frequencies of positive NTC and MeB in *E. coli* qPCR analysis, but not enterococci qPCR analysis, Environmental Master Mix contamination by *E. coli* DNA is likely present. However, the degree of contamination is low, and likely would have little impact on beach monitoring.
- Using TSM and ROC analyses, we were able to derive different candidate BAV threshold values for *E. coli* qPCR method using two USEPA recommended FIB culture and qPCR methods and corresponding health-based BAV criteria.
- The finding that each method and reference FIB measurement yielded different *E. coli* qPCR BAV highlights a need for further USEPA guidelines about setting site-specific alternative standards using alternative indicator and/ or methods to improve clarity and refine assumptions of comparability of BAVs of different USEPA-approved methods for quantifying indicator bacteria.
- Given the availability of a qPCR method (enterococci) developed by USEPA, along with criteria values and BAVs calibrated directly to observed health risk in epidemiological studies, there is little reason to attempt developing BAV criteria for other testing methods in the absence of clear information about how results of such water testing methods predict health risk.

III. FECAL NON-POINT POLLUTION SOURCE CHARACTERIZATION AT LAKE MICHIGAN BEACHES IN CHICAGO

A. Introduction

Recreational waterborne illness (RWI) is a significant public health problem. Recreational water activities such as swimming, boating, fishing has been established to result in more than 90 million illnesses per year in the United States with an estimated annual cost of $2.9 billion (DeFlorio-Barker, Wing, Jones, & Dorevitch, 2018). Prevention of RWI is a major goal of beach monitoring and public notification when general fecal indicator bacteria (FIB) exceed Beach Action Values (BAV) established by the US Environmental Protection Agency (USEPA). In the past two decades, it has become clear that high concentrations of FIB in water samples do not necessarily indicate that sewage contamination of beaches has occurred as was once thought. In fact, it is reported that naturalized populations of these general FIB used for monitoring beaches- enterococci for all waters and *E. coli* for freshwater beaches- can proliferate in beach wracks, submerged aquatic vegetation, sediments and sands of marine and freshwater beaches (Badgley, Nayak, & Harwood, 2010; M. Byappanahalli & Fujioka, 2004; M. N. Byappanahalli et al., 2006; Imamura et al., 2011; Nevers et al., 2016; Whitman & Nevers, 2003). Furthermore, fecal matter from non-human sources, such as dogs, birds, and other wildlife, also contain high levels of these bacteria (Wright et al., 2009). As a result, the presence and concentration of general FIB in beach water does not necessarily imply a sewage spill or wastewater infrastructure failure, because general FIB measurements cannot differentiate between sources of fecal pollution (human, bird, dog, wildlife, or agricultural animals). Studies have shown that fecal matter from multiple non-human

animals can equally contribute to high FIB levels in recreational waters (Converse et al., 2012; Ervin et al., 2014; Wang et al., 2010; Wright et al., 2009). While current beach-monitoring programs make use of only general FIB, developing pollution control initiatives and remediation strategies at large lakes may be beneficial and more effective if the animal (host) sources of the fecal pollution were known.

Beach water quality criteria and BAVs were developed from epidemiological studies that involved linking measures of general FIB at beaches to rates of occurrence of gastrointestinal illness among beachgoers. The largest and most recent of those studies – the National Environmental and Epidemiological Assessment of Recreational Water (NEEAR) - was conducted at several beaches that were selected in part because they were thought to be impacted by effluent from a nearby wastewater treatment plant (WWTP), a known "point source" of human fecal pollution. It is not known whether the observed associations between levels of general FIB and illness risk applies to beaches which are not impacted by such point sources of fecal contamination. Furthermore, relatively little is known about the presence and concentration of human fecal pollution at beaches that are thought to not be impacted by WWTPs. Likewise, the health risks of swimming in water contaminated by bird or dog fecal pollution has been described in observational studies, and the presence of human pathogens in the fecal matter of other animals is well established (Field & Samadpour, 2007; J. A. Soller, Schoen, et al., 2010). Because some pathogenic human viruses (such as norovirus) are present only in fecal matter of humans, human sources of fecal pollution may present a greater human health risk than fecal matter of other animals.

Microbial sources tracking (MST) methods have been used to identify sources of fecal waste in water samples. Many MST methods target bacterial or viral DNA sequences relatively unique to a particular animal host such as human, dog, ruminant, and bird (Bernhard & Field, 2000; Green, Dick, Gilpin, Samadpour, & Field, 2012; Green, Haugland, et al., 2014; Green, White, Kelty, & Shanks, 2014). MST methods for human, dog, bird, ruminant and other animal fecal waste characterization have been developed and tested in research settings (Harwood et al., 2014; Krentz, et al., 2013; Roslev & Bukh, 2011) and some have been applied to characterize fecal contamination sources at fresh and marine recreational waters around the United States (Harwood et al., 2014). These host-associated MST genetic markers may allow the development of more cost-effective and focused recreational beach water management and public health protection efforts, especially in locations where the likelihood of waste originating from known stormwater and sewage conveyances is minimal. Lake Michigan beaches in Chicago present a unique scenario where stormwater and wastewater flows are engineered to discharge in the nearby Chicago River diverting pollution away from the lake and its recreational sites. Nevertheless, BAVs are exceeded with some regularity at several of Chicago's Lake Michigan beaches (Dorevitch et al., 2017; Nevers & Whitman, 2011). Whether these exceedances reflect human fecal contamination - which would be unexpected given Chicago's wastewater management system – is unknown. MST testing would be an opportunity to answer that question.

Much of the application of MST methods to surface water research has been in investigations of high FIB levels at specific locations (Ervin et al., 2014; Heaney et al., 2015; Mika et al., 2014; Staley & Edge, 2016). In those focused investigations, some

studies found no association between the general FIB tested and the MST markers (Flood et al., 2011; McQuaig, Griffith, & Harwood, 2012; Mika, Ginsburg, Lee, Thulsiraj, & Jay, 2014; Santoro & Boehm, 2007; Shibata et al., 2010; Staley, Vogel, Robinson, & Edge, 2015), while other studies found that the detection of human MST markers was significantly associated with higher levels of enterococci measured using culture or quantitative polymerase chain reaction (qPCR) methods (Eichmiller et al., 2013; Gordon et al., 2013; Hughes et al., 2017). Conversely, Boehm et al. (2009) reported that the presence and concentration of the human-associated HF183 marker were inversely associated with the concentration of *E. coli* and enterococci in marine water. We are aware of no publications that describe MST monitoring as a supplement to routine monitoring of general FIB at beaches.

The objectives of our study were to 1) evaluate the presence and concentration of host-associated genetic markers for human (HF183/BacR287 and HumM2), dog (DG3 and DG37), and bird (GFD) fecal pollution sources at select Chicago beaches, 2) characterize association between MST results and general FIB measurements generated for beach monitoring and notification, and 3) explore whether MST testing results might complement the information obtained in routine beach monitoring.

B. Material and methods

1. Site description and water sample collection

Chicago has 26 miles of lakeshore with 27 public beaches that attract an estimated 20 million visits each summer (Chicago Park District; USEPA, 2011). During the summer of 2016, routine beach monitoring using enterococci qPCR was conducted five days a week (Wednesday- Sunday) at nine Chicago beaches

(**Figure 8**, Appendix B and **Table XX,** Appendix B). At the same times and locations of sampling for qPCR analyses, water samples were also collected for *E. coli* culture analyses (only during weekdays). Samples were transported to University of Illinois at Chicago Water Microbiology Research Laboratory (UIC) within approximately 1.5 hours of collection for immediate qPCR analysis as described in Dorevitch et al., 2017. In addition to the filters that were analyzed immediately for enterococci qPCR, additional filter sets from each beach water sample were archived at -80°C in sterile, pre-loaded glass bead tubes for future analyses.

2. *E. coli* culture (cEC)

E. coli culture analyses were performed by a commercial laboratory, STAT Analysis Corporation (STAT) laboratory (Chicago, IL) using the defined substrate test, Colilert® (IDEXX Laboratories, Westbrook, ME). The upper limit of quantification (uLOQ) for this method is 2,419 MPN/100 mL. Results above the uLOQ were assigned the value of 2,420 MPN/ 100 mL for analysis purposes.

3. Enterococci qPCR (qENT) analysis

The procedures outlined in Method 1609.1: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay (United States Environmental Protection Agency, 2015) were followed for the extraction, amplification, and quantification of enterococci DNA. Briefly, for genomic DNA extraction, bead beating with 0.3g of acid-washed glass beads (Generite, LLC, North Brunswick, NJ) was done. For amplification, Applied Biosystems™ TaqMan™ Environmental Master Mix

2.0 (Thermo Fisher Scientific, Waltham, MA) was used along with primers and TaqMan® probes (**Table II, Appendix B**) purchased from Integrated DNA Technologies (Coralville, IA). All reactions were performed in duplicates on the Applied Biosystems StepOnePlus Real-Time PCR platform (Applied Biosystems, Foster City, CA) with settings as shown in **Table III, Appendix B**. Following the calculations described in Method 1609.1, the comparative cycle threshold (ΔΔCt) method was used to calculate calibrator cell equivalents (CCE) for enterococci, which were reported as CCE /100 mL water sample.

Quality control for qENT consisted of replicate reactions for all samples, use of no template controls (NTC), method blanks (MeB), positive control calibrator samples, salmon DNA (SSDNA) sample processing control (SPC) and periodic standard curves runs. For the MeB, 30 mL of sterile PBS was filtered before the samples were filtered and the MeB filters were extracted in similar way as the samples. Each qPCR 96-well plate contained replicate reactions of NTC, MeB, and calibrator samples [known concentration spike of laboratory-cultured *E. faecalis* (ATCC® 29212™)], as positive controls set up daily with each sample run. A composite standard curve (n=9 independent instrument runs) using linear regression analysis (Sivaganesan et al., 2010) was generated from serially diluted, purified, RNA-free and spectrophotometrically quantified *E. faecalis* (ATCC® 29212™) genomic DNA in target sequence concentration (TSC). Each standard curve included triplicate analyzes of genomic enterococci DNA in the following range of concentrations: 3.6×10^4 TSC/5 µL, 3.6×10^3 TSC/5 µL, 3.6×10^2 TSC/5 µL, 36 TSC/5 µL, and 10 TSC/5 µL.

4. qPCR analysis for MST markers

A total of 195 samples were selected for MST testing based on general FIB levels (cEC and qENT). The goal of sample selection for MST analysis was not to characterize the frequency of MST marker presence at beaches, but rather to efficiently contrast the likelihood of marker presence at beach-days when general FIB was relatively high versus relatively low. For that reason, rather than analyzing a random sample of archived water samples (filters), we selected samples with a qENT CCE >320/100mL (which is 50% of the USEPA's BAV meant to limit recreational waterborne illness to 32 cases/1000 bathers). Because it is not known whether MST marker presence at Chicago beaches are more strongly associated with qENT or cEC measures, we also selected, for MST analysis, filters from beach-days in which cEC MPN>160/100mL (50% the 32 illnesses/1000 bathers BAV) though qENT was <320 CCE/100mL. The third category was composed of the filters from the beach-days that had both the lowest qENT and cEC values. The distribution of water quality categories among the 195 samples selected for analysis were 95 samples (48.7%) in the relatively high qENT category, 42 samples (21.5 %) were samples which were high cEC but low qENT, and 58 samples (29.7%) were the ones with low cEC and low qENT. Because no samples from two beaches (North Avenue and South Shore) exceeded the qENT or cEC BAVs, samples from those beaches were excluded from the MST analysis.

Two canine markers, DG3 and DG37 (Green et al., 2014), one general avian marker, GFD (Green et al., 2012), and two human-associated markers,

HF183/BacR287(Green et al., 2014) and HumM2 (Shanks et al., 2009), were used for this study. Genomic DNA extracted from the archived samples was used for the MST analysis using method described previously (Shanks et al., 2016). Briefly, all DNA extractions were performed using a DNA-EZ RW02 kit (Generite, LLC, North Brunswick, NJ) following the manufacturer's extraction protocol (Kelty et al., 2012). For all test filters including MeB controls, 600 µL of 0.2 µg/mL warm SSDNA diluted in AE buffer (Qiagen, Valencia, CA) was added to each bead tube prior to extraction (Haugland et al., 2010). Three set of MeB filters were prepared for each batch by filtering 100 mL of PCR-grade water. The genomic DNA extracts from the filters were stored at 4°C in 1.5 mL low-retention microcentrifuge tubes (Sarstedt, Inc., Newton, NC) until the time of analysis (<24 hours storage time).

Two microliters of purified genomic DNA extracted from the archived samples was added, in triplicate, to 23 µL of PCR master mix. The PCR master mix included 12.5 µL of ABI Environmental Master Mix 2.0, 3 µL forward and reverse primers and a TaqMan probe (except GFD), 2.5 µL of BSA and 5 µL of ultra-pure water. The human and dog markers used TaqMan chemistry while the bird marker used SYBR green. Moreover, the PCR master mix for HF183/BacR287 and HumM2 also included 2 µL of IAC plasmid in the PCR master mix as internal assay controls to test for amplification inhibition. MST analyses were performed at the USEPA's National Risk Management Research Laboratory (Cincinnati, OH) on the Applied Biosystems QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Information on the

primers, probes and thermal cycling settings for all MST markers are summarized in **Tables XXI** and **XXII**, Appendix B. Triplicate reactions of all MeB and six replicate reactions of NTC were included in every MST qPCR instrument run as routine quality control procedure.

The MST target concentrations were estimated using data acceptance metrics previously described in Shanks et al., 2016 with one minor modification to the SPC proficiency metric requirement. We used an SPC proficiency acceptance threshold ≤ 0.71 Ct standard deviation in the extraction blank measurement instead of $\leq .62$. The lower limit of quantification (LLOQ) threshold for each MST assay was defined as the upper bound of the 95% credible interval corresponding to the respective master standard curve model at 10 copies/reaction. Standard curves for the MST markers were generated from serially diluted plasmid standards (10^1, 10^2, 10^3, 10^4 and 10^5 copies/reaction). Data from six independent instrument run standard curves for each MST assay, every run consisting of triplicate reactions at each concentration, were pooled to generate a master standard curve using a Bayesian approach (Sivaganesan et al., 2008).

