ABSTRACT

USING TRADITIONAL AND MOLERCULAR TECHNIQUES TO QUANTIFY *Campylobacter jejuni* IN BEACH WATER SAMPLES

By Bhargavi Meka

Campylobacter jejuni is considered one of the most common causes of gastroenteritis in the world. Although, the GI illness caused by this bacterium is self-limiting, the post-infection complications that follow the gastroenteritis are rather serious. To estimate the prevalence of this bacterium in the environment, both culture and qPCR techniques were performed on water samples collected from Lake Michigan beaches (Otumba , Sunset and Whitefish Dunes beaches) and Lake Winnebago beach (Menominee beach). The mean concentrations of the pathogen target sequences (*C. jejuni*) and the fecal indicator bacteria (enterococci) were found to be 2,136.33 CFU's and 257,383 CFU's per 100mL of water from Menominee Park beach. The concentrations of *C. jejuni* were above the minimum number to cause infection (500 organisms) at Otumba, and Sunset beaches and below the minimum number at Whitefish Dunes beach. No *C. jejuni* was detected (except on the June 22nd) when the culture technique was performed but with qPCR the pathogen was detected. Enterococci concentrates were more than 10 fold greater by qPCR when the two methods were compared. We conclude that the qPCR technique has better sensitivity, for detection of bacteria in recreational water than the culture techniques, for both fecal indicator bacteria (FIB) and a pathogen, *Campylobacter jejuni.*

USING TRADITIONAL AND MOLERCULAR TECHNIQUES TO QUANTIFY *Campylobacter jejuni* IN BEACH WATER SAMPLES

by

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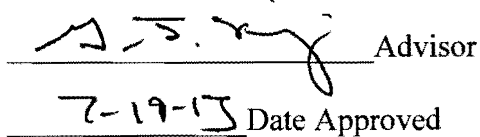

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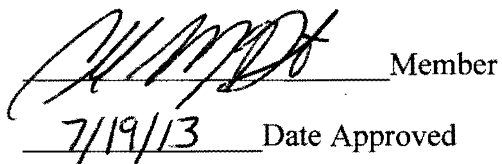

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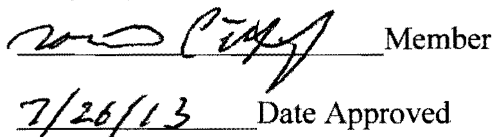

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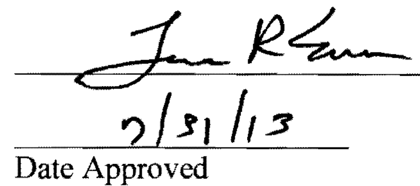

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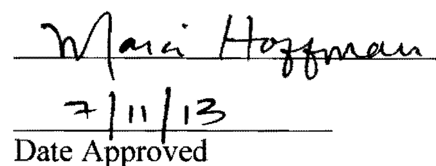

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TABLE OF CONTENTS

Page #

LIST OF TABLES IV

LIST OF FIGURES VI

INTRODUCTION 1

MATERIALS AND METHODS 17

Collecting Water Samples 17

Bacterial Recovery and Quantification of *Campylobacter jejuni* 17

qPCR Analysis 18

TaqMan qPCR Assay 19

qPCR Primers and Probe 20

SYBR Green qPCR Assay 22

Recovery and Quantification of Enterococci 25

Statistical Analyses 28

RESULTS 29

Detection *C. jejuni* in Environmental Samples 29

Using qPCR for the Detection and Quantification of *C. jejuni* 29

***Campylobacter jejuni* Standard Curve Dilutions** 31

Wilcoxon t-test 35

Analysis of Variance Among qPCR Target Sequences/ 100 mL for the Four Beaches 43

Detection of Enterococci in Environmental Water Samples 45

EPA Method 1611 qPCR on the Beach Water Samples for the Detection of Enterococci 46

Standard Curve Experiment for Enterococci 47

Paired t-test 61

ANOVA of Culture *Enterococcus* Enumerations 62

ANOVA of qPCR *Enterococcus* Enumerations 63

Comparison of *Enterococcus* and *C. jejuni* Concentrations Each Determined by qPCR Method 64

DISCUSSION 68

Specific Detection and Quantification of *C. jejuni* from the Selected Beaches 68

Detection and Quantification of Enterococci from the Selected Beaches 71

Comparison of *C. jejuni* and Enterococci Concentrations from Beach Waters 74

CONCLUSIONS 76

FUTURE WORK 77

REFERENCES 79

LIST OF TABLES

Page #

Table 1: *Campylobacter jejuni* forward, reverse primer and probe sequences along with melting temperatures (Tm) (From Nogva et al., 2000)....................... 20
Table 2: Enterococci forward, reverse primer and probe sequences along with melting temperatures (Tm). 26
Table 3: Salmon forward, reverse primer and probe sequences along with melting temperatures (Tm). 26
Table 4: Log Transformed values of target sequences of *C. jejuni* oxidoreductase gene for standard curve experiment... 31
Table 5: *C. jejuni* qPCR target sequences/ 100 mL for the four beaches. 34
Table 6: Otumba Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples... 36
Table 7: Sunset Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples... 38
Table 8: Whitefish Dunes Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples. 40
Table 9: Menominee Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water sample............................... 42
Table 10: ANOVA of qPCR enumeration of four beaches. 44
Table 11: Concentration of enterococci at selected beaches (MPN/100mL). 45
Table 12: Log transformed values of target sequences of lsrRNA gene for standard curve experiment. 46
Table 13: Enterococci concentrations per 100 mL of water sample by qPCR method from the four beaches in Summer, 2012.. 48
Table 14: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Otumba Park beach. 49
Table 15: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Sunset Park Beach.. 52

Table 16: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Whitefish Dunes Park Beach. 55
Table 17: MPN and target sequences (/100mL) of enterococcus using Enterolert and qPCR methods from Menominee beach. 58
Table 18: Paired t-test comparing mean of enterococci enumerations using Enterolert and qPCR enumeration at the four beaches. 61
Table 19: ANOVA test on Enterolert and qPCR enumeration to estimate the variance among the four beaches 62
Table 20: ANOVA test on *Enterococcus* qPCR enumeration to estimate the variance among the four beaches. 63
Table 21: Mean of number of target sequences of *C. jejuni* quantified from the water samples (qPCR). 70
Table 22: Mean concentrations of enterococci quantified from water samples using culture and qPCR techniques. 73

LIST OF FIGURES

Page #

Figure 1: The mechanism of qPCR showing different steps involved in the reaction- Polymerization(A), Strand Displacement(B), Cleavage(C) and Completed polymerization(D) 9

Figure 2: Map of Wisconsin with the sites selected for beach water investigation 14

Figure 3: Map shows the Lake Michigan Beaches- ‘Otumba Park Beach, Sunset Park Beach and Whitefish Dunes Park Beach’ selected for this study 15

Figure 4: Map shows Lake Winnebago Beach- Menominee Park Beach selected for this study 15

Figure 5: The primer and probe binding regions on the 86 bp region of *C .jejuni* genome. The forward and reverse primer binding areas are green in color and the probe binding region is blue in color 21

Figure 6: Melt curve analysis of beach water sample contaminated with *C. jejuni* showing the melting temperature of the amplicon at 75.51^{o}C whose melt curve is ideal 23

Figure 7: The melt curve analysis of the beach water samples and ‘No Template Control’ showing the possible primer dimer formation with the melting temperature of 70°C whereas the melting temperature of amplicon was 74.86^{o}C 24

Figure 8: Standard curve of *C. jejuni* oxidoreductase gene at 1x 10^2, 2x 10^2, 4x 10^2, 4x 10^3, 4x 10^4 and 4x10^5 dilutions of target sequences. 32

Figure 9: Standard curve of enterococci lsrRNA gene at 1x 10^2, 2x 10^2, 4x 10^2, 4x 10^3, 4x 10^4 and 4x10^5 dilutions of target sequences. 47

Figure 10: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Otumba water samples 50

Figure 11: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Otumba Park using scatter plot 51
Figure 12: Comparison of MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Sunset water samples 53
Figure 13: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Sunset Park using scatter plot 54
Figure 14: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Whitefish Dunes Park Beach water samples 56
Figure 15: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Whitefish Dunes Park using scatter plot 57
Figure 16: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Menominee water samples 59
Figure 17: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Menominee Park using scatter plot 60
Figure 18: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Otumba Park beach. 64
Figure 19: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Sunset Park beach. 65
Figure 20: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Whitefish Dunes Park beach. 66
Figure 21: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Menominee Park beach. 67

INTRODUCTION

Swimming in coastal waters is a favorite pastime in United States. In a survey of more than 75,000 households conducted by National Survey on Recreation and Environment (NSRE), 42% of the respondents reported swimming in coastal waters annually (Leeworthy and Wiley 2005). With so many recreational users of these waters it is important to understand the risks associated with use of this resource. Recreational water quality is subjected to many temporal changes in chemical, biological and physical parameters. Discharges from septic tanks, agricultural runoff, storm water runoff or feces of waterfowl can contaminate the recreational waters (Wade *et al*., 2010).

Human sewage can leak from Private Onsite Water Treatment Plants (POWTS) and enter beach water. Varieties of pathogens are associated with the digestive tract of humans and can enter and contaminate water bodies. Agricultural runoff is the rain water or irrigation water than can flow over fields and can reach beaches (Gaffield *et al.,* 2003). Storm water is the untreated rain water which passes over the ground from roads and parking lots that can enter into lakes, estuaries or storm water conveyance systems. Avian feces may be deposited directly into beach water or to beach sand and can carry pathogenic and fecal indicator bacteria into beach waters, thus contaminating the bathing water (Bolton *et al.,* 1999) (Kleinheinz *et al.,* 2006).

Contaminated water can cause illnesses in humans upon exposure. In recent years, the reported incidence of gastrointestinal illnesses due to exposure to the contaminated recreational water has increased (Dwight *et al.,* 2005). Different types of microorganisms, such as protozoans, viruses and bacteria can cause gastrointestinal illnesses Bacteria-associated illnesses are the most common (Pond, 2005). While bacteria genera such as *Salmonella, Listeria, E. coli, Yersinia, and Campylobacter* can cause GI tract illness in humans, *Campylobacter jejuni* -associated gastroenteritis has been found to be the most common cause of the bacterial gastrointestinal sickness in the world (Friedman *et al.,* 2000; Savill *et al.,* 2001 and Schaffter and Parriaux 2002). Since *Camplylobacter* can be carried to recreational water via overland or storm water runoff, it is a concern as a bacterial pollutant in recreational water. *Campylobacter jejuni* is a gram-negative, microaerophilic bacterium which is very sensitive to temperature fluctuations. The slightest changes in ambient temperature can hinder the growth and survival of these bacteria (Mihaljevic *et al.,* 2007). It is a curve-shaped bacterium with a polar flagellum (Sherburne *et al.,* 1985). It is commonly found in bird feces, and after *Campylobacter* associated illness subsides, humans and ruminants can act as reservoirs of this organism for a few days.

The most common type of illness caused by this bacterium is Campylobacteriosis (Wilson *et al.,* 2008). The minimum number of organisms required to cause an infection is approximately 500 organisms (Robinson, 1981).

After ingestion, the bacterium moves through the intestinal lumen using its polar flagellum and reaches the distal ileum and colon. In this region, it multiplies to large numbers and causes disease. It damages the gut epithelial cells directly by invading the cells or by indirectly initiating the inflammatory response that leads to bloody diarrhea with cramping (Ketley, 1997)

Virulence factors of *C. jejuni* include a polar flagellum which is important in host colonization and cell invasion. Recently identified are two methyl-accepting chemotaxis receptors important for colonization and invasion of the epithelial cells. The lipopolysaccharide (O-antigen) stimulates the immune response against the neural gangliosides which causes Flaccid Paralysis and the capsule which contributes to serum resistance, facilitates the invasion of the epithelial cells and colonization of the organism (extensive variation in the capsule structure probably has a key role in the evasion of the host immune response too) (Bereswill *et al.,* 2003). Campylobacteriosis has common symptoms such as nausea, fever, headache and diarrhea with blood in stools (Paisley *et al.,* 1982). The post infectious complications of the Campylobacteriosis, however, are often more serious than the primary infection. Although Campylobacteriosis does not usually lead to death, it is estimated that as many as 730 people in Unites States die of *Campylobacter* infections annually, due to secondary complications (Saleha *et al.,* 1998). The most important secondary complications are Guillain–Barré syndrome (GBS), Reactive arthritis, Irritable Bowel Syndrome and

Immunoproliferative small intestinal disease (IPSID) (Adedayo and Kirkpatrick 2008).

GBS is an autoimmune disease which is seen in 36% to 38% of the people with Campylobacteriosis as the primary infection (Mishu B *et al.,* 1993). This is a cross-reaction autoimmune disease associated with acute flaccid paralysis, linked to the molecular mimicry. These antibodies produced against the *C. jejuni* antigens of lipopolysaccharide mimic the terminal sugar residues on the peripheral nerves in humans. These antibodies cross react with the sugars found on the gangliosides of the peripheral nerves which lead to the demyelination of axons, resulting in flaccid paralysis (Kuroki S *et al.,* 1993).

Reactive arthritis is seen in 2% to 7% of the people infected with *C. jejuni* (Hannu, 2004). Genetic factors of the individual plays an important role in the development of reactive arthritis following *C. jejuni* infection. Individuals who are positive for human leukocyte antigen HLA-B27 are most likely to develop this complication. Between 65% to 95% of Caucasian patients and 50% of the African-Americans with Campylobacteriosis are at great risk for reactive arthritis (Petersel and Sigal 2005). The mechanism underlying this complication is not properly understood but is believed to be an autoimmune phenomenon due to the immune response against bacterial antigens mistargeted at autologous joint peptides presented by HLA-B27 (Yu D and Kuipers 2003). Longer duration of the

diarrheal symptom is seen in patients suffering with this condition and antibiotic therapy is usually required in these cases.

Postinfectious irritable bowel syndrome is a persistent diarrheal illness that may result after acute Campylobacter gastroenteritis (Thorney *et al.,* 2001). It is observed in 9% of the patients affected previously with *C. jejuni* infection. The mechanism involved is not well understood but the inability to down regulate the inflammatory markers after the persistent inflammation is believed to be a reason behind the development of the condition (Spiller *et al.,* 2000).

IPSID is a rare carcinoma in gut mucosa associated lymphoid tissue seen in *C. jejuni* patients (Leciut *et al.,* 2004). This condition mainly involves the malabsorption, diarrhea and abdominal pain of the proximal small intestine (Al-Saleem and Al-Mondhiry 2005). An elaborated model of the IPSID following the *Campylobacter* gastroenteritis has not been successfully studied yet, but *C. jejuni* may be a part of a multifactorial process of the whole IPSID development (Parsonnet and Isaacson 2004).

