

ABSTRACT

AN EVALUATION OF U.S. EPA qPCR METHODS AT SELECT DOOR COUNTY, WI BEACHES

By Aaron Campbell

The United States Environmental Protection Agency (EPA) has adopted numerous techniques for enumerating fecal indicator bacteria, each with varying advantages and disadvantages. Traditional culture techniques require 18-24 hours of incubation and do not provide results in a time sufficient manner to adequately protect public health. To help solve this issue, the EPA has developed rapid bacterial indicator methods based on real-time quantitative polymerase chain reaction (qPCR) technology. Some results from these rapid methods (calibrator cell equivalents, CCE) have shown to correlate with results from culture based methods (most probable number, MPN), accurately predicting risk for gastro-intestinal illness. However, there are no comparative studies that have been done in the Great Lakes or at beaches that are not directly impacted by human fecal contributions. In this study, *Enterococcus* qPCR Method 1609 and *E. coli* qPCR Method C were compared to the defined substrate methods Enterolert and Colilert, respectively, at four beaches in Door County, Wisconsin. Baileys Harbor beach does not exhibit any significant correlations ($p > 0.05$) between the qPCR methods and defined substrate methods ($r^2 = 0.025$ (*Enterococcus*) and 0.002 (*E. coli*)). Fish Creek beach shows a positive correlation between *Enterococcus* qPCR and culture methods ($r^2 = 0.387$, $p < 0.01$), while method C does not show any significant correlation ($r^2 = 0.01$, $p > 0.05$). Otumba beach shows weakly positive correlations for both qPCR assays and their associated cultures (*Enterococcus* $r^2 = 0.141$ ($p = 0.0169$), *E. coli* $r^2 = 0.363$ ($p < 0.01$)). Correlations for Sunset beach have r^2 values of 0.021 for *Enterococcus* ($p > 0.05$) and 0.104 for *E. coli* ($p = 0.0307$).

In addition to the correlations, the agreement between methods was recorded and compared to current regulatory standards. If adopted as the regulatory method, qPCR methods would result in a minimum increase of beach closures of 10 percent and a maximum increase of 47 percent for either method. This suggests that qPCR would be more protective of public health than current culture based enumeration methods. These increases in beach closures may have potential social and economic ramifications in local communities. While it is clear much research is needed to distinguish which method is more appropriate for the Great Lakes Region and this study suggests a site by site evaluation of qPCR testing methods before they are adopted for use in monitoring recreational water quality.

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WI BEACHES

by

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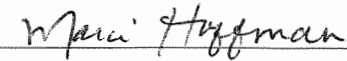
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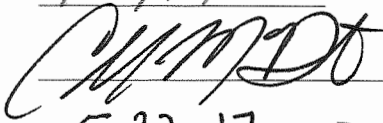
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INTRODUCTION

As a public recreational resource, beaches provide many people the opportunity to enjoy activities such as swimming and sunbathing. In addition to providing the public with recreational opportunities, public swimming beaches also have been shown to be essential to many local economies (Sohngen et al., 1999). While the exact amount varies from person to person, day trips to Great Lakes public swimming beaches can cost an average of \$15-\$25 (Sohngen et al., 1999) to approximately \$50 depending on location (Kleinheinz, unpublished data). The overall quality of a particular beach also influences the decisions of potential visitors to visit or not visit a particular beach. When questioned about beach characteristics, overall beach cleanliness and maintenance, along with overall water quality, influenced visitor decisions the strongest. With beach closings occurring occasionally due to poor water quality, it is important to keep the public informed of the current water quality at public swimming beaches, to protect public health.

In the 1960's, the US Public Health Service recommended using fecal coliforms as fecal indicator bacteria, which was recommended by the Environmental Protection Agency in 1976 (US EPA, 1976). In 1986, the US EPA released water quality standards for recreational waters (US EPA, 1986) based on epidemiological studies that related microbial water quality, assessed by culture based enumeration methods, to overall health effects experienced by individuals exposed to high microbial contamination (Cabelli, 1983; Dufour, 1984). In those studies, enterococci and *Escherichia coli* had the strongest correlations to swimming associated gastrointestinal illness (US EPA, 2012a). Though

both bacteria correlate with increased illness, the US EPA recommended that enterococci be used as the approved indicator in both marine and fresh water settings, while *E. coli* was only approved for use in fresh water. The World Health Organization (WHO) recommends the use of only enterococci as an indicator of water-quality in recreational waters (WHO, 2003). Epidemiological studies conducted worldwide suggest dramatic improvements in the accuracy of prediction over the previously favored indicators, total and fecal coliforms (US EPA, 1986; US EPA, 1998; US EPA, 2010; US EPA, 2012b). In October 2000, Congress passed the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) designed to reduce the risk of disease to users of the nation's coastal recreational waters (US EPA, 2000) and the US EPA began to regulate fecal contamination in recreational water shortly after. The aim of the BEACH Act is to better inform the public of health concerns at beaches by requiring states with coastal beaches (including Great Lakes beaches) to follow an approved plan for monitoring microbial contamination and for informing the public when established standards are exceeded (Dufour, 1984; NRDC, 2008). Shortly after, in 2002, the EPA initiated the National Epidemiological and Environmental Assessment of Recreational Water study program, a series of epidemiological studies to determine a relationship between gastrointestinal illness and rapid detection techniques that produce results within 2-3 hours, rather than 18-24 hours traditional culture based methods require.

As previously stated, the US EPA approved two indicator organisms, enterococci and *E. coli*, to be used in recreational water to help protect the public from fecal contamination. Enterococci were approved for use in both fresh water and marine

environments. This is because data analysis of the National Epidemiological Environmental Assessment of Recreational (NEEAR) water studies of culture densities shows that there are no compelling differences between measurements taken in marine and freshwater environments (Noble et al., 2004; US EPA, 2012a). The most notable influences in the fate of enterococci in the environment are sunlight, temperature, and predation, rather than salinity (Noble et al., 2004). Though enterococci are an approved indicator to be used in freshwater systems, in the Great Lakes Region the indicator of choice is *E. coli* (WI DNR, 2004). In studies completed by the EPA, *E. coli* levels were found to have the best correlation with cases of gastrointestinal illness in freshwater systems, like the Great Lakes (US EPA, 1986). While FIB are reliable indicators in environments with point sources of fecal pollution, in areas with nonpoint sources, FIB have been found to be less reliable, suggesting a need for alternative indicators of water quality where nonpoint sources are the largest contributors to fecal pollution (Colford et al., 2007; Boehm et al., 2009; Wong et al., 2009). Studies completed on water quality indicators have suggested that indicators may need to be site specific (Griffith et al., 2016). Until more research can be completed, traditional fecal indicators are currently the best option to protect public health.

FIB have specific relationships that are utilized to help quantify the presence of other fecal based pathogens that may be present in recreational water (US EPA, 2012a). While most FIB are not pathogenic themselves, they often demonstrate similar characteristics to the pathogens of interest, making them reliable indicators of fecal contamination. Specifically the origin of these FIB and the pathogens are typically the

same, but the enumeration of the FIB have much simpler methods of detection (US EPA, 2012a). Using this information, FIB are classified as pathogen indicators; which are defined as “a substance that indicates the potential for human infectious diseases” (CWA §502(23)). The usefulness of FIB extends further as there are many pathogens of interest, but pathogen-specific enumeration methods were not available at the time of the NEEAR water study and thus, health relationships with specific pathogens were not established (US EPA, 2012a), making the enumeration of FIB such as enterococci and *E. coli* the best option with current technologies.

Currently FIB culture-based enumeration methods are used widely in routine monitoring programs. In culture-dependent methods, cultivation conditions are manipulated to encourage growth of the target microorganisms (Mesquita and Noble, 2013). In the 1920s, multiple tube fermentation was developed to detect fecal coliforms present in drinking water (Edberg and Edberg, 1988). This method required adding ten milliliters of water to five test tubes containing a protein base with the fermentable carbohydrate, lactose, and after 48 hours of incubation, the number of coliforms per 100 milliliters of water was calculated from the number of positive tubes, yielding a most probable number (MPN) result (Edberg and Edberg, 1988). The MPN value is an estimate of the number of bacteria present in a sample based on the number of positive tubes and negative tubes inoculated (Edberg and Edberg, 1988). Due to the need for balance between sensitivity and selectivity, many other alternative methods for sample processing have arisen. In addition to multiple tube fermentation, there are two other widely used culture based methods for quantifying fecal indicators in water based

samples, membrane filtration and the defined substrate method (Mesquita and Noble, 2013). In the 1950s, the membrane filtration technique was developed, where a set amount of water is passed through a bacterial exclusion membrane. After, the membrane is then placed on an agar-based medium containing a protein base, lactose, and a pH indicator. Much like the multiple tube fermentation technique, confirmation must be performed as the primary plate can yield false-positive results (Edberg and Edberg, 1988). The defined substrate method, the most widely used culture method, is an extremely user friendly, inexpensive method, that can quantify total fecal coliforms and enumerate indicator bacteria (Edberg and Edberg, 1988, Edberg et al., 1990). The defined substrate method is a method adapted from the medical field, used to identify urinary tract pathogens (Edberg and Edberg, 1988). Unlike the multiple tube fermentation, which allows for growth of all aerobic microbes, the defined substrate technology targets only microbes of interest by providing essential nutrients only to those particular microorganisms. In this particular scenario, a defined substrate is used as the essential nutrient, where digestion of the substrate produces a product that interacts with a chromogen, indicating the presence of the target organisms after a 24 hour incubation period (Edberg and Edberg, 1988, Edberg et al., 1990).

Though these culture based enumeration methods are reliable, they do not provide results in timely manner, requiring 18-24 hour incubation periods. This prevents beach managers from making same day beach management decisions, hindering public health protection efforts (Sheth et al., 2016). In addition to the reduced protection of public health, traditional culture methods can have negative impacts on the local

economy, keeping beaches closed when they should be reopened (Ali, 2011). For these reasons, there is a strong need for a method that provides real-time or near real-time results (Whitman and Nevers, 2004). Predictive models are faster than culture methods and correctly classify results at a high percentage, but have difficulty correctly identifying instances when fecal indicator bacteria counts exceed the health standard thresholds, in addition to identifying new or unusual fecal inputs that would cause elevated FIB (Telech et al., 2009). Predictive models can use a range of methods for FIB enumeration, typically either linear regression or the least squares method (Nevers and Whitman, 2005; Franczy et al., 2006). The least squares method is used to establish a functional relationship that could be used to predict exceedances of fecal indicator bacteria, thus supporting issued advisories. Many authors have employed the partial least squares model in order to reduce the large number of variables for predictive purposes. Holtschlag et al. (2008) used a least squares model in order to establish the relationship between the specific pathogen indicator and the unique meteorological variables relevant to each beach. These variables often include cloud cover, wave height and direction, wind direction, turbidity, pH, water temperature, rainfall and the number of waterfowl on or near each beach (Telech et al., 2009; Nevers et al., 2014). With the input of these variables into the models, the model is then used to forecast fecal contamination. Although predictive modeling eliminates the time-lag in issuing beach advisories, predictive models have shown that they have the ability to correctly classify results a high percentage of the time, but had difficulty correctly identifying instances when fecal indicator bacterial counts exceed the health standard thresholds shown to correlate with

increased risk for gastrointestinal illness (Telech et al., 2009). In addition to predictive models, the US EPA has also examined more rapid laboratory methods to alleviate the time lag problem between sample collection and sample result.

The United States Environmental Protection Agency has conducted numerous studies on alternative methods for detection of fecal contamination. qPCR has received a significant amount of attention from research scientists, and public health professionals have shown significant correlations between the risk of gastrointestinal illness and qPCR based enumeration methods for each EPA approved indicator bacterium (US EPA, 2012; Wade et al., 2006). These studies have indicated that qPCR based enumeration methods are also highly predictive of the risk of gastrointestinal illness, but concluded that more research needed to be completed to investigate how different environmental variables affect qPCR's reliability, forecasting rates of inhibition in qPCR analysis (Wade et al., 2006; Byappanahalli et al., 2010;). This method has the ability to provide results within 3 hours of sample collection, greatly reducing the time of previous methods. This allows for public health professionals to better protect the public from exposure to unnecessary risk associated with gastrointestinal illness, but with greater expense and increased training need for those running the tests. With the development of these new techniques and continuous research, the protection of public health will increase, allowing for patrons of public bathing beaches to make better informed decisions.

As previously mentioned, the US EPA has developed more rapid bacterial indicator methods based on real-time qPCR technology, and have also identified significant relationships between these results and the risk of gastrointestinal illness in

beachgoers. These rapid test methods have the advantage of providing laboratory results within two or three hours of sample receipt, enabling beach managers to take action to protect public health on the same day that water samples are collected. However, due to the price of the reagents and equipment needed, along with the complexity of running these tests, many beach managers opt for alternative tests at the expense of quicker results (Griffeth et al., 2011). In addition to increased cost, qPCR is often considered to be extremely technical, making it difficult for untrained personnel to run this method. In addition qPCR also has a deficiency in the ability to distinguish between viable and dead cells as both cell types would contribute to the overall amount of target DNA sequences present in the sample, along with any ambient DNA present in the sample that matches the target DNA sequence. This can lead to artificially inflated results, which could greatly influence beach management decisions. Artificially inflated results coming from the increase in DNA from dead cells could lead to an overprotection of public health. For these reasons the US EPA has conducted a series of beach epidemiological studies to further examine the relationship between the densities of fecal indicator bacteria enumerated by real-time qPCR and the risk for gastrointestinal illness (Brenner et al., 2010, Wade et al., 2006, 2008, 2010). With more information about the relationship between qPCR methods and traditional culture based methods, more beach managers and public health professionals may opt for this rapid detection technique as it is the most developed rapid detection technique currently available (Colford et al., 2012).

Though qPCR is technical, according to Sheth et al. (2016) it was possible to implement water quality testing with the method at a remote qPCR testing lab (Sheth et

al., 2016). Results from numerous studies conducted at freshwater and marine beaches show that qPCR data has a good correlation with traditional culture technique results, and with the overall risk for gastrointestinal illness (Ferretti et al., 2011; Griffith et al., 2004; Griffith and Weisberg, 2006; Haugland et al., 2005; Kephart and Bushon, 2009; Sivaganansan et al., 2012, 2014), reporting correlation coefficients of approximately 0.45 to approximately 0.90. Additional studies, however, have noted that with qPCR methods the relationship between calibrator cell equivalents (CCE) values (the result of qPCR analysis) and colony forming units (CFU, membrane filtration) increases in uncertainty in waters with low numbers of FIB (Whitman et al., 2010). This increase in variability can lead to type I and type II statistical errors when issuing advisories. This could result in the closure of beaches when they should remain open and beaches remaining open when they should be closed (Ali, 2011).

Studies to address this problem have utilized a combination of qPCR testing and tracking of predetermined environmental factors, such as wave height and precipitation. Bacterial indicator levels can be elevated for 24-28 hours after heavy rains, typically as the result of surface runoff or increased tributary contributions, while large waves can suspend fecal indicator bacteria associated with bottom sediments (Lavender and Kinzelman, 2009). Lavender and Kinzelman (2009) employed these techniques and were able to utilize quantitative polymerase chain reaction to accurately predict beach status and quantify bacterial indicator density within four hours of sample collection (Lavender and Kinzelman, 2009). Quantitative polymerase chain reaction has shown great promise

to serve as an alternative for culture-based techniques by being able to provide reliable results within 2-3 hours of sample receipt and by increasing protection of public health.

