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The implementation of qPCR beach monitoring methods: Analysis of a multi-lab validation study and the role of environmental parameters on a comparison of Colilert and qPCR methods

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THE IMPLEMENTATION OF qPCR BEACH MONITORING METHODS: ANALYSIS OF A MULTI LAB VALIDATION STUDY AND THE ROLE OF ENVIRONMENTAL PARAMETERS ON A COMPARISON OF COLILERT AND qPCR METHODS

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Dedication

This work is dedicated to my grandparents, Thelma and Ev Lane and Margaret Bryant; and to my parents, Jerry Lane and Tina Lynch. As well as to the rest of my loved ones who have whole-heartedly supported me through this journey and without whom I would not have had the courage to attempt.

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Preface

The beaches along the eastern shore of Lake Michigan are something to be marveled at. Their serenity, calmness, fury and glittering reflections make an impression on all who take the time to embrace their beauty. To some, they are places of solitude or reflection; to others, they are a playground. Whatever role they may play in each of our lives, it is important we feel secure in that they are a safe place to spend our time.

Abstract

Public beaches are routinely tested for potentially pathogenic bacteria to protect beachgoers from possible illness. An EPA approved method, Colilert™, used for testing *E. coli* in recreational water requires 18 – 22 hours before a result is reported but, recreators have already contacted unsafe water before the beach is closed. My study focused on a U.S. EPA proposed qPCR method (Draft Method C) to quantify *E. coli* in recreational waters that can provide same-day results. In Chapter 2, I examined the calibration procedure used to validate Draft Method C and compared standard curve intercept and slope estimates calculated with a Bayesian model to estimates generated from a simpler weighted linear regression (WLR) model to determine if it can replace the complicated Bayesian model for method implementation. A < 1% difference in the overall mean and median intercept and slope was observed between the two models, demonstrating that the WLR model results were comparable. I also analyzed inter-lab variability in intercept, slope, and R^2 estimates produced by passing curves from the WLR model in a multi-lab 2018 data set. Significant pairwise differences were detected in 11% of the 36 inter-lab intercept comparisons; no differences were detected in the slope or R^2 parameters. I concluded that the proposed standard curve acceptance criteria showed minimal variation between labs, thus ensuring reported results are accurate and reliable. In Chapter 3, I measured *E. coli* concentrations with Colilert™ and Draft Method C in water samples collected during the summer of 2018 from 14 inland lake and 6 Lake Michigan beaches in Muskegon County, MI. Kaplan-Meier distribution curves and log-rank trend tests were used to identify categorized environmental variables that significantly impacted *E. coli* concentrations based on the quantification method and the lake type. Bird count and birds present/absent significantly impacted *E. coli* levels at beaches of both lake types and quantification methods. The remaining variables significantly impacted *E. coli* concentrations depending on lake type and quantification method. Therefore, predictive models for beach water quality should

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Abbreviations

ANCOVA: Analysis of Covariance

BCI: Bayesian Credible Interval

BEACH: Beaches Environmental Assessment and Coastal Health Act

BNV: Beach Notification Value

BSA: Bovine Serum Albumin

cfu: Colony forming units

CI: Confidence Interval

Coefficient of Determination: R^2

Ct: Threshold Cycle Value

DNA: Deoxyribonucleic Acid

E. coli: *Escherichia coli*

EGLE: Department of Environment, Great Lakes & Energy

EU: European Union

EV(s): Environmental Variable(s)

FAM: TaqMan probe reporter molecule

FIB: Fecal Indicator Bacteria

FRET: Fluorescence Resonance Energy Transfer

GE: Genomic Equivalent

GI: Gastrointestinal

IA: Index of Agreement

KM: Kaplan-Meier

LOQ: Limit of Quantification

mL: milliliters

MM: Master Mix

MPN: Most probable number

MSC: Master Standard Curve

MUG: 4-methyl-umbelliferyl β-D-glucuronide

NTC: No Template Control

NTU: Nephelometric Turbidity Unit

PAR: Photosynthetically Active Radiation

PBS: Phosphate Buffer Saline

qPCR: Quantitative Polymerase Chain Reaction

RW: Recreational Water

TAMRA: TaqMan probe quencher molecule

Taq: *Thermus aquaticus* DNA polymerase

TaqMan: Thermo Fisher Taq DNA polymerase

µg: microgram

µL: microliter

U.S. EPA: United States Environmental Protection Agency

WLR: Weighted Linear Regression

Chapter 1 : Introduction

Coastal communities throughout the world are impacted by the waters they border. These communities rely heavily upon these waters for employment opportunities, drinking water, and tourism, which in turn supports local businesses. Furthermore, coastal waters provide recreation opportunities such as boating, kayaking, swimming, and observing wildlife, all of which improve the quality of life for those who live near them. Because of the significant role these waters play in our lives, it is important to ensure they are safe for use. One way municipalities address this is by routinely monitoring the water at public beaches for potentially pathogenic fecal indicator bacteria. Exposure to fecal contaminated water has long been known to cause illness (Stevenson 1953; Cabelli and Dufour, 1982; Dufour 1984). Diseases associated with exposure to fecal contaminated water include respiratory or ear infections, skin irritations and gastrointestinal (GI) illness (Seyfried, et al., 1985 (I); Wade et al., 2008) and the economic impact from getting them can be substantial. Recently, it was estimated between $2 – 4 billion was spent annually as a result of contracting these illnesses (Deflorio-Barker et al., 2018).

A commonly used United States Environmental Protection Agency (U.S. EPA) approved method to monitor recreational water for fecal bacteria, specifically *Escherichia coli* (*E. coli*), is called IDEXX Colilert-18™. This method measures culturable *E. coli* cells and requires 18 – 22 h before results are obtained (Rice et al., 2012). Consequently, water quality results are not disseminated to county officials, beach managers or the public until the following day. If elevated *E. coli* levels are present, the beach closure or advisory occurs the day after the water sample(s) were obtained and exposures occurring on the day of sampling are not prevented. Since *E. coli* concentrations can vary greatly from day to day (Whitman, 1999; Dorevitch, 2017) monitoring programs that identify and prevent exposure on the same day are needed. One such method being developed uses a

Quantitative Polymerase Chain Reaction (qPCR) assay first introduced, and described by, Holland et. al. (1991). This "rapid" qPCR technique is different from the Colilert method in that it measures a specific *E. coli* DNA sequence resulting in the quantification of both culturable and non-culturable *E. coli*. The use of qPCR as a replacement method has been validated repeatedly (Frahm and Obst, 2003; Haughland et al., 2005; Lavender and Kinzelman, 2009; Whitman et al., 2010) and in 2014, the U.S. EPA submitted a standardized draft method for the use of qPCR to measure *E. coli* in water samples called Draft Method C. Unlike current approved methods, no incubation is needed, which reduces turn-around time. Once the samples are collected and processed in the morning, only 2 – 3 hours are theoretically needed to complete analysis, making it possible to sample and assess *E. coli* levels prior to peak beach usage times in the afternoon.

Before the new standardized qPCR method can be approved, it must be shown that laboratories with varying degrees of qPCR expertise can produce reliable and accurate results upon its implementation. One of the underlying components of Draft Method C is using pooled data from multiple individual standard curves to generate lab-specific composite standard curves. The individual standard curves are created from a series of known concentrations of a target analyte (Pfaffl et al., 2001; Rutledge et al., 2003), *E. coli* for our research, and the lab-specific standard curves are then used to determine the amount of *E. coli* DNA in water samples. To ensure the lab-specific composite curves will produce accurate results, individual standard curve acceptance criteria have been proposed. The purpose of Chapter 2 was two-fold: to assess the model chosen for generating standard curves and to determine the amount of inter-lab variation present after controlling for curves that passed the acceptance criteria proposed for Draft Method C. I hypothesized that: 1) selection of a simpler, more user-friendly model (i.e. Weighted Linear Regression) for generating a composite standard curve to quantify *E. coli* in recreational water samples is comparable to a more sophisticated Bayesian Master Standard Curve model used to set the standard curve acceptance

criteria for Draft Method C, and 2) the standard curve acceptance criteria proposed in Sivaganesan et al., (2019) for the intercept, slope and, R^2 will reduce the amount of inherent method and analyst variability associated with qPCR techniques to ensure data obtained with Draft Method C are reproducible and accurate. Our analysis is one of the first steps in demonstrating whether Draft Method C will be usable across the country as a rapid standardized method for testing *E. coli* in recreational water. If the outcome of our analysis shows that multiple labs can reliably produce similar results when implementing Draft Method C, it documents that the proposed standardized qPCR method could be a replacement for the more time intensive Colilert method. The expedited qPCR method would provide same-day bacterial water quality results thus protecting recreators from potentially contacting harmful pathogens in the water on the day where the risk is high versus the following day when the risk may be lower.

Another aspect of introducing a new standardized method is to understand how it compares to the already approved method. Environmental variables such as rainfall or air and water temperature are used in models that predict microbial water quality exceedances based on already approved quantification methods (Frick et al., 2008; Byappanahalli et al., 2010). The purpose of Chapter 3 was to better understand how environmental variables observed during sampling would impact *E. coli* concentrations measured with the Colilert and qPCR methods at 14 inland lake and 6 Lake Michigan beaches within Muskegon County, MI. I hypothesized that, although there are differences in the underlying methodologies of the two methods, environmental variables would impact *E. coli* concentrations measured with the Colilert and qPCR methods similarly. More specifically, I predicted that (1) higher turbidity, amounts of rain, and number of birds will be associated with higher *E. coli* concentrations; (2) higher air and water temperatures and longer periods of time since rain occurred prior to sampling, will be associated with lower *E. coli* concentrations; (3) *E. coli* concentrations measured with Colilert would be lower than when

measured using qPCR; and (4) inland lakes will have higher *E. coli* concentrations than Lake Michigan beaches when quantified using both the Colilert and proposed qPCR methods, all with respect to their *E. coli* concentration distributions. To perform these analyses, I assumed environmental variables recorded at the center sampling site of Lake Michigan beaches were the same at the north, center, and south sample sites, and therefore the same measurement or recorded observation was used for all three sites at the same beach. Additionally, because *E. coli* concentrations measured at the same beach on a daily and hourly basis are largely variable (Whitman and Nevers, 2004; Wyer et al., 2018), the variability in *E. coli* concentrations measured on a weekly basis at the same site on the same beach in our study could be treated as independent from each other and repeated measures analysis was unnecessary. This information will inform predictive models created for microbial water quality about whether the two analytical methods impact *E. coli* concentrations similarly, thereby protecting the public's health from exposure to unsafe swimming conditions. Since up to 40% of observations contained no detectable *E. coli*, statistical analysis that allowed censored data to be included was needed. Therefore, survival analysis was used to provide a more representative picture of the impact of environmental parameters on *E. coli* distributions than studies that replace or remove the censored data.

The scope of this research includes a large data set from a 2016 and 2018 multi-lab Draft Method C validation study. A diverse group of labs took part in both the 2016 (21 labs across the Midwest and Southeast United States) and 2018 (9 labs across Michigan) studies, and included government, health department, and academic labs where analysts had varying degrees of experience with Draft Method C and qPCR. This research also included *E. coli* concentration data collected from 14 inland lake and 6 Lake Michigan public beaches within Muskegon County, MI. The county has approximately 42 km (26 miles) of Lake Michigan shoreline which plays a large role in its

economy. Following Chapters 2 and 3 are an extended review of the literature, extended methodology and bibliography.

Chapter 2 : A multi-laboratory comparison study of proposed standard curve acceptance criteria for U.S. EPA Draft Method C

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2.1 Abstract

Draft Method C is a proposed standardized method for the absolute quantification of *Escherichia coli* (*E. coli*) in recreational waters. It uses a lab-specific composite curve generated from pooled results of individual standard curves. Three standard curve acceptance criteria proposed for individual curves to ensure results obtained with this method are of high quality are: intercept (36.66 to 39.25), slope (−3.23 to −3.74), and R^2 (≥ 0.98). To test if Draft Method C will provide reproducible and accurate results, we compared intercept and slope estimates calculated with Bayesian Master Standard Curve (MSC) and Weighted Linear Regression (WLR) models using data generated by twenty laboratories in a 2016 method validation study. There was < 1% difference between the two models in the overall group mean and median intercept and slope estimates and when comparing individual labs' intercept estimations from the two models. Percent differences in slope estimates between the WLR and MSC models of individual labs ranged from 0.0 – 2.2%. We also analyzed inter-lab variability in the three acceptance criteria produced by passing curves from the WLR model in a 2018 data set. Significant pairwise differences were detected in four of the 36 (11%) inter-lab intercept comparisons and no differences were detected in the slope or R^2 parameters. Our results support the use of Draft Method C as a rapid method for *E. coli* enumeration in recreational water samples because little variation was seen in the standard curve

acceptance criteria between labs, suggesting that the method can produce reliable and consistent results.

Keywords: qPCR, standard curve model, multi-laboratory study, Draft Method C, *E. coli*, absolute quantification, beach monitoring, fecal pollution, recreational water

2.2 Introduction

Quantitative real-time Polymerase Chain Reaction (qPCR) has become a valuable tool for scientific research due to its specificity, analysis speed, and sensitivity. The technique has been implemented in environmental science and public health fields, where it is currently under regulatory consideration as a means for the rapid testing of fecal indicator bacteria (FIB) in recreational water (RW) samples. Since contact with fecal contaminated water increases the likelihood of developing RW illnesses (Dufour, 1984; Wade et al., 2006; Wade et al., 2008) such as ear infections, swimmer's itch, and gastrointestinal issues (Wade et al., 2008; Seyfried et al. a, 1985; Seyfried et al., b, 1985), FIBs are used to alert beach managers and goers to the presence of fecal contamination (DEQ, 2006; U.S. EPA, 2012). Furthermore, current bacterial recreational water quality criteria are based on the risk of contracting gastrointestinal illness after contact with fecal-contaminated water (Dufour, 1984; Cabelli & Dufour, 1982; U.S. EPA, 1986, 2012). Therefore, to reduce the risk of recreational exposure, public beaches are routinely monitored for the presence of FIB.

Enforcing recreational water quality guidelines across many localities requires a universal method—a method robust to the varying environmental conditions intrinsic to water bodies (i.e. turbidity and organic content), easily performed by laboratory analysts, and shown to produce reliable, consistent and accurate results. Draft Method C is being developed by the United States Environmental Protection Agency (U.S. EPA) as a universal, standardized qPCR assay for absolute quantification of *Escherichia coli* (*E. coli)*, a potentially pathogenic FIB specific to the gut of

endothermic ("warm-blooded") animals (Whitman et. al., 1999). Absolute quantification by qPCR is achieved by fitting a standard curve, also called a calibration curve, to data consisting of the base-10 logarithms (log10) of a series of known concentrations of target DNA (the standards) and the corresponding measured threshold cycles (Ct values; Pfaffl et al., 2001; Rutledge et al., 2003). The fitted standard curve is used to estimate the initial copy number of the target DNA sequence in RW samples based on their measured Ct values.

Traditionally, qPCR assays include a single standard curve per plate and apply the fitted standard curve only to the samples on the same plate (Converse et al., 2009; Noble et al., 2010; Shanks et al., 2016). This procedure ensures the standard curve used to estimate initial copy numbers in the unknowns reflects the identical conditions, which differ from run to run, under which the samples were prepared and processed (e.g. small differences in master mix preparation). However, this reduces the number of samples that can be analyzed on each plate. A typical standard curve uses fifteen to eighteen wells, making it inefficient in terms of time and supply costs when large numbers of samples will be analyzed. An alternative approach is to have each lab analyze several independent standard curves prior to running samples, then create a single lab-specific composite standard curve by pooling the results (U.S. EPA, 2012, 2013, 2014). Subsequent plates are then populated with samples and quality control measures, such as sample processing controls and positive/negative controls, to determine if the sample was analyzed correctly or to identify matrix interferences which would inhibit the qPCR reaction. Samples are analyzed separately from the lab-specific composite standard curve, increasing sample processing efficiency. When using this method, uncertainty in concentration estimates increases since the lab-specific composite standard curve used to calculate concentrations in samples no longer accounts for the exact conditions during each instrument run. Thus, there is a trade-off between precision and efficiency.

Two methods for generating a lab-specific composite curve are: 1) a hierarchical Bayesian method to generate a Master Standard Curve (MSC) (Sivaganesan et. al., 2008; Green et al., 2014), and 2) a classical Weighted Linear Regression (WLR) model (U.S. EPA, 2014). The Bayesian MSC method (Sivaganesan et. al., 2008) requires specialized statistical software and therefore is not suitable for use by labs with limited statistical capabilities. Instead, Draft Method C will use Microsoft Excel software to generate a lab-specific composite standard curve from pooled results of independently analyzed standard curves within each lab implementing the method using a WLR model to quantify *E. coli* concentrations in RW samples. The WLR model was chosen over the Bayesian MSC method mainly because its simplicity permits implementation of the method using the more familiar Excel software.

Once an appropriate absolute quantification calibration model has been selected, it is important to assess model performance (i.e. reliability and consistency) because its reliability and robustness must be demonstrated before the method can become U.S. EPA approved. The use of quality assurance and quality control procedures, such as standard curve acceptance criteria, will help to ensure data quality and composite standard curve performance are acceptable (Shanks et. al., 2016). Standard curve acceptance criteria have been proposed for Draft Method C which include ranges for the y-intercept (hereafter referred to as intercept) and slope of individual standard curves as determined by a Bayesian MSC model (Sivaganesan et al., 2019). Additionally, Draft Method C has a designated acceptance criterion for a standard curve's R^2 value.

The goals of the present study were two-fold: 1) determine if the WLR standard curve model selected for Draft Method C yields results similar to the Bayesian MSC method and 2) determine the amount of variation produced after controlling for curves passing the standard curve acceptance criteria. To achieve these goals, we 1) used the WLR model to calculate the mean intercept, slope, and 95% confidence intervals (CI) of lab-specific composite curve estimates with Ct values

produced during a 2016 Draft Method C validation study and compared them with the mean estimates obtained using the Bayesian MSC for each lab by visually assessing side-by-side plots and calculating the percent difference in mean estimates between the WLR and MSC models and 2) evaluated inter-lab variability of the intercept, slope, and R^2 parameters estimated with the WLR model for individual standard curves analyzed in 2018.

2.3 Materials and Methods

Both the 2016 and 2018 studies used the *E. coli* EC23S857 qPCR assay, a specific region on the 23S rRNA gene of *E. coli* that is detected and amplified, and the same methods of DNA extractions and qPCR analysis. Methods used to construct standard curves for the 2016 Draft Method C validation study (Bayesian MSC) are described in detail by Sivaganesan et al. (2019). Methods used to construct the 2018 standard curves (WLR) are described below.

Participants

A diverse group of 21 labs from across the midwestern and southeastern United States participated in studies conducted in 2016, and 9 labs from across the state of Michigan participated in 2018 (Table S2.1). Government, university and county health department labs were represented in both studies. In each study, labs were assigned a unique code: '1' through '21' in 2016 (Sivaganesan et al., 2019) or 'A' through 'I' in 2018 to maintain anonymity when referring to them.

Standards Instructions

Standards (estimated copy numbers: standard 1 = 25,822.6; standard 2 = 3,396.25; standard 3 = 417.83; standard 4 = 54.83; standard 5 = 11.61) were prepared and verified at the U.S. EPA Cincinnati laboratory, as described in Sivaganesan et al. (2019), then shipped to a central lab that managed their distribution to the remaining labs. Standards were shipped overnight on dry ice. The U.S. EPA recommended that standards should be stored in 20 µL aliquots at −80°C until ready for

use. Each time a standard curve was analyzed, a set of the five concentrations of aliquoted standards were removed from the freezer and any unused standard was discarded.

qPCR Assay

In addition to the EC23S857 *E. coli* qPCR assay, a Sketa22 (salmon DNA) qPCR assay also was used. The Sketa22 assay amplifies a segment of the internal transcribed spacer region 2 (ITS2) of the salmon rRNA gene operon and was used as a sample processing and reaction inhibition control. The reporter molecule was FAM and the quencher molecule was TAMRA. Assay master mix (MM) was prepared by combining TaqMan™ Environmental MM 2.0 (Thermo Fisher Scientific, Grand Island, NY) (12.5 µL), 2.0 mg/mL stock solution bovine serum albumin (BSA) from fraction V powder (Sigma B-4287 or equivalent) (2.5 µL) dissolved in PCR-grade water, 500 µM stock solution of *E. coli* or sketa22 forward and reverse primers combined with 100 µM stock solution of *E. coli* or Sketa22 probes (3.0 µL) (Invitrogen or equivalent) (Table 2.1), and qPCR-grade water (2.0 µL). Volumes in parentheses are volumes specified in Draft Method C to use per 25 µL qPCR reaction being carried out. Two standard curves were permitted to be analyzed on the same 96-well plate (Thermo Fisher Scientific) provided separate batches of MM were prepared for each curve. A single lot of TaqMan™ Environmental MM 2.0 (Table 2.1) was used by all eight labs (Lot#180115) and confirmed to have no underlying *E. coli* contamination by analyzing six No Template Controls (NTC) of AE buffer (Qiagen), run in duplicate (Saginaw Valley State University). All NTC runs resulted in an 'Undetermined' Ct value; meaning no fluorescence was detected above the background 'noise' or fluorescence signal, indicating the absence of *E. coli* DNA template in the TaqMan™.

Table 2.1 *E. coli* and salmon DNA forward ('F')/reverse ('R') primer sequences and TaqMan™ probe sequence used in Draft Method C.

Assay	Primer/probe name	Locus	Forward & reverse primer sequence (5' to 3')	TaqMan™ probe sequence
E. coli	EC23S857	16S rRNA	F: GGTAGAGCACTGTTTTGGCA	(6-FAM)-5'-TCATCCCGACTTACCAACCCG-TAMRA
			R: TGTCTCCCGTGATAACTTTCTC	
Salmon DNA	Sketa22	23S rRNA	F: GGTTTCCGCAGCTGGG	(6-FAM)-5'-AGTCGCAGGCGGCCACCGT-TAMRA
			R: CCGAGCCGTCCTGGTC	

DNA Extraction (Filter blanks and calibrators only)

Each standard curve required three calibrator filters (positive controls; 1 x 10^4 *E. coli* cell equivalents) and three filter blanks (negative controls) prepared on separate sterile disposable MicroFunnel™ Filter Funnels (Pall Corporation, Ann Arbor, MI or equivalent) or re-useable filter funnel units. Calibrators were prepared by filtering 1 mL of a 1 x 10^4 *E. coli* suspension in sterile Phosphate Buffer Saline (PBS) (pH = 7.4 ± 0.2), through a polycarbonate 47 mm diameter with 0.45 μm pore size filters (Millipore or equivalent) seated on a sterile filtration unit. The same procedure was used to filter 20 mL of PBS for filter blank preparation. Filters from calibrators and filter blanks were folded in half four times and placed in a 2.0 mL sterile semi-conical, screw-cap microcentrifuge tube containing 0.3g (± 0.01g) of acid-washed glass beads. Next, 600 μL of AE Buffer spiked with 0.2 μg/mL salmon DNA (sketa22) was added to each extraction tube and tightly sealed. Extraction tubes were bead milled for 1.0 min at 5,000 rpm then centrifuged at 12,000 x g for 5 min. Approximately 400 μL of the crude DNA extract was removed and transferred into a clean correspondingly labeled, sterile 1.7 mL low retention micro-centrifuge tube and centrifuged again for 1 min at 12,000 x g, then ~ 100 μL of the clarified supernatant was transferred into a second sterile centrifuge tube. Calibrator and filter blank extracts were analyzed shortly after extraction along with each standard curve.

Plate Setup

Extracted DNA from calibrator and filter blanks were analyzed in duplicate with both *E. coli* and salmon MM separately. NTC and standards 1–5 were tested in triplicate solely with *E. coli* MM. (Figure S2.1). Each well contained 25 µL of final reaction volume; 20.0 µL of prepared MM, as described in section 2.3, and 5.0 µL of the supplied *E. coli* DNA standard or positive/negative control or NTC.

Instrument Run Method

Thermocycling consisted of an initial 'holding stage' (50.0°C, 2 min; 95.0°C, 10 min) followed by 40 cycles of DNA denaturation and primer/probes annealing (95.0°C, 15 s; 56.0°C, 1 min). At the end of each of the 40 cycles, fluorescence was measured by the instrument (StepOnePlus, Applied Biosystems). The fluorescence threshold, or the level of fluorescence in which the signal rises above the background level, was manually set to 0.03 ΔRn and baseline cycles were set to AUTO determination (Sivaganesan et al., 2019).

Standard Curve Generation

Upon completion of the instrument runs, data were exported from the StepOne™ Software (v2.3) and the resulting Ct values were copied into an accompanying Draft Method C Excel workbook where they were fitted to the WLR model of the form:

$$X_{ijk} = \alpha_i + \beta_i \log_{10}(X_j) + \varepsilon_{ijk} \quad (1)$$

where X_{ijk} is the observed Ct value for replicate k of standard j in run i; α_i and β_i are the intercept and slope, respectively, for run i; X_j is the known copy number in standard j, and ε_{ijk} is the statistical error in the observed threshold cycle. A separate WLR was fitted to data for each standard curve run, and the externally Studentized residuals (Cook and Weisberg, 1983; p. 20) were examined to identify and remove up to two outliers from each data set if needed. The WLR model was then re-

fitted to the retained data for each run, and the intercept, slope, and R^2 values for each standard curve were assessed for acceptability based on the proposed standard curve acceptance criteria developed for Draft Method C (Table 2.2). When all three parameters met the acceptance criteria, an individual curve was considered 'passing' but, if any one of the three parameters failed, a curve was considered 'failing'. Draft Method C requires a minimum of four passing individual cures to generate the lab-specific composite curve used to analyze RW samples therefore, if there were fewer than four passing, labs were requested to analyze additional curves. Only passing curves were considered for further statistical analysis of the 2018 data.

Table 2.2 Standard curve acceptance criteria for Draft Method C

Parameter	Criteria	Reference
Intercept	36.66 to 39.25	(Sivaganesan et al., 2019)
Slope	−3.23 to −3.74	(Sivaganesan et al., 2019)
R-squared (R^2)	≥ 0.98	(Draft Method C and U.S. EPA, 2014)

Data Analysis

Bayesian MSC and WLR Standard Curve Model Comparisons

Labs participating in the 2016 validation study analyzed four to five standard curve assays each, as described in Sivaganesan et. al, (2019) for a total of ninety-one curves analyzed. To obtain the WLR model values, intercept and slope data for each lab's separate passing standard curve runs were assessed with the Draft Method C Excel workbook which uses an analysis of covariance (ANCOVA) to determine if there was evidence ($\alpha = 0.01$) that any parameter estimates differed among runs. If not ($p \geq 0.01$ for both parameters), results from the individual curves were pooled, and a WLR was performed to estimate the lab-specific composite curve intercept, slope and 95% CI for each lab. Mean intercept and slope estimates from the Bayesian MSC model for individual labs were taken from the 2016 validation study (Sivaganesan et al., 2019); the 95% Bayesian Credible Intervals (BCIs) are provided in the supplemental material (Table S2). The ANCOVA evaluation

was used in the WLR model only, and not the Bayesian MSC model. Intercept and slope estimates, and corresponding 95% CIs and BCIs, from the WLR and Bayesian MSC models, respectively, for each lab were plotted and compared visually. Comparisons also were made by examining the relative percent difference between the WLR and MSC models of the intercept and slope estimates for each lab. Percent differences were calculated by dividing the absolute value of the difference between the two models' mean estimates by the average of the absolute values of the two models' estimates and multiplying by one hundred.

Inter-lab variability of 2018 standard curve WLR estimated acceptance criteria

Each of the nine labs participating in 2018 used the WLR calibration model in the Draft Method C Excel workbook to analyze the instrument-determined Ct values for each individual standard curve and the workbooks were then shared with the authors of this study. Each lab produced between four and ten standard curves for a total of eighty-two. Of these curves, only those that passed all three acceptance criteria were considered for further statistical analysis.

Potential differences between acceptance criteria estimates produced by individual labs were assessed by determining the statistical significance of parameter differences and, where a statistically significant difference was detected, the relative magnitude of the difference on a percent scale was determined, as described above. The main goals of the assessment were to determine the proportion of labs for which a statistically significant difference was detected for each parameter and, more importantly, the relative magnitude of any detected differences.