Campylobacter infection can be suspected in a person with fever and abdominal pain associated with bloody diarrheal stools. Gram staining of the diarrheal stool shows gram negative curved cells with darting motility under dark-field microscope (Wang and Murdoch 2004). As *C. jejuni* is a sensitive organism, its growth conditions are very specific. The optimum temperature required for its

growth is 42°C and it can be grown in the lab in the presence of 10% carbon dioxide (Paisley *et al.,* 1982).

The presence of *C. jejuni* has been reported in raw milk, raw chicken, raw clams, untreated water and other environmental samples. Waterborne outbreaks of *Campylobacter jejuni* infection associated with drinking water had occurred in the past (O' Reilly *et al.,* 2007). For example, in South Bass Island, Ohio, an outbreak of gastroenteritis was reported when 1450 individuals who travelled to the island became ill (O' Reilly *et al.,* 2007). Among the 1450 persons reporting illness, *C. jejuni, Giardia, Norovirus* and *Salmonella enterica* were identified in the patients. An environmental study was conducted to identify the source of contamination after receiving reports of illness by the individuals who travelled to this island. The sources were identified as ground water from wells which were contaminated by improperly managed sewage system which contained fecal microbes such as, *C. jejuni*, *Giardia, Norovirus, Salmonella* and *E. coli*.

The reported incidence of recreational water associated *C. jejuni* infections are low when compared to other sources, such as consumption of undercooked chicken, raw milk or contaminated drinking water. This could be due to a lower incidence, or it could be that recreational water infections are not as easy to track, due to the transient nature of tourists using beaches. Gulls carry *C. jejuni* in their cloaca and can release the organisms into the environment through their feces. Gull droppings on beach sand or in beach waters can

release the organisms which can increases the risk of contracting gastroenteritis. In 2010, Schoen and Ashbolt studied the incidence of gastrointestinal sicknesses associated with gull feces and improper practices in POWTS that can lead to the contamination of the water bodies. An important observation was that the probability of developing a GI illness was higher upon exposure to gull feces-contaminated recreation water when compared to sewage leaks.

In public health laboratories two methods are typically used to quantify *C. jejuni* in recreational water. These include microbiology enrichment and culture or molecular techniques. Selective plate count requires growing the organism at 42°C in 10% CO_2 on a specific medium, such as Blaser's agar, Skirrows agar, or Campy CVA agar (Blaser *et al.,* 1980 and Lovett *et al.,* 1983). Water samples are filtered through nitrocellulose membrane with the help of a vacuum source and the filters. After 24hrs of incubation, the number of colonies is counted are applied to the selective medium. Molecular methods involve DNA-based methods such as qPCR whose sensitivity or the limit of detection can be as low as one organism or copy of an organism's DNA (Nass *et al.,* 2011).

The basic mechanism of qPCR involves: polymerization, strand displacement, cleavage and elongation (Figure 1). The double stranded target DNA is denatured resulting in two single DNA strands. The forward and reverse primers designed to complement the target DNA attach to the target DNA. The DNA probe that has fluorescent molecule at one end and a quencher at the other

end also hybridizes to the specified nucleotide sequence which is in between the complementary sequence of the forward and reverse primers. The TaqMan polymerase helps in the synthesis of the second strand by elongating it with the addition of appropriate nucleotides. The exonuclease activity of DNA polymerase removes the probe upon encounter. As a result of this activity, the nucleotides having the reporter and the quencher are separated and the fluorescence signal is now detected by the thermo cycler which is connected to a fluorometer. As the number of cycles of the amplification increase, the intensity of the fluorescent signal is also increased and is displayed on the computer (Applied Biosystems qPCR Manual). The relationship between the number of cycles and intensity of the fluorescent signal are proportional, allowing for quantification of the number of DNA copies when compared to controls.

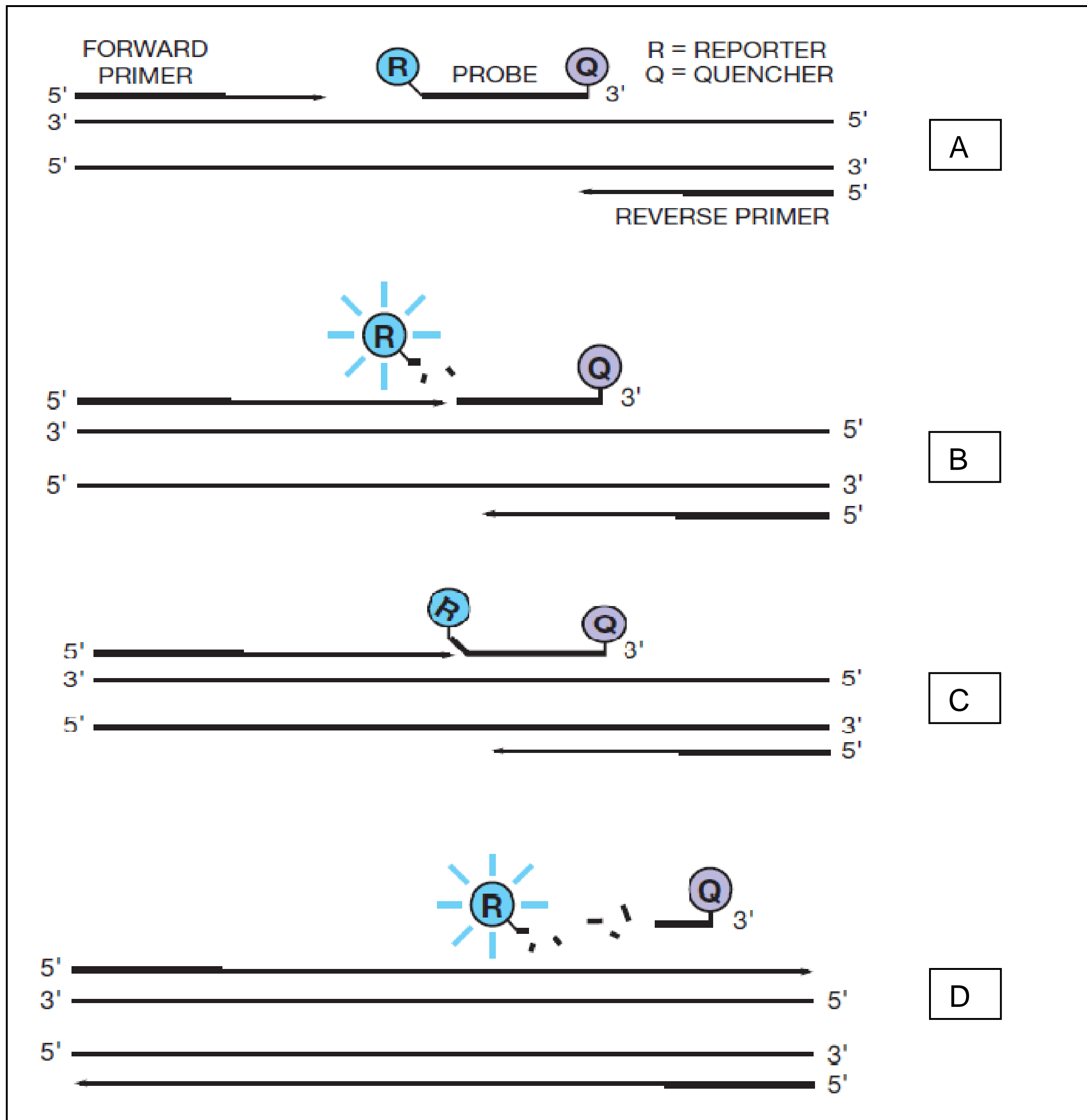

Figure 1: The mechanism of qPCR showing different steps involved in the reaction- Polymerization(A), Strand Displacement(B), Cleavage(C) and Completed polymerization(D).

There are advantages in using the qPCR technique over the culture-based methods. Culture based methods are time consuming, laborious, requires additional preparation work and prolonged incubation. It takes over 24 hours to

get the result which makes it impossible to notify the public of contaminations on the same day of sample collection. These problems are solved with qPCR as the technique is rapid, sensitive and results can be obtained in less than 3 hours. *Campylobacter* can enter the viable but non-culturable state (VBNC) where the organism stays viable and dormant but does not grow on the media, due to environmental stress (Rollins and Colwell 1986). As a result, the enumeration obtained by using culture techniques can often be misleading due to inaccurate results. Again this is not a problem with qPCR as it targets the nucleic acid of the organism rather than the organism itself. Techniques like qPCR are essential to determine the low incidence of *C. jejuni* in the environment (Hutchison *et al.,* 2004, Stanley and Jones 2003). However, there are limitations to the qPCR technique. This method can detect and amplify extracellular DNA from the environment and DNA from dead cells. These problems may be overcome in part, by using DNases that cleave non-cellular DNA. DNases also degrade the DNA of the dead cells but not of viable bacterial cells because of their intact cell membrane and cell wall (O'Brien and Bolton 1995). With qPCR, there is need for expensive equipment and the need to have highly trained staff to successfully execute the procedures.

The quality of recreational water has been traditionally monitored by using fecal indicators such as enterococci, *E. coli* and other fecal coliforms (Hanninen *et al.,* 2003). US EPA initiated the BEACH Act and its subsequent monitoring program (Beaches Environmental Assessment, Closure, and Health) in order to

monitor the health of Great Lakes beaches (Boehm *et al.,* 2009). The quality of the beach water is tested for the fecal indicator *E. coli* in the Great Lakes. However, there are important considerations that have to be taken into account when using fecal bacteria as indicators. The main purpose of using the fecal indicator bacteria is to assess the health risk posed by other pathogenic organisms which are also of fecal origin. One consideration is that indicator bacteria have different survival than the pathogens in a given environment (McFeters *et al.,* 1974 and Schaffter and Parriaux 2002). Thus, the incidence of the indicators may not reflect the concentration of the pathogens in recreational water and will change disproportionally with time from discharge. Ideally, the input and transport of the indicator bacteria should be at the same rate as pathogenic organisms (Anderson *et al.,* 2005). If the fate and transport rates are different, the concentration of the indicator bacteria does not correlate with the risk of illness and could be completely different from the concentration of the pathogens. Another fact to consider is that, lower concentration of one pathogen (For eg: *Campylobacter* species) do not indicate lower concentration of other pathogens (For eg: *Salmonella*, *Norovirus etc.*) that can also cause respiratory, ear, nose, eye, skin and GI illness (Griffin *et al.,* 2003). Due to this, the information on the concentrations of a single pathogen obtained through the quantification technique may not be reliable to derive conclusions about the quality standards of the tested recreational waters. Also, feces from different human or animal sources can pose different levels of risk as they carry different

pathogens at different concentrations (Abdelzaher *et al.,* 2009). That is, fecal indicator organisms from gulls, humans, or bovine sources will have different associated risks because the host-specific pathogens are different for each species. Considering these facts, it is advantageous to develop techniques that detect multiple pathogens that commonly pose health risk to swimmers.

Previous studies that investigated the presence of *C. jejuni* in environmental samples used PCR assays (Kirk and Rowe 1994). Different PCR approaches such as semi-nested PCR, PCR- Enzyme Linked Immunosorbent Assay (ELISA) were conducted to detect the prevalence of *C. jejuni* in water samples (Sails *et al.,* 2002 and Waage *et al.,* 1999). Waage *et al.,* 1999, conducted a comparative study where they developed a PCR assay whose sensitivity was between 3 to 30 cells per 100mL of water. Another group developed a PCR assay that had the sensitivity of 10-100 CFU's per 100mL of water (Oyofo and Rollins 1993). However, this level of sensitivity using PCR was achieved with artificially contaminated water and not with environmental water samples. This is a very important difference since spiked lab water samples will not contain all the interferences found in surface water. Even though this sensitivity range is good for traditional PCR assays, an advanced molecular technique like qPCR assay could provide a sensitivity range as low as one organism or one copy of an organism's DNA in a given sample (for eg: per 100 mL) (Naas *et al.,* 2011). Most of the studies conducted assays using culture enrichment techniques rather than directly using the filter concentrated samples

of naturally contaminated environmental water which was tested in this study. A thorough comparative study for the enumeration of both pathogen (i.e. *C. jejuni*) and indicator bacteria (i.e. enterococci) has not been conducted using filter concentrated samples by the previous investigators.

The overall objective of this project is to investigate the concentrations of fecal indicator bacteria, enterococci, and the pathogenic bacterium, *Campylobacter jejuni,* using both culture and qPCR techniques. Water samples were collected from Lake Michigan beaches at Whitefish Dunes Park beach (W Dunes), Otumba Park beach, Sunset Park beach and from a Lake Winnebago beach, Menominee Park beach during summer, 2012 (Figures 2- 4).

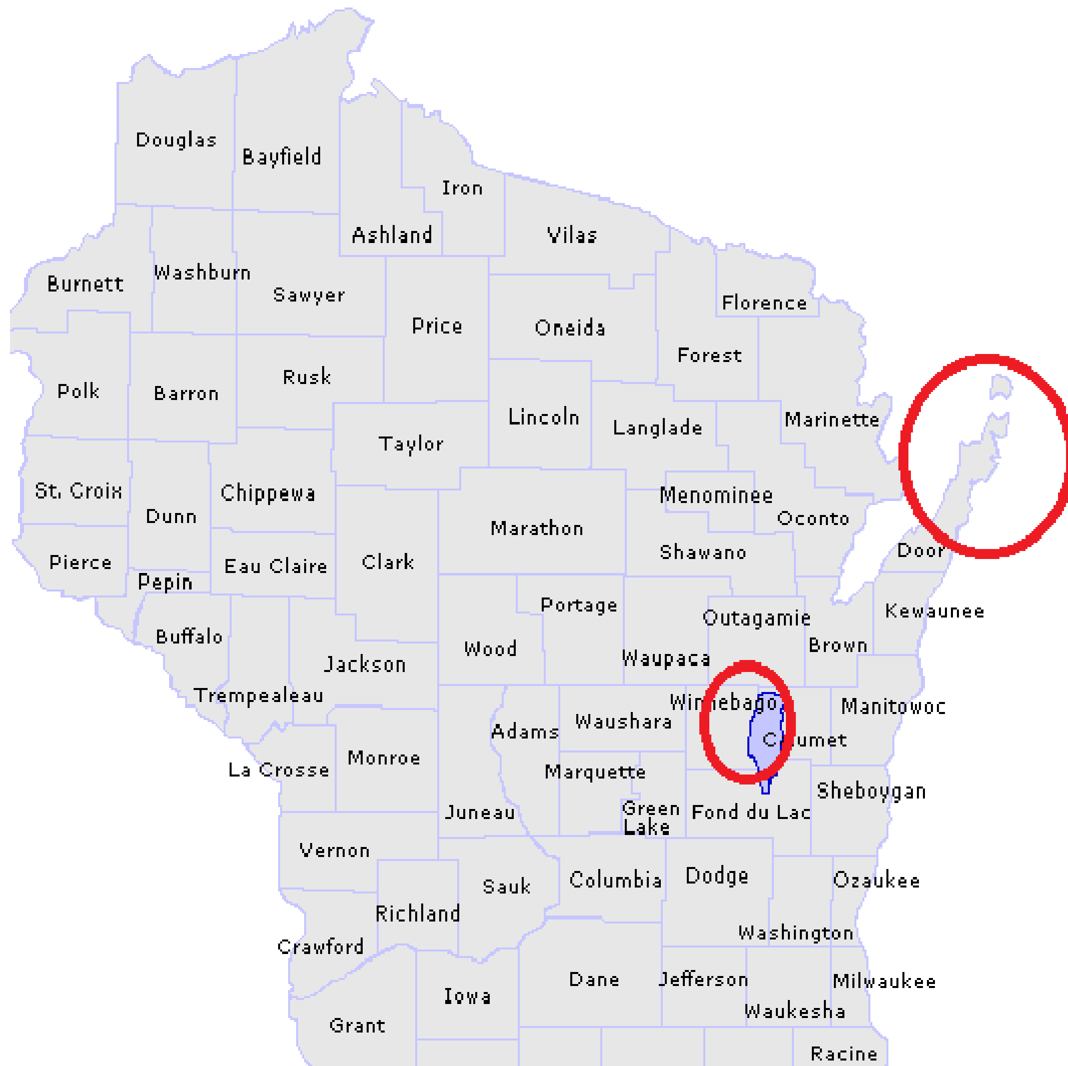

Figure 2: Map of Wisconsin with the sites selected for beach water investigation.

