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Performance of Colilert-18 and qPCR for Monitoring *E. coli* Contamination at Freshwater Beaches in Michigan

James N. McNair [1,*], Richard R. Rediske [1], John J. Hart [1,†], Megan N. Jamison [2] and Shannon Briggs [3]

[1] Robert B. Annis Water Resources Institute, 740 West Shoreline Dr., Muskegon, MI 49441, USA; redisker@gvsu.edu (R.R.R.); john.hart@geosyntec.com (J.J.H.)
[2] Department of Civil, Environmental and Geodetic Engineering, Ohio State University, 2070 Neil Avenue, Columbus, OH 43210, USA; jamison.171@buckeyemail.osu.edu
[3] Michigan Department of Environment, Great Lakes and Energy, 525 W. Allegan St., Lansing, MI 48909, USA; briggss4@michigan.gov
* Correspondence: mcnairja@gvsu.edu
† Current address: Geosyntec Consultants, Inc., 500 West Wilson Bridge Road, Suite 250, Worthington, OH 43085, USA.

Abstract: Fecal contamination is a common cause of impairment of surface waters. In monitoring studies, it is usually assessed by measuring concentrations of fecal indicator bacteria such as *Escherichia coli* (*E. coli*), a common monitoring target in freshwater systems. In this study, we assess the advantages and disadvantages of two common and previously validated methods for monitoring *E. coli* concentrations at freshwater beaches: Colilert-18®, with a turnaround time of ca. 18 h, and real-time quantitative PCR (qPCR), with a turnaround time of ca. 3–4 h. Based on data comprising 3081 pairs of Colilert-18 and qPCR estimates of *E. coli* concentrations in split samples from Michigan's annual beach monitoring program in 2019 and 2020, we found that qPCR monitoring detected a high percentage of exceedances of the state's water quality standard for *E. coli* contamination that went undetected on the day of sampling with Colilert-18 monitoring because qPCR concentration estimates were available on the day of sampling but Colilert-18 estimates were not. However, Colilert-18 data were more useful than qPCR data for the statistical comparison of contamination levels at different beaches, probably in part because Colilert-18 data showed a much lower percentage of concentration estimates outside the method's range of quantification.

Keywords: beach monitoring; recreational water quality; fecal indicator bacteria (FIB); *Escherichia coli* (*E. coli*); real-time quantitative polymerase chain reaction (qPCR); Colilert-18; EPA draft method C; censored data

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1. Introduction

Fecal pollution is one of the most common causes of impairment of surface waters in countries throughout the world. Bain et al. [1], for example, estimate that 1.8 billion people worldwide drink water from sources that are contaminated with feces. And the U.S. Environmental Protection Agency (USEPA) found that 30% of the cumulative 1.94 million kilometers of river and stream segments assessed across the United States by the National Rivers and Streams Assessment 2013–2014 were impaired by levels of fecal contamination that made them unsafe for total-body contact recreation [2].

Fecal contamination is typically assessed by measuring concentrations of fecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and *Enterococcus* spp. [3]. FIB are bacteria that are abundant in the intestines of endothermic ("warm-blooded") vertebrates such as

humans, farm animals, and waterfowl but are typically scarce in the external environment, and whose presence at elevated levels in surface water therefore provides evidence of fecal contamination. Monitoring studies usually employ a single FIB species or genus that is easy to quantify in the lab and is either not pathogenic or only mildly so and therefore is safer to work with than the primary pathogens of interest, especially when culture-based methods of quantification are used. Elevated levels of FIB in surface water indicate that fecal pathogens (viruses, bacteria, protozoa) are likely to be present, as well [4]. These pathogens, not the FIB, are the main concern.

Epidemiological studies have shown that total-body contact with recreational waters exhibiting elevated FIB concentrations is associated with an elevated incidence rate of gastrointestinal illnesses [4–9]. USEPA [4] has therefore established recreational water quality criteria that include suggested limits for *E. coli* contamination that are believed to protect human health for total-body contact recreation, based on available epidemiological evidence. Each state is required to develop and enforce recreational water quality standards (RWQS) that are based on, but not necessarily identical to, USEPA's recommended criteria. These RWQS are then the basis for each state's monitoring program for recreational waters and are used by county health departments in deciding whether particular beaches should be temporarily closed for total-body contact recreation or a warning should be posted (we will refer to such decisions as *beach notification decisions*).

In addition to supporting state RWQS, USEPA [4] also supports the use of two types of what it calls "supplemental elements for enhanced protection of recreational water": Beach Action Values and rapid monitoring methods. Beach Action Values are numerical thresholds intended for use in making precautionary beach notification decisions and therefore are set at concentrations somewhat lower than the corresponding RWQS. Rapid monitoring methods, such as qPCR (real-time quantitative polymerase chain reaction), are methods of quantifying FIB that have much shorter turnaround times than do the culture-based methods typically required by state RWQS.

Michigan's RWQS for fecal contamination are stated in terms of the number of *E. coli* per 100 mL of sample (Michigan Recreational Water Quality Sandards—Part 4 rules, Water Quality Standards, promulgated under Part 31, Water Resources Protection, of the Natural Resources and Environmental Protection Act, 1994 PA 451, as amended). Thus, they require the use of *E. coli* as the FIB, and also require that the level of *E. coli* contamination be quantified with a "standard method" that produces estimates of the number of *E. coli* per 100 mL. Standard methods are defined as methods that appear in the authoritative reference, *Standard Methods for the Examination of Water and Wastewater* [10]. Various colony-count methods that produce estimates of the number of colony-forming units per 100 mL (CFU/100 mL) are therefore accepted, as are enzyme substrate methods, such as Colilert-18, that produce estimates of the most probable number of colony-forming units per 100 mL (MPN/100 mL). Genetic methods based on quantitative forms of the polymerase chain reaction, such as qPCR and ddPCR (droplet digital PCR), produce estimates of the number of copies of a target DNA sequence instead of the number of cells or colony-forming units and therefore are not accepted by the RWQS as standard methods for estimating *E. coli* concentrations. It is important to understand, however, that all of these methods produce estimates that apply to *the time at which the field samples were collected*, not the time at which the results become available. Since the standard methods require 18–24 h to produce results, they cannot be used as the basis for making beach notification decisions on the sampling day, which is the day to which their concentration estimates actually apply.

To address this serious deficiency of the standard methods as tools for monitoring recreational waters, Michigan has proposed what is essentially a combination of USEPA's two supplemental elements: a qPCR-based Beach Action Value for *E. coli* contamination,

which we will call the qPCR threshold value (qTV). The proposed numerical value of Michigan's qTV, developed in collaboration with USEPA [11], is 1.863 $\log_{10}$ gene copies per reaction (GC/reaction), which is equivalent to about 73 GC/reaction on a linear scale. A detailed assessment of this qTV by McNair et al. [12], based on state-wide split-sample Colilert-18® and qPCR beach monitoring data for Michigan beaches for the years of 2016–2020, supports its validity as a rapid monitoring tool for determining whether a beach is safe for recreation on the day of sampling.

The main reason for employing qPCR-based beach monitoring is that it produces results in only 3–4 h, compared to 18–24 h for culture-based methods such as Colilert-18 and colony-count methods that require significant incubation periods before producing results [11,13,14]. With qPCR-based monitoring, it is both possible and feasible to make beach notification decisions early enough on the same day that samples are collected so that warnings can be issued or beaches closed before most recreators have entered the water. Making and posting these decisions on the same day that samples are collected is crucial, because *E. coli* concentrations in samples collected from a given beach on consecutive days typically show little or no correlation [15–20] (Figure 1); in other words, today's *E. coli* concentration is not a reliable indicator of what tomorrow's concentration will be.

