ABSTRACT

DETECTION OF *ESCHERICHIA COLI* IN NORTHERN

LAKE MICHIGAN WATERS USING QPCR METHOD C

By Roland Hernandez Jr.

The defined substrate method, Colilert® (IDEXX, Westbrook, ME), is currently the most widely used method approved by the United States Environmental Protection Agency (US EPA) to detect *Escherichia coli* and total coliforms in surface water. One use for the test is to determine the risk of exposure to waterborne pathogens by beach users after contact with recreational water. However, results are not available until 18 to 24 hours after sampling because the microbiological cultures need to incubate. This could mean beach goers could be exposed to contaminated water while lab testing proceeds. To help alleviate this shortcoming the US EPA has recently investigated a quantitative polymerase chain reaction (qPCR) method for use in recreational waters. This qPCR method is called Method C (US EPA, 2015) and can reduce the analysis time to four hours when identifying *E. coli* DNA. My overall goal was to determine how qPCR Method C compares to traditional culture-based enumeration methods at Lake Michigan beaches. I conducted a comparison of both techniques (Method C and Colilert®) at six beaches in Door, Kewaunee, and Manitowoc counties in Wisconsin throughout the 2016 and 2017 summer beach seasons. Six beaches were selected and placed in three groups: high, moderate and low mean *E. coli* concentrations based on Colilert® historical data since 2003. More than 30 sampling events for each beach were used to help elucidate relationships between these two methods. My work will provide critical knowledge for beach management as practitioners consider a transition to a rapid qPCR method for *E. coli* detection.

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by

Roland Hernandez Jr.

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COMMITTEE APPROVAL

_____ Advisor

5-8-18 Date Approved

_____ Member

5-8-18 Date Approved

_____ Member

5-10-18 Date Approved

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My thesis is dedicated to my parents Elaine and Roland Hernandez Sr. who have always been there for me since the beginning. From their support towards my academic success, I am where I am today. Also, to my fiancée Whitney who has been by my side throughout my entire research project. You have provided endless support.

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INTRODUCTION

Currently, conventional culture-based microbiology is the standard for bacterial quantification in United States health departments when testing recreational beach water. Since October 10th, 2000, the United States amended the Clean Water Act (CWA) and funded the Environmental Protection Agency (EPA), approximately $149 million since 2002, to implement the Beach Environmental Assessment and Coastal Health (BEACH) Act (US EPA, 2018). The goal of this act is to help protect recreational beach users from waterborne infection by monitoring bacterial concentrations (US EPA, 2002). The BEACH Act required states, as well as some territories and tribes, that contain beaches on the Great Lakes or coastline to implement new water quality standards by 2004 (Figure 1).

Each year US marine and freshwater beaches exceed microbiological water quality standards thousands of times each beach season. In 2011 and 2012, the CDC's Waterborne Disease and Outbreak Surveillance System (WBDOSS) reported 90 recreational waterborne outbreaks in 32 states and Puerto Rico (Hlavsa *et al*, 2015). These outbreaks resulted in 1,788 cases of illness, 95 hospitalizations and one death due to exposure to contaminated recreational water. Monitoring bacterial concentrations in beach water and issuing beach advisories or closures can reduce the number of people encountering contamination at United States beaches. Although most microorganisms in aquatic ecosystems are not harmful, the small amount that are able to infect humans usually comes from fecal matter or other waste material (Illinois EPA, 2004). Common bacterial waterborne pathogens can include *Salmonella enterica*, *Campylobacter* spp., *Leptospira* spp., *Shigella sonnei*, *Streptococcus* spp. and pathogenic strains of

Escherichia coli. Usual symptoms of bacterial infection include diarrhea, abdominal pain, nausea and vomiting. Additionally, parasites and viruses such as *Giardia lamblia*, *Schistosoma* spp., *Cryptosporidium parvum*, hepatitis A virus, and rotavirus can be present in contaminated recreational water and can cause similar illness (Illinois EPA, 2004). Pathogens such as these can even cause death in children or immunocompromised persons.

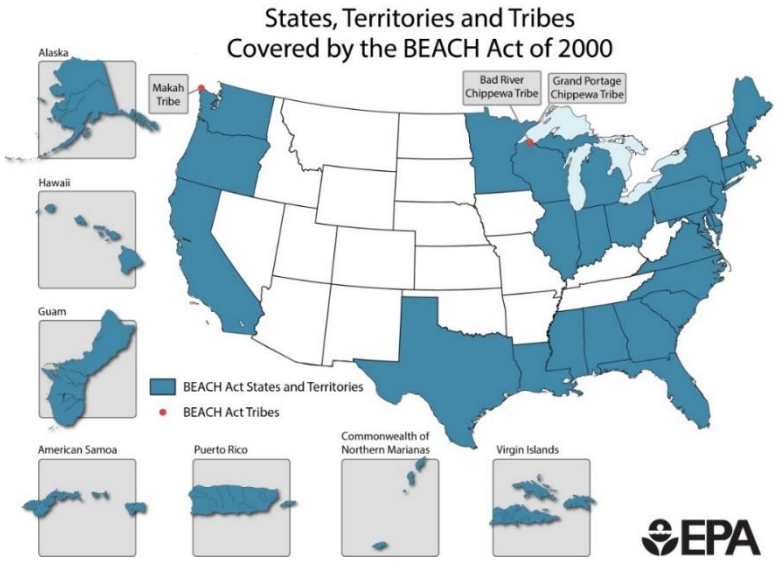

Figure 1. Map of the United States showing where the BEACH Act provides annual funds to coastal and Great Lakes states, territories and tribes (US EPA, 2018).

The Need for Beach Monitoring

When communities do not provide sufficient funding to monitor or mitigate recreational water contamination, beach water contamination can reach dangerous levels. An example of this occurred during the 2016 summer Olympics in Rio de Janeiro, Brazil where the coastal waters were heavily polluted with sewage weeks before the opening ceremony. Of the 146 water samples tested along the coast before the event, 95.9%

contained viruses similar to the ones found in sewage contamination (Staggemeier *et al*, 2017). During the Olympics, about 350,000 foreign beach goers were potentially in danger of contracting harmful pathogens (Brooks, 2015). Ingesting 15mL of water from these polluted beaches was enough to contract an illness.

A less extreme, but closer to home, example of recreational water pollution occurred July of 2002 on Lake Michigan. 68 people fell ill after swimming at Nicolet Beach in Peninsula State Park in Door County Wisconsin (Door County Pulse, 2008). The Nicolet Beach outbreak produced fear in the Door County community that ultimately affected the overall county income from tourism that summer beach season. Many local businesses in Wisconsin depend on the summer season to maintain revenue since over half the year the area is covered in ice and snow. On average, the cost of spending the day at the beach is between $15-$25 per person depending on location (Sohngen *et al*, 2000). Up to 70% of the money spent goes into the local economy. Nationally, Americans take more than 900 million trips to coastal areas each year, spending approximately $44 billion annually during these visits (US EPA, 2018). With hundreds of beach goers in Door County every day during the summer, a bacterial outbreak can greatly affect the total revenue and human health in a community. Furthermore, the 2002 Nicolet Beach outbreak was one year before Wisconsin implemented its' beach monitoring program from the BEACH Act, so there was no warning that the beach water was contaminated. The state of Wisconsin did not start annual beach monitoring until the 2003 summer season, and with this beach monitoring the Nicolet Beach outbreak may have been prevented or at least reduced (Door County Pulse, 2008).

Sources of Contamination

The release of harmful pathogens into recreation water supplies can come from multiple sources. Much of this contamination stems from sewage contamination, with the US EPA estimating that 3.5 million people fall ill due to sewer overflow contamination in coastal waters and water supplies (NRDC, 2018). Globally, that number increases to 120 million cases of gastrointestinal (GI) disease each year specifically caused by swimming in waste-water polluted coastal waters (Shuval, 2003). Other contaminants can come from microbes introduced into the water from storm runoff. Since the cause of infection usually occurs from microbes introduced by storm runoff, waste products or sewage, the classification commonly used for bacterial water contamination are fecal indicator bacteria (FIB) (Dufour, 1986).

To better identify sources of recreational water contamination, an approach called microbial source tracking (MST) is being used to identify the origins of contamination. In general, approximately 95% of water quality advisories within the Great Lakes are not attributed to a known contamination source (Busse, 2014). Current research into source tracking using a Beach Sanitary Survey (BSS) tool has found that common sources can include animal or livestock waste, wildlife excreta, and waterfowl droppings on beach environments (Pandey, 2014). Additionally, when using a BSS at a beach site, there is a higher chance that this survey can identify the source of contamination to prevent future contamination spikes. All these sources of contamination can introduce a variety of harmful pathogens into recreational waters. Currently, *Escherichia coli* is used as the indicator organism for freshwater beaches throughout the Great Lakes (Dufour, 1986).

The Use of Indicator Organisms

An indicator organism is used to easily examine the overall "health" of an environment or sample. According to Blonde's (1966) Criteria for an Ideal Indicator, the indicator organism should:

1) Be present whenever the pathogens are present;
2) Be present only when the presence of pathogens is an imminent danger;
3) Occur in much greater numbers than the pathogens;
4) Be more resistant to disinfectants and to the aqueous environment than the pathogens;
5) Grow readily on simple media;
6) Yield characteristic and simple reactions enabling as far as possible an unambiguous identification of the group;
7) Be randomly distributed in the sample to be examined, or it should be possible to obtain a uniform distribution by simple homogenization procedures; and
8) Grow widely independent of other organisms present, when inoculated in artificial media (Bonde, 1966).

Although *E. coli* is used as the indicator organism in freshwater beaches, Enterococci was chosen for marine beaches. The EPA evaluated both *E. coli* and Enterococci as indicator organisms based on measurements of Highly Credible Gastrointestinal Illnesses (HCGI). Results showed strong correlations in freshwater with *E. coli* concentrations and HCGI ($R^2 = 0.80$) (Dufour, 1986). For marine beaches, the EPA uses Enterococci since in marine environments *E. coli* showed weaker correlations with HCGI ($R^2 = 0.52$), while Enterococci provided stronger correlations for marine beaches ($R^2 = 0.75$). The EPA's evaluation for using these fecal coliform indicators estimated that 8 illnesses per 1,000 swimmers at freshwater beaches and 19 illnesses per 1,000 swimmers at marine beaches

are acceptable illness rates for an open beach according to the Quality Criteria for Water (Dufour, 1986).

Testing for *E. coli*

In order to detect *E. coli* in freshwater environments, the EPA uses a conventional culture-based method to find both qualitative and quantitative results. This culture-based enumeration process is done by measuring the Most Probable Number (MPN/100mL) of *E. coli* using the IDEXX Colilert® defined substrate method. This method works by promoting the growth of both total coliforms, and specifically, *E. coli* cells. The growth of coliforms releases the enzyme β-galactosidase. Additionally, *E. coli* specifically produces the enzyme β-glucuronidase, while other coliform bacteria do not. After incubation for 18 or 24 hours, depending on the Colilert® media, fecal indicator numbers can be reported. The Colilert® media contains two nutrient indicators, *ortho*-Nitrophenyl-β-galactoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG). The breakdown of ONPG and MUG indicator molecules from bacterial growth creates a yellow coloration and fluorescence under UV light respectively (George *et al*, 2001). This method is used for annual beach monitoring in the United States to test for *E. coli* and total coliforms. The State of Wisconsin issues a beach advisory when 235-999 *E. coli* MPN/100 ml water are detected, and a beach closure when 1,000 or more *E. coli* MPN/100 ml of water are detected (Wisconsin DNR, 2001).

Rapid Methods for Testing *E. coli*

Multiple problems arise when using the Colilert® defined substrate method. Since this method is not considered a rapid microbiological method (RMM), the concern stems from the 18-24 hour delay between sample collection, incubation and reporting results. This delay allows recreational beach goers to encounter contaminated water while sample analysis is being completed. One solution to this delay in is a new US EPA qPCR draft method called Method C (Aslan, 2015). This RMM can potentially be used by public health agencies to identify beach advisories/closures more promptly. This method takes 4-6 hours to have results reported to the public, compared to 18-24 hours for Colilert®. Compared to the EPA approved culture-based standard, the ability to use qPCR provides same day results that could be used as the new alternative standard method (Aslan, 2015).

The use of RMMs is becoming more prevalent in clinical and industrial laboratory settings as the need for faster real-time results rises. In general, laboratories that have expanded to use RMMs benefit from reducing the time, cost and labor of sample analysis (Duguid, 2011). Apart from real-time data analysis, other benefits of using RMMs include increased detection, enumeration and identification of microorganisms when compared to conventional methods (Moldenhauer, 2008). Laboratories no longer require multi-day sample analysis for identifying colonies on growth medium or using microscopy on bacterial Gram stains. A prevalent RMM that is used is quantitative polymerase chain reaction (qPCR), which is a nucleic acid-based detection method that can detect and amplify regions in a single strand of deoxyribonucleic acid (DNA) (NLEP, 1991). Additional benefits of using qPCR over conventional methods also include the ability to detect viable but non-culturable (VBNC) organisms and to detect a multitude of

species in a sample (Duguid, 2011). Although the small test sample volumes may require a skilled technician to perform RMMs, the number and level of detection of organisms able to be identified in a sample is far greater than using conventional methods. However, for beach monitoring purposes, the detection of dead organisms or extra DNA in the water that present no risk to public health must also be accounted for.

qPCR Basics

The process of qPCR requires a DNA template with the presence of two oligonucleotide primers that bind around the target sequence, deoxynucleotide triphosphates (dNTPs), a heat stable polymerase, and magnesium ions in the buffer (Kubista, 2006). The process begins with the denaturation of the DNA double helix at 94-98°C for 20-30 seconds followed by a decrease in temperature to 50-65°C for another 20-40 seconds to allow primers to bind to the target sequence. Elongation of a newly synthesized DNA copy then begins around 75-80°C, which is the optimum temperature for the Taq (*Thermus aquaticus*) polymerase (Chien, 1976). The steps of denaturing, annealing and elongation is considered a cycle and after numerous cycles the DNA target amplifies to millions of copies. The equation is "$2^{n} = x$", where x equals the number of DNA copies obtained and with n equating to the numbers of cycles. Normally, 30-40 cycles are performed in a qPCR reaction.

The traditional polymerase chain reaction (PCR) was invented in 1983 by Kary Mullis and is able to detect the DNA concentration of a sample once the reaction is complete by measuring absorbance, florescence or running an agarose gel (Promega, 2018). Since then, numerous types of PCR have been created for even more rapid and

accurate results such as the qPCR used in this beach water study. The main difference between the traditional form of PCR and qPCR is that qPCR can detect the amount of DNA during each cycle of the PCR reaction (Higuchi, 1992). qPCR does this by attaching additional fluorophore-quencher probes to the target sequence. These probes break down as the qPCR reaction amplifies the target sequence and allows the florescence to be detected real-time by the thermocycler (Aslan, 2015).

By using qPCR, microbial DNA can be used to detect the quantity of the desired indicator organism, compared to measuring living cells, when testing recreational beach water (Griffith & Weisburg, 2011). Using qPCR takes less than six hours for reporting beach advisories and could reduce the number of people coming in contact with contaminated beach water by promptly notifying beach goers of unsafe levels of microbes in water. One drawback to using qPCR is that live cells, non-cultural cells, dead cells and free DNA can be detected (Aslan, 2015). This drawback means there may be beach sampling sites that display false positives even when pathogen levels are low, since free *E. coli* DNA can have high concentrations in a water sample and be detected by the qPCR reaction. On the other hand, this result could provide more protection to the community since a beach advisory or closure would be issued due to high levels of DNA in the water.

The overarching objective of this study was to compare the EPA standardized Colilert® method during the annual beach monitoring season in Door, Kewaunee and Manitowoc counties in Wisconsin, while simultaneously implementing qPCR Method C when testing the same beach samples. A comparative analysis was done between both methods to test if correlation of *E. coli* detection occurs. Comparing beach sampling

results from the Colilert® and qPCR methods can help determine if qPCR Method C can be used as an alternative and allow posting of beach advisories and closures sooner to protect public safety.

OBJECTIVES

The overarching objective of this project was to evaluate the relationship between US EPA's qPCR Method C and the defined substrate Colilert® method for *E.coli* enumeration and possible future use in beach monitoring and management in northeast Wisconsin. The steps in this study were to:

- Utilize qPCR Method C and Colilert® at six different Lake Michigan beaches to determine concentrations of *E. coli* using each method.
- Conduct comparative analysis of each method's results at beaches with relatively high, moderate and low historical concentrations of *E. coli*.
- Examine the management implications of changing to Method C for beach monitoring in northern Lake Michigan beaches.

METHODS

Sample Sites and Lab Facilities

Along eastern Wisconsin's 500 miles of coast lies Lake Michigan where millions of people visit each summer season to enjoy the recreational beaches. These beach sites play an important role in summer tourism. For this study, a total of six beaches located in Door, Kewaunee, and Manitowoc counties were sampled and *E. coli* concentrations were enumerated using Colilert® and qPCR Method C. These six beaches were chosen because of our historical knowledge of *E. coli* concentration seasonal averages since 2003. The six beaches were arranged into three groups of two beaches each. These groups were based on high, moderate or low total *E. coli* concentrations (Table 1, Figures 2). Sample analysis occurred at Crossroads at Big Creek Nature and Education Center in Sturgeon Bay, WI during the summers of 2016 and 2017 (Figure 3).

Table 1. Beach locations chosen for this study with their historical MPN/100mL grouping.

Beach Name	**County**	**Historical *E. coli* Concentration**
Crescent Beach	Kewaunee	High
Red Arrow Beach	Manitowoc	High
Otumba Beach	Door	Moderate
Ephraim Beach	Door	Moderate
Ellison Bay Beach	Door	Low
Europe Bay Beach	Door	Low

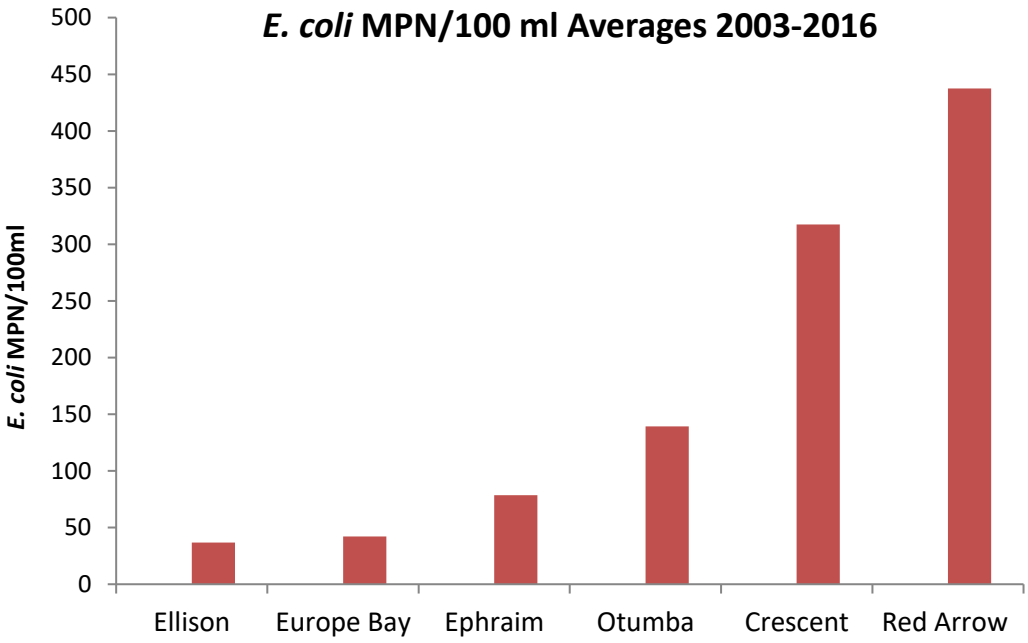

Figure 2. The historical mean *E. coli* MPN/100mL for the six beaches that were chosen for this study during the 2016 and 2017 summer beach seasons. Prior to this study, three groups of two beaches each were created based on historical mean *E. coli* concentrations. Low: Ellison Bay and Europe Bay, Moderate: Ephraim and Otumba, and High: Crescent and Red Arrow.

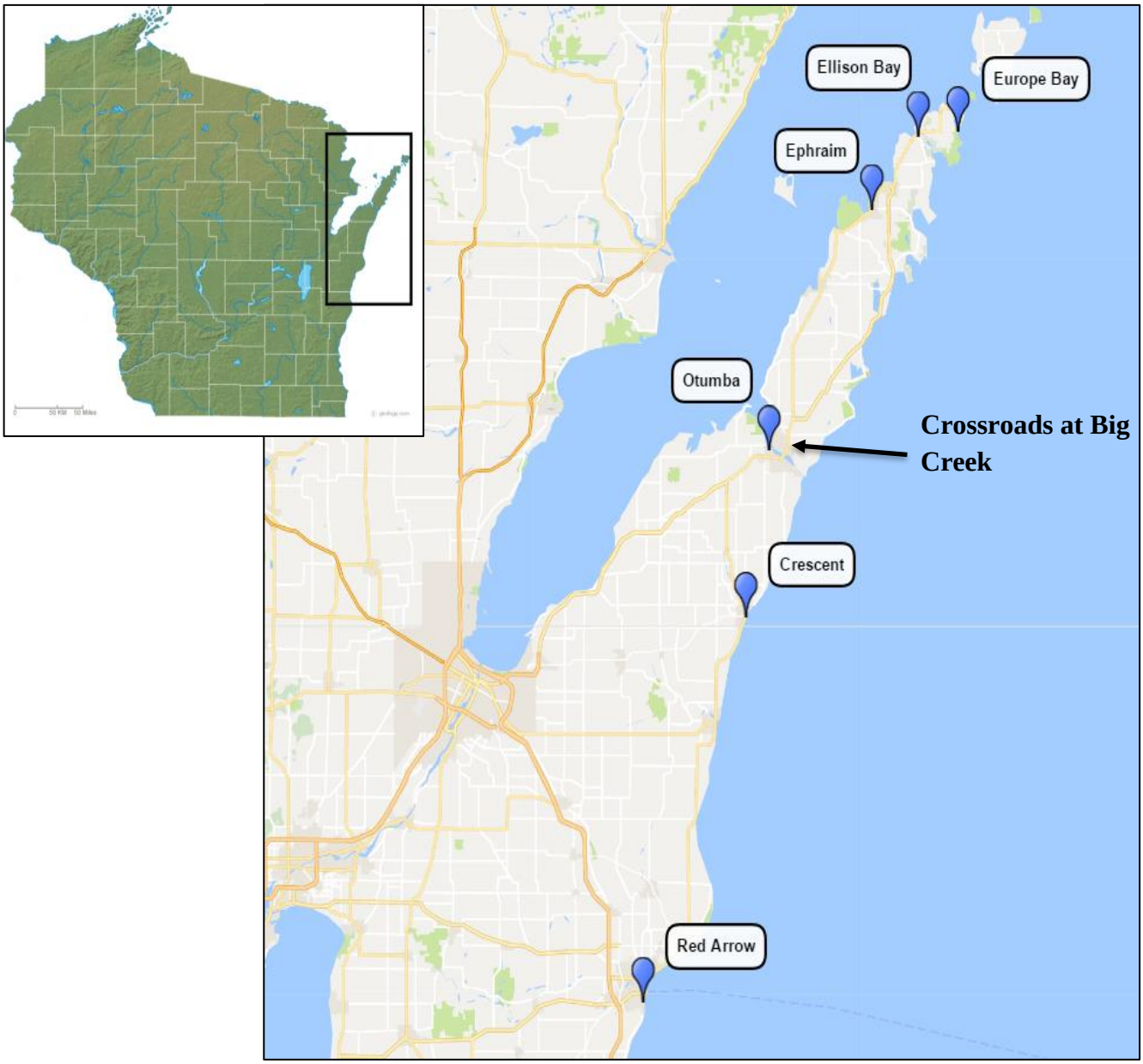

Figure 3. The locations of the six northern Lake Michigan beaches tested for *E. coli* concentrations using Colilert® and qPCR Method C during the 2016 and 2017 summer beach seasons.

Sample Site Descriptions

Crescent Beach



Figure 4. Aerial photo of Crescent Beach in Algoma, WI.

Crescent Beach is located in downtown Algoma, WI, which has a population of 3,167 people. It is bounded on the north by a large break wall and has impounded waters. A storm water outfall is located near the beach area. A major roadway and multi-purpose trail runs parallel to the beach (Figure 4).

Red Arrow Beach



Figure 5. Aerial photo of Red Arrow Beach in Manitowoc, WI.

Red Arrow Beach is located in Manitowoc, WI, which has a population of 37,736 people, the largest municipal area for this study. Red Arrow Beach is a large flat beach below a bluff skirted with impervious surfaces. There is a large outfall with extensive dry and wet weather flow, which is one source of beach contamination. A redesign has recently been done to prevent overland flow from the park, but there are still high amounts of storm water discharge on the south side of the beach (Figure 5).