These tests to validate each qPCR method set forth by the US EPA has led to the development of new methods for both the indicator bacteria, enterococci (Method 1611 and Method 1609) and *Escherichia coli* (Method C). Method 1611, the first qPCR method developed by the US EPA, was revised to include an internal amplification control assay (IAC), which is co-amplified simultaneously with the target sequences, to help detect inhibition, specifically polymerase inhibition (US EPA, 2013; Haugland et al. 2016). These methods amplify specific regions of the large subunit ribosomal ribonucleic acid (RNA) gene (1srRNA and 23S rRNA) from the target organisms (US EPA, 2012b; US EPA, 2013). Method 1611, 1609 and draft Method C utilize the comparative cycle threshold method to calculate the ratio of target sequences (1srRNA) recovered in total DNA extracts from water samples (US EPA, 2014b). These unknown samples are then compared to known concentration of calibrator samples. The target sequence ratio is then multiplied by the number of cells present in the whole cell calibrator samples to obtain Calibrator Cell Equivalents (CCE) (Sivaganesan et al., 2011). Attempts to further standardize these methods have been completed, including the use of thermocyclers. By further standardizing qPCR methods, results will be able to be compared between studies. Studies completed to standardize these methods have included standardizing calibrator samples and instrumentation, along with establishing thresholds and beach action values to be used in routine monitoring (Sivanganesan et al., 2011, 2012; Haugland et al., 2014)

While much research has been completed to standardize rapid detection techniques in marine systems, and freshwater beaches directly impacted by human fecal pollution, little work has been completed at nonpoint source beaches in the Great Lakes system to establish the relationships between traditional culture methods (defined substrate method) and rapid detection methods such as qPCR. Since there is ample history correlating traditional culture methods (defined substrate method) and the risk of gastrointestinal illness, relationships between rapid detection methods (qPCR) and traditional culture based detection methods (defined substrate method) should be established.

While comparison of US EPA method 1609 and the defined substrate method was included in this study, the US EPA also has recently released *E. coli* qPCR method Draft Method C. This study will not only include comparisons between each qPCR method and their respective defined substrate methods (Method 1609 and Enterolert, Draft Method C and Colilert), but between the two qPCR methods also. This is because in freshwaters systems, like the Great Lakes, the indicator of choice is currently *E. coli* because it has greater reliability to accurately represent the risk of gastrointestinal illness in freshwater systems (WDNR, 2004). However, with the introduction of new rapid monitoring techniques, such as Draft Method C, these past trends may not necessarily hold true. In this study, US EPA Method 1609 and Draft Method C are compared to their respective defined substrate methods to investigate which indicator better correlation, along with investigating the impacts of switching to qPCR detection methods would have on beach management decisions for each indicator.

The overarching objective of this study was to determine the relationship between qPCR detection methods (Method 1609 and Draft Method C) and their respective culture methods (defined substrate methods). The underlying hypotheses are H1: US EPA Draft Method C will have a more significant positive correlation with its define substrate method than method 1609, and H2: qPCR methods would result in higher numbers of closures than defined substrate methods. The specific objectives of this project were:

1. Determine the relationships between Method 1609 and Draft Method C to their respective defined substrate methods (Enterolert and Colilert, respectively).
2. Compare the relationship between Method 1609 and Enterococci defined substrate method (Enterolert) to the relationship between Method C and *E. coli* defined substrate method (Colilert) to compare the effectiveness of each qPCR method.
3. Determine the impacts of qPCR in routine monitoring programs on beach management decisions.

MATERIALS AND METHODS

Study Site and Lab Facilities

Door County, WI, USA is bordered by Lake Michigan to the east and Green Bay to the west. It has over 500 km of shoreline with over 30 swimming beaches and is an important tourist destination, with more than two million visitors per year. Beaches are located on both sides of the Door County peninsula, on Washington Island, along the Sturgeon Bay Canal, and at three inland lakes present throughout the peninsula (Sheth et al., 2016). For this study, four sites were selected for defined substrate and qPCR analysis for both enterococci and *E. coli* twice each week from Memorial Day to Labor Day during the summers of 2015 and 2016. The four beaches used for this study were Baileys Harbor Ridges (*Enterococcus* n=37; *E. coli* n=41), Fish Creek (*Enterococcus* n=41; *E. coli* n=45), Otumba (*Enterococcus* n=40; *E. coli* n=45), and Sunset (*Enterococcus* n=39; *E. coli* n=45) (Figure 1).

Baileys Harbor Ridges beach and Fish Creek beach are both located in smaller villages along the Door County coastline, with populations of approximately 1,000 people. Baileys Harbor Ridges beach is located on the outer edge of Baileys Harbor, WI, while Fish Creek beach is located near the center of town of Fish Creek, WI. Otumba and Sunset Beaches are both located in the town of Sturgeon Bay, WI, which has a population of approximately 10,000 individuals, with Otumba beach being located near downtown Sturgeon Bay and Sunset beach being located near the industrial park. In addition, Baileys Harbor Ridges, Otumba, and Sunset beaches have had remediation

projects completed to reduce fecal contamination present at each beach. Fish Creek and Otumba beaches both have outfall pipes discharging at or near the beach.

All lab analyses were completed in a state-certified laboratory operated by University of Wisconsin Oshkosh researchers at Crossroads at Big Creek Nature and Education Center in Sturgeon Bay, WI in 2015 and 2016 (Figure 1). This lab is primarily staffed by undergraduate and graduate college students, with varying degrees of molecular biology laboratory training. Any students associated with the beach monitoring program are trained in proper pipetting techniques and undergo method specific trainings (EPA Method 1609, Draft Method C, and defined substrate method). Using the above techniques, students collected water samples to enumerate fecal indicator bacteria (FIB).

Surface Water Sample Collection

Surface water samples were collected in the center of each beach, in approximately 24 inches (knee depth) water, and 12 inches below the surface, in the center of each beach, using a sterile 120 mL polystyrene IDEXX bottle. While wading slowly in the water, the sampler avoided kicking up bottom sediment at the sampling site. The sampling bottle was opened while grasping it at the base with one hand and the bottle mouth was plunged downward into the water to avoid introducing surface scum. The bottle mouth was positioned into the current, away from the sampler's hand. If the water body was static, an artificial current was created by moving the bottle horizontally with the direction of the bottle pointed away from the sampler. The bottle was tipped slightly upward to allow air to exit and the bottle to fill. The bottle was completely filled before

removing it from the water. The bottle was removed from the water body and a small portion of water was poured out to allow an air space of 2 centimeters for proper mixing of the sample before analyses. The cap was closed tightly and the bottle was labeled. The sample was stored in a cooler filled with ice immediately following collection. Samples were collected twice a week during the traditional monitoring season (approximately Memorial Day to Labor Day), with 5 bottles being collected. The bottle breakdown is as follows: 1 bottle for *E. coli* defined substrate methods, 1 bottle for enterococci defined substrate methods, 3 bottles for *E. coli* and enterococci qPCR methods. The extra bottles for the qPCR detection methods are to allow for repeated analysis if inhibition was suspected.

***E. coli* Defined Substrate Method**

Samples arrived at the laboratory on an ice slurry within 6 hours of sample collection. Samples were analyzed within 8 hours of collection according to EPA standards. Bench tops were properly sanitized prior to analysis. The sample bottle was shaken approximately 25 times or for 2 minutes to homogenize the water sample. The excess water was poured out to the marked 100 milliliter (mL) line to produce a 100 mL sample of water. Once the sample was at proper volume, contents of one snap pack (Colilert or Colilert-18, IDEXX, ME) were added to the 100 mL water sample in the sterile vessel. The vessel was capped and shaken until dissolved (approximately 2 minutes). If the Colilert did not readily dissolve, the sample was warmed to room temperature and mixing began again. The sample/reagent mixture was poured into a sterile Quanti-Tray®/2000. The tray was placed into the IDEXX Quanti-Tray® Sealer

(IDEXX, Main). It was then heated and completely sealed by the time the tray was removed from the sealer. The sealed tray was placed in a $35^{\circ} \pm 0.5^{\circ}\text{C}$ incubator for a minimum of 24 hours (18 hours if using Colilert-18). The Quanti-Tray was observed for color changes (yellow) and fluorescence. The numbers of positive wells (small and large) were counted separately and the MPN table was used to obtain a Most Probable Number (MPN). Fluorescence was observed with a 6-watt, 365 nm, UV light held within five inches of the sample in a dark environment. The results were reported as *E. coli* MPN/100 mL of water.

Enterococci Defined Substrate Method

Samples arrived at the laboratory on an ice slurry within 6 hours of sample collection. Samples were analyzed within 8 hours of collection according to EPA standards. Bench tops were properly sanitized prior to analysis. The sample bottle was shaken approximately 25 times or for 2 minutes to homogenize the water sample. The excess water was poured out to the marked 100 milliliter (mL) line to produce a 100 mL sample of water. Once the sample was at proper volume, contents of one snap pack (Enterolert, IDEXX, ME) were added to the 100 mL water sample in the sterile vessel. The vessel was capped and shaken until dissolved (approximately 2 minutes). If the Enterolert did not readily dissolve, the sample was warmed to room temperature and mixing began again. The sample/reagent mixture was poured into a sterile Quanti-Tray®/2000. The tray was placed into the IDEXX Quanti-Tray® Sealer (IDEXX, Main). It was then heated and completely sealed by the time the tray was removed from the sealer. The sealed tray was placed in a $41^{\circ} \pm 0.5^{\circ}\text{C}$ incubator for a minimum of 24 hours.

The Quanti-Tray was observed for fluorescence. The numbers of positive wells (small and large) were counted separately and the MPN table was used to obtain a Most Probable Number (MPN). Fluorescence was observed with a 6-watt, 365 nm, UV light held within five inches of the sample in a dark environment. The results were reported as Enterococci MPN/100 mL of water.

***E. coli* qPCR Detection Method**

Samples were collected in triplicate and arrived at the laboratory on an ice slurry within 6 hours of sample collection. All sample and reagent preparation was completed at separate workstations, with reagent preparation occurring within a high efficiency particulate air (HEPA)-filtered laminar flow PCR-Workstation hood with an ultraviolet (UV) light source and separate supplies (e.g., pipettors, tips, gloves, etc.) (US EPA, 2014b). All reagents were purchased from Applied Biosystems (ABI), and were prepared as recommended by the manufacturer. In addition, all solutions were adequately mixed using a vortex mixer before being combined with other solutions. After preparation, all stock reagents were aliquoted into smaller amounts to prevent contamination. After properly sanitizing all workspaces, samples were filtered onto sterile, white, 47 mm diameter, Millipore polycarbonate membrane filters, with a 0.45 μm pore size, using sterile disposable filter bases and funnels (Pall Gelman 4242 and Nalgene CN 130-4045) (US EPA, 2014b). Once filtered, filter membranes were placed into sterile 1.7 mL tubes containing 0.3 grams of acid washed glass beads. All filters were then stored in a -20°C , with one replicate being kept for analysis.

After filtration, DNA was extracted from each sample using salmon extraction buffer, as instructed by Draft Method C (US EPA, 2014b). Once completed, primer and probe working solutions were prepared for each assay (*E.coli* and Sketa22) in a separate workspace. Primer and probe working solutions were prepared by diluting 10 μ L of forward and reverse primers and 4 μ L of probe with PCR-grade water for each assay (US EPA, 2014b). The primer and probe sequences are as follows with the appropriate reporter and quencher dyes; *E. coli* Forward Primer: 5'-GGTAGAGCACTGTTTTGGCA; *E. coli* Reverse Primer: 5'-TGTCTCCCGTGATAACTTTCTC; *E.coli* TaqMan® probe: [6-FAM]-5'-TCATCCCGACTTACCAACCCG-TAMRA; Salmon Forward Primer: 5'-GGTTTCCGCAGCTGGG; Salmon Reverse Primer: 5'-CCGAGCCGTCCTGGTC; Salmon TaqMan® probe: [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA (US EPA, 2014b). After the primer and probe working solutions were prepared, a final master mix containing all reagents was made. This master mix contained 2.0 μ L of PCR-grade water, 2.5 μ L of Bovine Serum Albumin (BSA), 12.5 μ L of ABI TaqMan® mastermix, 3.0 μ L of the appropriate primer/probe working stock solution (US EPA, 2014b). These amounts were multiplied by the total number of wells required to run all samples. 20 μ L of each master mix was then aliquoted into each well, along with 5 μ L of each sample, making the final volume 25 μ L.

All samples were plated in triplicate, using a 96-well Life-Technologies MicroAmp plate and were spun for approximately 1 minute in a salad spinner to ensure all liquid was together in the bottom of each well. The plate was then placed into a Life

Technologies StepOnePlus™ Real-Time PCR System. All samples were analyzed using a two-step cycling using the Quantitation-Standard Curve setting using standard TaqMan® reagents. Target reporter and quencher dyes for each assay (*E. coli* and Salmon) were defined as specified within Method C (US EPA, 2014b). The final reaction volume was set to 25 µL, and the number of cycles was changed to 45 as advised by staff at the ERIC at the University of Wisconsin Oshkosh. The thermal profile was set to the following holding and cycling stages: Holding Stage 1: 50.0° C for 2:00 minutes; Holding Stage 2: 95° C for 10:00 minutes, Cycling Stage: 95° C for 0:15 seconds (US EPA, 2014b). The second step of the Cycling Stage is 60° C for 1:00 minute (US EPA, 2014b). The settings for each run were setup in accordance with Method C Appendix B (US EPA, 2014b). Each run was analyzed in Microsoft Excel, using the Method C spreadsheet recommended by the EPA. Standard curves were generated by trained staff from the Environmental Research and Innovation Center (ERIC) at the University of Wisconsin Oshkosh.

Enterococci qPCR Detection Method

Samples were collected in triplicate and arrived at the laboratory on an ice slurry within 6 hours of sample collection. All sample and reagent preparation was completed at separate workstations, with reagent preparation occurring within a high efficiency particulate air (HEPA)-filtered laminar flow PCR-Workstation hood with an ultraviolet (UV) light source and separate supplies (e.g., pipettors, tips, gloves, etc.) (US EPA, 2013a). All reagents were purchased from Applied Biosystems (ABI) or MidSci Scientific (master mix), and were prepared as recommended by the manufacturer. In

addition, all solutions were adequately mixed using a vortex mixer before being mixed with other solutions. After preparation, all stock reagents were aliquoted into smaller amounts to prevent contamination. After being properly sanitizing all workspaces, samples were filtered onto sterile, white, 47 mm diameter, Millipore polycarbonate membrane filters, with a 0.45 μm pore size, using sterile disposable filter bases and funnels (Pall Gelman 4242 and Nalgene CN 130-4045) (US EPA, 2013). Once filtered, filter membranes were placed into sterile 1.7 mL tubes containing 0.3 grams of acid washed glass beads. All filters were then stored in a -20°C freezer, with one replicate being kept for analysis.

After filtration, DNA was extracted from each sample using salmon extraction buffer, as instructed by Draft Method 1609. Once completed, primer and probe working solutions were prepared for each assay (Enterococci/IAC and Sketa22) in a separate workspace. Primer and probe working solutions were prepared by diluting 10 μL of forward and reverse primers and 4 μL of probe with PCR-grade water for each assay (US EPA, 2013). An additional 4 μL of IAC probe was added to the Enterococci working solution, displacing 4 μL of PCR-grade water (US EPA, 2013). The sequences for primers and probes, along with the appropriate reporter and quencher dyes are as follows; Entero Forward Primer: 5'-GAGAAATTCCAAACGAACTTG ; Entero Reverse Primer: 5'-CAGTGCTCTACCTCCATCATT ; Entero TaqMan® Probe: [6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA; Salmon Forward Primer: 5'-GGTTTCCGCAGCTGGG; Salmon Reverse Primer: 5'-CCGAGCCGTCCTGGTC; Salmon TaqMan® probe: [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA; IAC

UC1P1TaqMan® probe: [VIC]-5'-CCTGCCGTCTCGTGCTCCTCA-TAMRA. After the primer and probe working solutions were prepared, a final master mix containing all reagents was made. This master mix contained 2.0 µL of the appropriate Internal Amplification Control (IAC) plasmid or PCR-grade water, 2.5 µL of Bovine Serum Albumin (BSA), 12.5 µL of MidSci TaqMan® mastermix, 3.0 µL of the appropriate primer/probe working stock solution (US EPA, 2013a). For the Enterococci master mix, PCR-grade water was replaced with an equal amount of IAC plasmid at a 1×10^3 dilution. 20 µL of each master mix was then aliquoted into each well with 5 µL of each sample, making the final volume 25 µL.