5. Reference sample analysis

In order to verify host-association of genetic markers in reference samples from the Chicago area, fecal and sewage samples were collected as previously described (Shanks et al., 2010). Animal fecal samples consisted of dog (N=21), gull (N=5) and goose (N=5). Primary effluent sewage sample from a local wastewater treatment plant (N=1) was used as a surrogate for human fecal

reference. Reference sample DNA extractions were performed using a DNA-EZ RW02 kit (Generite, LLC, North Brunswick, NJ) according to the manufacturer's instructions as previously described (Green et al., 2014; Kelty et al., 2012).

6. Data analysis

All data analyses were performed in SAS software for Windows, version 9.4 (SAS Institute, Cary, NC) unless noted otherwise. For each MST qPCR assay, sample results were categorized as follows: 1) non-detect (ND), 2) detect but non-quantifiable (DNQ), or 3) quantifiable (Q). A non-detect result was defined as two or more Ct values for a given assay and sample replicates combination were "undetermined". A DNQ occurred when two or more replicate reactions had Ct values above the respective assay LLOQ. Samples yielding replicate reactions with all Ct values ≥ LLOQ were categorized as Q. Quantifiable MST results were reported as $\log_{10}$ copies per 2 µL reaction (or 100 mL sample). Descriptive statistics of the MST results were summarized as percentage of ND, DNQ and Q for each MST marker tested. The normality of distribution of *E. coli* culture and enterococci qPCR results were determined by Kolmogorov-Smirnov tests, using $p>0.05$ to indicate normality. Since neither cEC nor qENT results were normally distributed, data were $\log_{10}$ transformed.

Two approaches were used to assess associations between MST markers and general FIB measurements. Firstly, logistic regression assessed the relationship between the detection/non-detection of the MST markers in selected sample filters with the general FIB concentrations (expressed as $\log_{10}$ *E. coli* MPN/100 mL and $\log_{10}$ enterococci CCE/ 100 mL). The magnitude of the effect

of a unit increase in the predictor (independent) variable (log_{10} general FIB concentrations) on the outcome (dependent) variable (presence or absence of an MST marker) was described by the odds ratio (OR). Additionally, agreement between exceedance of BAVs (cEC or qENT) and detection of MST markers were characterized using Cohen's Kappa statistic which accounts for the expected agreement due to chance alone. The Kappa statistic for agreement were interpreted as following: 0–0.20, none; 0.21–0.39, minimal; 0.40–0.59, weak; 0.60–0.79, moderate; 0.80–0.89, strong; ≥0.90, near perfect (McHugh, 2012).

Since most of the MST qPCR results included a large proportion of non-detects, a second approach using a recently reported qPCR censored-data method as described in Cao et al., 2018 was utilized to generate weighted-average fecal scores (log_{10} copies per 100 mL) for each MST marker. Briefly, prior to calculating the fecal scores for MST markers, the mean Ct for each sample (no amplification was set to 40 Ct) was classified into a ROQ group (if mean Ct ≤ LLOQ) or MPN group (if mean Ct > LLOQ). After classification of each sample into either the ROQ or MPN groups, overall fecal scores and fecal scores for different FIB categories was calculated using a most-probably number approach, by beach, by target. This was calculated as described in Cao et al., 2018 with the following modifications. We evaluated whether fecal scores (which utilizes all data, including non-detects) were different among ordinal categories of general FIB levels. The categories were defined as either FIB <10% percent of the BAV, 10% of the BAV ≤ FIB < BAV, and FIB ≥ BAV). Basically, the weighted-average fecal scores for the MST assays can be defined as an estimate of the level

of fecal contamination from a particular source (dog, bird or human) present at a given FIB category based on the average concentration of the source-specific gene observed in all the water samples in the study.

C. Results

1. Data quality

All quality control (QC) requirements for the USEPA Method 1609.1 were met or exceeded. The linearity of the standard curves for the enterococci target and all the five MST markers tested was high, with an overall R^2 above 0.991 for all the assays. Amplification efficiencies for MST assays ranged from 92.3% to 96.5% and LLOQ values ranged from 35.09 to 37.35 Ct based on repeated measures from six instrument runs. Calibration model performance parameter information from the pooled standard curves for individual assays are summarized in **Table XXIII,** Appendix B**.** Out of the 780 NTC and MeB total reactions, 99.5% were DNA-free (N = 4 false positives) suggesting minimal DNA contamination (**Table XXIV,** Appendix B).

A total of 71 sets of enterococci calibrator samples were analyzed throughout the study period. We observed high precision, with very little variability of Ct values in measurement on different days. The coefficients of variation (CV) for the SPC and for the enterococci calibrator cells were low (**Table XXV,** Appendix B). There was no amplification inhibition observed in any of the 195 archived filters DNA extracts tested over the study period. SPC threshold Ct for each of the assays is presented in **Table XXVI,** Appendix B.

2. Reference fecal sample results

A standardized concentration (1 ng/reaction) was tested for each sample and assay combination as previously reported (Kelty et al., 2012). Human-associated (HF183/BacR287 and HumM2) genetic markers were detected in all replicates of the sewage sample but were not detected in any dog or goose fecal samples. Bird-associated (GFD) genetic marker was detected in 50% of goose sample reactions, with no false positives observed in dog fecal or sewage samples. Poor DNA recovery from gull fecal samples prevented GFD performance with local reference samples, though previous studies report the presence of the GFD marker in 90% to 100% of gull fecal samples from the US Midwest region (Green et al., 2012). Dog-associated genetic markers were detected in 41.3% (DG3) to 76.2% (DG37) reactions, with no false positives observed in goose samples. Both DG3 (66.7%) and DG37 (33.3%) were detected in the primary effluent sample suggesting that the local sewage samples from Chicago may contain dog waste as a result of its combined sewer systems. Occurrence of dog markers in sewage samples from various other locations have been described previously (Green et al., 2014).

3. MST qPCR results and their associations with *E. coli* culture and enterococci qPCR

The MST marker detected most frequently was GFD (bird), in 40% of samples. Dog marker (DG3) was detected in 14% of samples, while human marker HF183/BacR287 was detected in 10% of samples (**Table IV**). Out of the 195 samples, host-associated genetic markers were in the quantifiable (Q) range

in 4% (n=8) of samples for HF183/BacR287, 1% (n=3) for HumM2, 6% (n=12) for DG3, 2% (n=4) for DG37 and 23% (n=45) of samples for GFD. Among the dog makers, DG3 was detected more frequently, and among the human markers, HF183/BacR287 was detected more frequently. Considerable variability in marker detection by beach was observed (**Table IV**). The human makers were detected at six of the seven beaches and they were detected and quantifiable on ten different sampling dates. MST marker detection patterns varied by beach. Some beaches showed concordant detection among markers (Montrose: relatively frequent detection of all markers), concordant non-detection of markers (Rainbow: no quantifiable dog or human markers and relatively infrequent bird marker in the quantifiable range). Other beaches showed discordance (Ohio Street: frequent DNQ and Q range bird and human markers, with no quantifiable DG3 marker; at 12th Street bird maker was frequently detectable or quantifiable but human markers were not). Dog markers were in the DNQ or Q range almost exclusively at one beach, Montrose, which is the only beach with a designated "bog beach" area, where dogs are allowed into the water. At that beach, mean concentrations of DG37 and DG3 among quantifiable samples ranged from 1.1 to 1.4 $\log_{10}$ copies per reaction and 1.3 to 2.7 $\log_{10}$ copies per reaction respectively.

The detection of the human markers, HF183/BacR287 and HumM2, and one of the dog markers, DG37, was not frequent enough to model by beach. Beach-specific logistic regression models demonstrated that general FIB concentrations (on a $\log_{10}$ scale) were predictive of the presence of avian (GFD) and dog (DG3) markers **(Table V**). Overall, the odds of detecting the GFD

marker increased with $\log_{10}$ concentrations of enterococci CCE (OR=2.3; 95% CI =1.5-3.4) and *E. coli* MPN (OR=2.2; 95% CI =1.5-3.3). The odds of detecting the DG3 marker also increased with $\log_{10}$ concentrations of enterococci CCE (OR=2.2; 95% CI =1.5-3.3). Beach-specific regression analysis showed that the relationships between general FIB and MST target detection varied by beach, though the associations were quite similar whether qENT or cEC was used to predict MST target detection (**Table V)**. After accounting for chance alone, the degree of agreement between detection of most MST markers and exceedance of general FIB BAV exceedances was "none" (Kappa <0.2) and "minimal" agreement between detection of DG3 and qENT BAV exceedance (Kappa=0.22) only.

4. <u>Beach-specific fecal scores and their comparisons at different FIB levels</u>

Overall average fecal scores with 95% Bayesian confidence interval (BCI) for each marker with beach-specific fecal scores based on general FIB concentrations are presented in **Table VI** and **Table VII**. While variability of fecal scores was relatively small among beaches, the mean scores differed substantially among targets, with much higher scores for GFD than the other MST targets. An exception to this is the very high fecal score for DG3 at the beach with an area for dogs to swim (Montrose).

Using a weighted-average fecal score approach we were able to compare whether the occurrence of different pollution sources differed at various general FIB levels. Again, the FIB level was considered high when results were at or above the BAV, medium when the results were lower than the BAV but higher

than 10 percent of the BAV and low when the general FIB levels were below 10 percent of the BAV. The weighted-average host-associated $\log_{10}$ copies per 100 mL sample concentrations for GFD (avian) was 8.4 times higher and DG3 (dog) was 4.2 times higher in samples that exceeded the enterococci qPCR BAV (1,000 CCE/100mL) compared to samples that were < 100 CCE/100mL as shown in **Figure 3**. A similar pattern was observed in samples that exceeded the *E. coli* culture-based BAV (235 MPN/100mL) compared to samples < 23.5 MPN/100mL for GFD (9 times higher) and DG3 (3.5 times higher) as seen in **Figure 4**. However, weighted-average $\log_{10}$ copies per 100 mL sample concentrations for GFD for the medium category for *E. coli* was significantly higher than the low and high *E. coli* groups. In contrast, both human-associated marker average concentrations were not significantly different, regardless of general FIB BAV sample groupings **Figure 3** and **Figure 4**.

D. Discussion

Identifying sources of fecal contamination in beaches is crucial for mitigation strategies in order to make meaningful beach management decisions to better protect health of the beachgoers and water recreators. In the first study of MST testing linked to routine beach monitoring, we found that two host-associated markers (dog, bird) were strongly associated with enterococci qPCR results generally, and specifically, associated with exceedance of USEPA's ambient water quality criteria recommendations for recreational waters or BAVs. The avian marker, GFD, was most frequently detected among the MST targets, and it was detected regularly at all beaches, consistent with visual observations reported in sanitary surveys that birds are present, and often in large

numbers, at local beaches (Chicago Park District, unpublished data). In contrast, human fecal markers, HF183/BacR287 and HumM2, were detected relatively infrequently. The more frequently detected of the two, HF183/BacR287, was detected in fewer than 10% of samples, and quantifiable in only 4% of samples, consistent with the understanding that Chicago's beaches are not impacted by wastewater due to the engineering of the local wastewater management system (local WWTPs do not discharge into Lake Michigan). While several prior publications describe associations between general FIB and host-associated markers, in those studies the number of samples and beaches were small, and testing was generally conducted as part of an investigation into unexplained high concentrations of general FIB. Unlike previous studies which found significant association between the detection of human MST markers in surface waters with higher enterococci levels measured using qPCR (Eichmiller et al., 2013; Gordon et al., 2013; Hughes et al., 2017), weighted-average human fecal score results from the present study suggests that human-associated marker average concentrations in our setting are low and did not vary when BAVs (either based on the enterococci qPCR or *E. coli* culture) were exceeded or not.

Several previous studies have found that dog fecal pollution can impact general FIB levels and water quality at recreational beaches (Ervin et al., 2014; Walker et al., 2015; Wang et al., 2010; Wright et al., 2009; Zhu et al., 2011). In our study the DG3 marker was only detected in 14% of all samples. The detection of the other dog marker, DG37, was even lower (4% of the samples). The dog marker, DG3, was quantifiable exclusively in samples from the one beach that has a section where dogs are allowed in the water (and on the beach). It is likely that the dog fecal contamination originated from

the dog beach. The overall low detection rate of the dog markers at other beaches suggest that the beachgoers generally adhere to posted signs and lifeguard communications about keeping dogs off of beaches during beach season (Memorial Day- Labor Day).

Recreational water quality criteria and BAVs were designed to limit the occurrence of acute gastrointestinal illness among bathers at beaches impacted by WWTPs. Our finding that human targets were rarely in the quantifiable range and generally not detectable suggests that those threshold values are likely conservative (more protective of public health) at Chicago beaches than in those at WWTP-impacted settings. However, 1) we did not measure health outcomes in our study, 2) fecal matter from other species can contain pathogens capable of infecting human, and 3) we do not know how frequently the human MST markers we studied would have been detected in the NEEAR epidemiologic from which criteria and BAV were derived. Thus, we would not recommend relaxing criteria or BAV definitions in our setting.

Because MST targets in beach water samples often include a large proportion of non-detects, the ‘weighted-average fecal score’ statistical censored-data approach for MST qPCR data (Cao et al., 2018) is useful. Until now there have been no real-world examples implementing this tool in a recreational water setting. Our study demonstrated how this novel fecal score approach, which makes use of data in the ND, DNQ, and Q ranges, can be applied to MST qPCR technologies to identify links between general FIB used for beach monitoring and host-associated MST markers at non-point source impacted recreational beach settings.

This study was an initial exploration of the role of MST in the context of routine beach monitoring and notification. We found that general FIB were predictive of bird

marker (GFD) and dog marker fecal marker presence. We did not observe such an association with the human markers, but the overall rate of detection of those markers was low, resulting in low statistical power to detect such an association.