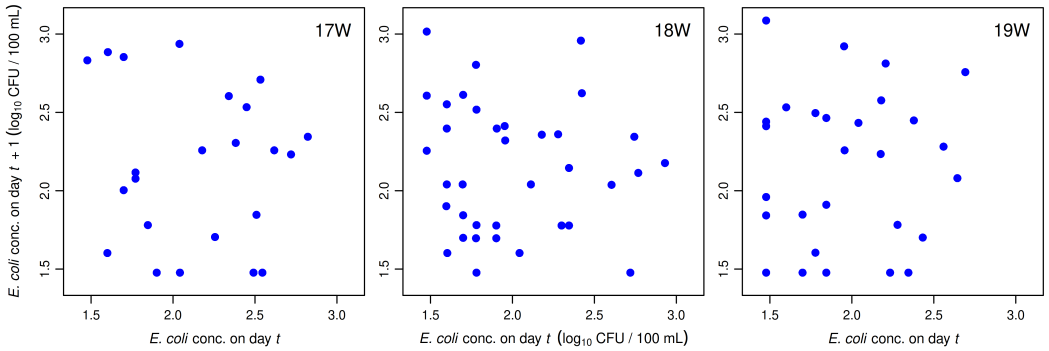

Figure 1. *E. coli* concentrations as $\log_{10}$ colony-forming units (CFU) per 100 mL for day $t + 1$ (vertical axis) versus day t (horizontal axis) at three locations (Sunnyside Beach 17 W, 18 W, and 19 W) on a Lake Ontario beach in Toronto, Canada. Data digitized from Figure 2 of Saleem et al. [20].

Since 2016, Michigan's annual state-wide beach-monitoring program has included the analysis of split samples using both Colilert-18 and qPCR quantification of *E. coli* contamination. As is typical of analytical methods, both of these methods have upper and lower limits of quantification that serve as reporting limits (note that the lower reporting limit is the lower limit of quantification, not the limit of detection). Sample concentrations outside the range of quantification for each method are by definition too uncertain to report as valid measured concentrations, so they are reported only as falling below the lower limit of quantification or above the upper limit, along with the corresponding numerical values of the limits. Such data are called *censored data* in the statistical literature. These limits are fixed for Colilert-18 (but are adjusted appropriately if samples are diluted or concentrated) but must be determined for each new standard curve with qPCR analysis. Because of the wide range of sample concentrations encountered in monitoring studies, a significant proportion of samples typically have concentrations below the lower limit of quantification for each method of analysis. It is also common for some samples to exceed the upper limit of quantification for Colilert-18 (unless samples are diluted and re-analyzed, which often is not feasible). With current methods of qPCR analysis for surface waters, it is common in our experience for more than 25% of samples to have concentrations below the lower limit of quantification, while typically none have concentrations exceeding the upper limit of quantification. By contrast, Colilert-18 data typically contain a much lower proportion of censored data (usually well below 10%), which mostly comprise concentrations below

the lower limit of quantification but may also include a small percentage of concentrations above the upper limit.

A rigorous assessment of spatial or temporal patterns with censored data requires specialized statistical methods from the discipline variously known as survival analysis, reliability analysis, failure-time analysis, or time-to-event analysis [21]. These methods make it possible to validly estimate and compare *E. coli* concentration distributions for different beaches, counties, or dates, estimate percentiles and confidence intervals, assess spatial or temporal trends, assess potential relationships with explanatory variables, and so on. However, their ability to detect differences, trends, or relationships diminishes as the proportion of censored data increases. Thus, while estimates of *E. coli* concentration can be obtained in much less time when qPCR is used instead of Colilert-18, the resulting data may be less useful for detecting differences between levels of *E. coli* contamination at different beaches or sampling times and for exploring hypotheses regarding their potential causes, due to the much higher proportion of censored data.

The purpose of this paper is to address three issues regarding the performance of Colilert-18 and qPCR in monitoring studies of *E. coli* contamination of recreational beaches:

1. The relative merits of Colilert-18 and qPCR as the basis for beach notification decisions, viewing decisions based on Colilert-18 estimates of *E. coli* concentrations for the day of sampling as the benchmark;
2. The relative merits of Colilert-18 and qPCR estimates of *E. coli* contamination for rigorous statistical assessment of differences in levels of contamination at different beaches or groups of beaches;
3. The relative levels of *E. coli* contamination at Michigan's inland-lake and coastal beaches during 2019 and 2020, as judged by Colilert-18 and qPCR.

An earlier paper by McNair et al. [12] uses quantitative performance measures based on Michigan's beach-monitoring data for 2016–2020 to address the first issue in considerable detail. For completeness, the present paper briefly addresses the first issue by applying some of the same methods to Michigan's 2019 and 2020 beach monitoring data, but our main focus is on the second and third issues.

With regard to the first issue, the State of Michigan accepts Colilert-18 as a standard method of *E. coli* quantification in beach monitoring, and the estimates it produces have served as the main basis for making beach notification decisions for many years. Because it is an accepted method in Michigan and also the method on which nearly all beach notification decisions during 2016–2020 were based, McNair et al. [12] treat this method as a benchmark for assessing the performance of qPCR-based quantification. We note, however, that while Colilert-18 quantification of *E. coli* levels is an accepted method for making these decisions in Michigan, it is by no means fully satisfactory. Most importantly, as noted above, the estimates of *E. coli* concentration it produces are not available until the day after sampling, meaning they cannot be used to make real-time beach notification decisions on the only day for which they provide reliable estimates of the level of contamination (i.e., the sampling day).

To circumvent this serious deficiency of both Colilert-18 and standard colony-count methods of quantifying *E. coli* contamination in beach monitoring, our present assessment of Colilert-18 and qPCR as tools for making sound and timely beach notification decisions (the first issue in the above list) follows McNair et al. [12] and uses existing data from previous years so that we are able to retrospectively assign Colilert-18 concentrations to the day of sampling. Thus, the benchmark for assessing qPCR-based decisions is actually the Colilert-18 decisions *that would have been made* if those data had been available on the day of sampling.

As already noted, the main focus of the present paper is on the second and third issues listed above. More specifically, while we do use quantitative performance measures to assess the degree to which beach notification decisions based on qPCR estimates of *E. coli* contamination and Michigan's proposed qTV correspond to the benchmark decisions based on Colilert-18 estimates and Michigan's RWQS, our main focus is on comparing these performance measures for Michigan's inland-lake and coastal beaches, comparing the abilities of Colilert-18 and qPCR data to detect differences between levels of *E. coli* contamination at these two types of beaches, and comparing estimated levels of *E. coli* contamination at Michigan's inland-lake beaches with those at its coastal beaches. All assessments utilize Michigan's state-wide beach monitoring data for 2019 and 2020, which comprise paired Colilert-18 and qPCR estimates of *E. coli* concentration in split samples. It is our hope that the results of these comparisons will be useful to investigators in choosing between Colilert-18 and qPCR methods of quantifying *E. coli* contamination when planning monitoring studies.