Otumba Beach



Figure 6. Aerial photo of Otumba Beach in Sturgeon Bay, WI.

Otumba Beach is located in Sturgeon Bay, WI, which has a population of 9,144 people. Two storm water outfall pipes are located at this beach, with one of them almost directly in the center of the beach. A waste water treatment plants is also located nearby (Figure 6).

Ephraim Beach



Figure 7. Aerial photo of Ephraim Beach in Ephraim, WI.

Ephraim Beach is located in Ephraim, WI, which has a population of 288 people. The beach is located in the center of Eagle Harbor bay (Figure 7).

Ellison Bay Beach



Figure 8. Aerial photo of Ellison Bay Beach in Ellison Bay, WI.

Ellison Bay Beach is located in Ellison Bay, WI, which has a population of 165 people. The beach is enclosed by a jetty on the westside and multiple docks on the east side. Previous infiltration and vegetation projects have been implemented to reduce storm water runoff (Figure 8).

Europe Bay Beach



Figure 9. Aerial photo of Europe Bay Beach in northern Door County, WI.

Europe Bay Beach is located in the Europe Bay Woods State Natural Area, on the Lake Michigan side of the Dorr County Peninsula and is the most remote beach site for this study (Figure 9).

Surface Water Sample Collection

Surface water samples were collected in the geographic center of each beach from water that is knee depth (24 inches or 60 cm). Four 120 ml sterile polystyrene IDEXX bottles were used at each test site to collect beach water samples 12 inches (30 cm) below the surface. Introducing sediment or any debris into the surface water samples was avoided by positioning the mouth of the bottle away from the sampler's hand and plunging the bottom of the open bottle straight down into the surface (Bordner *et al*, 1978). The four samples were taken directly to the lab on ice for analysis with Colilert® and qPCR Method C two times each week during both 2016 and 2017 summer seasons. Three of the sample bottles were used for qPCR Method C and one was used for Colilert® beach monitoring. Observational data were also collected for each sample and included surface water temperature, wind direction, wave height, rainfall, number of bathers and number of animals.

E. coli Defined Substrate Method (Colilert®)

Samples arrived to the lab at Crossroads at Big Creek within six hours of collection to remain compliant with both protocols. Excess water was removed from the IDEXX bottles until the marked 100ml line. The Colilert® defined substrate (18 or 24-hour packet media) was added to one sample bottle per site for traditional microbiological culturing. Samples were then homogenized for approximately two minutes for substrate to dissolve. The sample-substrate mixture was then placed into a sterile IDEXX Quanti-Tray™/2000 and placed into a sealer (Figure 10). The sealed tray was then placed into an incubator at 35ºC for 18 or 24 hours depending on the substrate

used. Quanti-Trays™ were then observed for color changes, specifically florescence under UV light (IDEXX, 2017). The number of changed small or large wells were counted for *E. coli* quantification using the most probable number statistical method (MPN/100mL). Results were reported in the Wisconsin Beach monitoring site (Wibeaches.us) for the public to view. Beaches with 235-999 *E. coli* MPN/100mL were placed under a contamination advisory and with 1000 or more *E. coli* MPN/100mL under a beach closure.



Figure 10. Products used for the Colilert® method. (A) Sterile 120ml water collection bottle. (B) Box containing Colilert® substrate packets. (C) IDEXX Quanti-Tray/2000. (D) Quanti-Tray sealer.

E. coli qPCR Method C Detection

Samples had to arrive to the lab at Crossroads at Big Creek within six hours of collection based on the EPA Method C protocol (Aslan, 2015). The excess water in the triplicate samples were removed until the 100ml mark. The water samples were then filtered through a polycarbonate filter with a pore size of 0.45 µm. Additionally, a 1ml

$5x10^4$ cells/ml of *E. coli* suspension (positive control) was filtered as the calibrator, as well as a 20ml PBS sample as a filter blank (negative control).

All triplicate filtered samples per site were transferred to a sterile labeled bead tube containing 0.3g of glass beads. One of the triplicate samples for each site had 600µl of salmon DNA (0.2 µg/ml) extraction buffer added for bead mill extraction for 1 minute. The additional two filters per site were frozen in -80ºC for further testing if needed. The salmon extraction buffer was transferred from glass bead tubes and prepared for qPCR loading. Reaction master mix was prepared under a high efficiency particulate air (HEPA)-filtered laminar flow PCR-Workstation hood. The master mix contained 2 parts nuclease-free water, 2.5 parts Bovine Serum Album (BSA), 3 parts either *E. coli* (EC23S) or Salmon (Skeeta22) primer probe (100µM) and 12.5 parts TAQMAN master mix. The reaction master mix and sample DNA were prepared in triplicate on a 96-well Life-Technologies MicroAmp plate before entering the thermocycler. All reagents and equipment were purchased from Applied Biosystems (ABI). After aliquoting 20µl of master mix reagent into reaction plates, 5µl of extracted DNA was added for a 25µl total volume for each qPCR reaction well. Reaction plate was sealed and placed in the ABI StepOnePlus™ thermocycler instrument.

The number of cycles was set to 40 and the thermal profile was set to the following: Holding stage 1: 50.0ºC for 2:00 minute; Holding stage 2: 95ºC for 10:00 minutes; Cycling Stage: 95ºC for 0:15 seconds. The Cycling Stage 2: 60ºC for 1:00 minute. These stages were run according to the qPCR Method C Appendix B guidelines. Amplification curves data was analyzed under Microsoft Excel and Method C spreadsheet recommended by the EPA.

The *E. coli* forward ad reverse primers (500µM) contained the sequences 5'-GGTAGAGCACTGTTTTGGCA and 5'-TGTCTCCCGTGATAACTTTCTC, respectively. The TaqMan probe (100µM) contained the sequence [6-FAM]-5'-TCATCCCGACTTACCAACCCG-TAMRA. The salmon forward and reverse primers contained the sequences 5'-GGTTTCCGCAGCTGGG and 5'-CCGAGCCGTCCTGGTC, respectively. While the salmon TaqMan probe contained the sequence [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA (US EPA, 2014b).

Statistical Analysis

A minimum of 30 sampling events per beach for both 2016 and 2017 combined summers were collected. The *E. coli* MPN/100mL and CCE/100mL data were analyzed using Pearson product-moment correlation coefficients (R^2) and an EPA Index of Agreement to see the relationship between Colilert® and qPCR Method C. A one-way ANOVA and Tukey's HSD test was done between all six beaches to see which beaches had significant differences between each other. Boxplots for all six beaches were made to visualize beach means, variance and outliers. The correlation coefficients (R^2) and Index of Agreement also had $\log_{10}$ transformed values to reduce scatter and were produced using Microsoft Excel. The beach boxplots, ANOVA and Tukey's HSD test were performed using the statistics program R and had $\log_{10}$ transformed values. Additional figures were made between rainfall, wave height and temperature between all six beaches, as well as sampling date graphs to visualize results from each sampling date for both summers.

The Index of Agreement is an EPA method used for correlating alternative indicator methods in bodies of water (US EPA, 2014).

$$IA = 1 - \frac{\frac{1}{N}\sum_{i=1}^{N}(x_i - y_i)^2}{\frac{1}{N}\sum_{i=1}^{N}(|x_i - \bar{x}| + |y_i - \bar{x}|)^2}$$

In the equation, x represents the density of the microorganisms determined by Method 1 (Colilert®) and y represents Method 2 (qPCR Method C). Additionally, *i* is a counter, *N* is the total number of data points in the set and IA values range from 0 to 1 (US EPA, 2014).

RESULTS

The overarching objective for this study was to evaluate the relationship between Colilert® and qPCR Method C when enumerating *E. coli* in recreational beach water in northeast Wisconsin. The six Lake Michigan beaches tested during the 2016 and 2017 beach seasons showed unique relationships between qPCR and Colilert® (Table 2). All beaches showed a positive correlation between both methods for same day beach samples. This was expected since both methods have the ability to detect *E. coli* in water if present. The main difference observed was the extent of the positive correlation between each beach.

Table 2. Correlation coefficients (R^2) and Index of Agreement values for all beaches and beach groups. According to the US EPA, an R^2 of >0.60 and an IA of >0.70 provides a sufficient relationship to accept qPCR Method C as an alternative method for a certain beach site.

	Correlation Coefficient (0.6)	Index of Agreement (0.7)
Crescent	0.57	0.56
Red Arrow	0.31	0.52
Otumba	0.40	0.61
Ephraim	0.43	0.70
Ellison Bay	0.28	0.44
Europe Bay	0.30	0.52
Low Beaches	0.30	0.48
High/Mod Beaches	0.42	0.62
All Beaches	0.56	0.67

In this project, 41 sampling dates took place between June 8th – August 22nd, 2016 and June 1st – August 16th, 2017 (Figure 11 and 12). Twenty sampling events for 2016 and 21 sampling events for 2017. A total of six beaches were tested resulting in approximately 246 samples collected and ran with Colilert® between both summers. When running qPCR Method C, the DNA extract for each beach was ran in triplicate and averaged after the qPCR reaction, while only one water sample was tested when using Colilert®. Thirty-two samples experienced inhibition when running qPCR Method C, and seven sampling dates ran Colilert® without running qPCR leaving a total of 207 samples analyzed between all six beaches between both methods (Figure 13).

Beach Classification

After analyzing the *E. coli* MPN/100mL data for the 2016 and 2017 summers, three beaches (Crescent, Red Arrow and Ellison Bay) had a lower *E. coli* MPN/100mL mean than their historical values, while the other three beaches (Otumba, Ephraim and Europe Bay) had a higher *E. coli* MPN/100mL mean (Table 3). From the 2016 and 2017 data, the high and moderate concentration beaches observed similar averages (between 100-200 MPN/100mL), while the low concentration beaches were significantly lower (below 75 MPN/100mL) (Table 3). The low concentration beaches had significantly lower *E. coli* MPN/100mL mean because 17/41 of Ellison Bay and 14/41 of Europe Bay sampling dates resulted in non-detection of *E. coli* when using Colilert®, while the other four beaches combined had four non-detections. This resulted in two distinct groups when analyzing the 2016 and 2017 data, four beaches (high and moderate) and two beaches (low).

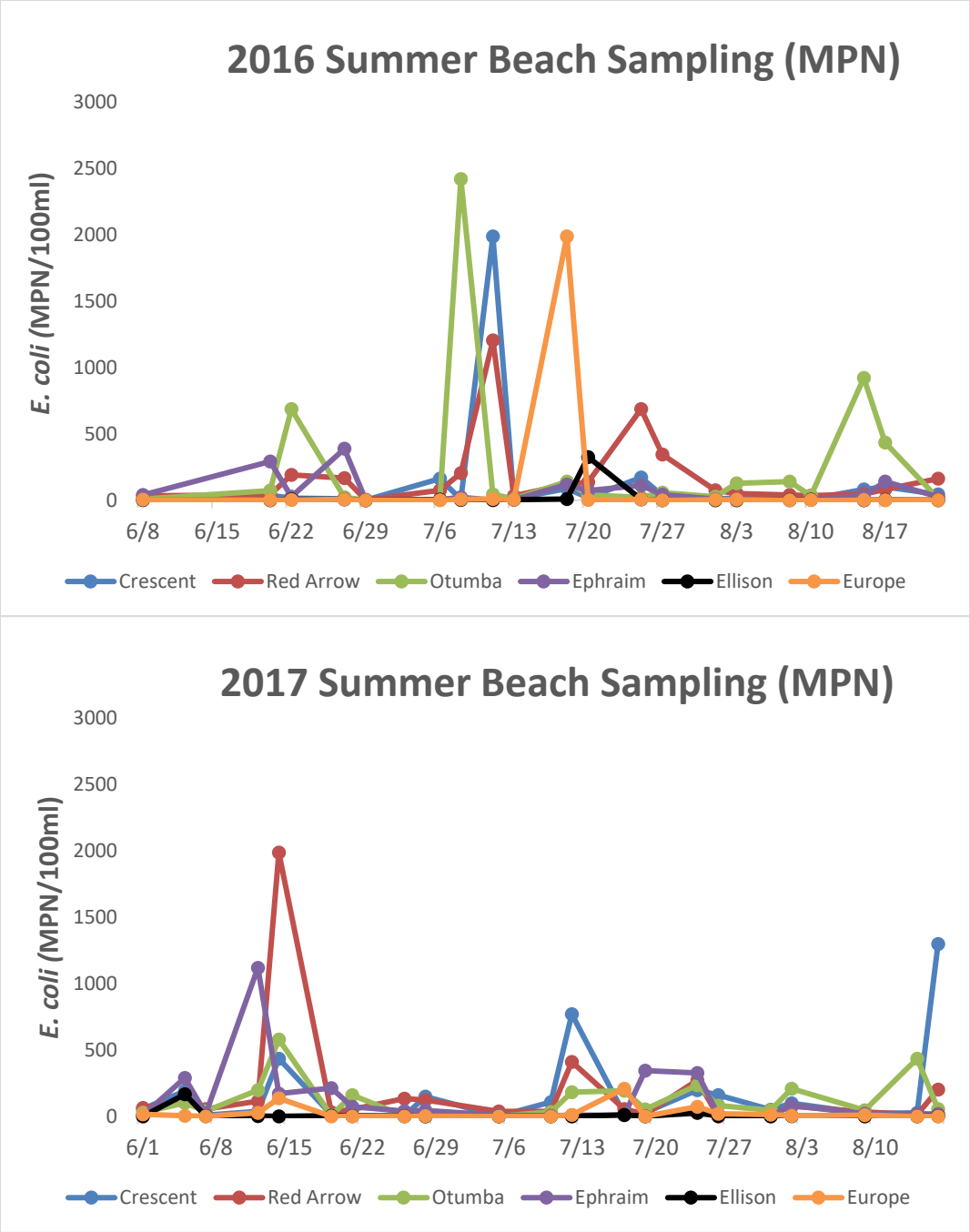

Figure 11. Concentrations of *E. coli* MPN/100mL during both 2016 and 2017 summer sampling events for all six beaches tested with Colilert®.

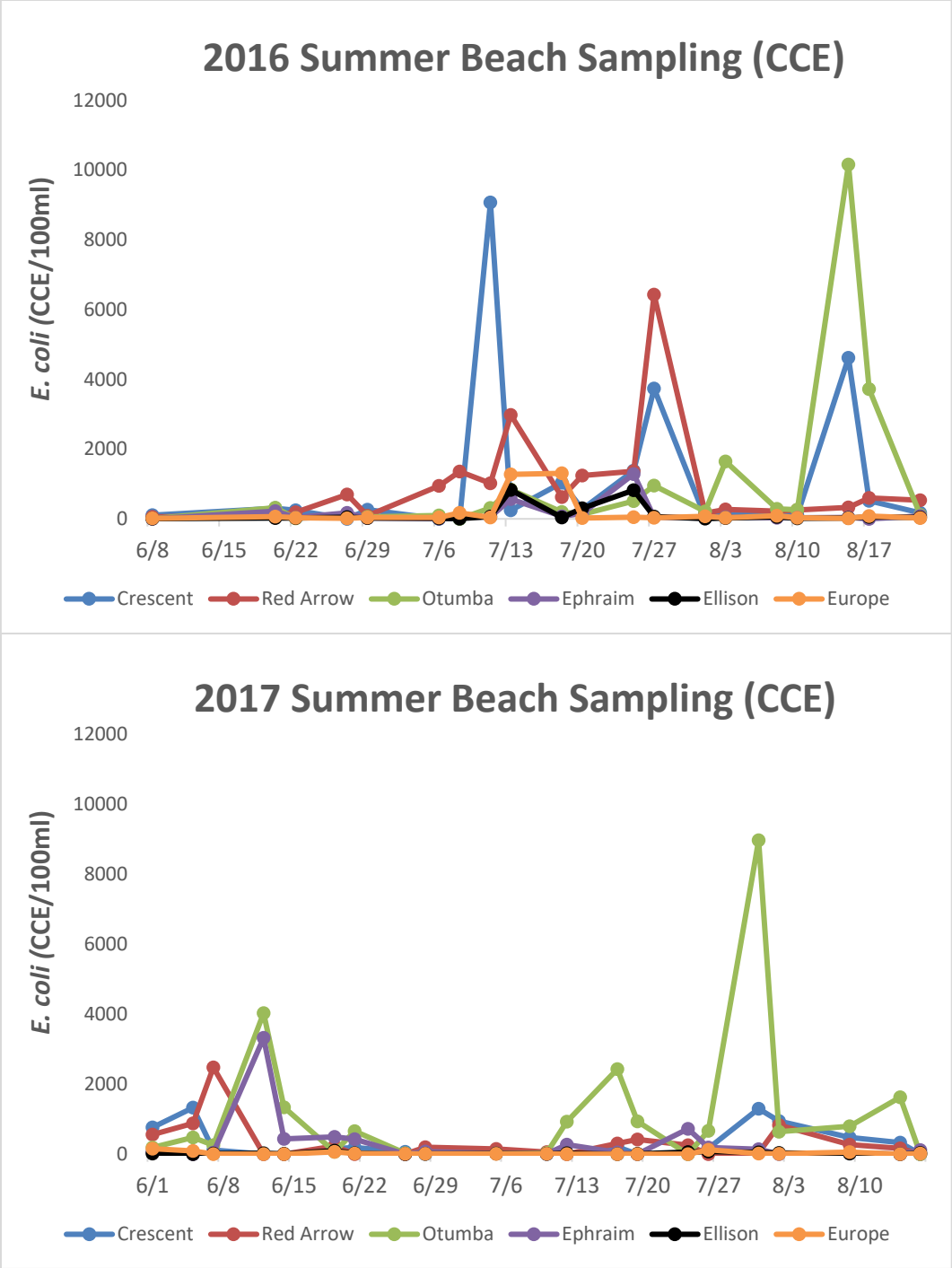

Figure 12. Concentrations of *E. coli* CCE/100mL during both 2016 and 2017 summer sampling events for all six beaches tested with qPCR Method C.

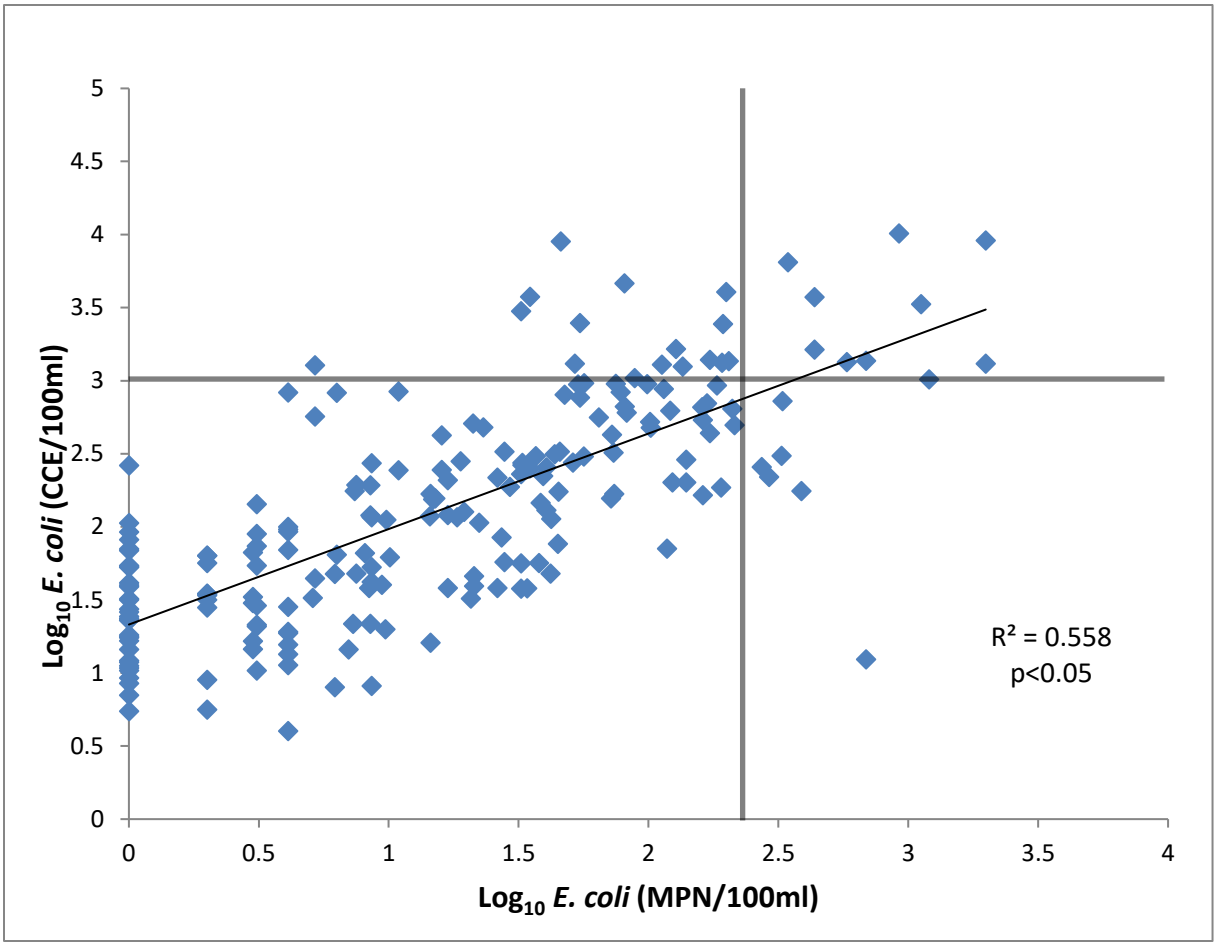

Figure 13. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) for all six beaches. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 207.

Table 3. Beach mean and median MPN/100mL historical values (2003-2016) and for both summers of this study (2016 & 2017). One-way ANOVA for Colilert® Method for all six beaches (2016 & 2017): $F = 2.239$, $\alpha = 0.05$, ($p>0.05$).

	Historical Mean ± Stdev	Median	Mean ± Stdev
Crescent	317.5 ± 704.7	35.0	130.3 ± 331.7
Red Arrow	417.8 ± 806.7	74.4	189.2 ± 366.7
Otumba	139.2 ± 392.9	53.6	196.7 ± 413.7
Ephraim	78.7 ± 144.7	26.2	104.0 ± 197.3
Ellison Bay	36.7 ± 83.5	2.0	15.6 ± 56.7
Europe Bay	42.2 ± 95.0	3.6	66.4 ± 318.0

Table 4. Beach mean and median CCE/100mL values both summers (2016 & 2017). One-way ANOVA for qPCR Method C for all six beaches: $F = 4.368$, $\alpha = 0.05$ ($p<0.05$).

	Median	Mean ± Stdev
Crescent	253.1	943.9 ± 1856.5
Red Arrow	313.4	744.0 ± 1078.2
Otumba	316.6	1159.6 ± 2217.9
Ephraim	68.3	275.1 ± 599.6
Ellison Bay	26.0	82.3 ± 186.8
Europe Bay	33.9	119.7 ± 299.6

In addition to calculating the mean *E. coli* values for all beaches, median values were also calculated due to the high standard deviations surrounding the means. Previous studies comparing Colilert® and qPCR on recreational beach water used median values as well (Dorevitch, 2017). Median values for beaches were significantly lower than the mean values due to the number of outliers from *E. coli* spikes affecting the mean (Table 3 and 4). The majority of the samples taken from each beach were below the threshold for beach advisories and closures (Table 5). However, the *E. coli* concentration spikes in all

beaches were from unknown environmental factors in this study that resulted in high variance of sample values. The *E. coli* spikes in beach water normally returned to levels under the advisory or closure threshold after a few sampling dates later (Figure 11 and 12). This occurred for all beaches for both summers of the study. When measuring the median, the beaches are grouped in the same manner as for the mean. The high and moderate beaches had similar median values (25-75 MPN/100mL), while the low beaches both have a median value below 5 MPN/100mL. The mean and median values for both methods can be seen in Tables 3 and 4. Boxplots for each beach were also made depicting the quartile range for each beach as well as outliers from spikes of *E. coli* (Figure 14 and 15). Additionally, performing a one-way ANOVA and Tukey HSD test on the $\log_{10}$ Colilert® data showed significant differences between the four high and moderate concentration beaches with the two low concentrated beaches. These differences between beaches are better visualized in the boxplots.

Table 5. Exceedance table 2016 & 2017 showing the number and percent of sampling dates that experienced an advisory or closure with Colilert®. Samples were taken for each beach for both summers combined. N=41 for each beach.