To the best of our knowledge, this is the first study to evaluate the value of MST data in the interpretation of general FIB levels and BAV exceedance (though our MST analyses were not conducted in 'real time' while our cEC and qENT analyses were). On one hand, the MST results reflected findings apparent on visual inspection of beaches: birds are common at all locations and dogs swim at one of the beaches. While those results are consistent with expectations, the finding of discordant MST results at several beaches suggests a need for further exploration of pollution sources. Discordant findings of MST detection patterns across beaches might be due to differences in sources, hydrology, or marker persistence at different beaches. Likewise, while human target detection did not appear to cluster spatially or temporally, further investigation is warranted for the presence of local source of fecal pollution at those sites (pluming cross connections, children not toilet trained using the beach, dirty diapers on the sand, etc.). Moreover, after accounting for chance alone, we learnt that BAV exceedances (cEC or qENT) had no significant agreement with most of the MST markers detection. Since we do not know whether pathogen levels are higher when general FIB BAV is exceeded compared to when MST markers are more frequently detected or vice versa, additional research is necessary to establish which of the two, MST marker detection or BAV exceedance, would pose higher health risk to the beachgoers and water recreators at these beaches.

A strength of the present study is that it is the first study to utilize the novel weighted-average fecal score approach for characterizing the associations between measures of general FIB in the context of routine beach monitoring and different host-associated MST markers. Another strength of our study is that we examined several beaches with multiple days of monitoring per beach and we ensured a wide range of FIB results values by our sample selection procedure. However, findings from the present study are subject to several limitations. Chicago's engineered system of wastewater and stormwater drainage infrastructure to protect Lake Michigan from urban discharges makes its beaches relatively unique. Hence, findings from our study may not be generalizable to other settings. Our study also looked at general FIB and MST results from a single beach season. It is not known whether the observed associations would vary at different beach locations or in the same locations at a different point in time. Moreover, while cEC and qENT was done in real time, MST analysis was performed using archived filters stored at -80ºC so the results may not be comparable. Finally, the specific source tracking markers also have their own limitations (sensitivity and specificity), so if different MST molecular methods were used, findings of this study would likely have been different.

TABLE IV

RESULTS OF MST QPCR ANALYSIS, BY BEACH (N=195)

Beach	N	DG3			DG37			GFD			HF183/BacR287			HumM2		
		ND (%)	DNQ (%)	Q (%)	ND (%)	DNQ (%)	Q (%)	ND (%)	DNQ (%)	Q (%)	ND (%)	DNQ (%)	Q (%)	ND (%)	DNQ (%)	Q (%)
CL	29	100	0	0	100	0	0	59	7	34	83	10	7	93	7	0
MB	28	93	7	0	100	0	0	57	18	25	100	0	0	100	0	0
MN	28	21	36	43	78	11	11	53	18	29	78	11	11	89	7	4
OS	30	97	3	0	97	0	3	63	20	17	83	10	7	97	0	3
RB	27	96	4	0	100	0	0	74	15	11	96	4	0	100	0	0
ST	31	97	3	0	100	0	0	58	13	29	97	3	0	94	3	3
TS	22	100	0	0	100	0	0	50	36	14	95	0	5	100	0	0
All Sites	**195**	**86**	**8**	**6**	**96**	**2**	**2**	**60**	**17**	**23**	**90**	**6**	**4**	**96**	**3**	**1**

[a] Abbreviations: ND = Non-detect.; DNQ = Detect but not quantifiable; Q = Quantifiable.

TABLE V

LOGISTIC REGRESSION: ASSOCIATION BETWEEN MST PRESENCE AND LOG_{10} FIB CONCENTRATIONS

Beach	General FIB- ENT			General FIB- EC		
	N	GFD-OR (95 % CI)	DG3-OR (95 % CI)	N	GFD-OR (95 % CI)	DG3-OR (95 % CI)
CL	29	4.9* (1.1-22.9)	N/A	26	4.7* (1.0-20.8)	N/A
MB	28	1.2 (0.5-2.9)	3.0 (0.3-28.4)	24	2.6 (0.95-7.2)	2.4 (0.4-16.6)
MN	28	92.9* (2.5- >999)	2.4 (0.7-8.5)	22	4.8 (0.6-36.2)	0.4 (0.03-3.9)
OS	30	2.8* (1.2-6.7)	8.7 (0.1-866.4)	26	7.7* (1.5-40.4)	7.8 (0.4-159.6)
RB	27	1.3 (0.4-4.3)	0.2 (0.01-3.5)	26	1.3 (0.5-3.5)	N/A
ST	31	1.4 (0.5-4.3)	0.5 (0.03-8.6)	27	1.6 (0.4-6.5)	1.7 (0.03-86.9)
TS	22	2.7 (0.7-10.5)	N/A	19	2.7 (0.8-9.7)	N/A
All Sites	**195**	**2.3* (1.5-3.4)**	**2.3* (1.3-4.1)**	**170**	**2.2* (1.5-3.3)**	**1.98* (1.1-3.6)**

* Statistically significant ($p<0.05$).

[a] Abbreviations: FIB = Fecal Indicator Bacteria; ENT = Enterococci; EC = *E. coli*; OR = Odds Ratio; CI = Confidence Interval.

TABLE VI

BEACH-SPECIFIC ENTEROCOCCI BAV EXCEEDANCE AND WEIGHTED-AVERAGE FECAL SCORES WITH 95% BCI FOR MST ASSAYS (N=195)

Beach	N	BAV Exceedance N (%)	Weighted-Average Fecal Score in copies/ 100 mL (95 % BCI)				
			DG3	DG37	GFD	HF183/ BacR287	HumM2
CL	29	2 (6.9)	NA	NA	62.56 (37.61-92.16)	8.42 (4.08-14.30)	NA
MB	28	8 (28.6)	NA	NA	64.66 (42.52-89.13)	NA	NA
MN	28	9 (32.1)	326.4 (254.4-406.4)	15.43 (8.77-23.74)	68.99 (47.31-91.01)	15.34 (8.20-24.20)	5.67 (2.33-10.44)
OS	30	6 (20.0)	NA	NA	35.15 (22.97-49.48)	8.45 (4.10-14.34)	NA
RB	27	4 (14.8)	NA	NA	26.63 (16.64-38.85)	NA	NA
ST	31	7 (22.6)	NA	NA	66.07 (43.43-92.86)	NA	2.78 (0.79-5.98)
TS	22	1 (4.5)	NA	NA	42.52 (30.60-50.93)	NA	NA
All	**195**	**37 (19.0)**	**7.63 (5.79-9.70)**	**1.32 (0.71-2.10)**	**49.93 (42.67-57.71)**	**5.38 (3.98-7.00)**	**1.97 (1.22-2.90)**

[a] Abbreviations: BAV = Beach Action Value; BCI = Bayesian Confidence Interval.

Table VII

BEACH-SPECIFIC *E. COLI* BAV EXCEEDANCE AND WEIGHTED-AVERAGE FECAL SCORES WITH 95% BCI FOR MST ASSAYS (N=195)

Beach	N	BAV Exceedance N (%)	Weighted-Average Fecal Score in copies/ 100 mL (95 % BCI)				
			DG3	DG37	GFD	HF183/ BacR287	HumM2
CL	26	13 (50.0)	NA	NA	56.36 (31.65-86.61)	7.70 (3.31-13.81)	NA
MB	24	11 (45.8)	NA	NA	82.93 (53.41-115.40)	NA	NA
MN	22	16 (72.7)	327.6 (248.5-415.0)	17.61 (9.91-27.11)	57.36 (35.95-81.48)	15.95 (8.15-25.94)	7.74 (3.21-14.08)
OS	26	4 (15.40	NA	NA	35.39 (21.38-52.54)	9.31 (4.27-16.07)	NA
RB	26	13 (50.0)	NA	NA	23.54 (13.91-35.56)	NA	NA
ST	27	14 (51.8)	NA	NA	79.65 (52.47-110.10)	NA	3.29 (0.95-6.99)
TS	19	4 (21.0)	NA	NA	35.80 (24.62-43.52)	NA	NA
All	**170**	**75 (44.1)**	**7.27 (5.44-9.38)**	**1.40 (0.75-2.24)**	**48.46 (40.63-56.79)**	**5.48 (3.96-7.21)**	**2.30 (1.40-3.40)**

[a] Abbreviations: BAV = Beach Action Value; BC = Bayesian Confidence Interval.

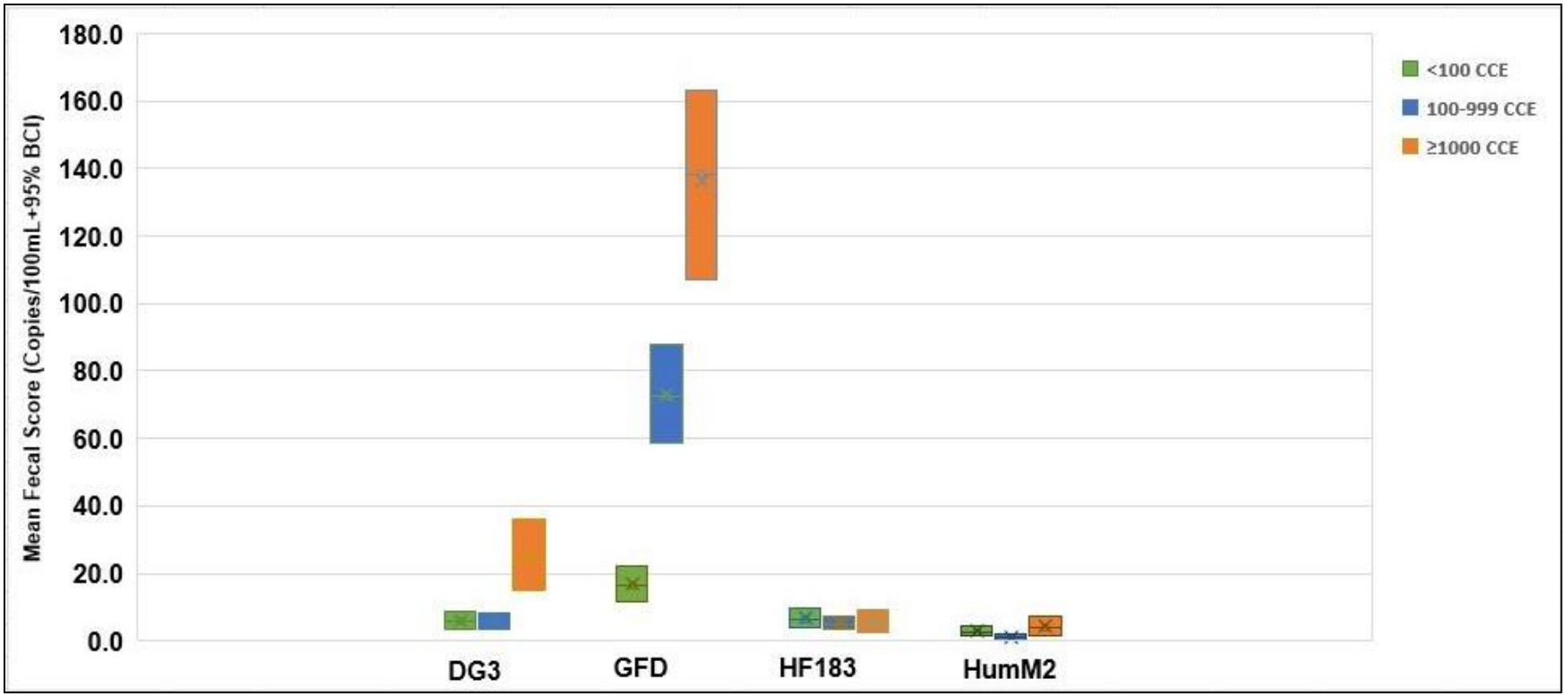

Figure 3: Weighted-average fecal scores for MST markers for low, medium and high enterococci CCE levels
[a] Abbreviations: CCE = Calibrator Cell Equivalent; BCI = Bayesian Confidence Interval.

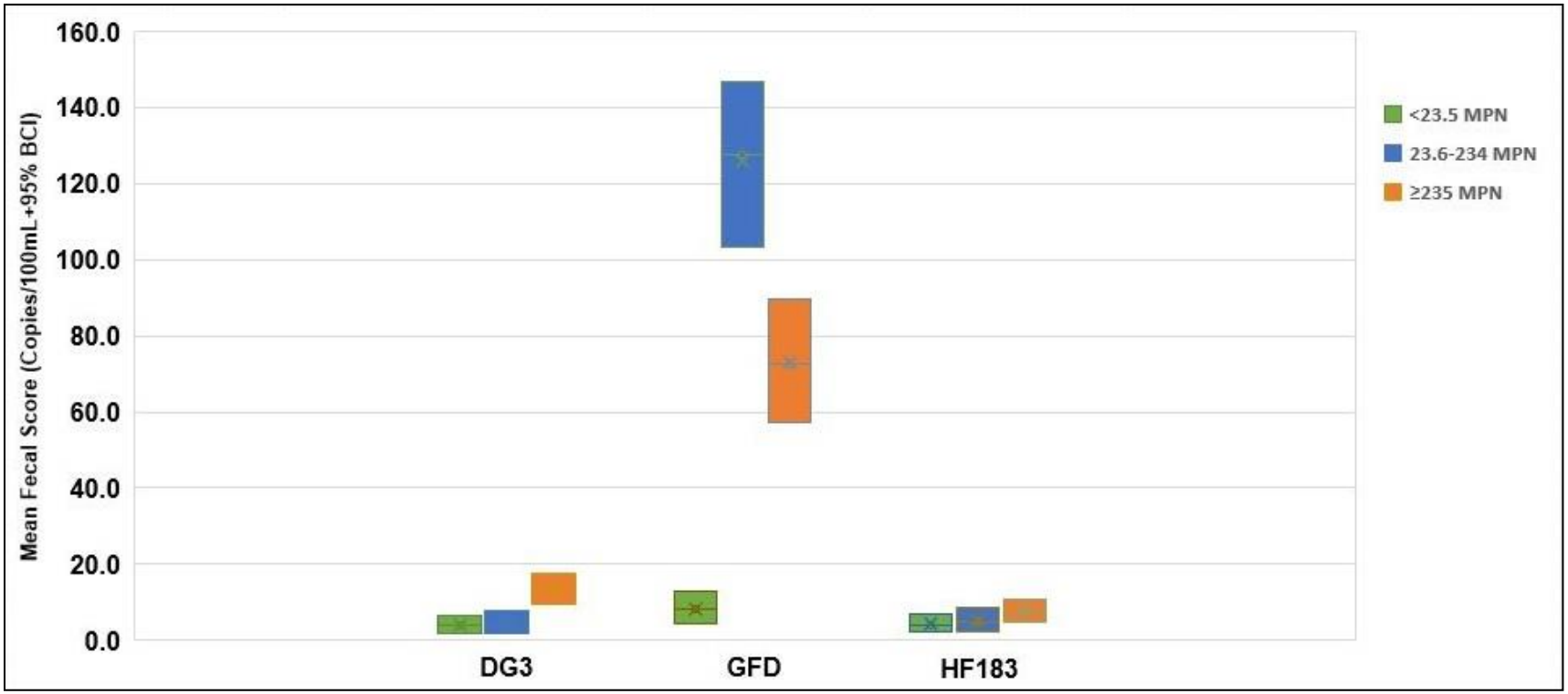

Figure 4: Weighted-average fecal scores for MST markers for low, medium and high *E. coli* MPN level
[a] Abbreviations: MPN = Most Probable Number; BCI = Bayesian Confidence Interval.