2. Materials and Methods

The data employed in the present assessments were produced in 2019 and 2020 by multiple laboratories across the state of Michigan as part of Michigan's annual beach monitoring program. The sampling locations, participating laboratories, methods of sample collection and preparation, and methods of *E. coli* quantification with Colilert-18 and qPCR are described in detail by McNair et al. [12] and therefore will be only briefly outlined here. The methods of data analysis, however, will be described in more detail, since most of them have not been employed in any previous study involving qPCR or Colilert-18 quantification of microbial contamination.

2.1. Sampling Locations

The data comprise 3081 pairs of Colilert-18 and qPCR estimates of *E. coli* concentration from split samples that were collected from freshwater beaches in Michigan during 2019 and 2020. Of the total of 102 beaches with paired data, 69 were located on inland lakes and 33 were coastal beaches, by which we mean beaches on Lake Superior, Lake Michigan, Lake Huron, or Lake St. Clair (Figure 2).

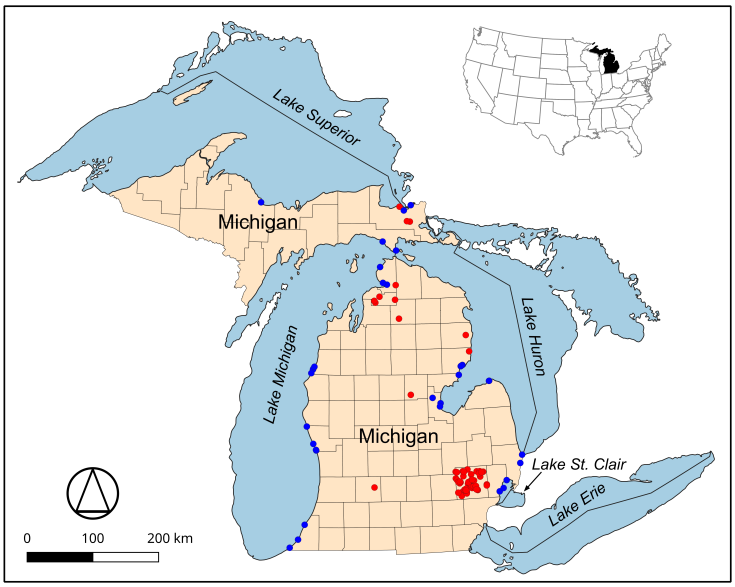

Figure 2. Locations of Michigan recreational beaches for which paired Colilert-18 and qPCR beach monitoring data were available. Red dots: inland-lake beaches. Blue dots: coastal beaches. Base map: Michigan Geographic Framework.

2.2. Participating Laboratories

Seven Michigan laboratories contributed paired Colilert-18 and qPCR beach monitoring data in 2019 and 2020 (Appendix A Table A1).

2.3. Sample Collection and Preparation

McNair et al. [12] describe the methods of sample collection and preparation employed. Briefly, beaches were sampled every 2 to 7 days from May to October. Sampling frequency was set by county health departments and varied between counties. Water samples were collected in sterile water bottles from 3 to 6 representative locations at each beach. They were stored at 4 °C during transport to the lab and were analyzed within 4–8 h (typically 6 h) of collection. In most cases, each sample was split before analysis, and one half was analyzed with Colilert-18 and the other with qPCR. To reduce analytical costs, some counties with large numbers of beaches or unusually long beaches composited samples from an individual beach and sampling date, then split and analyzed the composites.

2.4. Methods of *E. coli* Quantification

E. coli contamination was quantified with both Colilert-18 and qPCR. Colilert-18 analysis employed the Colilert-18 Quanti-Tray/2000® system (IDEXX Laboratories, Westbrook, ME, USA) and followed manufacturer instructions. With this method, estimates of *E. coli* concentration are obtained in 18 h and are in units of the most probable number of colony-forming units per 100 mL (MPN/100 mL). The lower and upper limits of quantification (LLOQ and ULOQ) are fixed at 1.0 and 2419.6 MPN/100 mL (unless samples are concentrated or diluted, which was not done).

As noted in the Introduction, the state of Michigan has accepted Colilert-18 as a standard method for estimating *E. coli* concentrations on the day of sampling in beach monitoring. Its estimates in units of MPN/100 mL may be compared directly with the state's recreational water quality standards (Section 2.5), which are stated in terms of the number of *E. coli* per 100 mL of sample. Because Colilert-18 has been extensively validated and is quicker and easier for non-research technicians to implement properly than are any of the standard colony-count methods, it has long been the method of choice in Michigan's beach monitoring program. Thus, Colilert-18 is accepted by the State of Michigan as a standard method and is also the method that produces the data on which the vast majority of beach notification decisions are made. For both of these reasons, we treat Colilert-18 as the benchmark for assessing beach notification decisions in Michigan, but we use existing data from previous years so that we can assign the Colilert-18 concentrations to the day to which they actually apply (the sampling day) and use these values to correctly determine whether the *E. coli* concentration on that day did or did not exceed the RWQS for total-body contact recreation.

qPCR analysis followed EPA Draft Method C [12,22–24], with certain exceptions to reduce analytical costs. With this method, estimates of *E. coli* concentration are obtained in 3–4 h and are in units of $\log_{10}$ gene copies per reaction ($\log_{10}$ GC/reaction). The qPCR quantification of field samples relies on classical calibration and therefore requires a standard curve. Ideally, a set of calibration standards for fitting a standard curve would be included with each set of field samples, but to reduce analytical costs of Michigan's statewide beach-monitoring program, an instrument-specific and analyst-specific composite standard curve based on five or six separate runs was created once during each beach-monitoring season for each combination of analytical instrument and analyst in a given lab. A calibrator and positive control with known *E. coli* concentration (e.g., MultiShot-1E8 BioBalls™, BioMèrieux, Lombard, IL, USA, Reference #56146) was run with each set of samples. Each standard curve yields its own LLOQ [25], so this procedure produces one

instrument/analyst-specific LLOQ for each combination of analytical instrument and analyst in each lab during each beach-monitoring season. LLOQs for 2019 and 2020 ranged from about 0.36 to 0.91 $\log_{10}$ GC/100 mL. ULOQs were not formally estimated, because the maximum standard concentration was chosen well above the range of gene copy concentrations encountered in beach monitoring and also yielded the lowest variability in observed values of the threshold cycle in calibration data.

2.5. Michigan's Recreational Water Quality Standards

The relevant part of Michigan's RWQS for the present study requires that estimated *E. coli* concentrations must not exceed 300 *E. coli*/100 mL at any sampling event (Michigan Recreational Water Quality Sandards—Part 4 rules, Water Quality Standards, promulgated under Part 31, Water Resources Protection, of the Natural Resources and Environmental Protection Act, 1994 PA 451, as amended). This standard requires culture-based quantification of *E. coli* concentration using either classical colony counts that yield estimates of colony-forming units per 100 mL (CFU/100 mL) or a simpler enzyme-substrate method such as Colilert-18 that yields estimates of the most probable number of colony-forming units per 100 mL (MPN/100 mL). Following USEPA guidance [4], Michigan also permits the rapid monitoring of *E. coli* concentrations using qPCR as a supplemental method, with beach notification decisions based on a proposed qTV of 1.863 $\log_{10}$ GC/reaction that was developed in collaboration with USEPA.