	Advisories	Closures
Crescent	2 (4.9%)	2 (4.9%)
Red Arrow	4 (9.8%)	2 (4.9%)
Otumba	6 (14.6%)	1 (2.4%)
Ephraim	5 (12.2%)	1 (2.4%)
Ellison Bay	1 (2.4%)	0 (0%)
Europe Bay	0 (0%)	1 (2.4%)

This grouping also worked well when comparing the Colilert® and qPCR Method C results (Figure 16 and 17). This was mainly due to the fact that both low concentration beaches (Ellison Bay and Europe Bay) resulted in numerous non-detections when using Colilert®, as stated above (Figure 16). This is because Colilert® is only able to detect living *E. coli* cells suspended in the water, (Garcia-Armisen *et al*, 2005). For all sample sites that retrieved a non-detection with Colilert®, *E. coli* DNA was still detected when using qPCR Method C.

Beach Correlation

The correlation of *E. coli* concentrations between the two methods were done by viewing the Pearson product-moment correlation coefficients (R^2) and the Index of Agreement. R^2 correlation coefficient graphs between Colilert® and qPCR Method C were made for each beach for both 2016 and 2017 summers. A black line is added on each axis signifying where a beach advisory threshold is for each method. However, since no public beach advisory threshold is currently in use for qPCR Method C, the 1,000 CCE/100mL threshold is used because that is the advisory threshold for EPA approved Enterococci qPCR methods (Table 6). Samples that lie in the upper right and lower left are considered true positive and negatives, while samples located in the upper left and lower right are considered false positives and negatives. The ratio between true and false values is considered percent agreement (Table 7). All correlations coefficient graphs are in $\log_{10}$ transformation for both methods.

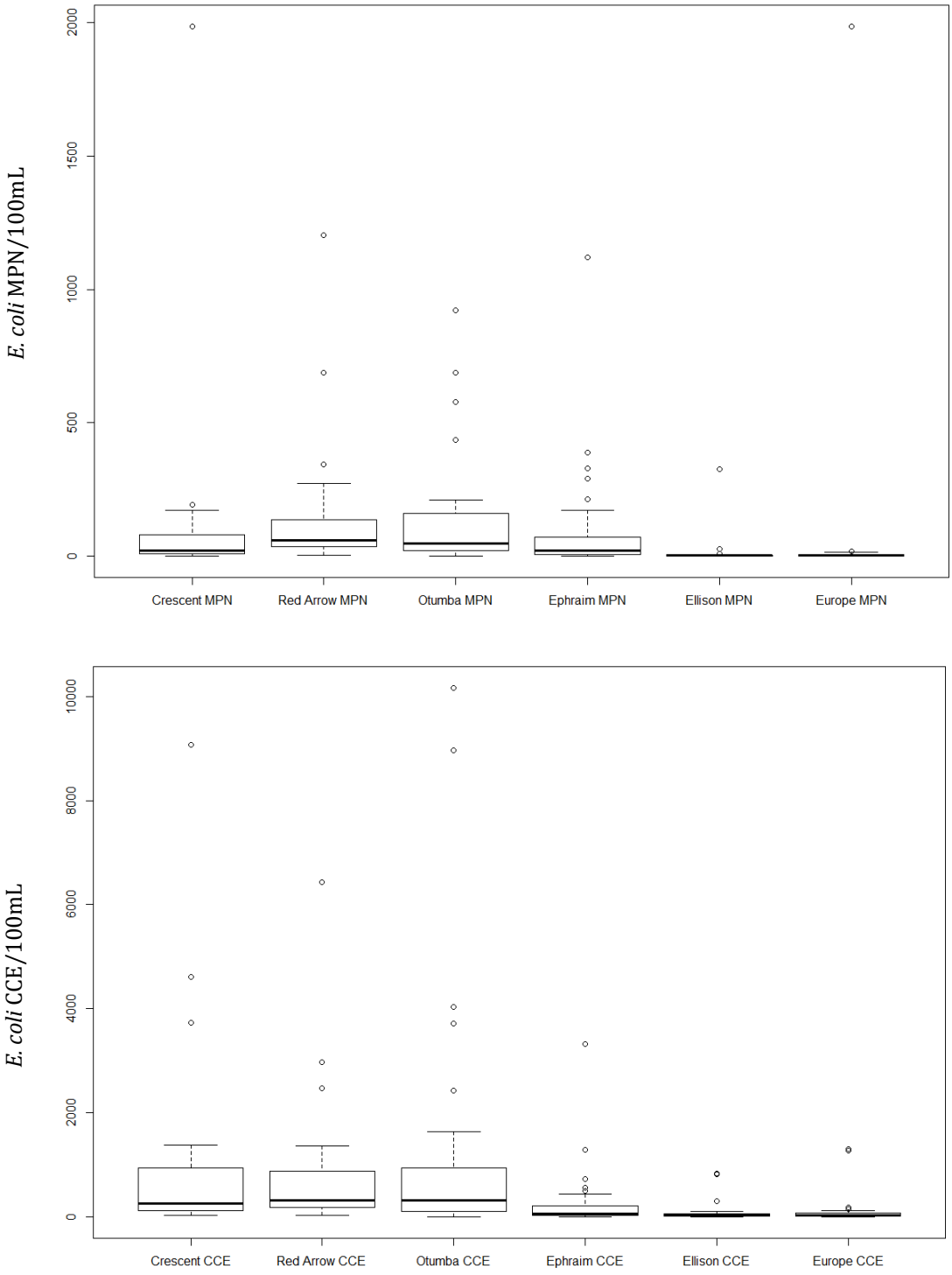

Figure 14. Boxplots of the six beaches tested using Colilert® (Top) and qPCR Method C (Bottom) for the 2016 and 2017 summer beach seasons.

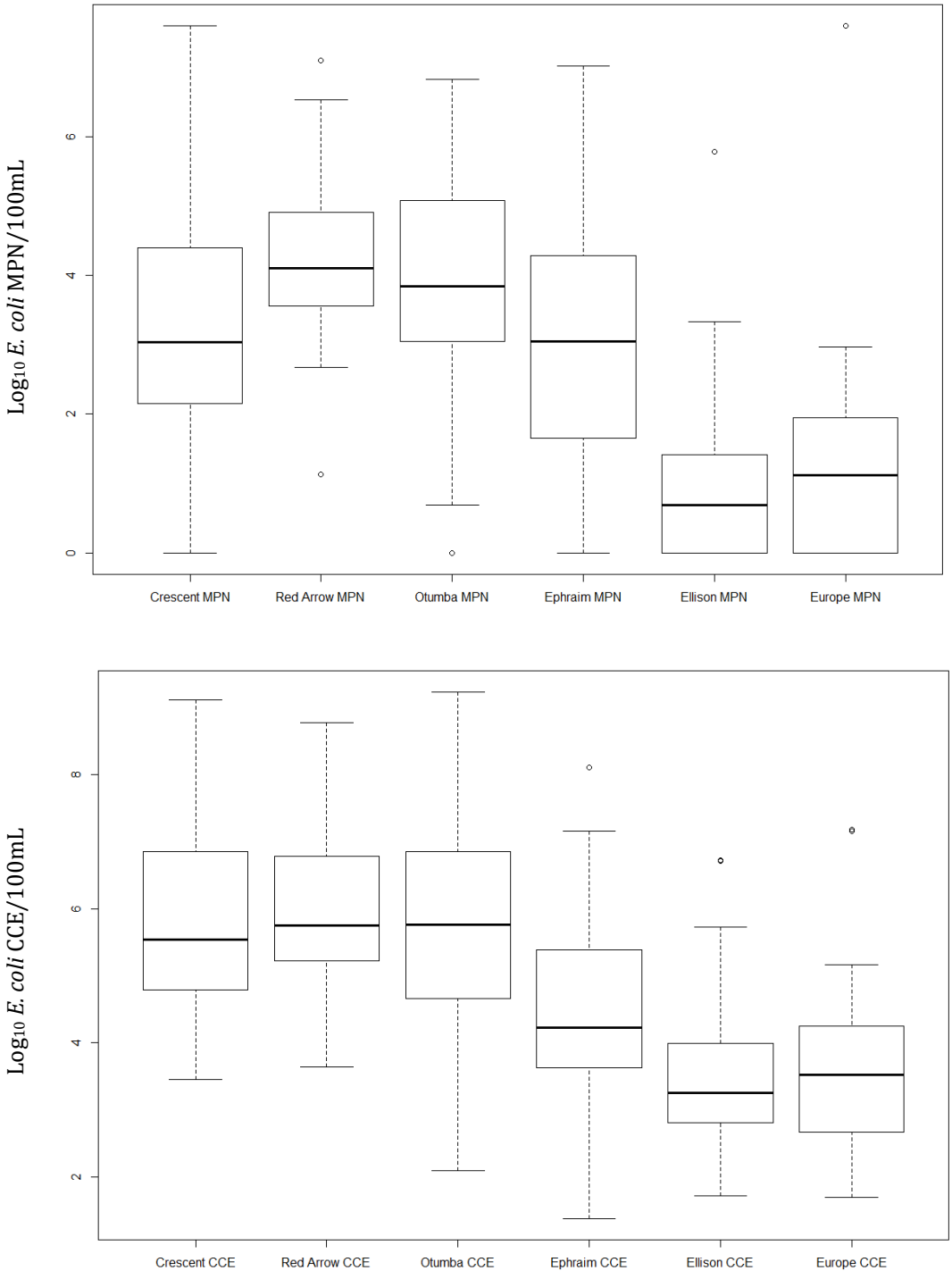

Figure 15. Boxplots of the six beaches tested using Colilert® (Top) and qPCR Method C (Bottom) for the 2016 and 2017 summer beach seasons transformed into Log_{10} values.

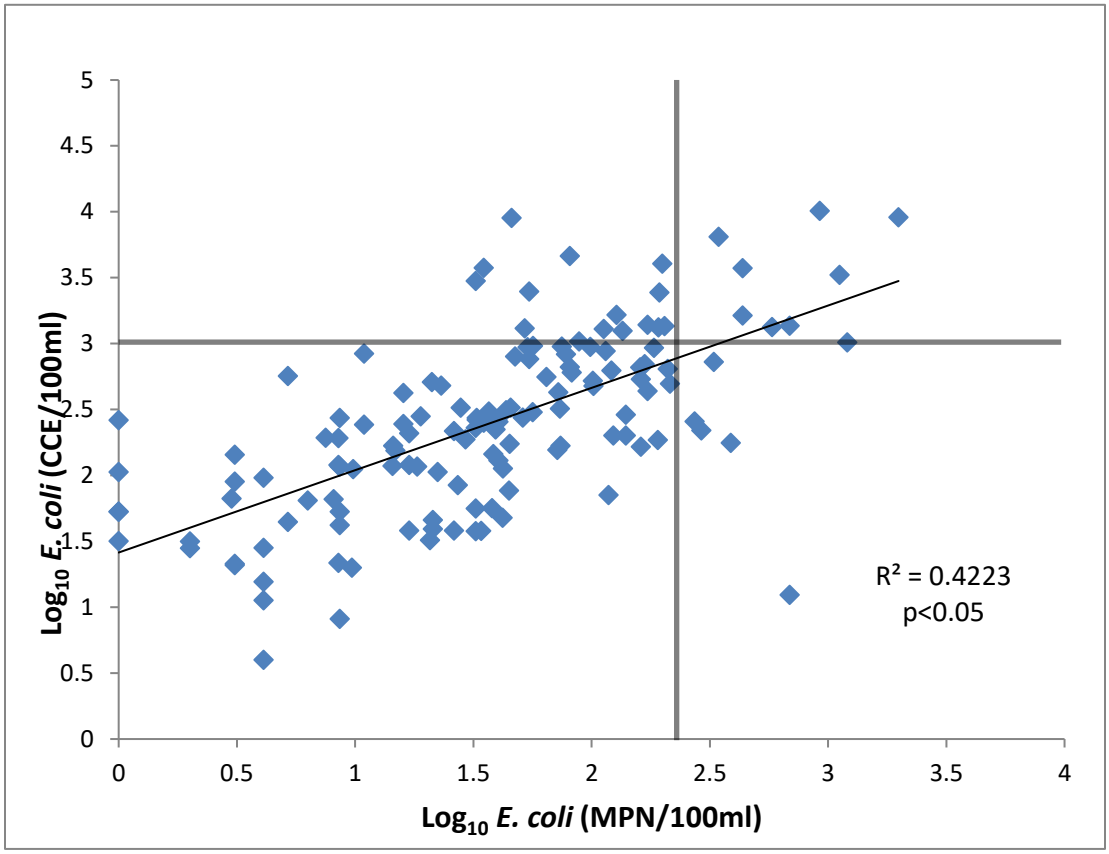

Figure 16. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) for Crescent, Red Arrow, Ephraim and Otumba beaches (high and moderate concentration beaches). Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 136.

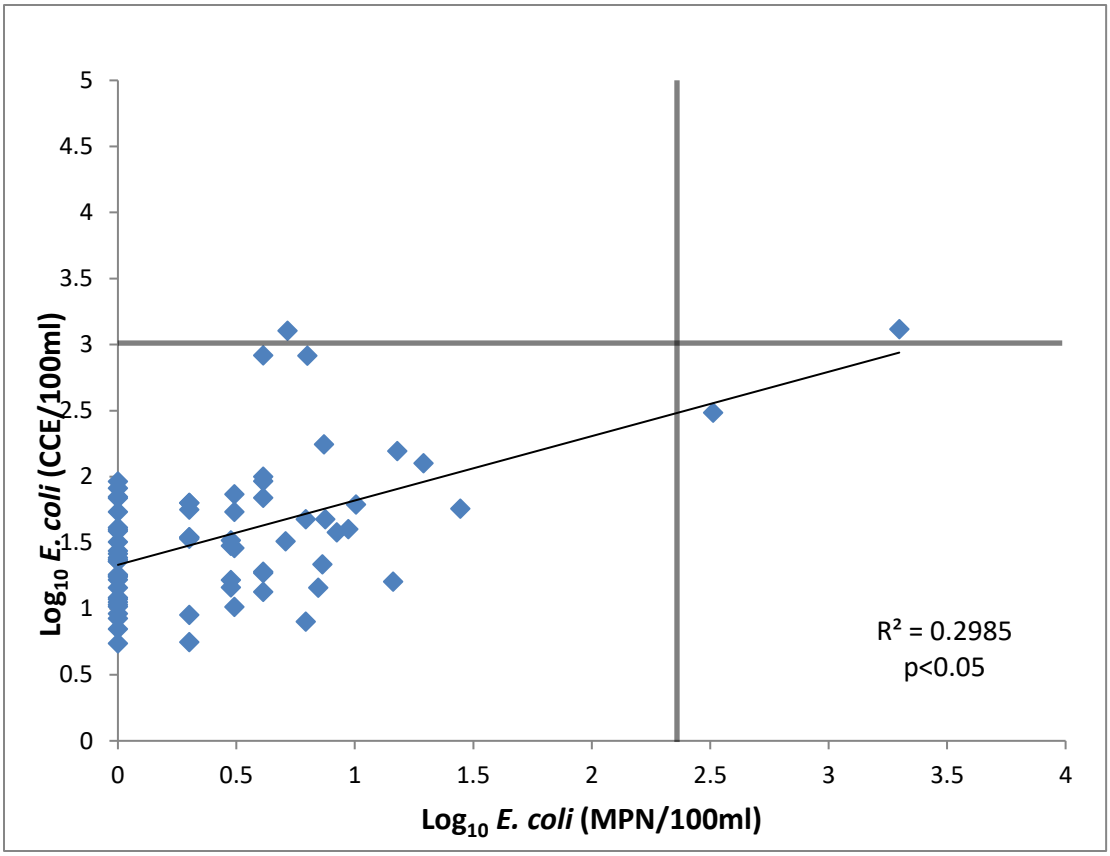

Figure 17. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) for Ellison Bay and Europe Bay beaches (low concentration beaches). Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 71.

Table 6. Exceedance table 2016 & 2017 showing the number and percent of sampling dates that experienced an advisory or closure with qPCR Method C. Due to inhibition, less than 41 samples were analyzed.

	Samples Analyzed	Advisories	Closures
Crescent	30	3 (10.0%)	4 (13.3%)
Red Arrow	34	2 (5.9%)	5 (14.7%)
Otumba	38	0 (0%)	8 (21.1%)
Ephraim	34	0 (0%)	2 (5.9%)
Ellison Bay	37	0 (0%)	0 (0%)
Europe Bay	34	0 (0%)	2 (5.9%)

According to the US EPA, correlation of two methods is sufficient if the Index of Agreement between sampling events is greater than 0.7 or if the R^2 is greater than 0.6. From the data collected for 2016 and 2017, only Ephraim beach showed sufficient correlation with an Index of Agreement score at 0.7 (Table 2). Beaches that fell just below this threshold were Crescent ($R^2 = 0.57$) and Otumba (IA = 0.61). However, when combining data from all six beaches and correlating both methods, the (R^2) value becomes 0.56 and the Index of Agreement becomes 0.67. These values are very close to allowing qPCR Method C to be used as a sufficient alternative method according to US EPA initial considerations. When performing a one-way ANOVA on all six beaches for both methods, using Colilert® resulted in no significant differences between beaches ($F = 2.20$, $\alpha = 0.05$) compared to the results from qPCR which did have significant differences between beaches ($F = 4.37$, $\alpha = 0.05$). However, when transforming the data into $\log_{10}$ values, the one-way ANOVA showed differences between both tests ($p<0.05$). Better visualized on figures 14 and 15.

Physical/Chemical Data

The physical and chemical data (temperature, rainfall and wave height) collected from each sample site was also compared with the *E. coli* MPN/100mL and CCE/100mL values (Appendix A). Figures with secondary axis for MPN/100mL and CCE/100mL vs temperature, wave height and rainfall were made showing the sampling date. For both summers, the overall trend for water temperature increased towards the end of the summer. However, water temperature did not have an impact on *E. coli* levels for either the Colilert® or qPCR Method C (Mean $R^2 = 0.03$, $p>0.05$) (Appendix A). Furthermore, wave height had minimal impact when measuring *E. coli* from beach sample sites and seemed to be site specific (Mean $R^2 = 0.11$, $p>0.05$) (Appendix A). There was a pattern between rainfall events and spikes in *E. coli* concentrations at Crescent ($R^2 = 0.72$, $p<0.05$). Yet, the mean R^2 for all beaches combined for rainfall was 0.11 and had no significant relationship when combined, meaning the geography or urban area of each beach site individually may have an effect on introducing *E. coli* contamination into the water. The effect of increased *E. coli* concentrations appears to result from rain on the same date, or within the past 48 hours (Appendix A). Additionally, rainfall has been shown to affect some beach sites in previous studies (Ackerman and Weisburg, 2003).

Inhibition with qPCR

During the filtration step of qPCR Method C, samples that contained large amounts of algae or debris were more likely to experience inhibition, which did not result in a qPCR result from that sampling date. Inhibition occurred 32 times (13.4%) throughout both summers (Figure 18). Beach samples for which QC failed with qPCR

Method C were rerun with the -80ºC stored duplicate filters, but almost half of the rerun qPCR samples also had QC failure (47%), due to unknown factors from this study that impacted the qPCR reaction. Each beach experienced some sort of inhibition, hinting at additional sources of pollution or contamination other than microbial contamination in the water samples for those sample dates. Interestingly, inhibition occurred more frequently in the summer of 2017 than in 2016 (Figure 18).

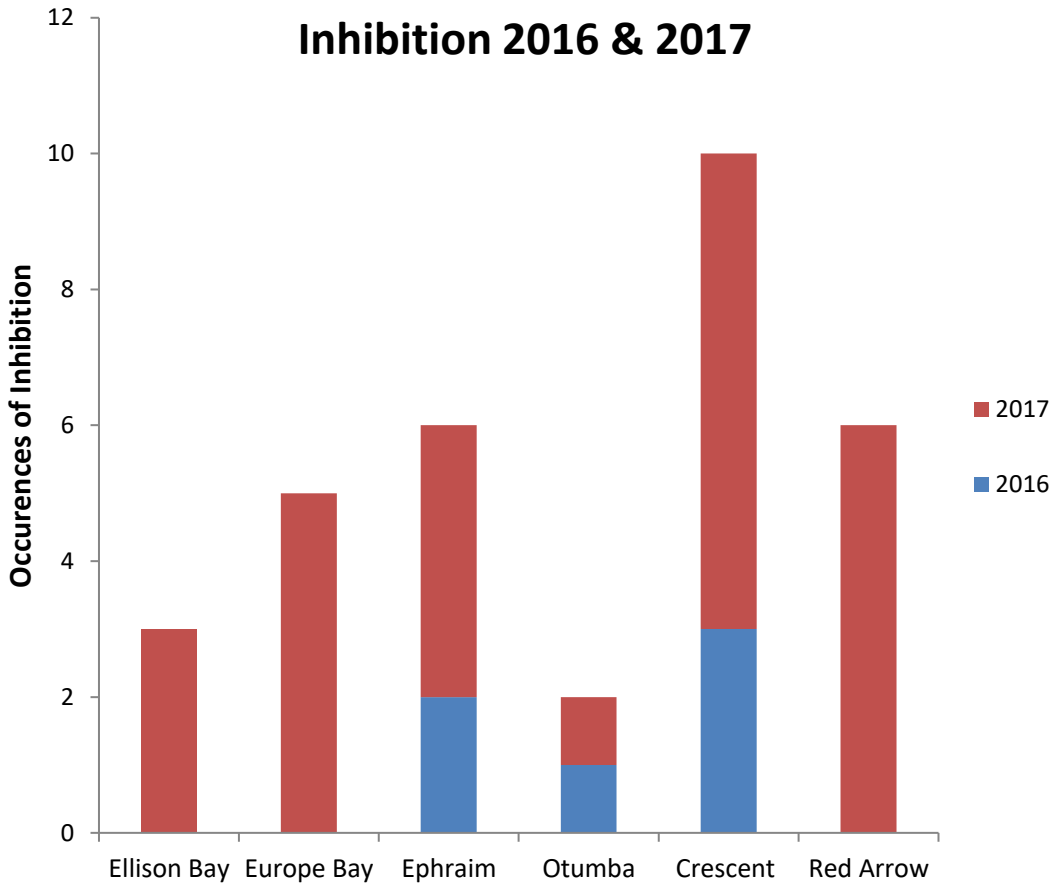

Figure 18. Number of occurrences of inhibition between the six beaches when using qPCR Method C for both summer beach seasons.

Table 7. Percent agreement for beaches for combined 2016 & 2017 summers showing the number of false positives or negatives when comparing qPCR Method C with Colilert®.

	Samples Analyzed	False positives with CCE/100mL	False negatives with CCE/100mL	% agreement
Crescent	30	5	0	83.3
Red Arrow	34	4	1	85.3
Otumba	38	4	1	86.8
Ephraim	34	1	3	88.2
Ellison Bay	37	0	1	97.3
Europe Bay	34	1	0	97.1

Specific Beach Results

Crescent Beach

Located in downtown Algoma, WI, Crescent beach had the 2nd highest historical *E. coli* concentration average since 2003 at 317.5 *E. coli* MPN/100mL, which is over the 235 *E. coli* MPN/100mL advisory level (Table 3). Algoma Wisconsin has a small population of 3,167 people with a density of 48,700 persons/km^2. For this study the mean *E. coli* MPN/100mL was 130.3 and the median *E. coli* MPN/100mL was 35.0. When testing Crescent Beach with qPCR Method C, Crescent Beach had the greatest number of occurrences of inhibition of any beach, with inhabitation detection in 10/41 of the sampling events (Figure 18). The correlation between methods for Crescent Beach had the highest R^2 value of any of the other beaches at 0.57 (Figure 19). There were two outliers with Colilert® and three outliers with qPCR to spikes in *E. coli* concentration (Figure 14). During 2016, one sampling event on July 11th showed strong correlation between rainfall and *E. coli* concentration. A spike in *E. coli* for both Colilert® and qPCR Method C was seen after the largest raining event for Crescent Beach (Appendix A).

Wave height was at its maximum height at that same time (Appendix A). Crescent Beach had five false positives when using qPCR Method C, giving it a method agreement of 83.3% when measuring in CCE/100mL (Table 7). For 2016 Crescent Beach had an *E. coli* CCE/100mL average above the closure threshold at 1316. However, the *E. coli* CCE/100mL average for 2017 dropped significantly to 466.8 (Figure 20).