E. Conclusions

- Non-human fecal pollution sources including dogs and birds may influence recreational water quality at Chicago beaches. For that reason, corrective water management actions targeting canine and avian non-point fecal pollution sources may be helpful in improving water quality at these Chicago area Great Lake beaches.
- Infrequent detection and low concentrations of human fecal markers in the samples tested indicate that human waste generally does not contaminate Chicago beaches. Our findings suggest that Chicago's engineered system of wastewater and stormwater drainage infrastructure to protect Lake Michigan from urban discharges may be adequate to protect the health of the public at these beaches from human fecal contamination.
- Finally, simultaneous use of MST and general FIB used for routine beach monitoring and notification may be useful to supplement or confirm sanitary survey data for remediation and management of recreational waters to better protect public health.

IV. EFFECTS OF PRECIPITATION ON MICROBIAL WATER QUALITY AT CHICAGO BEACHES

A. Introduction

Recreational waterborne illness (RWI) is a significant public health problem. High concentrations of fecal indicator bacteria (FIB) in recreational water have been linked to increased cases of acute gastrointestinal illnesses among water recreators (DeFlorio-Barker et al., 2018; Wade et al., 2008). Environmental factors, particularly rainfall, have been known to influence the concentration and distribution of FIB in recreational surface waters. After heavy rainfall, concentrations of FIB typically have been found to increase at Lake Michigan beaches (Kleinheinz et al., 2009; Olyphant, Thomas, Whitman, & Harper, 2003). Although a strong positive correlation between precipitation and general FIB concentrations in surface waters have been repeatedly observed in surface freshwater for *E. coli* (Ackerman & Weisberg, 2003; Dwight et al., 2011; Kirs et al., 2017; Kleinheinz et al., 2009; McLellan et al., 2007; Nevers & Whitman, 2011), the correlation is not found as consistently for enterococci (Cordero, Norat, Mattei, & Nazario, 2012; Jennings, Chern, O 'donohue, Kellogg, & Boehm, 2018; Laureano-Rosario, Symonds, Rueda-Roa, Otis, & Muller-Karger, 2017; Z. R. Staley, Chase, Mitraki, Crisman, & Harwood, 2013) a study conducted by Sampson, et al. (2006) did not show any significant relationship between amount of rainfall and bacterial concentrations (*E. coli* and coliform) at 15 beaches along Lake Superior. In addition to impacts on bacteria concentrations, a multi-year study revealed that extreme precipitation occurring the previous day to be significantly associated with beach closures resulting from elevated *E. coli* levels at several Great Lake beaches (Bush et al., 2014).

Observed increase in bacterial concentrations could occur through multiple mechanisms, including but not limited to, wastewater discharge, stormwater or wastewater infrastructure failure, a combined sewer overflow event, or simply resuspension of bacteria already in the water due to the stirring up of sand and sediment by high waves (Kleinheinz et al., 2009; McLellan et al., 2007; Whitman et al., 2006). Given the general finding that precipitation is followed by elevated levels of *E.coli* concentrations, real-time statistical models of high FIB levels generally include precipitation as a predictor (Farnham & Lall, 2015; Jones et al., 2013; Nevers & Whitman, 2008; Safaie et al., 2016).

In Chicago, stormwater and treated wastewater discharge into the Chicago River, system which has been engineered to flow out of the Great Lakes watershed, and into the Mississippi River system. Therefore, under dry and most wet weather conditions, Chicago beaches should not be impacted by point sources of fecal contamination. However, several Chicago beaches often exceed the U.S. Environmental Protection Agency recommended Beach Action Values (BAV) (Dorevitch et al., 2017; Nevers & Whitman, 2011). While the effects of heavy rainfall on concentrations of general FIB measured using culture methods have been well explored, there is limited data available on the relationship between precipitation and water quality monitoring with respect to the general FIB enterococci qPCR method. A study by Santiago-Rodriguez et al. (2012) revealed that rainfall was associated with higher enterococci concentrations using both culture and qPCR methods in certain water bodies, though not at an inland lake. In another study conducted at several urban marine recreational waters, Jennings et al. (2018) found precipitation to also be a significant predictor for both enterococci culture

and qPCR concentrations. Few studies in the past have also investigated the influence of precipitation on exceedance of USEPA's recommended criteria for recreational water quality (Bush et al., 2014; Jennings et al., 2018; Z. R. Staley et al., 2013). However, these studies have been mostly limited to either marine beaches and/or sites affected by point sources of fecal contaminations. Thus, not much is known about the role of precipitation on exceedance of USEPA's water quality criterion using enterococci qPCR BAV at non-point source impacted beaches.

Current beach monitoring approaches using general FIB such as *E. coli* or enterococci do not discriminate whether the pollution is coming from animal sources such as dogs, birds, ruminants or from human sewage. It is important to be able to distinguish between these different sources of fecal pollution and learn about their fate and transport in order to make meaningful beach management decisions for better public health protection. Microbial sources tracking (MST) methods have been used to identify sources of fecal waste in recreational waters. These MST methods are host-associated; meaning they are capable of differentiating various sources of fecal pollution. By providing information relevant to pollution sources, these MST tools may help in the development of more cost-effective and focused recreational beach water management and public health protection efforts. Furthermore, recognizing how environmental factors such as precipitation affects the fate and transport of these host-associated markers in recreational waters could allow for more focused mitigation efforts directed towards that specific source and the pathway through which fecal pollution moves from sources to the near-shore waters. While a number of studies have assessed the association between precipitation and general FIB from specific sources of fecal pollution using MST

methods, especially human markers, results have been inconsistent and in some cases even contradictory (Haack et al., 2013; Z. R. Staley et al., 2013; Zachery R Staley et al., 2018).

The objectives of this study were 1) to assess the effect of rainfall on general FIB concentrations and BAV exceedance frequency at non-point source impacted Chicago beaches, 2) to examine associations between precipitation and MST markers at these beaches, and 3) to evaluate whether the observed associations vary by beach.

B. Material and methods

1. Study site description and water sample collection

Chicago has 26 miles of lakeshore with 27 public beaches that attract an estimated 20 million visits each summer (Chicago Park District; USEPA, 2011). During the summer of 2016, routine beach monitoring using enterococci qPCR was conducted five days a week (Wednesday- Sunday) at nine Chicago beaches (**Figure 9,** Appendix C and **Table XXVII,** Appendix C). At the same times and locations of sampling for qPCR analyses, water samples were also collected for *E. coli* culture analyses (only during weekdays). Samples were transported the samples to University of Illinois at Chicago School of Public Health Water Microbiology Research Laboratory (UIC) at within approximately 1.5 hours of collection for immediate qPCR analysis as described in Dorevitch et al., 2017. In addition to the filters that were analyzed immediately for enterococci, additional sets of filters from each beach water samples were archived at -80°C in sterile, pre-loaded glass bead tubes for future analyses.

2. *E. coli* culture (cEC)

E. coli culture analyses were performed by a commercial laboratory, STAT Analysis Corporation (STAT) laboratory (Chicago, IL) using the defined substrate test, Colilert® (IDEXX Laboratories, Westbrook, ME). The upper limit of quantification (uLOQ) for this method is 2,419 MPN/100 mL. Results above the uLOQ were assigned the value of 2,420 MPN/ 100 mL for analysis purposes. Out of the total 195 selected samples, only 170 samples had corresponding *E. coli* culture results since qPCR analysis was done Wednesday through Sunday while corresponding *E. coli* culture results were only available Wednesday-Friday.

3. Enterococci qPCR (qENT) analysis

The procedures outlined in Method 1609.1: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay (United States Environmental Protection Agency, 2015) were followed for the extraction, amplification, and quantification of enterococci DNA. Briefly, for genomic DNA extraction, bead beating with 0.3g of acid-washed glass beads (Generite, LLC, North Brunswick, NJ) was done. For amplification, Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA) was used along with primers and TaqMan® probes (**Table XXVIII,** Appendix C) purchased from Integrated DNA Technologies (Coralville, IA). All reactions were performed in duplicates on the Applied Biosystems StepOnePlus Real-Time PCR platform (Applied Biosystems, Foster City, CA) with settings as shown in **Table XXIX,** Appendix C. Following the calculations described in Method 1609.1, the comparative cycle threshold

(ΔΔCt) method was used to calculate calibrator cell equivalents (CCE) for enterococci, which were reported as CCE /100 mL water sample.

4. qPCR analysis for MST markers

A total of 195 samples were selected for MST testing based on general FIB levels (cEC and qENT). Two canine markers, DG3 and DG37 (Green et al., 2014), one general avian marker, GFD (Green et al., 2012), and two human-associated markers, HF183/BacR287(Green et al., 2014) and HumM2 (Shanks et al., 2009), were used for this study. Genomic DNA extracted from the archived samples was used for the MST analysis using method described previously (Shanks et al., 2016). Briefly, all DNA extractions were performed using a DNA-EZ RW02 kit (Generite, LLC, North Brunswick, NJ) following the manufacturer's extraction protocol (Kelty et al., 2012). For all test filters 600 µL of 0.2 µg/mL warm SSDNA diluted in AE buffer (Qiagen, Valencia, CA) was added to each bead tube prior to extraction (Haugland et al., 2010). The genomic DNA extracts from the filters were stored at 4°C in 1.5 mL low-retention microcentrifuge tubes (Sarstedt, Inc., Newton, NC) until the time of analysis (<24 hours storage time).

Two microliters of purified genomic DNA extracted from the archived samples was added, in triplicate, to 23 µL of PCR master mix. The PCR master mix included 12.5 µL of ABI Environmental Master Mix 2.0, 3 µL forward and reverse primers and a TaqMan probe (except GFD), 2.5 µL of BSA and 5 µL of ultra-pure water. The human and dog markers used TaqMan chemistry while the bird marker used a SYBR green assay. Moreover, the PCR master mix for the

human markers, HF183/BacR287 and HumM2, also included 2 µL of IAC plasmid in the PCR master mix as internal assay control to test for qPCR inhibition. MST analyses were performed at the USEPA's National Risk Management Research Laboratory (Cincinnati, OH) on the Applied Biosystems QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Information on the primers, probes and thermocycler settings for all the MST markers are summarized in **Tables XXVIII** and **XXIX** in Appendix C.

The MST target concentrations were estimated using the methods previously described in Shanks et al., 2016. Results of sample analysis for each MST target were categorized as being either, 1) non-detect (ND), 2) detect but non-quantifiable (DNQ), or 3) quantifiable (Q). Each MST marker was considered detected or present if two out of the three replicate reactions of the sample had a Ct below 40. Samples with two or more replicate reactions with Ct=40 ("Undetermined") were characterized as ND. A detected sample was considered DNQ if all of its replicate reactions had Ct above (meaning, concentrations below) the LLOQ. Detected samples with all three of its replicate reactions with Ct at or below the LLOQ were categorized as Q. Quantifiable MST results were reported as $\log_{10}$ copies per 2 µL reaction (or 100 mL sample).

5. <u>Precipitation Data</u>

Precipitation data for all the beaches were downloaded from the National Weather Service (Midway Airport, Station 14819). Based on the hourly rainfall data, the total precipitation amount (mm) during the 24 hours preceding sample collection was calculated, assuming each sample was taken at 8:00 a.m. every

day. For assessing the effect of precipitation on the general FIB concentrations, samples were categorized as wet and dry weather samples. If there was two or more millimeter of rainfall within the last 24-hours of sample collection, it was classified as "wet" weather. Rainfall less than 2 mm in preceding 24 hours of sample collection was categorized as "dry" weather. We examined cumulative rainfall cutoffs 24-hour prior to time water samples were collected.

6. Statistical analysis

Datasets were generated in Microsoft Excel 2010 and exported to SAS software for Windows, version 9.4 (SAS Institute, Cary, NC) for analyses. All results and relationships were considered statistically significant at alpha (α) <0.05. The Shapiro-Wilks test was used to determine the normality of the distributions of *E. coli* culture and enterococci qPCR results, using $p>0.05$ to indicate normality. Since neither cEC nor qENT results were found to be normally distributed, Mann-Whitney U tests was performed to determine if there were significant differences in the ranked general FIB concentrations (*E. coli* MPN and enterococci CCE) under different weather conditions. Chi-square analysis, stratified by dry and wet weather, was used to examine how general FIB BAV exceedance and non-exceedance varied during different weather conditions at Chicago beaches. Additionally, sensitivity analysis, using various cutoffs of rainfall totals in the 24-hour period prior to sample collections, was performed to characterize the effects of different amounts of precipitation on the exceedance of general FIB BAVs.