Inter-lab comparisons were carried out using a pairwise Wilcoxon rank sum test (Hollander et al. 2014) on each parameter; *p*-values adjusted with a Holm correction were used to account for multiple comparisons to test the null hypothesis that the locations of the distributions of reported intercept, slope, and R^2 estimates were the same for each pair of labs against the two-sided alternative hypothesis that they were different. The Wilcoxon rank sum test is a nonparametric test

and does not assume a specific distribution for the individual estimates. All statistical analyses were performed using R Software (v3.5.2; R Core Team, 2018). Prior to any analysis, two data points from one lab (code H) were removed due to knowledge of errors during plate sealing.

2.4 Results

Bayesian and Weighted Linear Regression Standard Curve Model Comparisons

All passing standard curves from the 2016 validation study had intercept and slope estimates that passed the ANCOVA test which allowed for a WLR composite standard curve to be produced for every lab. However, lab code 21 was excluded from the model comparisons since it was not used in the Bayesian MSC analysis (Sivaganesan et al., 2019).

Intercept: Estimates of the intercept and the corresponding 95% CI and BCI calculated from WLR and Bayesian MSC models were similar within each lab (Figure 2.1). The overall means of the WLR and MSC Ct intercept estimates across all twenty labs were 38.01 and 37.98, respectively; the overall medians of the WLR and MSC estimates were 37.97 and 37.96, respectively. Thus, there was < 1% difference between the two models for the overall mean and median intercept Ct estimates. Across all twenty labs there was a < 1% average difference between the WLR and MSC intercept estimates and there was a < 1% difference between the WLR and MSC intercept estimate when comparisons were made within each of the individual labs (Table 2.3).

Slope: The slope estimates calculated from the WLR and Bayesian MSC models also exhibited similar means and 95% CIs and BCIs, respectively (Figure 2.2). The overall means of the WLR and MSC slope estimates were −3.50 and −3.49, respectively; the overall medians of the WLR and MSC slope estimates were −3.51 and −3.50, respectively. As determined for the intercept estimates above, there again was < 1% difference between the overall mean and median of the two models. Assessment of the overall average difference of individual labs was < 1% difference

between the WLR and MSC slope estimates. Percent differences between slope estimates calculated with two models ranged from 0.0 – 2.2% (Table 2.3).

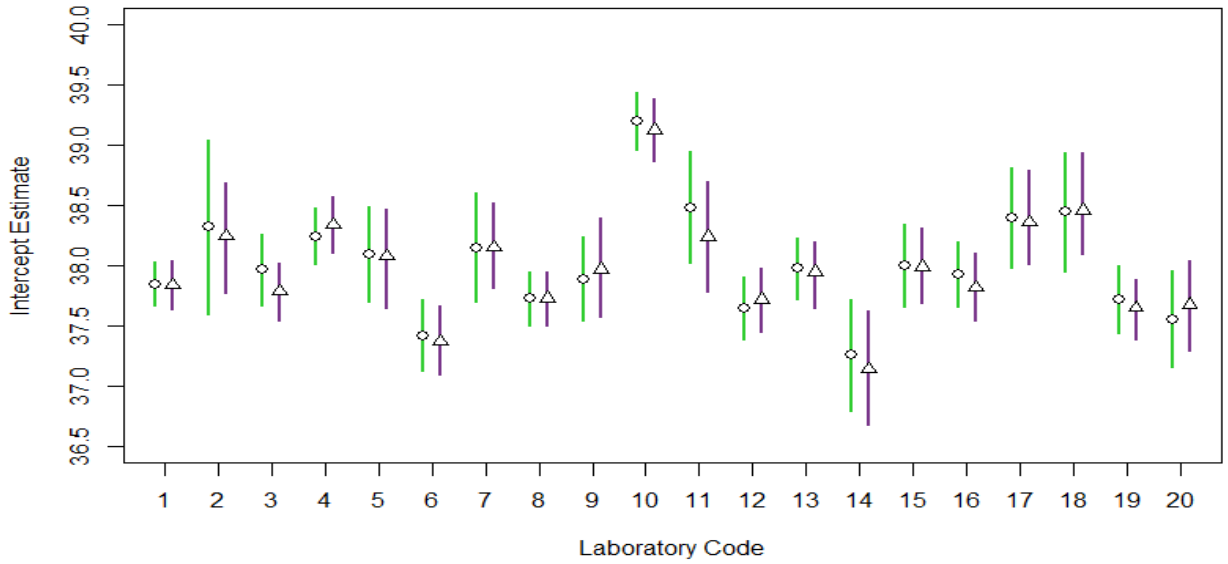

Figure 2.1 Comparison of mean intercept estimates for the 2016 standard curve composite WLR (green bars with open circles) and Bayesian MSC (purple bars with open triangles) models. Open circles and triangles on bars indicate the mean least-squares and Bayesian intercept estimates, respectively. Bars represent the WLR 95% CI (green) and MSC 95% BCI (purple).

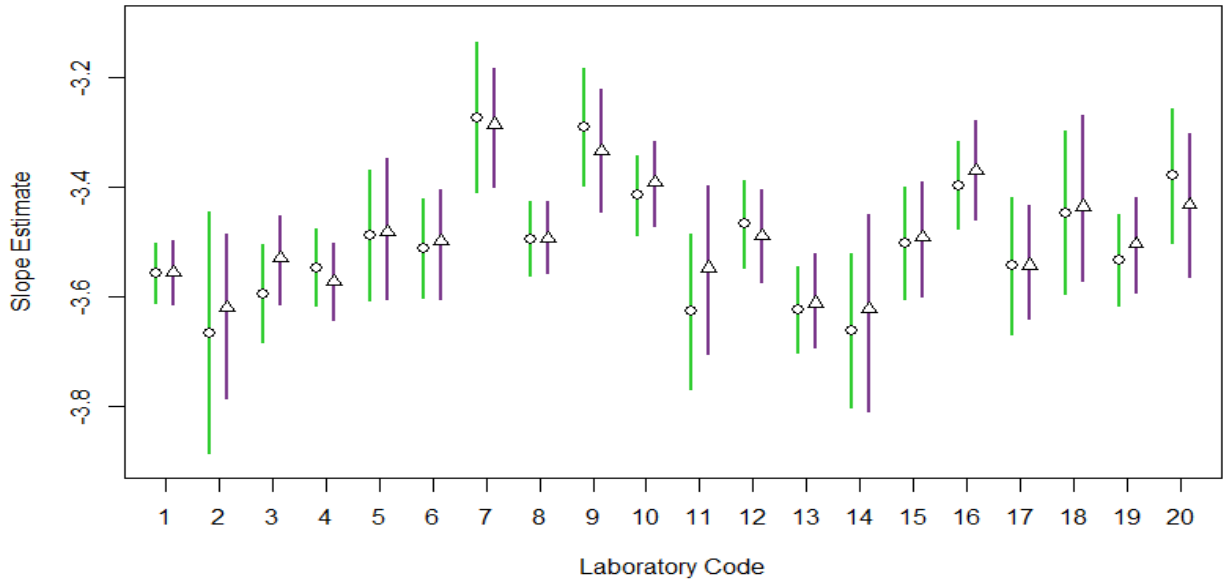

Figure 2.2 Comparison of slope estimates of the 2016 standard curve composite WLR (green bar with open circle) and Bayesian MSC (purple bar with open triangles) models. Open circles and triangles on bars indicate the mean least-squares and Bayesian slope estimates, respectively. Bars represent the WLR 95% CI (green) and Bayesian MSC 95% BCI (purple).

Table 2.3 Percent differences between WLR and Bayesian MSC estimates of intercept and slope parameters for individual labs.

Lab Code	Percent (%) Difference: Intercept	Percent (%) Difference: Slope
1	0.02	0.03
2	0.18	1.3
3	0.47	1.8
4	0.25	0.76
5	0.03	0.20
6	0.13	0.37
7	0.00	0.37
8	0.01	0.00
9	0.21	1.3
10*	0.18	0.71
11	0.64	2.23
12	0.20	0.63
13	0.07	0.33
14*	0.31	1.1
15	0.10	0.46
16	0.29	0.77
17	0.10	0.03
18	0.04	0.32
19	0.18	0.85
20	0.33	1.6

*Denotes labs using BioRad instrument

Inter-lab variability of 2018 standard curve WLR estimated acceptance criteria

Overall, sixty-three of the eighty-two (76.8%) standard curves analyzed in 2018 passed all three acceptance criteria for intercept, slope, and R^2. The intercept criterion was most frequently met, with seventy-four curves (90.2%) meeting the proposed criterion (Figure 2.3). Seventy-three curves passed the R^2 criterion (89.0%), and seventy-two curves met the slope acceptance criterion (87.8%) (Figure 2.3). Statistical comparisons of the reported estimates of intercept, slope, and R^2 were restricted to sixty-three curves that met all three criteria.

Four of the thirty-six (11.1%) inter-lab intercept estimate (Figure S2.2) pairwise comparisons resulted in statistically significant differences ($p \leq 0.05$) (Table S2.3a). Percent differences between labs whose WLR estimates of intercept were statistically significantly different are shown in Table

2.4. No inter-lab pairwise differences in mean slope (Figure S2.3) and R^2 (Figure S2.4) estimates were detected ($p > 0.05$) (Tables S2.3b and S2.3c).

Table 2.4 Percent differences between mean intercept estimates of passing standard curves for pairs of labs in the 2018 study. Only pairs of labs that showed a statistically significant difference are shown.

Lab Codes	Percent (%) Difference: Intercept
A–G	1.4
E–H	1.9
F–G	1.8
G–H	2.1

2.5 Discussion

To the best of our knowledge, this was the first study to compare intercept and slope estimates obtained with the Bayesian MSC model to those obtained with the WLR model and assess inter-lab variability of three proposed Draft Method C standard curve acceptance criteria across multiple labs within different regions. Comparisons of the two calibration models showed a < 1% difference between the mean and median intercept and slope estimates. Additionally, all differences between the Bayesian MSC and WLR estimates of intercept and slope for individual labs were below 3%. Inter-lab pairwise comparisons of WLR 2018 data detected the highest number of statistically significant differences in intercept estimates among labs, with four of the thirty-six pairwise comparisons showing differences and a relative percent difference between labs of only ≤ 2.1%. Statistically significant pairwise differences of slope and R^2 were not detected.

While a well-studied qPCR method for measuring *Enterococcus* (U.S. EPA, 2012; U.S. EPA, 2013; Sivaganesan et al., 2014; Haugland et al., 2005; Whitman et al., 2010) already exists and is associated with gastrointestinal illness in marine waters (Colford et al., 2012), *E. coli* enumeration may be a better predictor of gastrointestinal illness in freshwater systems (Pruss et al., 1998). A meta-

analysis of RW quality literature published from 1950 to 2003 reported that most studies indicated *E. coli* as being a more suitable predictor of gastrointestinal illness in freshwater than other bacterial indicators (Wade et al., 2003). Therefore, a standardized qPCR method for quantification of *E. coli* in freshwaters, such as Draft Method C, is needed to protect public health.

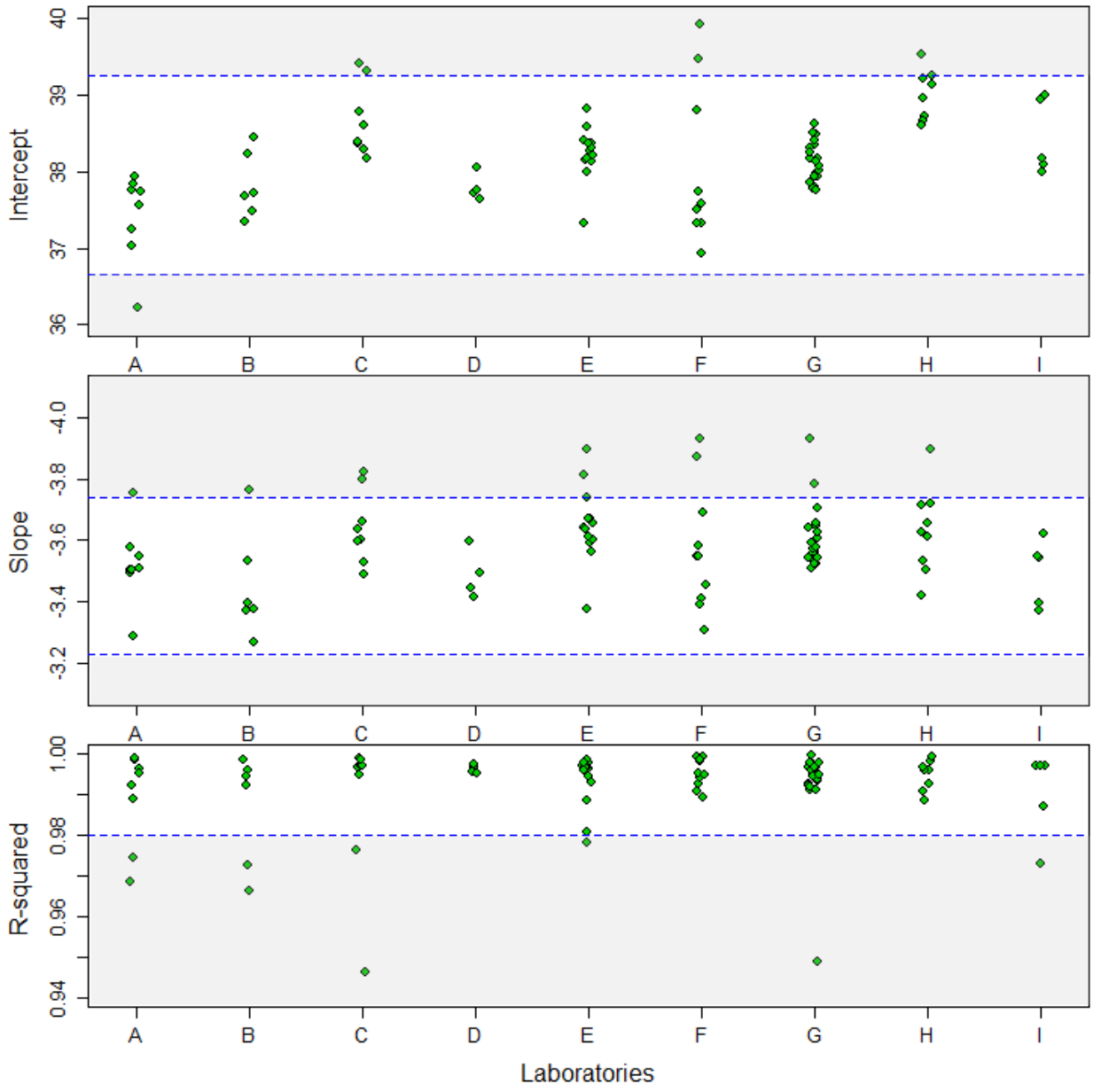

Figure 2.3 Summary of all 2018 standard curve acceptance criteria for participating labs (codes A-I). Blue dashed horizontal lines represent the acceptance criteria value(s). Points in shaded area did not meet the acceptance criteria.

Calibration Model Selection

Absolute quantification of *E. coli* by qPCR requires a standard curve model. A recent study compared gene copy estimates in water samples obtained with the Bayesian MSC model to those obtained using the delta-delta Ct (ΔΔCt) model, the absolute quantification method used to enumerate *Enterococcus* in RW (U.S. EPA, 2012, 2013), and found no statistically significant differences in estimates, although the MSC model exhibited overall higher gene copy estimates in all samples (Aw et al., 2019). Similarly, our study compared two standard curve models, the Bayesian MSC model, used to set the Draft Method C acceptance criteria, to those obtained with the WLR model, used in the current Draft Method C Excel workbook, and found very little variability in the means and medians of intercept and slope estimates (< 1%) calculated with both models indicating that parameter estimates from the two quantification models are quite similar. This result supports selection of the simpler, more user-friendly WLR model to be used in Draft Method C for RW sample analysis.

Although the intercept and slope estimates produced with the two different standard curve models are similar within a single lab, visual assessment of Figures 2.1 and 2.2 indicate the presence of some variability between labs. In 2016, all labs did not have −80°C storage, which may have caused the variation (Aw et al., 2019), compared to 2018 when all labs did have the recommended sample storage capabilities. Proper storage and handling of DNA standards is important to qPCR method performance (Sivaganesan et al., 2010; Sivaganesan et al., 2008; Shanks et al., 2016; Bustin et al., 2009), therefore suboptimal storage conditions of standards could cause DNA to degrade and subsequently impact the standard curve assay outcome (Dhanasekaran et al., 2010).

Interlaboratory Comparisons of 2018 Acceptance Criteria

The theory underlying Eq. (1) suggests that variation should be more pronounced in the intercept than in the slope, because the slope is smaller in absolute value and reflects inter-run

variation in only a single underlying parameter (the amplification factor) while the intercept is larger and reflects inter-run variation in two underlying parameters (the amplification factor and fluorescence of the passive reference dye) thus more variability in the intercept criterion was expected. However, calibration curves in our study were analyzed in multiple labs on different instruments and were therefore more likely to exhibit greater variability from instrumental and analyst factors (Sivaganesan et al., 2008) than because of the underlying standard curve model theory.

The coefficient of determination (R^2) for a linear ordinary least-squares regression model indicates the proportion of total variation in the response variable that is attributable to (or explained by) the predictor variable. The lack of detected significant differences in R^2 values indicated there was little variation among labs in this measurement of curve acceptance and that the predictor variable ($\log_{10}$ of the initial copy number) consistently accounted for a very high proportion of total variation in the response variable (Ct value). Overall, 89% of standard curves analyzed in 2018 had a passing R^2 acceptance criterion, indicating that the proposed 0.98 acceptance value was replicable in practice by multiple labs.

Future Considerations

Variation and error can be introduced during each step of qPCR analysis (Baker et al., 2011). Factors such as storage time, storage temperature of standards and reagents, precision of dilutions, and pipette calibration can influence the accuracy of standard concentration measurements used to create the calibration model (Sivaganesan et al., 2008; Sivaganesan et al., 2010). Additionally, differences in the equipment used within laboratories and the ability of analysts to pipette uniformly contribute to variation in absolute quantification by qPCR. Visible differences are seen in the 2016 intercept acceptance criterion values of labs 10 and 14 that used a different brand of thermocycler than the other participants (Figure 2.1) therefore, we agree with Ruijter et al. (2013) that in-depth

studies are needed to evaluate variability introduced to standard curves when using different thermocycler instruments. Also, for some labs participating in 2016, the standards storage temperature was not at the U.S. EPA recommended temperature of −80°C, therefore we also recommend that labs implementing Draft Method C use a −80°C storage freezer. Future work evaluating the variation in the resulting lab-specific composite curves of individual labs are also recommended along with epidemiological studies to show that bacteria concentrations measured with Draft Method C are associated with predicting recreators' risk of illness.

Further experimental studies are needed to evaluate intra-laboratory variation when implementing Draft Method C to better understand how individual analysts perform based on their experience. Pipetting is a fundamental element of qPCR and an analyst's ability to repeatedly and consistently pipette small volumes is vital to getting accurate results when performing Draft Method C. Lippi et al. (2017) tested whether an analyst's pipetting experience impacted their performance and saw no correlation between an analyst's age, sex or years of experience (varying from nine to forty-seven years of experience) and their pipetting precision. Conversely, Aw et al. (2019) concluded that prior experience with qPCR appeared to contribute to failure of quality control measures like those examined in this study. However, aside from knowing analyst experience with Draft Method C in the 2018 study, the extent of prior experience with qPCR or pipetting was unknown. Another key finding in the Lippi et al. study was a comparison of inter- and intra-analyst imprecision when pipetting volumes of 10 μL, 100 μL, and 1 mL of water which determined that the smaller the volume pipetted, the more error there was (Lippi et al., 2017). Draft Method C requires pipetting volumes as low as 5 μL, much lower than previously examined. This reinforces the use of acceptance criteria to ensure analysts are performing within a defined acceptable range and subsequently providing correct estimates of bacteria concentrations in RW samples.

The variability introduced by the plethora of environmental factors which occur during sampling also needs be considered when developing a standardized method. Items such as sampling frequency or time of day, number of samples collected per site, sampling season and sample depth can all introduce variability in bacterial quantification (Whitman et al., 2004). Likewise, spatial and temporal variation arise when quantifying bacterial concentrations (Whitman et al., 2004). For these reasons, a standardized method for absolute quantification of *E. coli* by qPCR is vital for ensuring results are consistent and reliable while reducing variability where possible.

Not only is it important to understand where variation occurs when implementing a standardized method such as Draft Method C, it is equally essential to understand how the variation impacts the reported *E. coli* values. The proposed individual standard curve acceptance criteria serve as a screening process where poor quality data are rejected. Therefore, it is important to know if the set criteria prevent large variation in the final *E. coli* concentrations. For example, the largest percent difference of 2.1% between lab codes G and H (Table 2.4) is not substantial in terms of percentages, but further studies are needed to better understand what a 1 – 2% difference may mean to the final *E. coli* concentration. Similarly, although no statistically significant differences were detected in passing slope or R^2 estimates generated by labs, the range of values (Slope: −3.31 – −3.71; R^2: 0.98 – 0.99) could potentially result in reportable *E. coli* quantities falling above or below the bacteria exceedance level. Analyses looking at the effect this variability could have on reported *E. coli* quantities is important because it may be the difference between a beach remaining open or an exceedance causing a beach closure, at the detriment of recreators and the surrounding businesses. If the proposed standard curve acceptance criteria need to be more stringent, the acceptance range can be reduced to limit variation in final measured *E. coli* quantities.

2.6 Conclusion

The results of this study provide evidence that absolute quantification of *E. coli* by qPCR using Draft Method C can provide reliable and reproducible results for RW samples. Our analyses of the mean intercept and slope estimates from the 2016 multi-laboratory study demonstrated that the WLR calibration model produces comparable results to the Bayesian MSC model used to set the acceptance criteria. Our pairwise comparisons of passing individual standard curve intercept, slope and R^2 estimates produced by multiple laboratories, which are used to generate the lab-specific composite curves, demonstrated little variation among labs as well. To advance these findings, further analysis is needed to determine what these detected differences mean to the magnitude of reported *E. coli* quantities measured with Draft Method C. Our findings support the use of Draft Method C as a rapid, standardized protocol for *E. coli* enumeration at public beaches.

2.7 Supplementary Materials

Supplemental Figures

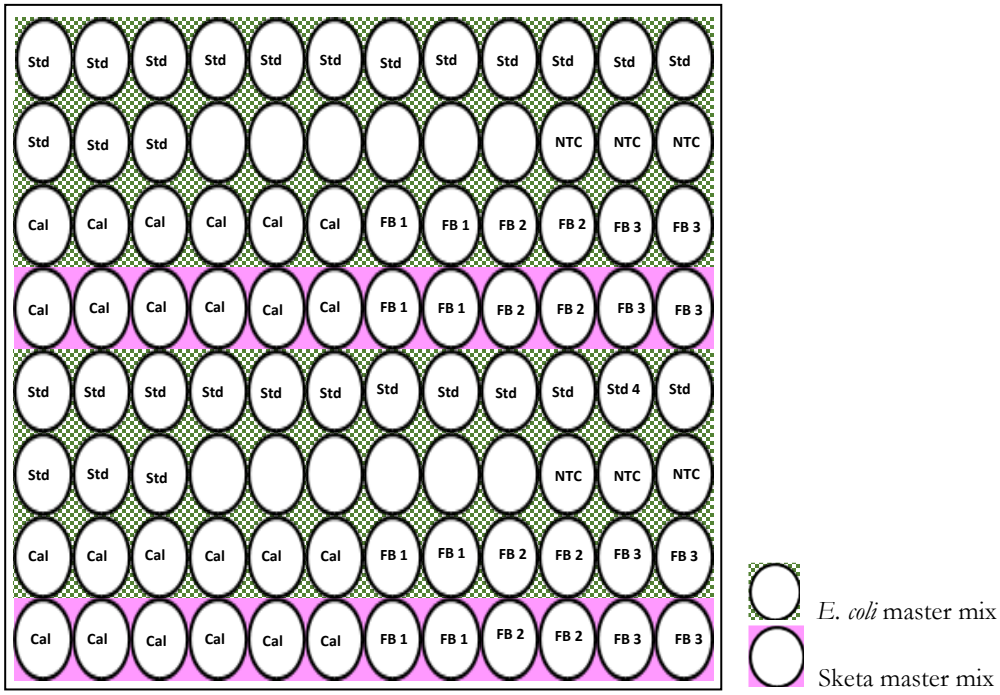

Figure S2.1 96-well plate setup for 2 standard curves per plate. NTC = No Template Control; Cal. = Calibrator; FB = Filter Blank.

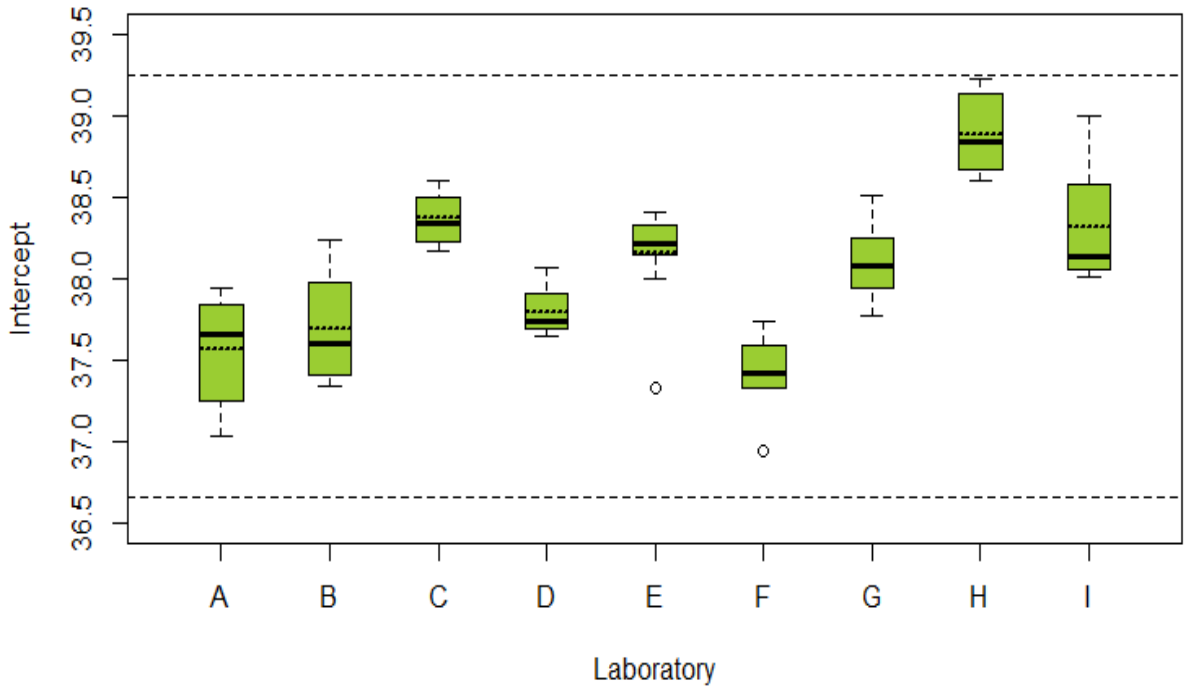

Figure S2.2 Individual labs (x-axis, codes A-I) WLR intercept estimates (y-axis) values from passing standard curves in the 2018 study. Solid and dotted lines inside boxes represents the median and mean values, respectively. Intercept acceptance criteria range (36.66 to 39.25) is shown with horizontal dashed lines across the plot area.