Figure 3: Map shows the Lake Michigan Beaches- 'Otumba Park Beach, Sunset Park Beach and Whitefish Dunes Park Beach' selected for this study.

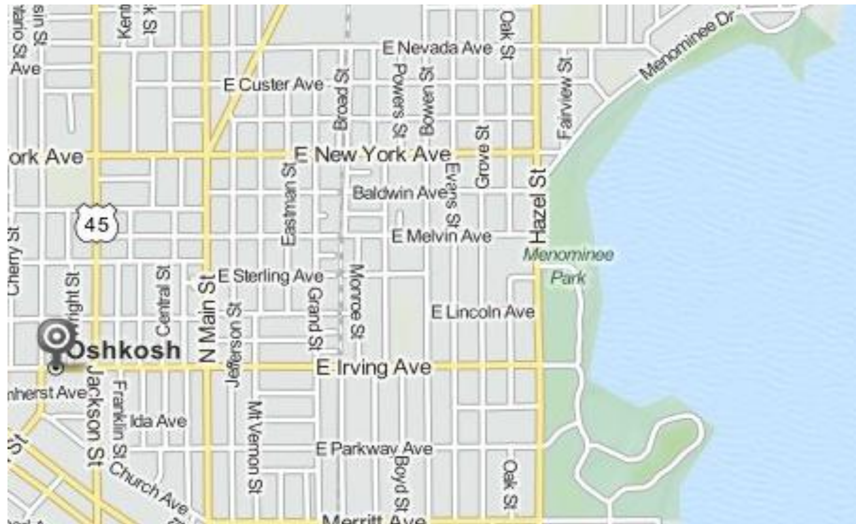

Figure 4: Map shows Lake Winnebago Beach- Menominee Park Beach selected for this study.

The specific objectives of the project include:

1) To utilize *C. jejuni* specific primers and probe with qPCR no enumerate this organism in recreational water (Nogva *et al.,* 2000)

2) To compare qPCR results with traditional, culture based enumeration of *Campyloabacter jejuni* from beach waters.

3) To quantify enterococci in the water samples using traditional microbiological techniques, and

4) To compare qPCR results with culture method for enumeration of enterococci in recreational water

MATERIALS AND METHODS

Collecting Water Samples

Water samples were collected from Otumba Park Beach, Sunset Park Beach, Whitefish Dunes Park Beach which are Lake Michigan beaches and Menominee Park Beach which is a Lake Winnebago beach (Figures 2-4). Approximately 3.5 liters of water was collected from each beach during June, August and September, 2012. A total of 64 samples were collected and were transported to the lab on ice, where they were filtered.

Bacterial Recovery and Quantification of *Campylobacter jejuni*

For each sampling date, 300 mL Lake Michigan water samples were filtered using 0.45 μm x 47 mm nitrocellulose filters in duplicates. Only 200mL of water was filtered from the Menominee Park Beach due to the turbidity of the water in duplicates. Sample concentrations were corrected for volume later. Filters were then incubated on a BD Campy CVA agar, Franklin Lakes, NJ for 24hrs at 42°C. The number of distinct types of colonies on each plate was noted and pure cultures were recovered by isolating and streak plating *Campylobacter* -like colonies onto Campy CVA agar. Later gram staining was performed to determine if the cells from the pure culture were gram negative curved to rod shaped cells. Later biochemical tests such as oxidase and catalase tests were done and the positive colonies were then subjected to *Campylobacter* antibody

test using Microgen Bioproducts Campylobacter Latex Kit, Frederick, MD for the presence of *Campylobacter jejuni*. The confirmation of the presence of *C. jejuni* was made when clumping was observed when the colony reacted with the *Campylobacter* specific antibody. If the culture tested positive for all of the above tests, a single colony of confirmed *C. jejuni* was inoculated into Thioglycollate broth and incubated for 24hrs at 42°C (Stern, 1982). This media is a differentiating media that provides different oxygen concentrations throughout the media. This allows the detection of different microorganisms whose oxygen requirement varies from each other. *C. jejuni* is a microaerophilic organism and grows below the surface of the broth where the oxygen levels are lower. A 10-fold serial dilution method was performed using 1mL of the original solution for the enumeration of the total number of cells. The total number of cells in the original solution was determined to be approximately 3.8 x 10^{7} cells per 1mL of solution. Then, 500μL of this broth was added to 30% glycerol in a 2mL cryovial for cryopreservation.

qPCR Analysis

For qPCR analysis, 200mL of the water sample was filtered from Lake Michigan beaches and 100mL of Menominee beach water was filtered using 0.45μm x 47mM polycarbonate filters. All the filters were then aseptically placed in microcentrifuge tubes and stored at -80°C until use. For qPCR calibrators, *C. jejuni* ATCC 29428, ATCC, Manassa, VA was used.

TaqMan qPCR Assay

As stated above, a qPCR assay was run using primers and probe adopted from Nogva *et al.,* 2000 (Table 1 and Figure 5). The 25µL reaction mixture contained 300nM of forward and reverse primers, 200nM of the probe, 12.5µL of the TaqMan Master Mix and 1µL of the DNA sample. Different concentrations of probe were used ranging from 20nM- 200nM in experiments trying to identify the appropriate concentration of the probe suitable for the complete reaction. The experiments were started by using the DNA extracted from the pure cultures of *C. jejuni* ATCC strain 29428. The concentration of the DNA in the positive control or calibrator was 3.5ng/µL. The results were not satisfactory and included several problems such as: poor amplification of the positive control in spite of the high target sequence concentration, and inability to replicate the former Ct values in the next series of experiments. Similar problems have been encountered by other researchers using TaqMan chemistry (Julie Kinzelman, City of Racine Personal Communication). In order to circumvent the issues with TaqMan chemistry, SYBR Green was used in place of the TaqMan, which helped in determining the amount of *C. jejuni* DNA in the beach water samples collected from June-September, 2012.

qPCR Primers and Probe

The primers and the probe sequences for the *Campylobacter jejuni* quantification were adopted from Nogva *et al.,* (2000).

Table 1: *Campylobacter jejuni* forward, reverse primer and probe sequences along with melting temperatures (Tm) (From Nogva et al., 2000).

Primer/ Probe	Sequence (5'- 3')	Melting temp. in ^{o}C
Forward	CTGAATTTGATACCTTAAGTGCAGC	60.4
Reverse	AGGCACGCCTAAACCTATAGCT	60.3
Probe	FAM- TCTCCTTGCTCATCTTTAGGATAAATTCTTTCA CA-TAMRA	66.6

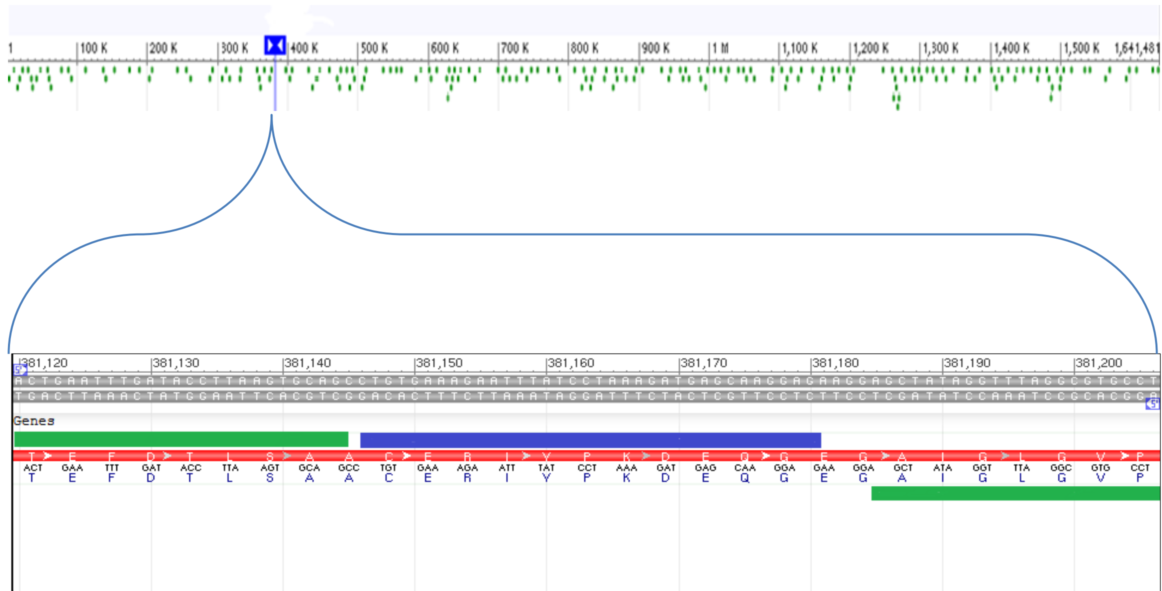

Figure 5: The primer and probe binding regions on the 86 bp region of *C. jejuni* genome. The forward and reverse primer binding areas are green in color and the probe binding region is blue in color

SYBR Green qPCR Assay

SYBR Green is a fluorescent DNA binding dye that fluoresces when it binds to the double stranded DNA. The reaction mixture using SYBR Green had a volume of 25μL which contained 300nM of the forward and reverse primers (Table 2), 12.5μL of the SYBR Green master mix, 1.5mM of additional $MgCl_2$ and 1μL of the DNA sample. The thermo cycling conditions included a denaturation step of 10 minutes at 95°C, and 40 cycles of 95^oC for 20 seconds and 60^oC for 1 minute. Eight different samples were run at a time in quadruplicates including positive controls, no template controls and method blanks. Melt curve analysis was done each time to look out for false positives and primer dimers which could result in erroneous amplification values. Typical examples of ideal melt curve and melt curve with possible primer dimer are shown in Figure 6 and Figure 7, respectively.

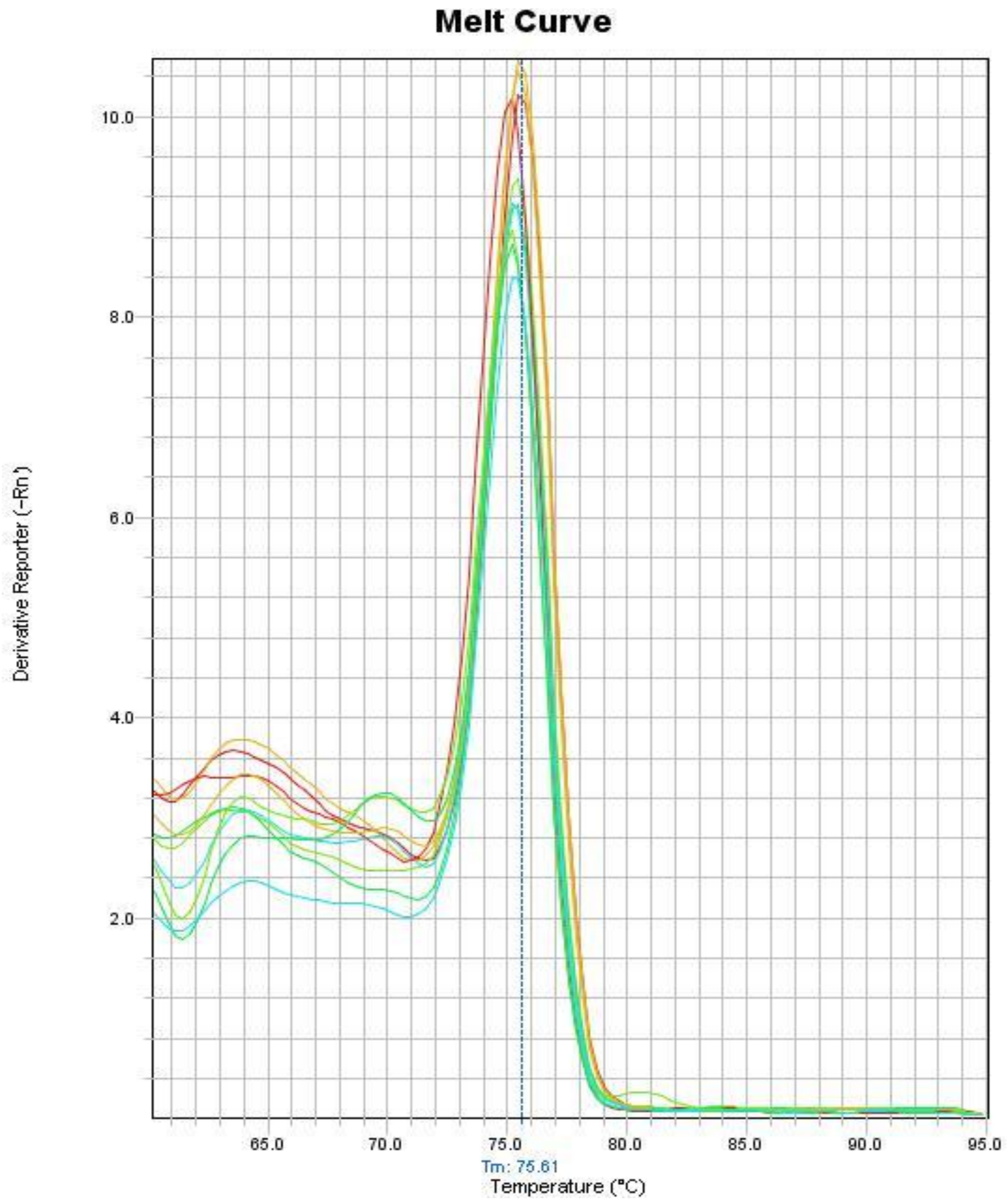

Figure 6: Melt curve analysis of beach water sample contaminated *with C. jejuni* showing the melting temperature of the amplicon at 75.51°C whose melt curve is ideal