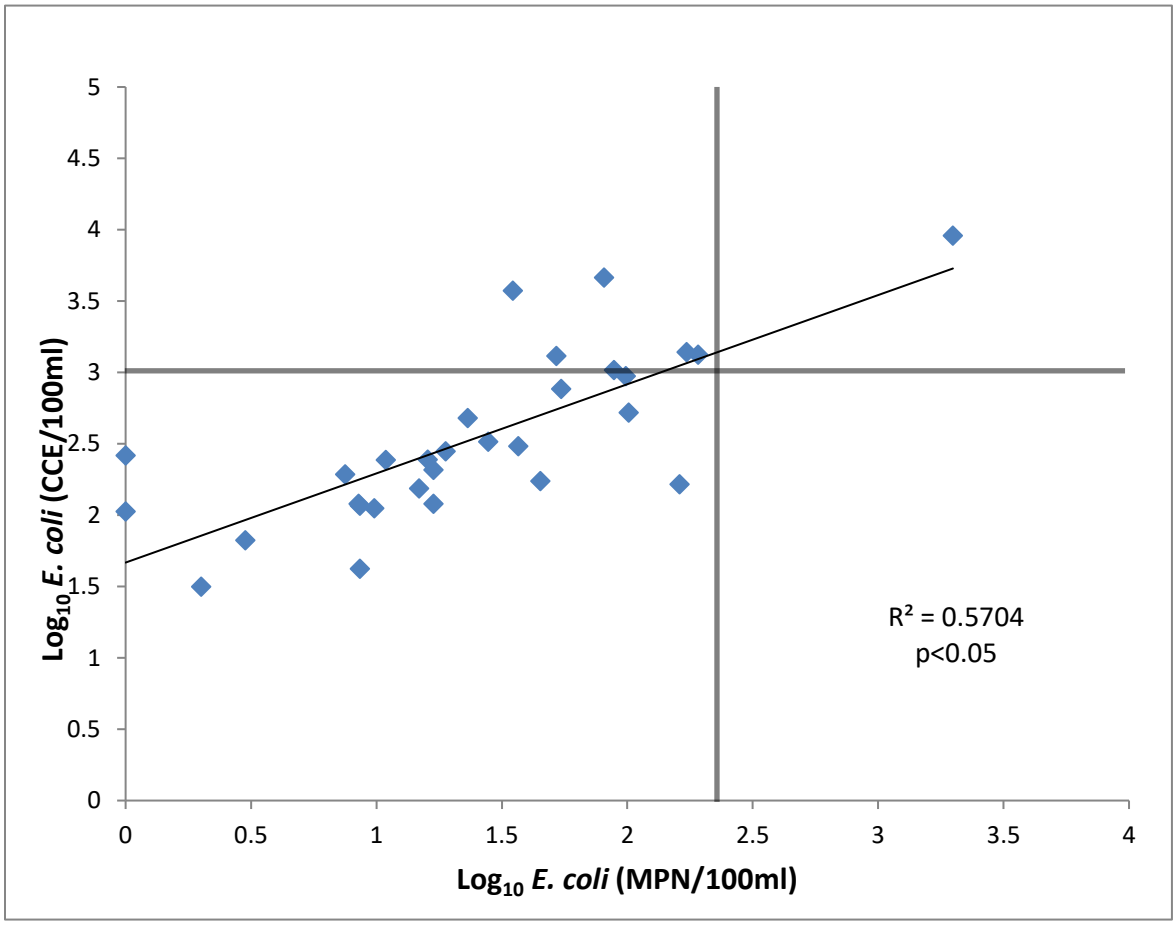

Figure 19. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Crescent Beach. Black lines indicate threshold values for beach advisories for each method showing the number of true and false negatives. All beach values were transformed into $\log_{10}$ values. N=30.

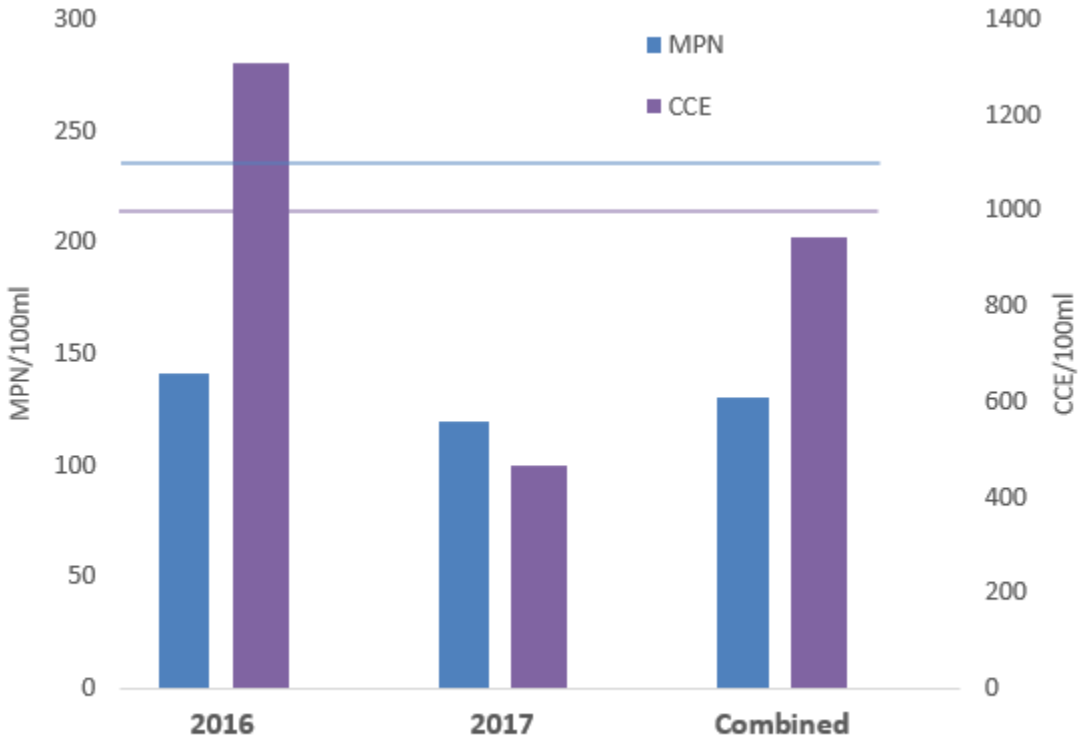

Figure 20. Mean concentrations between both Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Crescent Beach for 2016 (N=17), 2017 (N=13) and combined summers (N=30). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

Red Arrow Beach

Located in downtown Manitowoc, Wisconsin, Red Arrow Beach had the highest historical *E. coli* concentration since 2003 at 417.8 MPN/100mL for the beaches in this study (Table 3). Manitowoc has a population of 33,736 people with a density of 72,404 persons/km^2. This beach is also the most urban beach in this study. For the summers of 2016 and 2017 the *E. coli* MPN/100mL value was 189.2 for Red Arrow, which was the 2nd highest value for the study. For having high levels of *E. coli*, Red Arrow surprisingly had a lower correlation coefficient value ($R^2 = 0.31$) (Figure 21). This R^2 value is lower when compared with the other high/moderate *E. coli* concentration beaches for the relationship between Colilert® and qPCR Method C results. Additionally, Red Arrow experienced no inhibition for the summer of 2016, but had the 2nd greatest number of inhibition for 2017 (6/21) (Figure 18). Four sampling events resulted in false positives and one false negative, giving Red Arrow a method agreement value of 85.3% when measuring in CCE/100mL (Table 7). Red Arrow was the only beach in this study not to have a non-detection with the Colilert® method.

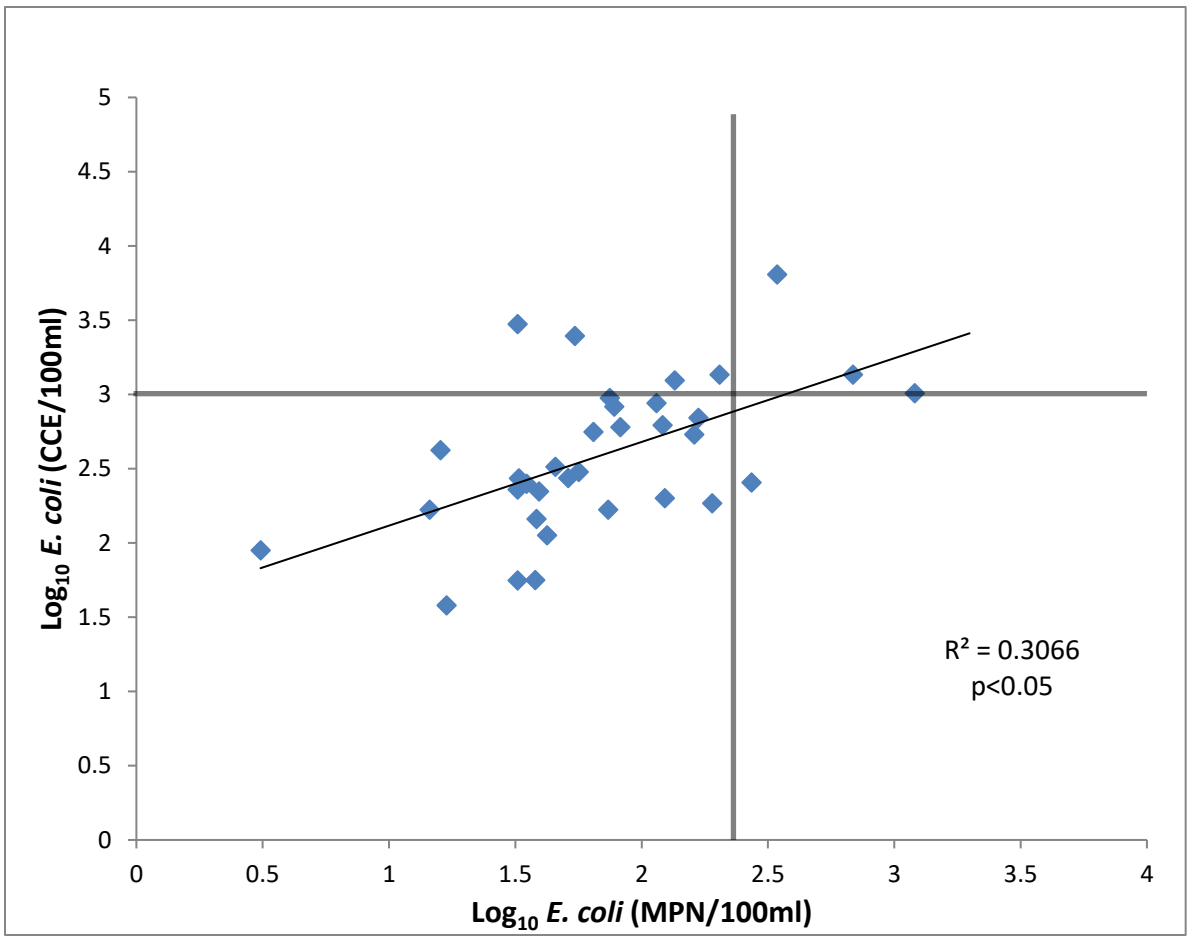

Figure 21. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Red Arrow Beach. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N=34.

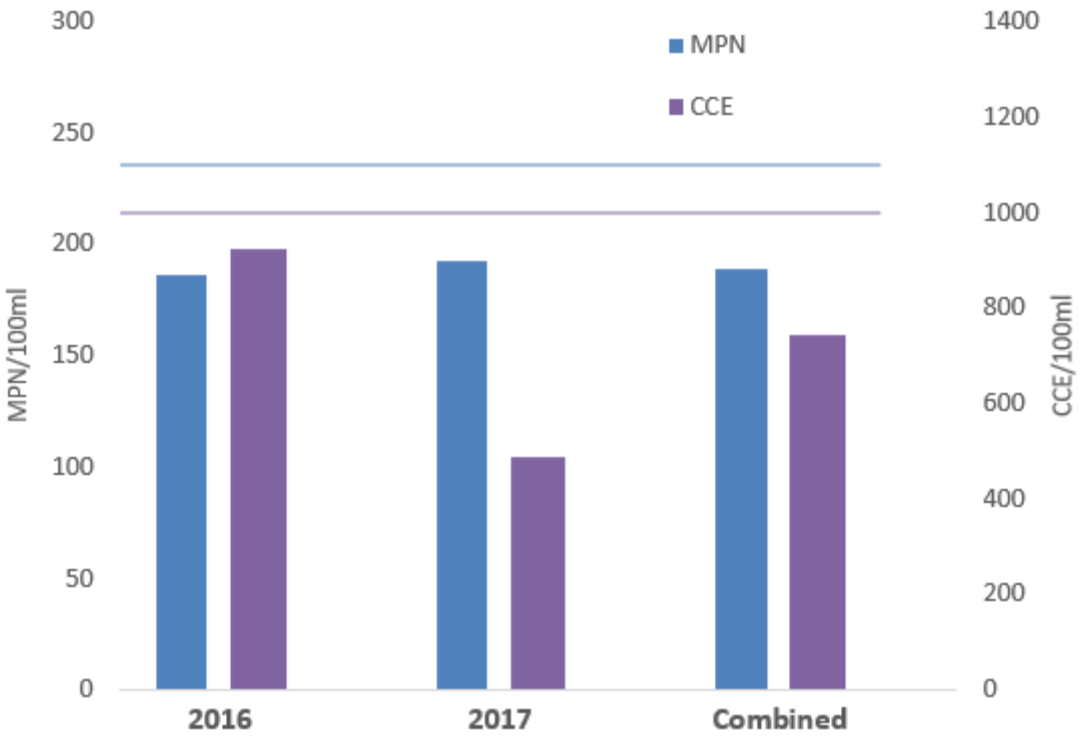

Figure 22. Mean concentrations of *E. coli* for both Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Red Arrow Beach for 2016 (N= 20), 2017 (N=14) and combined summers (N=34). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

Otumba Beach

Located near downtown Sturgeon Bay, Wisconsin Otumba Beach had a historical *E. coli* MPN/100mL mean of 139.2, which was lower than the average from this study, 197.6 (Table 3). Sturgeon Bay has a population of 9,144 people with a density of 30,287 persons/km^2. Otumba had the highest mean value of any beach from this study. The *E. coli* MPN/100mL average for Otumba in 2016 was above the threshold for an advisory at 261.2 MPN/100mL, yet the average for 2017 was significantly less (132.3 MPN/100mL) giving the average for both summers at 189.2. When looking at the results from qPCR Method C, Otumba had eight closures with Method C instead of one closure with Colilert®. Four false positives and one false negative were seen when measuring CCE/100mL giving it a method agreement value of 86.8% (Table 7). Otumba Beach experienced the least amount of inhibition at only one occurrence per year (Figure 18). Otumba had four outliers with the Colilert® method and five outliers with qPCR Method C (Figure 14 and 15). Measurements with qPCR Method C resulted in CCE/100mL averages above the *E. coli* CCE/100mL advisory threshold for both summers at 1050.4 for 2016 and 1268.0 for 2017 (Figure 24).

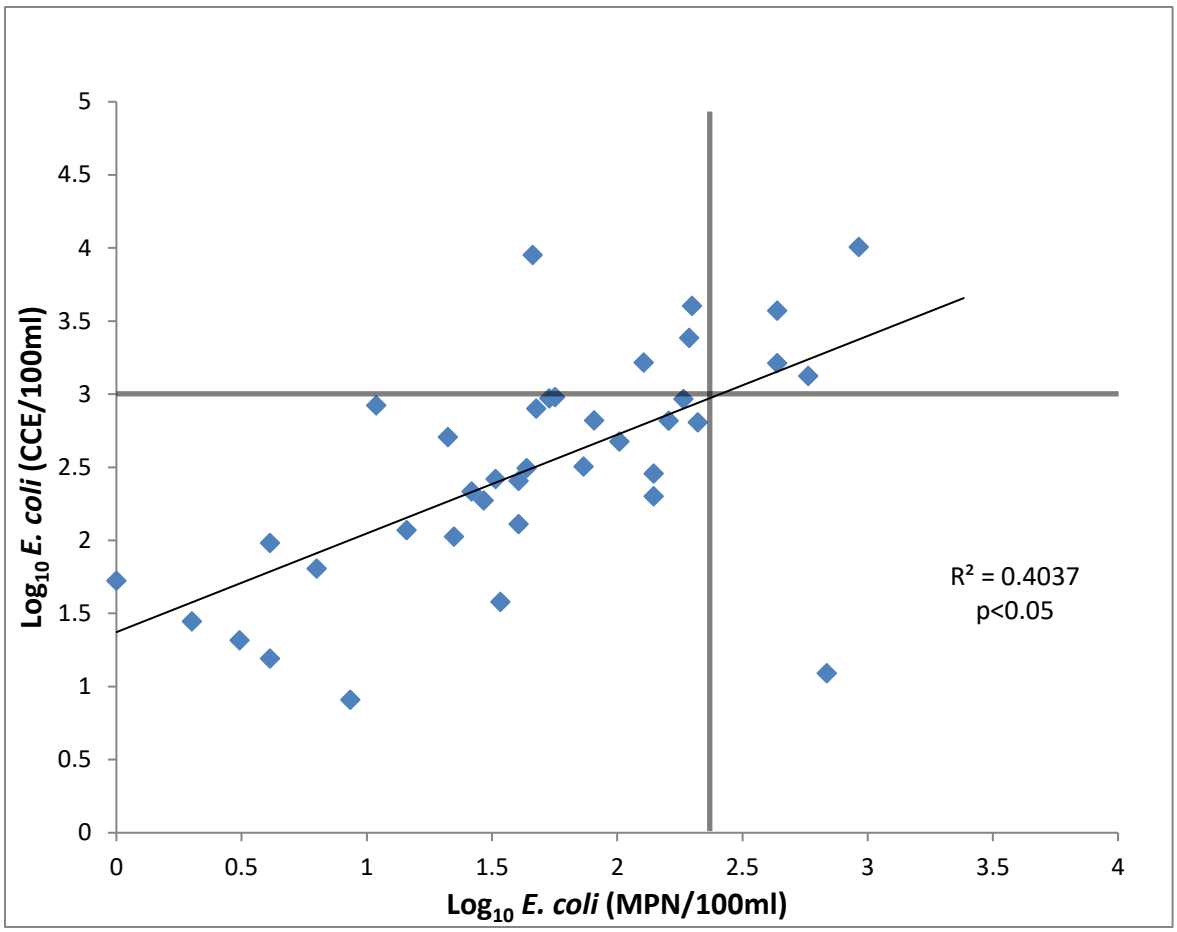

Figure 23. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Otumba Beach. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N=38.

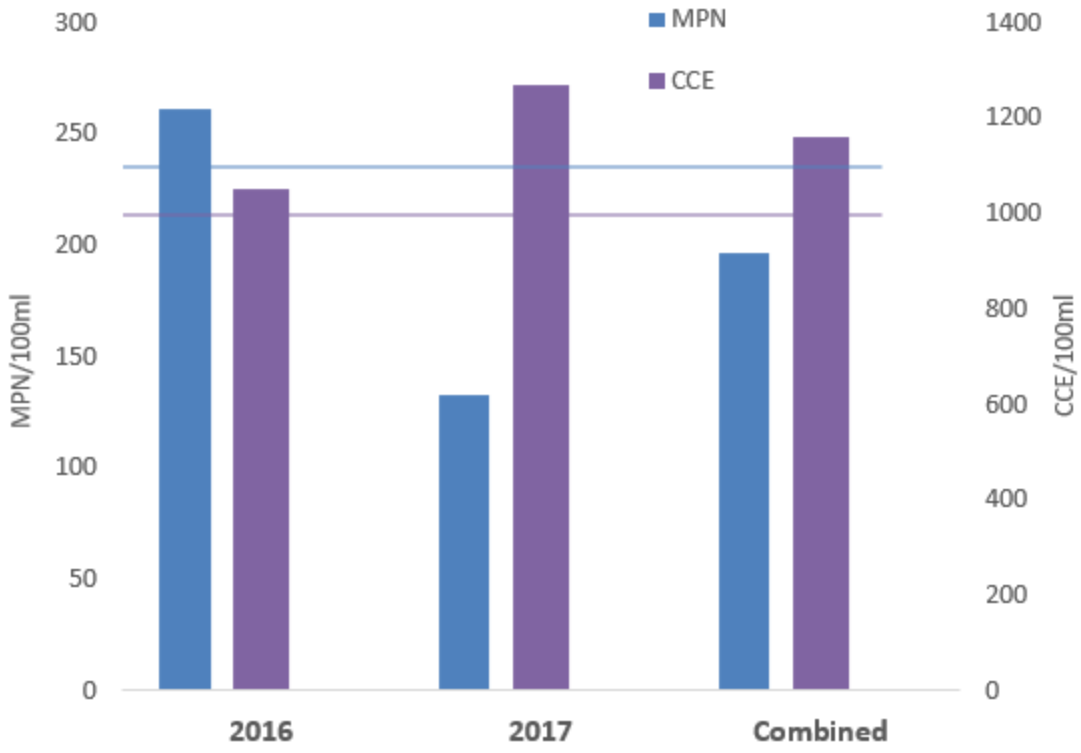

Figure 24. Mean *E. coli* concentrations for Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Otumba Beach for 2016 (N=19), 2017 (N=19) and combined summers (N=38). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

Ephraim Beach

Located in Ephraim, Wisconsin, Ephraim Beach had a historical *E. coli* concentration mean of 78.7 MPN/100 ml and a mean of 104.0 for this study (Table 3). Ephraim has a population of 288 people with a density of 1,554 persons/km^2. Ephraim had the lowest *E. coli* concentration means of the high/moderate beach groups. However, Ephraim beach had the highest IA value at 0.7, giving this Beach a sufficient value for using qPCR as an alternative method (US EPA, 2014) (Table 2). Ephraim had five outliers with Colilert® and four outliers with qPCR Method C (Figure 14 and 15). Ephraim experienced one false positive and three false negatives when using qPCR Method C giving it a method agreement value of 88.2% (Table 7). No patterns with observational data and *E. coli* concentration were seen with Ephraim Beach.

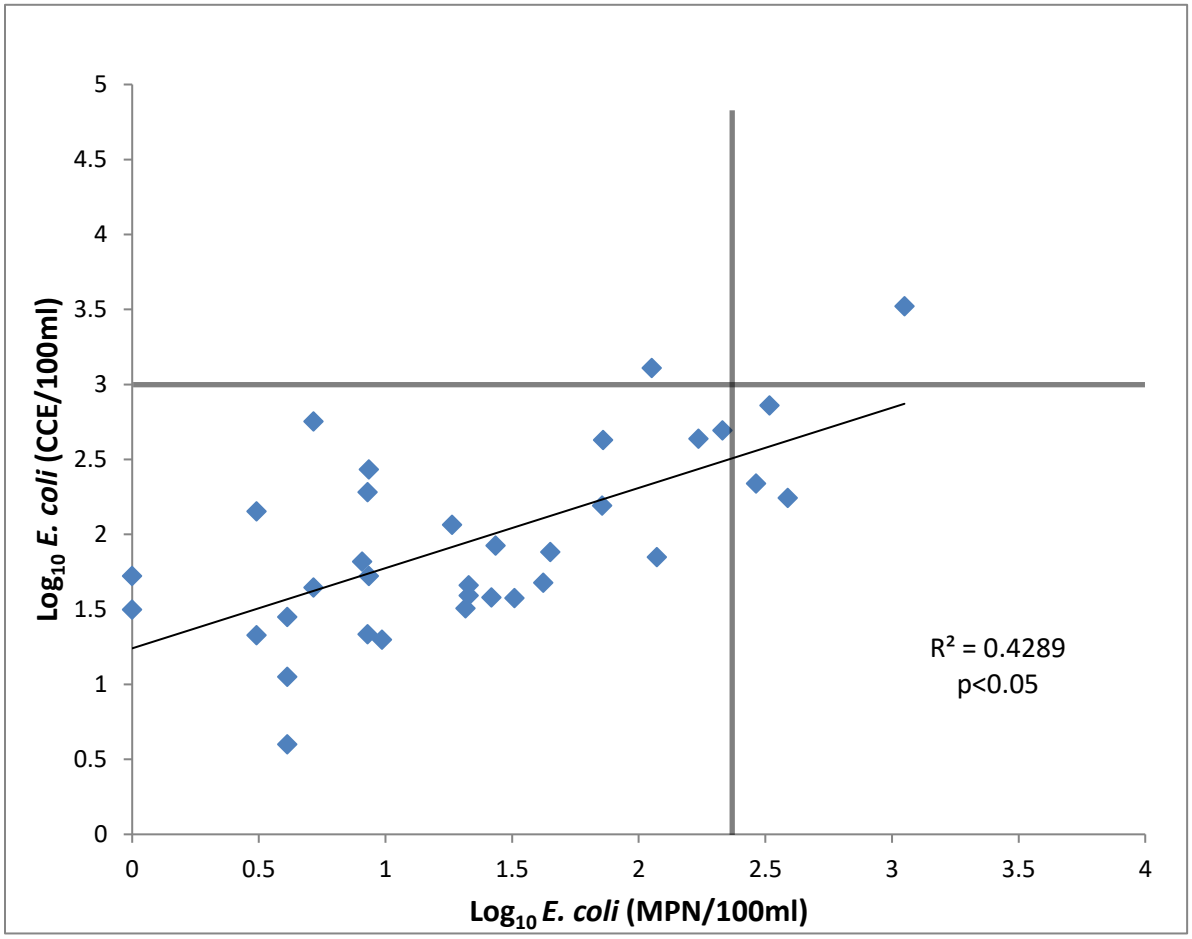

Figure 25. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Ephraim Beach. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 34.