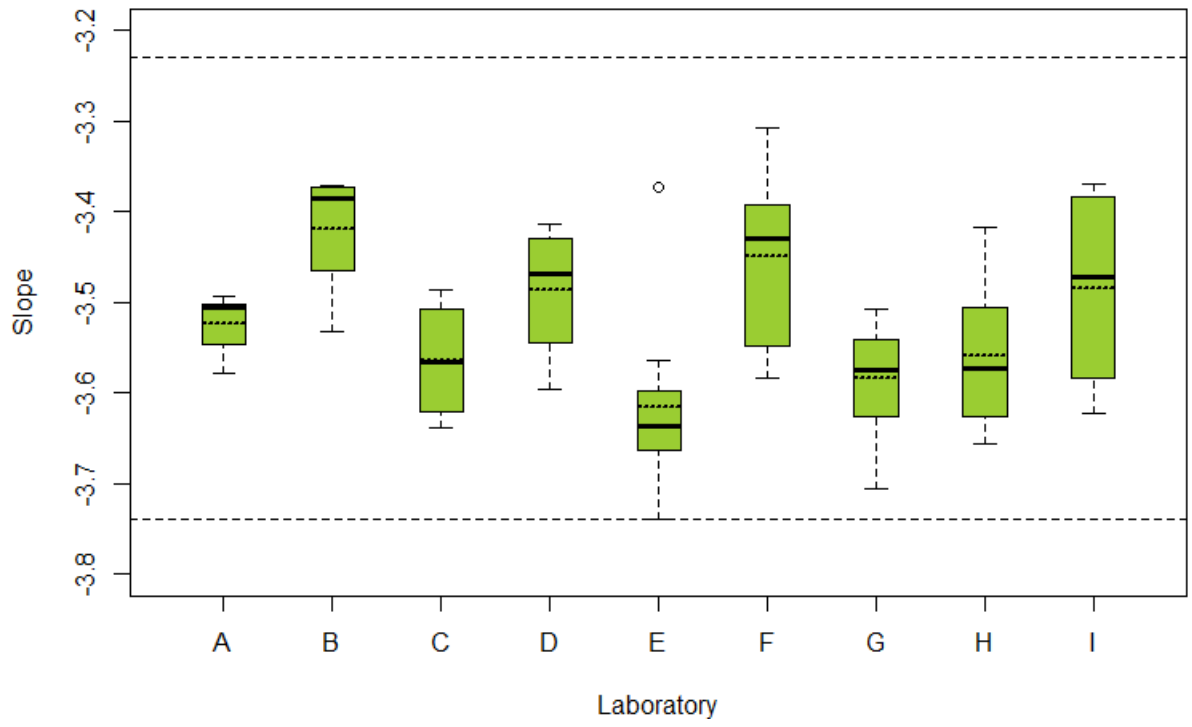

Figure S2.3 Individual labs (x-axis, codes A-I) WLR slope estimates (y-axis) from passing standard curves in the 2018 study. Solid and dotted lines inside boxes represents the median and mean values, respectively. Slope acceptance criteria range (−3.23 to −3.74).

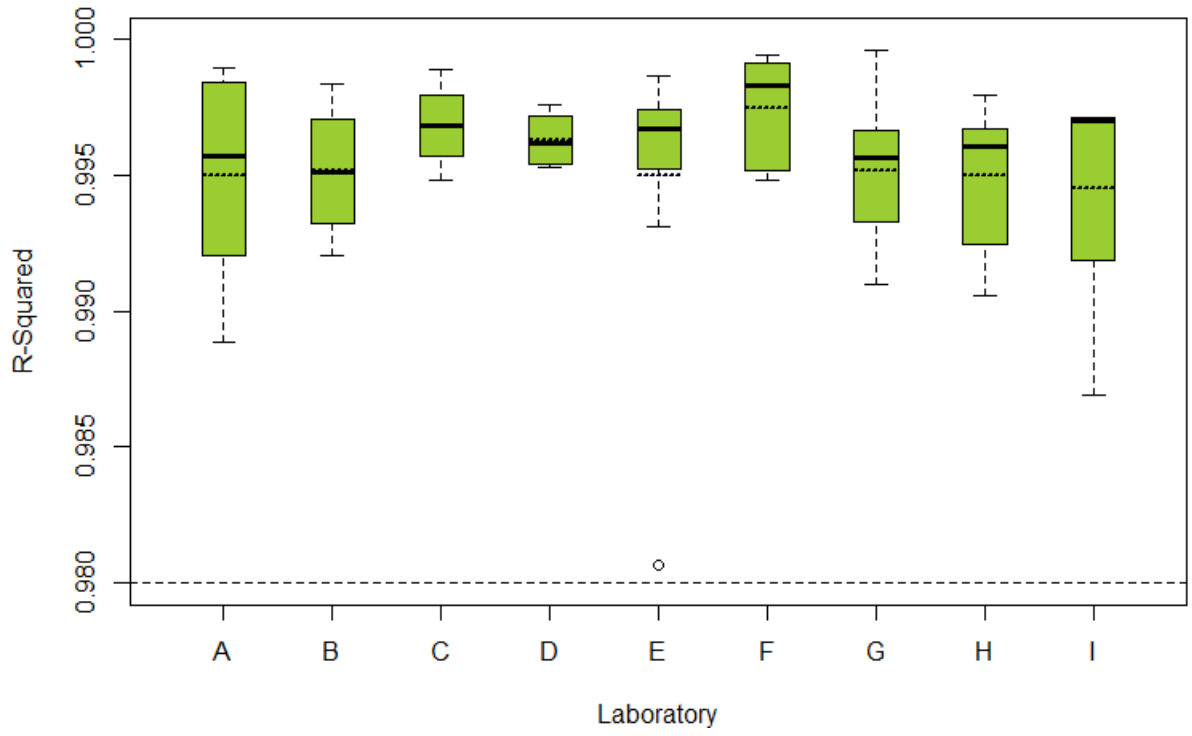

Figure S2.4 Individual labs (x-axis, codes A-I) WLR R^2 estimates (y-axis) values from passing standard curves in the 2018 study. Solid and dotted lines represent the median and mean values, respectively. R^2 acceptance criterion (0.98) is shown by a horizontal dashed line across the plot area.

Supplemental Tables

Table S2.1 Labs participating in the 2016 and 2018 standard curve studies. An 'x' indicates the year a lab participated in the study.

Lab	Location	2016	2018
Central Michigan District Health Department, Assurance Water Laboratory	Gladwin, MI	X	X
City of Racine Public Health Department	Racine, WI	X	
Ferris State University, Shimadzu Core Laboratory	Big Rapids, MI	X	X
Georgia Southern University, Department of Environmental Health Sciences	Statesboro, GA	X	
Grand Valley State University, Annis Water Resources Institute	Muskegon, MI	X	X
Health Department of Northwest Michigan, Northern Michigan Regional Laboratory	Gaylord, MI	X	
Kalamazoo County Health and Community Services Laboratory	Kalamazoo, MI	X	
Lake Superior State University, Environmental Analysis Laboratory	Sault St. Marie, MI	X	X
Marquette Area Wastewater Facility	Marquette, MI	X	X
Michigan State University, Department of Fisheries and Wildlife	East Lansing, MI	X	
Northeast Ohio Regional Sewer District, Environmental and Maintenance Services Center	Cuyahoga Heights, OH	X	
Oakland County Health Division Laboratory	Pontiac, MI	X	X
Oakland University, HEART Laboratory	Rochester, MI	X	X
U.S. EPA National Exposure Research Laboratory	Cincinnati, OH	X	
United States Geological Survey, Upper Midwest Water Science Center	Lansing, MI	X	X
U.S. National Parks Service, Sleeping Bear Dunes Water Laboratory	Empire, MI	X	
Saginaw County Health Department Laboratory	Saginaw, MI	X	
Saginaw Valley State University, Department of Chemistry	University Center, MI	X	X
University of Illinois at Chicago, School of Public Health	Chicago, IL	X	
University of North Carolina at Chapel Hill, Institute of Marine Sciences	Morehead City, NC	X	
University of Wisconsin-Oshkosh, Environmental Research Laboratory	Oshkosh, WI	X	

Table S2.2 95% Bayesian MSC Credible Intervals (BCIs) for intercept and slope parameters in the 2016 validation study. LB = Lower Bound; UB = Upper Bound.

Lab Code	Intercept		Slope	
	LB	UB	LB	UB
1	37.64	38.04	−3.615	−3.497
2	37.77	38.69	−3.786	−3.486
3	37.54	38.02	−3.615	−3.452
4	38.10	38.57	−3.643	−3.501
5	37.65	38.47	−3.604	−3.348
6	37.09	37.67	−3.604	−3.404
7	37.81	38.52	−3.399	−3.182
8	37.50	37.95	−3.558	−3.426
9	37.57	38.40	−3.446	−3.220
10	38.87	39.39	−3.472	−3.316
11	37.78	38.70	−3.705	−3.398
12	37.45	37.98	−3.574	−3.404
13	37.65	38.20	−3.692	−3.522
14	36.68	37.62	−3.810	−3.450
15	37.69	38.31	−3.601	−3.391
16	37.54	38.11	−3.460	−3.277
17	38.01	38.79	−3.641	−3.433
18	38.09	38.94	−3.571	−3.269
19	37.38	37.89	−3.592	−3.418
20	37.29	38.04	−3.563	−3.301

Table S2.3 *p*-values from the 2018 inter-laboratory (codes A−I) pairwise Wilcoxon comparisons of intercept (a), slope (b), and R^2 (c). *P*-values were adjusted for multiple comparisons using the Holm correction.

a.

Intercept	A	B	C	D	E	F	G	H
B	1.00	-	-	-	-	-	-	-
C	0.267	0.902	-	-	-	-	-	-
D	1.00	1.00	0.543	-	-	-	-	-
E	0.062	1.00	1.00	0.527	-	-	-	-
F	1.00	1.00	0.267	0.686	0.089	-	-	-
G	**0.044**	0.902	0.902	0.319	1.00	**0.005**	-	-
H	0.067	0.267	0.400	0.267	**0.005**	0.067	**<0.005**	-
I	0.267	1.00	1.00	0.902	1.00	0.267	1.00	1.00

b.

Slope	A	B	C	D	E	F	G	H
B	1.00	-	-	-	-	-	-	
C	1.00	1.00	-	-	-	-	-	
D	1.00	1.00	1.00	-	-	-	-	
E	0.242	0.338	1.00	1.00	-	-	-	
F	1.00	1.00	1.00	1.00	0.170	-	-	
G	0.379	0.069	1.00	1.00	1.00	0.379	-	
H	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
I	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

c.

R^2	A	B	C	D	E	F	G	H
B	1.00	-	-	-	-	-	-	
C	1.00	1.00	-	-	-	-	-	
D	1.00	1.00	1.00	-	-	-	-	
E	1.00	1.00	1.00	1.00	-	-	-	
F	1.00	1.00	1.00	1.00	1.00	-	-	
G	1.00	1.00	1.00	1.00	1.00	1.00	-	
H	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
I	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

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2.9 Author Contributions

Conceptualization: James McNair, Igor Mrdjen, & Molly J. Lane; Methodology: Igor Mrdjen, James McNair, Molly J. Lane; Software: James McNair & Molly J. Lane; Writing: Original Draft Preparation: Molly J. Lane, Richard Rediske, James McNair.

2.10 Conflicts of Interest

None

2.11 References

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Chapter 3 : Assessing the impact of environmental variables on *E. Coli* concentrations measured with Colilert® and Draft Method C (qPCR) at inland lake and Lake Michigan beaches within Muskegon County, MI.

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3.1 Abstract

To provide a safe environment for beachgoers, public beaches are tested for *E. coli* which alerts the public to the presence of potentially pathogenic bacteria in the water. We used Kaplan-Meier distribution curves paired with log-rank trend tests to analyze eight categorized environmental variables and their relative impact on *E. coli* concentrations measured with Colilert® and qPCR (Draft Method C) at 14 inland and 6 Lake Michigan beaches in Muskegon County, MI. Additionally, we compared the two methods' *E. coli* results and, *E. coli* levels of the two lake types with Kaplan-Meier distribution curve analysis to allow for the inclusion of all data (i.e. censored (below the limit of quantification) and quantifiable data) without any alterations. Of the eight environmental variables analyzed, only bird abundance and presence significantly impacted *E. coli* levels ($p < 0.001$) across all scenarios tested. Impacts on *E. coli* concentrations by other tested variables (i.e. turbidity, wind speed etc.) depended on the quantification method used and lake type, thus our results indicate that predictive models unique to the monitoring method being used and general lake type are necessary because the quantification method changes the manner in which environmental variables impact *E. coli* quantities. Furthermore, our analysis showed a statistically significant difference between Kaplan-Meier distribution curves of *E. coli* levels measured with Colilert and qPCR which supports the development of distinct water quality criteria for the two *E. coli* quantification methods explored in this study.

Keywords: Draft Method C, left-censored data, *E. coli*, recreational water quality, environmental variables, Colilert®, fecal indicator bacteria, Kaplan-Meier distribution curve, log-rank trend test

3.2 Introduction

Public beaches provide many opportunities to enjoy the outdoors. Throughout the summer months tourists visit communities with public beaches, benefitting the local economy. A Forest Service's National Survey on Recreation and the Environment (NSRE) report estimated that in 2008 approximately 61% of the United States' (US) population over 16 years of age participated in non-motorized water activities (i.e. swimming, snorkeling, visiting beaches) and almost 17% participated in canoeing, kayaking or rafting activities (Cordell, H., 2012). Furthermore, it was estimated that a typical beachgoer would spend approximately $13.13 within 10 miles of a beach per visit (Murray et al., 2001), which, when coupled with the number of recreators engaging in water activities at beaches, can potentially result in millions of dollars invested at local businesses. The state of Michigan has over 1,200 public beaches (www.deq.state.mi.us/beach). A 2017 tourism summary reported 122.4 million visitors to Michigan—which helped support 6% of all jobs—and spent $3.4 billion on recreation (www.medc.app.box.com (a)). Visitors to Muskegon County, MI spent over $313 million in 2017 (www.medc.app.box.com (b)). Although these reports do not distinguish tourism dollars spent specifically on or around water activities, one can assume that with millions of people using water as a form of recreation a large portion of the dollars spent was due to the presence of a nearby beach, thereby helping to sustain local economies.

With the vast number of people enjoying recreational waters and local businesses depending on them, it is important that visitors be confident the water is safe to use. One way local municipalities address this is by testing the water for fecal indicator bacteria (FIB), specifically *Escherichia coli* (*E. coli*) in Michigan, which alerts public health officials and beach managers to the

possibility of fecal contamination. The presence of fecal contamination has long been known to increase the risk of contracting recreational water illnesses (Dufour, 1984; Seyfried et al., 1985 (II)), such as respiratory or ear infections, skin irritations and gastrointestinal (GI) illness (Seyfried et al., 1985 (I), Wade et al., 2008). GI illnesses can range from being acute (mild discomfort or nausea) to severe (continuous vomiting and diarrhea leading to hospitalization for dehydration) (Deflorio-Barker et al., 2016) and are what the current recreational water quality criteria are based upon. Furthermore, recreational water illnesses were estimated to cost beachgoers between $2 – 4 billion dollars annually (Deflorio-Barker et al., 2018). By testing for FIB in recreational water, municipalities can reduce the risk of beachgoers contracting an illness and instill confidence that the water they are recreating in is safe.

Considering the possible health risks and economic impacts associated with exposure to fecal contaminated water, it is important to test and report unsafe conditions as quickly and as accurately as possible. Two common methods for quantifying *E. coli* in recreational water are: 1. The United States Environmental Protection Agency (U.S. EPA) approved IDEXX Colilert-18® Quanti-Tray 2000, hereafter referred to as Colilert, and 2. quantitative polymerase chain reaction (qPCR), a proposed molecular quantification method. Colilert is a defined substrate method, reported as most probable number (MPN) and requires an incubation period of 18 – 22h before results can be reported. The current recreational water quality standard for *E. coli* in Michigan is the geometric mean of three sample sites at a single beach or a single composited sample from three sample sites at a single beach cannot exceed 300 MPN per 100 mL daily, or a 30-day geometric mean of 130 MPN per 100 mL (EGLE, Water Resources Division, 2019). Alternatively, a standardized qPCR assay has been proposed by the U.S. EPA for quantification of *E. coli* in recreational water titled 'Draft Method C' which can provide recreational water *E. coli* concentrations within a few hours of samples being received at the lab. qPCR methods are becoming more widespread and one has already been

approved for the quantification of *Enterococcus* (U.S. EPA 2012 & 2013), another commonly used FIB. Recently, a beach notification value (BNV) has been established in Michigan for Draft Method C as 8,760 DNA copies of an *E. coli*-specific gene sequence per 100 mL. This BNV is being used across Michigan to monitor bacterial water quality at select beaches in Oakland County and Kent County (Dr. S. Briggs, EGLE. Pers. comm.). The underlying methodology of these two *E. coli* quantification methods is quite distinct in that Colilert only measures culturable *E. coli* cells and qPCR measures both culturable and non-culturable *E. coli.* Colilert relies on an *E. coli* specific enzyme (β-glucuronidase) to react with a patented substrate to produce fluorescence upon exposure to ultra-violet light (www.idexx.com). In contrast, qPCR amplifies a DNA sequence on the 23S rRNA gene specific to *E. coli* which is then used to calculate the number of DNA gene copies present.

As an alternative to collecting recreational water samples from beaches and testing for FIB, some scientists have created computer predictive models to anticipate when a microbial water quality exceedance may occur (Nevers and Whitman 2005; Frick et al., 2008). This approach does not require expensive instrumentation or a laboratory and, fewer personnel are needed to provide water quality monitoring to the community. Microbial concentration predictive models, such as the U.S. EPAs "Virtual Beach" (V3.0.6; Cyterski et al., 2016) use environmental variables (EVs) collected at the beach or from nearby weather stations to predict an exceedance (Frick et al., 2008, Byappanahalli et al., 2010). Common EVs used in these models and that were included in our study were: turbidity, water and air temperature, wind speed, the number of birds, the presence or absence of birds, rain amount, and time since the last rain fall. Each variable can potentially contribute to *E. coli* densities in recreational water in different but interrelated ways.

The effect different environmental variables can have on *E. coli* concentrations can vary significantly. A direct relationship between turbidity, wind speed, or birds and fecal contamination

can be predicted. Wind speed can drive increased wave action, which causes waves to break higher into the swash zone where fecal matter potentially stored in the sand (Alm et al., 2003) or bird feces along the shore (Goodwin et al., 2017; Kelly et al., 2018) is carried into the water column as the wave recedes. On the other hand, an inverse relationship between air or water temperature and *E. coli* densities can be expected since reduced cloud cover can result in higher air temperatures, and more ultraviolet light penetrating the water column thereby causing solar inactivation or die off of *E. coli* (Noble et al., 2004; Boehm et al., 2018). This trend would particularly be seen when *E. coli* was measured with Colilert since this method only quantifies culturable bacteria compared to qPCR which measures culturable and non-culturable *E. coli*. Consequently, even if warmer temperatures caused a decrease in culturable *E. coli*, qPCR would measure the inactivated or dead cells. Finally, low rainfall and an antecedent rainfall prior to sampling would correspond to lower *E. coli* concentrations. Rainfall produces terrestrial stormwater runoff which eventually enters the beach environment through tributaries or as it moves from the backshore across the sand potentially carrying fecal matter into the swim area. Additionally, during intense rainfall, the lake bottom in the shallower foreshore area could be disturbed, causing *E. coli* stored in the sand to be released into the water column (Whitman and Nevers, 2003; Ishii et al., 2007). Given that each of these environmental variables can contribute to increased fecal contamination at beaches, understanding how they can be used to predict *E. coli* concentrations across different environments and with different quantification methods will be vital to predictive models used to issue beach closures.

The goal of our study was to examine the association between eight categorized environmental variables (EVs) and *E. coli* concentrations quantified with the approved (Colilert) and proposed (qPCR Draft Method C) methods at fourteen inland lake and six Lake Michigan beaches to inform models aimed at predicting microbial water quality as to which variables play a significant role. We also compared *E. coli* results measured with Colilert to those obtained with qPCR for inland

and Lake Michigan beaches to assess the need for separate water quality criteria for the different methods. Finally, we compared the *E. coli* concentrations found at inland lake beaches with those at Lake Michigan beaches to determine if there was an overall difference in microbial water quality. Inland lake beaches are less frequently studied than the coastal waters of the Great Lakes, therefore our results will contribute much needed information regarding inland lake beach environments. A novel component of this study is that we used survival analysis methods to assess *E. coli* concentrations measured with an approved (Colilert) and proposed (Draft Method C) quantification method. Unlike most statistical analyses used for environmental studies, this method allows for all data to be included in the analysis, including data that is below the range of detection and normally removed from analysis. The information provided by this study will be useful to beach managers to focus recreational water mitigation efforts and delegate resources where they would be most beneficial.

3.3 Methods

Sampling

Sampling occurred once a week over five weeks (June 4th through August 6th, 2018) within Muskegon County, Michigan. Water samples were stored on ice until transported to the lab and processed for either Colilert analysis or filtered for the Draft Method C qPCR assay within six hours of collection.

Inland Lake Beaches: 100 mL samples were collected in 125 mL Security-Snap™ BacT (Thermofisher Scientific) sterile polypropylene bottles at each of the left, center, and right sample sites (Figures S3.1 – S3.5). The three 100 mL samples were composited in the lab into a sterile 500 mL HDPE bottle (Microtech Scientific), totaling 300 mL of a composite sample for each beach. One-100 mL aliquot was used for immediate Colilert analysis and two-100 mL aliquots were filtered for qPCR analysis then stored at −80°C for analysis at later date. Overall, five composite samples

from each of the fourteen inland lake beaches (Table 3.1) (n = 70) were analyzed. An ~50 mL sample also was collected from the center location of each beach in a Nalgene bottle for turbidity measurements.

Lake Michigan Beaches: A 500 mL sample was collected in sterile, tamper sealed HDPE bottles (Microtech Scientific) at each of the north, center, and south locations (Figures S3.6 – S3.8) and prepared individually for analysis as described above for both Colilert (1 x 100 mL) and the Draft Method C qPCR assay (2 x 100 mL). Overall, 15 water samples per Lake Michigan beach (3 samples x 5 sampling events) were analyzed over the course of the study (Table 3.1) (n = 90).

Environmental Variables

Eight environmental variables (EVs) were recorded for each beach during each sampling event to assess its impact on *E. coli* concentrations. They included: turbidity (NTU), wind speed (mph), air and water temperature (°C), number of birds, whether birds were present or absent, time (h) since the last rain fall, of any amount, and the amount of rain during the last rainfall (inches). Most variables were included on the Great Lakes Beaches Routine On-Site Sanitary Surveys (https://www.epa.gov/sites/production/files/documents/greatlakes_onsite.pdf), except for birds present/absent, as part of the beach monitoring program protocol. Wind speed, air and water temperature and samples for turbidity were collected from or measured at the center sample site. Bird counts were performed by visual observation as samplers walked from the center sampling point towards the north/right or south/left sampling points and counted the number of birds observed on the beach and in the water within the swim area (U.S. EPA 2008). Samplers would report the number of birds to the person at the center location and all birds observed were tallied and recorded on the Sanitary Survey. Wind speed and air temperature were measured with an anemometer by holding it above the sampler's head for approximately 10 s and recording the highest reading of wind speed and, once a consistent temperature was seen, air temperature.

Table 3.1 Lake type, beach name, location, and sampling event dates (month/day) in this study.

Lake Type	Beach Name	Location	Sample Dates
Inland	Blue Lake County Park	Big Blue Lake	7/11, 7/16, 7/25, 7/30, 8/6
	Duck Lake State Park	Duck Lake	6/6, 6/11, 6/18, 6/25, 7/2
	Fox Lake Park	Fox Lake	7/11, 7/16, 7/25, 7/30, 8/6
	Harbor Towne Beach	Muskegon Lake	6/4, 6/12, 6/19, 6/26, 7/5
	Maple Park	White Lake	7/11, 7/16, 7/25, 7/30, 8/6
	Mona Lake Park	Mona Lake	6/4, 6/12, 6/19, 6/25, 7/5
	Moore County Park	Half Moon Lake	6/13, 6/20, 6/27, 7/9, 7/19
	Muskegon State Park Channel Campground	Muskegon Lake	7/12, 7/18, 7/24, 7/31, 8/7
	Padley Park	West Lake	7/11, 7/16, 7/25, 7/30, 8/6
	Ross Park	Mona Lake	6/4, 6/12, 6/19, 6/26, 7/5
	Sunset Beach	Wolf Lake	6/13, 6/20, 6/27, 7/9, 7/19
	Twin Lake Park	Twin Lake	7/11, 7/16, 7/25, 7/30, 8/6
	Watersports Park	Muskegon Lake	7/12, 7/18, 7/24, 7/31, 8/7
	Wolf Lake County Park	Wolf Lake	6/13, 6/20, 6/27, 7/9, 7/19
Lake Michigan	Meinert County Park		6/6, 6/11, 6/18, 6/25, 7/2
	Old Channel		7/11, 7/16, 7/25, 7/30, 8/6
	Pere Marquette		7/12, 7/18, 7/24, 7/31, 8/7
	Pioneer Park		6/6, 6/11, 6/18, 6/25, 7/2
	PJ Hoffmaster Campground Beach		6/4, 6/12, 6/19, 6/26, 7/5
	PJ Hoffmaster Public Beach		6/4, 6/12, 6/19, 6/26, 7/5

Anemometer instructions directed measurements to be taken in the shade, however no shade was available at most center sampling sites thus for consistency, all readings were recorded at the center location regardless of the availability of shade. Water temperature was measured at knee depth with a thermometer (Component Design Northwest, Inc. DTQ450X) held near the surface of the water until a steady reading was observed. Turbidity samples were transported to the lab on ice and measured with a turbidimeter according to the manufacturer's instructions following analysis of three NTU standards (0, 10, and 100 NTU) as a quality control measure (Thermo Scientific Orion AQUAfast AQ4500). Time since the last rain and rain amounts were recorded from individual

weather stations (www.wunderground.com) chosen based on nearness to a beach and, in all instances except one, was the same throughout the sampling season (Table S3.1).

E. coli Quantification

Colilert: The composited inland lake beach samples and north, center, south Lake Michigan samples were all analyzed within six hours of collection using Colilert (Method 9233 B) (Rice et al., 2012). In accordance with this method, a Colilert reagent packet was emptied into and dissolved in 100 mL of water sample. The solution was poured into a Quanti-Tray and incubated for 18 – 22 h at 35°C. The Quanti-trays were then removed from the incubator and exposed to ultraviolet light. Based on the total number of fluorescing wells in the tray, *E. coli* concentrations were calculated and recorded as MPN. A more in-depth description of this method is provided in Kinzelman et al. (2005) and on the IDEXX website (www.idexx.com).

qPCR: Each composited inland lake beach sample and the three individual Lake Michigan samples per beach were analyzed according to the Draft Method C qPCR assay described in Sivaganesan et al. (2019) which uses TaqMan™ chemistry and the EC23S857 region of the 23S rRNA gene (Chern et al., 2011). Briefly, 100 mL of sample was filtered through 0.45 μm pore polycarbonate filter seated on a sterile disposable filtration unit (Pall Corporation, Puerto Rico, MicroFunnel™ Filter Funnel). The filters were folded four times and added to a 2.0 mL sterile semi-conical, screw-cap microcentrifuge tube containing 0.3 g (± 0.01 g) of glass beads. Working within a laminar flow hood, 600 μL of extraction fluid consisting of AE buffer with 0.2 μg/mL salmon (sketa) DNA was added to each extraction tube, tightly sealed then bead milled for 1.0 min at 5,000 rpm. The sketa DNA served as a sample processing control (SPC) to ensure the qPCR reaction occurred as expected. Because a known concentration of sketa DNA was added to each sample, a specific range (18.58 – 22.01) of cycle threshold (Ct) values was expected, and required, for designated Draft Method C quality control parameters to be met (Sivaganesan et al., 2019). The

extraction tubes were then centrifuged for 5 min at 12,000 x g and ~ 400 µL of the crude DNA extract was transferred into a correspondingly labeled sterile 1.7 mL low retention micro-centrifuge tube. The crude extract was centrifuged for 1 min at 12,000 x g, then ~ 100 µL of clarified supernatant was transferred to a second sterile 1.7 mL centrifuge tube. Tubes of clarified supernatant were then moved to a second laminar flow hood and set aside until sketa and *E. coli* Master Mixes (MM) were prepared (see chapter 2, section 2.3). Half of the 96-wells in the qPCR plate were filled with 20 µL of *E. coli* MM and the other half were filled with 20 µL of sketa MM. 5 µL of recreational water sample extracts were analyzed in triplicate using both the *E. coli* and sketa MM. Three separate negative (blanks) and positive (calibrator) controls, and a No Template Control (NTC) were analyzed in duplicate, on each tray of samples with *E. coli* and sketa MM. Once MM, samples, and quality controls were added, the plate was sealed and analyzed on a StepOnePlus™ Applied Biosystems thermocycler instrument using the StepOne™ Software (v2.3). Thermocycling consisted of an initial 'holding stage' (50.0°C, 2 min; 95.0°C, 10 min) followed by 40 cycles of DNA denaturation and primer/probes annealing (95.0°C, 15 s; 56.0°C, 1 min). At the end of each of the 40 cycles, fluorescence was measured by the instrument. The fluorescence threshold, or the level of fluorescence in which the signal rises above the background level, was manually set to 0.03 ΔRn and baseline cycles were set to AUTO determination (Sivaganesan et al., 2019). Instrument generated Ct values for water samples and quality control parameters were exported and entered in a U.S. EPA provided Draft Method C Excel Workbook where *E. coli* concentrations (log_{10}Copies/5 µL) were calculated, provided all quality control parameters were met. log_{10}Copies/5 µL were then converted to log_{10}Copies/100 mL to make the Colilert and qPCR quantification methods comparable. There were nine Lake Michigan beach samples whose sketa Ct values did not pass specified acceptance criteria. In these cases, the stored duplicate filter was extracted, and the crude extract was filtered through a OneStep™ PCR Inhibitor Removal Kit (Zymo Research) column, then re-analyzed with

the Draft Method C qPCR assay. The inhibitor removal kit does not impact dilution ratios although it is thought that it may bind some DNA within the column resulting in a lower measured concentration. The log_{10}Copies/100 mL results from five of the nine re-analyzed samples were used in statistical analysis, while four were unusable as the filter blank acceptance criteria was not met (Ct > lower limit of quantification as given in the Draft Method C Excel workbook) (Sivaganesan et al., 2019). All filtered qPCR samples were analyzed within 6 months after storage.