2.6. Assessing the Performance of Michigan's Proposed qTV

As noted in Section 2.4, Colilert-18 is both an accepted method for quantifying *E. coli* contamination in Michigan and by far the most commonly used method on which beach notification decisions are based. We therefore follow McNair et al. [12] in treating this method as the benchmark for making beach notification decisions. This means that if the Colilert-18 estimate of *E. coli* concentration as MPN/100 mL on the day of sampling at a particular beach (determined retrospectively) exceeds the state's RWQS for full-body contact recreation of 300 *E. coli*/100 mL, then the correct beach notification decision is to close the beach for recreation (or issue an advisory, at the discretion of the local health department); otherwise, the correct decision is to permit normal recreation. In practice, the correct decision can only be determined retrospectively, because the Colilert-18 results are not available until the day after sampling.

In the ideal case, the relationship between estimates of *E. coli* concentration based on qPCR and Colilert-18 would be one-to-one, so that a Colilert estimate would exceed Michigan's RWQS of 300 *E. coli*/100 mL if and only if the corresponding qPCR estimate exceeded the proposed qTV of 1.836 $\log_{10}$ GC/reaction. In reality, the relationship between these two types of concentration estimates is far from one-to-one, with any given qPCR estimate being associated with a wide range of Colilert-18 estimates and vice versa (Figure 3). As a result, the fact that a given qPCR estimate exceeds the qTV does not guarantee that the corresponding Colilert-18 estimate exceeds the RWQS, nor does the fact that a given qPCR estimate does not exceed the qTV guarantee that the corresponding Colilert-18 estimate does not exceed the RWQS.

Following McNair et al. [12], we will call qPCR estimates that exceed the qTV *q-positives* and qPCR values that do not exceed the qTV *q-negatives*. Similarly, we will call Colilert-18 estimates that exceed the RWQS for full-body contact recreation *c-positives* and Colilert-18 estimates that do not exceed this RWQS *c-negatives*. Recalling that we treat Colilert-18 as the benchmark for beach notification decisions, the percent of c-positives that qPCR classifies as q-positives is called the *true-positive rate* (TPR), while the complementary percent of c-positives that qPCR classifies as q-negatives is called the *false-negative rate*

(FNR). The percent of c-negatives that qPCR classifies as q-negatives is called the *true-negative rate* (TNR), and the complementary percent of c-negatives that qPCR classifies as q-positives is called the *false-positive rate* (FPR). Note that by definition, TPR + FNR = 100 and TNR + FPR = 100. The performance assessment of qPCR-based monitoring and Michigan's proposed qTV was based on TPR, FPR, TNR, and FNR. For example, uniformly excellent performance would be indicated by values of TPR and TNR close to 100% and by values of FPR and FNR close to 0%.

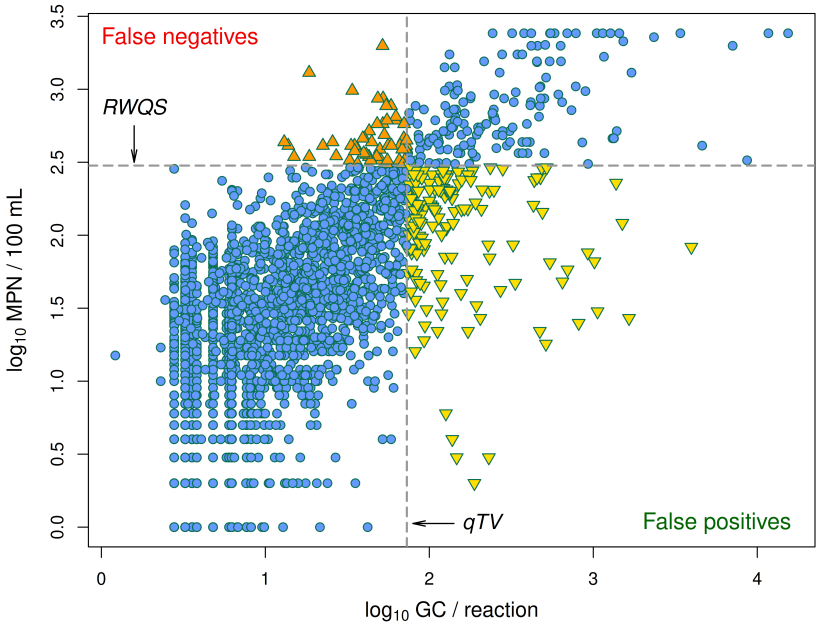

Figure 3. Relationship between Colilert-18 (vertical axis) and qPCR (horizontal axis) *E. coli* concentrations in split samples from Michigan beaches in 2019 and 2020. Blue dots are true positives ((**upper right**) quadrant) and true negatives ((**lower left**) quadrant). Orange upright triangles are false negatives ((**upper left**) quadrant) and yellow inverted triangles are false positives ((**lower right**) quadrant).

False-negative error rates, FNR, for coastal and inland-lake beaches, expressed as proportions (FNR/100), were compared using R statistical software (version 4.4.2) [26] and an exact two-sample test for proportions, as implemented by function `exact.test()` from the `Exact` package (version 3.2) [27], and this was the case similarly for false-positive error rates, FPR, expressed as proportions (FPR/100). In both cases, the null hypothesis of no difference was tested against the two-sided alternative hypothesis that a difference exists, since there was no compelling a priori reason to expect the FNR or FPR to be greater for one particular class of beaches than for the other.

2.7. Statistical Comparison of Concentration Distributions

As noted in the Introduction, Colilert-18 and qPCR data usually include a non-negligible proportion of censored data. An observed concentration is *left-censored* if it is less than the LLOQ for the analytical method and is *right-censored* if it is greater than the ULOQ for the analytical method. Data that include a mix of left-censored, right-censored, and uncensored observations are called *doubly-censored* data. Concentration estimates that fall outside a method's range of quantification are by definition too unreliable to accept as valid measurements, so the established practice in environmental chemistry is to report these values only as falling below the lower limit of quantification or above the upper limit of quantification, along with the numerical values of the limits.

The percentages of left- and right-censored data in the 2019 and 2020 beach monitoring data were as follows. At the statewide level (all counties), about 2.8% of the Colilert-18 data

and about 52.2% of the qPCR data were left-censored. Among the four counties for which paired Colilert-18 and qPCR data were available for both inland-lake and coastal beaches, the percentage of left-censored data ranged from about 1.9 to 9.0% for Colilert-18 and from about 44.1 to 71.4% for qPCR. At the statewide level, about 0.3% of the Colilert-18 data and none of the qPCR data were right-censored. Among the four counties with Colilert-18 and qPCR data for both inland-lake and coastal beaches, none of the Colilert-18 or qPCR data were right-censored.

Methods traditionally used by environmental scientists and engineers for analyzing censored data (usually, replacing them with fabricated values; e.g., replacing $<$ LLOQ values with the LLOQ or some arbitrary fraction thereof) are not statistically or scientifically defensible [21] and were not used in the present study. Instead, we employed rigorous, specialized methods from the statistical discipline known as survival analysis (also reliability analysis, failure-time analysis, and time-to-event analysis). Helsel [21] discusses various traditional methods of survival analysis (all of which can be applied only to right-censored data) that can be applied to left-censored data if the data are "flipped" by reversing the concentration scale, thus transforming left-censored values to right-censored values. The flipping approach is a kluge introduced by Ware and Demets [28] in the 1970s, prior to the development and dissemination of survival-analysis methods specifically designed for left- and doubly-censored data. In addition to its conceptual awkwardness, flipping suffers from the fact that it does not permit analysis of doubly-censored data.