All samples were plated in triplicate, using a 96-well Life-Technologies MicroAmp plate and were spun for approximately 1 minute in a salad spinner to ensure all liquid was together in the bottom of each well. The plate was then placed into a Life Technologies StepOnePlus™ Real-Time PCR System. All samples were analyzed using a two-step cycling using the Quantitation-Standard Curve setting using standard TaqMan® reagents. Target reporter and quencher dyes for each assay (Enterococcus, Salmonella, and IAC) were defined as specified within method 1609 (US EPA, 2013). The final reaction volume was set to 25 µL, and the number of cycles was changed to 45 as advised by staff at the ERIC at the University of Wisconsin Oshkosh. The thermal profile was set to the following holding and cycling stages: Holding Stage 1: 50.0° C for 2:00 minutes; Holding Stage 2: 95° C for 10:00 minutes, Cycling Stage: 95° C for 0:15 seconds. The second step of the Cycling Stage is 60° C for 1:00 minute (US EPA, 2013). All other settings for each run were setup in accordance with Method 1609 Appendix B (US EPA,

2013). Each run was analyzed in Microsoft Excel, using the Method 1609 spreadsheet recommended by the EPA. Standard curves were generated by trained staff from the ERIC at the University of Wisconsin Oshkosh.

Graphical and Statistical Analysis

Correlation coefficients (R^2) values were all generated in Microsoft Excel, using the basic statistical package on transformed (Log_{10}) data. All graphs also were created in Microsoft Excel. All comparisons of means (ANOVA), along with Tukey's HSD tests were completed in the statistical program, R.

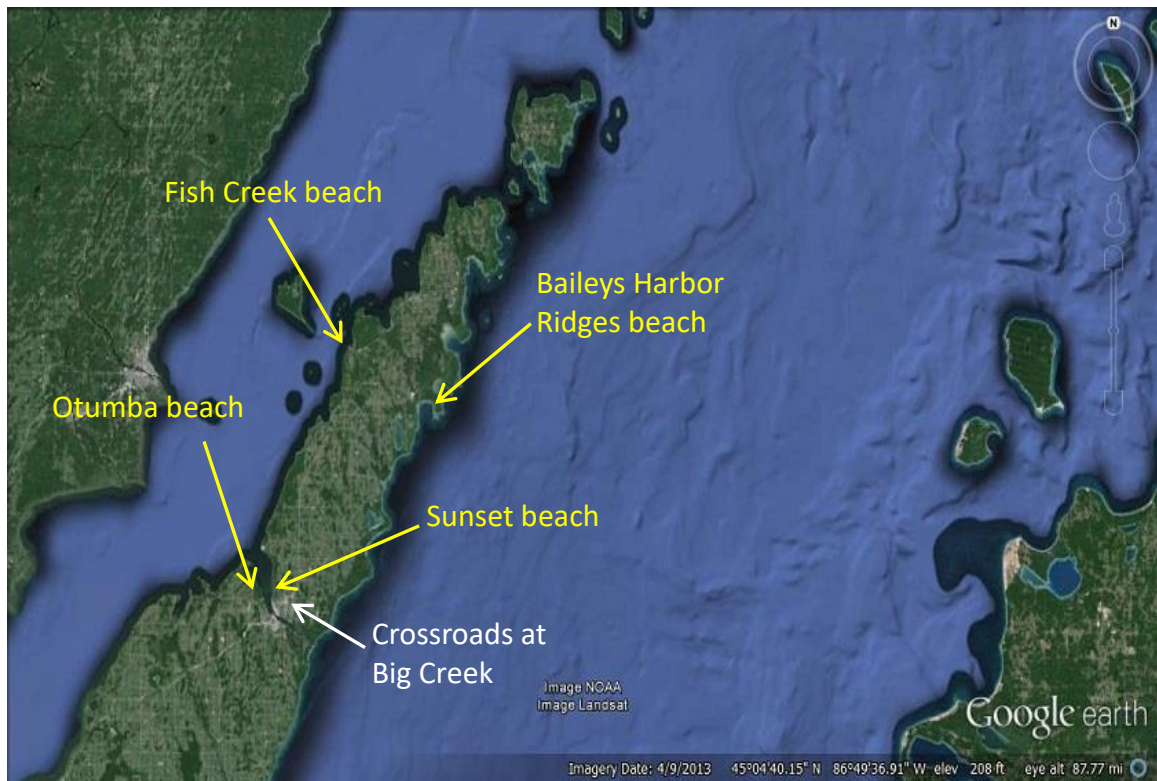


Figure 1. A map showing the location of the 4 beaches involved in the study along with the location of the remote laboratory at Crossroads and Big Creek.

RESULTS

The over-arching objective of this study was to determine how qPCR enumeration methods 1609 and Method C were related to their appropriate defined substrate methods, along with comparing each method to each other. Concentrations of FIB varied by analysis method and by beach (Table 1 and Table 2). Mean *Enterococcus* concentrations from beach water, as measured by Enterolert were similar at Baileys Harbor Ridges and Sunset beaches (Enterococci= 27.8 and 39.2 MPN/100 mL of water), while mean concentrations of *Enterococcus* were similar at Fish Creek and Otumba beaches (Enterococci= 264.2 and 139.3 MPN/100 mL of water, respectively).

E. coli was enumerated using the defined substrate method at each beach. Concentrations of *E. coli* measured by Colilert from beach water followed a similar pattern to *Enterococcus* measured by Enterolert. Mean *E. coli* concentrations were similar at Baileys Harbor Ridges and Sunset Park Beach beaches with means of 71.5 and 90.3 MPN/100 mL of water, respectively. Fish Creek and Otumba beaches had mean *E. coli* concentrations of 344.6 and 224.9 MPN/100 mL of water, respectively. It is important to note that for both indicators the same pattern emerges when using the defined substrate methods (Enterolert and Colilert).

Enumeration of enterococci using Method 1609 resulted in mean concentrations for all beaches of above 1,000 calibrator cell equivalents (CCE), the single-sample maximum allowable density (Haugland et al. 2014). Enterococci enumerated by Method 1609 yielded mean concentrations of 7,668.8 CCE/100 mL of water at Baileys Harbor and 4,871.8 CCE/100 mL of water for Fish Creek. Otumba yielded the highest

concentration of enterococci using Method 1609, with 2,1902.3 CCE/100 mL of water, and Sunset the lowest mean concentration of 3,611.3 CCE/100 mL of water (Table 1). When comparing these mean concentrations of enterococci with the mean concentrations measured by Enterolert, two different patterns emerged.

Much like Method 1609, Method C (*E.coli*) resulted in mean concentrations for all beaches of above 1,000 CCE. Unlike Method 1609, results from Method C do follow a similar pattern to that observed using the defined substrate method (Colilert). The mean concentration of *E. coli* as measured by Method C at Baileys Harbor was 2,124.4 CCE/100 mL of water, while the mean concentration of *E. coli* measured by Method C at Sunset was 3,655.3 CCE/100 mL of water. The CCE concentrations at Fish Creek and Otumba were much higher, with mean concentrations of 9,297.9 CCE/100 mL of water and 18,599.1 CCE/100 mL of water, respectively (Table 2).

Statistical comparisons of FIB concentrations by qPCR based detection methods and their respective defined substrate methods were all evaluated with a significance level of $p < 0.05$. Comparisons of *Enterococcus* measured by Method 1609 and Enterolert all yielded positive correlations, though to varying degrees (Figures 2-5). Baileys Harbor Ridges measurements resulted in a correlation coefficient (r^2 value) of approximately 0.025 (Table 3, Figure 2). Fish Creek and Otumba Park beaches measurements both resulted in significant correlations ($p < 0.05$) of approximately 0.39 and 0.14, respectively (Table 3, Figure 3 and 4). Sunset Park beach did not result in a significant correlation between Method 1609 and the defined substrate method (Enterolert), which had a r^2 value of 0.021 (Table 2, Figure 5). It is important to note that the only two beaches that

produced significant correlation coefficients (r^2 values) were the two beaches with the highest mean enterococci concentrations as measured by Enterolert (Table 1).

Comparisons of *E. coli* as measured by Method C and the defined substrate method (Colilert) were made. When qPCR results were plotted against the respective defined substrate methods, the following results are observed. All correlations were positive, though the exact degree varied greatly from beach to beach. Baileys Harbor Ridges and Fish Creek had no significant correlations between Method C and Colilert with r^2 values of 0.0015 and 0.0095, respectively (Table 3, Figures 6 and 7). Furthermore, Otumba had a significant correlation ($p < 0.05$, Table 3), with an r^2 value of 0.36 (Figure 8). Sunset Park beach had a significant correlation ($p < 0.05$) of 0.10 when using Method C and Colilert (Table 3, Figure 9). Unlike Method 1609, Otumba and Sunset beaches resulted in a significant correlation between Method C and Colilert, rather than for Fish Creek and Otumba.

The second objective of this study was to determine if Method 1609 or Method C correlated better to the defined substrate method than the alternative method. Using the information from above, it does not appear that either method correlated better to the defined substrate method than the other, with Method 1609 resulting in two significant correlations (Fish Creek and Otumba), while Method C resulted in only one significant correlation at Otumba (Table 3). These two beaches both had higher mean concentrations using the defined substrate methods, while the two beaches that did not result in any significant correlations between either qPCR both had low mean concentrations using the defined substrate method (Enterolert and Colilert). When mean

Enterolert concentrations were compared using an ANOVA, Fish Creek had significant differences ($p < 0.05$) when compared to Baileys Harbor and Sunset beaches, but not Otumba Beach (Table 4). While Otumba did have a significant correlation using Method 1609 and Enterolert, Otumba did not have any significant differences between its mean Enterolert concentration and the mean concentrations at any other beaches (Table 4). Mean *E. coli* concentrations as measured by Colilert followed the same pattern observed when using Enterolert to measure Enterococci concentrations, with Fish Creek being the only beach that has statistically different *E. coli* concentrations when compared to Baileys Harbor Ridges and Sunset beaches (Table 4). There was no statistical difference between Fish Creek and Otumba *E. coli* concentrations as measured by Colilert.

In order to determine how the use of each method would impact beach management decisions, results from each method (enterococci and *E. coli* defined substrate methods and qPCR methods) were compared with USEPA recommendations for single-sample allowable maximum-density guidelines of 1,000 CCE/100 mL for Method 1609 (Haugland et al. 2014). For Method C a single-sample allowable maximum-density threshold of 1,000 CCE/100 mL was used, as recommended currently by EPA. Single-sample allowable maximum densities for Enterolert and Colilert were set at 61 MPN/100 mL of water for enterococci and 235/100 mL of water for *E. coli* (Clesceri et al. 1998). Enterolert yielded the highest percentage of exceedances for all beaches in this study (Table 1) when compared to the percentage of exceedances obtained from using Colilert (Table 2). In all cases, qPCR based enumeration methods resulted in

highest percentages of exceedances, with a minimum increase of 10 percent and a maximum increase of 47 percent (Table 1 and Table 2).

Another important aspect to consider when comparing qPCR and defined substrate methods is the agreement between the two methods. Method 1609 tended to have a higher percent agreement with Enterolert when compared to Method C and Colilert (Table 1 and Table 2). Otumba beach had the lowest percent agreement when comparing Method 1609 and Enterolert, with a result of 48 percent agreement. Baileys Harbor, Sunset, and Fish Creek beaches all had higher percent agreements with values of 62, 69, and 71 percent agreement respectively (Table 1). Method C had lower percent agreement at all locations, except Otumba beach, which had an agreement of 53 percent when compared to Colilert (Table 2). Baileys Harbor, Fish Creek, and Sunset beaches had percent agreements of 42, 51, and 49 percent respectively (Table 2).

Finally, the traditional Colilert method used throughout the Great Lakes resulted in 22 beach closures at all beaches throughout the study period. Had these locations relied on Enterococci concentrations (Enterolert) for making regulatory decisions, there would have been 44 beach closures, which is a 200% increase in the total amount of beach closures. Furthermore, if qPCR based enumeration methods would have been utilized for management decisions, there would have been 77 closures using Method 1609, which is a 250% increase regulatory actions compared to Colilert. If Method C had been used as the regulatory method, there would have been 93 beach closures, which is an increase of 423% when compared to Colilert.

When considering adopting qPCR based enumeration methods in regulatory decisions at public swimming beaches it is important to consider the change in the number of false positive and false negatives (Figures 10-18). Method 1609 resulted in 0 false negatives at Baileys Harbor Ridges beach, but resulted in 14 false positives (Figure 10). Fish Creek beach had 5 false negatives, while only having 2 false positives (Figure 11). Otumba beach had the highest amount of false negatives using Method 1609, with 5, and the second lowest false positives with 9 (Figure 12). Furthermore, Sunset beach had a relatively low amount of false negatives, with only 2, but the second highest amount of false positives with 10 (Figure 13).

This pattern is not necessarily reflected when using Method C. Baileys Harbor Ridges beach had 18 false positives and only one false negative when using Method C as the regulatory method (Figure 14). Fish Creek beach had a similar number of false positives when compared to Baileys Harbor Ridges beach with 19 false positives and 7 false negatives (Figure 15). Otumba beach had the lowest number of false negatives using Method C, with 0, but had 22 false positives, as did Sunset beach (Figure 16 and 17). Sunset beach only had 1 false negative using Method C.

While many states use only one beach action value to make management decisions, Wisconsin utilizes a two-tiered beach monitoring system (WDNR, 2004). To evaluate a second tier beach action value, comparisons were made to the 1976 epidemiological studies from the NEEAR study to obtain a second tier beach action value of 1350 CCE. Using this value, all beaches saw a reduction in the number of advisories and dramatic increases in the number of closures, with a minimum decrease in advisories

of 3% and a maximum decrease of 8% when using the enterococci defined substrate method (Enterolert) and Method 1609 (Table 3). When using the enterococci defined substrate method and Method 1609, there also were dramatic increases in the rates of closures at all beaches, with a minimum increase of 17% and a maximum increase of 43% (Table 3). The *E. coli* defined substrate method (Colilert) and Method C had slightly different results when compared to enterococci methods. Both Baileys Harbor Ridges and Sunset beaches had increases in the number of advisories when using Method C as opposed to the defined substrate method (Colilert). These increases were of 2 and 3%, respectively for Baileys Harbor Ridges and Sunset beaches (Table 4). Both Fish Creek and Otumba beaches had decreases of 11 and 7%, respectively, in the number of advisories when going from the defined substrate method to Method C (Table 4). All beaches had dramatic increases in the number of closures when utilizing Method C over the defined substrate method. These increases ranged from a minimum increase of 38% for Baileys Harbor Ridges beach and a maximum increase of 51% for Otumba beach (Table 4).

Table 1. Mean concentrations of *Enterococcus spp.* and percent of beach water samples that exceeded the maximum allowable organism concentration at each beach based upon EntTaq (*Enterococcus* qPCR, CCE) and Enterolert (*Enterococcus* MPN).

Site	EntTaq (CCE/100 mL)		Enterolert (MPN/100 mL)		Comparison
	Mean Conc.	Percent exceedances (n=)	Mean Conc.	Percent exceedances (n=)	Percent Agreement
Baileys Harbor	7668.8	51 (37)	27.8	14 (37)	62
Fish Creek	4871.8	66 (41)	264.2	51 (41)	71
Otumba	21902.3	45 (40)	139.3	35 (40)	48
Sunset	3611.3	33 (39)	39.2	13 (39)	69

Table 2. Mean concentrations of *E. coli* and percent of beach water samples that exceeded the maximum allowable organism concentration at each beach based upon *E.coli*Taq (*E.coli* qPCR, CCE) and Colilert (*E. coli* MPN).