Statistical Analysis

It was assumed that the variation in EVs between north, center, and south sample locations at the same beach was negligible for Lake Michigan beaches, therefore the EV value recorded at the center sampling site was used for all three sample locations for analysis. Additionally, repeated measures statistical analysis was unnecessary because *E. coli* concentrations vary greatly from day to day and even hour to hour (Whitman and Nevers, 2004; Wyer et al., 2018) thus our weekly samples could be treated as independent of each other.

Kaplan-Meier distribution curves (KM) (Kaplan and Meier, 1958), a survival analysis statistical method, were used to analyze our EV data with respect to the *E. coli* concentration. This method allows all data to be included in analysis, particularly *E. coli* measurements whose values were determined to be below the instrument or method limit of quantification (LOQ), also called "left censored" data. For the purposes of our study, *E. coli* concentrations in the units of log_{10}(MPN/100 mL) for Colilert and log_{10}(Copies/100 mL) for qPCR were used in place of the traditional "time" variable, and the method or instrument LOQ was the "event". Colilert results recorded as < 1 MPN were below the LOQ and therefore considered a censored observation; qPCR results below 246 copies/100 mL (2.391 log_{10}Copies/100 mL) were considered censored.

EVs were categorized into two or three categories (e.g. "Low", "Medium", or "High") (Table 3.2) chosen to have at least 10 non-censored observations per group. KM distribution curves

were then generated using the categorized groups and *E. coli* concentrations from recreational water samples. For EVs categorized into three groups, KM curves were statistically compared with a log-rank trend test (Klein and Moeschberger 2006, section 7.4; Machin et al., 2006 section 3.5). The log-rank trend test tests the null hypothesis that the *E. coli* concentration distributions of the categorized EVs are equal against the alternative that there is a designated ordering among the KM curves. For example, if $K_1(x)$, $K_2(x)$, and $K_3(x)$ are the KM curves (as functions of concentration x) for the low, medium, and high categories of a particular EV, then the null hypothesis of the trend test is $K_1(x) = K_2(x) = K_3(x)$ for all concentrations x, and the "increasing form" of the alternative hypothesis is $K_1(x) \leq K_2(x) \leq K_3(x)$ for all x, with the inequality being strict (i.e., "<" instead of "≤") for at least one concentration and at least one pair of KM curves. The test outcome does not specify which KM distribution curve relationship is statistically different, just that a statistical difference in distribution curves was detected. For the bird present/absent and the method and lake type comparisons, a two-sample one-sided log-rank test (Hollander et al., 2013) (Ch. 11.7; p. 594 – 597) was used. Output from the log-rank test were used to calculate the original test statistic prior to it being squared for the chi-squared *p*-value in order to perform one-sided statistical analyses. These comparisons were performed to identify EVs that exhibited a statistically significant impact ($\alpha = 0.05$) on *E. coli* concentrations quantified with both methods (Colilert and qPCR) for inland and Lake Michigan beaches separately. All statistical analyses were performed using R Software (v3.5.2; R Core Team, 2018); survival analysis packages were "survival" (v2.44-1.1; Therneau, 2015) and "survMisc" (v0.5.5; Dardis, 2018).

Table 3.2 Categorized environmental (explanatory) variable ranges for Kaplan-Meier distribution curves. Instances where the two lake types grouping range varied, they were differentiated as: IL = inland lake range; LM = Lake Michigan range.

Environmental Variable	Category	Range
Turbidity (NTU)	Low	≤ 1.00 IL: 1.01 – 1.50
	Medium	LM: 1.01 – 1.99 IL: ≥ 1.51
	High	LM: ≥ 2.00
Wind Speed (mph)	Low	≤ 2.0
	Medium	2.1 – 4.9
	High	≥ 5.0
Air Temperature (°C)	Low	≤ 21.1 IL: 21.2 – 25.0
	Medium	LM: 21.2 – 24.9 IL: ≥ 25.1
	High	LM: ≥ 25.0 IL: ≤ 23.9
Water Temperature (°C)	Low	LM: ≤ 15.0 IL: 24.0 – 26.6
	Medium	LM: 15.1 – 22.0 IL: ≥ 26.7
	High	LM: ≥ 22.1 IL: ≤ 1
Bird Count	Low	LM: ≤ 3 IL: 2 – 5
	Medium	LM: 4 - 20 IL: ≥ 6
	High	LM: ≥ 21
Bird Presence	Present	≥ 1
	Absent	0
Time Since Last Rain (h)	≤ 24	≤ 24
	25 – 72	25 – 72
	≥ 72	≥ 72
Rain Amount (inches)	Low	≤ 0.10
	Medium	0.11 – 0.24
	High	≥ 0.25

The limitations of R-Software prevent it from performing survival analysis on left-censored data, like the data in our study, therefore trend tests and two-sample log-rank tests were carried out

on the 'flipped' *E. coli* concentration data set which is achieved by reversing, or 'flipping', the data by subtracting all measurements—*E coli* concentrations in our study—by an arbitrary number higher than the maximum observed value (Gillespie et al., 2010; Helsel, 2011; Dinse et al., 2014). Its primary benefit for our purposes is that it allows information from data below the LOQ to be included in the statistical analysis in a statistically rigorous way simply by changing data from left- to right-censored. All KM distribution curves presented below are based on the 'unflipped' or original *E. coli* concentration data scale.

3.4 Results

Trend tests of categorized EV on their corresponding KM distribution curves were tested in the same order for inland lake and Lake Michigan beaches and for both *E. coli* quantification methods (Colilert and qPCR) (see below). As previously mentioned, KM distribution curves were plotted on the original concentration scale (x-axis). Only KM distribution curves of categorized EVs where a statistically significant ordered difference was detected in the tested trend were shown below. Select statistically non-significant KM distribution curves were included in the supplemental material when referenced and all *p*-values for categorized EV curve comparisons are listed in Table S3.2.

Environmental Variables

Turbidity

The turbidity trend tested was 'low < medium < high' with respect to the *E. coli* concentration distribution. Specifically, the high turbidity category was hypothesized as having the greatest *E. coli* concentration distribution based on the original or 'unflipped' concentration scale. Categorized turbidity KM distribution curves for *E. coli* concentrations measured with Colilert at inland lake beaches were statistically significantly ordered ($p = 0.035$) (Figure 3.1a) with respect to the concentration distributions, however they did not follow the predicted order. For example, the

inland lake medium turbidity category (Figure 3.1a) had the highest *E. coli* concentration along the x-axis at a probability of 0.5 ($\log_{10}$ = 1.5 MPN/100 mL) indicating that the medium category had a higher distribution of *E. coli* concentrations than both the low ($\log_{10}$ = 0.7 MPN/100 mL) and high ($\log_{10}$ = 1.1 MPN/100 mL) groups. *E. coli* measured with qPCR at inland lake beaches did not result in statistically significant ordering (p = 0.606) based on turbidity.

Lake Michigan beaches also had statistically significantly ordered turbidity with respect to *E. coli* measured with Colilert (p < 0.001) (Figure 3.2a) and with qPCR (p < 0.001) (Figure 3.2b). The evidence to reject the null hypothesis at Lake Michigan beaches was stronger because the trend tested on the KM distribution curves with respect to *E. coli* concentration distributions was the same as that plotted (low < medium < high) (Figures 3.2a and 3.2b), where increasing turbidity was associated with increasing *E. coli* concentrations. Whereas, the order seen in the Figure 3.1a for inland lake beaches suggests a 'low < high < med' trend. Thus, turbidity had statistically significant impact on *E. coli* distributions when quantified with Colilert at inland lake beaches, albeit the trend observed was different than that tested. A statistically significant impact on *E. coli* measured with both methods at Lake Michigan beaches was observed.

Wind Speed

An increasing trend was tested for wind speed ('low < medium < high') with respect to the *E. coli* concentration distribution on the original concentration scale. Inland lake KM distribution curves of wind speed categories and *E. coli* quantified with Colilert were statistically significantly ordered (p = 0.018, Figure 3.1b), but not when measured with qPCR (p = 0.801). Therefore, wind speed impacted *E. coli* concentrations measured with Colilert but not qPCR at inland lake beaches.

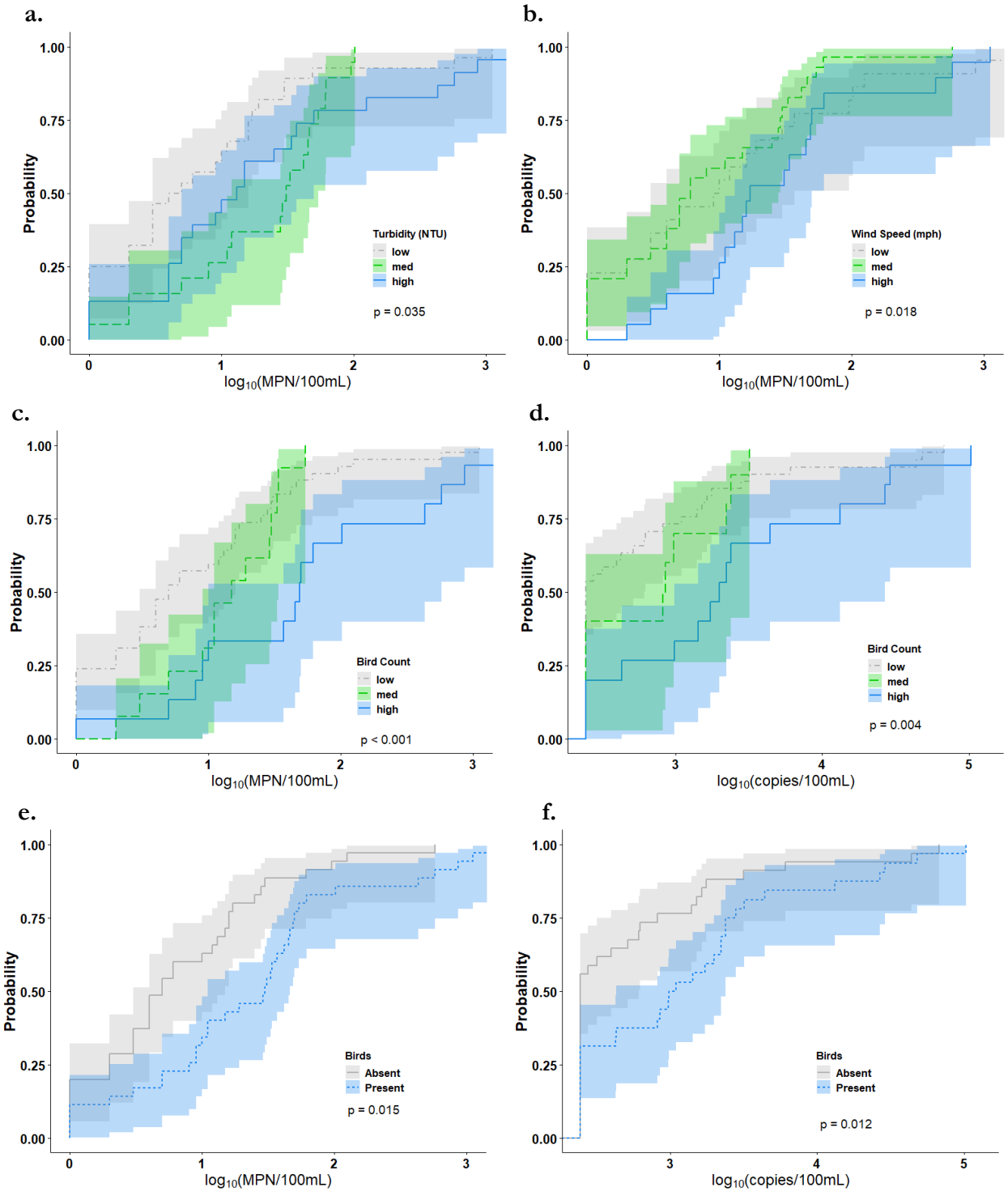

Figure 3.1 Inland lake beaches categorized environmental variables Kaplan-Meier distribution curves with 95% point-wise confidence intervals. X-axes are *E. coli* concentration quantified with Colilert (log_{10}(MPN/100 mL) or qPCR (log_{10}(copies/100 mL) on the original concentration scale; y-axis is the probability that a random sample selected from the data set will be less than or equal to the concentration along the x-axis.

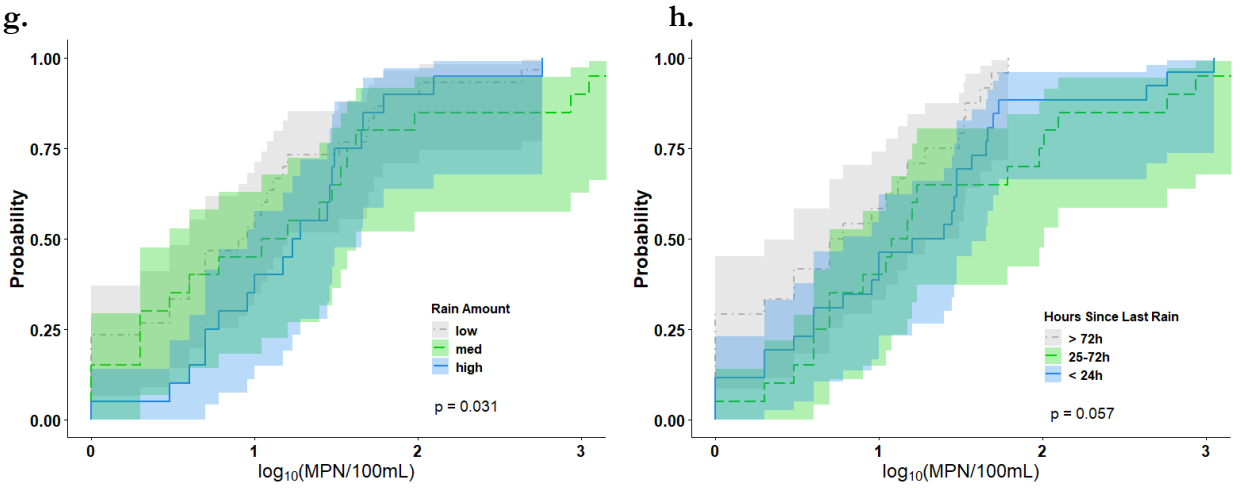

Figure 3.1 (Continued) Inland lake beaches categorized environmental variables Kaplan-Meier distribution curves with 95% point-wise confidence intervals. X-axes are *E. coli* concentration quantified with Colilert (log_{10}(MPN/100 mL) or qPCR (log_{10}(copies/100 mL) on the original concentration scale; y-axis is the probability that a random sample selected from the data set will be less than or equal to the concentration along the x-axis.

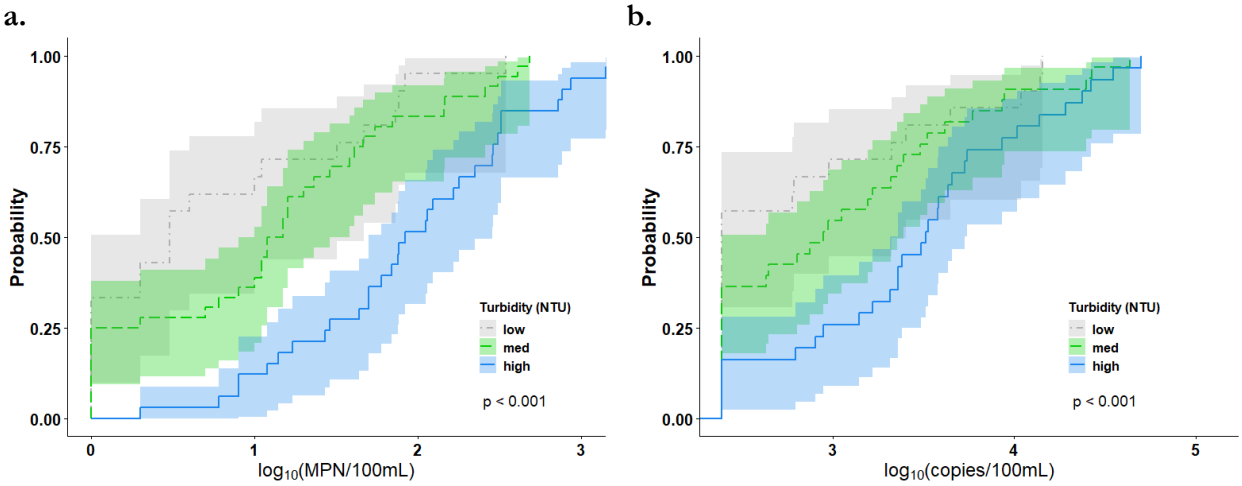

Figure 3.2 Lake Michigan beaches categorized environmental variables (EVs) Kaplan-Meier distribution curves with 95% point-wise confidence intervals. X-axis is the log base-10 of the observed *E. coli* concentration, on the original concentration scale, measured with either Colilert log_{10}(MPN/100 mL) or qPCR log_{10}(copies/100 mL); y-axis is the probability that the concentration of a random sample selected from the data set will be less than or equal to the log_{10}(concentration) on the x-axis.

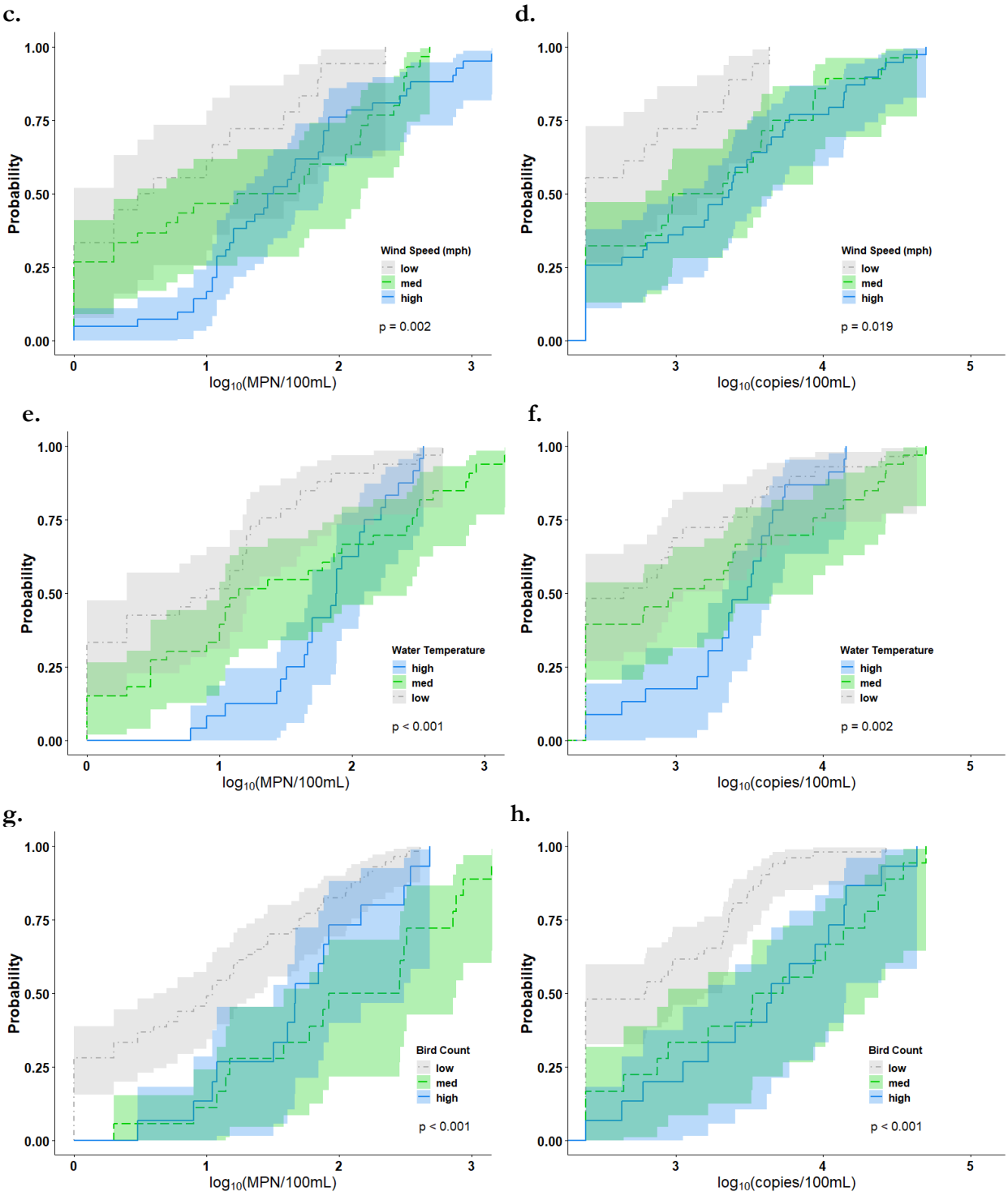

Figure 3.2 (Continued) Lake Michigan beaches categorized environmental variables (EVs) Kaplan-Meier distribution curves with 95% point-wise confidence intervals. X-axis is the log base-10 of the observed *E. coli* concentration, on the original concentration scale, measured with either Colilert $\log_{10}$(MPN/100 mL) or qPCR $\log_{10}$(copies/100 mL); y-axis is the probability that the concentration of a random sample selected from the data set will be less than or equal to the $\log_{10}$(concentration) on the x-axis.

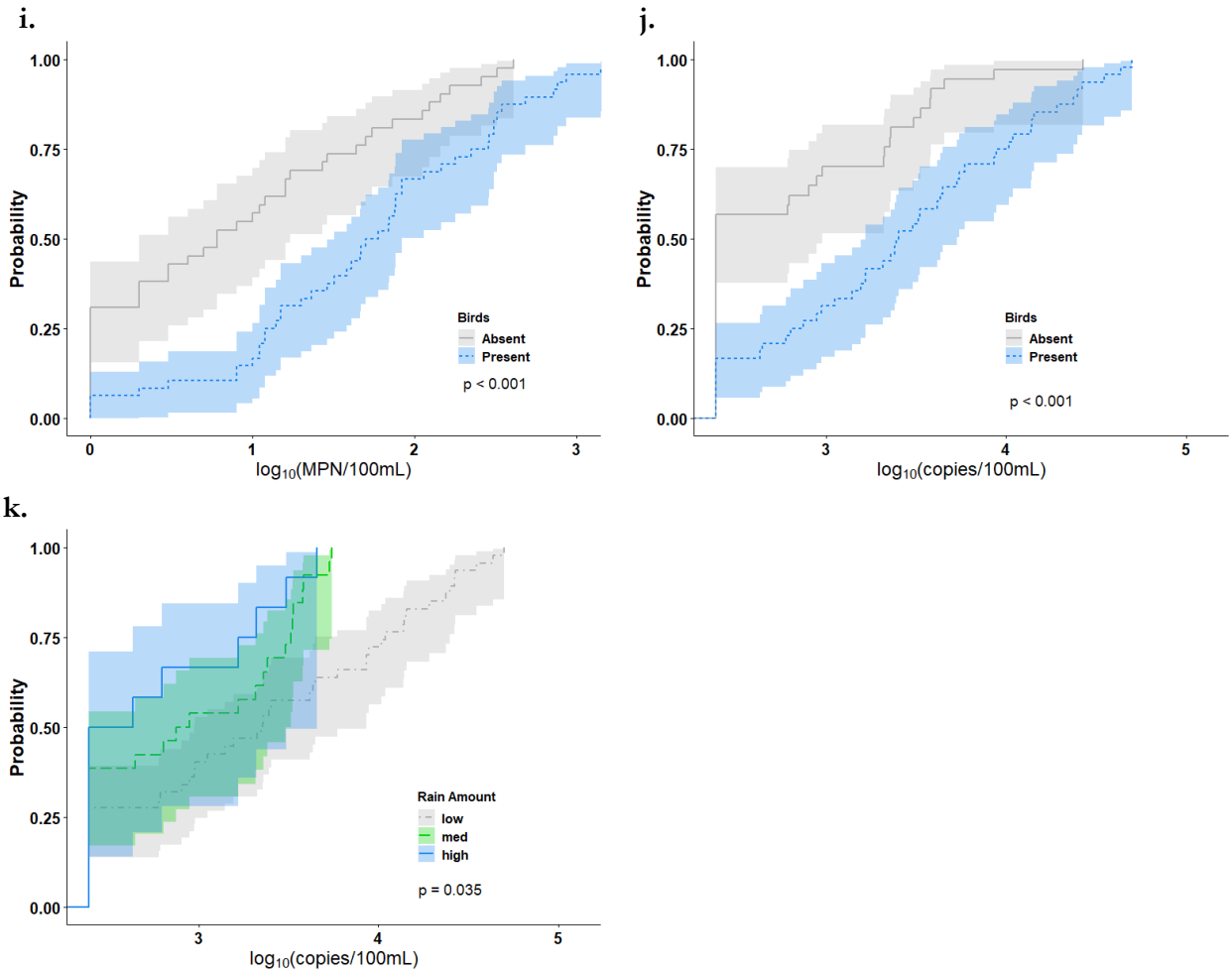

Figure 3.2 (Continued) Lake Michigan beaches categorized environmental variables (EVs) Kaplan-Meier distribution curves with 95% point-wise confidence intervals. X-axis is the log base-10 of the observed *E. coli* concentration, on the original concentration scale, measured with either Colilert $\log_{10}$(MPN/100 mL) or qPCR $\log_{10}$(copies/100 mL); y-axis is the probability that the concentration of a random sample selected from the data set will be less than or equal to the $\log_{10}$(concentration) on the x-axis.

In contrast, Lake Michigan beaches exhibited a statistically significant wind speed order for *E. coli* concentration distributions measured with both Colilert (p = 0.002, Figure 3.2c) and qPCR (p = 0.019; Figure 3.2d). There was some crossing of the medium and high category KM distribution curves in Figures 3.2c and 3.2d which affects the power of the tested trend (Klein and Moeschberger 2006, section 7.6), but the low category had a reduced *E. coli* concentration distribution throughout

the range. This observation suggested that wind speed, particularly lower wind speeds, influenced *E. coli* densities measured with either method at Lake Michigan beaches.

Air Temperature

The trend tested on air temperature categories was inversely ordered (high < med < low) with respect to *E. coli* distributions on the original scale. Meaning, we expected that high air temperatures would be associated with the lowest *E. coli* concentration distribution. A statistically significantly order was not detected for either method of *E. coli* quantification at inland lake (Colilert: $p = 0.684$; qPCR: $p = 0.364$) or Lake Michigan beaches (Colilert: $p = 0.418$; qPCR: $p = 0.478$). Thus, the way in which we categorized and ordered air temperature did not have a significant effect on *E. coli* measured with Colilert or qPCR at either type of lake.

Water Temperature

The trend test on KM distribution curves of categorized water temperatures at inland lake beaches were not statistically significantly ordered for Colilert ($p = 0.343$) or qPCR ($p = 0.942$) indicating water temperature at inland lake beaches did not significantly impact *E. coli* densities measured in this study. Trend tests for Lake Michigan beaches on the other hand, detected a significant order (Colilert: $p < 0.001$; qPCR: $p = 0.002$) (Figures 3.2e and 3.2f, respectively). However, the order of the plotted categories were directly, not inversely, related to *E. coli* quantities (Figures 3.2e and 3.2f) as predicted, where lower water temperatures had relatively reduced *E. coli* concentration distributions.

Bird Count and Presence/Absence

We hypothesized that the three bird count categories would directly impact *E. coli* concentration distributions (low < medium < high) in that low bird counts would have the lowest *E. coli* concentration distribution. Bird count KM distribution curve categories at inland lake beaches were statistically ordered for *E. coli* quantification with Colilert ($p < 0.001$) (Figure 3.1e) and with

qPCR (p = 0.004) (Figure 3.1d). Similarly, the number of birds at Lake Michigan beaches had a significantly ordered impact on *E. coli* concentrations measured with Colilert and qPCR (both p < 0.001) (Figures 3.2g and 3.2h, respectively). However, the category trend shown for quantification with the Colilert method (Figure 3.2g) was different than predicted. Both methods of quantification at Lake Michigan beaches had some degree of KM distribution curve crossing between the medium (4 – 20 birds) and high (≥ 21 birds) categories, but the low category had a reduced *E. coli* concentration distribution than the medium and high categories indicating that Lake Michigan beaches with ≤ 3 birds had a lower probability of high *E. coli* densities.