Our statistical analyses employ modern methods of survival analysis that are designed to be applied to left- or doubly-censored data on the original concentration scale. Probability distribution functions for Colilert-18 and qPCR data were characterized with the nonparametric Turnbull estimator for left- or doubly-censored data [29–31] using function `survfit()` from the R `survival` package [32]. The Turnbull estimator is essentially an extension of the traditional Kaplan–Meier estimator (which can be applied only to right-censored data) that can be applied to left-censored, right-censored, and doubly-censored data. The null hypothesis that two Turnbull distribution functions are identical was tested against the two-sided alternative hypothesis that they are not identical by using function `FHtesticp()` from R package `FHtest` [33].

The proportions of Colilert-18 data that exceeded Michigan's RWQS at inland-lake and coastal beaches were compared using an exact two-sample test for proportions, as implemented by function `exact.test()` from the R package `Exact` [27]. As in the case of comparisons of FNR and FPR estimates described above, the null hypothesis of no difference in exceedance percentage was tested against the two-sided alternative hypothesis, since there was no valid a priori reason to expect one alternative instead of the other.

3. Results

3.1. Beach Notification Decisions Based on qPCR vs. Colilert-18

The much shorter turnaround time for obtaining estimates of *E. coli* concentration with qPCR than with Colilert-18 is a meaningful advantage if the resulting beach notification decisions based on Michigan's proposed qTV agree satisfactorily with the decisions based on the state's RWQS that would have been made if Colilert-18 estimates had been available on the day of sampling. The paired qPCR and Colilert-18 data from split samples in 2019 and 2020 allowed us to judge retrospectively how well qPCR and the qTV performed in predicting decisions based on Colilert-18 and the RWQS. The necessary information is provided by the values of TPR, FNR, TNR, and FPR. Good performance is indicated by high values of "true" rates TPR and TNR and by low values of "false" rates FPR and FNR. However, as McNair et al. [12] point out, it is important to compare the values of these performance measures for qPCR and the qTV with those for Colilert-18 and the

RWQS rather than with values for perfect information, since Colilert-18 is the approved method (the benchmark) to which qPCR is an alternative. All values of the performance measures apply to the day of sampling; those for Colilert-18 reflect the fact that same-day estimates of *E. coli* concentration cannot be obtained with this method and therefore cannot be used to justify closing a beach or posting a warning on the sampling day (as noted in the Introduction, they also are not reliable as estimates of *E. coli* concentrations on the day after sampling, when they finally become available).

Table 1 shows the values of performance measures TPR, FPR, TNR, and FNR for both Colilert-18 and qPCR. Minimizing FNR is particularly important in protecting human health. Separate values for qPCR data are shown for inland-lake and coastal beaches; the Colilert-18 values are essentially the same for both types of beaches, because they simply reflect the inability to obtain Colilert-18 concentration estimates on the sampling day. Note that qPCR and the qTV provide much lower (better) values of the false-negative error rate FNR and only slightly higher (worse) values of the false-positive error rate FPR than do Colilert-18 and the RWQS. McNair et al. [12] found that this pattern also applies to the combined beach-monitoring data for all Michigan beaches and all years from 2016 to 2020.

The values of performance measures shown in Table 1 are the observed values for the full set of monitoring data from 2019 and 2020 and therefore accurately indicate the performance of Colilert-18 and qPCR at inland-lake and coastal beaches during those years. If, however, we view the monitoring data as a random sample from a larger population of beaches and sampling dates, then it is appropriate to estimate 95% confidence intervals for the difference between FNR at inland-lake and coastal beaches, and similarly for FPR (the values for TPR and TNR are implied by those for FNR and FPR). Using function `exact.test()` in R package `Exact` with the no-difference null hypothesis and two-sided alternative hypothesis, we find for FNR that $\mathrm{FNR}_{\mathrm{Inland}} - \mathrm{FNR}_{\mathrm{Coastal}} = -25.8\%$ with a 95% confidence interval $(-39.0\%, -12.9\%)$. For FPR, we find that $\mathrm{FPR}_{\mathrm{Inland}} - \mathrm{FPR}_{\mathrm{Coastal}} = 3.1\%$ with a 95% confidence interval $(1.4\%, 4.5\%)$. Note that neither confidence interval includes zero. Thus, there is strong evidence that FNR is markedly lower and FPR is slightly higher for inland-lake beaches than for coastal beaches.

Table 1. Performance measures for Colilert-18 and qPCR-based monitoring of *E. coli* contamination at Michigan's inland-lake and coastal beaches during 2019 and 2020. TNR, FNR: True- and False-Negative Rates; TPR, FPR: True- and False-Positive Rates. Values of these performance measures for Colilert-18 reflect the fact that concentrations for this method are never available for making decisions on the day to which they apply (the sampling day) and therefore are necessarily the same for inland-lake and coastal beaches.

Property	Colilert-18	qPCR	
		Inland-Lake	Coastal
Can beach decisions be made on the same day samples are collected?	No	Yes	Yes
% Sampling days on which recreation is prohibited when it is unsafe (TPR)	0	85.2	59.4
% Sampling days on which recreation is permitted when it is unsafe (FNR)	100	14.8	40.6
% Sampling days on which recreation is permitted when it is safe (TNR)	100	94.3	97.4
% Sampling days on which recreation is prohibited when it is safe (FPR)	0	5.7	2.6

3.2. Detecting Differences in E. coli Contamination Between Inland-Lake and Coastal Beaches

When the Colilert-18 data for all beaches in 2019 and 2020 are combined, Turnbull estimates of the distribution functions for *E. coli* concentrations at inland-lake and coastal beaches show a clear separation between the two classes of lakes (Figure 4). The distribution function for coastal beaches lies to the right of that for inland-lake beaches. More precisely, the distribution function for coastal beaches is related to that for inland-lake

beaches approximately as a stretching transformation (rather than a shift transformation). A Fleming–Harrington unweighted log-rank test of the null hypothesis of no difference between the two distribution functions against the alternative hypothesis that they differ at one or more concentrations provides strong evidence ($p < 0.001$) that the two distribution functions differ (note: the test actually applies to the complementary distribution functions $S_i(x)$ but is equivalent to a test on the distribution functions $F_i(x) = 1 - S_i(x)$).

A rather different result is obtained with the corresponding split-sample qPCR data. The forms of the Turnbull distribution functions for qPCR data deteriorate at concentrations at or below the range of LLOQs (Figure 4). By contrast, the distribution functions for Colilert-18 data remain reasonably smooth all the way down to the LLOQ of 1.0 MPN/100 mL. The qPCR data provide no evidence ($p = 0.814$) that the distributions for the two classes of lakes differ, even though the qPCR data come from the same field samples as the Colilert-18 data. This failure to detect the pattern that is so evident in the Colilert-18 data is likely due in part to the deterioration of the distribution function at low concentrations, but other factors that can reduce the accuracy of qPCR concentration estimates may be involved as well (e.g., variability in the DNA content of *E. coli* cells, inhibition of PCR amplification, poor fit of standard curves to calibration data).