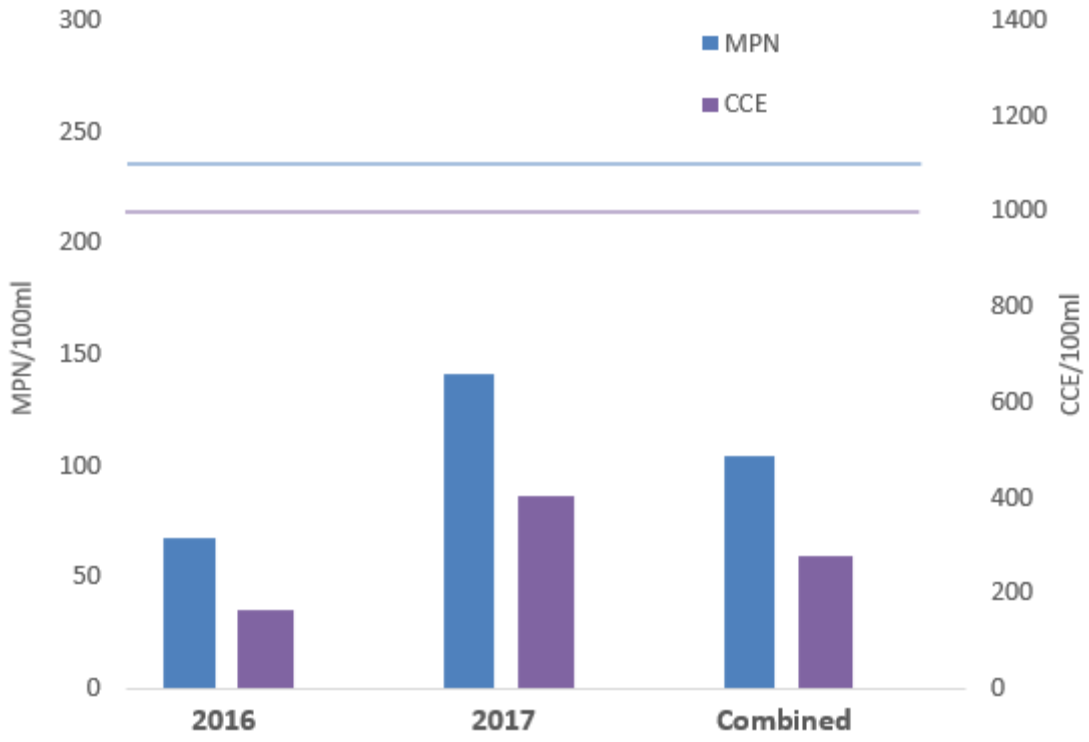

Figure 26. Mean *E. coli* concentrations for Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Ephraim Beach for 2016 (N=18), 2017 (N=16) and combined summers (N=34). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

Ellison Bay Beach

Located in Ellison Bay, Wisconsin, Ellison Bay Beach had a historical *E. coli* concentration value of 36.7 MPN/100mL, which dropped to 15.9 (Table 3). The population of Ellison Bay is 165 people with a density of 24 persons/km^2. The mean value for *E. coli* concentrations using qPCR was 82.3 CCE/100mL, giving Ellison Bay Beach the lowest average *E. coli* concentration for both methods tested (Figure 28). Ellison Bay experienced 17 non-detections with the Colilert® method, which was the most of all beaches (Figure 27). The correlation between Colilert® and qPCR Method C was the lowest for Ellison Bay at 0.28 R^2 and 0.44 IA (Table 2). Ellison Bay had two outliers for Colilert® and two outliers for Method C, yet these outliers had the smallest values when compared to other beaches (Figure 14 and 15). One false negative occurred for Ellison Bay Beach giving it a method agreement value of 97.3% (Table 7). Wave height was high with Ellison Bay beach in 2017, yet low concentrations of *E. coli* were seen for those same dates (Appendix A). Ellison Bay had no closures with either methods and only one advisory with Colilert® (Table 5 and 6).

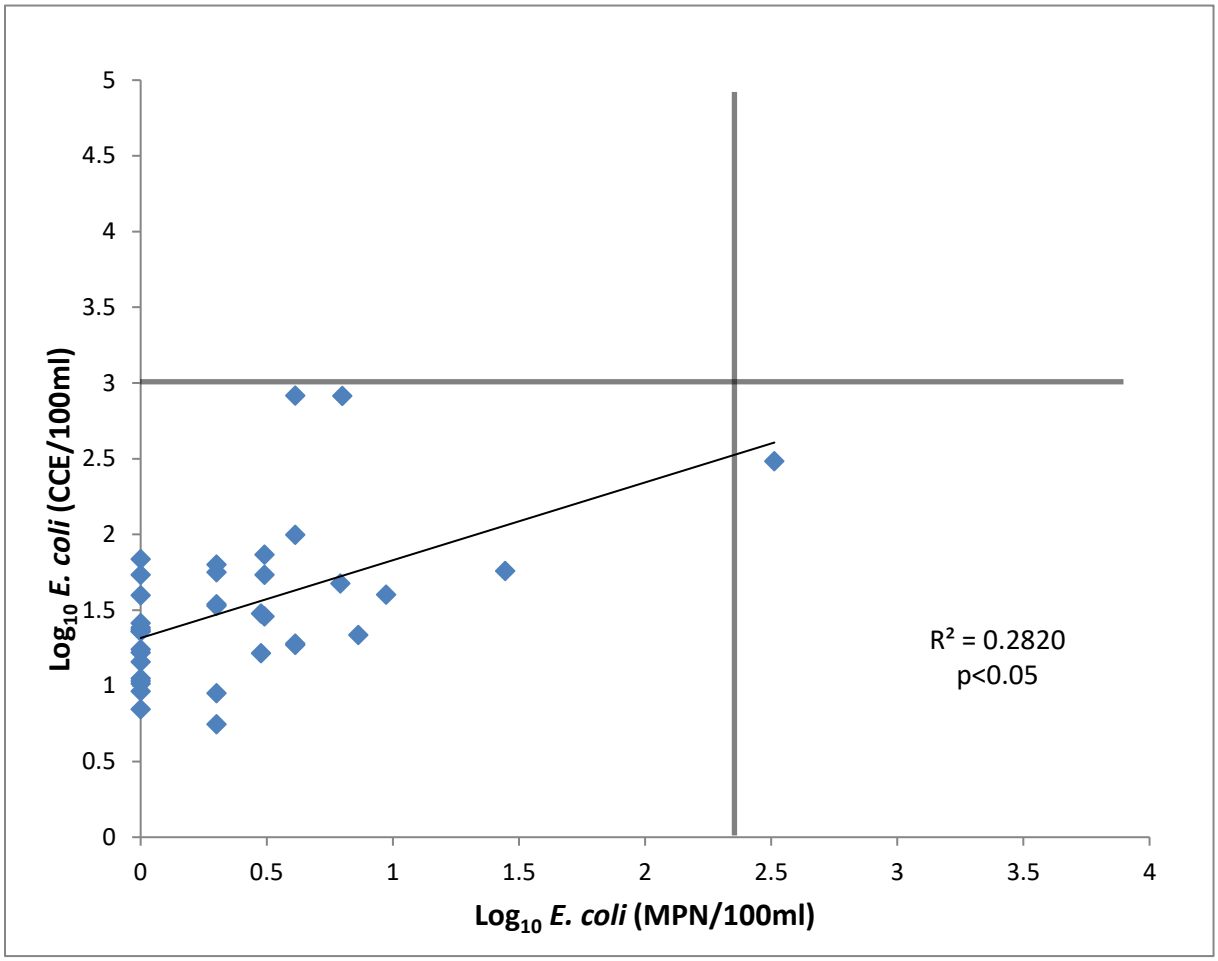

Figure 27. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Ellison Bay Beach. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 37.

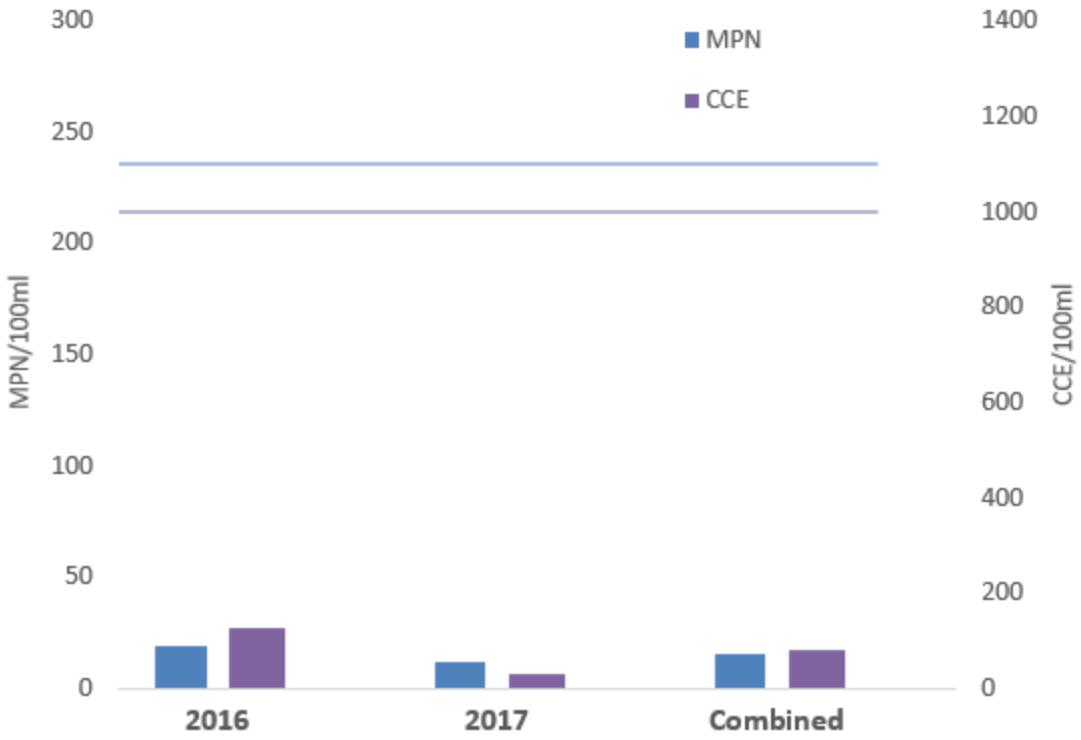

Figure 28. Mean *E. coli* concentrations for Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Ellison Bay Beach for 2016 (N=20), 2017 (N=17) and combined summers (N=37). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

Europe Bay Beach

Europe Bay Beach located in northern Door County Wisconsin is the most rural sampling site for this study, located within Europe Bay Woods State Natural Area. The historical *E. coli* concentration average for this beach using Colilert® was 42.2 MPN/100mL (Table 3). The mean *E. coli* MPN/100mL increased to 66.4 for this study. Europe Bay had the 2nd lowest R^2 at 0.30 (Figure 29). Europe Bay experienced one major spike in *E. coli* concentration (1986.3 MPN/100mL) on July 18th, 2016, which greatly affected the total average (Figure 11). This resulted in the only closure that Europe Bay experienced for this study. The only factor potentially explaining this spike was 1.25 inches of rain seven days prior. One false positive was observed at Europe Bay resulting in a method agreement of 97.0% (Table 7). With the Colilert® method, 14 sampling dates resulted in non-detections.

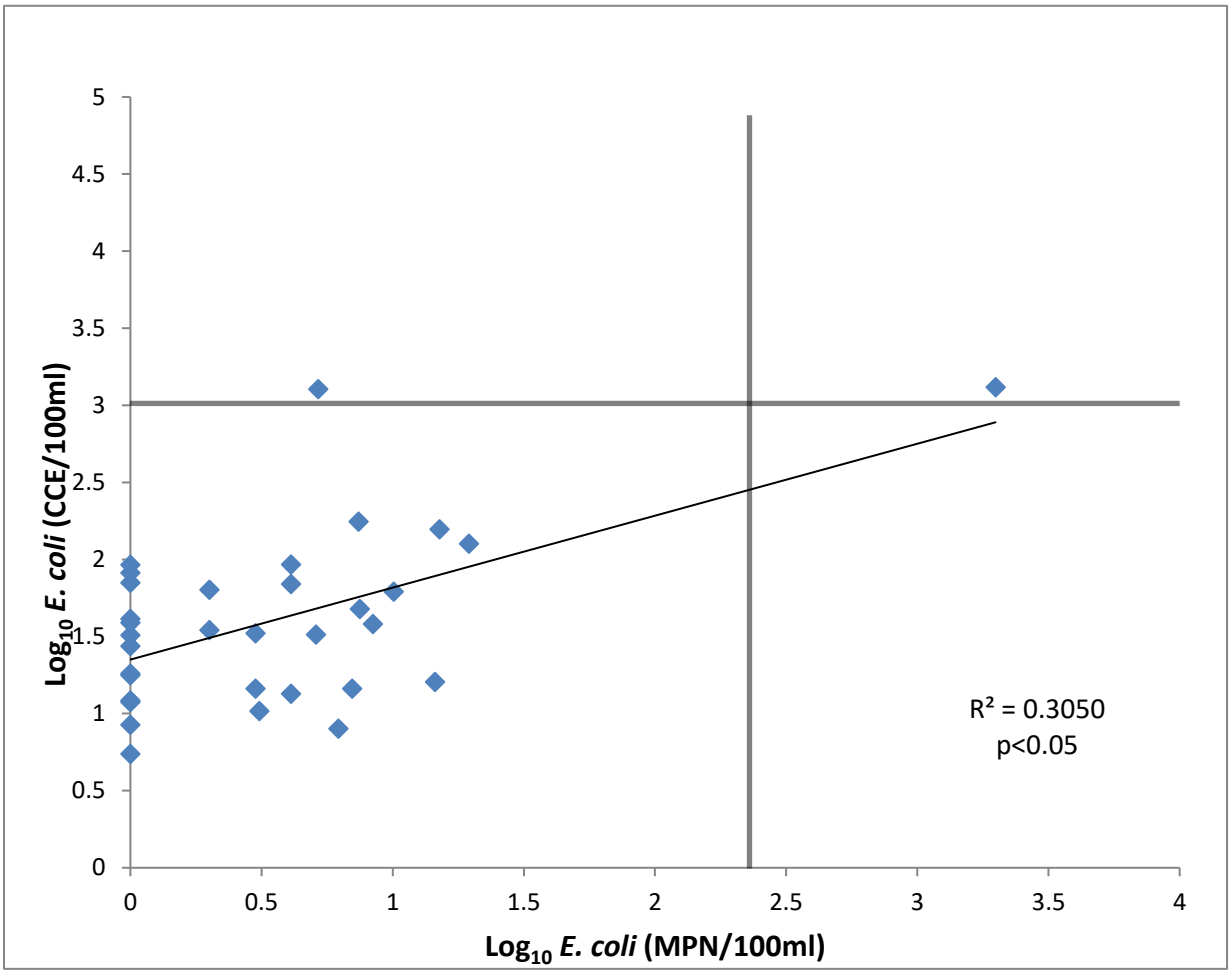

Figure 29. Comparison of Colilert® *E. coli* concentration (MPN/100mL) and qPCR Method C *E. coli* concentrations (CCE/100mL) at Europe Bay Beach. Black lines indicate threshold values for beach advisories for each method showing the amount of true and false negatives. All beach values were transformed into $\log_{10}$ values. N= 34.

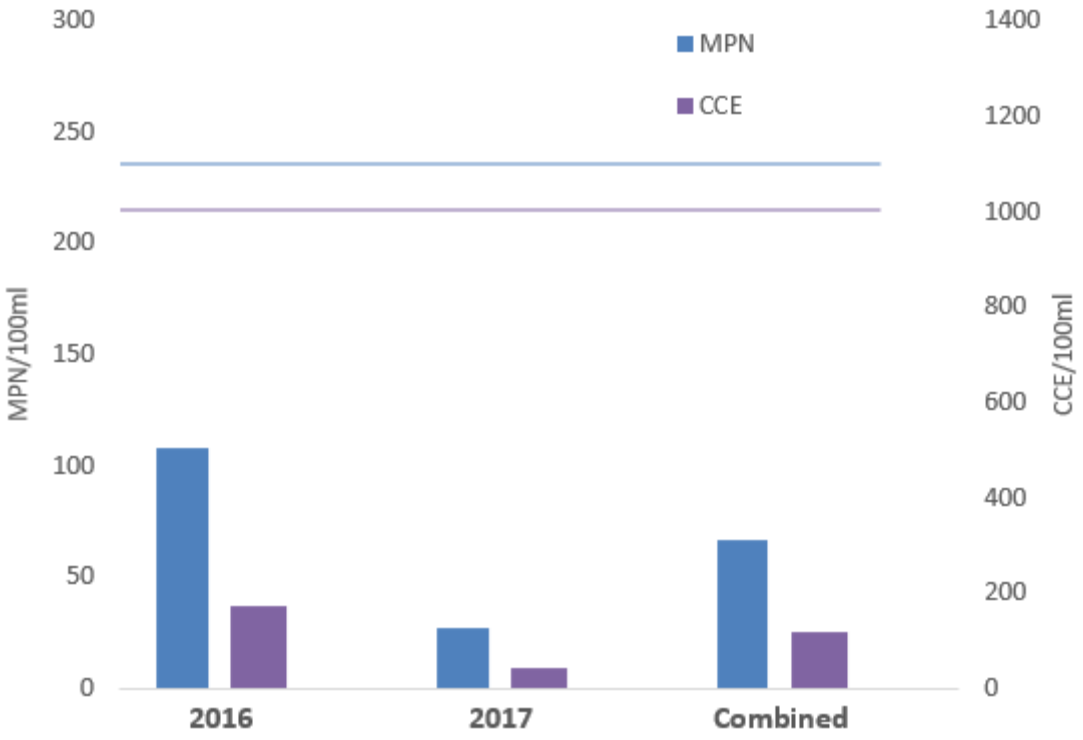

Figure 30. Mean *E. coli* concentrations using the Colilert® (MPN/100mL) and qPCR Method C (CCE/100mL) at Europe Bay Beach for 2016 (N=20), 2017 (N=14) and combined summers (N=34). Colored lines indicate advisory threshold for their respective method (235 MPN/100mL Colilert® & 1000 CCE/100mL qPCR). Combined standard deviations shown in Table 3 and 4.

DISCUSSION

The overarching objective for this study was to evaluate the relationship between Colilert® and qPCR Method C when enumerating *E. coli* in recreational beach water in northeast Wisconsin. The Colilert® method is currently the EPA standard for measuring *E. coli* in freshwater beaches and can only detect living *E. coli* cells. Both methods were successful in detecting *E. coli* concentrations in beach water, but the results indicated that the correlation between methods is better in some locations than at others. The historically high and moderate *E. coli* concentration beaches (Crescent, Red Arrow, Ephraim and Otumba) were grouped in this study based on similar mean and median *E. coli* MPN/100mL values, the number of non-detections with the Colilert® method and the results from the one-way ANOVA and Tukey HSD test. The low *E. coli* concentration beaches (Ellison Bay and Europe Bay) were grouped based on the same criteria. In general, using qPCR Method C tends to be more site specific than using Colilert® when testing *E. coli* concentrations and works better in for water that has a higher total *E. coli* concentration.

Beach Correlations

Sample sites that had higher mean and median *E. coli* concentrations also had a stronger positive correlation between qPCR Method C and Colilert® (Figure 16 and 17). When more *E. coli* cells were present in the beach water, more *E. coli* DNA was able to be extracted and detected, which is what was expected. However, qPCR Method C detected *E. coli* DNA at the low concentration beaches when Colilert® did not detect viable *E. coli* cells. This is because *E. coli* DNA or dead cells were floating in the water in the two low concentration beaches when viable cells were absent, which can also be

considered a false positive. The concentrations of *E. coli* DNA in the low concentration beaches were not great enough to cause an advisory or closure through qPCR (1000 or 1350 CCE/100mL). The higher number of false positives with the qPCR method also resulted in a reduction in the correlation for the low concentration beaches when compared with Colilert®. This is why using qPCR Method C for beaches with higher concentrations of *E. coli* would be more beneficial for the future. Although the values for percent agreement may be high in the lower concentration beaches, those beaches had the lowest method correlation and rarely had beach advisories or closures meaning using Method C would not be as impactful.

Furthermore, the relationship between viable and non-viable *E. coli* (DNA or dead cells) is still not well understood. Testing the ratio of viable microorganisms to nonviable for sampling sites can help predict if a site is prone to high levels of false positives when testing *E. coli* DNA. The ability for qPCR Method C to detect live, dead and floating DNA is why the CCE/100mL values and thresholds are higher (1000 CCE/100ml) for *E. coli* concentration and beach advisories when compared to defined substrate methods (235 MPN/100ml). Otherwise beaches would always be closed or have an advisory if using the same 235 and 1000 criteria used for Colilert®.

Since almost all the beaches studied do not have a high enough correlation coefficient or Index of Agreement, at this point Method C cannot be used as an alternative except at Ephraim Beach according to the US EPA preliminary recommendations. However, measuring beach water using qPCR Method C can still be beneficial, since conditions in beach water fluctuate overtime and sample sites may illustrate better correlations in the future. Thus, re-evaluating sites every three years is

what is recommended by the EPA if an insufficient correlation occurs between both Colilert® and qPCR Method C (US EPA, 2014). The Index of Agreement when compiling all six beaches for both summers is 0.67 and the correlation coefficient is 0.56, which are both just below the needed values produce a beach correlation sufficient to use qPCR Method C (Table 2).

Inhibition

When using qPCR Method C, most of the days throughout each summer (>73%) resulted in successful qPCR reactions. However, inhibition was a problem for some sample sites during parts of the summer. Inhibition of samples could be due to elevated levels of minerals, enzymes or pollutants in the beach water that can affect any step of the qPCR reaction (Pennington, 2014). When re-running the qPCR reaction with -80°C stored samples from the same sample date, inhibition still occurred in some samples, while others were able to be read on the second run. The frequency of inhibition was greater during the 2017 summer with every beach experiencing inhibition at least once during 2017. This could be due to an unknown factor in this study. The water quality of Lake Michigan affected the qPCR results differently in the 2016 beach season showing that water quality in Lake Michigan can be different for each summer season. In 2016 the only beaches to experience inhibition were Crescent, Ephraim and Otumba. Overall, 32 out of the 239 samples analyzed with both qPCR Method C and Colilert® experienced inhibition, or approximately 13.4% of samples.

Crescent Beach had the most occurrences of inhibition, hinting at a possible source of pollution from a large outfall pipe next to the beach affecting the qPCR reaction

for that sample date. One reason Crescent Beach had more incidents of inhibition is because Crescent Beach is somewhat urban from being located in downtown Algoma. Additionally, there is a large break wall in the water that can restrict water flow. This restriction can cause algae to build up. Based on previous observations, areas with filamentous green algal blooms may result in target non-detection (Aslan, 2015). During membrane filtration, Crescent Beach consistently had algae blocking the polycarbonate membrane 0.45 µm pores. Additionally, storm water from Algoma can be flowing into the Crescent Beach water supply as well affecting the qPCR reaction.

Other Factors Influencing Results

When comparing water temperature, wave height and rainfall with *E. coli* MPN/100mL and CCE/100mL, only rainfall had a strong correlation with *E. coli* concentration spikes. As temperatures increased for the summers, *E. coli* concentrations did not increase, indicating that external factors introduced into the beach water are likely the cause of contamination. It is possible, that for some sites, wave height had an impact, such as in Crescent, Red Arrow or Ephraim. However, wave height did not have a significant impact on *E. coli* concentration for all beaches. This could mean wave height may play a role in mixing contaminants in the water, or at the sand water interface, where some types of beach sand may be affected more than others. Previous studies have indicated that if concentrations of *E. coli* are located in the foreshore sand, wave intensity can be a contributor into the surface water (Vogel *et al*, 2016). After a rain event beach spikes were more likely to happen. This has been shown in previous studies (Ackerman

and Weisburg, 2003). This is most likely due to chemical or biological runoff into the water supply.

Physical factors at the beach site also play a role in *E. coli* beach contamination. Outfall pipes, jetties, geography and the current of Lake Michigan all have a role in affecting *E. coli* concentrations or the success of qPCR reaction. A possible reason why Otumba Beach had the highest MPN/100mL and CCE/100mL averages for this study is because a storm water discharge pipe is located almost in the center of the beach. Beach sites that have had previous remediation projects should be less prone to beach contamination. Shorelines with lower levels of vegetation can have higher levels of pollution enter their water supply. Greenspace around a beach can help reduce the runoff reaching the water (OCW, 2010). Additionally, high levels of urbanization cause more run off, while proximity to wastewater treatment plants can increase waterborne pathogens. For both methods, spikes in *E. coli* concentrations were observed at similar dates for all beaches, meaning factors related to Lake Michigan was affecting all beaches simultaneously (Figure 11 and 12).