Regarding the presence or absence of birds at beaches, we tested the null hypothesis that the KM distribution curve of *E. coli* concentrations at beaches where birds were present (≥ 1) was equal to concentrations at beaches without birds, with respect to the *E. coli* concentration distribution, against the alternative that beaches without birds would have lower *E. coli* densities than beaches with birds. Both methods of quantification at inland lake (Colilert: p = 0.015, z = −2.18; qPCR: p = 0.012, z = −2.24) (Figures 1e and f) and Lake Michigan beaches (both methods p < 0.001, Colilert: z = −4.12; qPCR: z = −4.04) (Figures 3.2i and 3.2j) resulted in the absence of birds exhibiting a significantly lower *E. coli* concentration distribution than when birds were present. Our findings strongly suggest that birds play a substantial role in *E. coli* concentrations at both lake types and with either method of quantification.

Hours Since the Last Rain

The trend interval tested was (>72 h) < (25 – 72 h) < (< 24h), with respect to the distribution of *E. coli* based on the original concentration scales, where *E. coli* was predicted to be the lowest when rain proceeded sampling more than 72 h (> 3d). Inland lake beach KM distribution curves of the ordered time categories were marginally significantly ordered when Colilert was used (p = 0.057) (Figure 3.1h) and not statistically significantly ordered when the qPCR quantification

method was used ($p = 0.658$). Although the curves for the 25 – 72 h group and the < 24 h group had a considerable amount of crossing, the > 72 h group had the lowest Colilert measured *E. coli* concentration distribution (Figure 3.1h), partially supporting our hypothesis. The amount of time since the last rainfall did not affect *E. coli* concentrations at Lake Michigan beaches when measured with Colilert ($p = 0.192$) or qPCR ($p = 0.099$). Our results indicated that hours since the last rainfall may have significantly contributed to *E. coli* concentrations measured with Colilert but not with qPCR at inland lake beaches and not at Lake Michigan beaches.

Amount of Rain

The tested trend for amount of rain with respect to the *E. coli* concentration distribution was 'low < medium < high'. The inland lake beach KM distribution curves of the categorized rain amount were significantly ordered ($p = 0.031$) (Figure 3.1g) when measured with Colilert but not with qPCR ($p = 0.887$). However, the Colilert method result should be interpreted with some caution because a substantial amount of crossing can be seen between all three KM distribution curves (Figure 3.1g) with the high category showing some indication of having the highest *E. coli* concentration distribution. Lake Michigan KM distribution curves for categorized rain amounts and *E. coli* measured with Colilert were not significantly ordered ($p = 0.613$). The KM curves of categorized rain amounts and *E. coli* measured with qPCR were statistically significantly ordered ($p = 0.035$). Noticeably, the opposite trend of what was expected when *E. coli* was measured with qPCR at Lake Michigan beaches is seen; high amounts of rain were associated with the lowest *E. coli* concentration distribution and low rain amounts had the highest concentration distribution (Figure 3.2k). Rain amounts were recorded and tested independently from the time since the last rain. Therefore, if rain occurred more than 72 h before sampling it would have the same impact on this category as rainfall within 24 h of sampling.

Method Comparison

We hypothesized that *E. coli* concentrations measured with Colilert would be lower than those measured with qPCR at both inland lake and Lake Michigan beaches because qPCR measures culturable and nonculturable *E. coli* DNA whereas Colilert only quantifies culturable *E. coli*. The KM distribution curve of *E. coli* quantified with Colilert was statistically lower than the qPCR method KM distribution curve (both $p < 0.001$; Inland: $z = -6.33$; Lake Michigan: $z = -7.56$) (Figures 3.3a and 3.3b) with respect to their *E. coli* concentrations on the original scale. This is further supported by the large area of no overlap between the two methods' KM distribution curves with the qPCR curve (blue dotted) exhibiting a higher *E. coli* concentration at 0.5 probability than the Colilert KM curve (green solid) for both lake types. Furthermore, the difference in the two censoring values, Colilert at 1 MPN/100mL ($\log_{10} = 0$ MPN/100mM) and qPCR at 246 copies/100mL ($\log_{10} = 2.391$ copies/100 mL), is visible by the lack of plotted qPCR *E. coli* observations before 2.391 along the x-axis.

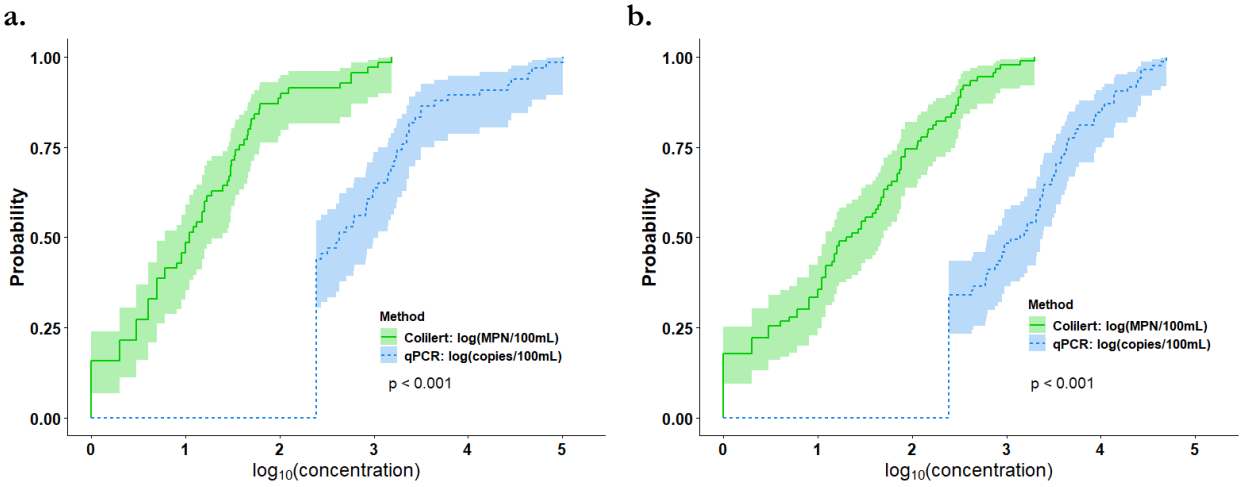

Figure 3.3 Method comparison of Kaplan-Meier distribution curves with 95% point-wise confidence intervals for inland lake (a) and Lake Michigan (b) beaches. X-axis is the log base-10 of the observed *E. coli* concentration, on the original concentration scale, measured with Colilert ($\log_{10}$(MPN/100 mL)) or qPCR ($\log_{10}$(Copies/100 mL)). Y-axis is the probability that the concentration of a random sample will be less than or equal to the $\log_{10}$(concentration) on the x-axis.

Lake Type Comparison

The null hypothesis that the KM distribution curves of the two lake types were equal was tested against the alternative that Lake Michigan beaches had lower *E. coli* concentration distributions than the inland lake beaches. Although the one-sided log-rank test did not support our hypothesis when *E. coli* was quantified with Colilert (p = 0.862, z = 1.09) (Figure 3.4a) or qPCR (p = 0.946, z = 1.61) (Figure 3.4b), the resulting inflated p-values were an indication that the reverse was more likely true in that *E. coli* concentrations at inland lake beaches were lower than those at Lake Michigan beaches. Examination of Figures 3.4a and 3.4b supported this theory since the Lake Michigan beaches KM distribution curve had a higher *E. coli* concentration distribution than the inland lake beaches.

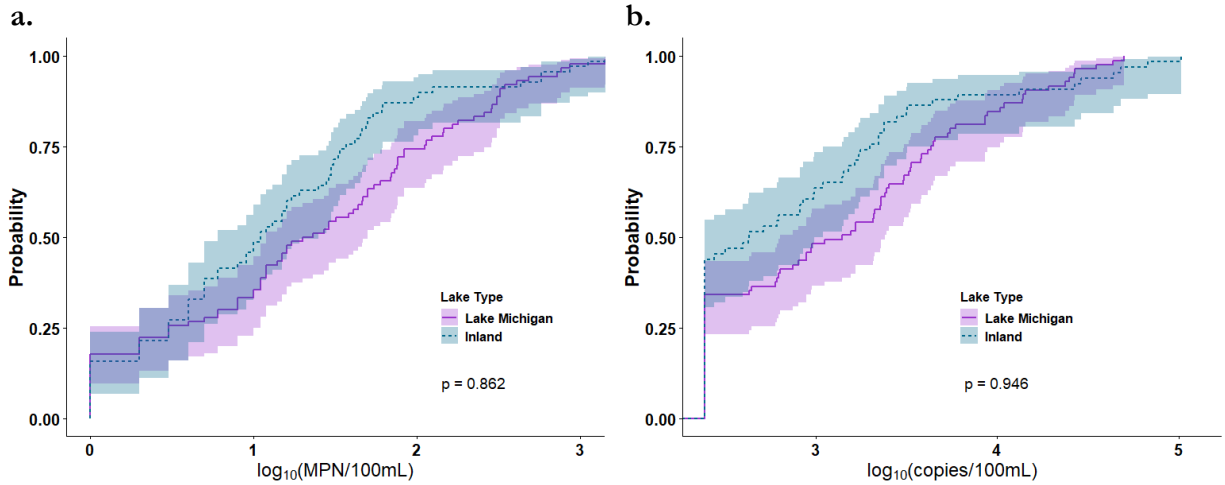

Figure 3.4 Kaplan-Meier distribution curves with 95% point-wise confidence intervals of inland lake beaches compared to Lake Michigan beaches when *E. coli* was quantified with Colilert (a) and qPCR (b). Log-transformed *E. coli* concentration are along the x-axis based on the original concentration scale; y-axis is the probability that the concentration of a random sample will be less than or equal to the $\log_{10}$(concentration) on the x-axis.

3.5 Discussion

The beach environment is a dynamic system affected by a multitude of environmental factors that exhibit both spatial and temporal variability. The factors examined in our study included turbidity, wind speed, air and water temperature, the number of birds present, whether birds were

present or absent, the number of hours prior to sampling that rain occurred, and rain amount. Using KM distribution curves, we were able to identify factors that significantly contributed to *E. coli* concentrations in four scenarios: two quantification methods (Colilert and qPCR) at beaches on two lake types (inland lake and Lake Michigan). Of the eight factors, only the number of birds and the presence/absence of birds significantly affected concentrations at both lake types and with both quantification methods. Turbidity and wind speed significantly influenced *E. coli* concentrations in three of the four scenarios tested. Two of the tested scenarios detected a significant impact by rain amount and water temperature, and air temperature and time since the last rain had no impact on *E. coli* concentrations for any tested scenario.

Environmental Variables

Turbidity

When water, potentially contaminated with fecal matter, is carried downstream by tributaries and discharges into the beach environment, turbidity increases along with *E. coli* concentrations. Three of the six Lake Michigan beaches had various sized tributaries near or within the swim area, while the inland lake beaches did not, which may have caused the trend in increasing turbidity to be more pronounced at Lake Michigan beaches than beaches on inland lakes. Turbidity also can be elevated from disturbance of the lake bottom when wave action is intense, which increases the likelihood that bacteria living or settled in the sand is resuspended into the water column (LaLiberte and Grimes, 1982; Ishii et al., 2007). Our study supports this since the wave action on Lake Michigan is more intense, resulting in higher turbidity measurements and *E. coli* measured with both quantification methods were found to be influenced by turbidity. Furthermore, without the intense wave action to resuspend dead *E. coli* cells in the water column, *E. coli* concentrations quantified with qPCR at inland lakes would be less impacted, as was observed in our study. Finally, turbidity can influence the amount of solar radiation penetrating the water column which can kill off bacteria cells

by solar inactivation (Weiskerger and Whitman et al., 2018). Our data showed that inland lake beach *E. coli* levels were significantly impacted by turbidity when measured with Colilert but not qPCR, supporting the solar inactivation theory because inactivation of *E. coli* would affect Colilert results and not qPCR due to the differences in their methodology (i.e. measuring culturable only versus culturable and nonculturable cells/DNA). Since turbidity was found to significantly influence *E. coli* quantities in three of the four scenarios tested and has been a good predictor of FIB measured with Colilert and qPCR using hydrometeorological models in other studies (Byappanahalli et al., 2010), we recommend including it as a factor in predictive models for forecasting microbial water quality in recreational water.

Wind speed

Wind speed impacts wave action in a similar manner as turbidity. Increasing wind speeds produce larger waves that break in the foreshore area of a beach. During periods of high-winds and intensified wave activity, waves breaking along the shoreline facilitate *E. coli* from bird feces or bacteria stored in the sand being washed into the beach water (Alm et al., 2003). *E. coli* concentrations at the Lake Michigan beaches in our study were affected by wind speed when both methods of quantification were used, whereas wind speed only influenced bacteria quantified with Colilert at inland lake beaches. Lake Michigan beaches experience higher wind speeds and higher energy waves than inland lake beaches thereby causing more bacteria, culturable and non-culturable, stored in the sand to enter the water. Both lake types were impacted by wind speed, although differently depending on the quantification method, therefore it is our recommendation that models used to predict microbial water quality should include wind speed as a factor. This supports the work by Haack et al. (2013) where wind speed was identified as a significant variable in all models tested.

Air and Water Temperature

We predicted that warmer air was connected to stronger solar radiation on less cloudy days resulting in more solar inactivation of *E. coli* within the water column reducing culturable but not nonculturable *E. coli*. However, air temperature did not significantly impact *E. coli* concentrations at inland or Lake Michigan beaches. This is most likely because air temperature and solar radiation are not necessarily directly related therefore, measuring the amount of light penetrating the water column or measuring solar radiation directly would likely be better predictors. One possible variable to use is photosynthetically active radiation (PAR) which measures light at 400 – 700 nm, however shorter wavelengths were more effective at killing *E. coli* cells (Vermeulen et al., 2008).

Water temperature impacted *E. coli* concentrations quantified at Lake Michigan beaches regardless of the quantification method, but the trend was in the opposite direction of what we expected. *E. coli* quantities can decrease as the day proceeds (Whitman et al., 2004) and, since most of our samples were collected between 8 am and noon, peak insolation may not have occurred until later in the day. Our results suggest that water temperature had more of an impact on *E. coli* levels than air temperature, particularly at Lake Michigan beaches. For this reason, we recommend using water temperature in predictive models for monitoring beach water quality.

Bird Abundance

Unquestionably, birds impact the microbial water quality at the beaches where they gather. Studies investigating before and after bird mitigation with dogs have seen significant reductions in *E. coli* concentrations measured with culture and qPCR methods (Converse et al., 2012). Our research included two ways of comparing beaches with or without birds. There was strong evidence in all tested scenarios that bird count categories and the presence or absence of birds significantly impacted *E. coli* concentrations at both lake types and with both quantification methods. In agreement with our findings, Kelly et al. (2018) saw that beaches where birds were present had a statistically significantly higher number of exceedances compared to beaches without. One

consideration regarding their study was that the number of beaches with birds outnumbered those without by almost one-hundred times and therefore may have biased their results. Our study on the other hand had several sampling events where birds were not seen at inland lake and Lake Michigan beaches and still had the same outcome. Because birds are a predictor of *E. coli* levels, we feel it is important that censored *E. coli* concentrations paired with bird counts are considered in predictive models to capture all facets of the system. For example, on two sampling occasions Pere Marquette beach had over 100 birds present, and the *E. coli* results were below the LOQ. Our use of survival analysis allowed the inclusion of these data when other current predictive models would not have. Also to be noted is the way in which we categorized our bird count categories could drive our results because the high category had a much larger range than the low or medium. However, the results of the binary present/absent KM distribution curves provide clear support that birds impact *E. coli* levels in the water at a beach. These conclusions substantiate including either the number of birds as a variable or, more simply, whether birds are present or absent in beach closure predictive models in addition to including censored data paired with the environmental variable observation.

Rainfall

Contrary to other studies which have shown that rain impacts microbial water quality (Dwight et al., 2011; Yakirevich et al., 2013), the tested trends for the two categorized rainfall EVs analyzed in this study did not have considerable impacts on *E. coli* concentrations at inland lake or Lake Michigan beaches. The expectation was that as time increased between a rain and sampling event, the *E. coli* concentration would decrease. Although rainfall and *E. coli* density impacts were not statistically significant based on the ordered groups in our analysis, there were some patterns that emerged. For instance, the > 72 h since the last rainfall KM curve where *E. coli* was measured with Colilert (Figure 3.1h) or qPCR (Figure S3.10) at inland lake beaches had the lowest *E. coli* levels, agreeing with our predictions. The same trend tested for Lake Michigan beaches revealed that the >

72 h category as being associated with the highest *E. coli* concentrations when either quantification method was used (Figure S3.11). We suspect these differences could be a result of the varying beach catchment areas of the two lake types. As previously discussed, inland lake beaches did not have large tributaries emptying stormwater into them like some beaches along Lake Michigan. Therefore, it was likely that inland lake beaches were more rapidly affected by rainfall than Lake Michigan beaches as it takes longer for the stormwater runoff to reach the Lake Michigan beach environment. Inland lakes also are inherently smaller and have less potential for dilution than Lake Michigan. Although our results did not show categorized rainfall as being significantly associated with *E. coli* quantities, we still advise it to be included in predictive models because multiple studies have identified rain as a good predictor of bacterial water quality exceedances (Dwight et al., 2011; Eregno et al., 2016; Staley et al., 2018). The lack of evidence in our study may simply be from the manner in which we categorized and tested rainfall or because the rain gauges were not situated directly on the beach.

Method Comparison

In addition to the improved result turn-around-time qPCR provides, qPCR methods quantify viable but non-culturable bacteria cells along with culturable cells. These viable but non-culturable organisms can still be pathogenic (Pruzzo et al., 2002) and possibly become culturable under certain environmental conditions (Lleo et al., 2005). Therefore, the use of qPCR data can prevent encounters with some potentially harmful pathogenic *E. coli* in recreational water when Colilert results predict safe conditions. The strength of the detected significant difference between the two quantification methods in our study is reflected in the KM distribution curves of both lake types (Figures 3.2a & 3.2b) where a large separation is visible between the two methods. Our results support the implementation of a separate water quality criterion for the proposed Draft Method C qPCR assay because it is clear the two methods perform differently when quantifying *E. coli*. Our

findings also emphasize that models created for predicting *E. coli* concentrations must incorporate the quantification method being used because different quantification methods produce vastly different *E. coli* results.

Lake Type Comparison

Beach type, such as those along an open coast or situated within a bay, played a role in *E. coli* levels at beaches in Florida where lower concentrations were observed at the coastal beaches (Kelly et al., 2018). Although not an exact comparison to the freshwater lake types in our study, their open-coast beaches are similar to the freshwater coastal beaches of Lake Michigan in that there are long stretches of open coast with no disruptions to the coastline. We hypothesized that *E. coli* densities in Lake Michigan beaches would be lower than those at inland lake beaches because stronger water currents cause a larger mixing effect within Lake Michigan, which in turn contributed to the dilution of fecal contaminates throughout the substantially larger water body. However, our Lake Michigan beaches were not associated with lower *E. coli* densities than inland lake beaches. In fact, the Lake Michigan beach KM distribution curves were associated with higher *E. coli* concentrations (Figures 3.4a and 3.4b). One explanation for our results departure from our hypothesis is that with over 1,600 miles (~2,600 km) of shoreline and a 176,007 km^2 catchment area that can contribute water potentially contaminated with fecal matter, Lake Michigan has more tributaries and river outlets impacting its water quality than any inland lake tested. Tributaries can transport fecal contamination from upstream locations (Haack et al., 2013, Weiskerger et al., 2019) along with potentially pathogenic *E. coli* when upstream land has cattle pastures (Bradshaw et al., 2016) or highly developed areas lacking pervious surfaces (Molina et al., 2014). Although a conflicting study by the United States Department of Agriculture found no significant increase in *E. coli* found in runoff from fields after dairy compost application, the maximum *E. coli* concentration at all three types of agricultural fields tested did occur when compost had been applied (Harmel et al., 2010). Given that

there are hundreds if not thousands of tributaries within the Lake Michigan watershed, identifying those with considerably high *E. coli* concentrations, then taking steps to reduce the contamination would improve the microbial water quality at Lake Michigan beaches. Furthermore, microbial water quality predictive models should include tributaries and the plumes they produce as a factor because the plumes produced from water emptying at their outlets has the potential to impact *E. coli* along the coast (Nekouee et al., 2015).

3.6 Conclusion and Future Studies

The only EVs that performed the same for *E. coli* quantification method and lake type were related to the abundance or presence of birds. The remainder of the EVs analyzed differed in their impact on *E. coli* depending on the method used to measure it and the lake type. This was further supported by Haack et al. (2013) who used beach catchment delineation as a grouping variable and found no set of shared variables that were able to predict *E. coli* levels. EVs impact *E. coli* concentrations in drastically different ways at inland lake beaches compared to freshwater coastal beaches like those found on Lake Michigan, as our study shows. Therefore, our overall recommendation is that separate microbial water quality predictive models should be created based on the FIB quantification method being used to monitor water quality and the lake type. Additionally, analyzing *E. coli* or other FIB levels in beach sand samples alongside recreational water samples could improve model accuracy by providing a more complete picture of the possible sources of fecal contamination.

E. coli is a commonly used indicator of fecal contamination to monitor for unsafe swimming conditions at beaches. Beaches with repeated bacterial exceedances can then use microbial source tracking (MST)—another emerging qPCR tool—to determine the source (i.e. human, dog, bovine etc.) of fecal contamination (Byappanahalli et al., 2015; Shanks et al., 2016; U.S. EPA 2019) and action can be taken to reduce the number of beach closures. Future studies also should use emerging

molecular methods to investigate specific pathogens to better understand the risk to recreators, like is being done for swimmer's itch (cercarial dermatitis) (Rudko et al., 2018).

Kaplan-Meier distribution curves—part of the survival analysis statistical methods suite—allowed us to incorporate all *E. coli* concentration data in analysis. This provides a more universal view of the system being studied than if these data were removed or replaced with a different value. These statistical methods can extend into other environmental studies such as those including nutrient data or FIB fate and transport models. Therefore, future studies should consider these types of statistical analyses when censored results occur. Our trend tests did not distinguish between the differences in EV groupings though. For example, turbidity categories at inland lake beaches measured with Colilert were statistically significantly ordered, but it is unknown what pair of categories was the driver in the difference. Future studies should employ higher levels of survival analysis, such as Cox Regression (Cox, 1972), to distinguish which categories are driving the impact on *E. coli* concentrations.

Protecting recreators from unsafe swimming conditions is the primary objective for monitoring microbial water quality at beaches. What must be remembered, is that good water quality at beaches is not only important for recreational use, but that same water is often used as a drinking water source and for our food supply hence, safe beach water is connected to good water quality for all aspects of our life.

3.7 Supplemental Materials

Supplemental Tables

Table S3.1 Weather stations reporting to www.wunderground.com and the beaches whose rain data were recorded from it. Weather stations were selected based on proximity to the beach.

Weather Station	Associated Beach
KMICONKL3/ KMIRAVEN5	Moore County Park
KMIMONTA1	Maple Park
KMIMONTA2	Old Channel
KMIMONTA5	Meinert County Park
KMIMONTA7	Duck Lake State Park
KMIMUSKE4	Mona Lake Park
	Ross Park
KMIMUSKE35	Sunset Beach
	Wolf Lake County Park
KMIMUSKE45	Pere Marquette
	Harbor Towne
KMINORTO8	PJ Hoffmaster Public
	PJ Hoffmaster Campground
KMINORTH13	Pioneer
	Muskegon State Park North Channel Campground Watersports Park
KMITWINL5	Blue Lake County Park
KMITWINL8	Twin Lake County Park
KMITWINL9	Padley Park
	Fox Lake Park

Table S3.2 Log-rank trend test and log-rank paired with two-sample test for censored data *p*-values and z-statistic, where appropriate, from comparisons of categorized environmental variable (EV) Kaplan-Meier distribution curves. Bolded values were statistically significantly different ($\alpha = 0.05$).

Lake Type	Inland		Lake Michigan	
EV	**Colilert**	**qPCR**	**Colilert**	**qPCR**
Turbidity (NTU)	**0.035**	0.606	**<0.001**	**<0.001**
Wind Speed (mph)	**0.018**	0.801	**0.002**	**0.019**
Air Temperature (°C)	0.684	0.364	0.418	0.478
Water Temperature (°C)	0.343	0.942	**<0.001**	**0.002**
Number of Birds	**< 0.001**	**0.004**	**<0.001**	**<0.001**
Birds Present/Absent*	**0.015** **z = -2.18**	**0.012** **z = -2.24**	**<0.001** **z = -4.12**	**<0.001** **z = -4.04**
Time Since Last Rain (h)	0.057	0.658	0.192	0.099
Rain Amount (inches)	**0.031**	0.887	0.613	**0.035**

*Tested birds absent < present

Supplemental Figures

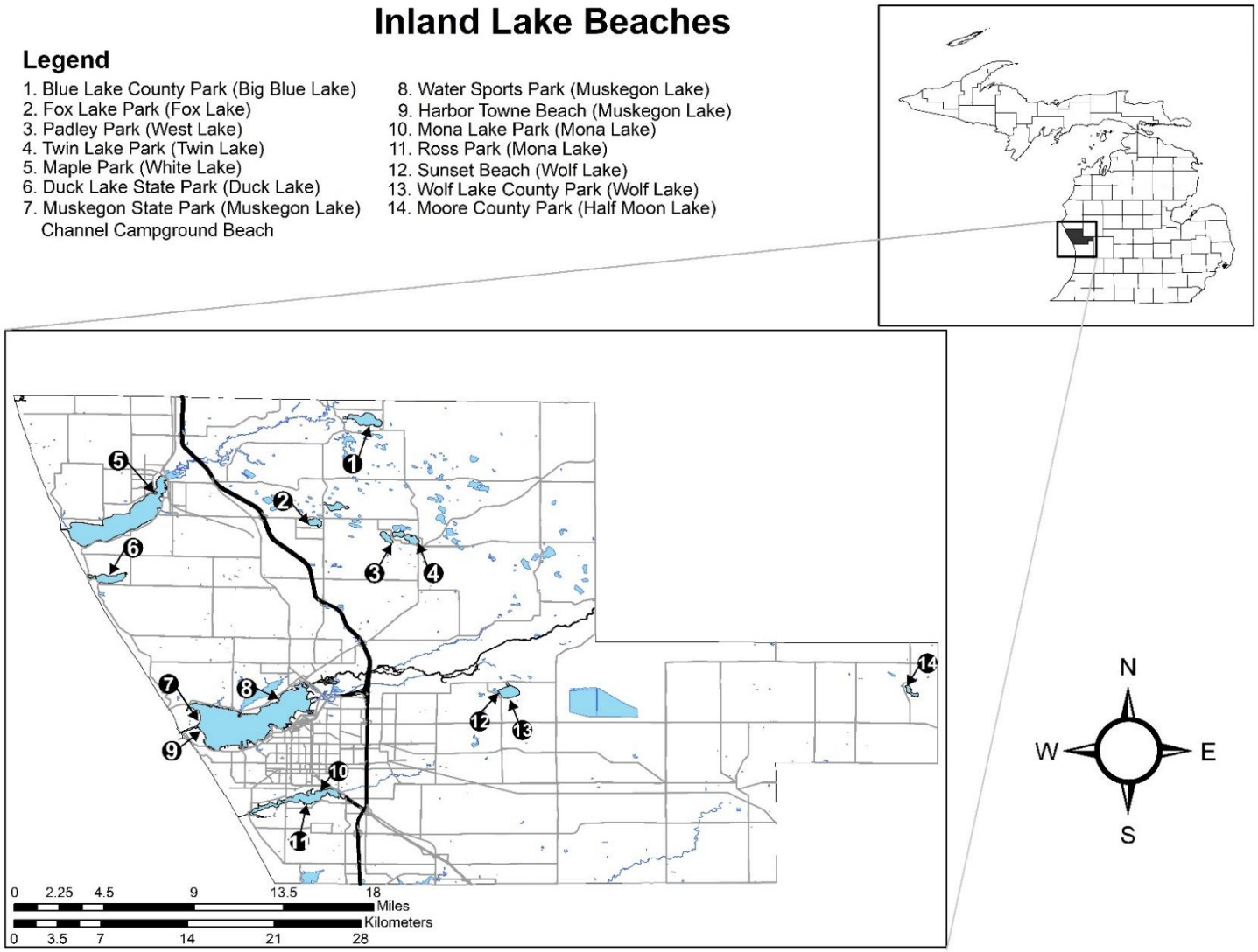

Figure S3.1 Inland lake beaches within Muskegon County, MI (inset) included in this study. Lake names where beaches are located are in parentheses.