Site	<i>E.coli</i> Taq (CCE/100 mL)		Colilert (MPN/100 mL)		Comparison
	Mean Conc.	Percent exceedances (n=)	Mean Conc.	Percent exceedances (n=)	Percent Agreement
Baileys Harbor	2124.4	46 (41)	71.5	5 (37)	51
Fish Creek	9297.9	51 (45)	344.6	24 (45)	42
Otumba	18599.1	62 (45)	224.9	18 (45)	53
Sunset	3655.3	51 (45)	90.3	4 (45)	49

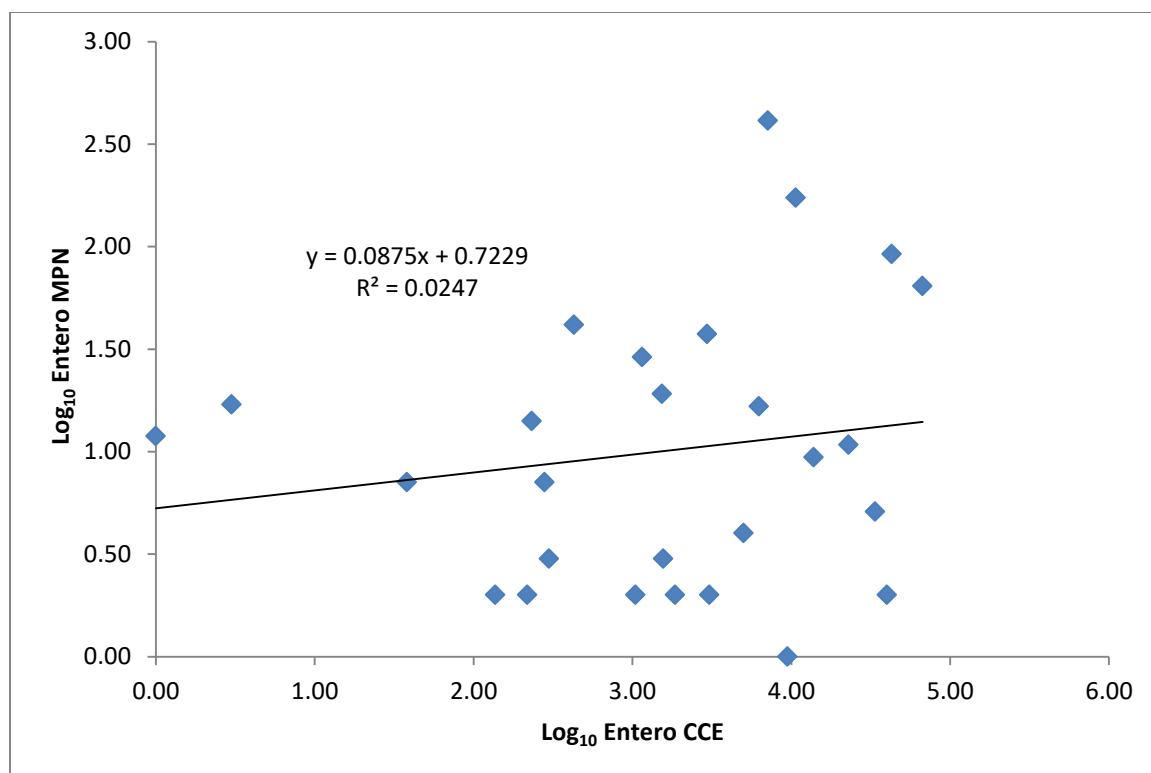


Figure 2. Correlation between Log_{10} enterococci concentrations using Method 1609 and Enterolert at Baileys Harbor Ridges beach.

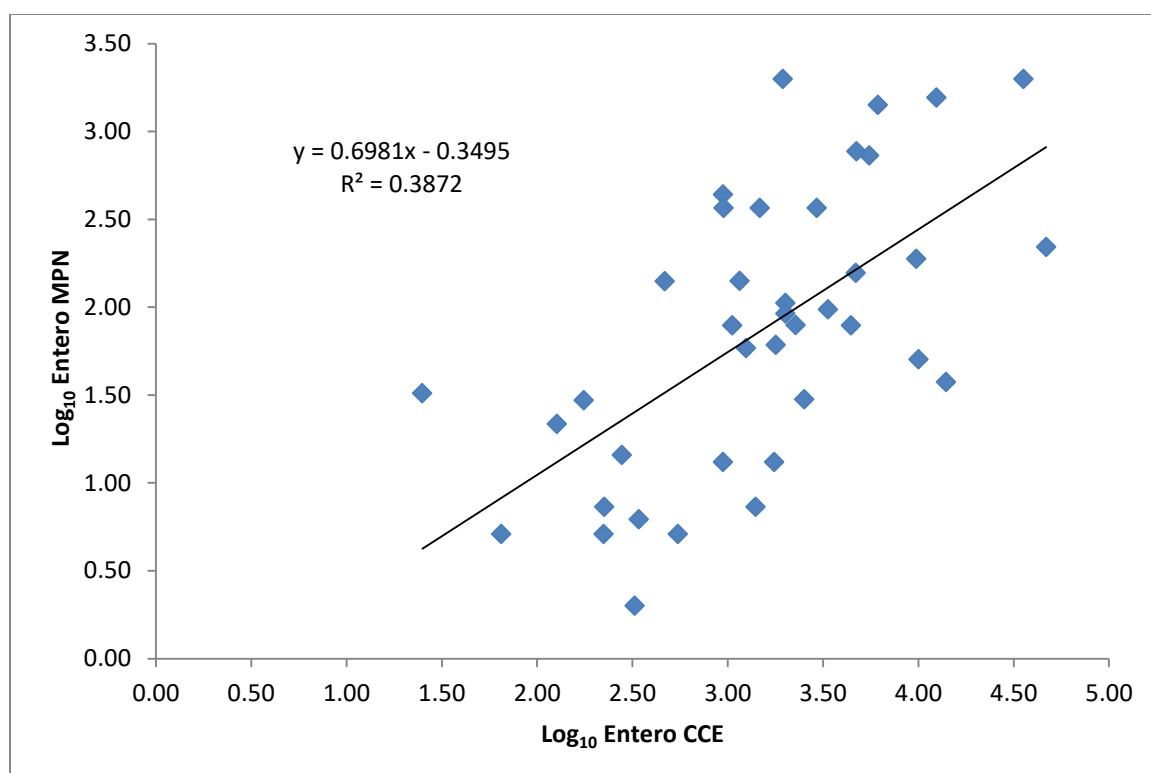


Figure 3. Correlation between Log_{10} enterococci concentrations using Method 1609 and Enterolert at Fish Creek beach.

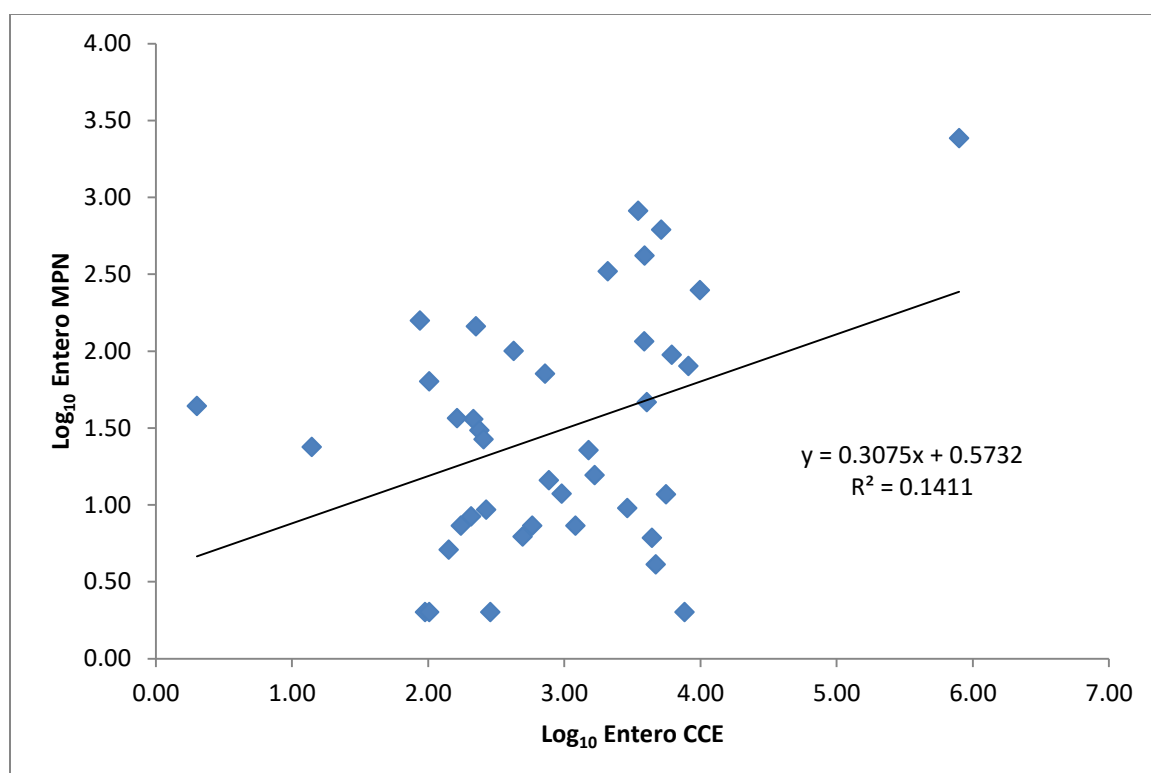


Figure 4. Correlation between Log_{10} enterococci concentrations using Method 1609 and Enterolert at Otumba beach.

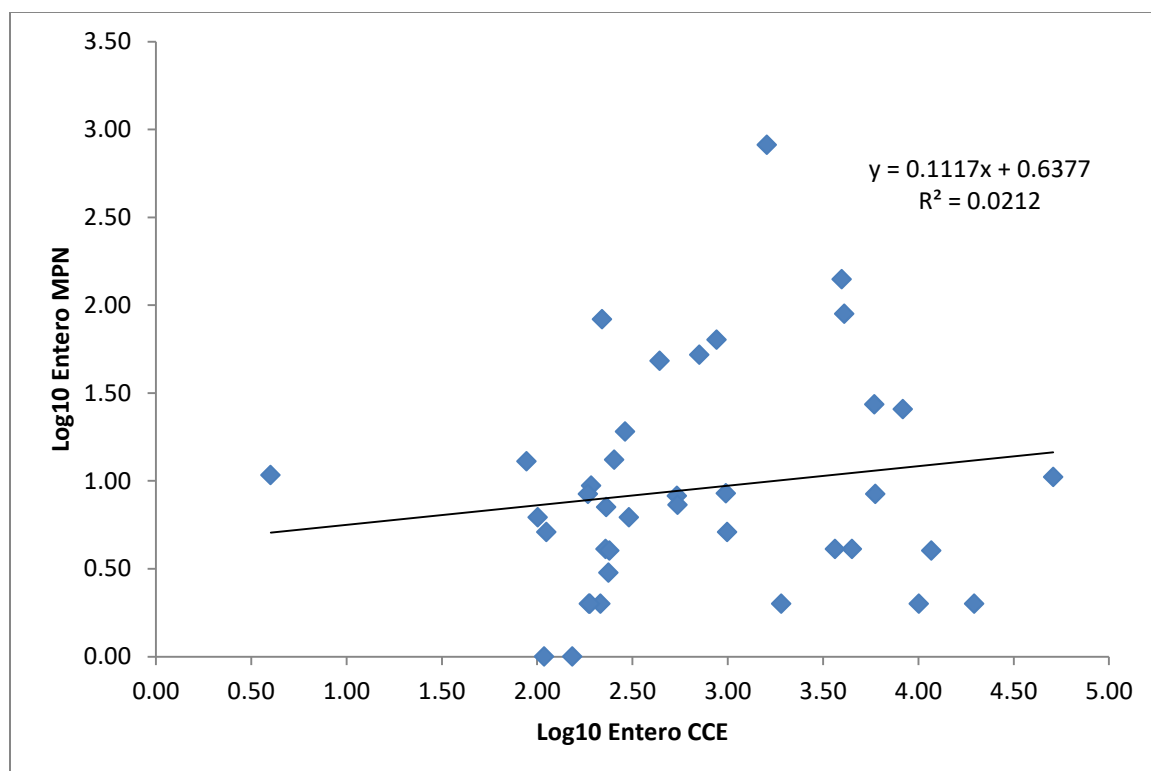


Figure 5. Correlation between Log₁₀ enterococci concentrations using Method 1609 and Enterolert at Sunset beach.

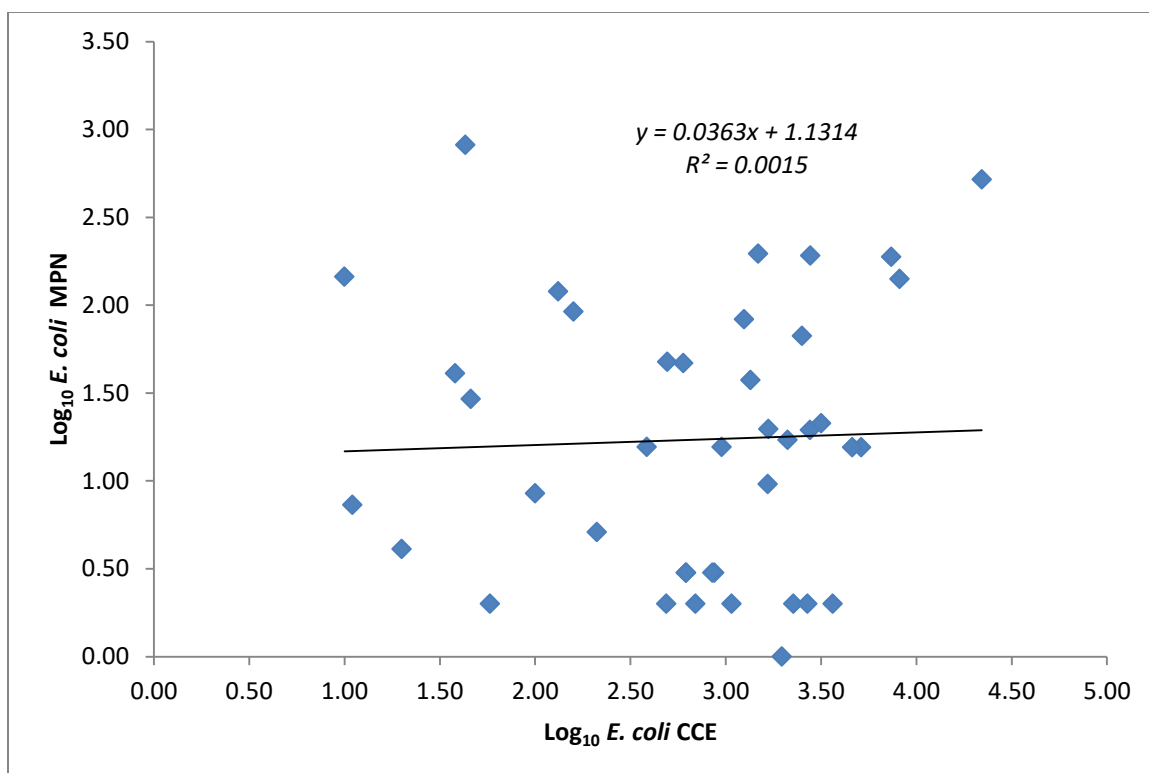


Figure 6. Correlation between $\text{Log}_{10} E. coli$ concentrations using Method C and Colilert at Baileys Harbor Ridges beach.

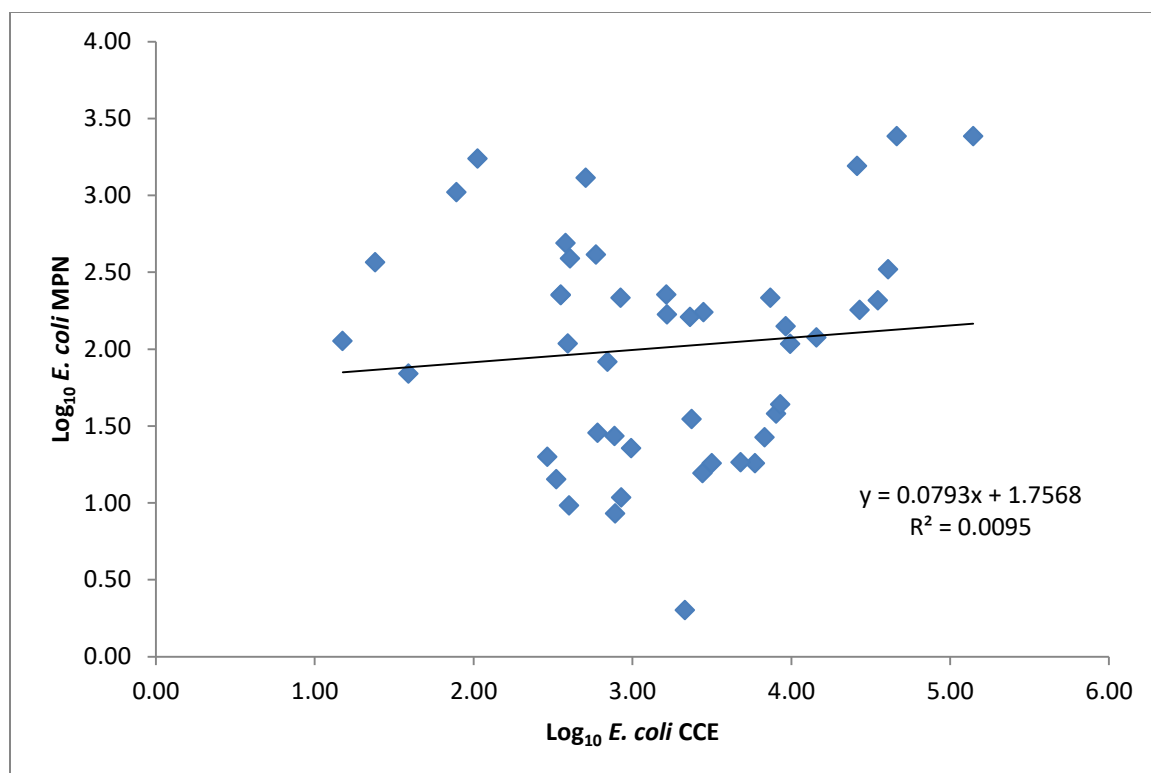


Figure 7. Correlation between $\text{Log}_{10} E. coli$ concentrations using Method C and Colilert at Fish Creek beach.