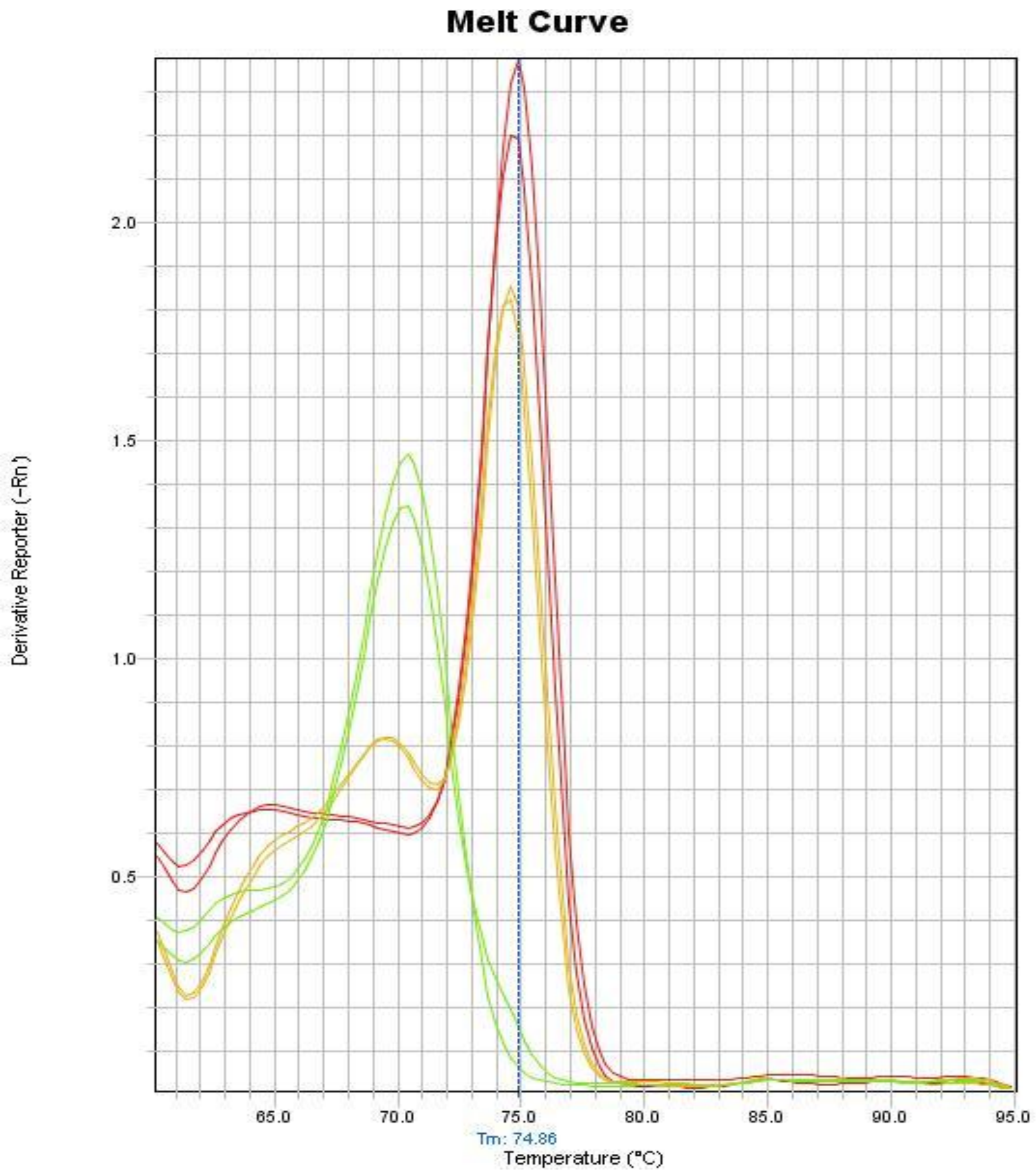

Figure 7: The melt curve analysis of the beach water samples and 'No Template Control' showing the possible primer dimer formation with the melting temperature of 70°C whereas the melting temperature of amplicon was 74.86°C

Recovery and Quantification of Enterococci

Quantitray.

For enterococci, 100mL of each water sample was mixed with one Enterolert capsule and incubated at 37°C for 24 hrs. This capsule contains the media and suitable for the growth of enterococci *species*. This mixture was carefully transferred to a Quantitray, which has large and small wells and was sealed. The number of large and small florescent colonies was counted using the Quantitray Most Probable Number (MPN) Table (Budnick *et al*., 1996).

EPA method 1161.

For enterococci, USEPA Method 1161 was followed to quantify this organism in recreational water. Water samples were filtered through 0.45µm x 47mm polycarbonate filters and aseptically transferred to the microfuge tubes to be stored at -80°C. The DNA from the sample filters was extracted by transferring the filter to an O-ring tube with 0.2µg/mL Salmon DNA extraction buffer and 0.3 gms acid washed glass beads. After beating the sample filter for 60seconds at 5000 rpm, the extracted DNA was carefully pipetted out and centrifuged at 12,000 x g for 1 minute. This step was repeated at the same speed and an extended time of 5 minutes to pellet the crude materials and to obtain clear supernatant. For calibrators, 10µL of the prepared enterococci undiluted pure culture with a concentration of 3.7 x 10^{8} CFU/mL was used. For qPCR analysis of *enterococci* EPA 1611 method, *Enterococcus fecalis* ATCC strain stock culture

was used to prepare the calibrators following the EPA 1611 for enterococci quantification via qPCR. (US EPA EPA-821-R-12-008).

Table 2: Enterococci forward, reverse primer and probe sequences along with melting temperatures (Tm).

Primer/ Probe	Sequence (5'- 3')	Melting temp. in ^{o}C
Forward	GAGAAATTCCAAACGAACTTG	48.5
Reverse	CAGTGCTCTACCTCCATCATT	52.4
Probe	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	60.1

Table 3: Salmon forward, reverse primer and probe sequences along with melting temperatures (Tm).

Primer/ Probe	Sequence (5'- 3')	Melting temp. in ^{o}C
Forward	GGTTTCCGCAGCTGGG	51.1
Reverse	CCGAGCCGTCCTGGTC	53.6
Probe	FAM-AGTCGCAGGCGGCCACCGT-TAMRA	59.7

Note: The terms cells and target sequences will be interchangeably used as theoretically, the number of target sequences corresponds to number of cells.

DNA from the positive controls was extracted using the same procedure as that of the samples. Three calibrators were extracted each time the analysis was conducted and used as positive controls. The qPCR assay was performed targeting *Enterococcus* and Salmon DNA. Salmon testes DNA sequence was selected as endogenous control in order to evaluate the performance of the qPCR reaction each time water sample analysis occurred. The same thermal cycling parameters used for enterococci were used to check the level of inhibition caused by any inhibitory materials that could be present in the environmental samples.

Each qPCR assay mix contained a total reaction volume of 25µL which included, 500mM of both forward and reverse primers, 100mM of the probe, 12.5 µL of the TaqMan master mix, 2.5µL of BSA and 1.5µL of sterile water. 5µL of the template DNA was added to each reaction well (Tables 2 and 3). Two types of negative controls, a 'No Template Control' (in quadruplicates) and a 'Method Blank' (in duplicates) were run during each analysis. The thermal cycling conditions used were: an initial holding step of 50°C for 2 minutes, denaturation step of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The reaction process took approximately 90 minutes to 2hrs to complete. The amplification plot was then observed for the concentration of the bacteria and cyclic threshold values (Ct) were used to obtain the total number of target sequences in the samples.

Statistical Analyses

The Wilcoxon t-test was used to test if the enumeration of the pathogen obtained through culture technique was similar to that of the qPCR. For FIB, a paired t-test was used to test the same hypothesis. A Two-way ANOVA was used to observe if the bacterial cell counts differed among the four beaches for both the organisms. Linear regression analysis was conducted to determine the correlation between the concentration of enterococci by culture and qPCR techniques, and also to establish a correlation between pathogen and FIB target sequence numbers obtained through qPCR.

RESULTS

Detection *C. jejuni* in Environmental Samples

Using the selective plate count method, *C. jejuni* was quantified by counting the number of colonies on CVA plates and then by confirming the colonies were *C. jejuni* performing biochemical tests on the unique colonies. *C. jejuni* was not detected with culture techniques except for the 1st week (June 22nd) of sampling where 8 CFU's were detected from Otumba Park beach.

Using qPCR for the Detection and Quantification of *C. jejuni*

A standard curve experiment was conducted each time a new gene was targeted in a qPCR assay. Ideally, a standard curve containing 5 to 6 different dilutions of the target sequence was run each week of the analysis. During the week of *C. jejuni* analysis from the environmental samples, 3 different standard curve experiments were conducted on ABI StepOne Plus instrument. This was conducted in order to evaluate the percentage of confidence in the method being used. In other words, the efficiency of the selected primers and other qPCR reagents (such as SYBR Green master mix, $MgCl_{2,}$ nuclease free water) were verified using this standard curve experiment. SYBR Green was used as a reporter dye as the assays using TaqMan chemistry did not work well showing interference problems. The target gene concentrations used in these experiments were $4x10^5$, 4×10^4, 4×10^3, 4×10^2, 2×10^2 and 1×10^2 per 1

microliter of the solution (Table 4). Originally, DNA was extracted from a calibrator whose number of CFU's (as determined by the regular plate count) was 15 CFU's in 10μL of the prepared pure culture. The initial concentration of the DNA extracted from the calibrator was 3.2775 μg/ mL. The dilutions were made accordingly to achieve the above standard target concentrations.

The number of target sequences each time was estimated using the absorbance of the DNA in each dilution prepared (Tables 4 and 5). These estimations were based on the assumption that the average weight of the base pair is 650 Daltons (standard number used in research laboratories). This means that one mole of a base pair weighs 650 g and that the molecular weight of any double stranded DNA template can be estimated by taking the product of its length (in bp's) X 650. Avogadro's number, 6.022×10^{23} molecules/ mole, was used to calculate the number of molecules of the template per gram:

mol/ g * molecules/ mol = molecules/ g

Finally, the number of molecules or number of copies of template in the sample can be estimated by multiplying with 1*109 to convert to ng and then multiplying by the amount of template (in ng).

The number of target sequences calculated was then adjusted to 100 mL of water sample. For *C. jejuni*, as there is only one target sequence per organism (NCBI), the number of target sequences obtained after the above adjustment

was believed to be the actual number of sequences in 100 mL of the water sample tested.

Campylobacter jejuni Standard Curve Dilutions

Table 4: Log Transformed values of target sequences of *C. jejuni* oxidoreductase gene for standard curve experiment.

Sample Number	Target DNA copy number/ 1 μL	Log transformed values	Avg Ct Value
1	1×10^2	2.0	37.64
2	2×10^2	2.30	35.44
3	4×10^2	2.60	30.49
4	4×10^3	3.60	26.19
5	4×10^4	4.30	23.36
6	4×10^5	5.60	18.51

A linear regression analysis was performed on the obtained log values and Ct values to analyze if the data is linearly related. The slope of the line can be achieved by obtaining the equation of the line connecting the points (Figure 8).

***Campylobacter jejuni* Standard Curve Graph:**

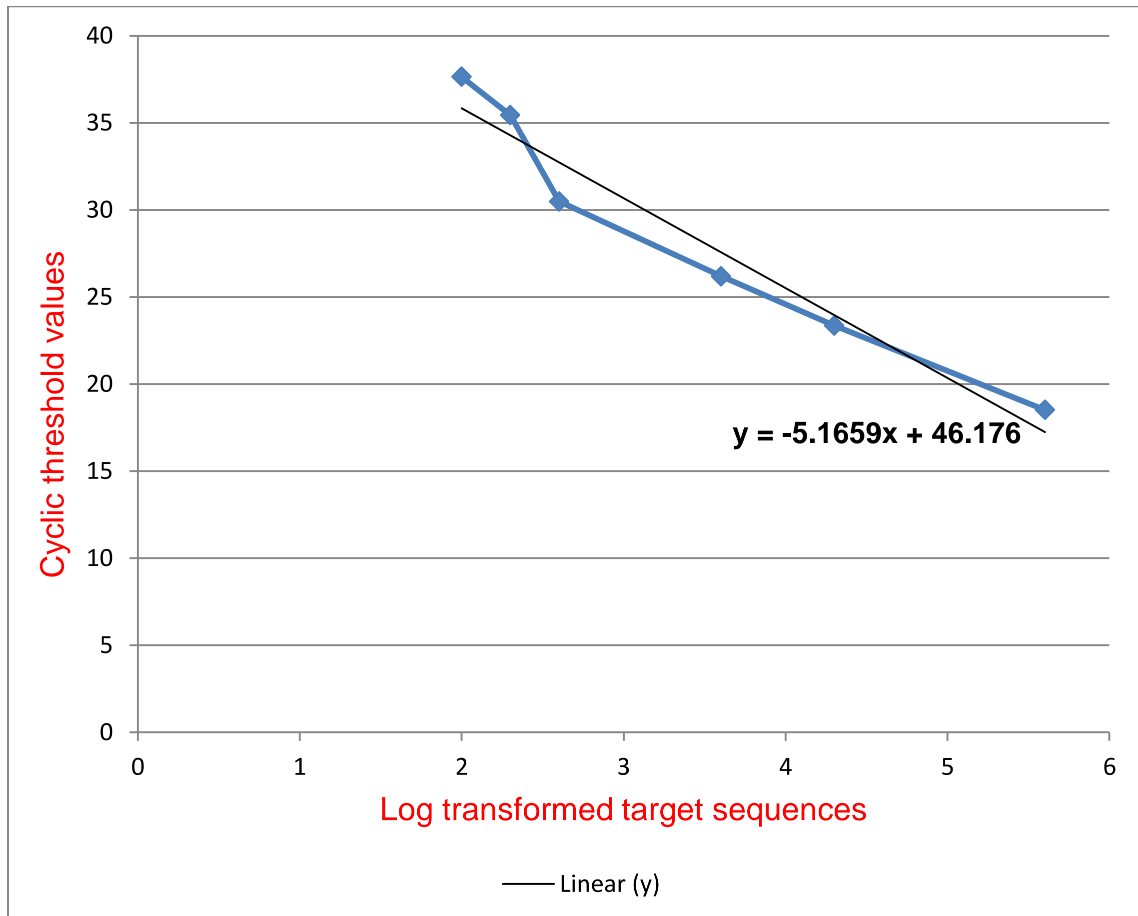

Figure 8: Standard curve of *C. jejuni* oxidoreductase gene at 1x 10^2, 2x 10^2, 4x 10^2, 4x 10^3, 4x 10^4 and 4x10^5 dilutions of target sequences.

The slope obtained from the plot was y= -5.1659x+ 46.176. Regression analysis was performed to obtain the square regression value of R^2= 0.9496. There was 95% confidence in the assay that was performed during the week of sample analysis. The R^2 value shows how well the x-axis (target sequences) explains the variance observed in the y-axis (Cyclic threshold values). The regression values lower than 0.99 could be due to the primer design, secondary

structures in the amplicons; whereas regression values higher that 1.00 could be due to primer dimers or unspecific amplification products. Though R^2 values of 0.99 – 1.05 are ideal, the values ranging from 0.90- 110 are also acceptable (Taylor *et al.,* 2011). Thus, this result was acceptable.