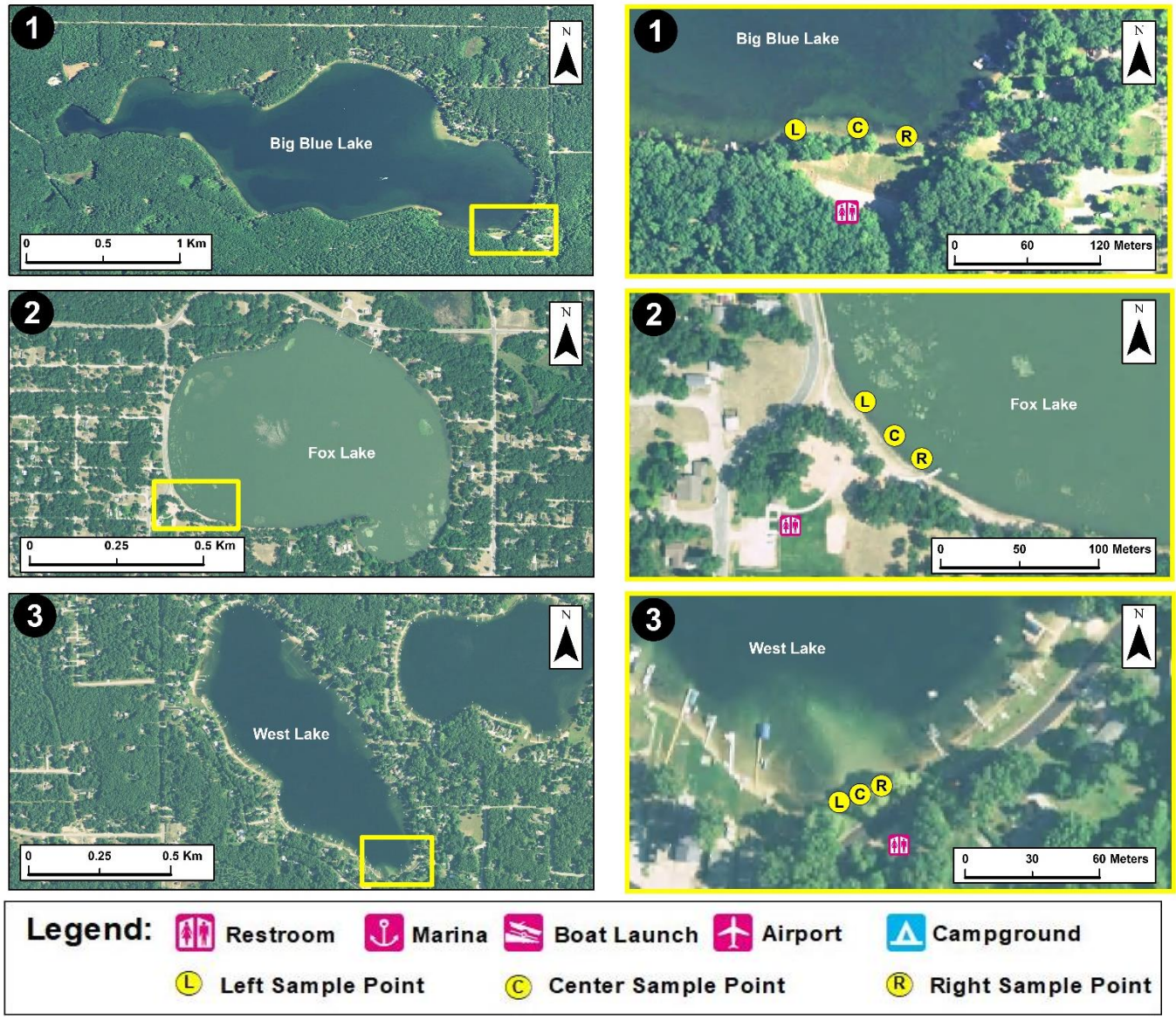

Figure S3.2 Blue Lake County Park (1), Fox Lake Park (2), and Padley Park (3) beach location on lake (yellow rectangle in left column) and sampling sites (right column).

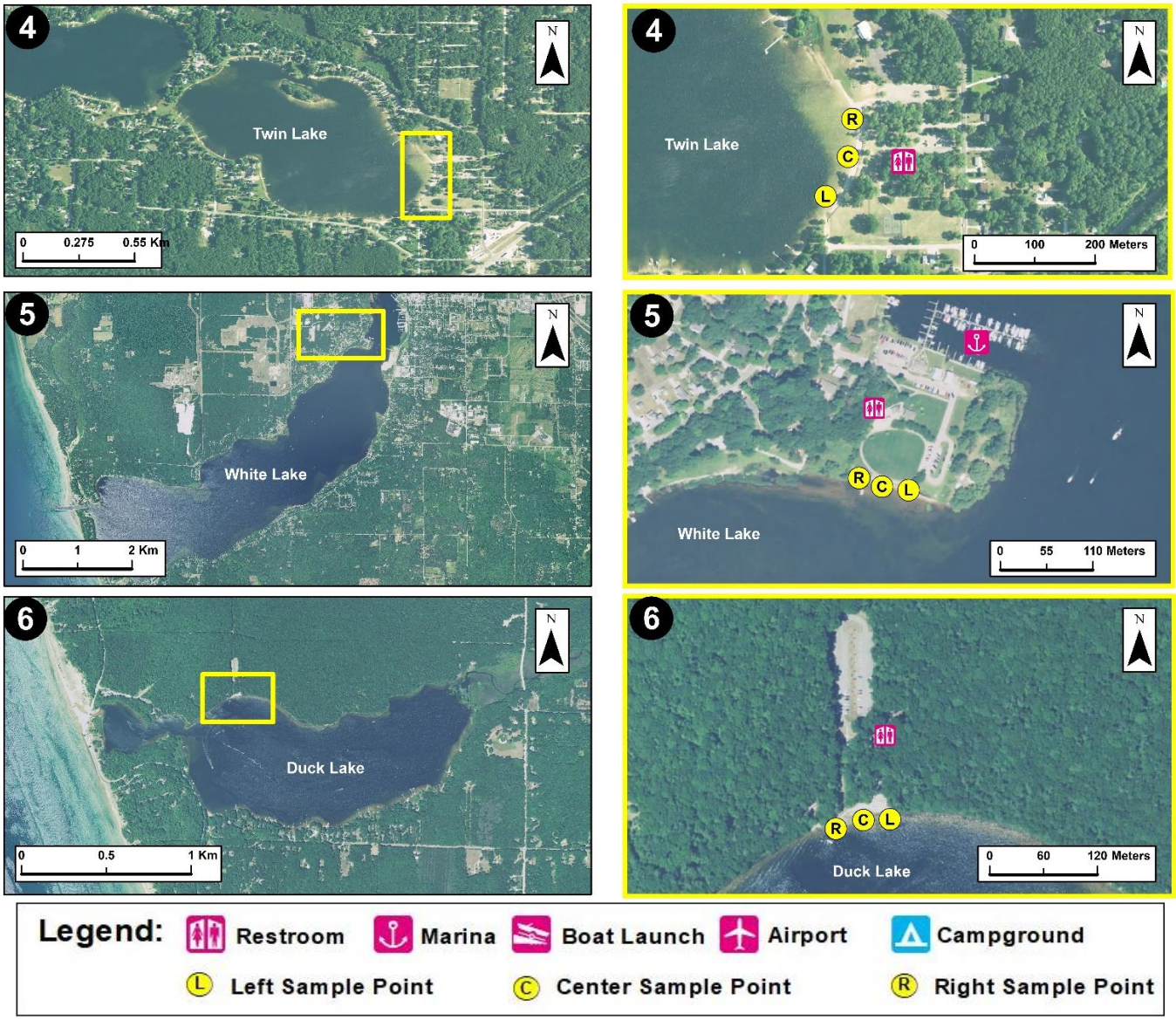

Figure S3.3 Twin Lake County Park (4), Maple Park (5), and Duck Lake State Park (6) beach location on inland lake (yellow rectangle in left column) and sampling sites (right column).

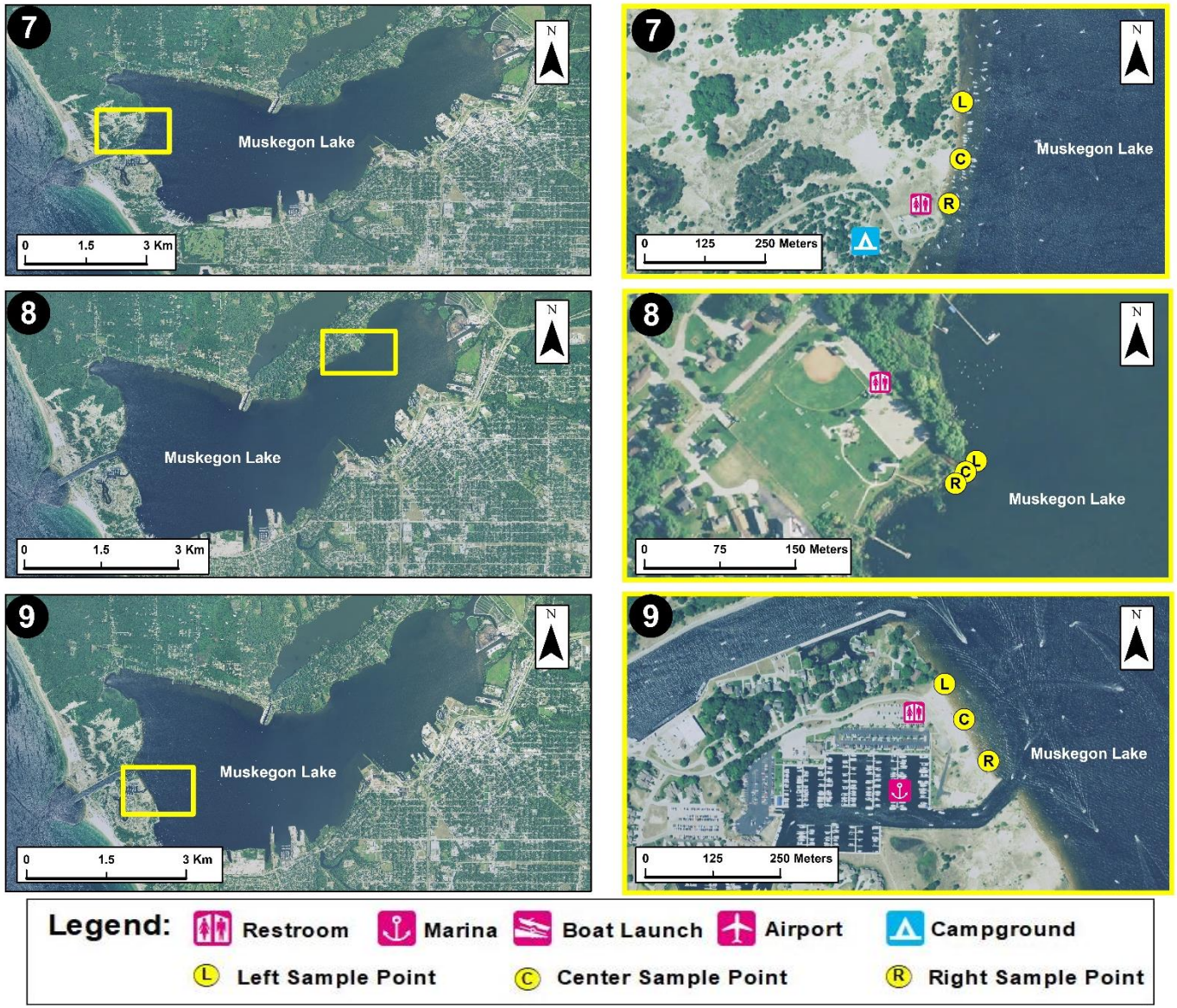

Figure S3.4 Muskegon State Park North Channel Campground (7), Watersports Park (8), and Harbor Towne Beach location on inland lake (yellow rectangle in left column) and sampling sites (right column).

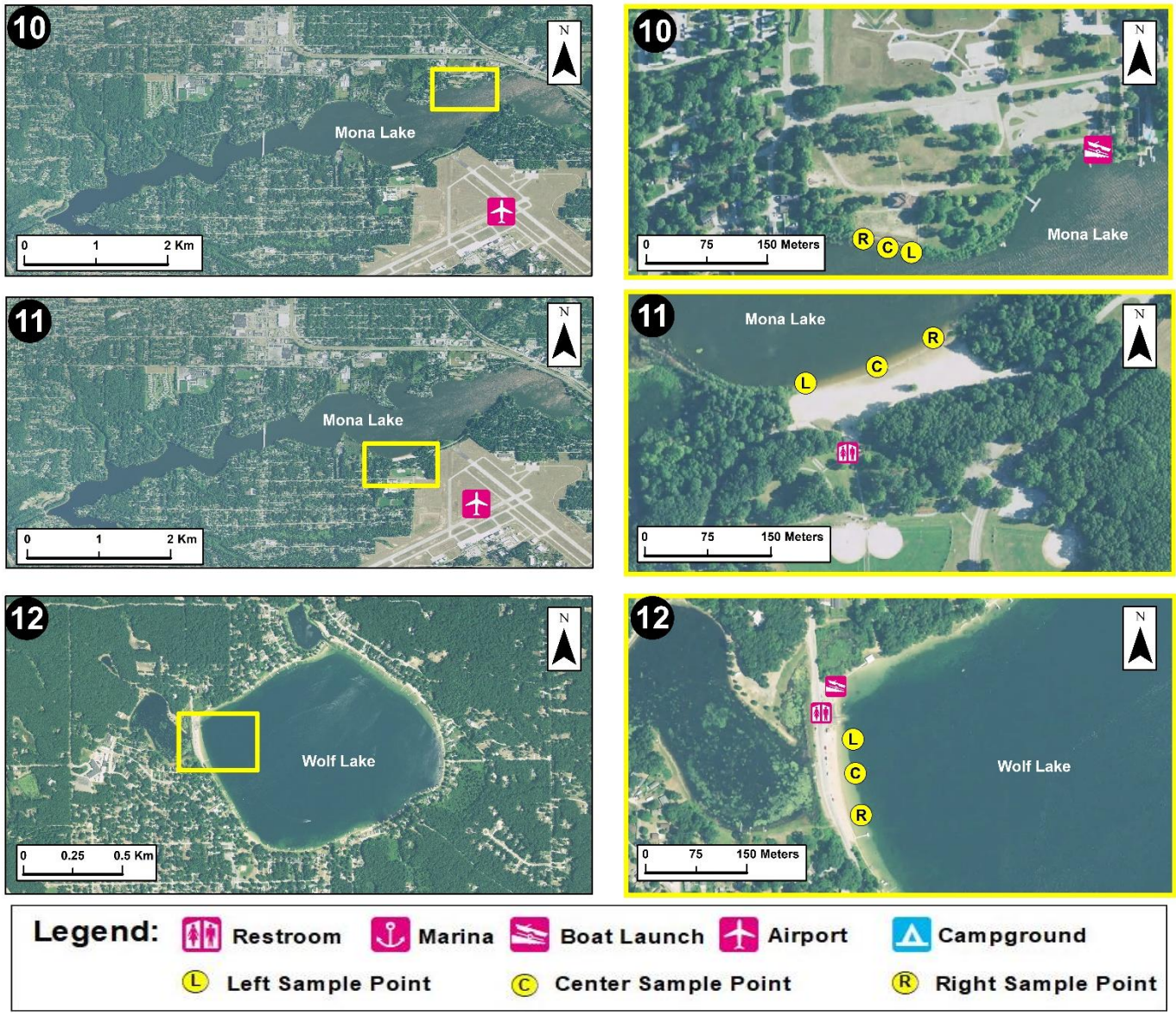

Figure S3.5 Mona Lake Park (10), Ross Park (11), and Sunset Beach (12) location on inland lake (yellow rectangle, left column) and sampling sites (right column).

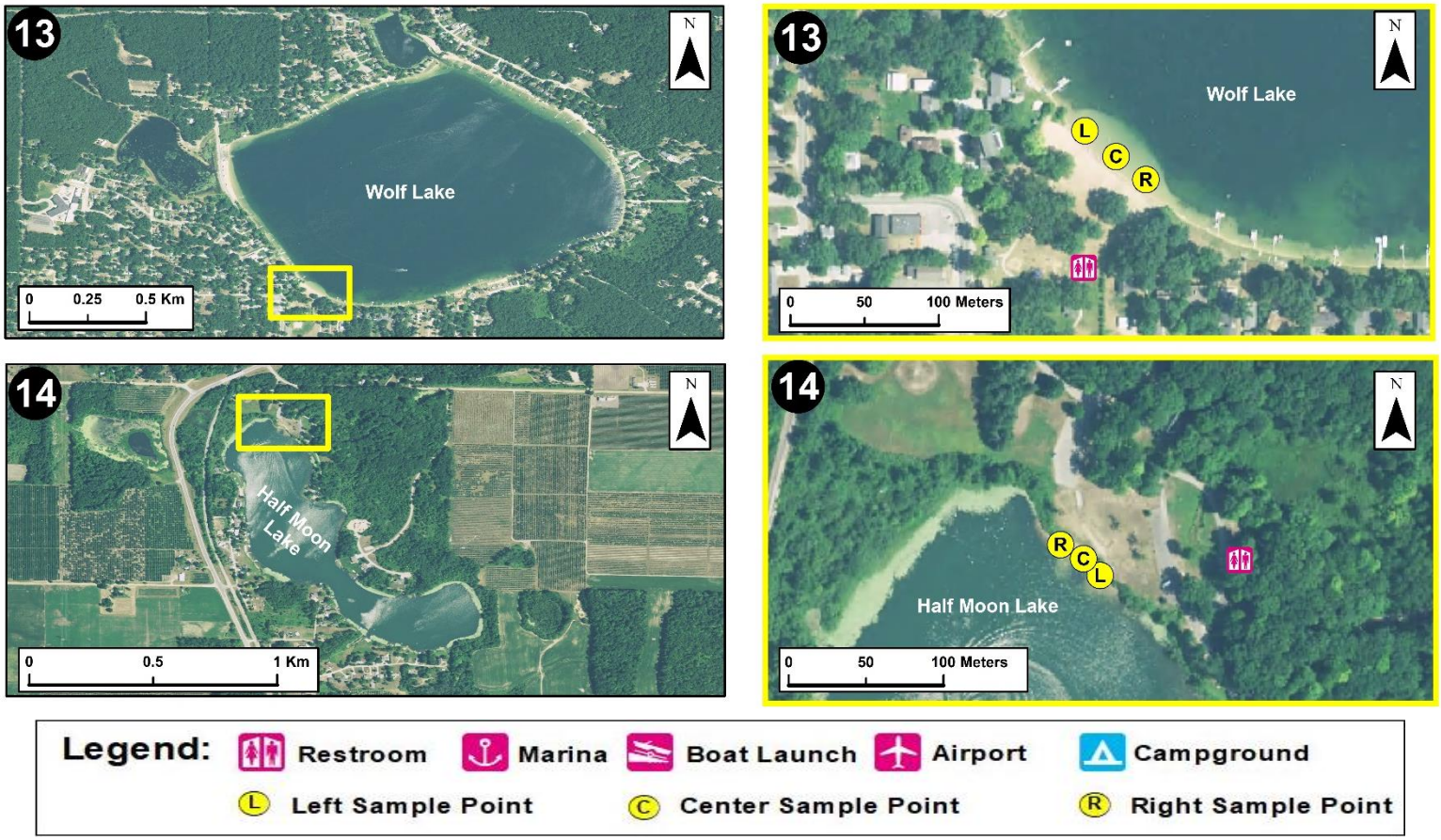

Figure S3.6 Wolf Lake Park (13) and Moore County Park (14) beach location on inland lake (yellow rectangle, left column) and sampling sites (right column).

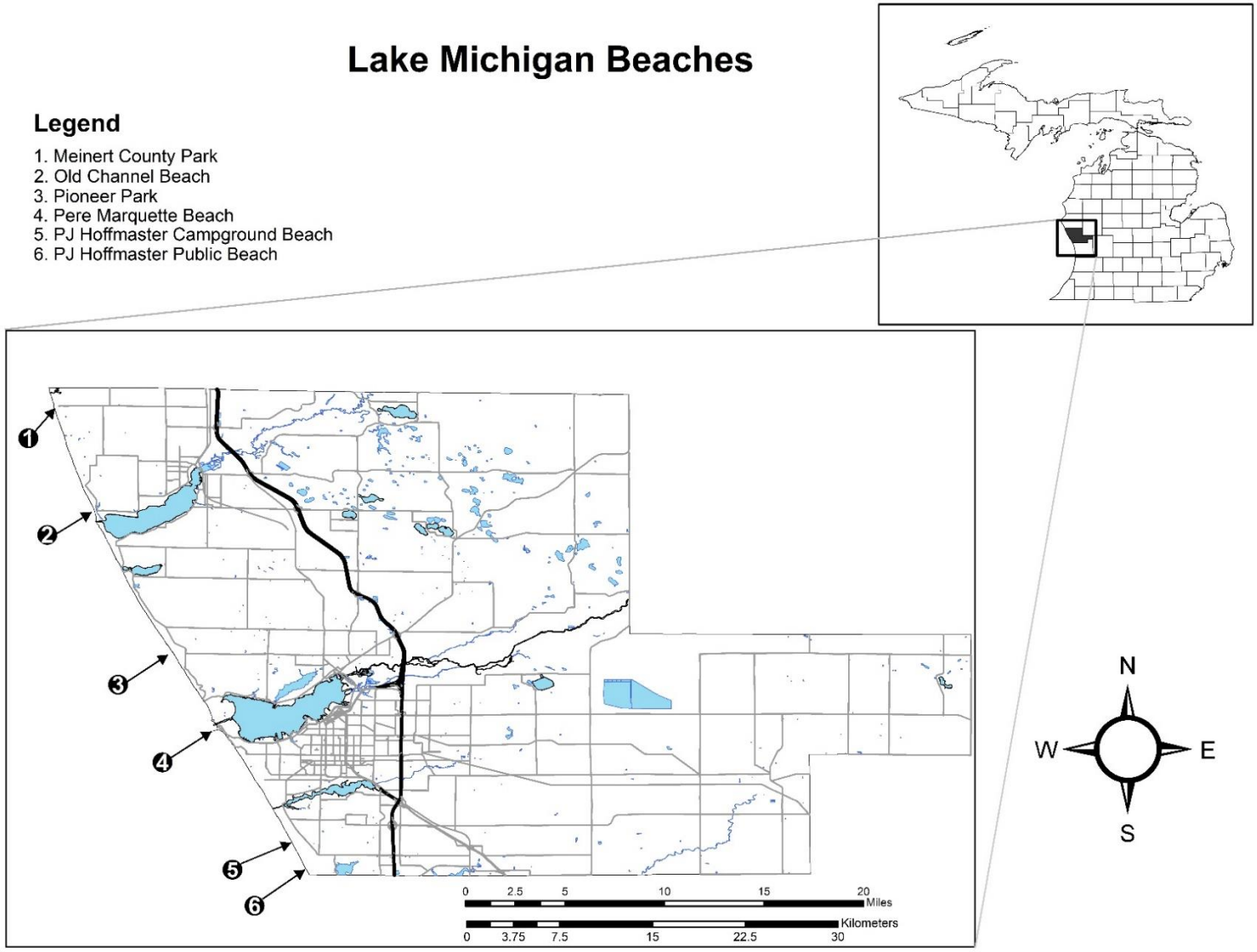

Figure S3.7 Lake Michigan beaches sampled for this study within Muskegon County, MI.

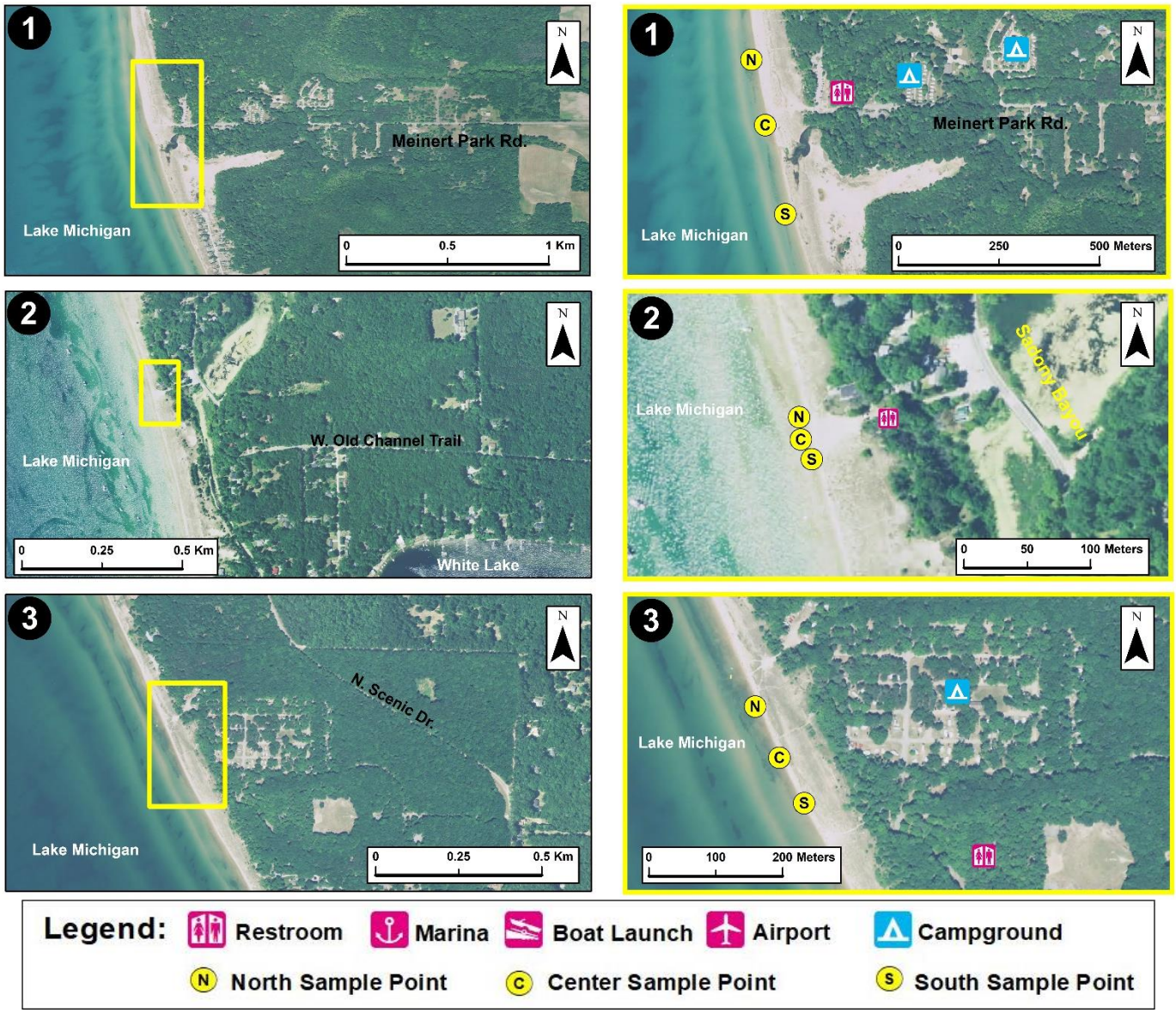

Figure S3.8 Meinert County Park (1), Old Channel (2), and Pioneer Park (3) beach location (yellow rectangle, left column) and sampling sites (right column).