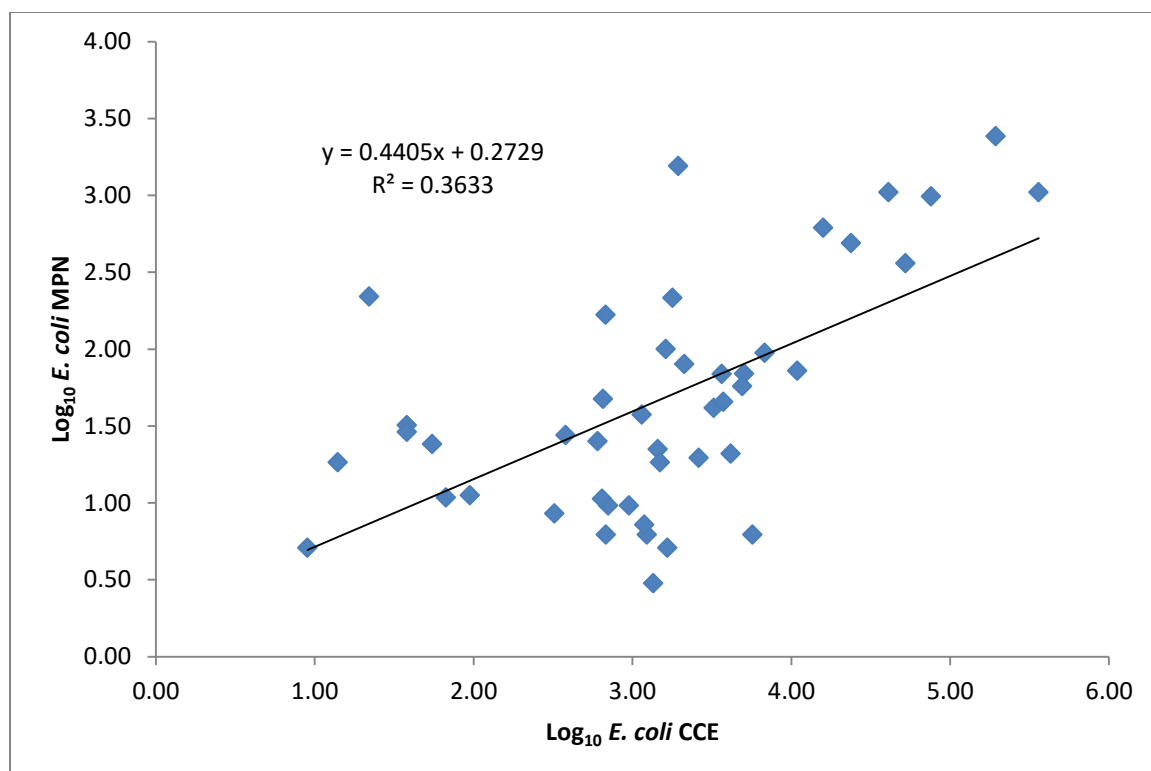


Figure 8. Correlation between Log_{10} *E. coli* concentrations using Method C and Colilert at Otumba beach.

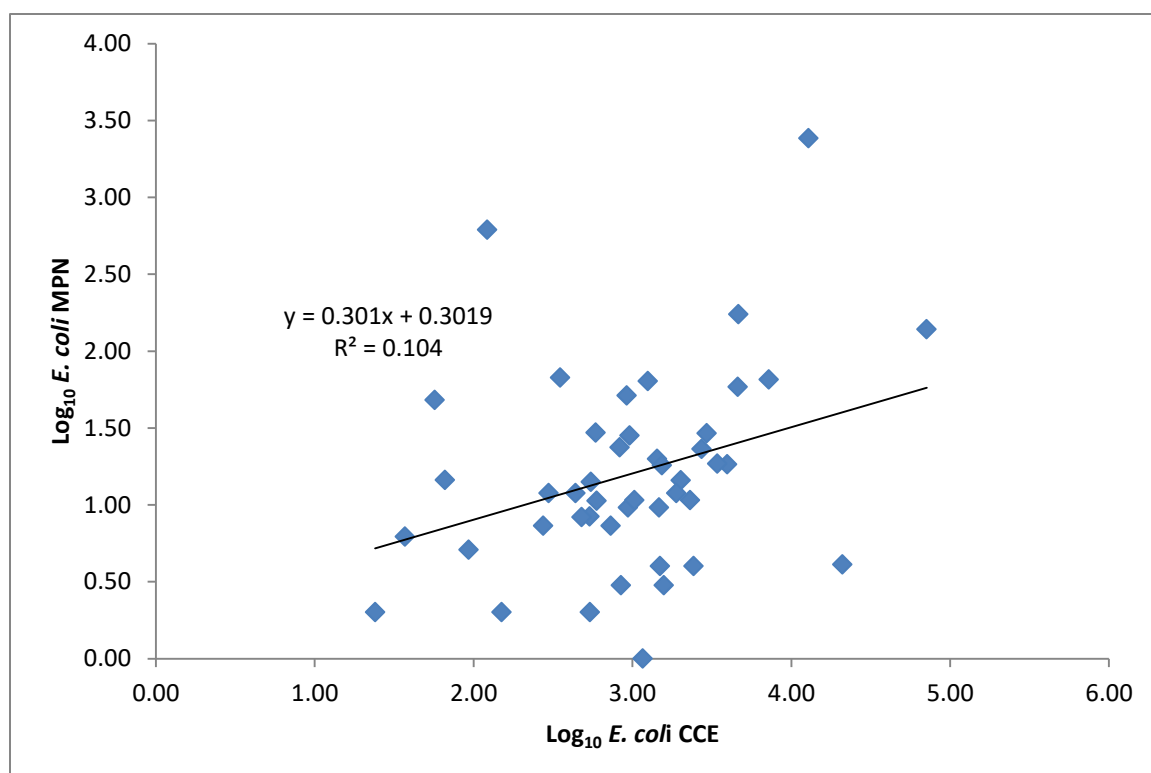


Figure 9. Correlation between $\text{Log}_{10} E. coli$ concentrations using Method C and Colilert at Sunset beach.

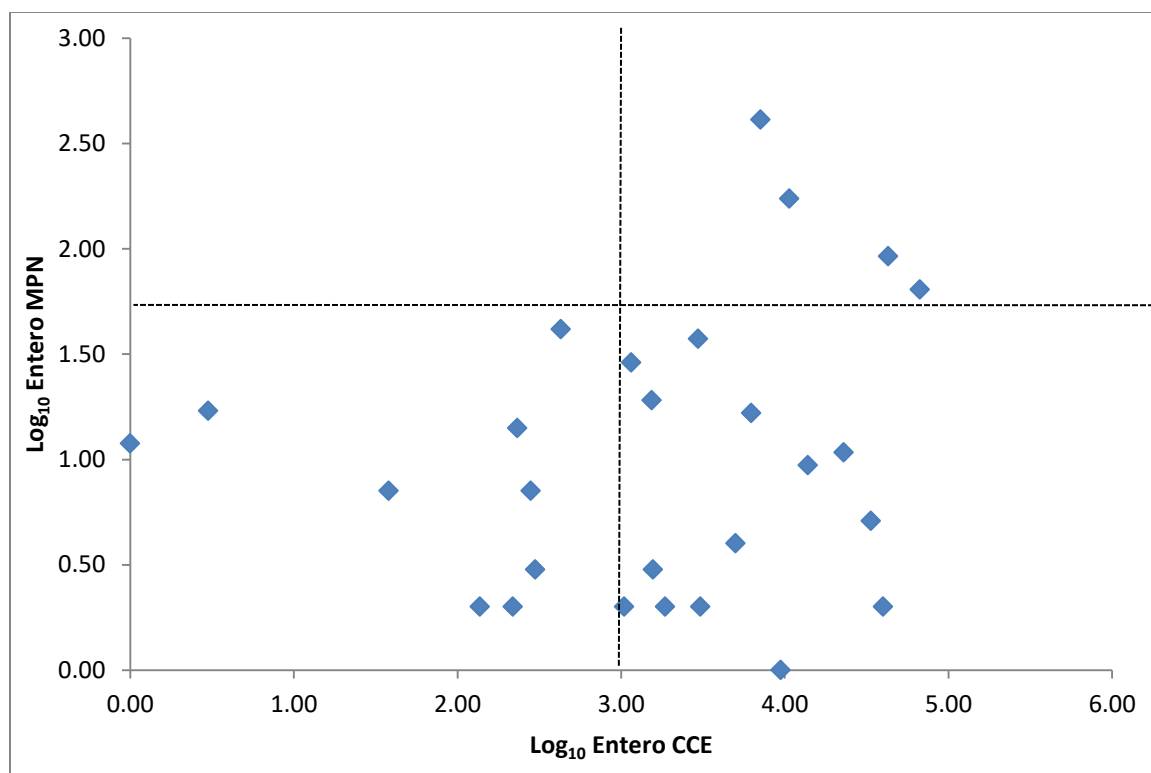


Figure 10. Comparison of culture-based enterococci concentrations (MPN/100 mL) and qPCR enterococci concentrations (CCE/100 mL at) at Baileys Harbor Ridges beach. Dashed lines indicate threshold values for closures using the method on that axis (Method 1609 and Enterolert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

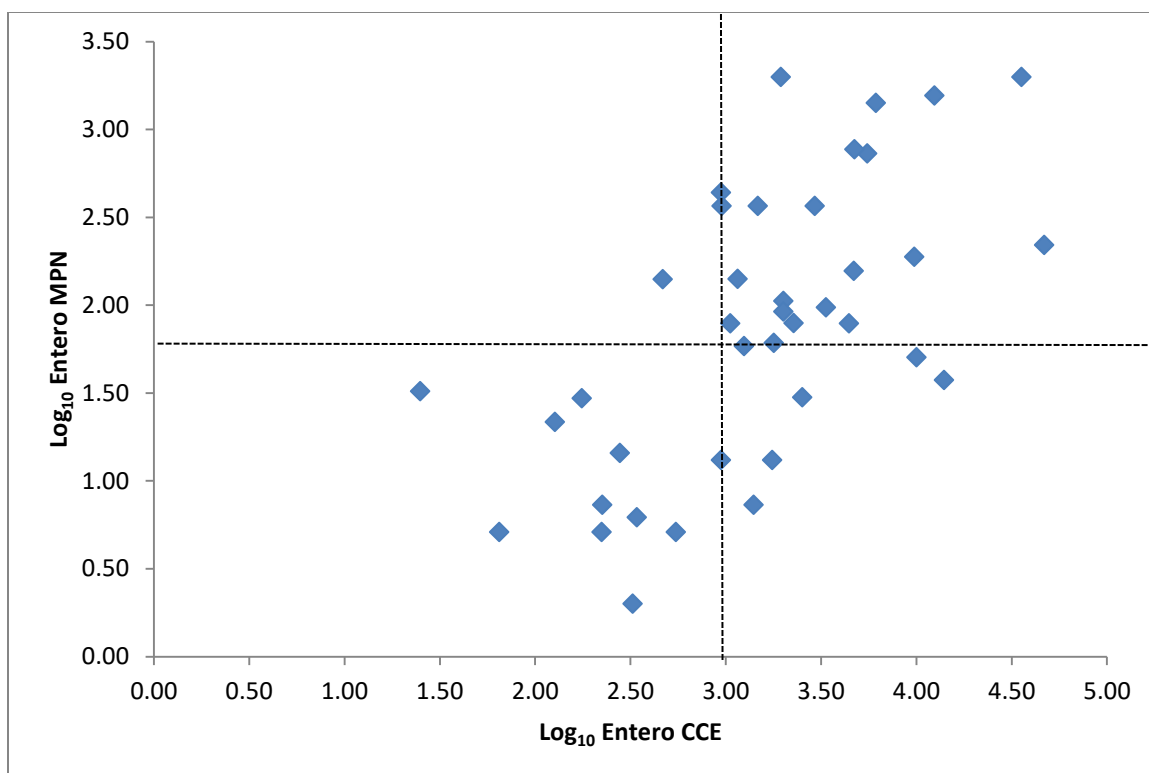


Figure 11. Comparison of culture-based enterococci concentrations (MPN/100 mL) and qPCR Enterococci concentrations (CCE/100 mL) at Fish Creek beach. Dashed lines indicate threshold values for closures using the method on that axis (Method 1609 and Enterolert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

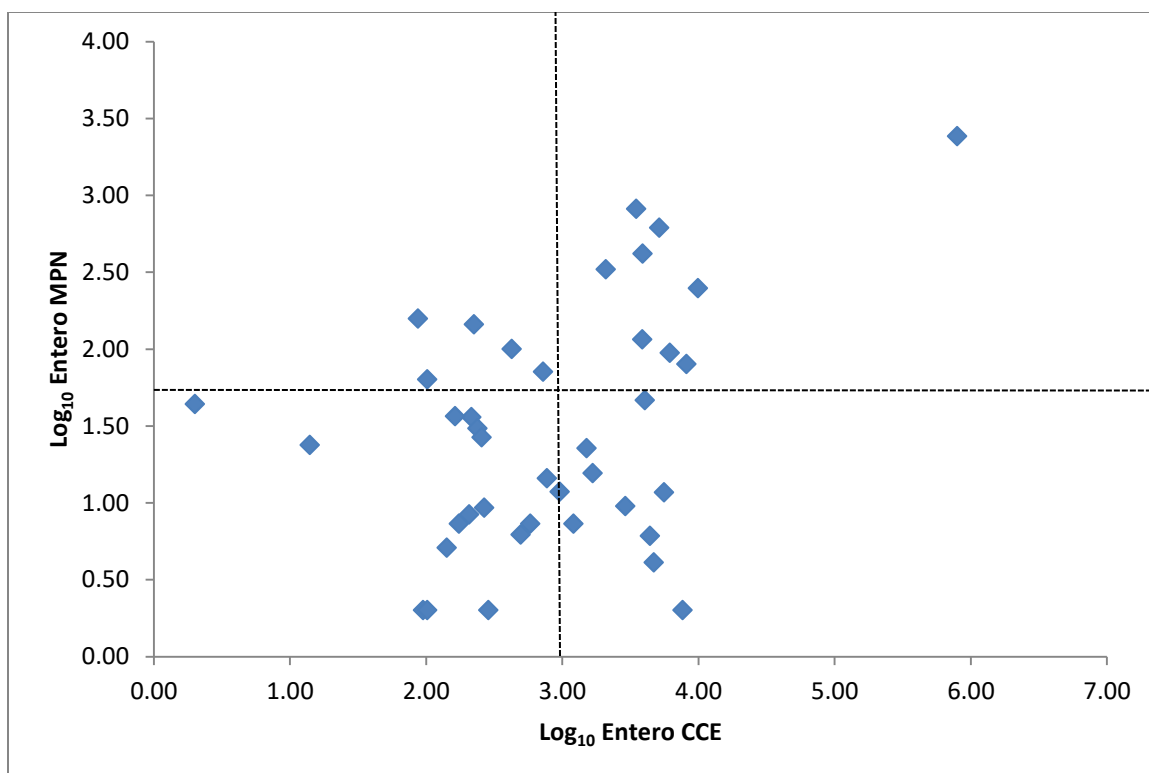


Figure 12. Comparison of culture-based enterococci concentrations (MPN/100 mL) and qPCR Enterococci concentrations (CCE/100 mL) at Otumba beach. Dashed lines indicate threshold values for closures using the method on that axis (Method 1609 and Enterolert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