Two separate approaches were used to examine the relationship between MST markers and weather conditions. Chi-square tests were performed to assess the relationship between the detection/non-detection of the different MST markers in samples collected under dry and wet weather conditions; these associations were described by odds ratios (OR). Since most of the MST qPCR results included a large proportion of non-detects, a second approach using a recently reported qPCR censored-data method as described in Cao et al., 2018 was utilized to generate weighted-average fecal scores (log_{10} copies per 100 mL) for each MST markers. Briefly, prior to calculating the fecal scores for MST markers, the mean Ct for each sample (no amplification was set to 40 Ct) was classified into a ROQ group (if mean Ct ≤ LLOQ) or MPN group (if mean Ct > LLOQ). After classification of each sample into either the ROQ or MPN groups, fecal scores for overall beaches were determined using a most-probably number approach, using information about the frequency of N, DNQ and Q categories of results, by target. This was calculated as described in Cao et al., 2018 with the following modifications. We evaluated whether fecal scores for each MST markers were different under dry vs. wet weather conditions if they met the following selection criteria. Each marker had ten or more samples in the ROQ and MPN categories combined with at least one data point in each of these categories and each weather conditions. Essentially, the host-associated weighted-average fecal scores can be described as an estimate of the level of fecal contamination from a particular source (dog, bird or human) present at a given weather condition (wet or dry)

based on the average concentration of the source-specific gene observed in all the water samples in the study.

C. Results

1. Association between precipitation and general FIB

Of the total 195 samples, 97 (49.7%) were dry weather samples while 98 (50.3%) were wet weather samples. On wet weather days, the mean (range) of precipitation was 25.4 mm (2.5 mm-65.0 mm). Overall (all beaches combined), the Mann-Whitney U tests showed that the ranked enterococci qPCR concentrations were not significantly different between dry and wet weather conditions (p=0.42) (**Figure 5** and **Figure 6**), while ranked *E. coli* concentrations were significantly higher during wet weather days than dry weather days (p=0.004). Differences in ranked *E. coli* concentrations at individual beaches between wet and dry weather were statistically significant only at 63rd Street beach (p=0.03).

While only 19% (N=37) of the samples exceeded the qENT BAV, 44% (N=75) of the samples exceeded the cEC BAV (**Table XXX** and **Table XXXI** in Appendix C). No association was observed between wet weather and ENT qPCR BAV exceedance (OR = 1.38, 95% CI: 0.67, 2.84), but wet weather was associated significantly with an increased cEC BAV exceedance (OR= 2.34, 95% CI: 1.26, 4.36) (**Table VIII**). While beach-specific analysis showed elevated point estimates of the odds of BAV exceedance, none reached statistical significance (**Table VIII)**. These observations were not sensitive to rainfall thresholds (≥2mm

vs. <2mm, ≥5mm vs. <5 mm, ≥5mm vs. 0 mm) in the 24 hours preceding sample collection (**Table IX**).

2. <u>Precipitation's association with MST markers</u>

The detection of MST markers varied with precipitation (**Table IX**). For all five MST targets the point estimate of the odds of detecting the MST markers were higher following wet weather (vs. dry weather), though none of these associations reached statistical significance. The concentration estimates (generated from the /weighted-average fecal score approach) of host-specific fecal markers following dry and wet weather differed between dry and wet weather conditions (**Table X**). Fecal scores for one of the human markers, HumM2, could not be calculated for the entire dataset as HumM2 was not detected under wet weather conditions. Specifically, host-associated $\log_{10}$ copies per 100 mL sample concentrations for DG3 (dog) were 2.4 times higher, DG37 (dog) were 2.1 times and GFD (bird) were 1.6 times higher in wet weather samples compared to dry weather samples (**Table X**). Conversely, overall HF183/BacR287 concentrations were slightly higher in samples during dry weather than wet weather, though this marker was detected much less frequently than the dog or bird markers.

Furthermore, beach-specific mean fecal scores for the MST markers showed very high fecal score for DG3 at the beach with an area for dogs to swim (Montrose), with concentrations 1.9 times higher in wet weather than dry weather. The beach-specific GFD values also varied among the different beaches with wet weather sample concentrations ranging from 3.8 to 1.2 times higher during wet weather when compared to dry weather with 63rd Street samples showing inverse

pattern (**Table X).** The two beaches that had sufficient data for beach-specific HF183/BacR287 fecal scores (Montrose and Ohio Street) suggest very different associations with precipitation. HF183/BacR287 concentrations in wet weather samples from Montrose was 4 times higher than in dry weather samples, with little overlap in the Bayesian confidence interval (BCIs). On the contrary, HF183/BacR287 concentrations in samples from Ohio Street were comparable in wet and dry weather conditions.

D. Discussion

We found that recent precipitation was strongly associated with exceedance of the *E. coli* (measured by culture) BAV and with cEC values (MPN/100mL) but not with metrics of qENT. We found suggestions that host-specific targets (two dog MST markers, two human MST markers, and a bird marker) tended to increase following precipitation as well. This suggests that in our setting, which does not have stormwater or wastewater point sources, surface flow in or after rain events conveys fecal pollution and contributes to BAV exceedance and to a great likelihood of fecal pollution from specific sources.

Our finding of a significant positive association between precipitation and *E. coli* concentrations and a higher likelihood of *E. coli* culture BAV exceedance following recent rain events is consistent with the current literature (Ackerman & Weisberg, 2003; Bush et al., 2014; Dwight et al., 2011; Kirs et al., 2017; Kleinheinz et al., 2009; McLellan et al., 2007). However, unlike one previous study that found significant association between rainfall and enterococci levels measured using qPCR (Jennings et al., 2018), our results revealed that recent rainfall events had no significant effect on the concentrations of enterococci (CCE/100mL) or prevalence of qENT BAV exceedances in our setting.

Selected beaches in many states in the US, including those in the Great Lakes region, have quantitative rainfall standards for issuing pre-emptive beach advisories and/or closures following heavy precipitation (Dorfman & Haren, 2013). Our data indicated that qPCR concentrations were not significantly different between dry and wet weather conditions and that ENT qPCR BAV exceedances were not necessarily triggered after rain events. Because Chicago beaches have been monitored exclusively with qPCR and not cEC since 2017, rainfall data alone would not be sufficient to pre-emptively trigger beach advisories at these Great Lake beaches.

Two pieces of information may be useful in reducing microbial measures of water quality at beaches: identification of pollutant sources and identification of pathways through which fecal pollution moves from sources to the near-shore waters. Results from the MST analysis suggest that dog and bird fecal matter might flow into beach water following rainfall. This information may allow local beach managers to develop targeted mitigation efforts that would interrupt the flow of fecal pollution to the nearshore areas of beaches at which water quality is most sensitive to effects of precipitation. Using a weighted-average fecal score approach, we observed that the dog marker concentrations almost doubled in wet weather compared to dry weather at one particular beach. Since this is the only beach with a designated "dog beach" area, where dogs are allowed in the beach and into the water, developing pollution prevention actions targeting better management of dog fecal pollution and its pathways following rain events should help in improving beach water quality at that beach. Moreover, recognizing beaches that are negatively impacted after heavy rainfall could also help in the identification of sources (e.g., impervious surfaces like paved streets, parking lots, illegal sewer connections,

malfunctioning sewage disposal systems, etc.) that could contribute to increasing bacteria level in surface water using detailed sanitary surveys at these beaches. As a result, appropriate remediation plans can be implemented for better health protection of beachgoers and communities adjacent to beaches.

Human markers were detected in much lesser frequency in our setting compared to previous studies; a study by Jennings et al. (2018) conducted at highly urban point source impacted marine beaches in northern California with detection frequency of 77% and a study by Cao et al. (2017) at various coastal drainages in southern California, in which HF183 detection frequencies were more than 2-times in wet weather. These substantial differences in detection frequencies of the human sewage marker in either weather conditions may be due to the engineering of the local wastewater management system in Chicago where local wastewater treatment plants do not discharge into Lake Michigan, protecting the lake from point source of human fecal pollution.

A strength of our study is that several beaches were evaluated with multiple sampling days per beach, as well as a relatively large number of "wet days." Likewise, the use of a suite of MST targets that were analyzed, and the use of two different general FIB provided an opportunity to evaluate differences in precipitation effects among the various microbial targets. There are number of limitations relating to the data and interpretations of results within the present study. First and perhaps most importantly, since this study was conducted in non-point source impacted beaches in Chicago, the results may not be generalizable to beaches impacted by point-source pollution. Since this research used data from only a single beach season, it is not known whether the observed associations of precipitation with *E. coli* culture, enterococci qPCR and MST

results would be different in other settings or in the same setting at a different point in time. Our study was also limited to small sample size and the observed associations might have been significant with more data points. The rainfall data were obtained from a single National Weather Service station approximately 15 km from the nearest beach might not be representative of the rainfall at each specific beach, biasing associations between precipitation and water quality toward the null. Moreover, when calculating the precipitation period of 24-hour period, we assumed that each sample was taken exactly at the same time for all the beaches, which was not the case and may not accurately represent the relationship calculated. The definition used for wet and dry weather was also arbitrary. Finally, rainfall might not be the sole factor for determining the microbial presence or concentration at these beaches. Overall, other environmental and non-environmental factors, not assessed in this study, could be potentially influencing the microbial level in the beach water at the tested locations.

TABLE VIII

CHI-SQUARE ANALYSIS RESULTS OF GENERAL FIB BAV EXCEEDANCES AND DIFFERENT RAINFALL THRESHOLDS

Beach	OR (95% CI)- ENT BAV Exceedance			OR (95% CI)- EC BAV Exceedance		
	Scenario 1	Scenario 2	Scenario 3	Scenario 1	Scenario 2	Scenario 3
CL	0.93 (0.05, 16.42)	1.25 (0.07, 22.13)	1.00 (0.06, 17.90)	3.60 (0.71, 18.25)	7.50* (1.31, 43.03)	5.25 (0.87, 31.55)
MB	0.82 (0.16, 4.23)	0.33 (0.05, 2.07)	0.45 (0.07, 3.07)	1.50 (0.29, 7.75)	1.92 (0.38, 9.80)	1.80 (0.32, 10.21)
MN	3.15 (0.52, 19.27)	2.22 (0.43, 11.60)	3.00 (0.47, 19.04)	2.20 (0.32, 14.97)	1.29 (0.20, 8.43)	1.80 (0.26, 12.5)
OS	0.59 (0.09, 3.86)	0.33 (0.03, 3.33)	0.36 (0.03, 3.79)	4.33 (0.39, 48.61)	2.14 (0.25, 18.50)	3.71 (0.28, 48.55)
RB	1.87 (0.22, 15.93)	3.60 (0.40, 32.37)	3.00 (0.33, 27.23)	3.89 (0.72, 21.06)	3.44 (0.53, 22.43)	4.17 (0.61, 28.62)
ST	1.87 (0.34, 10.25)	2.67 (0.48, 14.90)	2.17 (0.38, 12.30)	4.05 (0.81, 20.20)	4.44 (0.84, 23.58)	6.00* (1.02, 35.37)
TS	0.62 (0.44, 0.87)	2.28 (0.08, 62.43)	2.04 (0.07, 56.26)	0.50 (0.05, 4.67)	0.67 (0.07, 6.11)	0.56 (0.06, 5.24)
All sites	**1.38 (0.67, 2.84)**	**1.22 (0.59, 2.50)**	**1.29 (0.61, 2.75)**	**2.34* (1.26, 4.36)**	**2.37* (1.27, 4.43)**	**2.61* (1.35, 5.05)**

* Statistically significant ($p<0.05$).

[a] Abbreviations: OR = Odds Ratio; CI = Confidence Interval; ENT = Enterococci; BAV = Beach Action Value; EC = *E. coli.*

[b] Scenario 1: ≥2mm vs. <2mm rainfall in prior 24-hour; Scenario 2: ≥5mm vs. <5 mm rainfall in prior 24-hour; Scenario 3: ≥5mm vs. 0 mm in prior 24-hour.

TABLE IX

DETECTION OF DIFFERENT MST MARKERS UNDER DIFFERENT WEATHER CONDITIONS (N=195)

MST Target	Detect (%)		Non-Detect (%)		OR (95% CI)
	Dry weather	Wet weather	Dry weather	Wet weather	
DG3	9 (33)	18 (67)	88 (52)	80 (48)	2.20 (0.94, 5.18)
DG37	2 (29)	5 (71)	95 (51)	93 (49)	2.55 (0.48, 13.49)
GFD	37 (47)	42 (53)	60 (52)	56 (48)	1.22 (0.69, 2.16)
HF183/BacR287	11 (58)	8 (42)	86 (49)	90 (51)	0.70 (0.27, 1.81)
HumM2	6 (75)	2 (25)	91 (49)	96 (51)	0.32 (0.06, 1.61)

[a] Abbreviations: OR = Odds Ratio; CI = Confidence Interval.

TABLE X

BEACH-SPECIFIC WEATHER CONDITIONS AND WEIGHTED-AVERAGE FECAL SCORES WITH 95% BCI FOR THE MST ASSAYS (N=195)

Beach	Weather	N	Weighted-Average Fecal Score in copies/ 100 mL (95 % BCI)				
			DG3	DG37	GFD	HF183/ BacR287	HumM2
CL	Dry	14	NA	NA	30.65 (10.18-59.50)	NA	NA
	Wet	15	NA	NA	116.2 (68.88-163.5)	NA	NA
MB	Dry	13	NA	NA	42.25 (23.81-59.19)	NA	NA
	Wet	15	NA	NA	94.66 (52.44-141.1)	NA	NA
MN	Dry	11	204.7 (145.2-321)	NA	61.81 (36.05-82.92)	6.15 (0.93-15.63)	NA
	Wet	17	385.5 (274.1-505.3)	NA	73.17 (44.79-101.3)	24.68 (13.07-39.35)	NA
OS	Dry	17	NA	NA	25.33 (15.84-33.32)	10.97 (4.74-19.76)	NA
	Wet	13	NA	NA	43.14 (14.92-82.27)	6.92 (1.67-15.62)	NA
RB	Dry	17	NA	NA	25.52 (14.50-38.05)	NA	NA
	Wet	10	NA	NA	30.85 (12.85-53.35)	NA	NA
ST	Dry	17	NA	NA	74.80 (40.24-117.7)	NA	NA
	Wet	14	NA	NA	58.56 (32.93-84.67)	NA	NA
TS	Dry	8	NA	NA	NA	NA	NA
	Wet	14	NA	NA	NA	NA	NA
All	**Dry**	**97**	**4.71 (2.86-6.99)**	**0.95 (0.31-1.95)**	**39.53 (31.61-48.40)**	**6.11 (4.02-8.68)**	**NA**
	Wet	**98**	**11.37 (8.17-15.06)**	**1.95 (0.91-3.38)**	**63.89 (51.30-77.56)**	**4.95 (3.16-7.12)**	**NA**

[a] Abbreviations: BCI = Bayesian Confidence Interval; NA = Not Applicable.