Beach Management

The third objective in this study was to determine how well qPCR Method C can be implemented in the future at municipal health departments for routine beach monitoring. When viewing the percent exceedances with Colilert® and qPCR Method C, Method C tends to better protect beaches when using 1000 and 1350 CCE/100mL as advisory and closure thresholds (Table 6). On some occasions, qPCR Method C may result in issuance of an advisory or closure when just high amounts of *E. coli* DNA are present, rather than living cells. Using Method C on beaches with high concentrations of

total *E. coli* would be more beneficial because there is a better correlation between methods and the need for rapid results is greater since people are more likely to get sick in those beaches. Compared to low concentration beaches, many of them are rural and less visited, have cleaner water, and correlation with Colilert® is less. In order to save money, community health departments can maximize their budget if using qPCR Method C with high priority and high *E. coli* concentration beaches (Table 8).

Table 8. Differences between using Colilert® and qPCR Method C when testing recreational beach water.

Method	Sample Cost	Equipment Cost	Hours Until Results	-/+ Comments
Culture Based (Colilert®)	$8-$20	$5,000	18-24	Time Delay in results but easy to do
qPCR Method C	$35-$70	$20,000+	3-5	Expensive and technical but quick

Previous studies have been done in northern Lake Michigan beaches comparing qPCR and Colilert® (Sheth *et al*, 2016). From both their results and results from this study, communities need to evaluate the impact of changing to rapid methods for measuring *E. coli*. If health departments decide to use qPCR Method C for freshwater beach monitoring, it should be known that qPCR is more expensive but retrieves results in 4-6 hours compared to 18-24 hours. The rapid notification can be beneficial in communities where waterborne bacterial outbreaks are more common, or in communities that heavily depend on their recreational water supply as their source of tourism in order

to prevent waterborne outbreaks. Rapid notification of beach advisories and closures can help alleviate the amount of people coming in contact with waterborne pathogens.

FUTURE WORK

- Laboratories within the Great Lakes Beach Association have and will continue sampling numerous Great Lake beach sites comparing the performance of *E. coli* qPCR methods (Haugland *et al*, 2016).
- Research is still needed to determine which Great Lake beaches can use qPCR Method C for *E. coli* as an alternative to the culture-based methods. Multiple laboratories and local health departments are continuing to report results from using qPCR Method C to the US EPA to be able to determine the status of each beach on a site-by-site basis when using these new molecular methods.
- Once beaches on the Great Lakes are studied, future studies can analyze the use of qPCR Method C on inland lakes as well as improve source tracking in communities that experience high levels of beach contamination.

APPENDIX A

PHYSICAL/CHEMICAL DATA

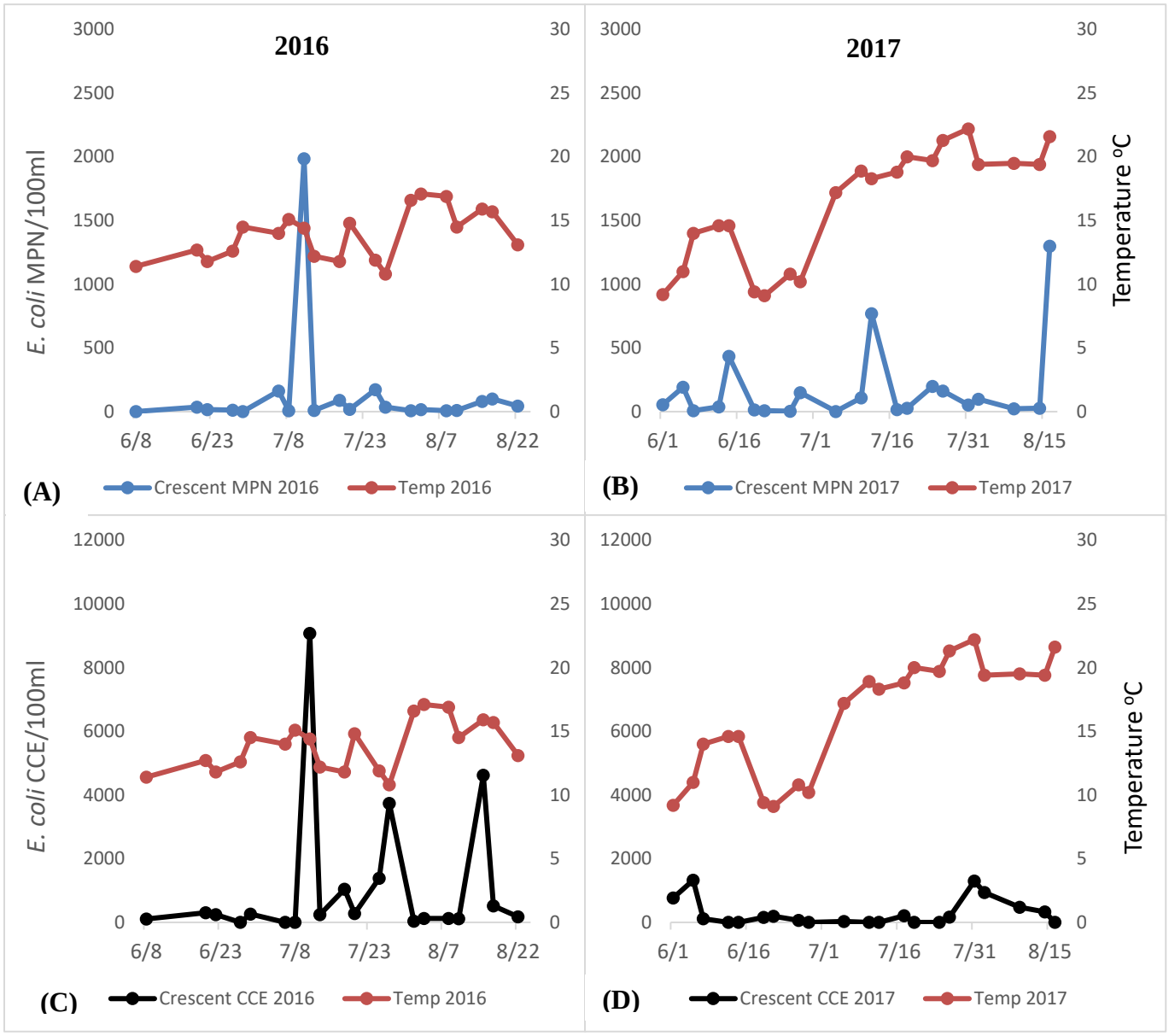

Figure 31. Crescent Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL $R^2 = 0.18$ ($p>0.05$) and CCE/100mL $R^2 = -0.04$ ($p>0.05$).

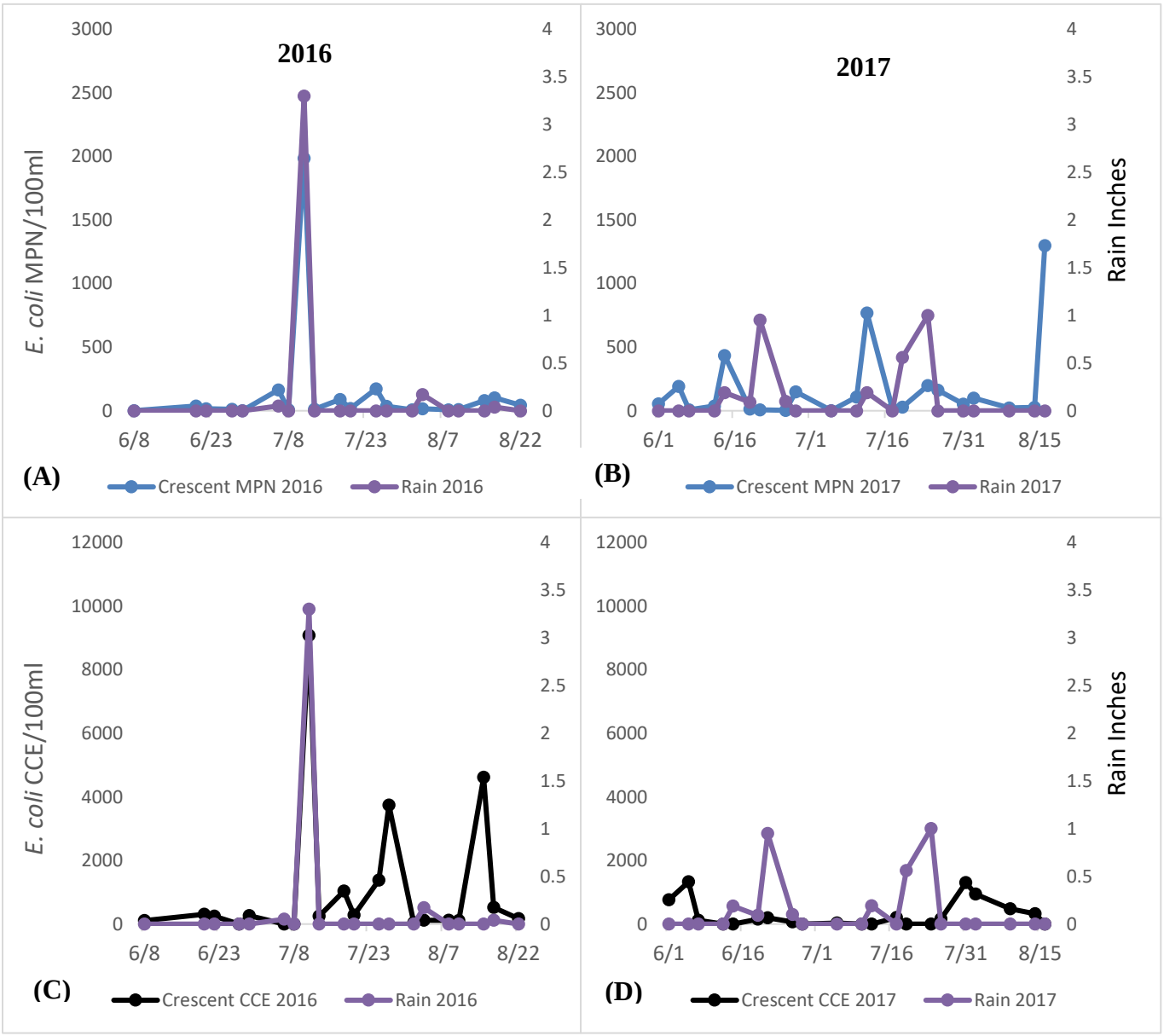

Figure 32. Crescent Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL $R^2 = 0.72$ ($p<0.05$) and CCE/100mL $R^2 = 0.77$ ($p<0.05$).

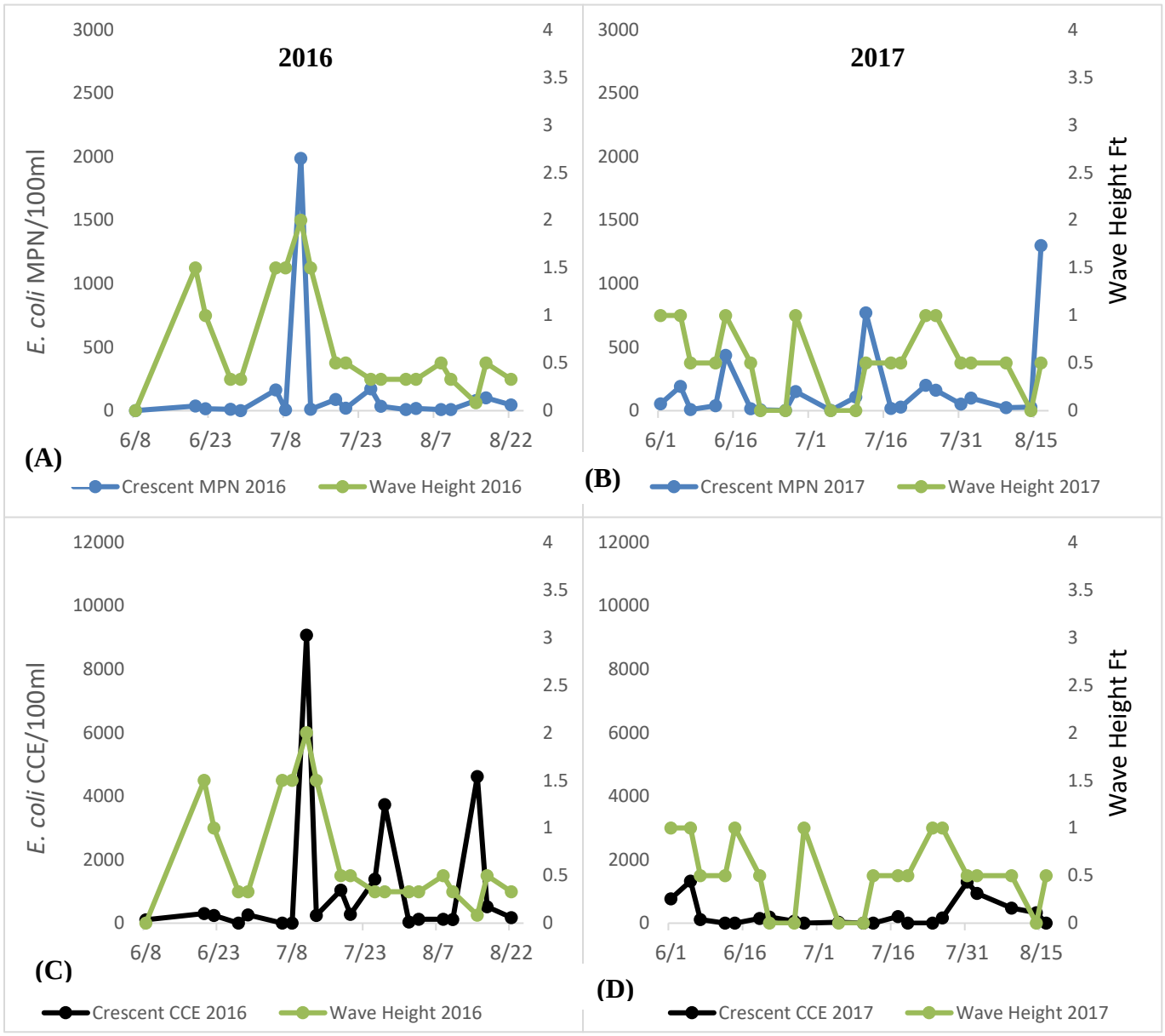

Figure 33. Crescent Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL $R^2 = 0.41$ ($p<0.05$) and CCE/100mL $R^2 = 0.41$ ($p<0.05$).

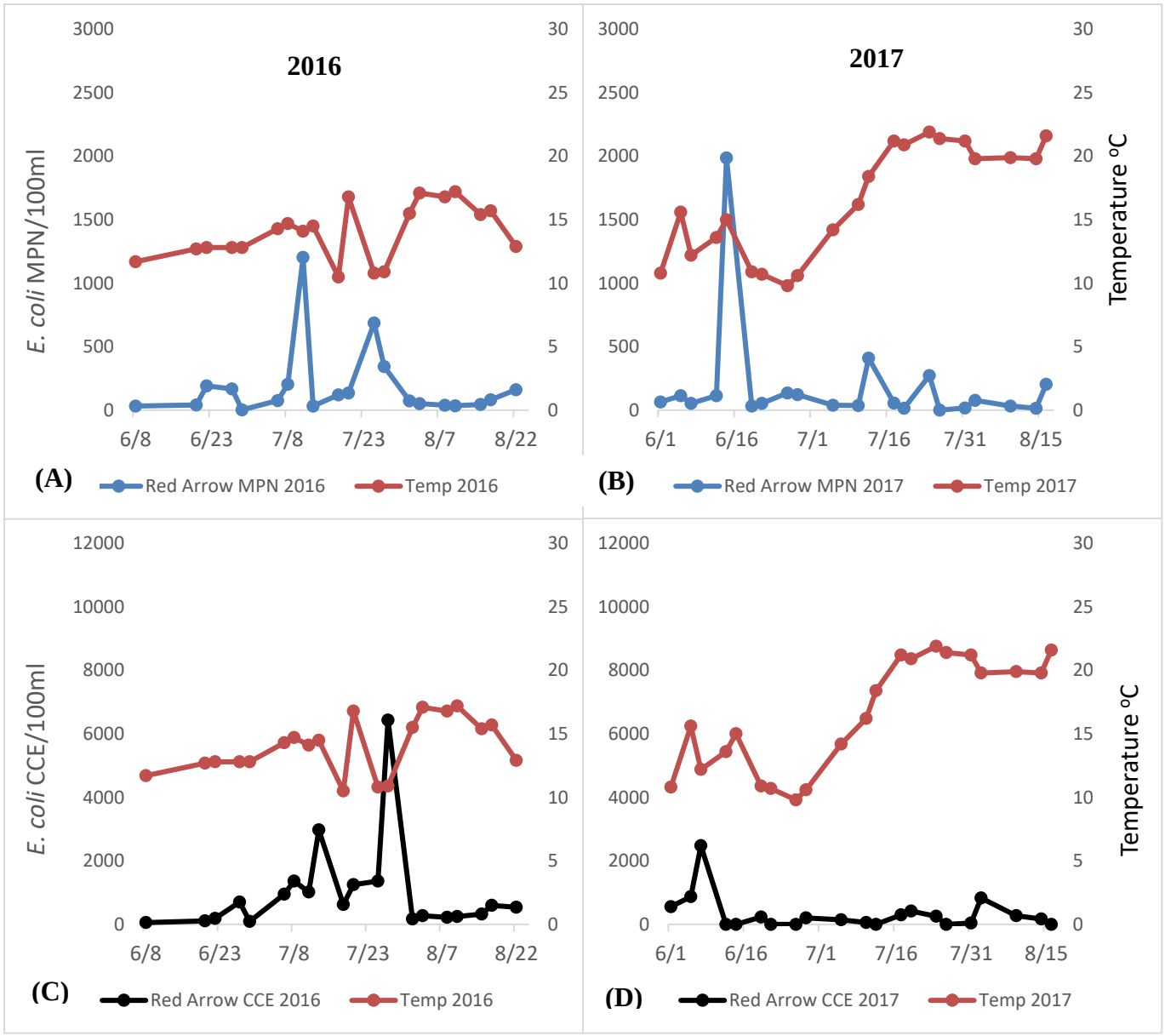

Figure 34. Red Arrow Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL R^2 = -0.08 ($p>0.05$) and CCE/100mL R^2 = -0.29 ($p>0.05$).

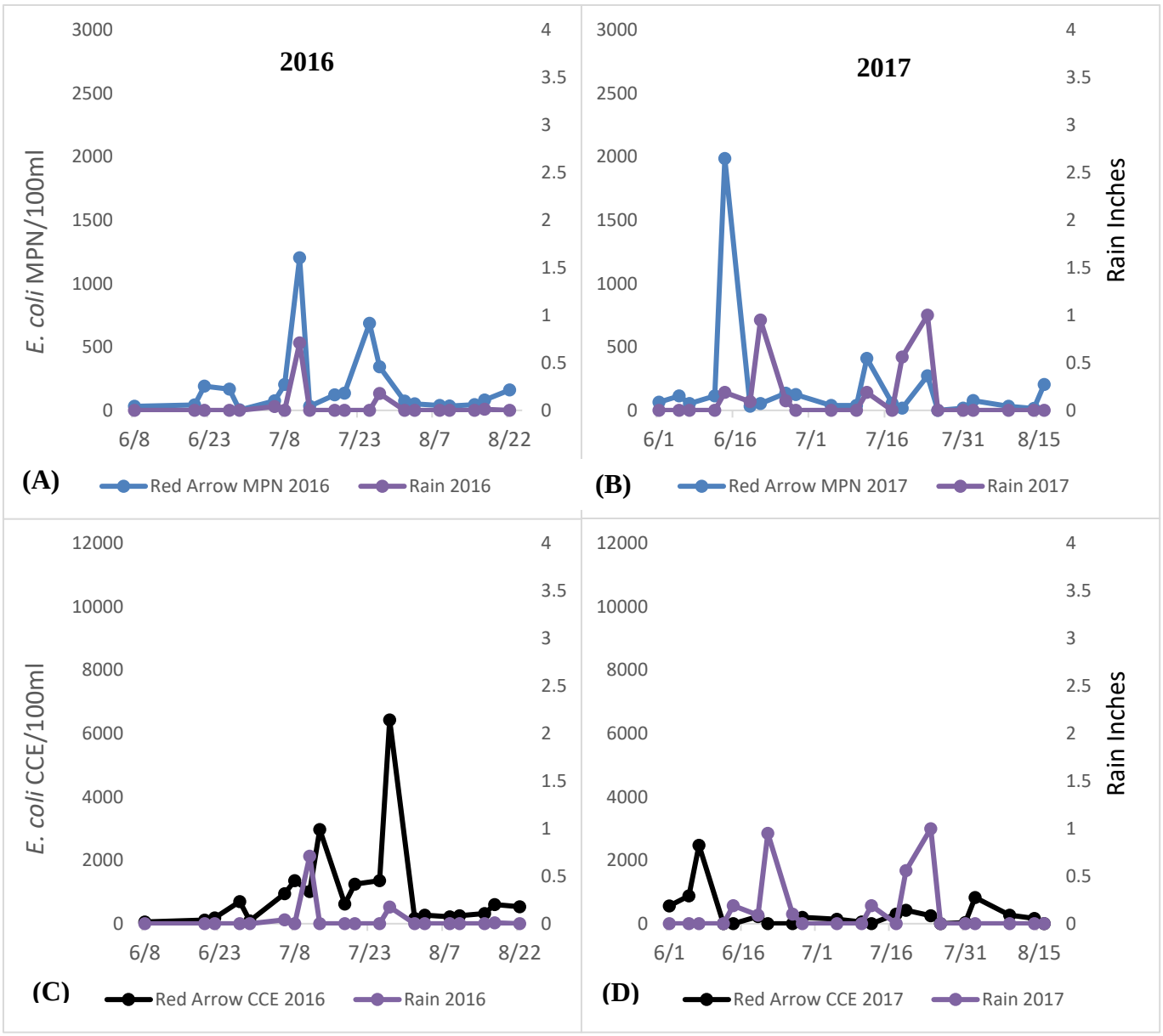

Figure 35. Red Arrow Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL $R^2 = 0.27$ ($p>0.05$) and CCE/100mL $R^2 = 0.05$ ($p>0.05$).

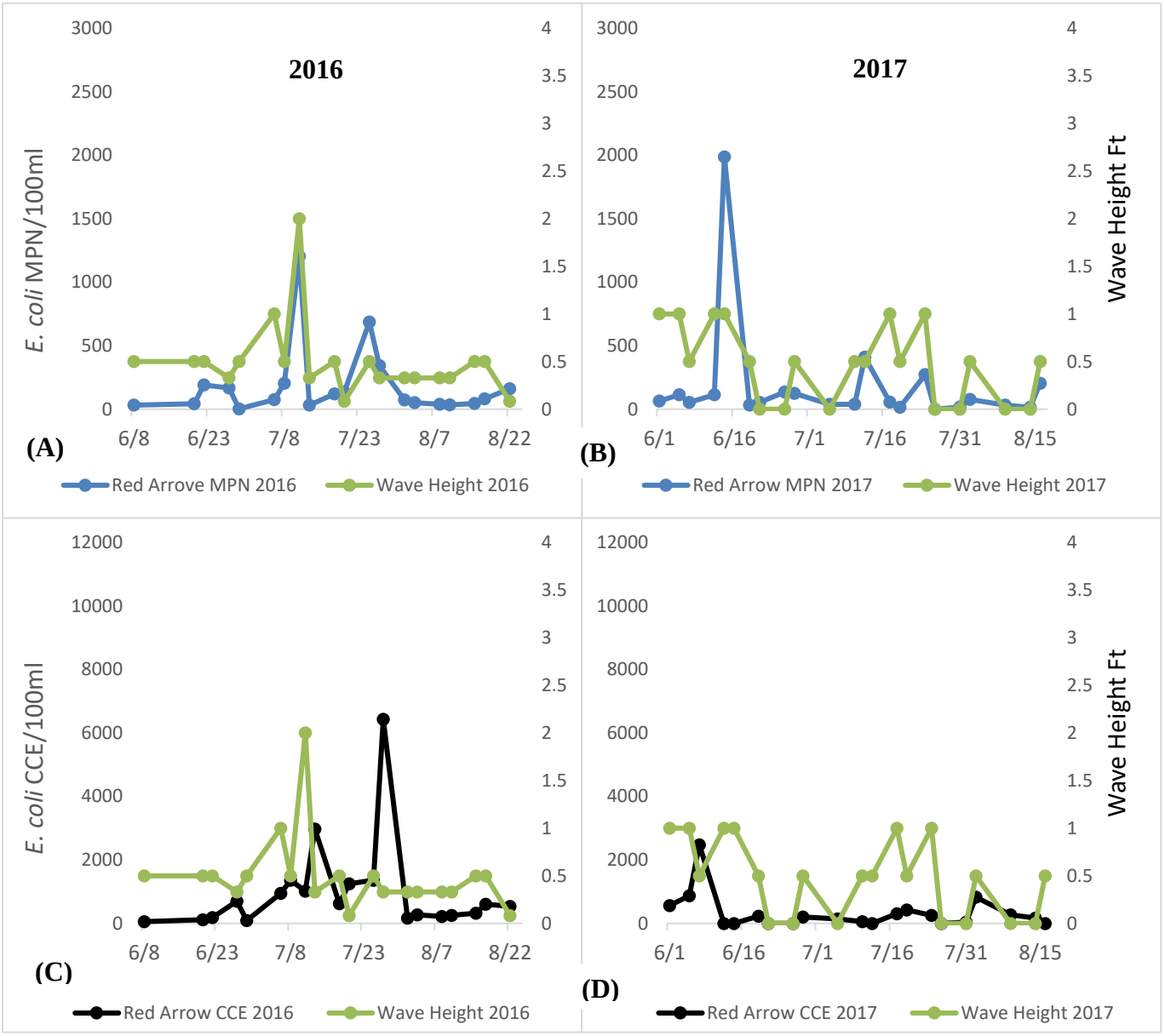

Figure 36. Red Arrow Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL $R^2 = 0.50$ ($p<0.05$) and CCE/100mL $R^2 = 0.01$ ($p>0.05$).