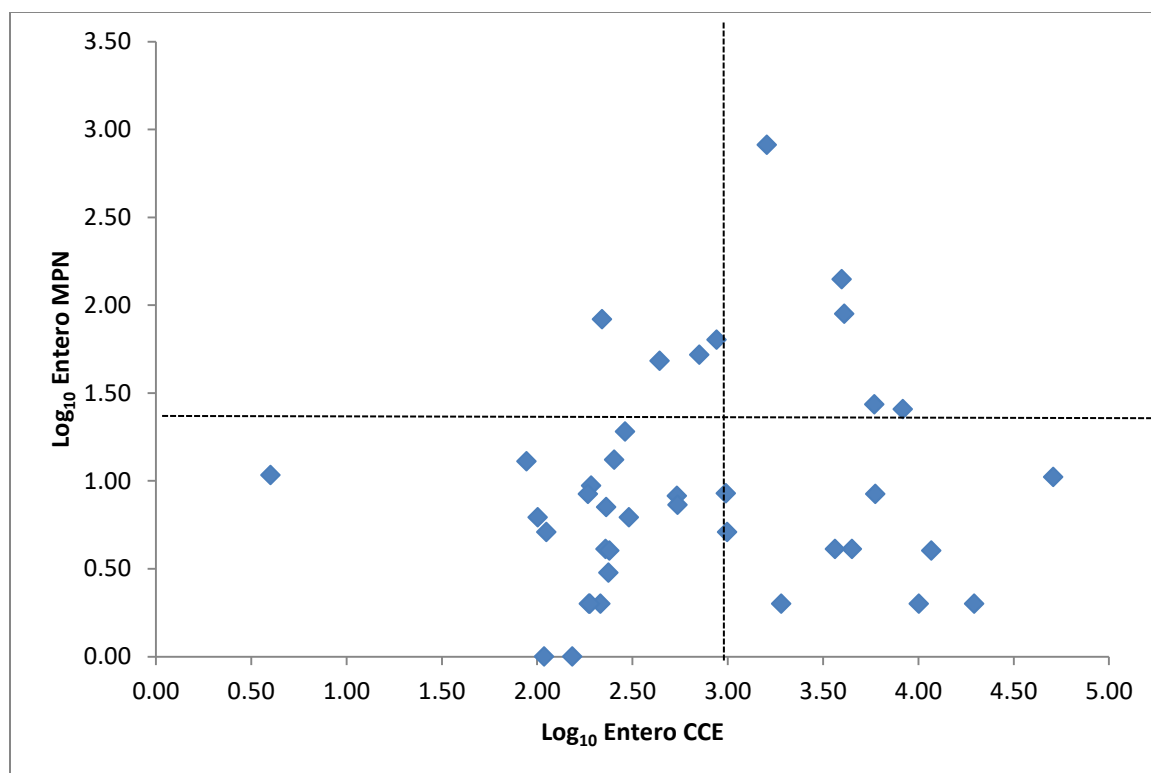


Figure 13. Comparison of culture-based enterococci concentrations (MPN/100 mL) and qPCR enterococci concentrations (CCE/100 mL) at Sunset beach. Dashed lines indicate threshold values for closures using the method on that axis (Method 1609 and Enterolert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

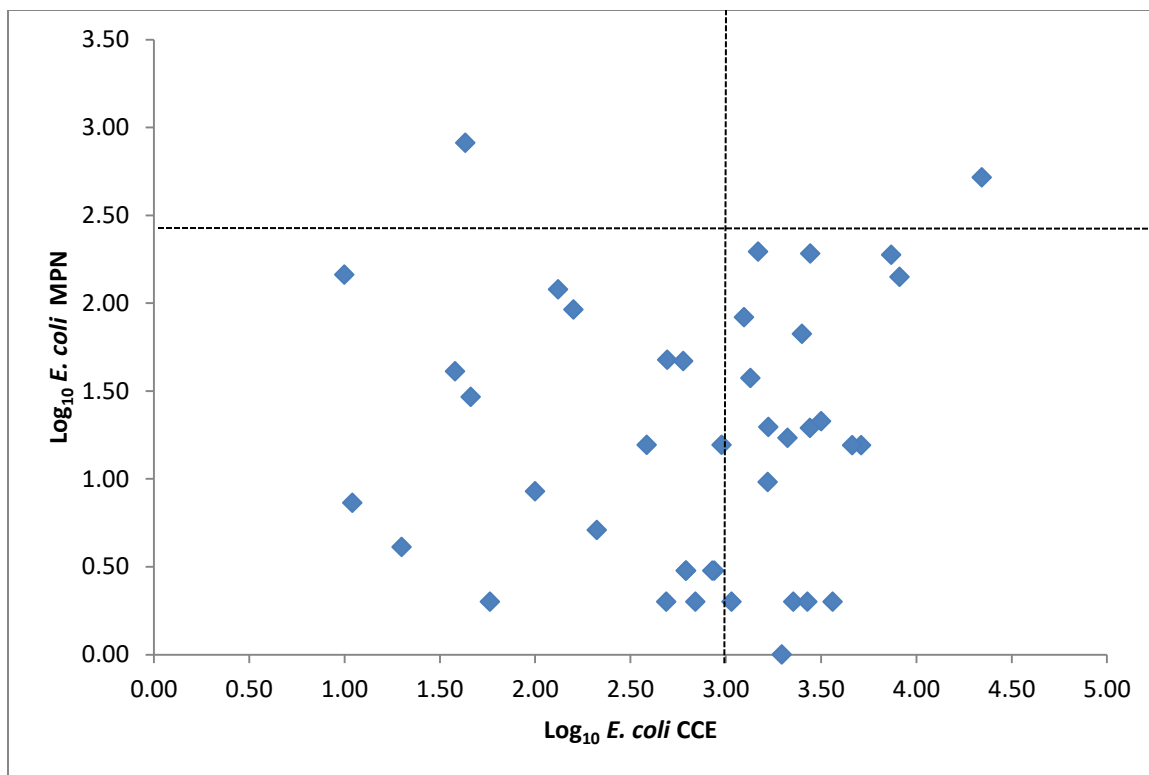


Figure 14. Comparison of culture-based *E. coli* concentrations (MPN/100 mL) and qPCR *E. coli* concentrations (CCE/100 mL) at Baileys Harbor Ridges beach. Dashed lines indicate threshold values for closures using the method on that axis (Method C and Colilert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

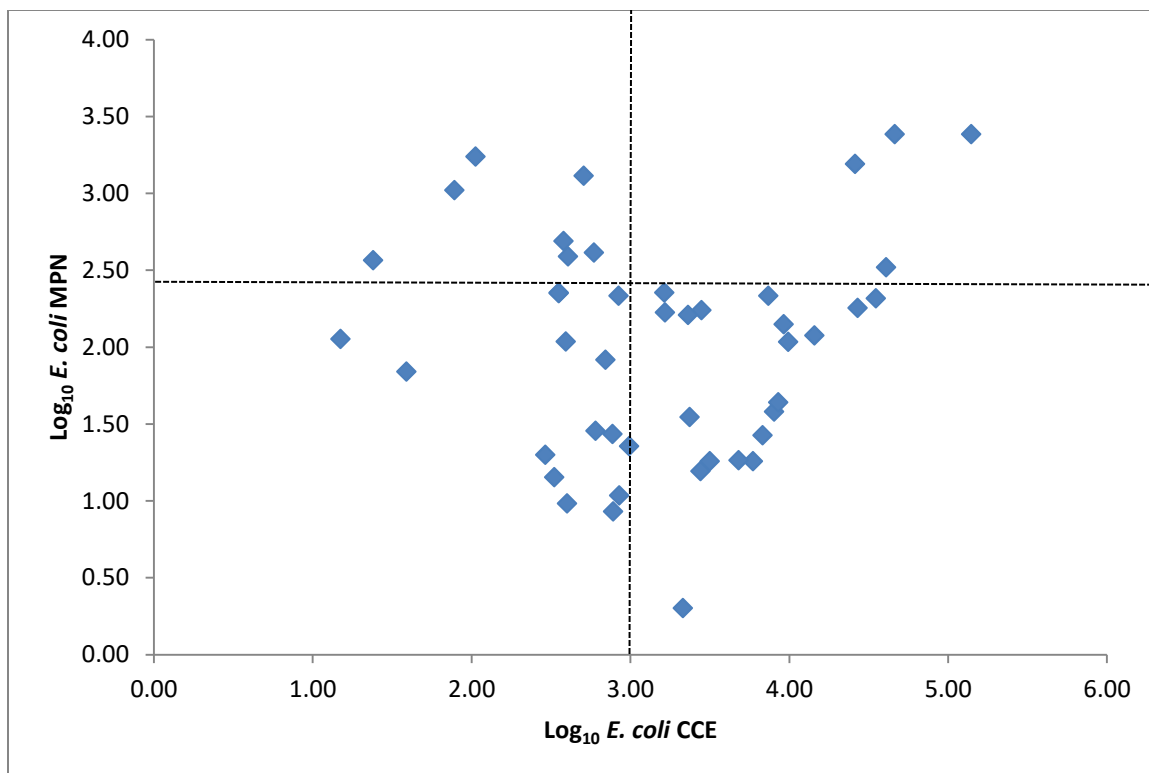


Figure 15. Comparison of culture-based *E. coli* concentrations (MPN/100 mL) and qPCR *E. coli* concentrations (CCE/100 mL) at Fish Creek beach. Dashed lines indicate threshold values for closures using the method on that axis (Method C and Colilert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

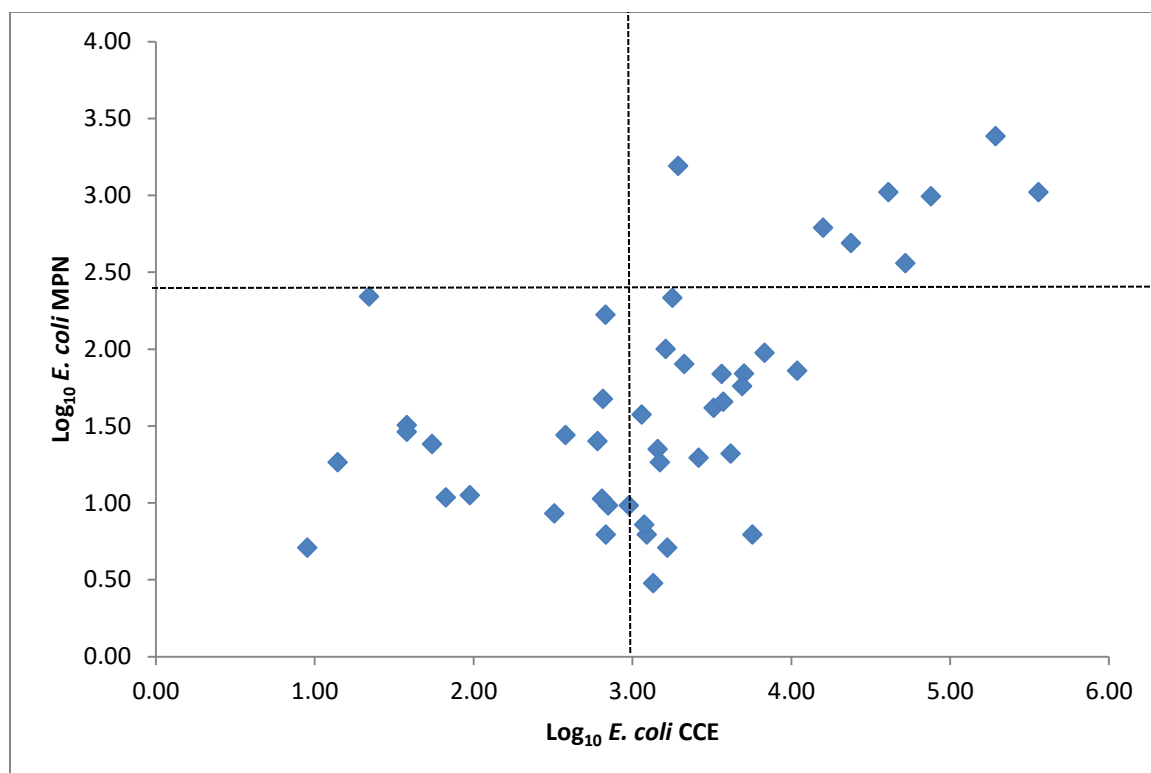


Figure 16. Comparison of culture-based *E. coli* concentrations (MPN/100 mL) and qPCR *E. coli* concentrations (CCE/100 mL) at Otumba beach. Dashed lines indicate threshold values for closures using the method on that axis (Method C and Colilert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

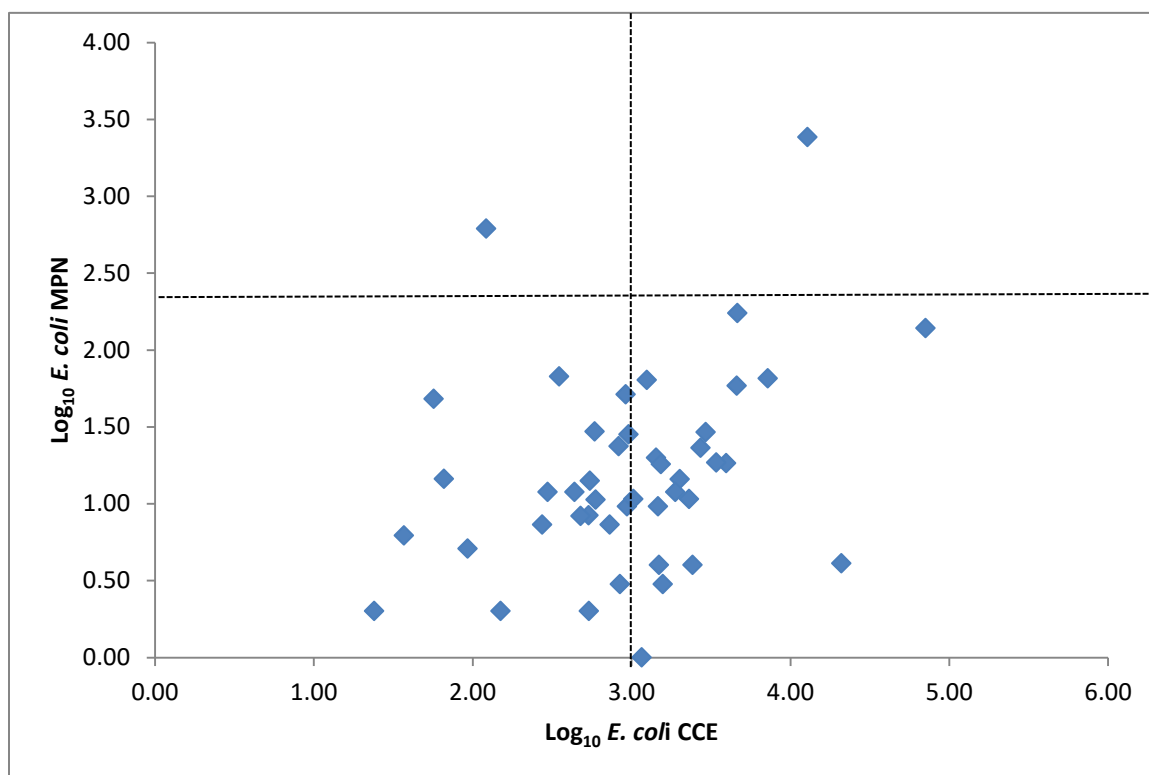


Figure 17. Comparison of culture-based *E. coli* concentrations (MPN/100 mL) and qPCR *E. coli* concentrations (CCE/100 mL) at Sunset beach. Dashed lines indicate threshold values for closures using the method on that axis (Method C and Colilert), showing amount of true positives, true negatives (top right and bottom left respectively) and false positives and false negatives (bottom right and top left).

Table 3. Differentiation between closures and advisories using Method 1609 and Enterolert.