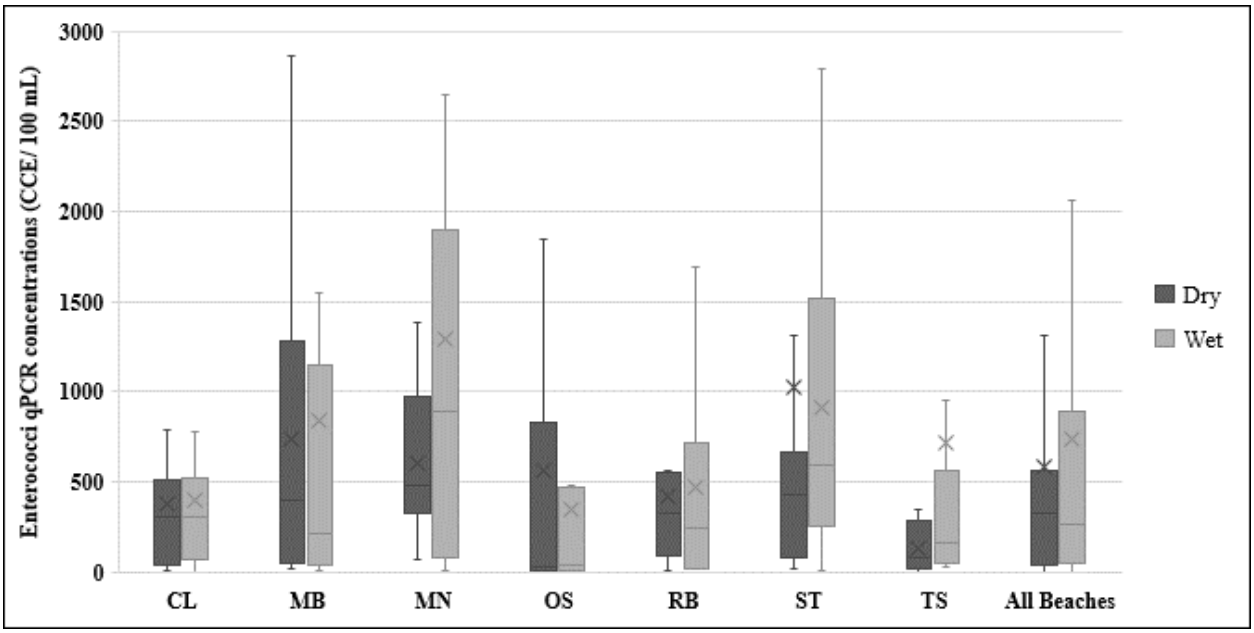

Figure 5: Distribution of enterococci qPCR concentrations at Chicago beaches under different weather conditions
[a] Abbreviations: CCE = Calibration Cell Equivalent.

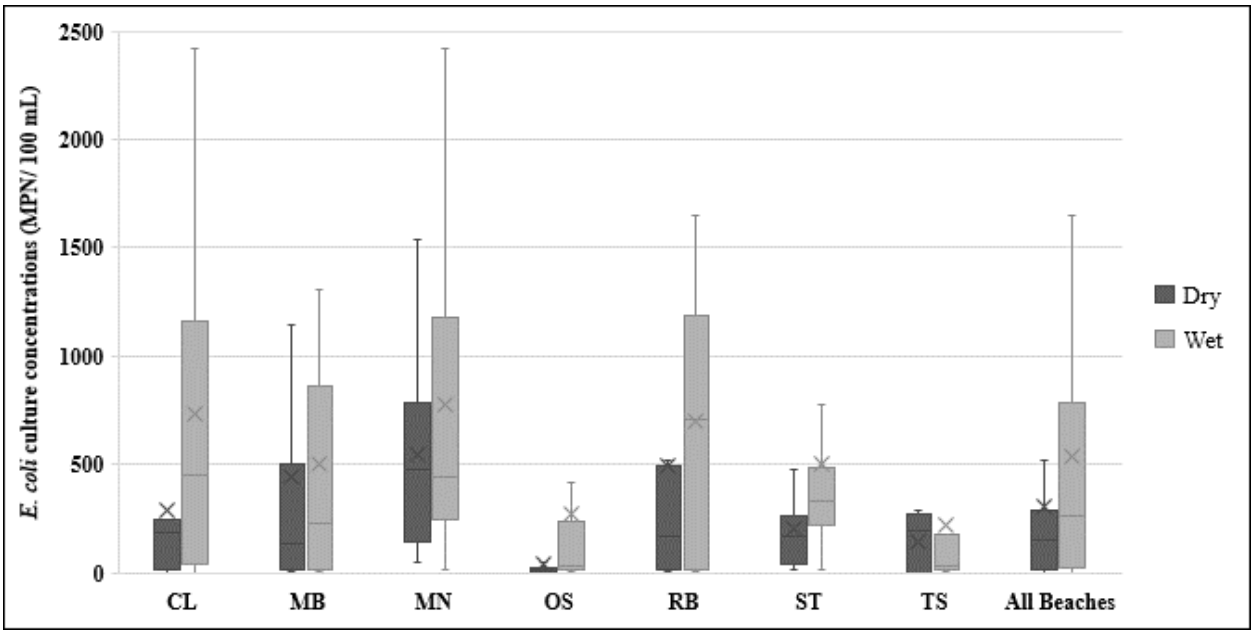

Figure 6: Distribution of *E. coli* culture concentrations at Chicago beaches under different weather conditions
[a] Abbreviations: MPN = Most Probable Number

E. Conclusions

- Although previous studies have reported significant positive relationship between precipitation and general FIB levels in beach water, at Chicago beaches we only found a significant relationship between precipitation and *E. coli* culture but not with enterococci qPCR.
- Our study found that enterococci qPCR CCE concentrations were not significantly different between wet weather conditions and dry weather conditions and that rain events do not necessarily trigger ENT qPCR BAV exceedances. In contrast, precipitation tends to increase *E. coli* culture results and the overall odds of an exceedance of the *E. coli* culture BAV did significantly increase following rain events.
- We were also able to identify Chicago beaches which might be at higher risk of elevated microbial concentrations from particular sources of fecal pollution after rain events. This information may be useful for remediation and management of Chicago's Lake Michigan recreational waters for better public health protection.
- Finally, MST findings coupled with precipitation information can provide a better picture of different sources of fecal pollution and their pathways and can further help implement appropriate remediation plans for recreational beach water quality management to better heath protection of beachgoers.

V. SUMMARY OF CONCLUSIONS

The overarching goal of this research was to improve the monitoring of and interpretation of microbial fecal pollution at freshwater beaches, so as to protect the health of water recreators. More specifically, this research: 1) evaluated a relatively new rapid *E. coli* qPCR method intended to be used for monitoring freshwater beaches, 2) explored the possible roles of fecal source characterization in the context of routine beach monitoring, and 3) described the influence of different levels of precipitation on microbial water quality at non-point source impacted beaches. Though this research represents a significant contribution to our understanding of beach monitoring methods and management, knowledge gaps remain to be addressed before major changes in beach monitoring and notification programs can be implemented.

This research is the first to compare beach monitoring results for *E. coli* obtained by a new qPCR method with *E. coli* and enterococci measured using traditional methods in the same water samples on a daily basis. We found that the qPCR method targeting *E. coli* demonstrated good technical performance, but that comparisons of *E. coli* measured by qPCR to the existing BAV for the other FIB measures yielded very different potential BAV for *E. coli* measured by qPCR. Currently, BAVs for different microbes (*E. coli* measured by culture, enterococci measured by culture, and enterococci measured by qPCR) are considered to be interchangeable at freshwater beaches, but the lack of consistency when these BAVs were extrapolated to *E. coli* measured by qPCR suggests that all BAVs may not be equivalent, at least the Lake Michigan beaches studied in this research. Furthermore, our finding that each statistical method and reference FIB measurement yielded different *E. coli* qPCR BAV further highlights a need for USEPA guidelines about setting site-specific alternative standards using alternative indicator and/ or methods to improve clarity and evaluate assumptions of comparability of BAVs of

different USEPA-approved methods for quantifying indicator bacteria. Finally, given the availability of a rapid qPCR method (enterococci) developed by USEPA in conjunction with epidemiological studies, there is little reason to attempt developing BAVs for other methods that cannot be calibrated directly to observed health risk. This is because predicting health risk based on predicting enterococci qPCR results would introduce an unnecessary source of error in the prediction of health risks.

The second study examining host-associated source tracking markers at Chicago beaches was one of the first attempts to explore the role of MST in the context of routine beach monitoring and notification. To the best of our knowledge, this was the first study to evaluate the value of MST data in the interpretation of general FIB levels and BAV exceedances. Additionally, our efforts demonstrate how a novel censored-data analysis approach using weighted-average fecal scores can be used to identify non-point sources of fecal pollution linked to local general FIB criteria using MST technologies in a recreational water setting. Findings from this study identified that non-human fecal pollution sources including dogs and birds may influence recreational water quality at Chicago beaches. Our study results suggest that prioritizing recreational water management actions targeting canine and avian non-point fecal pollution sources may be helpful in improving water quality at these Great Lake beaches. We also found that human fecal pollution is generally not measurable at Chicago beaches. Our findings support the assumptions that Chicago's engineered system of wastewater and stormwater drainage infrastructure to protect Lake Michigan and its recreational beaches from urban discharges may effectively prevent human fecal contamination at beaches. Our study indicates that simultaneous use of microbial source tracking markers along with the general FIB

may be useful to supplement or confirm sanitary survey data for mitigation and management of recreational waters to better protect health of the beachgoers and water recreators.

The potential implications of these studies on stormwater, wastewater, and beach management are significant on both local and national level. Our findings can provide valuable insights to local beach managers on possible mitigation strategies that could help combat fecal contamination in recreational waters. Furthermore, results from our precipitation study examining the influence of rainfall on general FIB and MST can prove useful for prioritizing Chicago beaches for intervention implementations so as to have the greatest impact towards improving recreational water quality at these beaches. This research might also be helpful to beach managers interested in exploring effects of precipitation on source tracking host-associated markers. Our study can further assist in developing a sample selection strategy for MST testing with host-associated MST genetic markers and for designing a tailored quantitative data interpretation plan that can identify any potential associations between general FIB and MST measurements using a recently reported qPCR censored-data approach.

The findings from all three studies are expected to add to the current literature on frameworks of beach monitoring and notification while suggesting future research on recreational water quality at large lakes. However, our studies also raise several questions. For example, in Chapter III we concluded that there is discordance between exceedance of BAV (cEC and qENT) and detection of all host-associated markers. Since we did not measure pathogen levels in beach water, we do not know about the associations between pathogen levels and either general FIB BAV exceedances or the detection of MST markers. For that reason, additional research is warranted to establish which of the two, BAV exceedance or MST marker detection, would pose higher health risk to the beachgoers and water recreators. Additionally,

Chapter IV only addressed a small part of a much greater picture about the effects of precipitation and microbial pollution at non-point source impacted beaches. Future research directions that might further clarify questions raised by this research might include studies examining larger sample numbers and more cut off points for different rainfall amounts which could provide a better understanding of the effects of rainfall on microbial pollution at these beaches. The amount of precipitation might not be the sole factor for determining changes in bacterial concentration at these beaches following rainfall. For that reason, beach catchment characteristics (slope, area, presence of non-permeable surfaces, etc.), intensity of precipitation (inches/hour) and other environmental factors should be considered and included in future studies.