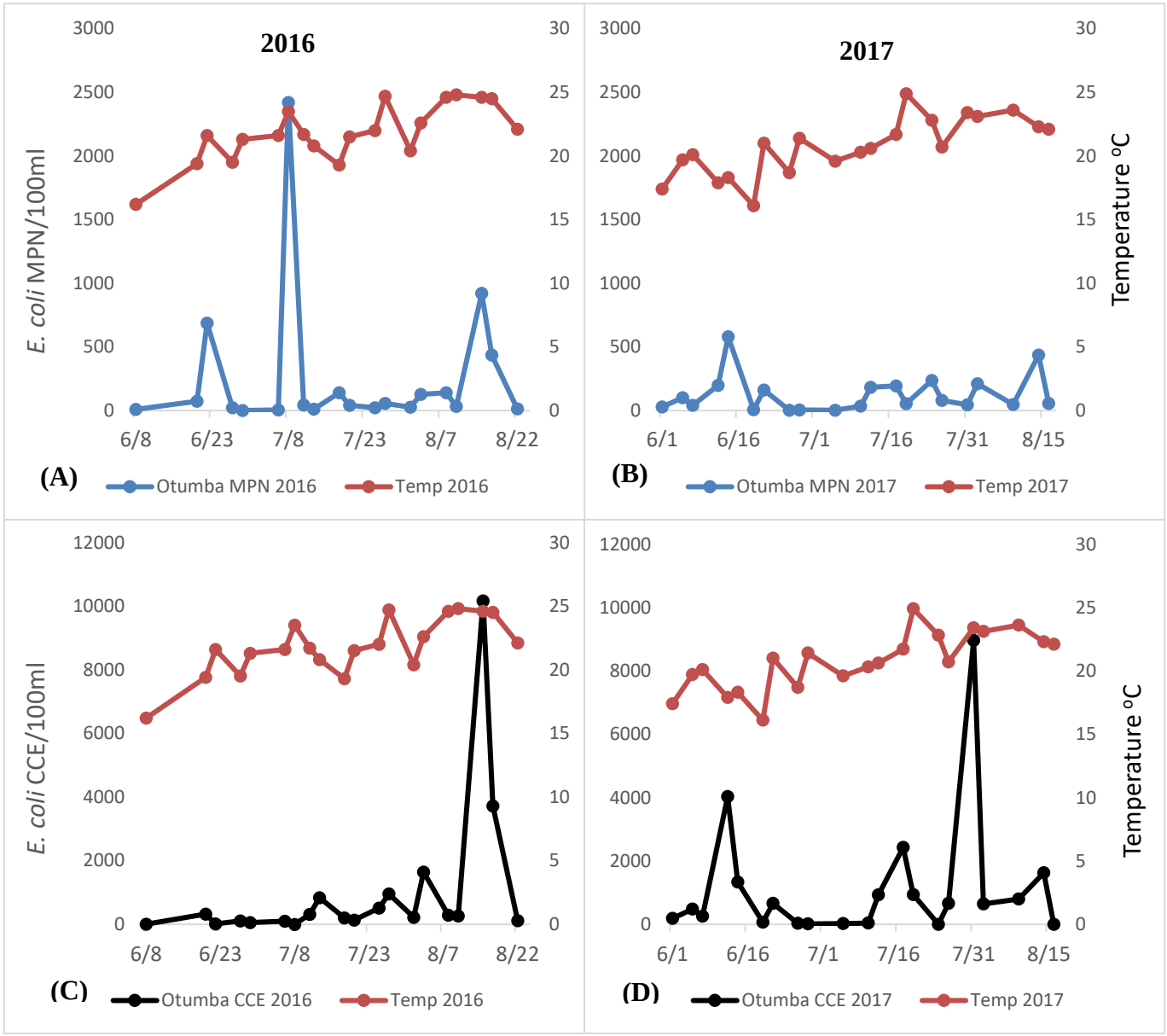

Figure 37. Otumba Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL $R^2 = 0.25$ ($p>0.05$) and CCE/100mL $R^2 = 0.34$ ($p>0.05$).

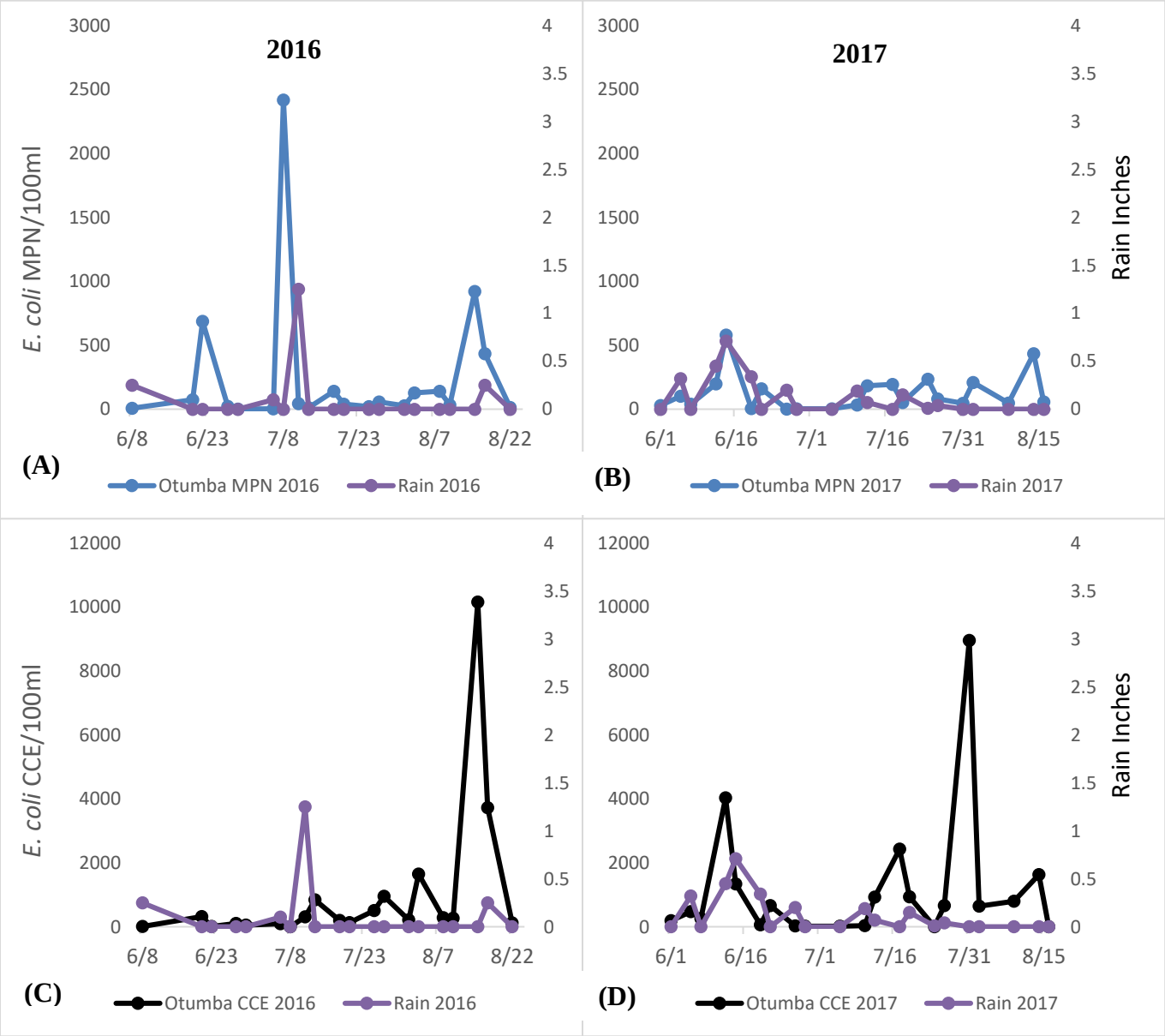

Figure 38. Otumba Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL R^2 = -0.03 ($p>0.05$) and CCE/100mL R^2 = -0.02 ($p>0.05$).

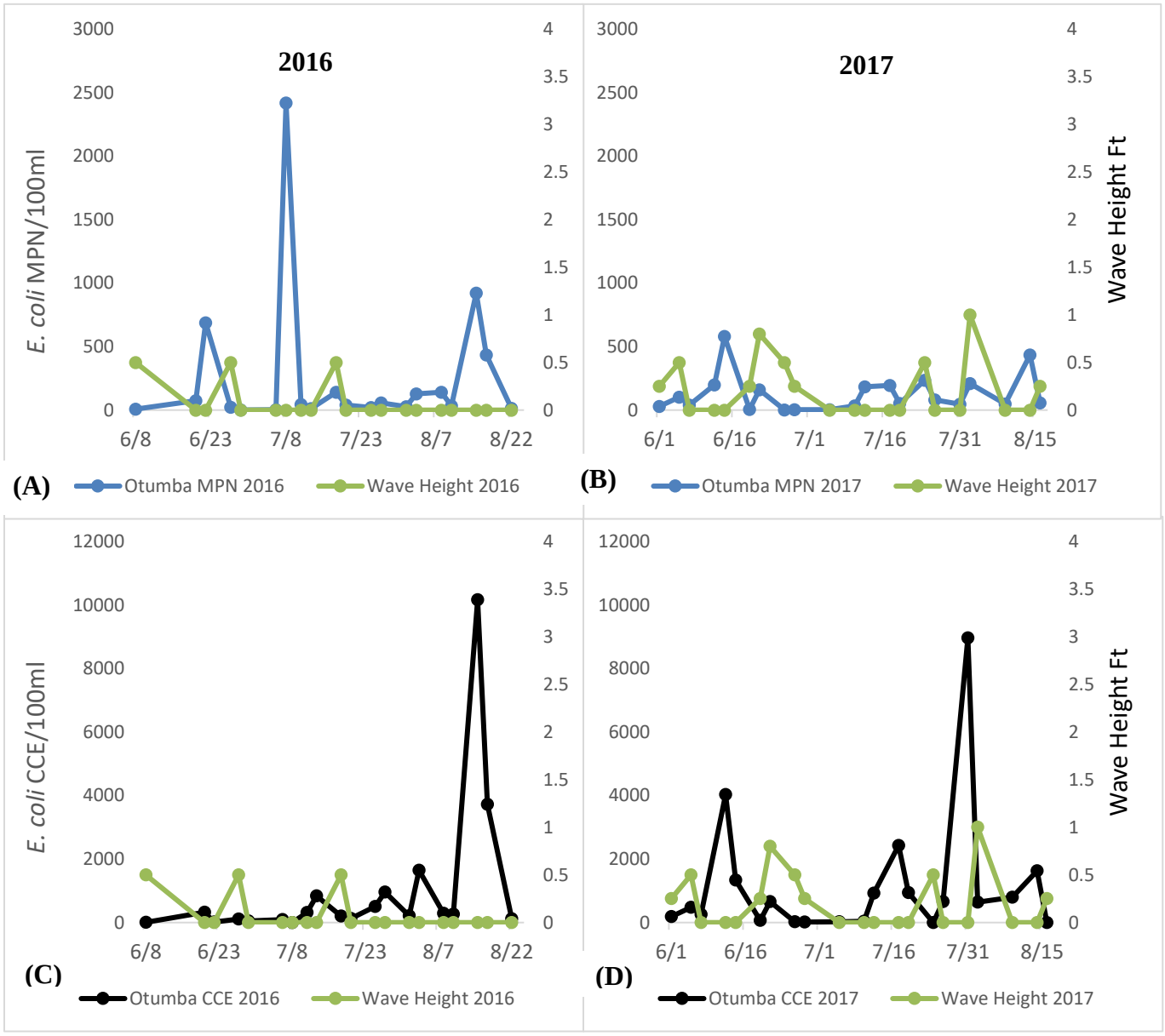

Figure 39. Otumba Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL R^2 = -0.12 ($p>0.05$) and CCE/100mL R^2 = -0.20 ($p>0.05$).

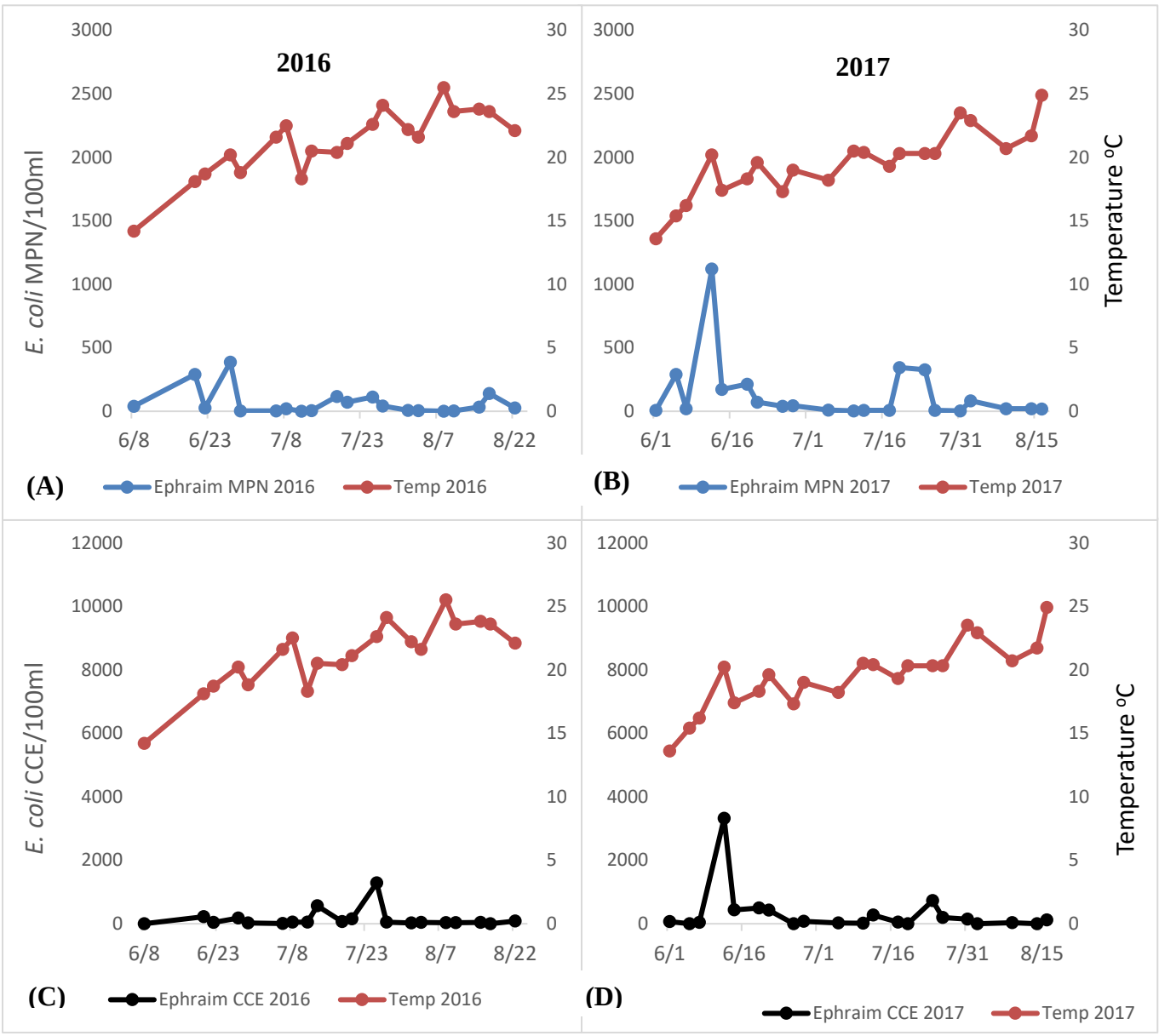

Figure 40. Ephraim Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL R^2 = -0.11 ($p>0.05$) and CCE/100mL R^2 = -0.03 ($p>0.05$).

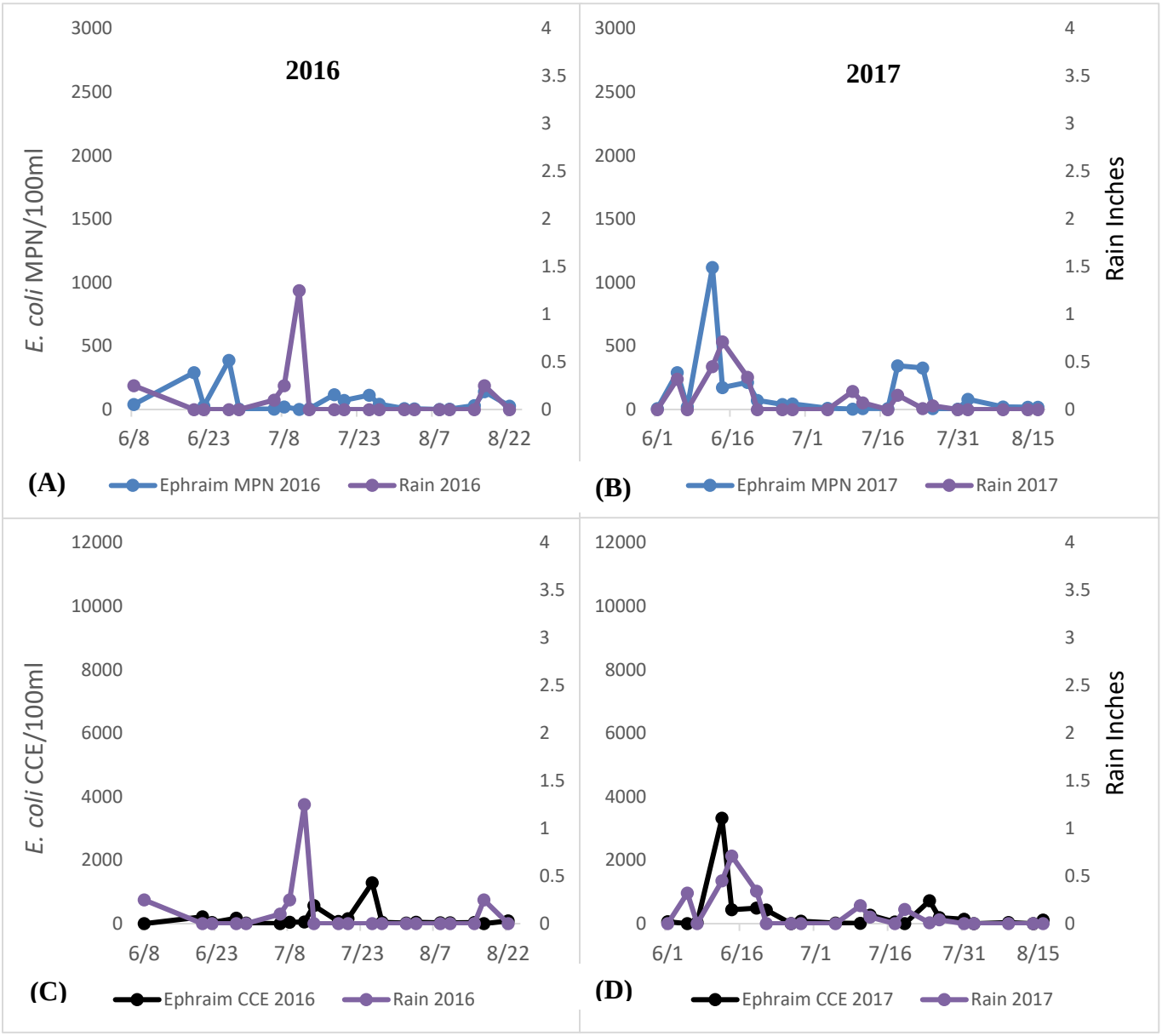

Figure 41. Ephraim Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL $R^2 = 0.24$ ($p>0.05$) and CCE/100mL $R^2 = 0.23$ ($p>0.05$).

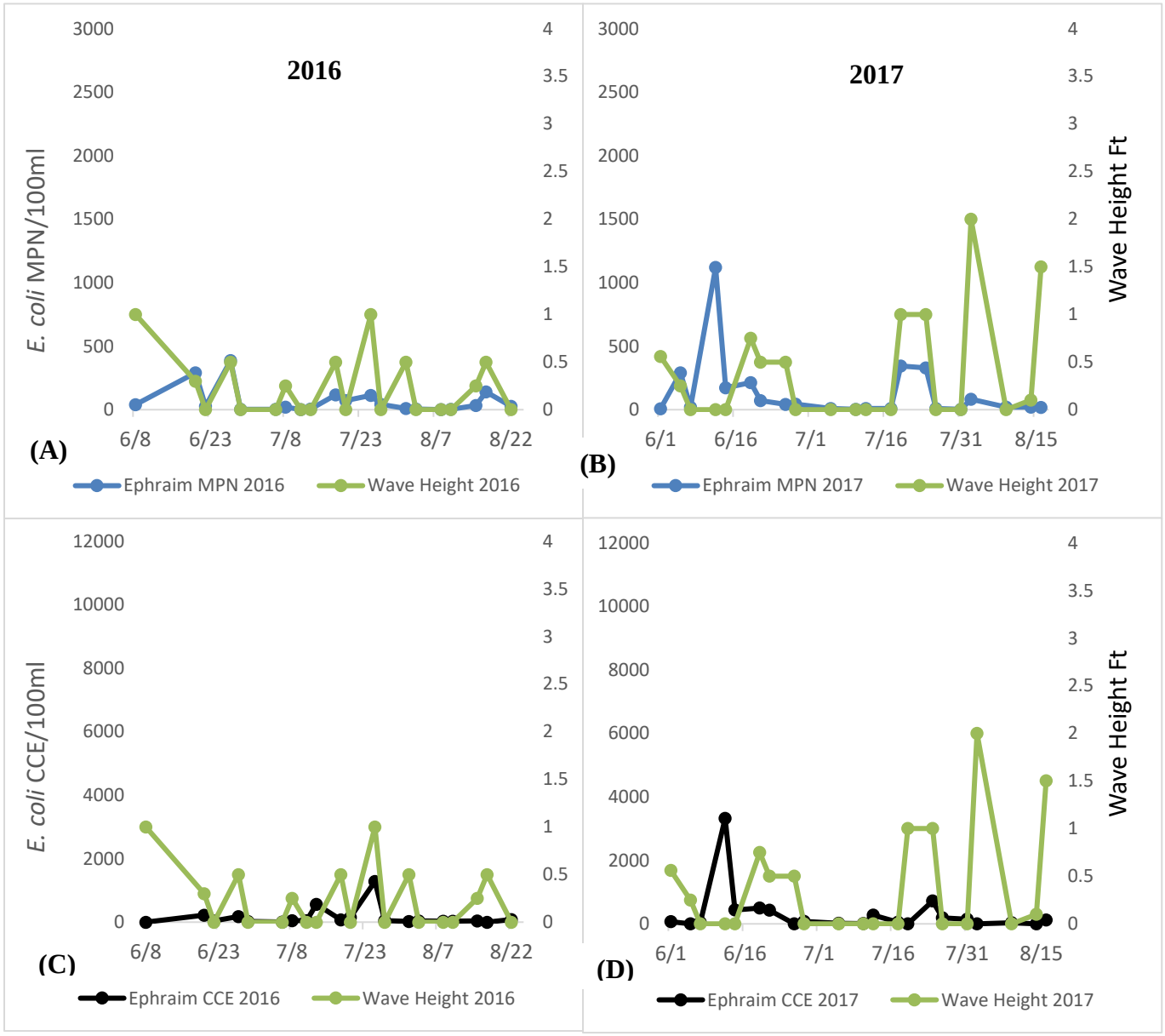

Figure 42. Ephraim Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL $R^2 = 0.11$ ($p>0.05$) and CCE/100mL $R^2 = 0.13$ ($p>0.05$).