Site	EntTaq (CCE/100 mL)			Enterolert (MPN/100 mL)		
	Percent exceedances (n=)	Percent Advisories (n=)	Percent Closures (n=)	Percent exceedances (n=)	Percent Advisories (n=)	Percent Closures (n=)
Baileys Harbor	51 (37)	5 (2)	46 (17)	14 (37)	11 (4)	3 (1)
Fish Creek	66 (41)	7 (3)	59 (24)	51 (41)	10 (4)	41 (17)
Otumba	45 (40)	3 (1)	42 (17)	35 (40)	10 (4)	25 (10)
Sunset	33 (39)	0 (0)	33 (13)	13 (39)	8 (3)	5 (2)

Table 4. Differentiation between closures and advisories using Method C and Colilert.

Site	<i>E.coli</i> Taq (CCE/100 mL)			Colilert (MPN/100 mL)		
	Percent exceedances (n=)	Percent Advisories (n=)	Percent Closures (n=)	Percent exceedances (n=)	Percent Advisories (n=)	Percent Closures (n=)
Baileys Harbor	46 (41)	7 (3)	39 (16)	5 (37)	5 (2)	0 (0)
Fish Creek	51 (45)	0 (0)	51 (23)	24 (45)	11 (5)	13 (6)
Otumba	62 (45)	2 (1)	60 (27)	18 (45)	9 (4)	9 (4)
Sunset	49 (45)	7 (3)	42 (19)	4 (45)	2 (1)	2 (1)

DISCUSSION

The overarching objectives of this study were to determine the relationships between enterococci concentrations determined by Method 1609 and Enterolert, and the relationships between *E. coli* concentrations determined by Method C and Colilert. Correlations between qPCR and traditional culture-based methods were generally low in this study, which is consistent with previous work conducted in the Door County area (Sheth et al., 2016). The correlations between Method 1609 and Enterolert at Fish Creek Beach and Method C and Colilert at Otumba Beach were substantially different from all other comparisons made between qPCR based enumeration methods and the appropriate defined substrate method. Fish Creek beach had a correlation of 0.387 between Method 1609 and the defined substrate method (Enterolert), while Otumba beach had a correlation of 0.363 between Method C and the defined substrate method (Colilert). These correlations are consistent with previous studies completed in the Door County area (Sheth et al., 2016). Correlation between molecular and culture methods at all other beaches were substantially less.

One factor that may be impacting these relationships at Otumba and Fish Creek is the outfall pipes that discharge at the beaches, resulting in higher mean concentrations of FIB at both beaches, compared to others in the study (Table 1 and 2). These outfall pipes likely contribute both viable and non-viable organisms to the near-shore waters, but the relationship between viable and non-viable, but genetically intact microbes, is not well understood. However, due to the rather high correlations at these particular beaches, it is

possible that a relatively large proportion of the DNA found in the qPCR testing comes from viable bacteria rather than partial DNA fragments or non-viable bacteria.

Baileys Harbor Ridges and Sunset beaches both have relatively low mean concentrations of enterococci and *E. coli* using the defined substrate methods and poor correlations between FIB concentrations detected by molecular vs. culture techniques. Due to the low mean concentration of FIB using the defined substrate methods at these beaches, a large amount of the DNA obtained from water samples collected at Baileys Harbor Ridges and Sunset beaches likely can be attributed to non-viable cells or free-floating DNA fragments. Thus, the ratio of viable microorganisms to amplifiable DNA fragments is likely a major factor in the relationship between molecular and culture-based methods. Additionally, the low mean concentrations at some locations will hinder the statistical resolving power of the correlations, as too many of the viable concentrations are at the very low range of detection.

The low level of FIB concentrations in some locations could be due to remediation projects designed to reduce the amount of fecal pathogens present. All beaches in the study, except Fish Creek beach, have undergone remediation. The remediation or disinfection at a wastewater plant targets viable organisms and it is likely that these treatment systems may result in discharge of non-viable cell parts or bacterial DNA that may be detected by qPCR testing. Baileys Harbor Ridges and Sunset beaches have undergone remediation and do not have outfalls, resulting in the relatively low concentrations of FIB as enumerated by the defined substrate methods. Otumba beach, like Fish Creek, has direct stormwater discharge causing high correlations and is

coincidentally located the closest to the Sturgeon Bay wastewater treatment plant. While the stormwater discharge is a likely a source of viable FIB, the proximity to the Sturgeon Bay wastewater treatment plant is likely a source of nonviable FIB, which could cause lower correlations observed at Otumba beach when compared to Fish Creek. It is unlikely however, that Otumba beach is largely impacted by non-viable FIB from the Sturgeon Bay wastewater treatment plant. If it was, lower correlations would be expected at Otumba beach than what was observed.

Further, Fish Creek beach is the only beach that has statistically different mean FIB concentrations from Baileys Harbor and Sunset beaches. Again, this is most likely due to the presence of the outfall and lack of previous remediation. The outfall pipe could be acting as a direct route to the beach for stormwater with high concentrations of FIB. If Fish Creek beach were to be remediated, there may be a reduction in FIB, with concentrations being similar to those observed at Otumba beach. Otumba beach is a remediated beach with an outfall, causing higher concentrations using the defined substrate methods, but not high enough to be statistically different from Baileys Harbor and Sunset beaches, as the stormwater is filtered for FIB before it reaches nearshore waters at the beach.

The second objective of this study was to compare the specific relationship of Method 1609 and Method C with their respective defined substrate in terms of beach monitoring criteria. Data from this study suggest that neither method correlates with its respective defined substrate method better than the other. Method 1609, however, tended to have a higher percent agreement with the Enterolert than did Method C with Colilert.

Method 1609 had a minimum percent agreement with Enterolert of 48% at Otumba beach and a maximum percent agreement of 71% at Fish Creek beach (Table 1). When examining all beaches collectively, Method C had a minimum percent agreement of 42% at Fish Creek beach and a maximum percent agreement of 53% at Otumba beach (Table 2).

This difference in percent agreements may potentially be explained by the addition of the internal amplification control (IAC) assay, which aids in detecting potential inhibition. The IAC assay is present in Method 1609 and absent in Method C. These percent agreements call into question whether the preferred freshwater indicator, *E. coli*, is the best indicator to be used if qPCR based enumeration methods are implemented in the region, as these percent correlations tend to be lower than other percent agreements published on Method 1609 (Haugland et al. 2014). The only location that had a higher percent agreement using Method C was Otumba beach, which only had a 5 percent difference between Method 1609 and Method C. These data also may indicate that no one method may be applied unilaterally across a geographical region and each beach location may require a different qPCR method to best correlate with traditional culture based methods.

Lastly, this study helps one understand how rapid molecular-based enumeration methods, such as qPCR, will impact beach management decisions. This study shows that, when compared to defined substrate methods, either qPCR method would result in higher rates of beach closures with a minimum increase of 10% and maximum increase of 47% (Table 1 and Table 2). Specifically, qPCR based enumeration methods, in most

cases, reduce the number of advisories and dramatically increase the number of closures when using 1,000 CCE and 1,350 CCE as advisory and closure beach action value, respectively. That is, the numbers using qPCR are much higher than the closure values would be and very rarely fall within the range between advisory and closure. This indicates that qPCR based enumeration methods are likely more protective of public health than the traditional defined substrate methods, but could be detecting dead or non-viable cells, along with partial DNA fragments. All of these sources of DNA could be causing qPCR results to be much higher than what one would expect from viable organisms at the beach. Exactly how each qPCR method relates to its respective defined substrate method also tends to vary by location, as each method had two beaches where it was considered “more protective” of public health, further complicating the use of qPCR based methods in regulatory decision making procedures. When considering rapid detection methods, it may be more beneficial to consider the overall percent agreement between the methods to maximize the protection of public health and minimize unnecessary closures.

While it is clear that the deployment of rapid detection methods may better protect public health, it is not clear if they close beaches unnecessarily. qPCR methods still need to be evaluated on a site-by-site basis in order to determine what the impacts may be on management. Additional studies on qPCR and their direct relationship to pathogens in non-human impacted waters is also desirable. This site-by-site evaluation may suggest re-evaluating the current preferred indicator (*E. coli*) used in the Great Lakes Region, as enterococci may prove to be more protective of public health than *E. coli*,

while minimizing unnecessary beach closures. While the predictive ability of molecular methods rivals those of traditional culture methods, qPCR methods may be site specific in their implementation as it is clear that these rapid detection methods have the ability to dramatically alter beach management, potentially leading to unwanted socioeconomic ramifications from excessive beach closures.

FUTURE WORK

In order to better understand qPCR and how it relates to the detection of fecal pathogens in recreational water the following work is recommended to be completed.

- Examine the relationships between qPCR methods and viable fecal indicator bacteria. Are qPCR methods more sensitive to method specific variability or are the increases in beach closures due to the presence of actual bacteria?
- Examine the fate and survival of DNA fragments in aquatic environments. How long does DNA survive outside a cell when exposed to the weather and water conditions?
- Examine the differences between method correlation and location. What environmental conditions control which qPCR method is going to correlate better at each location?
- Examine the impact of stormwater treatment on viable and non-viable indicators of fecal pollution and the relationship between these indicator types.

APPENDIX

Other Correlations Made Between qPCR Methods and Defined Substrate Methods

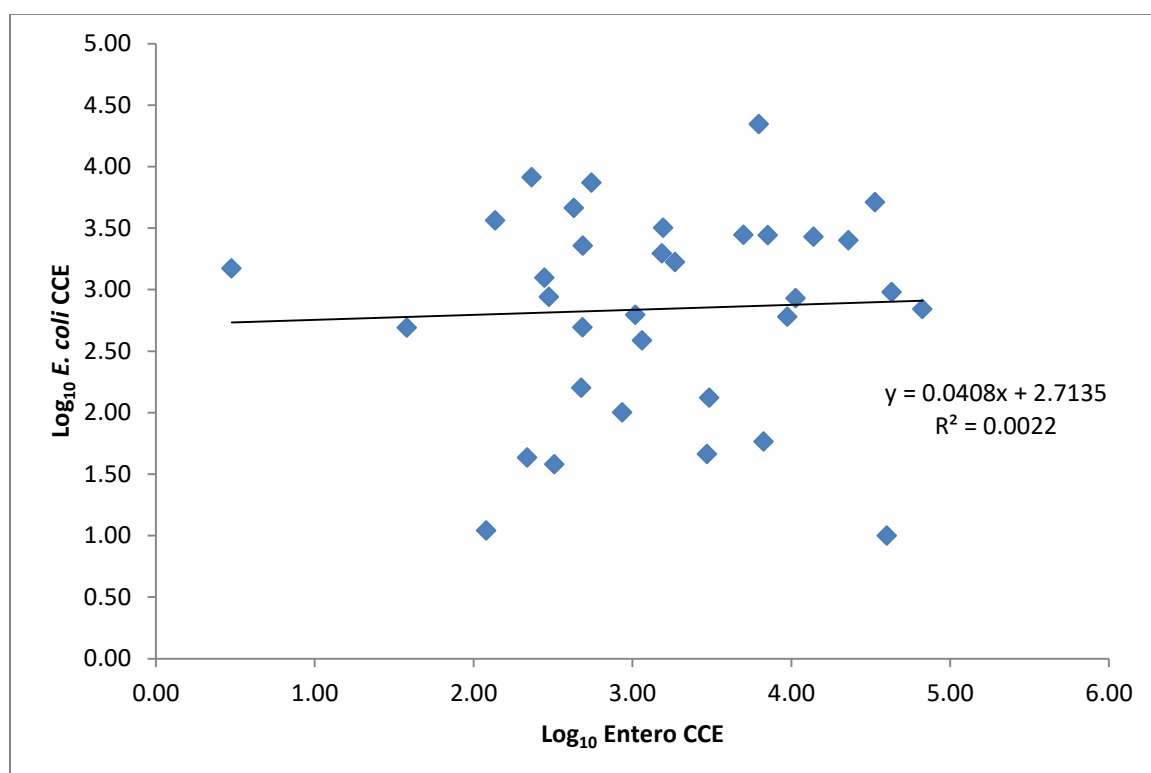


Figure A1. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Method 1609 and Method C at Baileys Harbor Ridges beach.

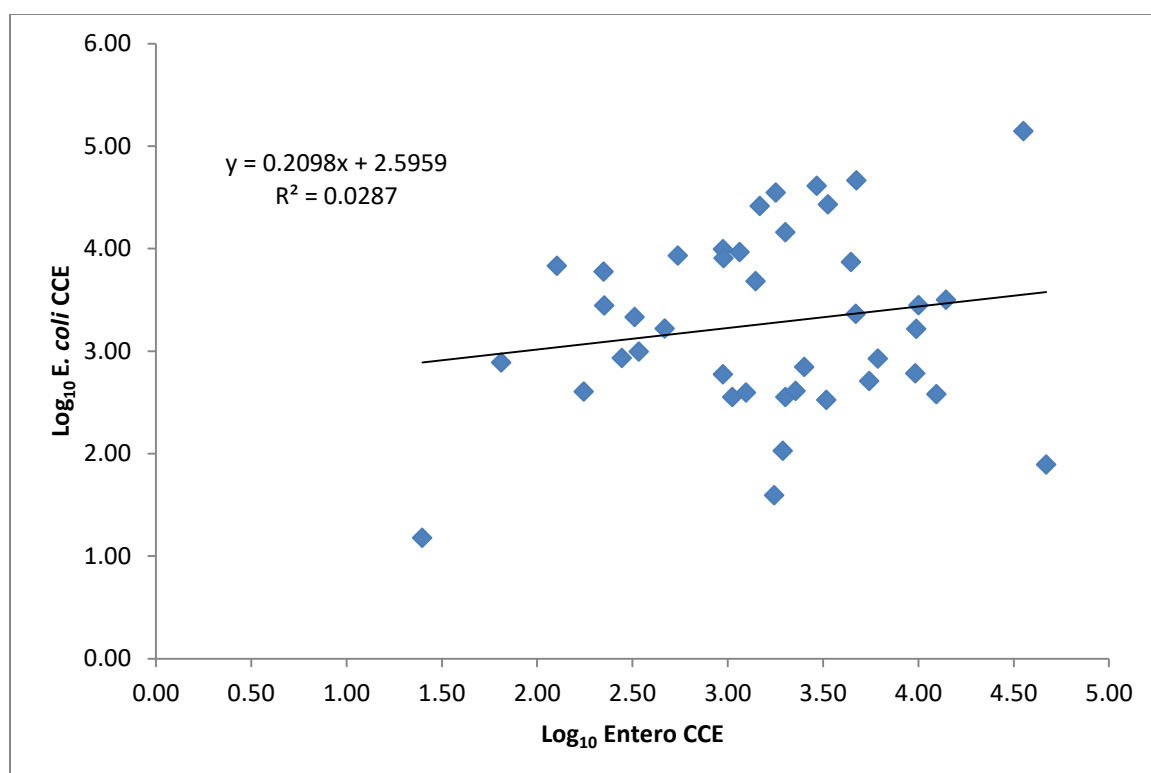


Figure A2. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Method 1609 and Method C at Fish Creek beach.

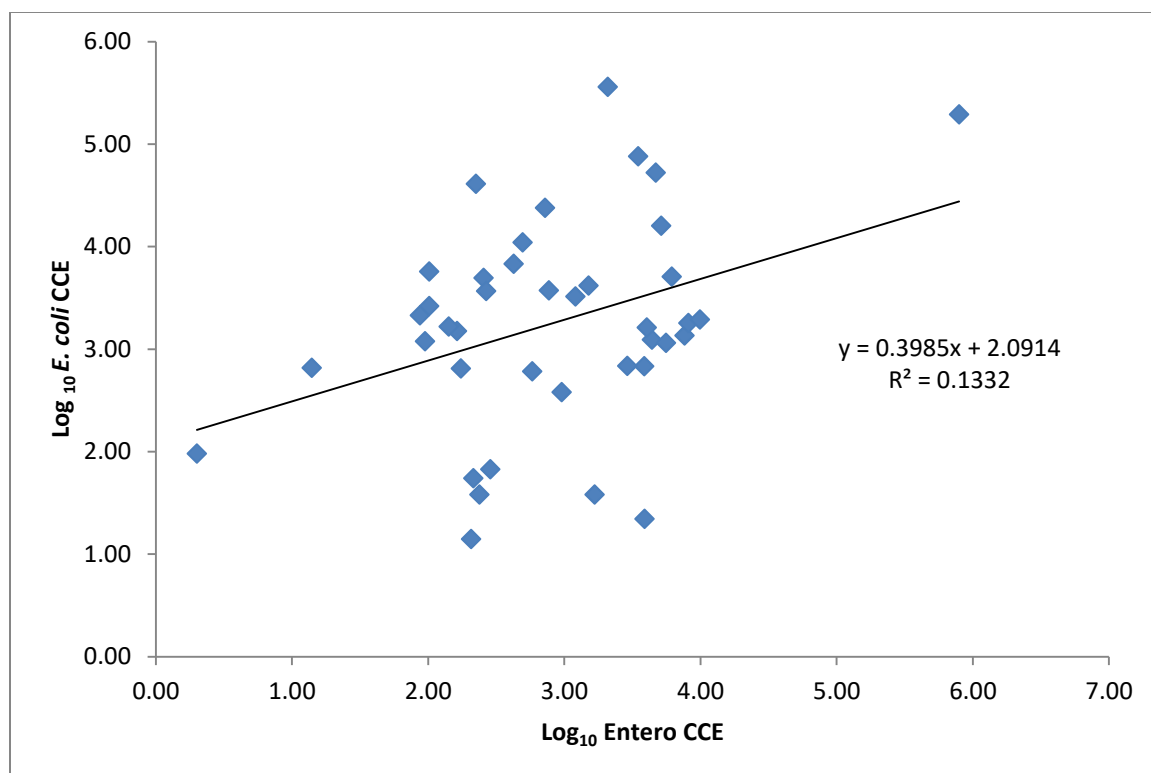


Figure A3. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Method 1609 and Method C at Otumba beach.