Table 5: *C. jejuni* qPCR target sequences/ 100 mL for the four beaches.

Sl. No.	Date of sampling	Otumba	Sunset	W Dunes	Menominee
1	6/22/2012	3500.31	348.61	326.43	576.88
2	6/28/2012	1626.13	507.50	379.42	893.87
3	8/9/2012	1985.09	1086.34	253.19	635.61
4	8/10/2012	2643.34	832.35	307.03	701.88
5	8/14/2012	2172.60	724.93	340.55	650.66
6	8/16/2012	1873.33	738.79	380.69	1543.15
7	8/20/2012	2404.46	110.76	347.45	1190.28
8	8/23/2012	5664.61	547.45	242.43	1098.52
9	8/27/2012	4005.56	400.27	304.64	1690.80
10	8/30/2012	816.73	402.06	231.08	3183.99
11	9/3/2012	1672.06	244.05	295.61	3651.71
12	9/7/2012	2652.19	280.84	371.48	7939.71
13	9/10/2012	2018.55	769.89	382.40	3299.57
14	9/13/2012	4835.60	832.35	300.93	1954.36
15	9/17/2012	4423.18	537.17	402.96	2573.58
16	9/20/2012	3079.31	1182.35	390.58	2596.63

Wilcoxon t-test

C. jejuni was not detected from the environmental water samples with selective plate count except for 1st week of sampling at Otumba Park beach, where there were 8 CFU/ 100 mL. A non-parametric paired test was chosen for these enumerations as this test does not assume the data to be normally distributed. Wilcoxon t-test was chosen to compare the culture enumeration with that of the qPCR enumeration.

H_O = The bacterial cell numbers calculated using the culture technique are equal to the qPCR technique

H_A = The bacterial cell numbers calculated using the culture technique are not equal to the qPCR technique

Otumba Park Beach:

Table 6: Otumba Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples.

Sample No.	Dates	Culture (CFU's/100mL)	qPCR (Tar. Seq's/100mL)	S/R
1	6/22/2012	8	3500.31	-12
2	6/28/2012	0	1626.13	-2
3	8/9/2012	0	1985.09	-5
4	8/10/2012	0	2643.34	-9
5	8/14/2012	0	2172.60	-7
6	8/16/2012	0	1873.33	-4
7	8/20/2012	0	2404.46	-8
8	8/23/2012	0	5664.61	-16
9	8/27/2012	0	4005.56	-13
10	8/30/2012	0	816.73	-1
11	9/3/2012	0	1672.06	-3
12	9/7/2012	0	2652.19	-10
13	9/10/2012	0	2018.55	-6
14	9/13/2012	0	4835.60	-15
15	9/17/2012	0	4423.18	-14
16	9/20/2012	0	3079.31	-11

CFU's =Colony forming units; S/R= Sum of ranks

N= 16; Calculated T- =-136; $T_{0.0005(2),16}$=15 (Here α= 0.0005, two-tailed test= (2), Number of samples =16)

$T- < T_{0.0005(2),16}$, H_0 is rejected that the bacterial cell numbers calculated using the culture technique are equal to the qPCR technique. qPCR technique detected greater number of *C. jejuni* than does the culture.

Sunset Park Beach:

Table 7: Sunset Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples.

Sample No.	Dates	Culture (CFU's/100mL)	qPCR (Tar. Seq's/100mL)	S/R
1	6/22/2012	0	348.61	-4
2	6/28/2012	0	507.50	-7
3	8/9/2012	0	1086.34	-15
4	8/10/2012	0	832.35	-13.5
5	8/14/2012	0	724.93	-10
6	8/16/2012	0	738.79	-11
7	8/20/2012	0	110.76	-1
8	8/23/2012	0	547.45	-9
9	8/27/2012	0	400.27	-5
10	8/30/2012	0	402.06	-6
11	9/3/2012	0	244.05	-2
12	9/7/2012	0	280.84	-3
13	9/10/2012	0	769.89	-12
14	9/13/2012	0	832.35	-13.5
15	9/17/2012	0	537.17	-8
16	9/20/2012	0	1182.35	-16

CFU's =Colony forming units; S/R= Sum of ranks

N= 16; Calculated T- =-136 ; $T_{0.0005(2),16}$=15 (Here α= 0.0005, two-tailed test= (2), Number of samples =16)

$T- < T_{0.0005(2),16}$, H_0 is rejected that the bacterial cell numbers calculated using the culture technique are equal to the qPCR technique. qPCR technique detected greater number of *C. jejuni* than does the culture.

Whitefish Dunes Park Beach:

Table 8: Whitefish Dunes Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water samples.

Sample number	Dates	Culture- (CFU's/100mL)	qPCR- (Tar. Seq's/100mL)	S/R
1	6/22/2012	0	326.43	-8
2	6/28/2012	0	379.42	-12
3	8/9/2012	0	253.19	-3
4	8/10/2012	0	307.03	-7
5	8/14/2012	0	340.55	-9
6	8/16/2012	0	380.69	-13
7	8/20/2012	0	347.45	-10
8	8/23/2012	0	242.43	-2
9	8/27/2012	0	304.64	-6
10	8/30/2012	0	231.08	-1
11	9/3/2012	0	295.61	-4
12	9/7/2012	0	371.48	-11
13	9/10/2012	0	382.40	-14
14	9/13/2012	0	300.93	-5
15	9/17/2012	0	402.96	-16
16	9/20/2012	0	390.58	-15

CFU's =Colony forming units; S/R= Sum of ranks

N= 16; Calculated T- =-136 ; $T_{0.0005(2),16}$=15 (Here α= 0.0005, two-tailed test= (2), Number of samples =16)

$T- < T_{0.0005(2),16}$, H_0 is rejected that the bacterial cell numbers calculated using the culture technique are equal to the qPCR technique. qPCR technique detected greater number of *C. jejuni* than does the culture.

Menominee Park Beach:

Table 9: Menominee Wilcoxon t-test comparing enumeration of *C. jejuni* from culture and qPCR techniques per 100 mL of water sample.

Sample number	Dates	Culture (CFU's/100mL)	qPCR (Tar. Seq's/100mL)	S/R
1	6/22/2012	0	576.88	-1
2	6/28/2012	0	893.87	-5
3	8/9/2012	0	635.61	-2
4	8/10/2012	0	701.88	-4
5	8/14/2012	0	650.66	-3
6	8/16/2012	0	1543.15	-8
7	8/20/2012	0	1190.28	-7
8	8/23/2012	0	1098.52	-6
9	8/27/2012	0	1690.80	-9
10	8/30/2012	0	3183.99	-13
11	9/3/2012	0	3651.71	-15
12	9/7/2012	0	7939.71	-16
13	9/10/2012	0	3299.57	-14
14	9/13/2012	0	1954.36	-10
15	9/17/2012	0	2573.58	-11
16	9/20/2012	0	2596.63	-12

CFU's =Colony forming units; S/R= Sum of ranks

N= 16; Calculated T- =-136 ; $T_{0.0005(2),16}$=15 (Here α= 0.0005, two-tailed test= (2), Number of samples =16)

$T- < T_{0.0005(2),16}$, H_0 is rejected that the bacterial cell numbers calculated using the culture technique are equal to the qPCR technique. qPCR technique detected greater number of *C. jejuni* than does the culture.

The calculated T minus was equal for all the beaches as the detection with culture enumeration was equal to 0. This means that enumeration of the bacterial cells using selective plate count method was not equal to the molecular enumeration (qPCR) as expected.

Analysis of Variance Among qPCR Target Sequences/ 100 mL for the Four Beaches

C. jejuni enumeration from Otumba, Sunset, Whitefish and Menominee beaches was subjected to ANOVA test in order to observe the difference in the pathogen numbers among the beaches.

H_0: The variance of the pathogen cell counts did not differ among the four beaches

H_A: The variance of the pathogen cell counts differed among the beaches

Table 10: ANOVA of qPCR enumeration of four beaches.

Sl. No.	Response	Df	Sum Square	Mean Square	F value	Pr(>F)	Result
1	Beaches	3	70001242	23333747	17.589	2.619e-08 ***	Reject H_0
1	Residuals	60	79594764	1326579			

This test was used to determine if *C. jejuni* target sequence numbers were different among beaches The calculated F is 17.589 was greater than the $F_{(0.05)(2),3,60}$ which is 3.34. Hence, H_O was rejected concluding the variances among the beaches are not equal which supports the finding that the water quality of each beach is different from others as it is subjected to different physical, chemical and environmental conditions. (Kinzelman *et al.,* 2008)

Detection of Enterococci in Environmental Water Samples

MPN of enterococci was determined using Enterolert[R] assay and MPN calculations in Summer, 2012.

Table 11: Concentration of enterococci at selected beaches (MPN/100mL).

Sl. No.	Date of sampling	Otumba	Sunset	W Dunes	Menominee
1	22-Jun-12	18.3	4.1	1.0	33.7
2	28-Jun-12	14.2	18.9	3.0	16.8
3	9-Aug-12	24.1	7.4	7.5	1,046.2
4	10-Aug-12	4.0	42.8	61.3	285.1
5	14-Aug-12	18.7	9.7	6.3	4.1
6	16-Aug-12	4.1	2	435.2	156.5
7	20-Aug-12	0	17.5	4.1	10.9
8	23-Aug-12	0	11	11.9	24.1
9	27-Aug-12	8.5	21.3	23.3	63.6
10	30-Aug-12	12.1	5.2	39.9	99.5
11	3-Sep-12	14.4	17.1	1.0	11.0
12	7-Sep-12	0	11.0	0	65.7
13	10-Sep-12	17.6	15.8	19.6	25.0
15	13-Sep-12	770.1	51.2	9.6	2.0
16	17-Sep-12	648.8	3.1	10.7	4.1

EPA Method 1611 qPCR on the Beach Water Samples for the Detection of Enterococci

Before conducting the experiments with beach samples, three standard curve experiments were conducted during the week of enterococci assay analysis for the beach samples (Table 12). The best standard curve experiment that gave a square regression value closest to 0.99 (as required by the EPA) was chosen (Figure 9).

***Enterococcus* standard curve:**

Table 12: Log transformed values of target sequences of lsrRNA gene for standard curve experiment.

Sample No.	Target DNA copy number/1µL	Log transformed values	Avg Ct Value
1	1×10^2	2	33.24
2	2×10^2	2.30	32.44
3	4×10^2	2.60	31.9
4	4×10^3	3.60	29.02
5	4×10^4	4.30	26.3
6	4×10^5	5.60	20.55

The number of target sequences is easy to calculate if there is an endogenous control involved in the experiment. The average target sequences per calibrator extract were calculated using the formula:

Avg. seq's/ calibrator extract = 6382 target seq's X 3000µL extract vol/ 5µL

Standard Curve Experiment for Enterococci

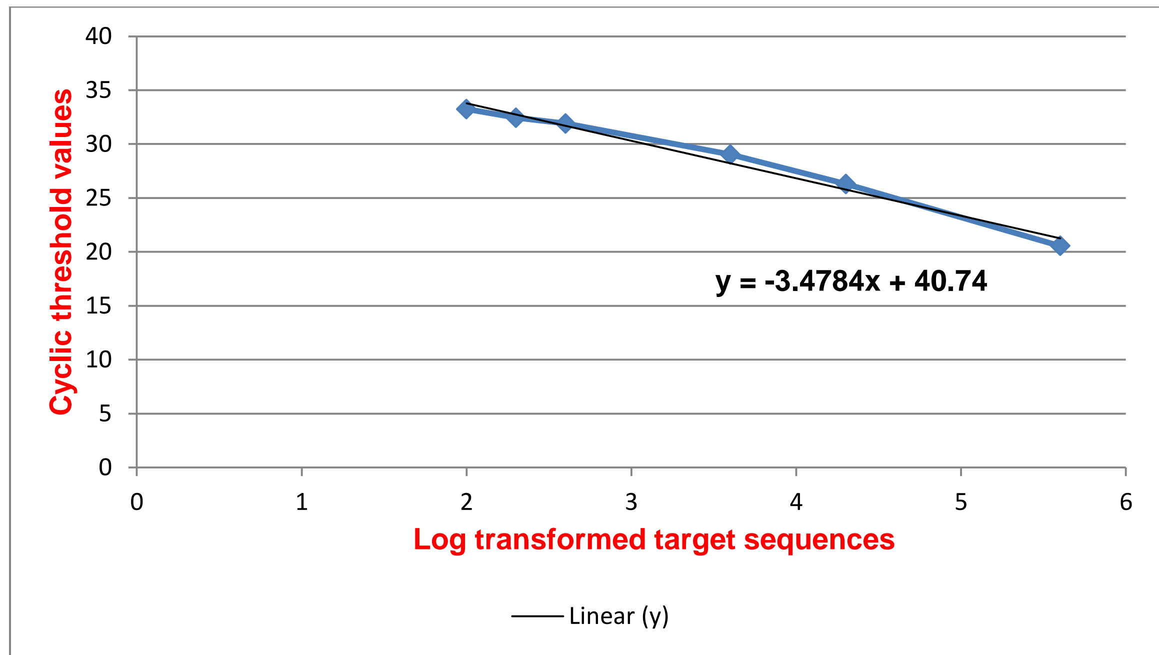

Figure 9: Standard curve of enterococci lsrRNA gene at 1x 10^2, 2x 10^2, 4x 10^2, 4x 10^3, 4x 10^4 and 4x10^5 dilutions of target sequences.

The slope of the line obtained was y= -3.4784x+ 40.74

Regression analysis was performed to obtain the square regression value:

R^2= 0.9841. So there is 98% confidence in the working of the assay.

Table 13: Enterococci concentrations per 100 mL of water sample by qPCR method from the four beaches in Summer, 2012.

Sl. No.	Date of sampling	Otumba	Sunset	W Dunes	Menominee
1	22-Jun-12	806.90	406.90	172.85	145.01
2	28-Jun-12	470.18	502.64	221.99	1,011,589.58
3	9-Aug-12	293.71	1101.02	293.70	1245.46
4	10-Aug-12	685.97	646.25	211.22	93.95
5	14-Aug-12	251.36	3,398.79	111.43	220.15
6	16-Aug-12	36,337.00	159,216.41	420.78	243.16
7	20-Aug-12	102,221.75	885.60	246.00	116.53
8	23-Aug-12	283.20	102,131.15	312.28	158.59
9	27-Aug-12	393.15	765.92	426.39	1,021,695.25
10	30-Aug-12	3,633.50	139,446.00	291.29	158.59
11	3-Sep-12	440.03	1,292.50	97.60	397.07
12	7-Sep-12	185.93	2,,106.29	155.21	233,863.54
13	10-Sep-12	248.05	1,986.61	3,87.33	470.18
14	13-Sep-12	359.50	957.40	5290.01	247.23
15	17-Sep-12	73.77	646.25	196.37	142.63
16	20-Sep-12	571.69	3,255.18	205.02	274.88

Quantification of Enterococci in Otumba Park Beach with Culture and qPCR techniques

Table 14: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Otumba Park beach.