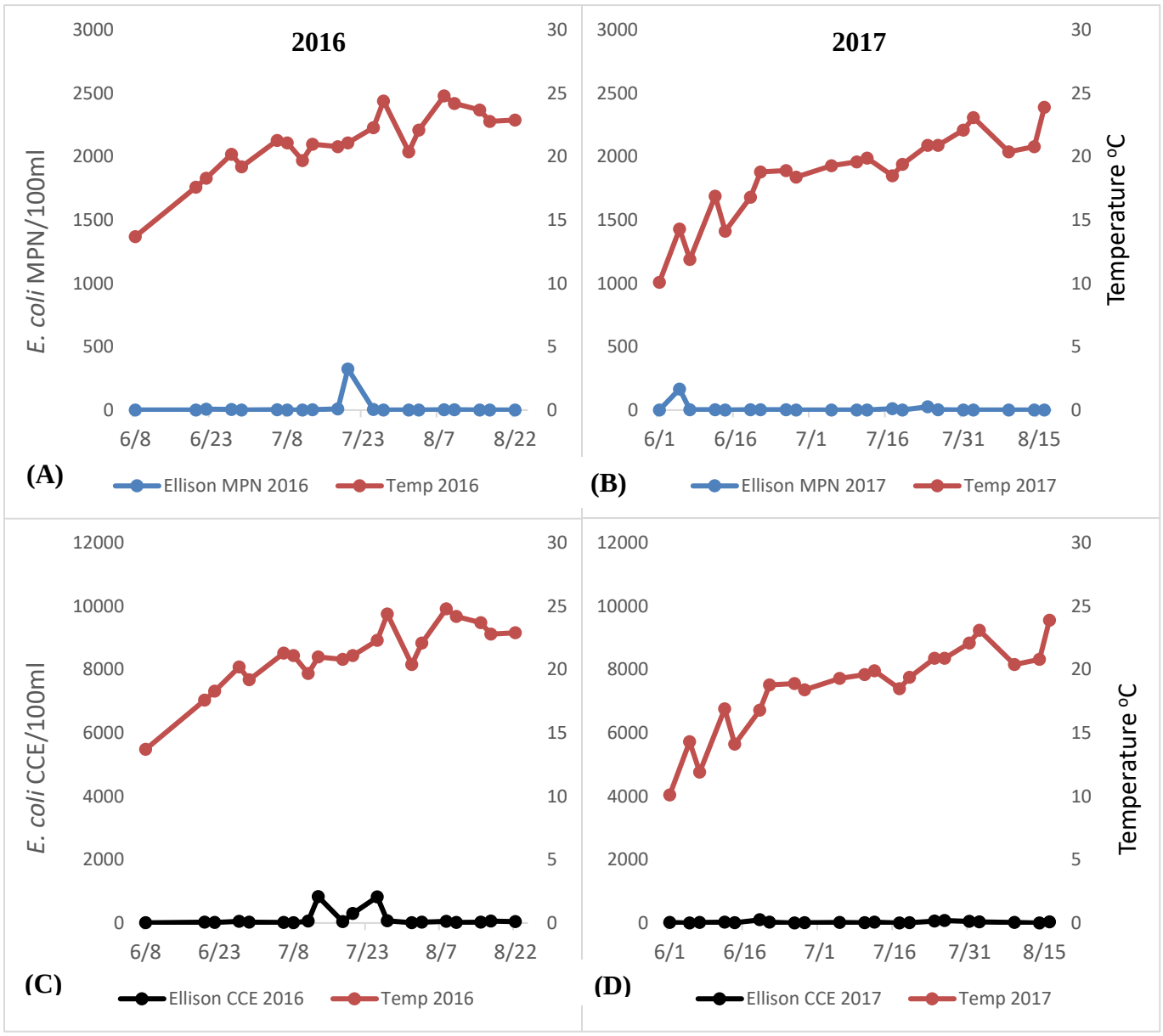

Figure 43. Ellison Bay Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL R^2 = -0.06 ($p>0.05$) and CCE/100mL R^2 = 0.17 ($p>0.05$).

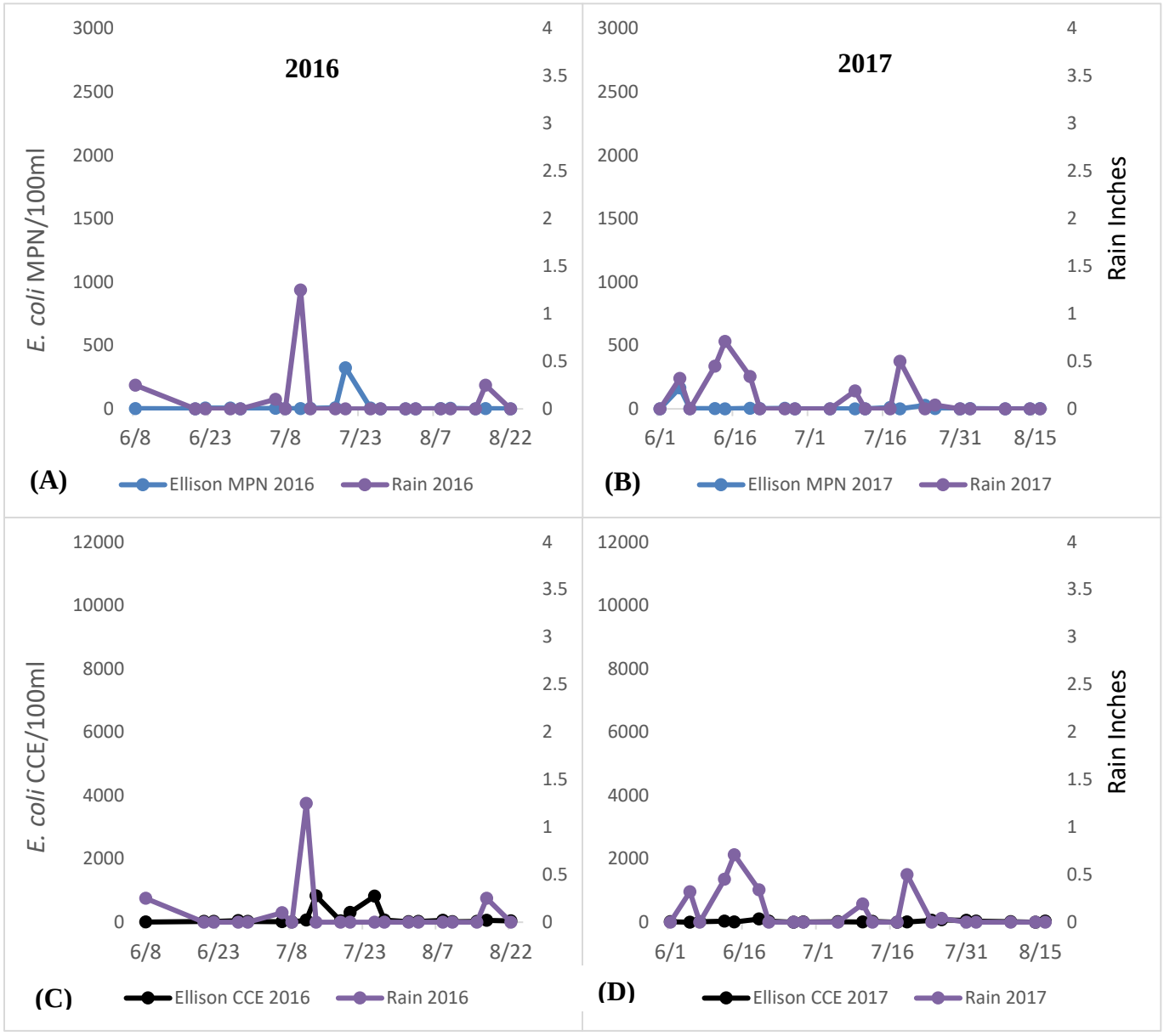

Figure 44. Ellison Bay Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL R^2 = -0.01 ($p>0.05$) and CCE/100mL R^2 = -0.10 ($p>0.05$).

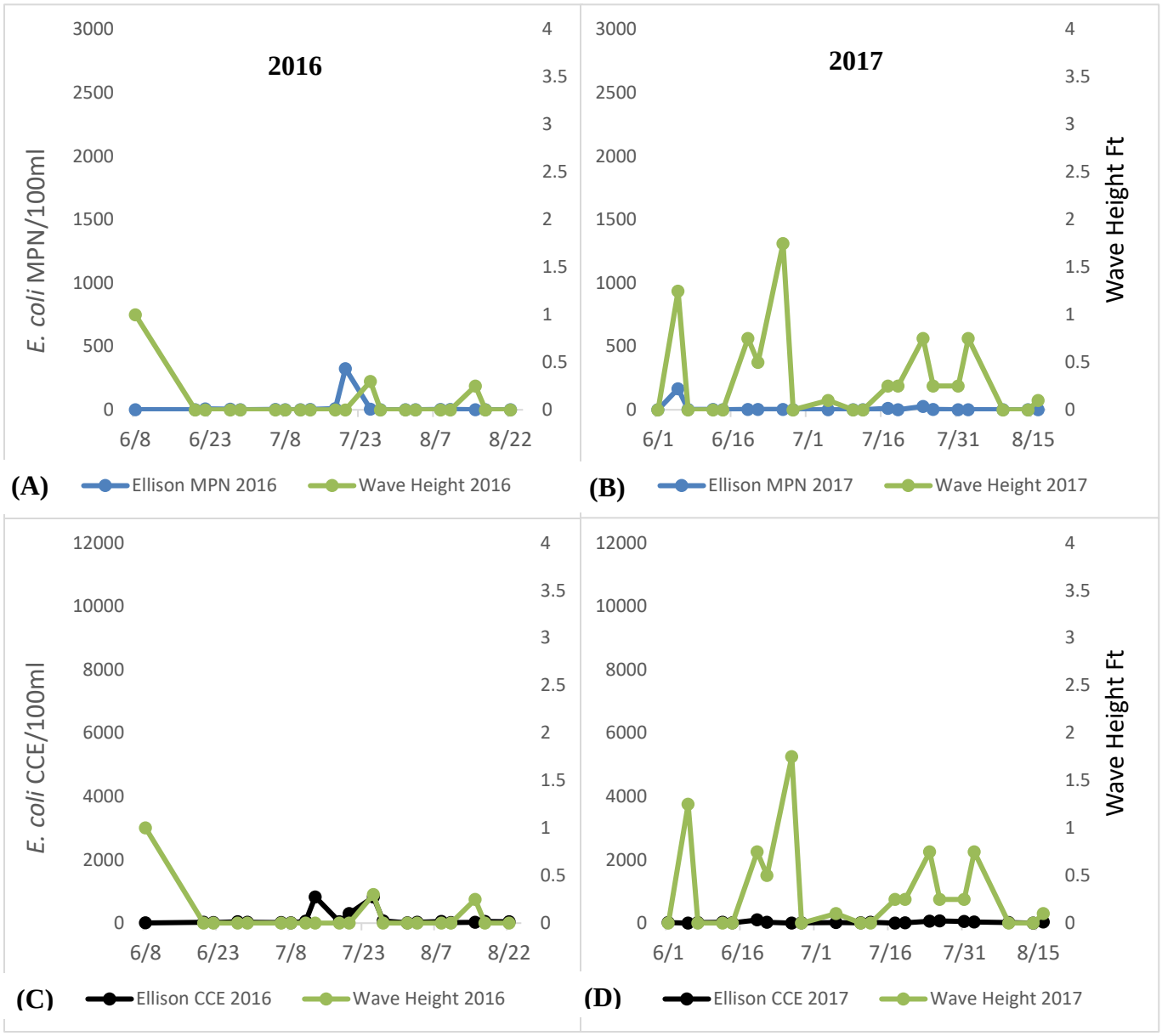

Figure 45. Ellison Bay Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL $R^2 = 0.14$ ($p>0.05$) and CCE/100mL $R^2 = 0.01$ ($p>0.05$).

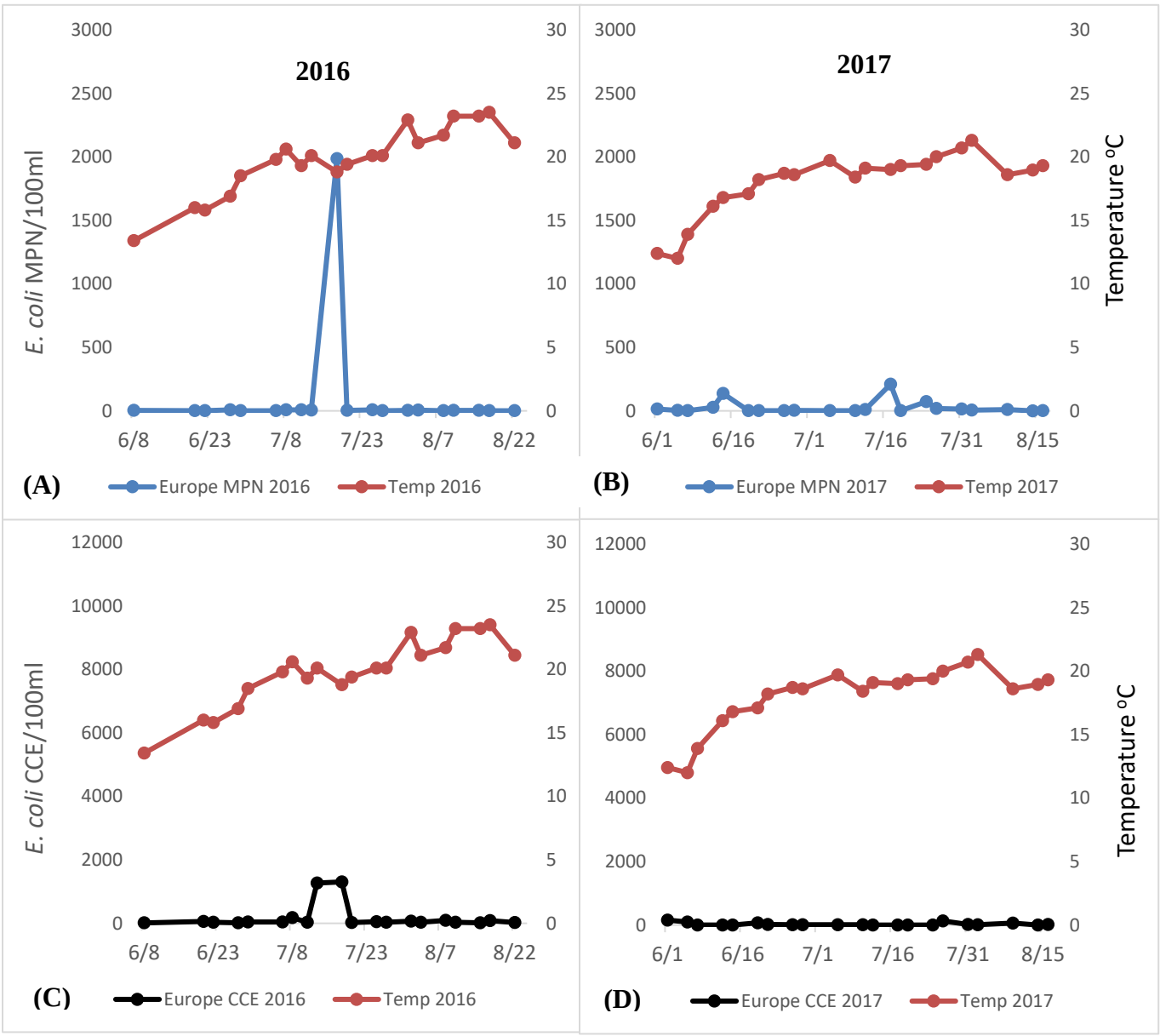

Figure 46. Europe Bay Beach *E. coli* concentration vs temperature for sampling dates collected. (A) 2016 MPN/100mL vs temperature. (B) 2017 MPN/100mL vs temperature. (C) 2016 CCE/100mL vs temperature. (D) 2017 CCE/100mL vs temperature. MPN/100mL R^2 = -0.01 ($p>0.05$) and CCE/100mL R^2 = 0.03 ($p>0.05$).

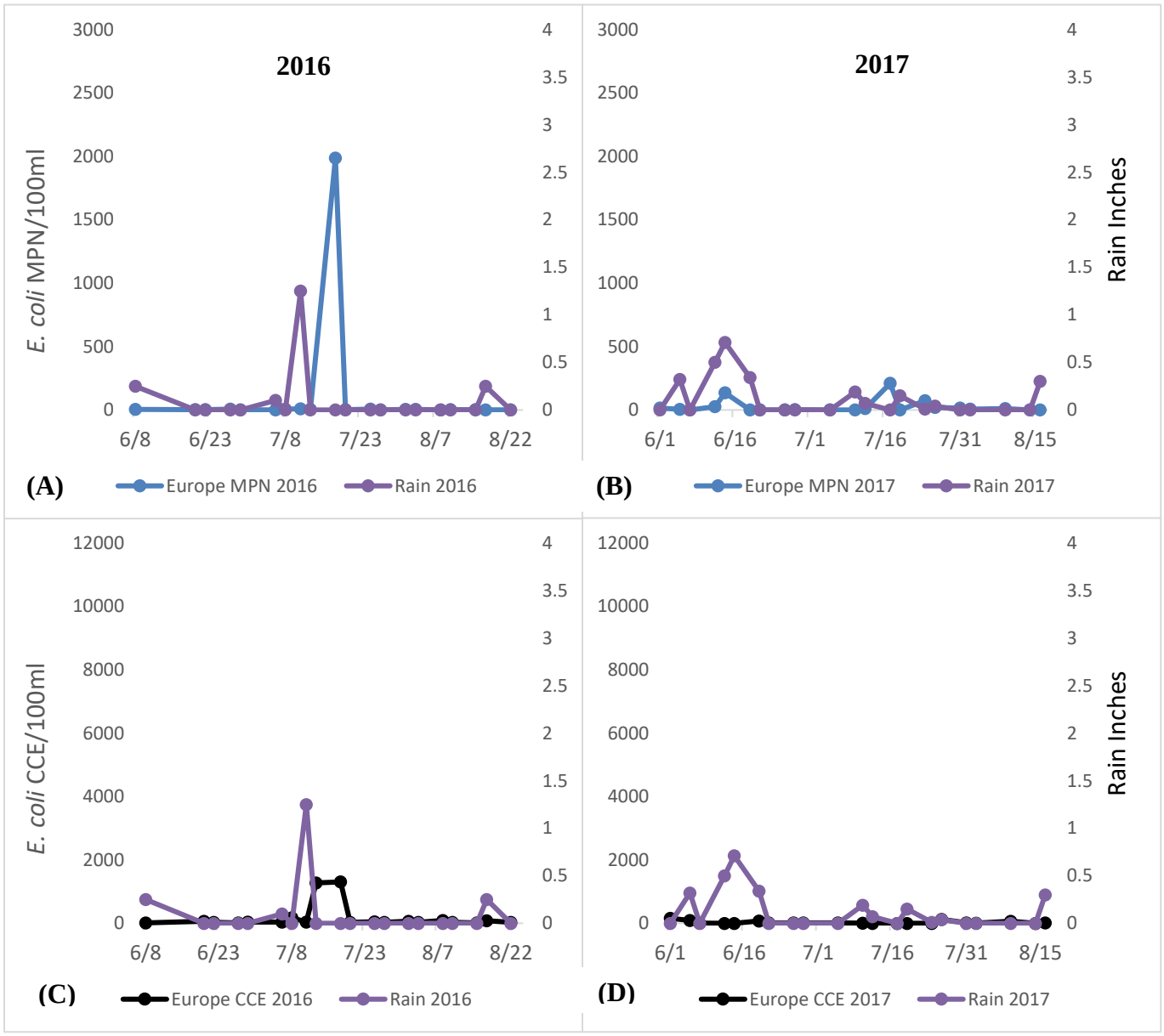

Figure 47. Europe Bay Beach *E. coli* concentration vs rainfall for sampling dates collected. (A) 2016 MPN/100mL vs rainfall. (B) 2017 MPN/100mL vs rainfall. (C) 2016 CCE/100mL vs rainfall. (D) 2017 CCE/100mL vs rainfall. MPN/100mL R^2 = -0.06 ($p>0.05$) and CCE/100mL R^2 = -0.11 ($p>0.05$).

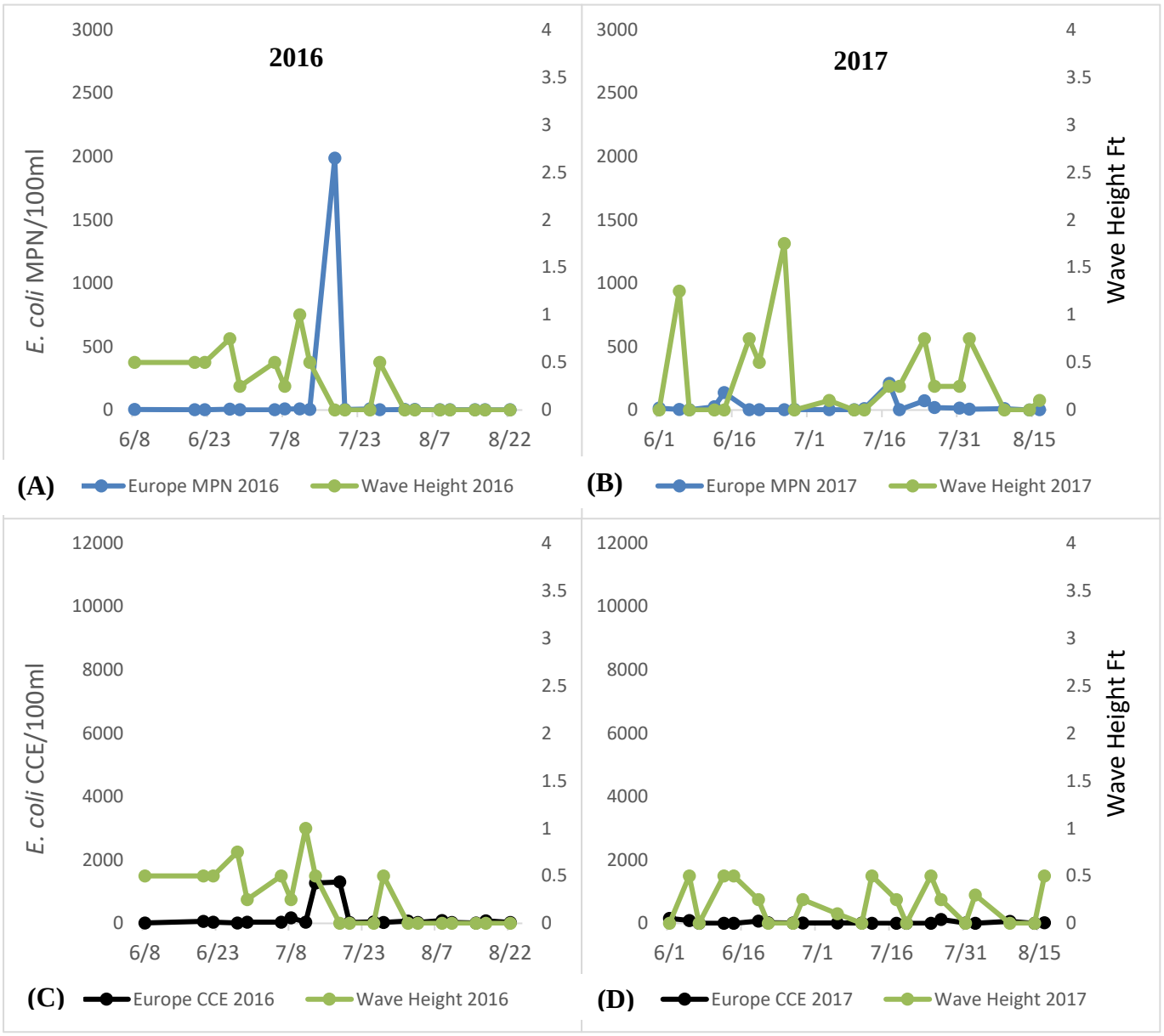

Figure 48. Europe Bay Beach *E. coli* concentration vs wave height for sampling dates collected. (A) 2016 MPN/100mL vs wave height. (B) 2017 MPN/100mL vs wave height. (C) 2016 CCE/100mL vs wave height. (D) 2017 CCE/100mL vs wave height. MPN/100mL R^2 = -0.13 ($p>0.05$) and CCE/100mL R^2 = 0.01 ($p>0.05$).

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