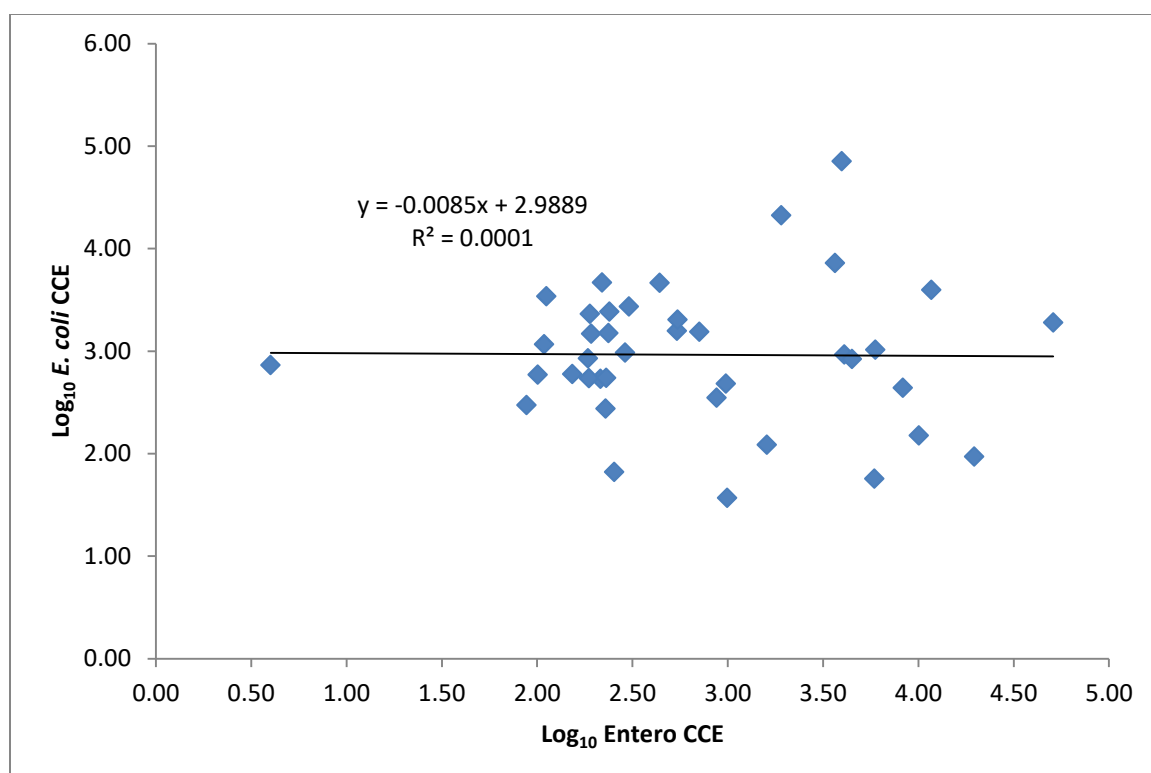


Figure A4. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Method 1609 and Method C at Sunset beach.

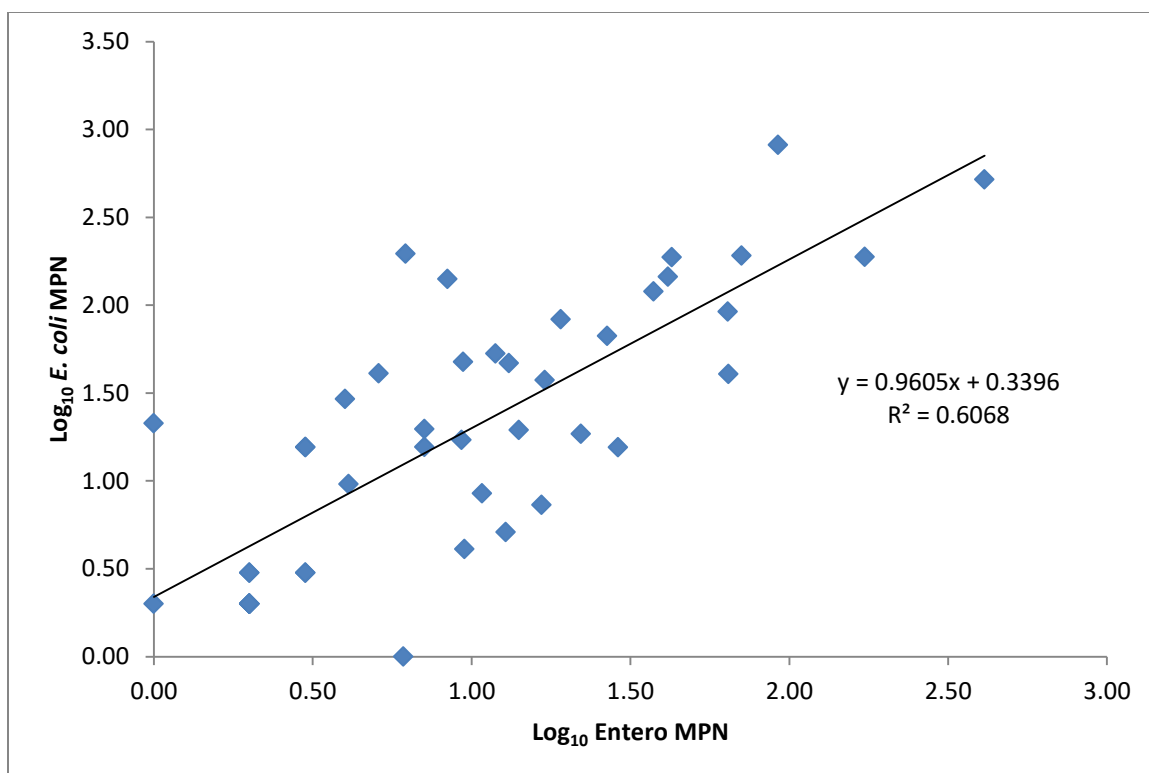


Figure A5. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Enterolert and Colilert at Baileys Harbor Ridges beach.

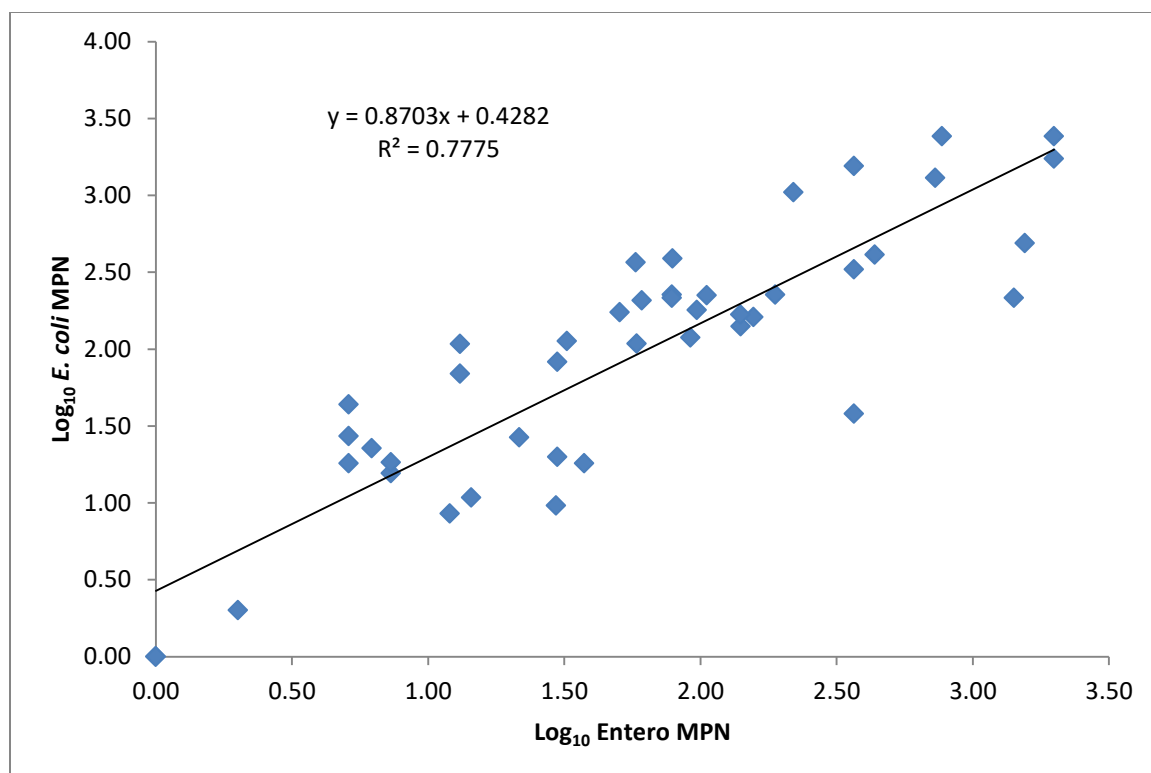


Figure A6. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Enterolert and Colilert at Fish Creek beach.

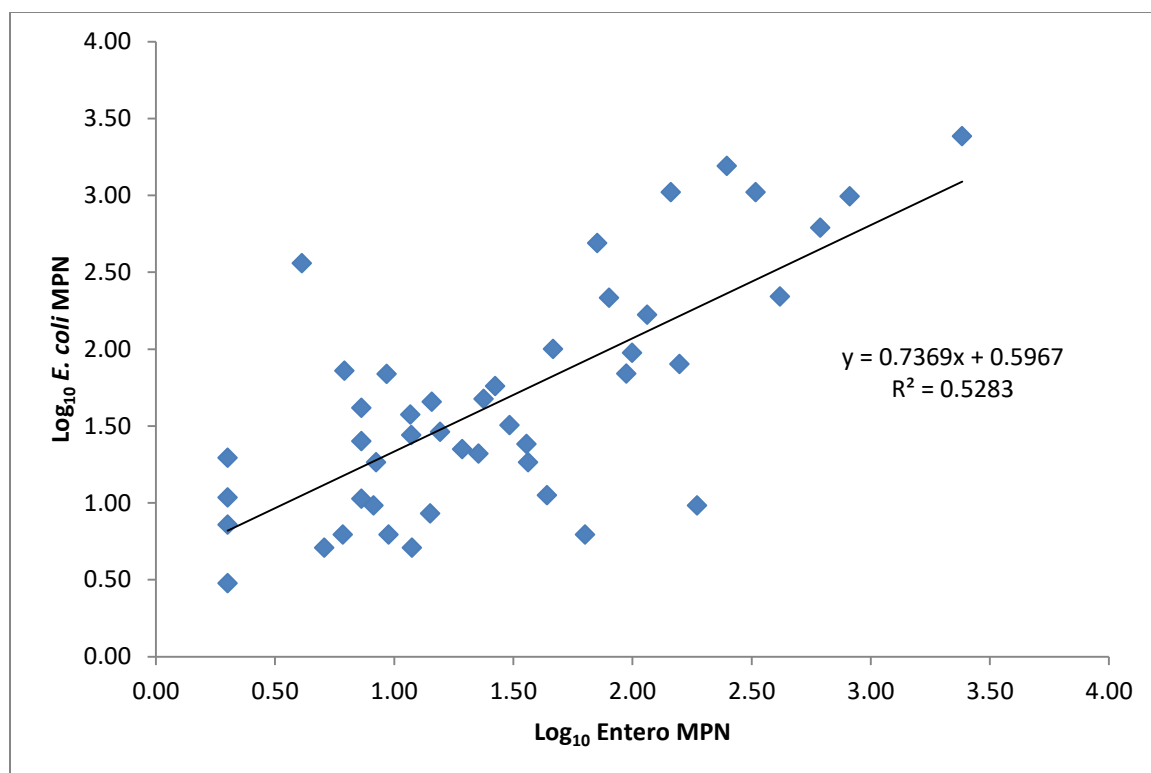


Figure A7. The correlation between Log_{10} enterococci concentrations and Log_{10} *E. coli* concentrations using Enterolert and Colilert at Otumba beach.

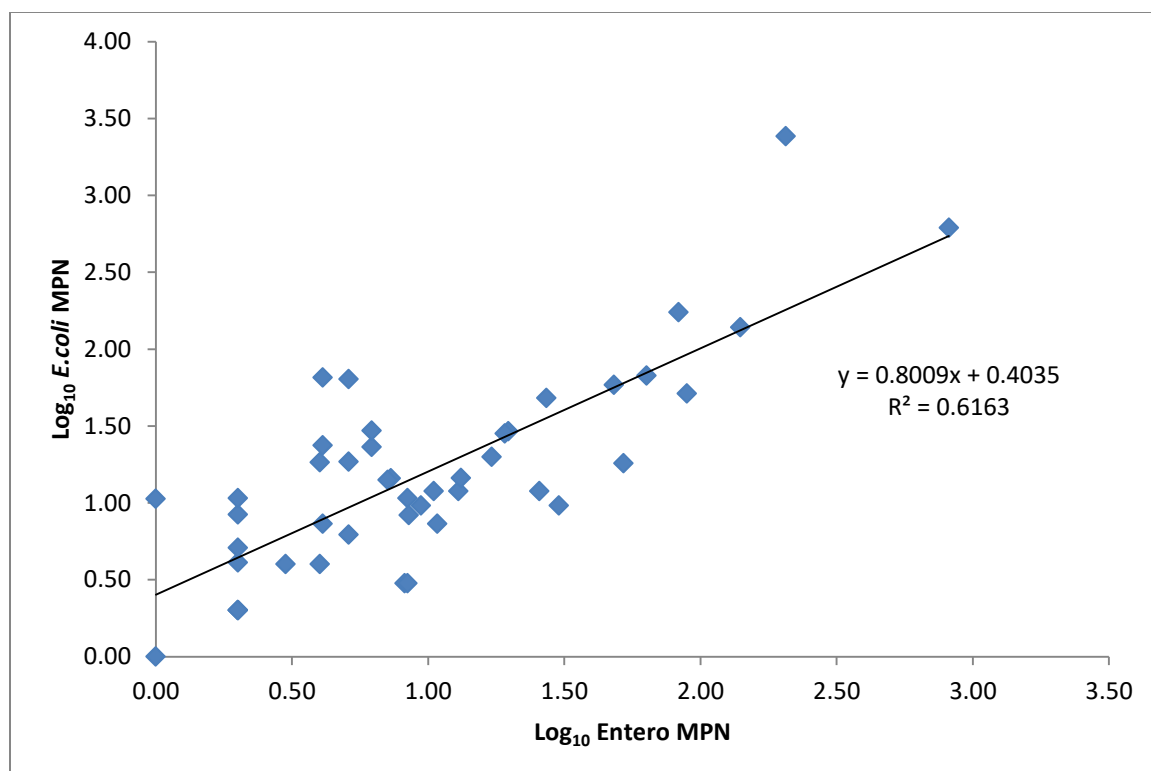


Figure A8. The correlation between Log₁₀ enterococci concentrations and Log₁₀ *E. coli* concentrations using Enterolert and Colilert at Sunset beach

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