Because Colilert-18 is accepted by Michigan RWQS as a standard method for estimating *E. coli* concentrations while qPCR is not, and also because of the anomolous form of qPCR Turnbull distribution functions for copy numbers within and below the range of LLOQs, we suggest that spatial and temporal patterns revealed by Turnbull distribution functions based on Colilert-18 data be accepted as a benchmark in comparisons with Turnbull distribution functions based on qPCR data. Thus, a spatial or temporal pattern would be considered real if detected with Colilert-18 data, even if it is not detected by qPCR data from the same samples.

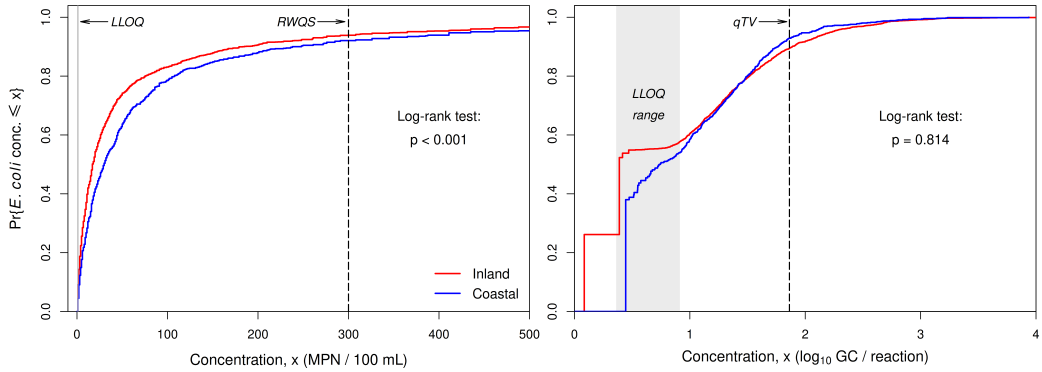

Figure 4. Turnbull distribution functions for coastal and inland-lake beaches based on Colilert-18 data (**left**) and qPCR data (**right**). The results of log-rank tests are shown for the null hypothesis of no difference between distributions against the alternative hypothesis of a difference for at least one concentration. LLOQ: lower limit of quantification, RWQS: Michigan's recreational water quality standard for total-body contact recreation, qTV: Michigan's proposed qPCR threshold value.

The clear difference between levels of *E. coli* contamination at coastal and inland-lake beaches that is revealed by the Colilert-18 data for all beaches combined could be produced by unknown geographic patterns other than inland-lake versus coastal, since most counties in Michigan with monitored beaches have only coastal or only inland-lake beaches. Four counties, however, have sufficient data for both beach classes to permit separate county-level assessments: Muskegon County (Inland: $N = 60$, Coastal: $N = 212$), Macomb County (Inland: $N = 127$, Coastal: $N = 197$), Iosco County (Inland: $N = 17$, Coastal: $N = 55$), and Chippewa County (Inland: $N = 171$, Coastal: $N = 84$). These county-level assessments partially control for geographic differences other than coastal versus inland-lake and for

differences in the laboratories performing the sampling and analysis, since other geographic properties are likely to be more similar within an individual county than across the entire state, and the laboratory that performed the sampling and analysis for beaches within each individual county was the same but differed between counties.

The separate analysis of the Colilert-18 data for Muskegon, Macomb, Iosco, and Chippewa counties provide strong support for the same pattern revealed by the combined data for all monitored beaches (Figure 5). In each county, the Turnbull distribution function for coastal beaches lies to the right of that for inland-lake beaches, and the differences are statistically significant ($p < 0.01$ in all four cases, with Holm adjustment for multiple comparisons). Thus, as with the combined data for all beaches, the Colilert-18 data for separate counties provide strong evidence that *E. coli* concentrations tend to be higher at coastal beaches than at inland-lake beaches.

The qPCR data for the individual counties also provide strong evidence for the same pattern in three of the four counties, despite the fact that the Turnbull distribution functions deteriorate at concentrations at or below the range of LLOQs. This result contrasts with that for the combined data, suggesting the possibility that geographic differences other than coastal versus inland may have contributed "noise" to the combined data that partially obscured the difference between *E. coli* concentrations at inland-lake and coastal beaches.

The percentages of Colilert-18 data that exceed Michigan's RWQS provide further evidence that the risk of total-body recreation to human health is higher at coastal beaches (Table 2). The percentage of Colilert-18 data that exceed the RWQS is consistently higher at coastal beaches, whether we look at all counties combined or at the four individual counties with sufficient data for both classes of beaches.

As in the case of the performance measures displayed in Table 1, the exceedance percentages in Table 2 are the observed values for the full set of monitoring data from 2019 and 2020 and therefore accurately indicate the patterns in those data. If, however, we again view the monitoring data as a random sample from a larger population of beaches and sampling dates, then it is appropriate to estimate 95% confidence intervals for the differences in exceedance percentages between inland-lake and coastal beaches. Using function `exact.test()` in R package `Exact` with the no-difference null hypothesis and two-sided alternative hypothesis that the values for inland-lake and coastal beaches differ, we find that the estimated differences $P_{\text{inland}} - P_{\text{coastal}}$ and (in parentheses) two-sided 95% confidence intervals for all 12 counties and for Muskegon, Macomb, Iosco, and Chippewa counties are, respectively: −1.8% (−4.0%, 0.1%), −4.2% (−8.0%, 1.9%), −7.6% (−12.7%, −2.6%), −3.6% (−12.8%, 15.2%), and −7.1% (−15.0%, −3.2%). Note that only for Macomb County and Chippewa County do the 95% confidence intervals exclude zero. This means that only for these two counties do the 2019 and 2020 data provide strong evidence that Colilert-18 concentrations for a randomly chosen pair of inland-lake and coastal beaches and a randomly chosen pair of sampling dates from a larger hypothetical population of beaches and dates would show a lower exceedance percentage at the inland beach than at the coastal beach. But the pattern does hold for the actual set of Colilert-18 concentrations in the 2019 and 2020 monitoring data.