Sl No.	Date of sampling	Culture-(MPN/100mL)	Culture Log_{10} values	qPCR-(Tar. Seq's/ 100mL)	qPCR Log_{10} Values
1	22-Jun-12	18.3	1.26	806.90	2.91
2	28-Jun-12	14.2	1.15	470.18	2.67
3	9-Aug-12	24.1	1.38	293.71	2.47
4	10-Aug-12	4.0	0.60	685.97	2.84
5	14-Aug-12	18.7	1.27	251.36	2.40
6	16-Aug-12	4.1	2.81	36,337.00	4.56
7	20-Aug-12	0	1.13	102,221.75	5.01
8	23-Aug-12	0	2.89	283.20	2.45
9	27-Aug-12	8.5	0.61	393.15	2.59
10	30-Aug-12	12.1	1.04	3,633.50	3.56
11	3-Sep-12	14.4	0.00	440.03	2.64
12	7-Sep-12	0	0.93	185.93	2.27
13	10-Sep-12	17.6	1.08	248.05	2.39
14	13-Sep-12	770.1	1.16	359.50	2.56
15	17-Sep-12	648.8	0.00	73.77	1.87
16	20-Sep-12	13.5	1.25	571.69	2.76

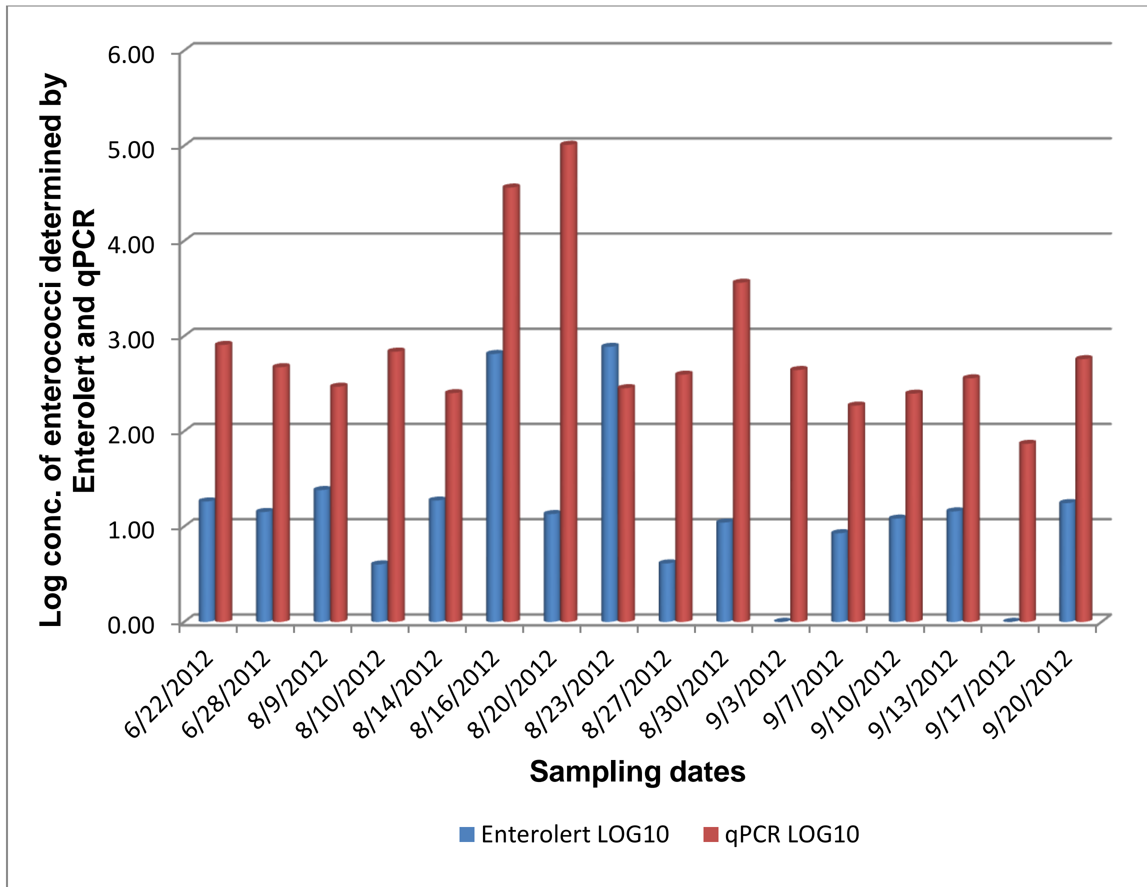

Figure 10: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Otumba water samples.

High enterococci enumeration was observed for all the samples with qPCR except for August 23rd water sample from Otumba Park beach which could be due to the errors or presence of inhibitory compounds in that water sample.

Correlation between Log_{10} transformed Enterolert enumeration vs. Log_{10} transformed qPCR enumeration from beach water at Otumba Park

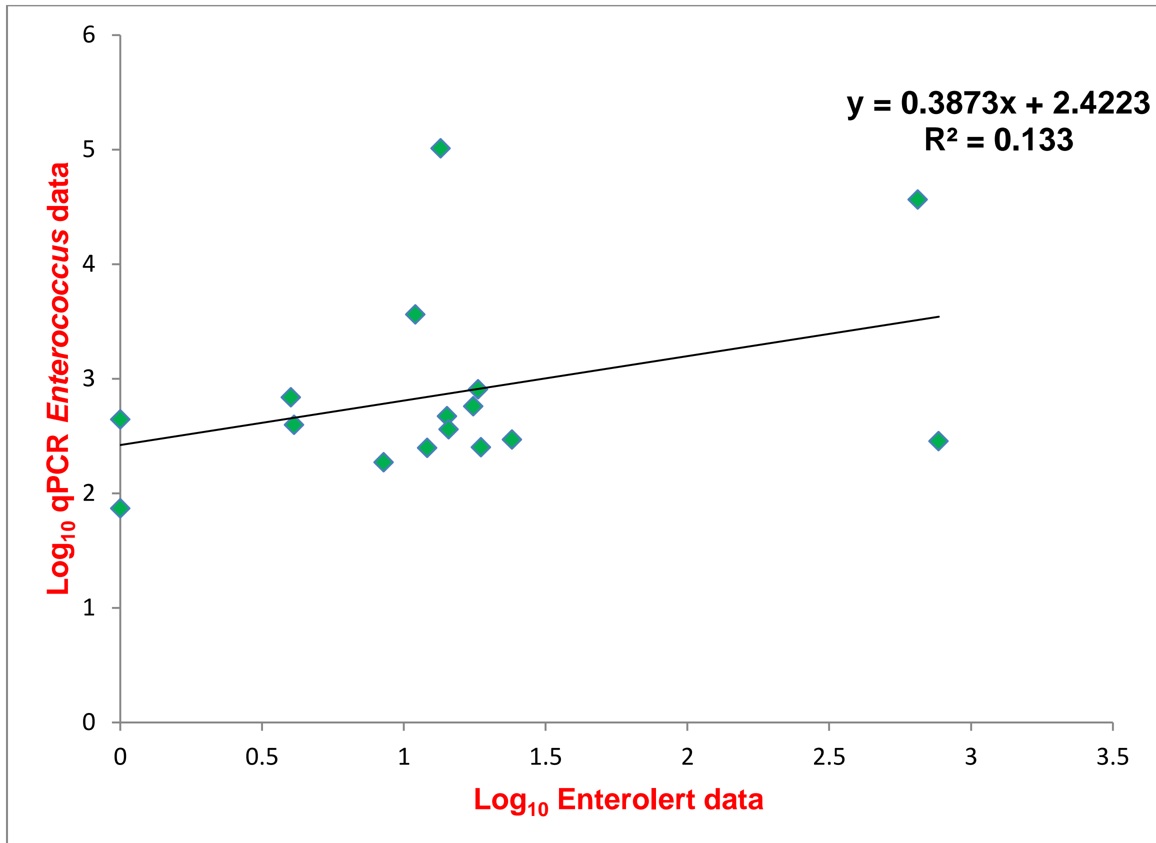

Figure 11: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Otumba Park using scatter plot.

The equation of the line is y= 0.3873x + 2.4223 and R^2= 0.133. A positive correlation between x and y axes was observed.

Quantification of enterococci in Sunset Park Beach with Culture and qPCR techniques

Table 15: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Sunset Park Beach.

Sl. No.	Date of sampling	Culture- (MPN/ 100mL)	Culture Log_{10} values	qPCR- (Tar Seq's/ 100mL)	qPCR Log_{10} values
1	22-Jun-12	4.1	0.61	406.90	2.61
2	28-Jun-12	18.9	1.28	502.64	2.70
3	9-Aug-12	7.4	0.87	1101.02	3.04
4	10-Aug-12	42.8	1.63	646.25	2.81
5	14-Aug-12	9.7	0.99	3398.79	3.53
6	16-Aug-12	2.0	0.30	159216.41	5.20
7	20-Aug-12	17.5	1.24	885.60	2.95
8	23-Aug-12	11.0	1.04	102131.15	5.01
9	27-Aug-12	21.3	1.33	765.92	2.88
10	30-Aug-12	5.2	0.72	139446.00	5.14
11	3-Sep-12	17.1	1.23	1292.50	3.11
12	7-Sep-12	11.0	1.04	2106.29	3.32
13	10-Sep-12	15.8	1.20	1986.61	3.30
14	13-Sep-12	51.2	1.71	957.40	2.98
15	17-Sep-12	3.1	0.49	646.25	2.81
16	20-Sep-12	20.6	1.31	3255.18	3.51

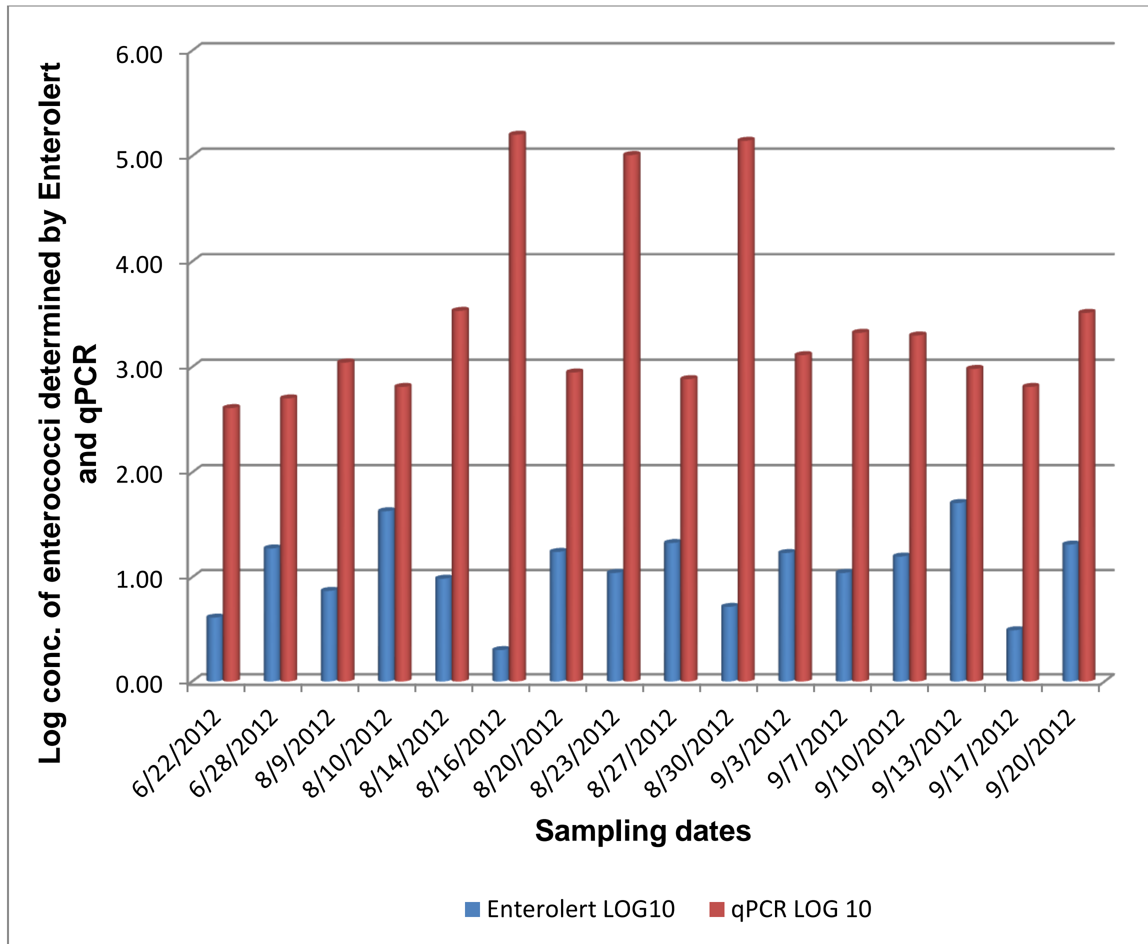

Figure 12: Comparison of MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Sunset water samples.

High enterococci enumeration was observed with qPCR when compared to the culture technique for Sunset Park beach.

Correlation between Log_{10} transformed Enterolert enumeration vs. Log_{10} transformed qPCR enumeration from beach water at Sunset Park

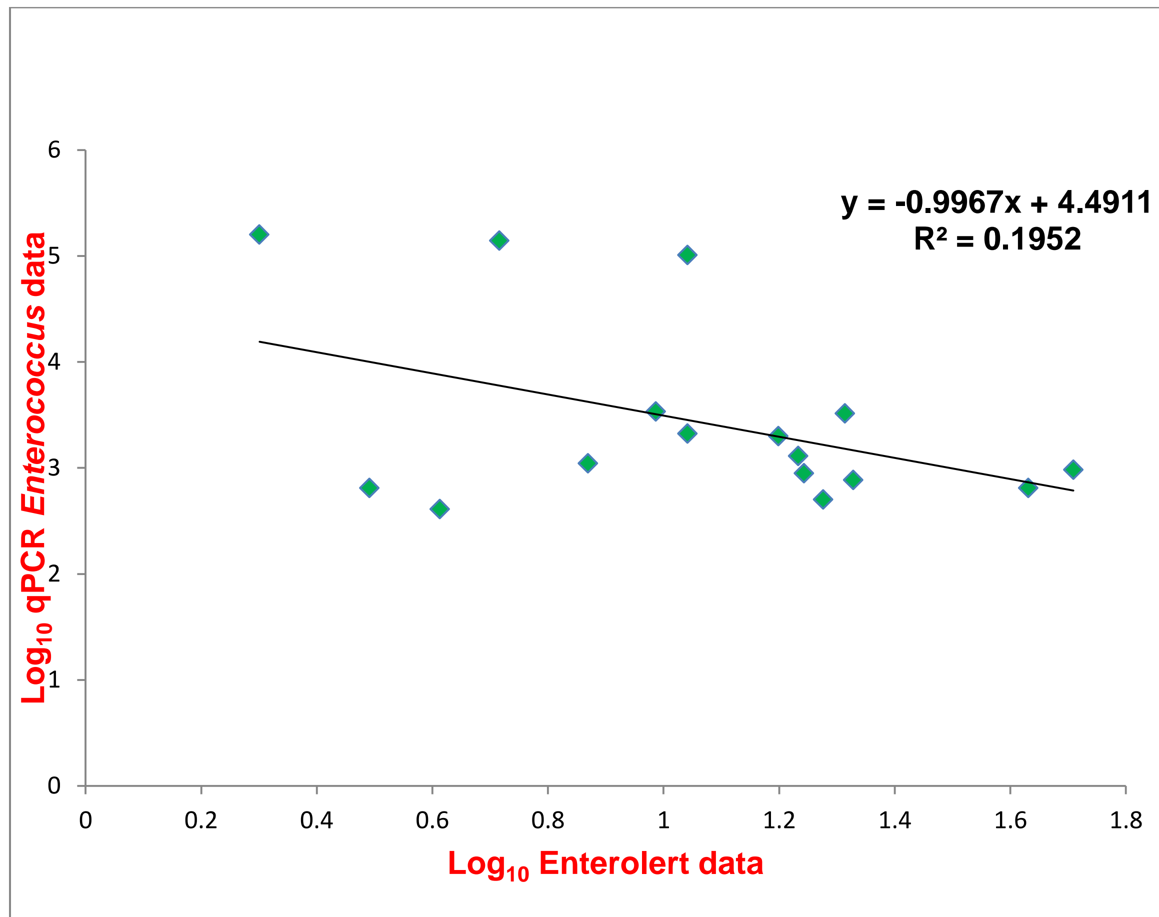

Figure 13: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Sunset Park using scatter plot.