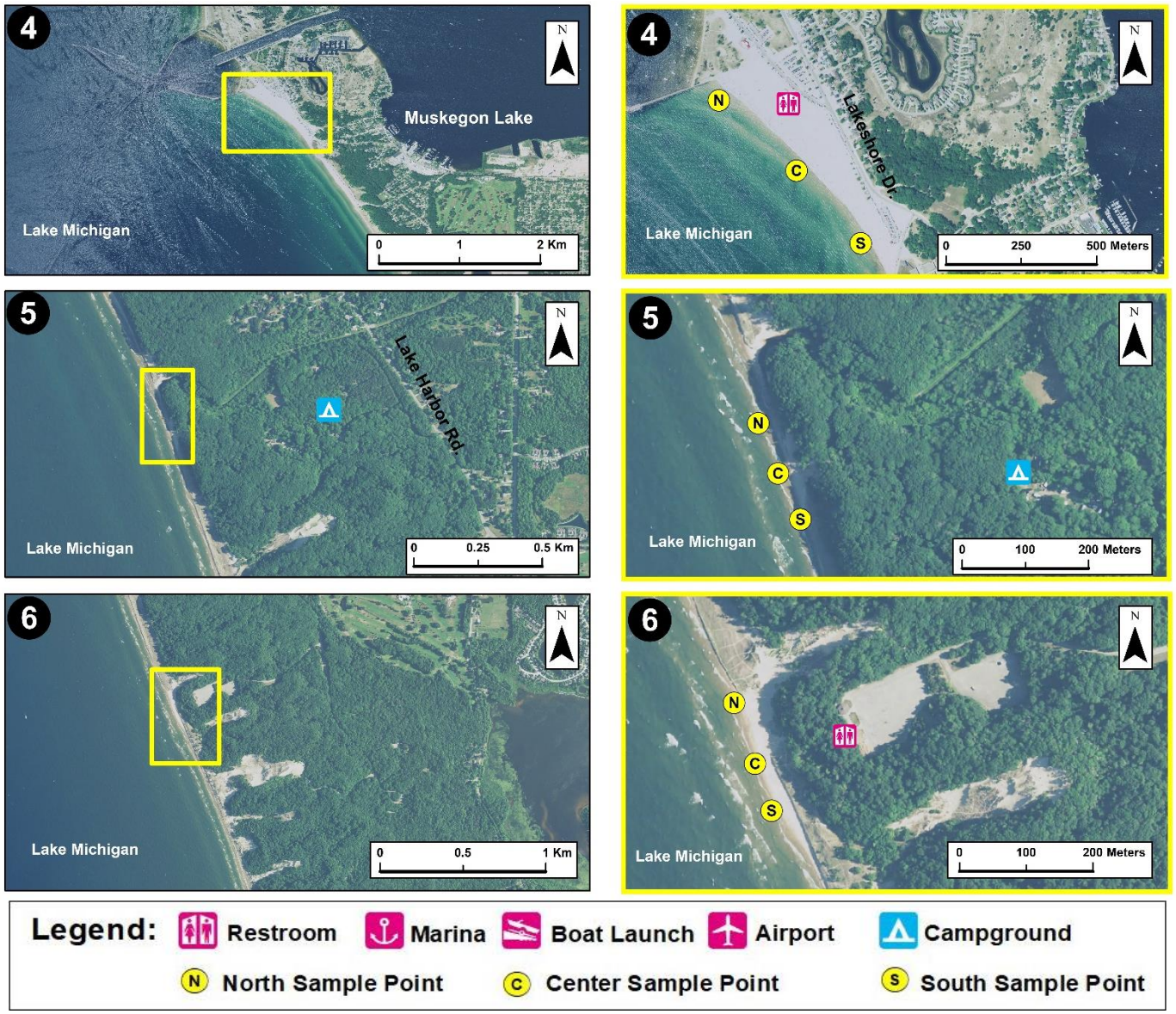

Figure S3.9 Pere Marquette (4), PJ Hoffmaster Campground (5), and PJ Hoffmaster Public (6) beach locations (yellow rectangle, left column) and sampling sites (left column).

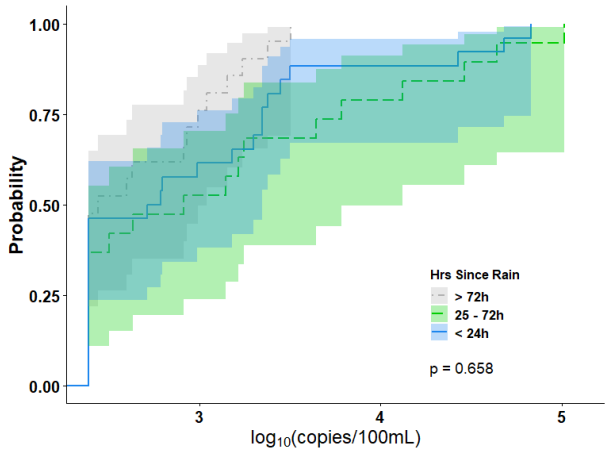

Figure S3.10 Kaplan-Meier distribution curves of hours since the last rain and *E. coli* concentrations measured with qPCR at inland lake beaches with 95% point-wise confidence intervals. Non-significant *p*-value ($p = 0.658$) indicates the tested trend did not significantly impact *E. coli* quantities measured with Colilert at inland lake beaches. Tested trend: (< 24 h) > (25 – 72 h) > (> 72 h); longer amount of time since the last rain would have lower *E. coli* concentrations.

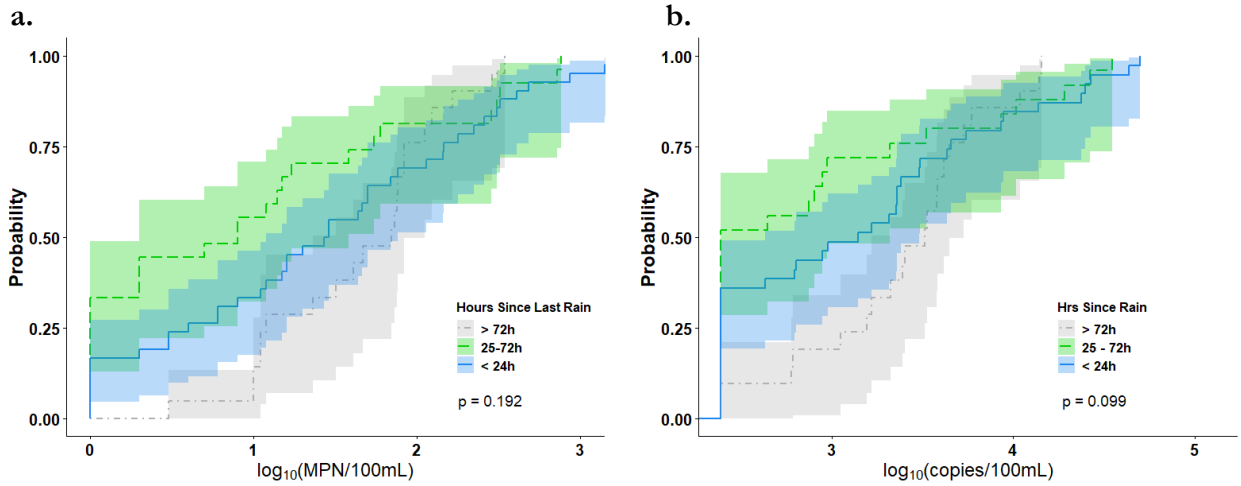

Figure S3.11 Kaplan-Meier distribution curves of hours since the last rain and *E. coli* concentrations measured with Colilert (a) and qPCR (b) at Lake Michigan beaches with 95% point-wise confidence intervals. Non-significant *p*-values indicate the tested trend did not significantly impact *E. coli* quantities. Tested trend: (< 24 h) > (25 – 72 h) > (> 72 h); longer amount of time since the last rain would have lower *E. coli* concentrations.

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Chapter 4 : Extended Review of Literature and Extended Methodology

4.1 Extended Literature Review

Recreational waters, such as public beaches, lakes, and rivers, are an economic resource for the local community. A Forest Service's National Survey on Recreation and the Environment (NSRE) report estimated that in 2008 approximately 61% of the United States' (US) population over 16 years of age participated in non-motorized water activities (i.e. swimming, snorkeling, visiting beaches) and almost 17% participated in canoeing, kayaking or rafting activities (Cordell, 2012). Furthermore, a single beachgoer will spend $13.13 within ten miles of the beach per visit (Murray et al., 2001), helping to support local businesses. Muskegon County is one of the communities that benefits from tourism. In 2016 approximately 1.7 million visitors traveled to Muskegon County and spent an estimated $244 million on leisure (B. Lukens, Community Development Director, Muskegon County Convention & Visitors Bureau, www.visitmuskegon.org, pers. comm.). One of the main tourist draws are the thirteen public beaches along the twenty-six miles of Lake Michigan shoreline and the fifteen public inland lake beaches that serve as places for recreation. On the other hand, a beach closure due to unacceptable water quality can result in lost tourism dollars. A one-time beach closure could cost, on average, $100,000 in lost revenue to businesses within a ten-mile radius of the closed beach (Jentes, 2000). Thus, the number of beach attendees in a single summer has the potential of adding up to millions of dollars invested in the community provided that the beaches remain open for use.

Recreational waters are also a public health concern. It has long been known that swimming in contaminated water can lead to illness (Stevenson, 1953; Cabelli et al. 1982; Dufour, 1984; Wade et al., 2008). Several types of illnesses have been reported as being associated with recreational waters. Respiratory infections, gastrointestinal illnesses (GI), ear and skin symptoms were higher

among swimmers than non-swimmers, and ear infections were higher among swimmers who had their head underwater (Seyfried et al., 1985 (I); Colford et al., 2007; Wade et al., 2008). Also to consider is how contact with polluted water affects the health of different groups of people. For instance, people with compromised immune systems or elderly individuals may be more susceptible to health issues arising from contact with contaminated water. Additionally, children ages 10 and under are more greatly affected by contact (Wade et al., 2008). Therefore, it is important that water quality guidelines protect everyone from exposure to contaminated water.

Testing recreational waters for fecal contamination is essential to public health, but the question of what organism to test for arises. There are numerous microbial pathogens associated with fecal contamination but testing for all of them would place a temporal and financial burden on the entities conducting the monitoring. Despite an early study showing that *staphylococci* were a better indicator of recreational water illnesses (Seyfried et al., 1985 (II)), the most commonly used fecal indicator bacteria (FIB) are total coliforms, fecal coliforms, *Escherichia coli (E. coli)* and *enterococci* (Meays et al., 2004). Of these, *E. coli*, a potentially pathogenic bacterium found in the intestines of endothermic ("warm-blooded") animals (Whitman et al., 1999), is considered to be the best indicator microorganism for freshwater (Prüss, 1998). A meta-analysis of recreational water quality literature published from 1950 to 2003 reported most studies indicated *E. coli* surpassed *enterococci* as a FIB for predicting GI illness in freshwater than other bacterial indicators (Wade et al., 2003).

E. coli is a well-studied FIB for recreational water monitoring. There are several routes through which it is believed to enter the beach environment: treated municipal wastewater discharge, untreated sewage during storm events due to combined sewer overflows, aging septic systems, agricultural runoff (Bradshaw et al., 2016), water vessel wastewater discharge, leakage from diapers on children, urban runoff (Molina et al., 2014), and as animal wastes from birds (Mathai et al., 2018),

dogs and other wildlife. Another possible introduction pathway for *E. coli* into the water column is from disturbed sand or sediment on the lake bottom when intense wave action occurs (Laliberte and Grimes, 1982; Staley et al., 2016). Sand in the foreshore area of the beach also can be an *E. coli* source as runoff from rain or waves carry *E. coli* stored in the sand towards the water (Whitman and Nevers, 2003; Alm et al., 2003; Ishii et al., 2007). Cladophora mats (Whitman et al., 2003; Quilliam et al., 2014) and submerged aquatic vegetation such as the invasive Eurasian watermilfoil (*Myriophyllum spicatum)* (Mathai et al., 2018), may also serve as introduction routes of *E. coli* intro recreational waters. Although the statistical methods used in the Mathai et al. study (2018), were unable to demonstrate the direction of *E. coli* movement; they assumed the bacteria moved from areas of higher to lower density (i.e. from the watermilfoil to the water column). Furthermore, tributaries that are near swim areas also may contribute *E. coli* as they carry storm water runoff into the beach area (Baral et al., 2018). Six years of hydrometeorological data were used to examine precipitation impacts on the coastal beaches of California and revealed peaks in precipitation levels, river discharge rates and bacteria quantities all happened concurrently (Dwight et al., 2011). Another study collected water samples downstream of a tarp-lined creek where high-water flow was simulated by releasing city water over the tarp. *E. coli* concentrations peaked when flow rates peaked, suggesting that *E. coli* stored in the benthos was resuspended in the water column during peak-flow, like that which occurs during rain events (Yakirevich et al., 2013); evidence that *E. coli* can be introduced into a beach through a contaminated tributary.

Because the waters of public beaches may be contaminated with pathogenic bacteria, they should be routinely sampled and analyzed during the summer when attendance is highest. Federal and local governments provide guidance and administer programs for monitoring the water quality at public beaches. An amendment to the Clean Water Act in 1986, section 304(a) (1), implemented

by the U.S. Environmental Protection Agency (U.S. EPA) set guidelines for bacterial densities in recreational waters. The guidelines, based on the research conducted by Cabelli (1982) and Dufour (1984), were determined by the likelihood of developing a GI illness through contact with fecal contaminated water. The current acceptable level of *E. coli* in a recreational water sample, as recommended by the U.S. EPA, is a 30-day geometric mean not to exceed 126 colony forming units (cfu) per 100 mL (U.S. EPA, 1986). This recommendation is explicitly a guideline for states and not an enforceable regulation. The decision to adopt it or develop a different one, based on scientifically determined water quality data, is left up to each individual state.

Michigan is one of the states with its own criteria for recreational water quality (EGLE, 2019). The Michigan Public Health Code (Act 368 of 1978) authorizes local health departments to monitor bathing beaches, but does not require it (EGLE, 1978). According to the health code, beaches must be sampled a minimum of five times in a 30-day period, with three samples taken at each beach during every sampling event. These three samples can be analyzed individually and a geometric mean calculated for the three samples. Alternatively, the three samples can be composited, and a single sample analyzed. For a beach to remain open for swimming the *E. coli* concentrations of the daily geometric mean, or single composited sample, must be below 300 cfu/100 mL. At the end of the 30-day period, a 30- day geometric mean is calculated for all individual samples collected during the 30-day sampling period. The results of the 30-day geometric mean must fall below 130 *E. coli* per 100 mL for the water to be designated as safe for swimming. A beach is closed if either of the following occur: 1. the single day result exceeds 300 cfu/100 mL or 2. the 30-day geometric mean is exceeded. The *E. coli* level results are then required to be reported to the Department of Environment, Great Lakes and Energy (EGLE) within 36 hours. Once the bacteria levels are back within the acceptable range, the waterbody is deemed safe for swimming and

the beach will reopen. The regulatory *E. coli* levels set by the State of Michigan are slightly higher than the U.S. EPA recommendation, but still provide a level of risk abatement when beaches are routinely monitored.

The above water quality criteria recommended by the U.S. EPA and established in Michigan were based on quantification by membrane filtration; one of the U. S. EPA approved methods for testing water quality. For the membrane filtration method, water samples are filtered, exposed to a modified thermo-tolerant *E. coli* agar and incubated for 22 ± 2 hours. *E. coli* colonies are counted based on a color change brought about by the culture media and are reported as cfu's. Another method, originally used for drinking water and wastewater testing, is the IDEXX Colilert-18® Quanti-Tray/2000 system, hereafter referred to as Colilert (Crane et al., 2006). Colilert relies on the enzyme β-glucuronidase to react with a patented chemical substrate containing 4-methyl-umbelliferyl β-D-glucuronide (MUG) which has a fluorescent molecule attached (www.idexx.com). Water samples are mixed with MUG, poured into a Quanti-Tray, sealed and incubated for 18 – 22 hours. The MUG substrate causes enzymatic activity in the β-D-glucuronidase enzyme, cleaving the fluorescent molecule. Following the incubation time, the tray is exposed to ultraviolet light, the number of large and small fluorescing wells are counted, and *E. coli* concentrations are reported as the most probable number (MPN). The U.S. EPA approved the Colilert method in 2003 (U.S. Federal Register-40 CFR Part 136 Vol. 68, No. 139, August 2003). Following its approval, a two-year comparison study was performed using water samples from five Lake Michigan beaches. A strong correlation in *E. coli* concentrations was found between the membrane filtration and Colilert methods, and in less than 0.5% of the samples would there have been a discrepancy in a beach closing or remaining open (Kinzelman et al., 2005). However, relying on the enzymatic activity of β-D-glucuronidase may not account for some of the most pathogenic strains of *E. coli*, such as the

O157:H7 because enzymatic activity is not exhibited (Fricker et al., 2008) and, other microorganisms, such as *Salmonella*, *Shigella* and *Staphylococcus* spp, also produce β-D-glucuronidase (Silva and Domingues; 2015). Furthermore, the Colilert method does not account for viable but non-culturable bacteria cells (Pruzzo et al., 2002) that may become viable, or culturable, under certain conditions (Lleo et al., 2005). Although the membrane filtration and Colilert methods are reliable, the time to obtain a result requires at least 18 hours. Consequently, water quality results are not disseminated to county officials, beach managers or the public until the day after the potential health risk occurred. This management practice is flawed in that *E. coli* levels can vary greatly from day to day (Whitman et al., 1999; Dorevitch 2017; Wyer et al., 2018) thus beaches are closed on days where there may be no risk but remain open on days where the risk was high. Both U.S. EPA approved methods provide some measure of protection from exposure to fecal contaminated water, but a new recreational water monitoring method was needed.

It is because of the temporal limitations and concerns about public health that the Beaches Environmental Assessment and Coastal Health (BEACH) Act was signed into law (Congress, 2000). The BEACH Act is an amendment to the Clean Water Act and instructs the U.S. EPA to conduct and fund research for faster testing, monitoring and reporting techniques for water quality in recreational waters. Additionally, the BEACH Act requests epidemiological research aimed at potential pathogens found in water and their effect on human health. The culmination of these studies would deliver revised water quality criteria to be included in the Clean Water Act.

Many studies have since been conducted to find a more expedited method of testing and reporting beach water quality results. One technique is microbial water quality predictive modeling, or nowcast modeling. Nowcast modeling offers a more cost-effective method since samplers and complex instruments for analysis are not needed. Nowcast modeling of *E. coli* outbreaks is a

statistical technique in which past water quality data and current environmental variables are entered in a "Virtual Beach" public domain software (Frick et al., 2008; Cyterski et al., 2016). Abiotic factors thought to impact *E. coli* densities in water include such things as water turbidity, wind direction and speed, wave height, rainfall and presence of tributaries emptying into the beach area (Frick et al., 2008, Byappanahalli et al., 2010) and can often be collected from nearby buoys and weather stations already in place. The software then uses a multiple linear regression model and predicts when *E. coli* concentrations would be high enough to warrant a beach-closure. Microbial water quality predictions were often more accurate than sampling (Frick et al., 2008) and, when used in combination with a microbiological quantitative method, the model was effective (Byappanahalli et al., 2010). In contrast, on five of eleven occasions a nowcast model failed to predict *E. coli* levels above the U.S. EPA limits (Nevers and Whitman, 2005). While microbial water quality predictive models are faster and cost-effective there is still research needed to determine how they can be applied at individual beaches with dynamic and differing environments. Another approach is to test for chemical compounds associated with anthropogenic waste as a way of identifying fecal contamination in waterbodies. Pharmaceuticals such as carbamazepine (for seizures, nerve pain and bipolar disorders) and diphenhydramine (an antihistamine for pain and itching) or caffeine (Glassmeyer, et al., 2005) could potentially act as indicators of human sewage. However, the concentrations of these pharmaceutical chemicals decreased as the distance from waste-water treatment plants increased. Therefore, their use may not be applicable for recreational waterbodies distant from waste-water treatment plants. Moreover, connecting the presence of human-associated chemical substances found in wastewater and increased risk of illness is poorly supported (Napier et al., 2018).

The BEACH Act also sparked research on an emerging technology for water quality analysis, common in microbiology, called Quantitative Polymerase Chain Reaction (qPCR). Sample is mixed with a DNA polymerase, individual nucleotides and a primer/probe mixture whose DNA sequence template is complimentary to the target gene of interest. The sample mixture is heated, causing DNA strands to denature, then cooled allowing the primers and probes to anneal to the target sequence. Finally, the DNA polymerase extends the primer using free nucleotides in the reaction mixture thereby building a complimentary strand of the target DNA (Figure 4.1). The heating and cooling cycles are carried out approximately 40 times, producing an exponential amplification of the target gene sequence to be measured and is completed in only 2 – 3 hours. This is a significant improvement over the 18 – 24 hours required for membrane filtration or Colilert methods currently in use for microbial water quality analysis. An early demonstration of this assay for use with water samples used the DNA polymerase *Thermus aquaticus* (*Taq*) (Holland et al., 1991). *Taq* is a DNA polymerase with 5'→ 3' exonuclease capabilities, meaning it can both extend a primer (DNA polymerase activity) and cleave nucleotides annealed to the target DNA strand (exonuclease activity). Through use of the exonuclease capabilities, the probes, which attach at a different segment of DNA than the primer, can be labeled with a fluorescent signal molecule (fluorophore) on the 5' end of the DNA and *Taq* will cleave it thereby releasing it into the reaction mixture. After each cycle, the cleaved fluorescent signal molecules are detected by the instrument and measured. When the signal reaches a pre-determined level, the instrument provides a threshold cycle value (Ct) associated with the cycle number it occurred at. This value is inversely related to the starting amount of the target gene or, the lower the number of target gene copies in the sample, the higher the Ct value. Much research has been done to demonstrate the effectiveness of qPCR at measuring *E. coli* and approved

methods are available for measuring enterococcus concentrations in beach water samples (U.S. EPA 2012 and 2013).

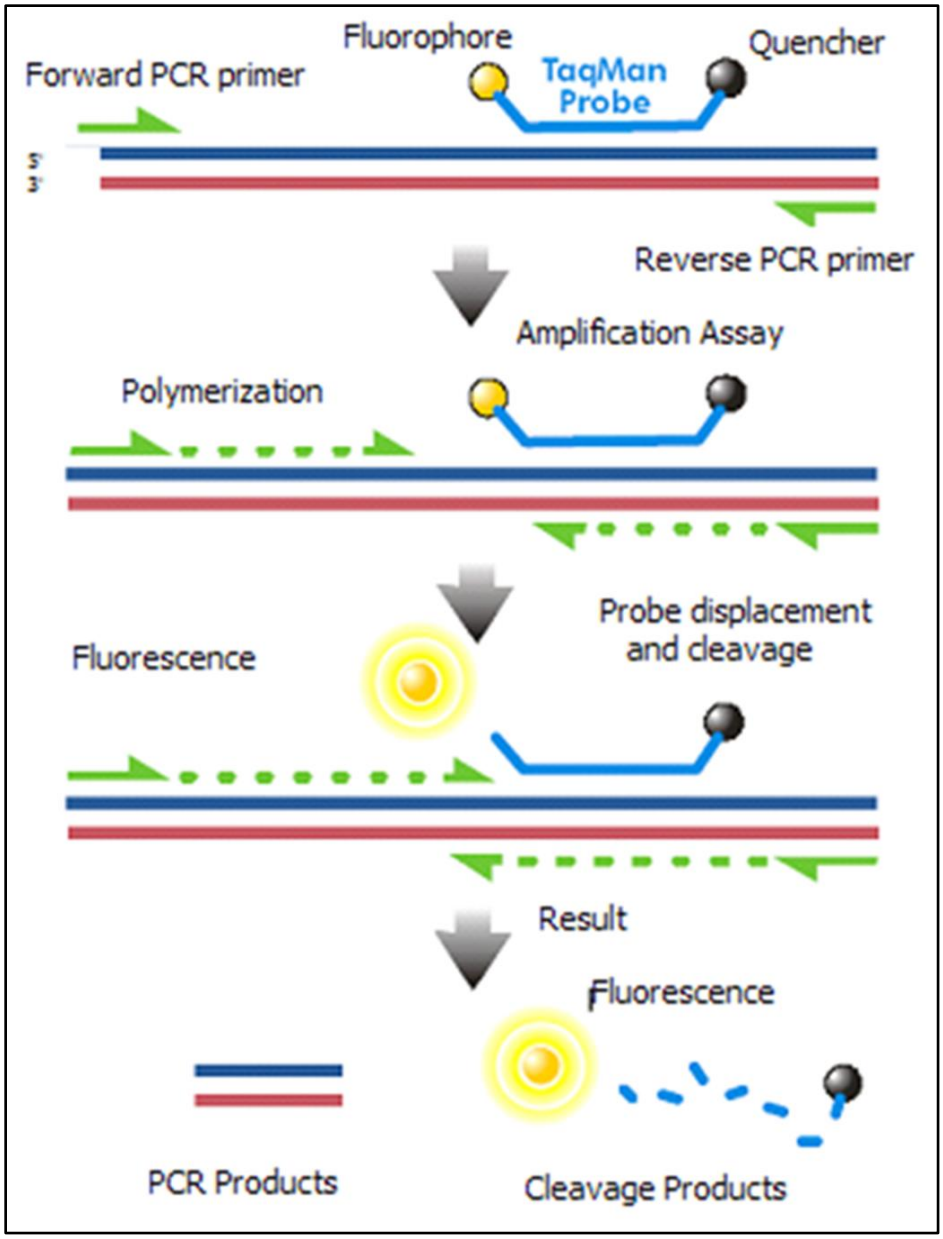

Figure 4.1 Diagram of a qPCR reaction cycle. The green arrow represents where the TaqMan™ DNA polymerase attaches and extends the primer. Photo courtesy of Wikipedia.org.

Quantification of *E. coli* by qPCR uses a fluorescent signal molecule called FAM, and a DNA polymerase called TaqMan™ (Applied Biosystems, StepOnePlus). The 23S rRNA gene, a specific

region on *E. coli* DNA, is amplified and used to calculate the number of *E. coli* DNA gene copies present in a water sample. A positive correlation between culture-based methods and qPCR has been repeatedly verified (Haughland et al., 2005; Lavender and Kinzelman, 2009; Lam et al., 2014). The correlation was even more pronounced at higher concentrations (Whitman et al., 2010) which is applicable to beach monitoring programs since regulatory levels are set to > 100 cfu. Confirmation of the qPCR method's ability to predict swimming-associated GI illnesses came in 2006 (Wade et al.) and was reported as being a better predictor of GI illnesses than the culture-based method (Wade et al., 2008). Preliminary studies showed that qPCR was a promising candidate for beach water quality monitoring since it would greatly improve sample turn-around time and because it can detect densities of *E. coli* in compliance within regulatory levels. However, there are many factors to be examined before it becomes a fully approved method.

Interactions within the reaction-mixture are one of the factors to consider when using qPCR. Factors such as detection of the fluorescent signal molecule while it is still attached to the probe and, preventing the *Taq* polymerase from cleaving the fluorescent molecule before it has annealed to the sample's *E. coli* target gene sequence. If either of these were to occur, an elevated fluorescent signal would be detected, and the method would not produce accurate results. To prevent signal detection before cleavage, The TaqMan™ assay uses a quencher molecule called TAMRA™. It is attached to the 3' end of the probe and while the quencher and fluorescent signal molecules are near one another, the fluorescent signal is "quenched" thereby preventing a false reading. This phenomenon is referred to as Fluorescence Resonance Energy Transfer (FRET) (Cardullo et al., 1988). It is only once the fluorescent signal molecule is released that the thermocycler instrument detects the fluorescence. Holland et al. (1991) undertook the second interaction and showed that the *Taq* polymerase only cleaved the fluorescent signal molecule while it

was attached to the target gene sequence. Factors involving laboratory technique also are important when implementing a qPCR assay. As discussed in Chapter 2.5, because qPCR requires pipetting volumes in the micro-liter (μL) range, it is important that analysts are adept at repeatedly pipetting small volumes. The cost and time to implement a new technology must also be addressed. Training of personnel, purchasing supplies and instrumentation necessary to implement a qPCR lab has been estimated to cost $100 thousand (Griffith and Weisberg, 2011). Grants awarded by the U.S. EPA under the BEACH Act will help defer costs, making the qPCR method available to a greater number of monitoring entities, thereby protecting a greater number of recreators from contact with fecal contaminated water.

Another factor which needs to be studied is qPCR's response to matrix interferences in varying water-body types. For it to replace current methods, it must provide accurate and reproducible results regardless of differing aquatic environments. Inland lakes tend to be more turbid then temperate water bodies such as the Great Lakes and turbidity is a cause of interference in qPCR analysis. Studies that have analyzed inland lake water samples (Siefring et al., 2008; Whitman et al., 2010; Haughland et al., 2012) did not extensively sample the inland waterbodies and, in some instances, only a single sample was taken from each inland lake. Another source of interference is organic material such as humic and tannic acids in the water sample (Opel et al., 2010). One effective approach to eliminating organic interferences and turbidity problems was to dilute water samples by five- or ten-fold (Haughland et al., 2005, 2012; Siefring et al., 2008; Noble et al., 2010). Another way to negate interference is to use a DNA purification step. DNA purification kits can be easily obtained and were found to be successful in samples exposed to heat prior to enumeration (Varma et al., 2009). However, for purposes of beach monitoring, heat-killing bacteria in samples before measurements are made is not a viable solution. Conversely, the purification step

did not improve results, and even caused DNA to be of poor quality leading to unmet method efficiency criteria (Noble et al. 2010). The amount of qPCR data on varying water-body samples is growing, but additional research is needed to make strong conclusions about how qPCR will handle the various water types and how to eliminate the related interferences.