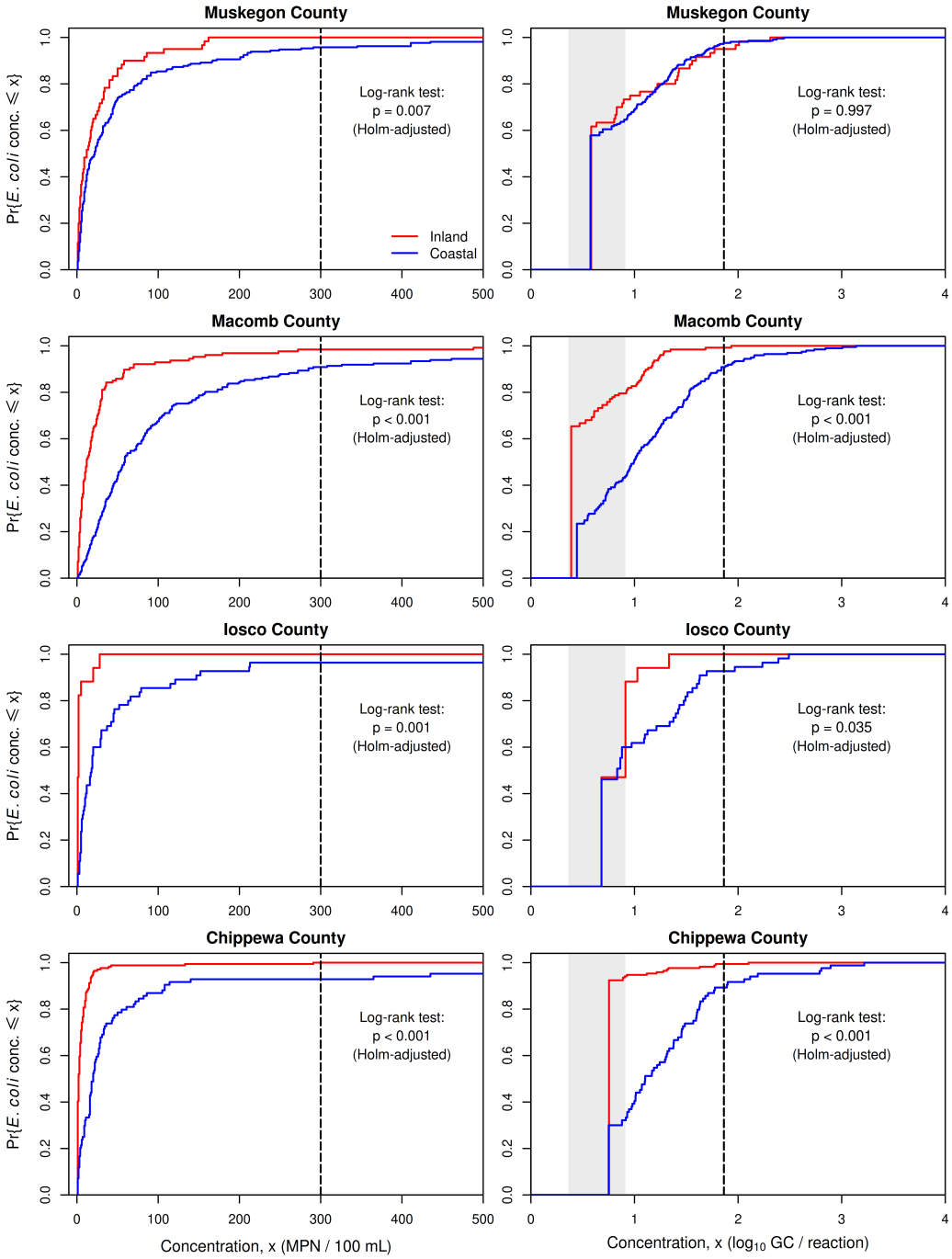

Figure 5. Turnbull distribution functions for coastal and inland-lake beaches, plotted separately for the four Michigan counties with sufficient data for both classes of beaches. Each row of panels corresponds to one county and shows distribution functions based on Colilert-18 data (**left**) and on qPCR data (**right**), plotted and annotated as in Figure 4.

The pattern is less clear for qPCR data and the proposed qTV: the percentage of qPCR data that exceed qTV is higher for coastal beaches in Macomb, Iosco, and Chippewa counties but is lower for coastal beaches in Muskegon County and in the combined data for all counties. The cases that are not consistent with the clear pattern in the Colilert-18 data are the same ones for which the qPCR data fail to show a clear separation between the Turnbull distribution functions for coastal and inland-lake beaches (Figures 4 and 5).

Table 2. Percentages of Colilert-18 and qPCR estimates of *E. coli* concentrations that exceed Michigan's RWQS for total-body contact recreation and the proposed qTV, respectively. The results for each method of quantification are shown for all counties combined and separately for each of the four counties for which both Colilert-18 and qPCR data were available.

County	Colilert-18		qPCR	
	Inland-Lake	Coastal	Inland-Lake	Coastal
All	6.1	7.9	10.5	7.1
Muskegon	0.0	4.2	5.0	2.4
Macomb	1.6	9.1	0.8	9.1
Iosco	0.0	3.6	0.0	7.3
Chippewa	0.0	7.1	0.6	10.7

4. Discussion

The results of this study address three main issues: (1) the relative merits of Colilert-18 and qPCR as the basis for beach notification decisions, viewing decisions based on Colilert-18 estimates of *E. coli* concentrations for the day of sampling as the benchmark, (2) the relative merits of Colilert-18 and qPCR estimates of *E. coli* contamination for rigorous statistical assessment of differences in levels of contamination at different groups of beaches, and (3) the relative levels of *E. coli* contamination at Michigan's inland-lake and coastal beaches during 2019 and 2020. The first and second issues deal with basic properties of Colilert-18 and qPCR methods of quantification and their use in monitoring programs, and so are likely to be relevant to monitoring programs in other geographic regions. By contrast, the third issue deals specifically with beach monitoring data for Michigan beaches and may or may not be relevant to other geographic regions.

Addressing the third issue first, the Colilert-18 results indicate a clear pattern of lower levels of fecal contamination at Michigan's inland-lake beaches than at its coastal beaches. This pattern—which is opposite to what we had expected to find—holds for the combined data from all twelve counties for which both Colilert-18 and qPCR data are available, and also holds individually for the subset of four counties that have such data from both inland-lake and coastal beaches. The reasons for this difference are as yet unknown, but potential explanations are currently being investigated. Examples include differences in bird density on beaches and the frequency and severity of resuspension of *E. coli* cells from wave action.

With regard to the first issue, the key advantage of qPCR quantification over Colilert-18 is that qPCR results can be available in only 3–4 h after collecting samples, compared to 18 h for Colilert-18, so it is possible to make beach notification decisions in time to prevent total-body contact recreation at unsafe beaches on the same day that the samples are collected. The ability to make same-day decisions is very important because, as we noted in the Introduction, several studies have shown that the levels of *E. coli* contamination at the same sampling locations on the same beach show little or no correlation on consecutive days. It follows that not only are Colilert-18 concentrations not available at all for making beach notification decisions on the sampling day, but they also are not reliable as estimates of contamination on the next day when they finally befome available, and therefore are not a reliable basis for decisions on that day, either.

One of the limitations of qPCR quantificaton is that the relationship between qPCR and Colilert-18 estimates of *E. coli* concentration on the day of sampling (based on retrospective analysis), though clearly an increasing one (Figure 3), shows substantial scatter that makes it impossible to find a qPCR threshold level for beach notification decisions that exhibits near-perfect agreement with decisions that would be made with Colilert-18 estimates and Michigan's RWQS if the Colilert-18 estimates were available on the sampling day.

However, several prior studies of *E. coli* and *Enterococcus* contamination in surface waters have compared qPCR and cultural quantification methods and found that qPCR-based results are sufficiently well correlated with culture-based results to be useful predictors in monitoring studies (e.g., [13,34]). Moreover, our results show that the false-negative error rate for qPCR-based beach notification decisions made on the sampling day is far lower than the false-negative error rate for real-time sampling-day decisions based on Colilert-18 (which is 100%, because warnings or beach closings are not issued without data indicating an exceedance), so qPCR quantification has a clear advantage.

Four disadvantages of qPCR quantification in beach monitoring are the far greater percentage of censored data it produces (compared to Colilert-18 quantification), its sensitivity to sample constituents that interfere with PCR amplification [35], its greater level of difficulty and hence susceptibility to lab error, and its dependence on calibration standards and the choice and implementation of a statistical method for fitting standard curves that underlie all sample concentration estimates [35–37]. If monitoring data are used exclusively for making beach notification decisions, these disadvantages are outweighed (in our opinion) by the key advantage of qPCR quantification: its results can be available early enough on the sampling day to prevent most recreators from entering unsafe water, whereas Colilert-18 results are not available until the next day, when the level of *E. coli* contamination is likely to be markedly different. However, if monitoring data are also to be used to identify patterns in contamination levels at different beaches or sampling times and to assess potential explanations for such patterns (e.g., as a basis for adjusting the qTV for particular beaches or groups of beaches to improve the balance between their qPCR false-negative and false-positive error rates), then the high censoring rate, greater susceptibility to lab error, and sensitivity to interference by sample constituents and to the details of statistical methodology for fitting standard curves are important disadvantages of qPCR quantification.