The equation of the line is $y = -0.9967x + 4.4911$ and $R^2 = 0.1952$. A negative correlation between x and y axes was observed.

Quantification of enterococci in Whitefish Dunes Park Beach with Culture and qPCR techniques

Table 16: MPN and target sequences (/100mL) of *enterococcus* using Enterolert and qPCR methods from Whitefish Dunes Park Beach.

Sl. No.	Date of sampling	Culture- (MPN/ 100mL)	Culture Log_{10} values	qPCR- (Tar. Seq's/ 100mL)	qPCR Log_{10} values
1	22-Jun-12	1.0	0	172.85	2.24
2	28-Jun-12	3.0	0.48	221.99	2.35
3	9-Aug-12	7.5	0.88	293.70	2.47
4	10-Aug-12	61.3	1.79	211.22	2.32
5	14-Aug-12	6.3	0.80	111.43	2.05
6	16-Aug-12	435.2	2.64	420.78	2.62
7	20-Aug-12	4.1	0.61	246.00	2.39
8	23-Aug-12	11.9	1.08	312.28	2.49
9	27-Aug-12	23.3	1.37	426.39	2.63
10	30-Aug-12	39.9	1.60	291.29	2.46
11	3-Sep-12	<1	0	97.60	1.99
12	7-Sep-12	<1	0	155.21	2.19
13	10-Sep-12	19.6	1.29	387.33	2.59
14	13-Sep-12	9.6	0.98	5290.01	3.72
15	17-Sep-12	10.7	1.03	196.37	2.29
16	20-Sep-12	8.4	0.92	205.02	2.31

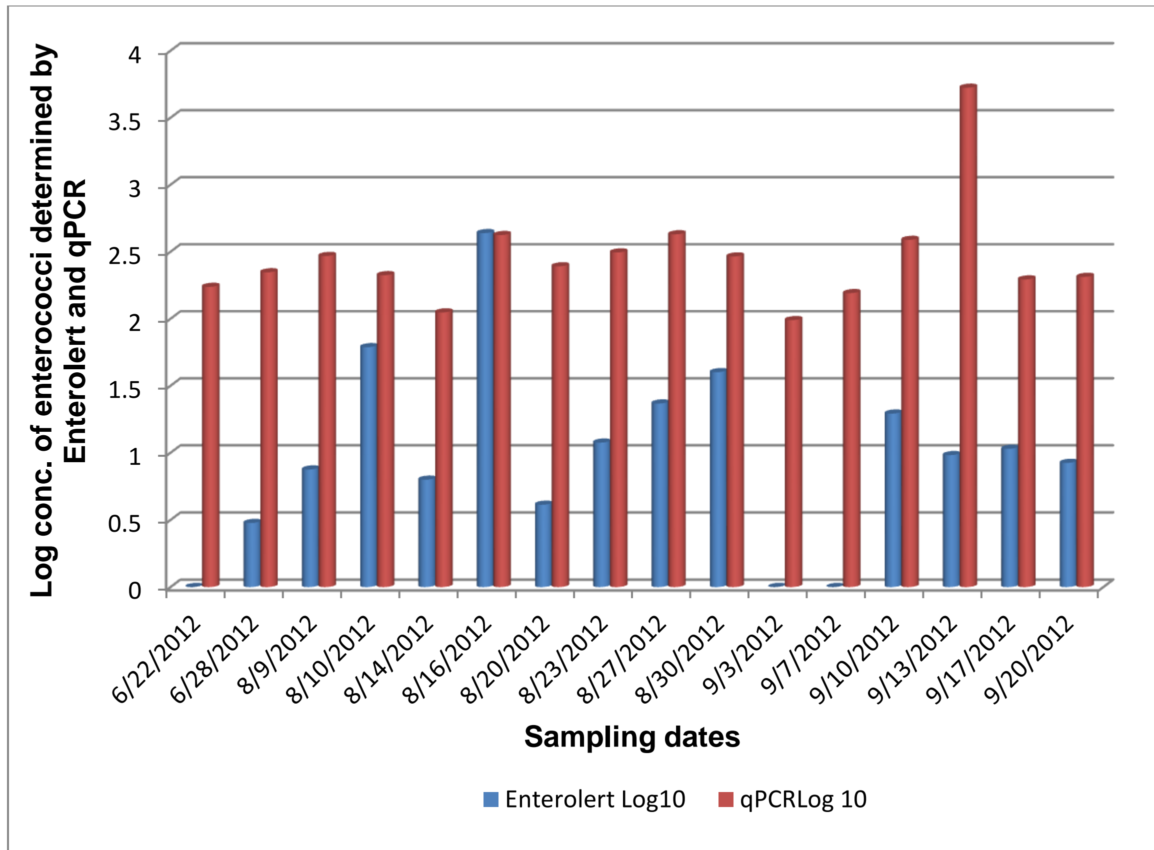

Figure 14: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Whitefish Dunes Park Beach water samples.

High enterococci enumeration was observed in most of the weeks with qPCR technique. The enumeration of the FIB with culture method was almost equal to that of the qPCR method for August 18th water sample from Whitefish Dunes Park beach. For three other weeks which are June 22nd, September 3rd and 7th, no enumeration was obtained with culture technique which could be due to the dormant state of the organism as bacterial species have the ability to enter dormancy upon environmental stress.

Correlation between Log_{10} transformed Enterolert enumeration vs. Log_{10} transformed qPCR enumeration from beach water at Whitefish Dunes Park:

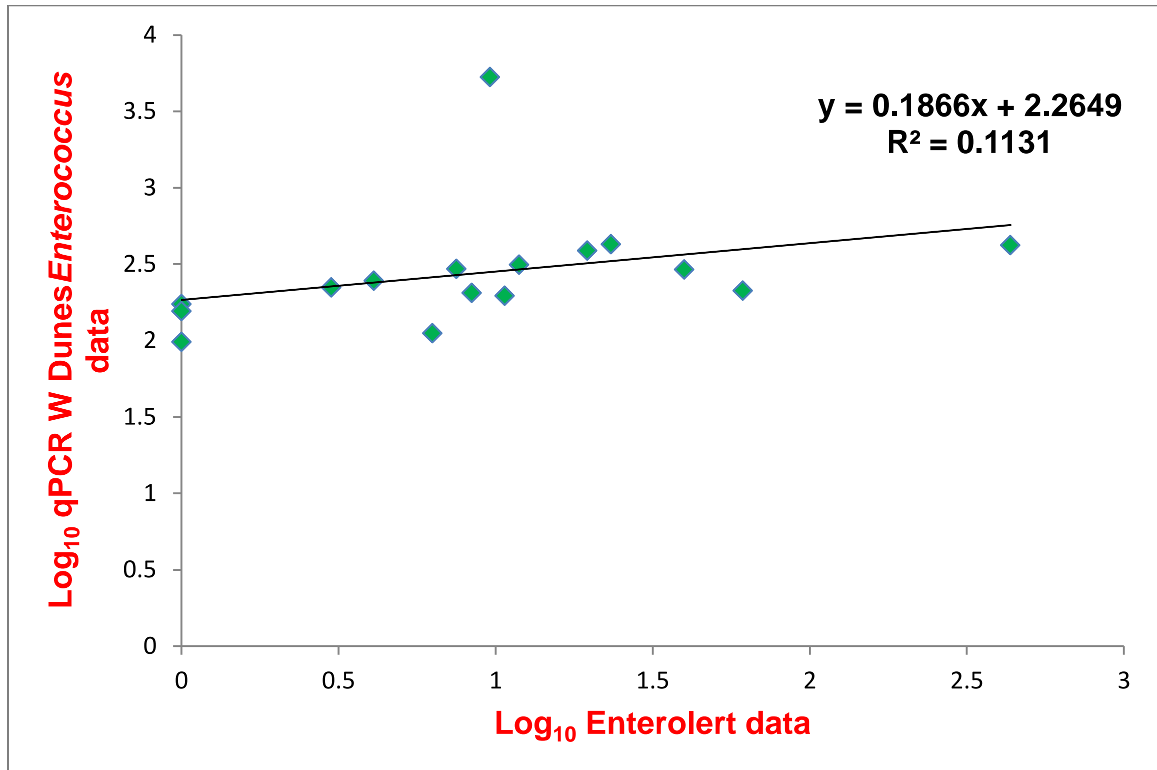

Figure 15: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Whitefish Dunes Park using scatter plot.

The equation of the line is $y = 0.1866x + 2.2649$ and $R^2 = 0.1131$. A positive correlation between x and y axes was observed.

Quantification of enterococci in Menominee Park Beach with Culture and qPCR techniques

Table 17: MPN and target sequences (/100mL) of enterococcus using Enterolert and qPCR methods from Menominee beach.

Sl. No.	Date of sampling	Culture- (MPN/ 100mL)	Culture Log_{10} values	qPCR- (Tar. Seq's/ 100mL)	qPCR Log_{10} values
1	22-Jun-12	33.7	1.53	145.01	2.16
2	28-Jun-12	16.8	1.23	1011589.58	6.01
3	9-Aug-12	1046.2	3.02	1245.46	3.10
4	10-Aug-12	285.1	2.45	93.95	1.97
5	14-Aug-12	4.1	0.61	220.15	2.34
6	16-Aug-12	156.5	2.19	243.16	2.39
7	20-Aug-12	10.9	1.04	116.53	2.07
8	23-Aug-12	24.1	1.38	158.59	2.20
9	27-Aug-12	63.6	1.80	1021695.25	6.01
10	30-Aug-12	99.5	2.00	158.59	2.20
11	3-Sep-12	11	1.04	397.07	2.60
12	7-Sep-12	65.7	1.82	233863.54	5.37
13	10-Sep-12	25	1.40	470.18	2.67
14	13-Sep-12	2	0.30	247.23	2.39
15	17-Sep-12	4.1	0.61	142.63	2.15
16	20-Sep-12	17.1	1.23	274.88	2.44

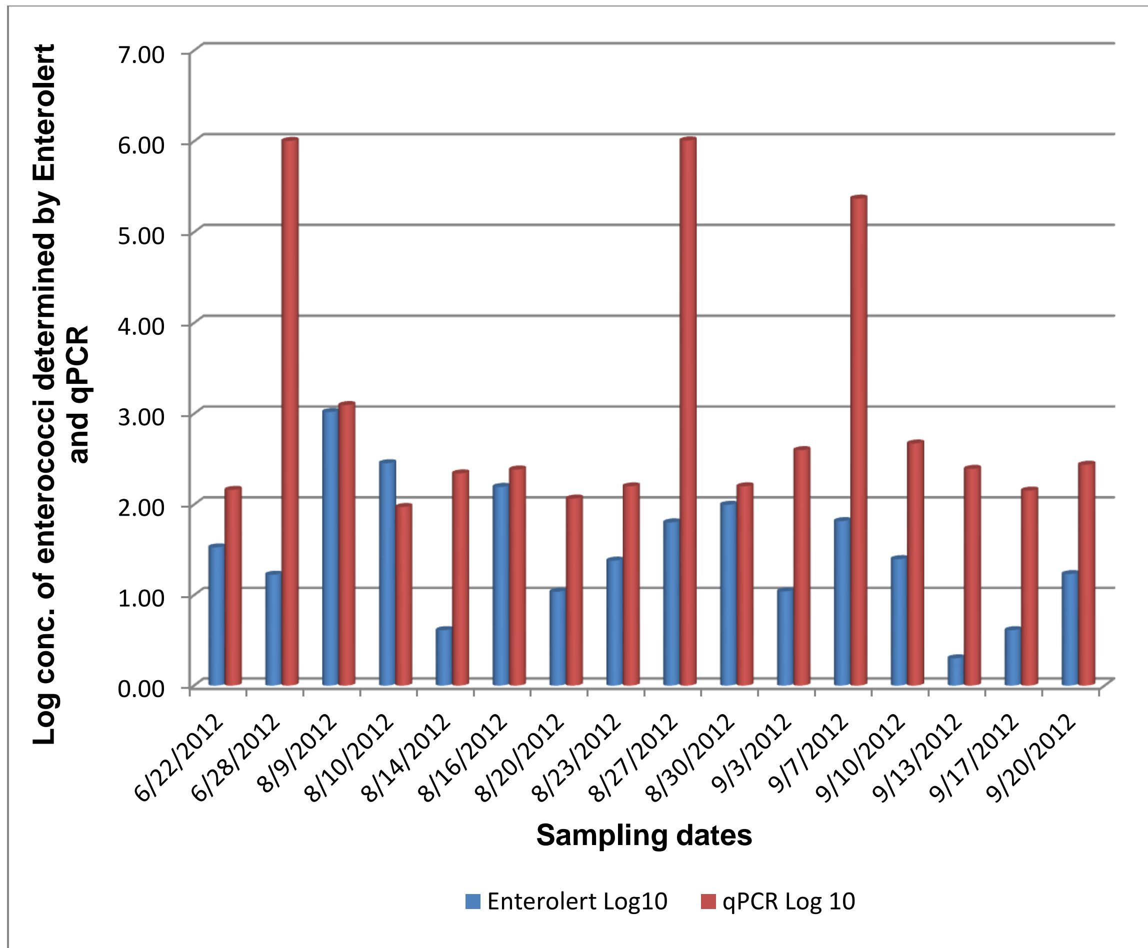

Figure 16: Comparison of the MPN and target sequences (/100mL) of enterococci obtained by performing IDEXX Enterolert and qPCR on Menominee water samples.