As a result of all the research on qPCR quantification methods, a draft method for quantification of *E. coli* in recreational water by qPCR that uses the Applied Biosystems TaqMan™ assay and StepOne Plus thermocycler has been proposed by the EPA, currently called Draft Method C. More details on the specific method steps can be found in Chapters 2 and 3 of this manuscript. There have been many changes to the method throughout its development. Initially, as per the Site-Specific Alternative Recreational Criteria Technical Support Materials for Alternative Indicators and Methods, hereafter referred to as TSM, (U. S. EPA, 2014) the U.S. EPA used Willmott's Index of Agreement (IA) (Willmott, 1981) to compare *E. coli* concentrations quantified with the Draft Method C qPCR method to the Colilert defined substrate method. According to the TSM, the IA or Pearson's Correlation Coefficient (R-squared; R^2) can be used to test if the association between a proposed and approved quantification method were equivalent; though, it was noted that squaring the error terms in the IA equation increased the sensitivity to outliers (Kneale et al., 2001) and Willmott later removed the squaring function from the error terms, thereby reducing outlier sensitivity (Willmott et al., 2012). The E. *coli* reporting units and quantification method also have changed during the development of Draft Method C. Originally, the reportable quantity was Calibrator Cell Equivalents (CCE) which were calculated using the comparative quantification method, or delta-delta Ct (ΔΔCt) quantification method (Life Technologies, Ch. 4.3, 2014; p. 46). The reporting unit then became the Genomic Equivalent (GE). GEs were calculated by dividing the CCE by seven, as it was assumed that each *E. coli* cell had seven copies of the target gene. By

dividing the number of target gene copies by seven, it would give the estimated number of *E. coli* cells present in the sample. However, it was later determined that the ratio of seven gene copies per cell was not ubiquitous for all samples (Dr. R. Haugland, U.S. EPA. pers. comm.) because qPCR amplifies the target gene sequence regardless of whether it is contained within a cell or free in the environment. The current version of Draft Method C uses a Weighted Linear Regression (WLR) calibration model and *E. coli* concentrations are reported as log base-10 gene copies per reaction. Lab-specific composite curves are generated from independently analyzed standard curves, as described in Chapter 2, and are then used to quantify *E. coli* concentrations in recreational water samples. Recently, beach notification value (BNV) has been established in Michigan for Draft Method C as 8,760 DNA copies of an *E. coli*-specific gene sequence per 100 mL; or as calculated in the Draft Method C provided Excel spreadsheet: 1.863 $\log_{10}$(copies/reaction). This BNV is being used to monitor bacterial water quality at select beaches in Oakland and Kent Counties in Michigan (Dr. S. Briggs, EGLE. pers. comm.).

Now that there is a scientifically tested standardized qPCR method for *E. coli* quantification, the question becomes how to handle the data produced from it. One of the many challenges that surface in data analysis is when the measured result, *E. coli* concentration in our study, is below the analytical limit of quantification (LOQ), often referred to as 'left-censored data'. Left-censored data are encountered when monitoring beaches if the water samples are relatively free from fecal contamination. Thus, having a statistical method that allows for the inclusion of such data are important to provide a complete picture of the beach environment. Historically accepted methods for handling censored data in the environmental sciences and microbiology fields include removing the censored values from the data set (Nie et al., 2010) or substituting LOQ/2 for the unknown value (Helsel, 2006; Chen et al., 2013). But both procedures ultimately alter the innate distribution of

the data set (Helsel, 2011). Even with as few as 5 – 10% of the data being censored, replacing values with LOQ/2 can introduce bias (Dinse et al., 2014; U.S. EPA, 2010, p. 10). Furthermore, by replacing censored values with LOQ/2 it is assumed that the data has a uniform distribution and that their median and mean are also LOQ/2 (Gillespie et al., 2010), which is unlikely the case. Thus, there is a need in the environmental sciences to use alternate statistical methods that allow for censored values to remain in the data set; because even an unquantifiable or zero result can inform us about the system being studied.

Survival analysis, also referred to as time-to-event analysis, offers such an alternative statistical method. Traditionally, it was used to analyze time-to-event data from medical and toxicology studies, however it has also been used in studies outside those disciplines (McNair et al., 2012; Sisson et al., 2013; Muñoz & Vermeiren, 2018; Tomazi et al., 2018). In our study, we used *E. coli* concentrations as the response variable in place of time. Survival analysis is typically used with right-censored data, because in many medical scenarios an 'event of interest', such as a diagnosis outcome or manifestation of a side-effect, is under investigation in response to some sort of treatment (e.g. variations in medication dosage). Often, the event of interest occurs after the completion of the study, so it is only known that the event time was greater than the study duration and this type of data are right-censored. Therefore, statistical software used to implement survival analysis testing methods, such as the trend tests used in our study (Chapter 3), do not have functions to handle left-censored data analysis. Fortunately, a simple data transformation allows one to employ survival analysis with a left-censored dataset. This is achieved by reversing, or 'flipping', the data by subtracting all measurements—*E coli* concentrations in our study—by an arbitrary number higher than the maximum observed value (Helsel, 2011; Dinse et al., 2014, Gillespie et al., 2010). Its primary benefit for our purposes is that it allows information from data below the LOQ to be

included in the statistical analysis in a statistically rigorous way simply by changing data from left- to right- censored. By using a survival analysis method like Kaplan-Meier distribution curves in Chapter 3, our results are more representative than those where data points were replaced or removed because no data were excluded, and our data set was not fundamentally altered.

Accurate quantification and analysis of *E. coli* concentrations in recreational waters is important to public health, but only if recreators understand the information provided to them. As pointed out by Oliver et al. (2016), how the public perceives and responds to swim warnings issued by beach managers is equally important. For example, full or part-time residents near a beach are more likely to view bacterial pollution as a measure of safe usage conditions (Jones et al., 2018). In contrast, non-resident visitors base water cleanliness on how they themselves perceive the quality by visual assessments such as the presence of garbage or clarity of the water (Jones et al., 2018). Analogous to beach monitoring in the United States, the European Union (EU) bathing waters are regulated by the Bathing Water Directive 2006/7/EC (Quilliam et al., 2019). Designated recreational waters are classified as being 'Excellent', 'Good', 'Sufficient' or 'Poor' based on monitoring data from the previous four years and the classification result is displayed at the beginning of the swim season (Quilliam et al., 2019). However, 60% of questionnaire respondents didn't recognize the signage used in the EU water quality classification system (Quilliam et al., 2019). It is only when recreators understand water quality warnings that they refrain from entering unsafe water thereby decreasing the risk of illness and improving the health of the community. Further research is needed to fully understand the best way(s) to inform the public.

4.2 Extended Methodology

Standard Curve Generation and Analysis

Both the 2016 and 2018 studies used the *E. coli* EC23S857 qPCR assay, a specific region on the 23S rRNA gene of *E. coli* that is detected and amplified, and the same methods of DNA extractions and qPCR analysis. Methods used to construct individual standard curves for the 2016 Draft Method C validation study (Bayesian MSC) are described in detail by Sivaganesan et al. (2019). Methods used to construct the 2018 individual standard curves (WLR) and lab-specific composite curve are described below.

Participants

A diverse group of 21 labs from across the midwestern and southeastern United States participated in studies conducted in 2016, and 9 labs from across the state of Michigan participated in 2018 (Table S2.1). Government, university and county health department labs were represented in both studies. In each study, labs were assigned a unique code: '1' through '21' in 2016 (Sivaganesan et al., 2019) or 'A' through 'I' in 2018 to maintain anonymity.

Standards Instructions

Standards (estimated copy numbers: standard 1: 25,822.6; standard 2: 3,396.25; standard 3: 417.83; standard 4: 54.83; standard 5: 11.61) were prepared and verified at the U.S. EPA Cincinnati laboratory, as described in Sivaganesan et al. (2019), then shipped to a central lab that managed their distribution to the remaining labs. Standards were shipped overnight on dry ice. The U.S. EPA recommended that standards should be stored in 20 μL aliquots at −80°C until ready for use. Each time a standard curve was analyzed, a set of the five concentrations of aliquoted standards were removed from the freezer and any unused standard was discarded.

qPCR Assay

In addition to the EC23S857 *E. coli* qPCR assay, a Sketa22 (salmon DNA) qPCR assay also was used. The Sketa22 assay amplifies a segment of the internal transcribed spacer region 2 of the salmon rRNA gene operon and was used as a sample processing and reaction inhibition control. The reporter molecule was FAM and the quencher molecule was TAMRA. Assay master mix (MM) was prepared by combining TaqMan™ Environmental MM 2.0 (Thermo Fisher Scientific, Grand Island, NY) (12.5 µL), 2.0 mg/mL stock solution bovine serum albumin (BSA) from fraction V powder (Sigma B-4287 or equivalent) (2.5 µL) dissolved in PCR-grade water, 500 µM stock solution of *E. coli* or sketa22 forward and reverse primers combined with 100 µM stock solution of *E. coli* or Sketa22 probes (3.0 µL) (Invitrogen or equivalent) (Table 2.1), and qPCR-grade water (2.0 µL). Volumes in parentheses are volumes specified in Draft Method C to use per 25 µL qPCR reaction being carried out. It was also recommended to prepare two additional reaction wells worth of MM to make sure enough was made for plate analysis. For example, if 24 wells of a 96 well plate were going to be used for *E. coli* quantification, 325 µL of TaqMan (24 wells + 2 additional; 12.5 µL x 26 wells) would be measured out. Two standard curves were permitted to be analyzed on the same 96-well plate (Thermo Fisher Scientific) provided separate batches of MM were prepared for each curve.

Reagents and Supplies

E. coli standards were provided by the US EPA; labs also purchased: *E. coli* and salmon primers, TaqMan™ Environmental MM 2.0, optical 96-well PCR plates (Thermo Fisher Scientific), optical adhesive PCR plate seals (Thermo Fisher Scientific), sterile disposable MicroFunnel™ Filter Funnels (Pall Corporation, Ann Arbor, MI or equivalent) or re-useable filter funnel units, polycarbonate 47 mm diameter with 0.45 µm pore size filters (Millipore or equivalent), AE Buffer

(Qiagen), sterile Phosphate Buffer Saline (PBS) (pH = 7.4 ± 0.2), BSA, 5 x 10^4 *E. coli* cell aliquots for calibrator preparation, sterile 2.0 mL semi-conical screw cap microcentrifuge tubes pre-filled with 212 – 300 μm acid-washed glass beads (0.3 ± 0.01 g) or purchased acid-washed glass beads separately and filled as indicated above, 1.7 mL low retention micro-centrifuge tubes, and PCR-grade water. A single lot of TaqMan™ Environmental MM 2.0 was used by all eight labs (Lot# 180115) and confirmed to have no underlying *E. coli* contamination by analyzing six No Template Controls (NTC) of AE buffer, run in duplicate (Saginaw Valley State University). All NTC runs resulted in an 'Undetermined' Ct value; meaning no fluorescence was detected above the background 'noise' or fluorescence signal, indicating the absence of *E. coli* DNA template in the TaqMan™.

DNA Extraction (Filter blanks and calibrators only)

Each standard curve required three calibrator filters (positive controls; 1 x 10^4 *E. coli* cell equivalents) and three filter blanks (negative controls) prepared on separate filtration units. Calibrators were prepared by filtering 1 mL of a 1 x 10^4 *E. coli* suspension in PBS through a 0.45 μm filter seated on a sterile filtration unit. The same procedure was used to filter 20 mL of PBS for filter blank preparation. Filters from calibrators and filter blanks were folded in half four times and placed in a 2.0 mL sterile semi-conical, screw-cap microcentrifuge tube containing 0.3g (± 0.01 g) glass beads. Next, 600 μL of AE Buffer spiked with 0.2 μg/mL salmon DNA was added to each extraction tube and tightly sealed. Extraction tubes were bead milled for 1.0 min at 5,000 rpm then centrifuged at 12,000 x g for 5 min. Approximately 400 μL of the crude DNA extract was removed and transferred into a clean correspondingly labeled, sterile 1.7 mL low retention micro-centrifuge tube and centrifuged again for 1 min at 12,000 x g, then ~ 100 μL of the clarified supernatant was transferred into a second sterile centrifuge tube. Calibrator and filter blank extracts were analyzed shortly after extraction along with each standard curve.

Plate Setup

Extracted DNA from calibrator and filter blanks were analyzed in duplicate with both *E. coli* and salmon MM separately. NTC and standards 1 – 5 were tested in triplicate solely with *E. coli* MM. (Figure S2.1). Each well contained 25 μL of final reaction volume; 20.0 μL of prepared MM, as described in section 2.3, and 5.0 μL of the supplied *E. coli* DNA standard or positive/negative control or NTC.

Instrument Run Method

Thermocycling consisted of an initial 'holding stage' (50.0°C, 2 min; 95.0°C, 10 min) followed by 40 cycles of DNA denaturation and primer/probes annealing (95.0°C, 15 s; 56.0°C, 1 min). At the end of each of the 40 cycles, fluorescence was measured by the instrument (StepOnePlus, Applied Biosystems). The fluorescence threshold, or the level of fluorescence in which the signal rises above the background level, was manually set to 0.03 ΔRn and baseline cycles were set to AUTO determination (Sivaganesan et al., 2019).

Standard Curve Generation

Upon completion of the instrument runs, data were exported from the StepOne™ Software (v2.3) and the resulting Ct values were copied into an accompanying Draft Method C Excel workbook where they were fitted to the WLR model of the form:

$$X_{ijk} = \alpha_i + \beta_i \log_{10}(X_j) + \varepsilon_{ijk} \quad (1)$$

where X_{ijk} is the observed Ct value for replicate k of standard j in run i; α_i and β_i are the intercept and slope, respectively, for run i; X_j is the known copy number in standard j, and ε_{ijk} is the statistical error in the observed threshold cycle. A separate WLR was fitted to data for each standard curve run, and the externally Studentized residuals (Cook and Weisberg, 1983; p. 20) were examined to

identify and remove up to two outliers from each data set if needed. The WLR model was then re-fitted to the retained data for each run, and the intercept, slope, and R^2 values for each standard curve were assessed for acceptability based on the proposed standard curve acceptance criteria developed for Draft Method C (Table 2.2). When all three parameters met the acceptance criteria, an individual curve was considered 'passing' but, if any one of the three parameters failed, a curve was considered 'failing'. Draft Method C requires a minimum of four passing cures to generate the composite curve used to analyze RW samples therefore, if there were fewer than four passing, labs were requested to analyze additional curves. Only passing curves were considered for further statistical analysis of the 2018 data.

Data Analysis

Bayesian MSC and WLR Standard Curve Model Comparisons

Labs participating in the 2016 validation study analyzed four to five standard curve assays each, as described in Sivaganesan et.al, (2019) for a total of ninety-one curves analyzed. To obtain the WLR model values, intercept and slope data for each lab's separate passing standard curve runs were assessed with the Draft Method C Excel Workbook which uses an analysis of covariance (ANCOVA) to determine if there was strong evidence ($\alpha = 0.01$) that any parameter estimates differed among runs. If not ($p \geq 0.01$ for both parameters), results from the individual curves were pooled, and a WLR was performed to estimate the lab-specific composite curve intercept, slope and 95% CI for each lab. Intercept and slope estimates from the Bayesian MSC model for individual labs were taken from the 2016 validation study (Sivaganesan et al., 2019); the 95% Bayesian Credible Intervals (BCIs) are provided in the supplemental material (Table S2.2). The ANCOVA evaluation was used only in the WLR model and not the Bayesian MSC model. Intercept and slope estimates, and corresponding 95% CIs and BCIs, from the WLR and Bayesian MSC models, respectively, for

each lab were plotted and compared visually. Comparisons also were made by examining the relative percent difference between the WLR and MSC models of the intercept and slope estimates for each lab. Percent differences were calculated by dividing the absolute value of the difference between the two model's estimates to the average of the absolute values of the two model's estimated means and multiplying by one hundred.

Inter-lab variability of 2018 standard curve WLR estimated acceptance criteria

Each of the nine labs participating in 2018 used the WLR calibration model in the Draft Method C Excel workbook to analyze the instrument-determined Ct values for each individual standard curve. The workbooks were then shared with the authors of this study. Each lab produced between four and ten standard curves for a total of eighty-two. Of these curves, only those that passed all three acceptance criteria were considered for further statistical analysis.

Potential differences between acceptance criteria estimates produced by individual labs were assessed by determining the statistical significance of parameter differences and, where a statistically significant difference was detected, the relative magnitude of the difference on a percent scale was determined, as described above. The main goals of the assessment were to determine the proportion of labs for which a statistically significant difference was detected for each parameter and, more importantly, the relative magnitude of any detected differences. Inter-lab comparisons were carried out using a pairwise Wilcoxon rank sum test (Hollander et al. 2013) on each parameter, *p*-values adjusted with a Holm correction to account for multiple comparisons, to test the null hypothesis that the locations of the distributions of reported intercept, slope, and R^2 estimates were the same for each pair of labs against the two-sided alternative hypothesis that they were different. The Wilcoxon rank sum test is a nonparametric test and does not assume a specific distribution for the individual estimates. All statistical analyses were performed using R Software (v3.5.2; R Core Team,

2018). Prior to any analysis, two data points from one lab (code H) were removed due to knowledge of errors during plate sealing.

Environmental Variables and *E. coli* Concentrations

Sampling

Sampling occurred once a week over five weeks (June 4th through August 6th, 2018) within Muskegon County, Michigan. Water samples were stored on ice until transported to the lab and processed for either Colilert analysis or filtered for the Draft Method C qPCR assay within six hours of collection.

Inland Lake Beaches: Fourteen inland lake beaches (Table 3.1) were sampled once a week over five weeks resulting in five samples per beach (n = 70). 100 mL samples were collected in 125 mL Security-Snap™ BacT (Thermofisher Scientific) sterile polypropylene bottles at each of three sample sites designated as left, center, and right (Figures S3.1 – S3.5). The three 100 mL samples were composited in the lab into a sterile 500 mL HDPE bottle (Microtech Scientific), totaling 300 mL of a composite sample for each beach. One-100 mL aliquot was used for immediate Colilert analysis and two-100 mL aliquots were filtered for qPCR analysis then stored in a −80°C freezer to be analyzed at a later date. An ~ 50 mL sample also was collected from the center location of each beach in a Nalgene bottle for turbidity measurements.

Lake Michigan Beaches: Six Lake Michigan beaches (Table 3.1) were sampled once a week over five weeks, three sampling sites per beach, for a total of 15 water samples per beach over the course of the study (n = 90). A 500 mL sample was collected in sterile, tamper sealed HDPE bottles (Microtech Scientific) at each of three locations (north, center, and south) (Figures S3.6 – S3.8) and

prepared individually for analysis as described above for both Colilert (1 x 100 mL) and the Draft Method C qPCR assay (2 x 100 mL).

Environmental Variables

Eight environmental variables (EVs) were recorded for each beach during each sampling event to assess its impact on *E. coli* concentrations. They included: turbidity (NTU), wind speed (mph), air and water temperature (°C), number of birds, whether birds were present (P) or absent (A), time (h) since the last rain fall, of any amount, and the amount of rain during the last rainfall (inches). Most variables were included on the Great Lakes Beaches Routine On-Site Sanitary Surveys (www.epa.gov/sites/production/files/documents/greatlakes_onsite.pdf), except for birds present/absent, as part of the beach monitoring program protocol. Wind speed, air and water temperature and samples for turbidity were collected from or measured at the center sample site. Bird counts were performed by visual observation as samplers walked from the center sampling point towards the north/right or south/left sampling points and counted the number of birds observed on the beach and in the water within the swim area (U.S. EPA 2008). Samplers would report the number of birds to the person at the center location and all birds observed were tallied then recorded on the Sanitary Survey. Wind speed and air temperature were measured with an anemometer by holding it above the sampler's head for approximately 10 s and recording the highest reading of wind speed and, once a consistent temperature was seen, air temperature. Anemometer instructions directed measurements to be taken in the shade, however no shade was available at the center sampling site of most beaches thus for consistency, all readings were recorded from the center location regardless of the availability of shade. Water temperature was measured at knee depth with a thermometer (Component Design Northwest, Inc. DTQ450X) held near the surface of the water until a steady reading was observed. Turbidity samples were transported to the

lab on ice and measured with a turbidimeter according to the manufacturer's instructions following analysis of three NTU standards (0, 10, and 100 NTU) as quality control assurance (Thermo Scientific Orion AQUAfast AQ4500). Time since the last rain and rain amounts were recorded from individual weather stations (www.wunderground.com) chosen based on nearness to a beach and, in all instances except one, was the same throughout the sampling season (Table S3.1).

E. coli Quantification

Colilert: The composited inland lake beach samples and north, center, south Lake Michigan samples were all analyzed within six hours of collection using Colilert (Method 9233 B) (Rice et al., 2012). In accordance with this method, a Colilert reagent packet was emptied into and dissolved in 100 mL of water sample. The solution was poured into a Quanti-Tray and incubated for 18 – 22 h at approximately 35°C. The Quanti-trays were then removed from the incubator and exposed to ultraviolet light. Based on the total number of fluorescing wells in the tray, *E. coli* concentrations were calculated and recorded as MPN. A more in-depth description of this method is provided in Kinzelman et al. (2005) and on the IDEXX website (www.idexx.com).

qPCR: Each composited inland lake beach sample and the three individual Lake Michigan samples per beach were analyzed according to the Draft Method C qPCR assay which uses TaqMan™ chemistry. Briefly, 100 mL of sample was filtered through 0.45 μm pore polycarbonate filter seated on a sterile disposable filtration unit (Pall Corporation, Puerto Rico, MicroFunnel™ Filter Funnel). The filters were folded four times and added to a 2.0 mL sterile semi-conical, screw-cap microcentrifuge tube containing 0.3 g (± 0.01 g) of glass beads. Working within a laminar flow hood, 600 μL of extraction fluid consisting of AE buffer with 0.2 μg/mL salmon (sketa) DNA was added to each extraction tube, tightly sealed then bead milled for 1.0 min at 5,000 rpm. The sketa DNA served as a sample processing control (SPC) to ensure the qPCR reaction occurred as

expected. Because a known concentration was added to each sample, a specific range (18.58 – 22.01) of cycle threshold (Ct) values was expected, and required, for designated Draft Method C quality control parameters to be met (Sivaganesan et al., 2019). The extraction tubes were then centrifuged for approximately 5 min at 12,000 x g and ~ 400 µL of the crude DNA extract was transferred into a correspondingly labeled sterile 1.7 mL low retention micro-centrifuge tube. The crude extract was centrifuged for 1 min at 12,000 x g, then ~ 100 µL of clarified supernatant was transferred to a second sterile 1.7 mL centrifuge tube. Tubes of clarified supernatant were then moved to a second laminar flow hood and set aside until sketa and *E. coli* Master Mixes (MM) were prepared (see chapter 2, section 2.3). Half of the 96-well qPCR plate was filled with 20 µL of *E. coli* MM and the other half was filled with 20 µL of sketa MM. 5 µL of recreational water sample extracts were analyzed in triplicate using both the *E. coli* and sketa MM. Three separate negative (blanks) and positive (calibrator) controls, and a No Template Control (NTC) were analyzed in duplicate, on each tray of samples with *E. coli* and sketa MM. Once MM, samples, and quality controls were added, the plate was sealed and analyzed on a StepOnePlus™ Applied Biosystems thermocycler using the StepOne™ Software (v2.3). Instrument generated Ct values for water samples and quality control parameters were exported and entered in an EPA provided Draft Method C Excel Workbook where *E. coli* concentrations (log_{10}Copies/5 µL) were calculated, provided all quality control parameters were met. log_{10}Copies/5 µL were then converted to log_{10}Copies/100 mL to make the two quantification methods comparable. There were nine Lake Michigan beach samples whose sketa Ct values did not pass specified acceptance criteria. In these cases, the stored duplicate filter was extracted, and the crude extract was filtered through a OneStep™ PCR Inhibitor Removal Kit (Zymo Research) column, then re-analyzed with the Draft Method C qPCR assay. The inhibitor removal kit does not impact dilution ratios although it is thought that it may bind some DNA within

the column resulting in a lower measured concentration. The log_{10}Copies/100 mL results from five of the re-analyzed samples were used in statistical analysis, while four were unusable as the filter blank acceptance criteria was not met (Ct > lower limit of quantification as given in the Draft Method C Excel workbook) (Sivaganesan et al., 2019). All filtered qPCR samples were analyzed within 6 months after storage.

Statistical Analysis

Data were organized by beach, sample date, sample site (for Lake Michigan Beaches), corresponding Colilert (MPN) and qPCR (log_{10}Copies/100 mL) *E. coli* concentrations and environmental variables collected or measured during each sampling event. Lake Michigan environmental variables were recorded at the center sampling site only and water samples were collected at three locations, therefore the same measurement or observation was filled in for all three sample locations the assumption being that the variation between north, center, south sample locations at the same beach was negligible. Additionally, repeated measures statistical analysis was unnecessary because *E. coli* concentrations vary greatly from day to day and even hour to hour (Whitman and Nevers, 2004; Badgley et al., 2011; Wyer et al., 2018) thus our weekly samples could be treated as independent of each other.

An indicator variable, or status, column was created for *E. coli* concentrations measured with both methods to indicate if the measurement was above the LOQ with a '1', or below the LOQ with a '0'. This is analogous to traditional survival analysis where a '1' is used for a known time of event and '0' is used for censored events. For the purposes of our study, *E. coli* concentrations in the units of log_{10}(MPN/100 mL) and log_{10}(Copies/100 mL) were used in place of the "time" variable, and the method or instrument LOQ was the "event". Colilert results were censored at 1 MPN, meaning any result recorded as < 1 MPN was below the method LOQ and considered a censored observation,

thus a status of '0' was given. qPCR results were censored at 246 copies/100 mL (2.391 $\log_{10}$Copies/100 mL) based on the lower limit of quantification Ct value (37.63) of a 6-pt standard composite curve generated from four individual standard curves analyzed. Values below 2.391 $\log_{10}$Copies/100 mL were considered censored ('0' status); values greater than 2.391 $\log_{10}$Copies/100 mL were given a '1' in the status column. To determine the LOQ, the LLOQ Ct value (37.63)— given in the Draft Method C Excel Workbook— was used as 'y', y-intercept (38.73), and slope (−3.53) in the $y = mx + b$ equation, where 'x' was the LOQ.

EVs were categorized into two or three groups (Table 3.2) chosen to have at least 10 non-censored observations per group. KM distribution curves were then generated using the categorized groups and *E. coli* concentrations from recreational water samples. For EVs categorized into three groups, KM curves were statistically compared with a log-rank trend test (Klein and Moeschberger 2006, section 7.4; Machin et al., 2006 section 3.5). The trend test tests the null hypothesis that the categorized EVs *E. coli* concentration distributions are equal against the alternative that there is a monotonic ordering among the KM curves. For example, if $K_1(x)$, $K_2(x)$, and $K_3(x)$ are the KM curves (as functions of concentration x) for the low, medium, and high categories of a particular EV, then the null hypothesis of the trend test is $K_1(x) = K_2(x) = K_3(x)$ for all concentrations x, and the "increasing form" of the alternative is $K_1(x) \leq K_2(x) \leq K_3(x)$ for all x, with the inequality being strict (i.e., "<" instead of "≤") for at least one concentration and at least one pair of KM curves. The test outcome does not specify which KM distribution curve relationship is statistically different, just that a statistical difference in distribution curves was detected. For the bird present/absent and the method and lake type comparisons, a two-sample one-sided log-rank test (Hollander et al., 2013; Ch. 11.7; p. 594 – 597) was used. Output from the log-rank test were used to calculate the original test statistic prior to it being squared for the chi-squared p-value in order to perform one-sided statistical

analyses. These comparisons were performed to identify EVs that exhibited a statistically significant impact ($\alpha = 0.05$) on *E. coli* concentrations quantified with both methods (Colilert and qPCR) for inland and Lake Michigan beaches separately. All statistical analyses were carried out using R Software (v3.5.2; R Core Team, 2018).

The limitations of R-Software prevent it from performing survival analysis on left-censored data, like the data in our study, therefore trend tests and two-sample log-rank tests were carried out on the 'flipped' *E. coli* concentration data set (further detail provided in the discussion). All KM distribution curves presented below are based on the 'unflipped' or original *E. coli* concentration data scale.

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