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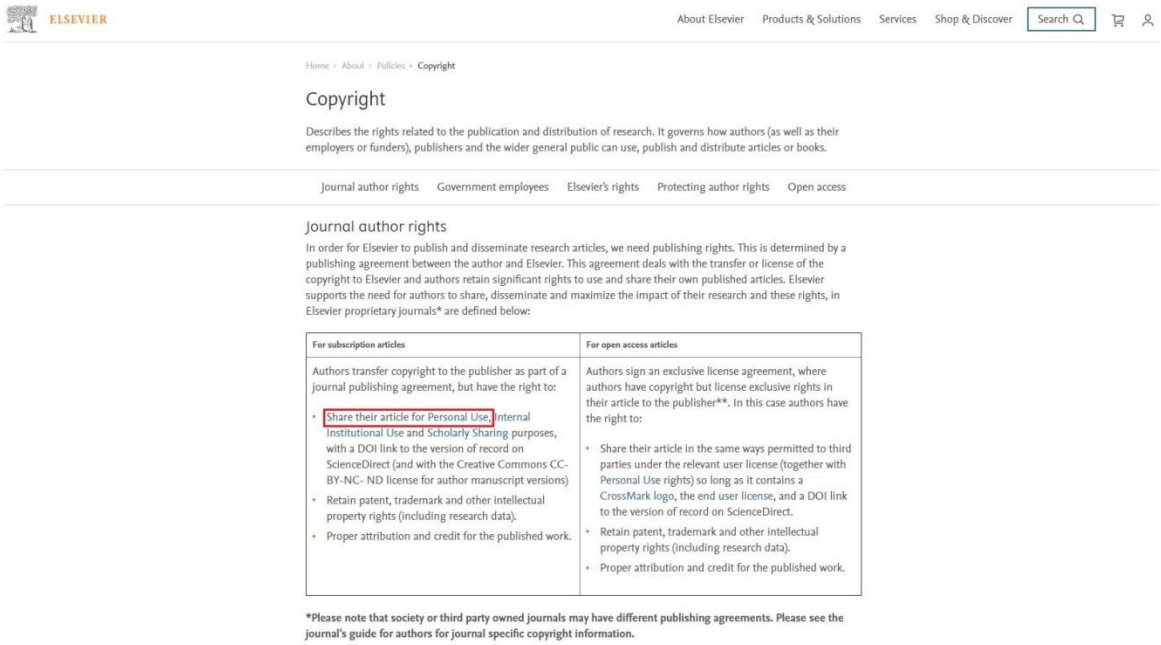
APPENDICES

Appendix A



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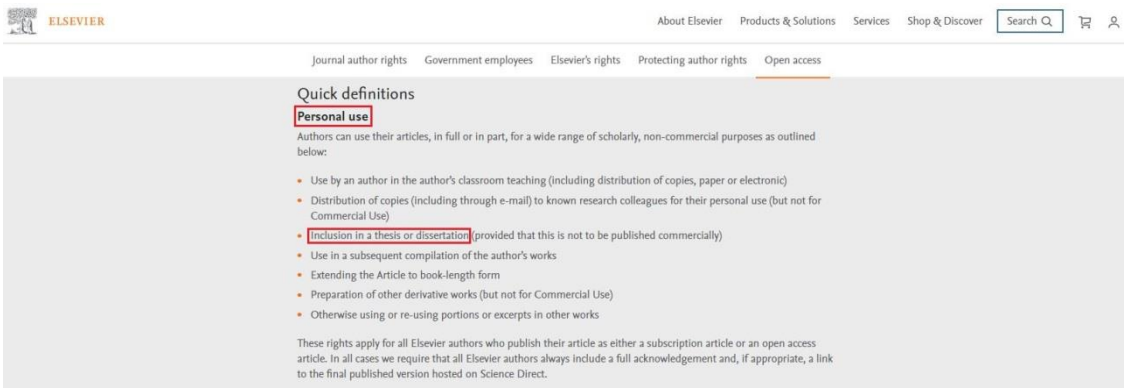

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Title: Evaluation of rapid qPCR method for quantification of E. coli at non-point source impacted Lake Michigan beaches

Author: Abhilasha Shrestha,Samuel Dorevitch

Publication: Water Research

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Figure 7: *E. coli* culture and qPCR testing beach locations

Appendix A (continued)

TABLE XI
COORDINATES OF BEACH LOCATIONS

SN	Beach Name	X_COORD	Y_COORD
1	Montrose Beach (MN)	-87.638819	41.96726
2	North Avenue Beach (NA)	-87.623949	41.914062
3	Ohio Street Beach (OS)	-87.61285	41.893437
4	Rainbow Beach (RB)	-87.550607	41.759468
5	South Shore Beach (SS)	-87.5617	41.768607
6	57th Street Beach (FS)	-87.578909	41.790913
7	63rd Street Beach (TS)	-87.572901	41.782273
8	Calumet Beach (CL)	-87.528468	41.714963

TABLE XII
PRIMERS AND PROBES USED IN MOLECULAR TESTS

Assay	Forward Primer	Reverse Primer	Probe	Reference
E. coli (EC23S857)	5'-GGTAGAGCACTGTTTTGGCA	5'-TGTCTCCCGTGATAACTTTCTC	[6-FAM]-5'-TCATCCCGACTTACCAACCCG-TAMRA	USEPA Draft Method C
Enterococci (Entero1a)	5'-GAGAAATTCCAAACGAACTTG	5'-CAGTGCTCTACCTCCATCATT	[6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	USEPA Method 1609.1
Sketa22	5'-GGTTTCCGCAGCTGGG	5'-CCGAGCCGTCCTGGTC	[6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA	USEPA Method 1609.1 and USEPA Draft Method C

Appendix A (continued)

TABLE XIII
THERMOCYCLER SETTING FOR THE DIFFERENT QPCR ASSAYS

Assay	Holding Stage 1	Holding Stage 2	Cycling Stage Step 1	Cycling Stage Step 2	# of cycles	Threshold Setting
E. coli	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	56.0°C for 1 min	40	0.03
Enterococci	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03

TABLE XIV
POOLED RESULTS OF STANDARD CURVES FOR *E. COLI* AND ENTEROCOCCI QPCR

Target	Parameter	Mean	Standard deviation	95% CI	Amplification factor (E)	R^2
E. coli	Slope	-3.332	0.069	-3.470, -3.195	1.00	0.999
	Intercept	38.321	0.217	37.890, 38.753	NA	
Enterococci	Slope	-3.403	0.055	-3.511, -3.296	0.98	0.965
	Intercept	37.065	0.155	37.760, 37.371	NA	

TABLE XV
PRECISION OF QPCR MEASUREMENT IN CALIBRATOR SAMPLES

Target	Variable	N	Ct Mean	Ct Standard deviation	Coefficient of variation
E. coli	Calibrator cells	36	28.82	0.33	1.14%
	SPC	36	19.98	0.26	1.31%
Enterococci	Calibrator cells	72	27.86	0.27	0.96%
	SPC	72	18.97	0.09	0.45%

Appendix A (continued)

TABLE XVI
DESCRIPTIVE STATISTICS OF CT FOR NTC AND MEB

Target	Variable	N	Ct ≥40 N (%)	Ct Mean	Ct Std dev	Range	CV
E. coli	No template control (NTC)	72	25 (34.7%)	38.65	1.20	36.11-40.00	3.11%
	Method blanks (MeB)	216	45 (20.8%)	38.30	1.14	36.00-40.00	3.09%
Enterococci	No template control (NTC)	144	136 (94.4%)	39.72	1.24	32.84-40.00	3.11%
	Method blanks (MeB)	144	131 (90.9%)	39.75	0.83	36.45-40.00	2.08%

TABLE XVII
BEACH-SPECIFIC CORRELATION MATRIX OF LOG_{10}-TRANSFORMED DATA

Beach	N	EC CCE vs. MPN	EC CCE vs. ENT CCE	ENT CCE vs. MPN
Montrose	36	r= 0.77*	r= 0.74*	r= 0.61*
North Avenue	36	r= 0.82*†	r= 0.69*	r= 0.59*†
Ohio Street	36	r= 0.85*	r= 0.60*	r= 0.60*
Rainbow	35	r= 0.84*	r= 0.71*	r= 0.68*
South Shore	36	r= 0.78*	r= 0.47**	r= 0.62*
57th Street	36	r= 0.87*	r= 0.61*	r= 0.59*
63rd Street	34	r= 0.86*	r= 0.73*	r= 0.72*
Calumet	36	r= 0.76*	r= 0.77*	r= 0.76*
Overall	**285**	**r= 0.83***	**r= 0.67***	**r= 0.67***

†Spearman; *p≤.0001; **0.01>p>0.0001.
[a] Abbreviations: EC = *E. coli*; CCE = Calibrator Cell Equivalents; MPN = Most Probable Number; ENT = Enterococci.

Appendix A (continued)

TABLE XVIII
BEACH-SPECIFIC ROC ANALYSIS FOR *E. COLI* QPCR VS. SAME-DAY CULTURE BAV EXCEEDANCE

Beaches	**N**	**Optimized BAV**	**Sensitivity**	**Specificity**	**AUC**	**95% CI [a]**	**R**
Montrose	36	650.1	70.0	88.5	0.81	0.64- 0.92	0.77*
North Ave.	36	239.5	100.0	81.5	0.93	0.80- 0.99	0.82*†
Ohio Street	36	NA	NA	NA	NA	NA	0.85*
Rainbow	35	168.2	100.0	52.0	0.80	0.64- 0.92	0.84*
South Shore	36	477.1	100.0	93.3	0.96	0.84- 1.00	0.78*
57th Street	36	512.7	83.3	86.7	0.86	0.71- 0.95	0.87*
63rd Street	34	942.9	75.0	93.3	0.90	0.75- 0.98	0.86*
Calumet	36	340.2	100.0	81.3	0.94	0.80- 0.99	0.76*
Overall	**285**	**410.4**	**82.0**	**80.4**	**0.88**	**0.84- 0.92**	**0.83***

†Spearman; *p≤.0001.
[a] Binomial exact method
[b] Abbreviations: BAV = Beach Action Value; AUC = Area Under Curve.

TABLE XIX
BEACH-SPECIFIC ROC ANALYSIS FOR *E. COLI* QPCR VS. ENTEROCOCCI BAV EXCEEDANCE

Beaches	**N**	**Optimized BAV**	**Sensitivity**	**Specificity**	**AUC**	**95% CI [a]**	**R**
Montrose	36	1102.0	77.8	96.3	0.86	0.70- 0.95	0.74*
North Ave.	36	548.2	100.0	93.9	0.98	0.87- 1.00	0.69*
Ohio Street	36	84.7	100.0	62.5	0.81	0.65- 0.92	0.60*
Rainbow	35	997.3	71.4	89.3	0.84	0.67- 0.94	0.71*
South Shore	36	251.7	100.0	69.0	0.84	0.68- 0.94	0.47**
57th Street	36	159.8	85.7	69.0	0.83	0.67- 0.93	0.61*
63rd Street	34	942.9	50.0	96.2	0.72	0.54- 0.86	0.73*
Calumet	36	499.0	85.7	100.0	0.95	0.82- 1.00	0.77*
Overall	**285**	**200.9**	**92.3**	**58.8**	**0.84**	**0.79- 0.88**	**0.67***

*p≤.0001; **0.004<p>0.0001.
[a] Binomial exact method.
[b] Abbreviations: BAV = Beach Action Value; AUC = Area Under Curve.

Appendix B

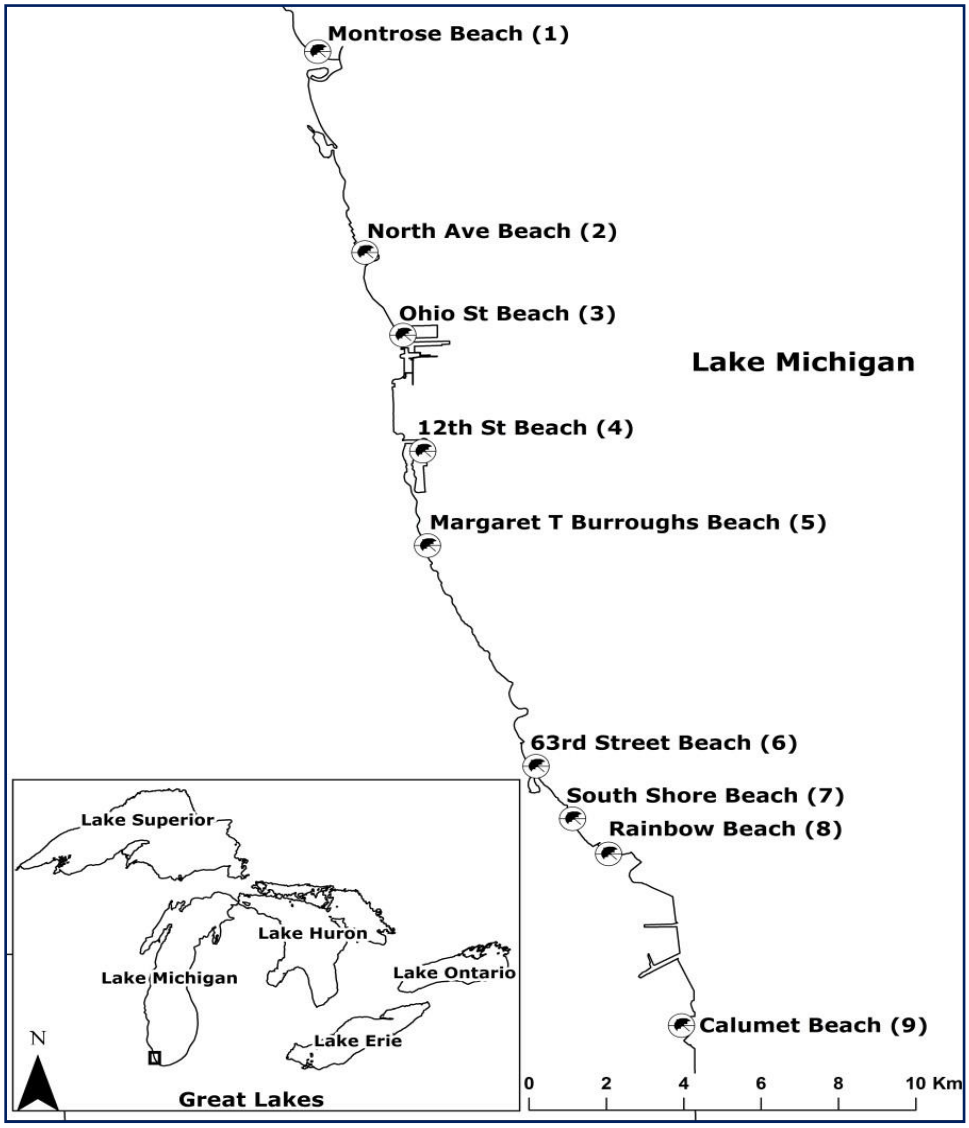

Figure 8: Enterococci qPCR and *E. coli* culture testing beach locations

TABLE XX

ENTEROCOCCI QPCR AND *E. COLI* CULTURE TESTING BEACH COORDINATES

SN	Beach Name	X_COORD	Y_COORD
1	Montrose Beach (MN)	-87.638819	41.96726
2	North Avenue Beach (NA)	-87.623949	41.914062
3	Ohio Street Beach (OS)	-87.61285	41.893437
4	12th Street Beach (TS)	-87.607405	41.863792
5	Margaret T Burroughs Beach (MB)	-87.606311	41.839194
6	63rd Street Beach (ST)	-87.572901	41.782273
7	South Shore Beach (SS)	-87.5617	41.768607
8	Rainbow Beach (RB)	-87.550607	41.759468
9	Calumet Beach (CL)	-87.528468	41.714963