Regarding the second issue that our results address, the shortcomings of qPCR-based monitoring data as a basis for comparing concentration distributions for different groups of beaches are evident in plots of the Turnbull distribution functions for the combined data from all counties (Figure 4) and for data from individual counties (Figure 5). In each case, the distribution function for qPCR data deteriorates at concentrations within and below the range of LLOQs, and in a few cases (the data for all counties combined and single-county data for Muskegon and Iosco counties), the separation between distribution functions for inland-lake and coastal beaches is less pronounced or entirely absent.

The relative degrees to which the high censoring rate, interference by sample constitutuents, and poor fits of standard curves contribute to the reduced separation between qPCR concentration distributions are unknown. The high censoring rate only affects the form of these distributions at concentrations well below the qTV and therefore does not affect the ability of qPCR data to correctly predict Colilert-18 beach notification decisions. It does, however, affect the ability to detect statistically significant differences between entire distributions. Interference by sample constituents that inhibit amplification lowers qPCR amplification curves (or stretches them to the right) and therefore increases estimates of the threshold cycle C_t, which in turn lowers estimates of sample concentrations. It can affect the full range of concentration estimates and is likely to vary among beaches from different waterbodies. The EPA Draft Method C workbook automatically adjusts concentration estimates (based on a sample processing control subject to inhibition and an external positive control with no inhibition: [11,25]) in an effort to reduce the effect of interference on sample concentration estimates, but there is no guarantee that the effect will be eliminated.

The EPA Draft Method C workbook fits standard curves to calibration data using weighted least-squares regression (because the calibration data typically are heteroskedastic), with weights automatically chosen as the standard concentrations. The squared errors (errors being the differences between observed and predicted C_t values) for higher concentrations are therefore weighted more heavily than those for lower concentrations, with the result that standard curves fitted by weighted least-squares often fit the trend in C_t values versus standard concentrations better at high concentrations that at low ones. Consequently, when a fitted standard curve is inverted to predict unknown sample concentrations from measured C_t values, the high concentration estimates are likely to be more accurate than the lower concentration estimates. This source of error, then, is somewhat similar to censoring in that it mainly affects low concentrations.

The differences between inland-lake and coastal beaches that were detected by comparing their *E. coli* concentration distributions and by comparing the percentage of concentrations that exceed Michigan's culture-based RWQS for total-body contact recreation indicate that the two classes of beaches tend to differ in one or more unknown ways that affect their levels of fecal contamination. We are currently exploring various explanatory variables (e.g., bird counts, wind speed, water turbidity, proximity of coastal beaches to river mouths, frequency and severity of resuspension events, algal/macroalgal biomass, and level of PCR inhibition) to gain insight into potential causes of the observed differences in contamination levels.

We also found that inland-lake and coastal beaches tend to differ in the false-negative and false-positive error rates for beach notification decisions based on qPCR data. This is one of several lines of evidence suggesting that it might be advisable to propose different qTV values for different beaches or classes of beaches in order to achieve a better balance between the false-negative and false-positive error rates. These two types of errors result in different kinds of costs: the main costs of false-negative errors are an increased incidence of gastrointestinal illnesses among beach recreators and the associated financial cost of treatment, while the main costs of false-positive errors are the financial loss suffered by local businesses that serve beach recreators and the dissatisfaction of recreators who are turned away from the beaches. If they can be measured accurately on the same scale, these costs can be incorporated in a decision framework and used to determine optimal decision boundaries. A potentially more practical way to choose an appropriate qTV value for a beach, if multiple years of paired Colilert-18 and qPCR data are available, is to choose a value that, when applied to prior data for the beach, makes the percentage of qTV exceedances by the qPCR data the same as the percentage of RWQS exceedances by the Colilert-18 data. We are currently working on developing and comparing this and other approaches to the problem of choosing qTVs that are specific to particular beaches or classes of beaches.

5. Conclusions

- The levels of *E. coli* contamination at Michigan's freshwater beaches during 2019 and 2020 tended to be higher at coastal (Great Lakes) beaches than at inland-lake beaches. This surprising pattern held for the combined state-wide data as well as for four individual counties across the state that had both coastal and inland-lake beaches.
- The main advantage of qPCR analysis for beach monitoring is that results can be available early enough on the day of sampling to prevent most recreators from entering the water if the *E. coli* level on that day exceeds the state RWQS for full-body contact recreation. By contrast, Colilert-18 results are not available until the day after sampling, by which time the *E. coli* level is likely to be markedly different.

- The main advantage of Colilert-18 data is the much lower frequency of censored *E. coli* concentrations. Estimated nonparameteric distribution functions for Colilert-18 typically are relatively smooth across the entire range of observed concentrations, while those for qPCR deteriorate into crude step functions at moderate to low concentrations, where most of the lower limits of quantification for qPCR analysis occur. Partly because of this deterioration in qPCR distribution functions, hypothesis tests for differences between *E. coli* distributions for coastal and inland lake beaches provided strong evidence for differences at both the state-wide and county levels more often with Colilert-18 data than with qPCR data.
- Overall, we conclude that each of the two analytical methods we compared has strengths and weaknesses that make it a more appropriate tool for certain tasks and a less appropriate tool for others.

Author Contributions: Conceptualization, J.N.M., R.R.R., J.J.H. and S.B.; methodology, J.N.M.; formal analysis, J.N.M.; resources, R.R.R. and S.B.; data curation, R.R.R. and S.B.; writing—original draft preparation, J.N.M.; writing—review and editing, J.N.M., R.R.R., J.J.H., M.N.J. and S.B.; visualization, J.N.M. and M.N.J.; funding acquisition, J.N.M. and R.R.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The beach monitoring data are not publicly available, due to ethical restrictions. Requests for data should be directed to Dr. Shannon Briggs, Michigan Department of Environment, Great Lakes and Energy, 525W. Allegan St., Lansing, MI 48909, USA (briggss4@michigan.gov).

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Conflicts of Interest: John J. Hart is currently employed by the company, Geosyntec Consultants, Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

E. coli	*Escherichia coli*
FIB	Fecal indicator bacteria
LLOQ	Lower limit of quantification
MPN	Most probable number
qPCR	Real-time quantitative polymerase chain reaction
qTV	qPCR threshold value
RWQS	Recreational water quality standard(s)
ULOQ	Upper limit of quantification
USEPA	United States Environmental Protection Agency

Appendix A

Table A1. Michigan laboratories that contributed Colilert-18 and qPCR estimates of *E. coli* concentrations to the 2019 and 2020 beach monitoring data used in the present study.

Laboratory
Assurance Water Laboratory, Central Michigan District Health Department
Shimadzu Core Laboratory, Ferris State University
Annis Water Resources Institute, Grand Valley State University
Environmental Analysis Laboratory, Lake Superior State University
Oakland County Health Division Laboratory
HEART Freshwater Field Station Laboratory, Oakland University
Department of Chemistry, Saginaw Valley State University

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