Appendix B (continued)

TABLE XXI
PRIMERS AND PROBES USED IN MOLECULAR TESTS

Assay	**Forward Primer**	**Reverse Primer**	**Probe**	**Reference**
Enterococci (Entero1a)	5'-GAGAAATTCCAAACGAACTTG	5'-CAGTGCTCTACCTCCATCATT	5'-FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	USEPA Method 1609.1
Sketa22	5'-GGTTTCCGCAGCTGGG	CCGAGCCGTCCTGGTC	[6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA	(Haugland et al., 2010)
HF183/BacR287	ATCATGAGTTCACATGTCCG	CTTCCTCTCAGAACCCCTATCC	[FAM] CTAATGGAACGCATCCC [MGB]	(Green, et al., 2014)
HumM2	CGTCAGGTTTGTTTCGGTATTG	TCATCACGTAACTTATTTATATGCATTAGC	[FAM] TATCGAAAATCTCACGGATTAACTCTTGTGTACGC [TAMRA]	(Shanks, et al., 2009)
DG3	TGAGCGGGCATGGTCATATT	TTTTCAGCCCCGTTGTTTCG	[FAM] AGTCTACGCGGGCGTACT [MGB]	(Green, et al., 2014)
DG37	CTTGGTTATGGGCGACATTG	TTTTCTCCCACGGTCATCTG	[FAM] TTGAACGTTTAAAGGAGCAGGTGGCAG [TAMRA]	(Green, White, et al., 2014)
GFD (SYBR)	TCGGCTGAGCACTCTAGGG	GCGTCTCTTTGTACATCCCA	N/A	(Green, et al., 2012)

Appendix B (continued)

TABLE XXII
THERMOCYCLER SETTING FOR THE DIFFERENT QPCR ASSAYS

Assay	Holding Stage 1	Holding Stage 2	Cycling Stage Step 1	Cycling Stage Step 2	# of cycles	Threshold Setting
Enterococci	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
Sketa22	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
DG3	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
DG37	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
GFD [a]	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	57.0°C for 1 min	39	0.08
HF183/BacR287	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
HumM2	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.08

[a] Melt curve for GFD at 57.0°.

TABLE XXIII
POOLED RESULTS OF STANDARD CURVES FOR ENTEROCOCCI AND MST MARKERS

Assay	# of curves	LLOQ	Intercept	Intercept SD	Slope	Slope SD	Amplification efficiency (E)	R^2
Enterococci	10	--	36.09	0.1187	-	0.0353	1.097559	0.9995
DG3	6	35.1	38.3	0.1531	-3.485	0.03068	0.936172493	0.994
DG37	6	35.42	38.69	0.08655	-3.408	0.02331	0.965292651	0.99558
GFD	6	36.53	39.8	0.1502	-3.52	0.03853	0.923494324	0.99121
HF183/BacR287	6	35.09	38.39	0.07754	-3.426	0.02357	0.958328665	0.9962
HumM2	6	37.35	40.63	0.09518	-3.45	0.02428	0.94919403	0.99395

Appendix B (continued)

TABLE XXIV
DESCRIPTIVE STATISTICS OF CT FOR NTC FOR MST ASSAYS

Assay	N	Min Ct	Ct ≥40 N (%)	Ct > LLOQ[a] < 40 N (%)	Ct < LLOQ N (%)
DG3	156	40	156 (100%)	0 (0%)	0 (0%)
DG37	156	40	156 (100%)	0 (0%)	0 (0%)
GFD	156	40	156 (100%)	0 (0%)	0 (0%)
HF183/BacR287	156	40	156 (100%)	0 (0%)	0 (0%)
HumM2	156	37.08	152 (97.5%)	3 (1.9%)	1 (0.6%)

[a] Lowest Limit of Quantification for each MST assay.

TABLE XXV
PRECISION OF QPCR MEASUREMENT BASED ON THE COEFFICIENT OF VARIATION (CV) OF ENTEROCOCCI AND SAMPLE PROCESSING CONTROL (SPC) TARGETS IN CALIBRATOR SAMPLES (N=71)

Assay	Ct Mean	Ct standard deviation	Coefficient of variation (CV)
Enterococci	26.5	0.35	1.33
SPC	18.8	0.27	1.41

TABLE XXVI
SPC THRESHOLD CT FOR THE MST ASSAYS

Assay	SPC Threshold Ct
DG3	24.362
DG37	25.06
GFD	23.99
HF183/BacR287	24.682
HumM2	26.832

Appendix C

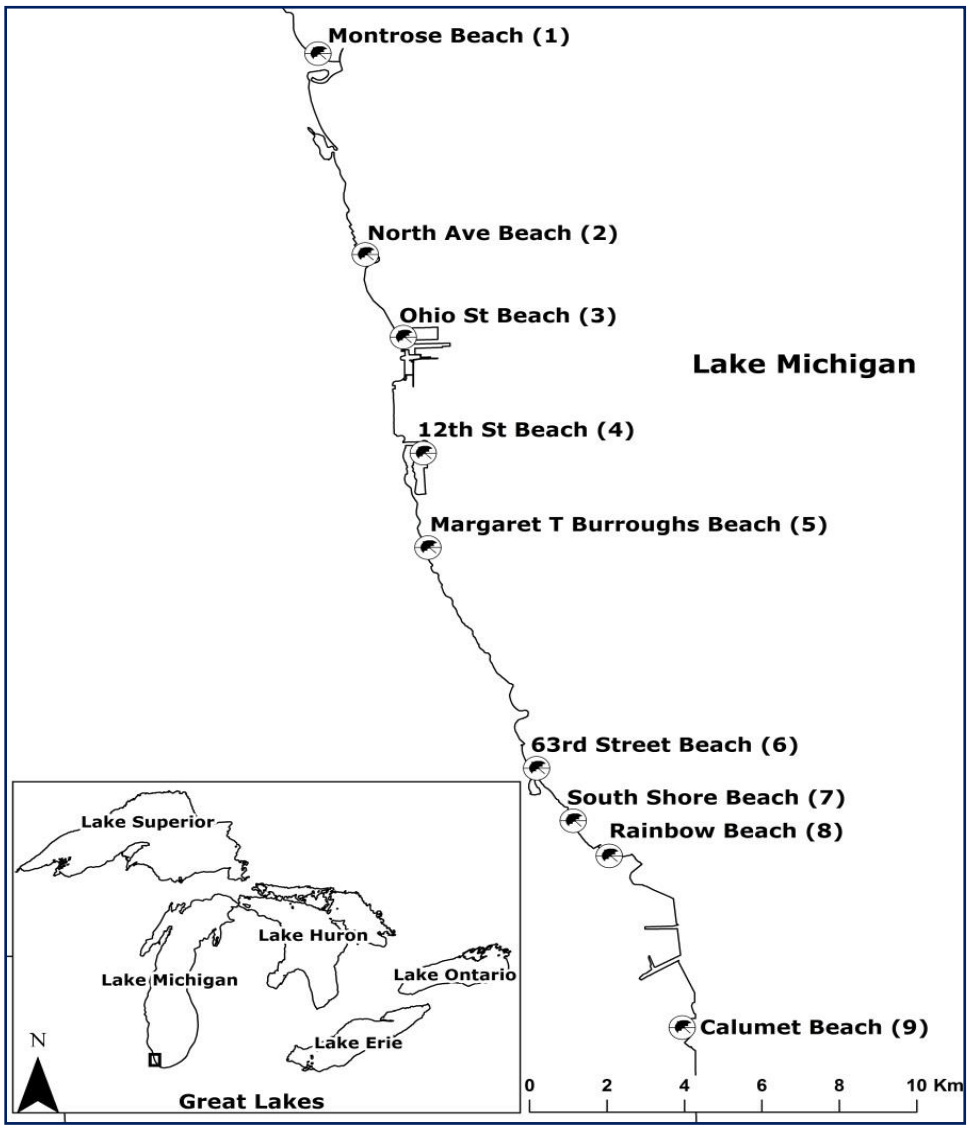

Figure 9: *E. coli culture* and enterococci qPCR testing beach locations

TABLE XXVII

CULTURE AND QPCR TESTING BEACH COORDINATES

SN	Beach Name	X_COORD	Y_COORD
1	Montrose Beach (MN)	-87.638819	41.96726
2	North Avenue Beach (NA)	-87.623949	41.914062
3	Ohio Street Beach (OS)	-87.61285	41.893437
4	12th Street Beach (TS)	-87.607405	41.863792
5	Margaret T Burroughs Beach (MB)	-87.606311	41.839194
6	63rd Street Beach (ST)	-87.572901	41.782273
7	South Shore Beach (SS)	-87.5617	41.768607
8	Rainbow Beach (RB)	-87.550607	41.759468
9	Calumet Beach (CL)	-87.528468	41.714963

Appendix C (continued)

TABLE XXVIII
PRIMERS AND PROBES USED IN MOLECULAR TESTS

Assay	Forward Primer	Reverse Primer	Probe	Reference
Enterococci (Entero1a)	5'-GAGAAATTCCAAACGAACTTG	5'-CAGTGCTCTACCTCCATCATT	5'-FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	USEPA Method 1609.1
Sketa22	5'-GGTTTCCGCAGCTGGG	CCGAGCCGTCCTGGTC	[6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA	(Haugland et al., 2010)
HF183/BacR287	ATCATGAGTTCACATGTCCG	CTTCCTCTCAGAACCCCTATCC	[FAM] CTAATGGAACGCATCCC [MGB]	(Green, et al., 2014)
HumM2	CGTCAGGTTTGTTTCGGTATTG	TCATCACGTAACTTATTTATATGCATTAGC	[FAM] TATCGAAAATCTCACGGATTAACTCTTGTGTACGC [TAMRA]	(Shanks, et al., 2009)
DG3	TGAGCGGGCATGGTCATATT	TTTTCAGCCCCGTTGTTTCG	[FAM] AGTCTACGCGGGCGTACT [MGB]	(Green, et al., 2014)
DG37	CTTGGTTATGGGCGACATTG	TTTTCTCCCACGGTCATCTG	[FAM] TTGAACGTTTAAAGGAGCAGGTGGCAG [TAMRA]	(Green, White, et al., 2014)
GFD (SYBR)	TCGGCTGAGCACTCTAGGG	GCGTCTCTTTGTACATCCCA	N/A	(Green, et al., 2012)

Appendix C (continued)

TABLE XXIX
THERMOCYCLER SETTING FOR THE DIFFERENT QPCR ASSAYS

Assay	Holding Stage 1	Holding Stage 2	Cycling Stage Step 1	Cycling Stage Step 2	# of cycles	Threshold Setting
Enterococci	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
Sketa22	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
DG3	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
DG37	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
GFD [a]	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	57.0°C for 1 min	39	0.08
HF183/BacR287	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.03
HumM2	50.0°C for 2:00 mins	95.0°C for 10:00 mins	95.0°C for 0:15 secs	60.0°C for 1 min	40	0.08

[a] Melt curve for GFD at 57.0°.

TABLE XXX
POOLED RESULTS OF STANDARD CURVES FOR ENTEROCOCCI AND MST MARKERS

Assay	# of curves	LLOQ	Intercept	Intercept SD	Slope	Slope SD	Amplification efficiency (E)	R^2
Enterococci	10	--	36.09	0.1187	-	0.0353	1.097559	0.9995
DG3	6	35.1	38.3	0.1531	-3.485	0.03068	0.936172493	0.994
DG37	6	35.42	38.69	0.08655	-3.408	0.02331	0.965292651	0.99558
GFD	6	36.53	39.8	0.1502	-3.52	0.03853	0.923494324	0.99121
HF183/BacR287	6	35.09	38.39	0.07754	-3.426	0.02357	0.958328665	0.9962
HumM2	6	37.35	40.63	0.09518	-3.45	0.02428	0.94919403	0.99395

Appendix C (continued)

TABLE XXXI
ENTERCOCCI QPCR BAV EXCEEDANCE UNDER WET AND DRY WEATHER CONDITIONS (N=195)

Beach	N	ENT qPCR did not exceed BAV		ENT qPCR exceeded BAV	
		Dry (%)	Wet (%)	Dry (%)	Wet (%)
Calumet	29	13 (8)	14 (9)	1 (3)	1 (3)
Margaret T Burroughs	28	9 (6)	11 (7)	4 (11)	4 (11)
Montrose	28	9 (6)	10 (6)	2 (5)	7 (19)
Ohio Street	30	13 (8)	11 (7)	4 (11)	2 (5)
Rainbow	27	15 (9)	8 (5)	2 (5)	2 (5)
63rd Street	31	14 (9)	10 (6)	3 (8)	4 (11)
12th Street	22	8 (5)	13 (8)	0 (0)	1 (3)
All beaches	**195**	81 (51)	77 (49)	16 (43)	21 (57)

[a] Abbreviations: ENT = Enterococci; BAV = Beach Action Value.

TABLE XXXII
E. COLI CULTURE BAV EXCEEDANCE UNDER WET AND DRY WEATHER CONDITIONS (N=170)

Beach	N	EC culture did not exceed BAV		EC culture exceeded BAV	
		Dry (%)	Wet (%)	Dry (%)	Wet (%)
Calumet	26	8 (8)	5 (5)	4 (5)	9 (12)
Margaret T Burroughs	24	6 (6)	7 (7)	4 (5)	7 (9)
Montrose	22	3 (3)	3 (3)	5 (7)	11 (15)
Ohio Street	26	13 (14)	9 (9)	1 (1)	3 (4)
Rainbow	26	10 (11)	3 (3)	6 (8)	7 (9)
63rd Street	27	9 (9)	4 (4)	5 (7)	9 (12)
12th Street	19	5 (5)	10 (11)	2 (3)	2 (3)
All beaches	**170**	**54 (57)**	**41 (43)**	**27 (36)**	**48 (64)**

[a] Abbreviations: EC = *E. coli*; BAV = Beach Action Value.

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