Mixed enumeration (qPCR enumeration being higher, lower and similar to that of the culture) of enterococci was observed with samples from Menominee Park beach. This could be due to the effect of PCR inhibition compounds such as clay particles (Menominee water was highly turbid throughout the field work) that could delay the amplification of the target sequences.

Correlation between Log_{10} transformed Enterolert enumeratoin vs. Log_{10} transformed qPCR enumeration from beach water at Menominee Park:

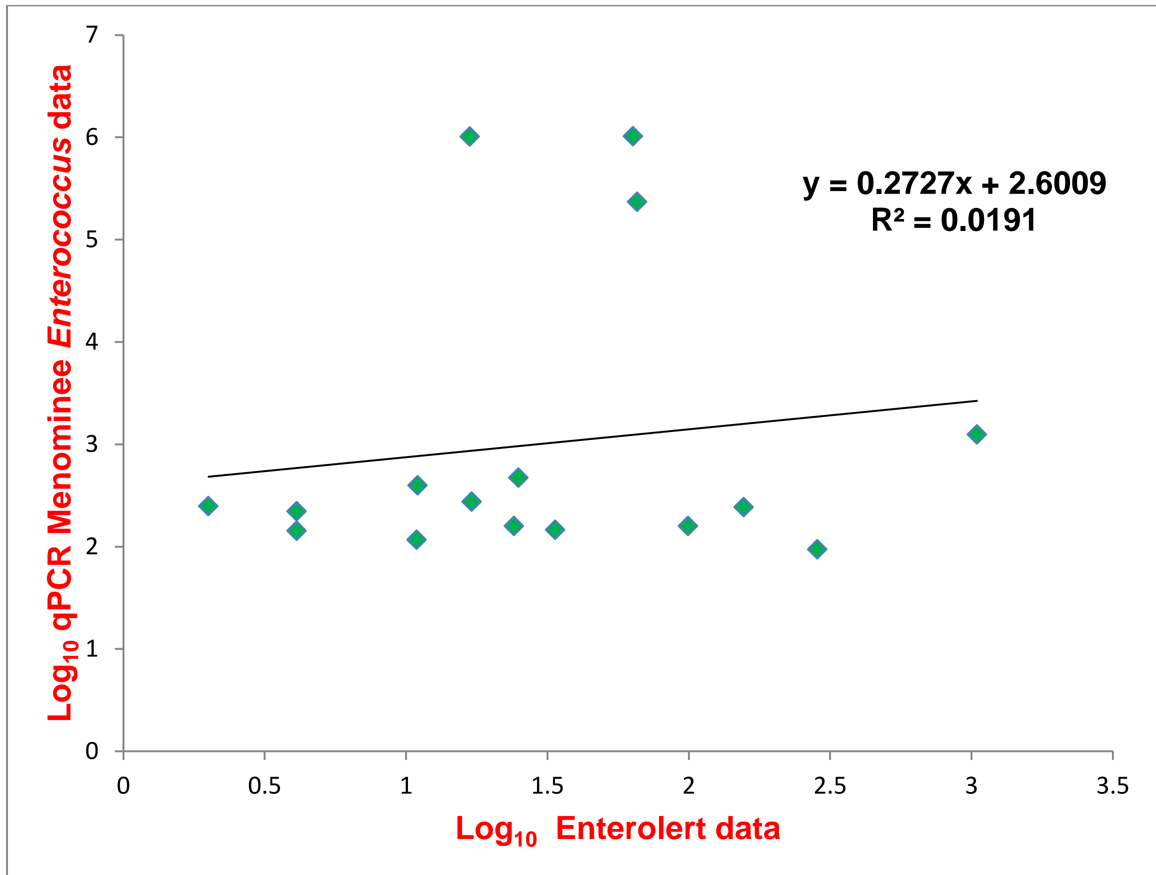

Figure 17: Correlation between Log10 enterococci enumerations obtained with Enterolert and qPCR from water collected at Menominee Park using scatter plot.

The equation of the line is $y = 0.2727x + 2.6009$ and $R^2 = 0.0191$. A positive correlation between x and y axes was observed.

Paired t-test

Paired t-test was used in order to test if the FIB enumeration obtained through culture technique was equal to the qPCR technique.

H_o= The different between the means is equal to zero

H_A= The difference between the means is not equal to zero

Table 18: Paired t-test comparing mean of enterococci enumerations using Enterolert and qPCR enumeration at the four beaches.

Sl. No.	Beaches	t- value	Degrees of Freedom	p-value	Result
1	Otumba	-1.9203	15	0.07404	Reject H_o
2	Sunset	-1.6392	15	0.122	Reject H_o
3	W Dunes	-1.0076	15	0.3296	Reject H_o
4	Menominee	-1.7614	15	0.09854	Reject H_o

Null hypothesis was rejected as the p-value calculated for all the four beaches was higher than 0.05.

ANOVA of Culture *Enterococcus* Enumerations

ANOVA test to compare the variances of the two MPN enumerations obtained by performing culture technique and qPCR was conducted to see how highly the bacterial cell number varied over the time of the field work.

H_o = Enumeration of the bacterial cells do not differ between the techniques throughout the sampling

H_A= Enumeration of the bacterial cells differs between the techniques throughout the sampling

Table 19: ANOVA test on Enterolert and qPCR enumeration to estimate the variance among the four beaches

Sl. No.	Beach	DF-T's	DF-Wks	Sum of Squares	Mean of Squares	F-values	Pr(>F)
2	Otumba	1	30	90585	90585	2.402	0.1317
4	Sunset	1	30	2.58e+12	2.58e+12	2.6869	0.1116
1	W Dunes	1	30	5.30e+15	5.30e+15	1.0153	0.3217
3	Menominee	1	30	39786214	39786214	3.1144	0.08778

T's =Techniques; Wks= Weeks and R=Result

Two-way ANOVA test was performed with α= 0.05 to test the consistency in the culture enumeration and qPCR in the weeks sampled from

June to September, 2012. As F calculated was lower than F expected, null hypothesis was not rejected in all the four cases.

ANOVA of qPCR *Enterococcus* Enumerations

Analysis of variance was conducted on qPCR enumeration of enterococci to analyze the variance in the indicator numbers throughout the field work. This was performed to see if the target sequences detected significantly differed among the four beaches.

H_0: The variance of indicator cell counts did not differ between the four beaches

H_A: The variance of indicator cell counts differed between the beaches

Table 20: ANOVA test on *Enterococcus* qPCR enumeration to estimate the variance among the four beaches.

Sl. No.	Response	Deg of Frdm	Sum of squares	Mean of Squares	F value	Pr(>F)	Result
1	Beaches	3	7.8141 e+15	2.6047 e+15	0.9978	0.4001	Do not reject H_O
2	Residuals	60	1.5662 e+17	2.6104 e+15			

The calculated F value 0.9978 is lower than $F_{(0.05)(2),3,60}$ which is 3.34.

Hence, the null hypothesis was accepted. So, the hypothesis that the variance of the FIB counts did not differ between the four beaches can be accepted.

Comparison of *Enterococcus* and *C. jejuni* Concentrations Each Determined by qPCR Method

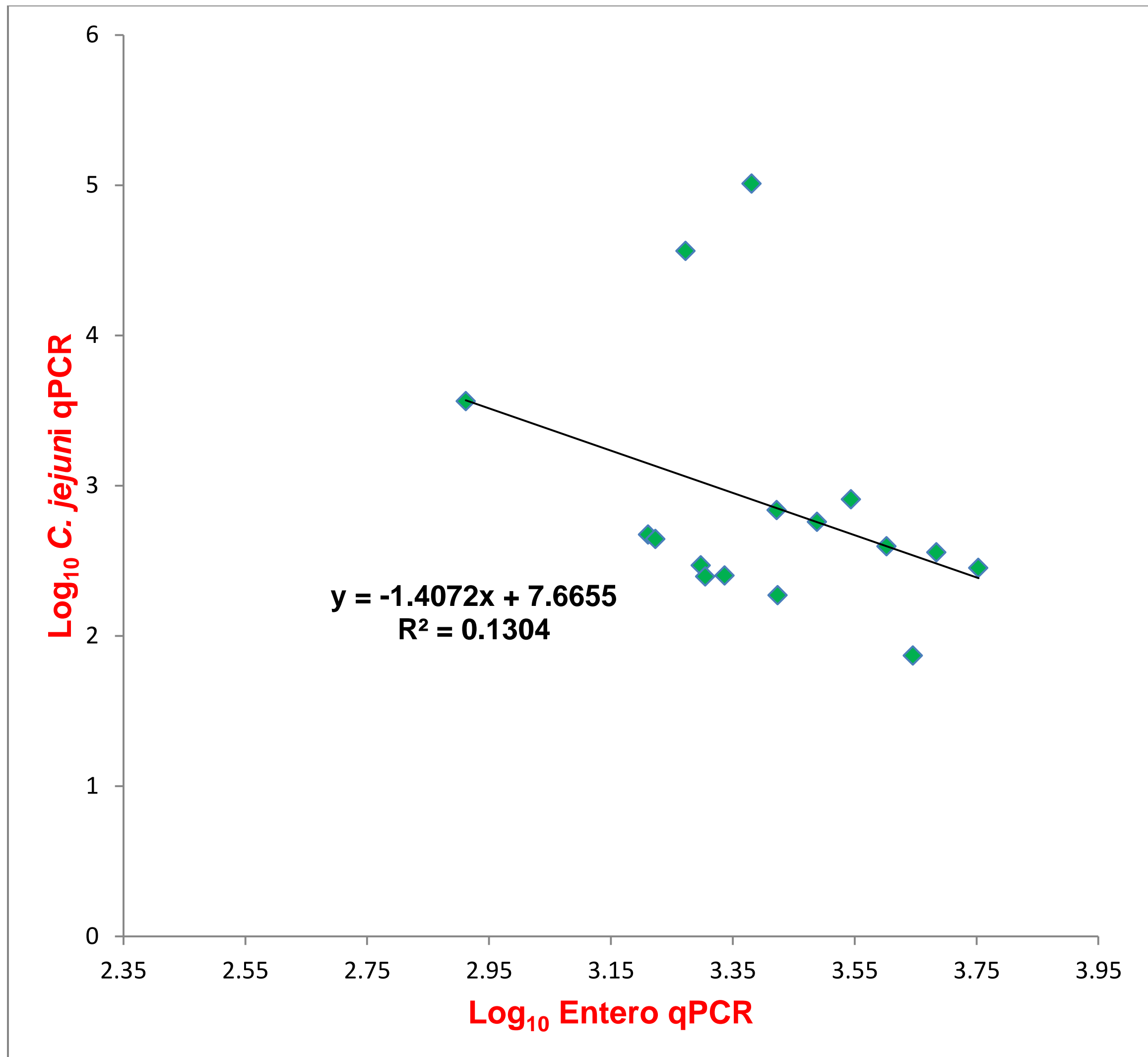

Figure 18: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Otumba Park beach.

The equation of the line is $y = -1.4072x + 7.6655$ and $R^2 = 0.1304$. A negative correlation between x and y axes was observed.

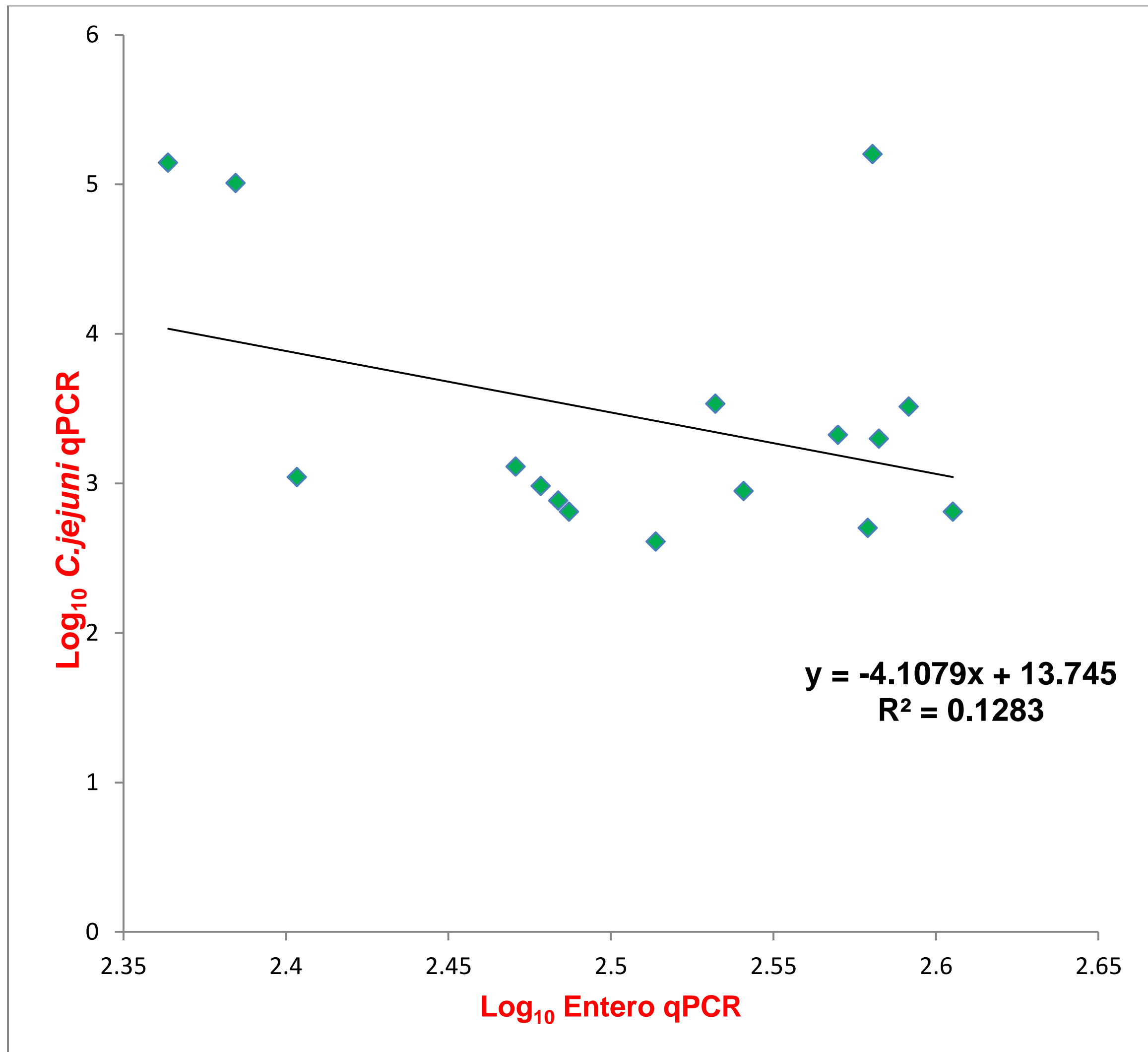

Figure 19: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Sunset Park beach.

The equation of the line is $y = -4.1079x + 13.745$ and $R^2 = 0.1283$. A negative correlation between x and y axes was observed.

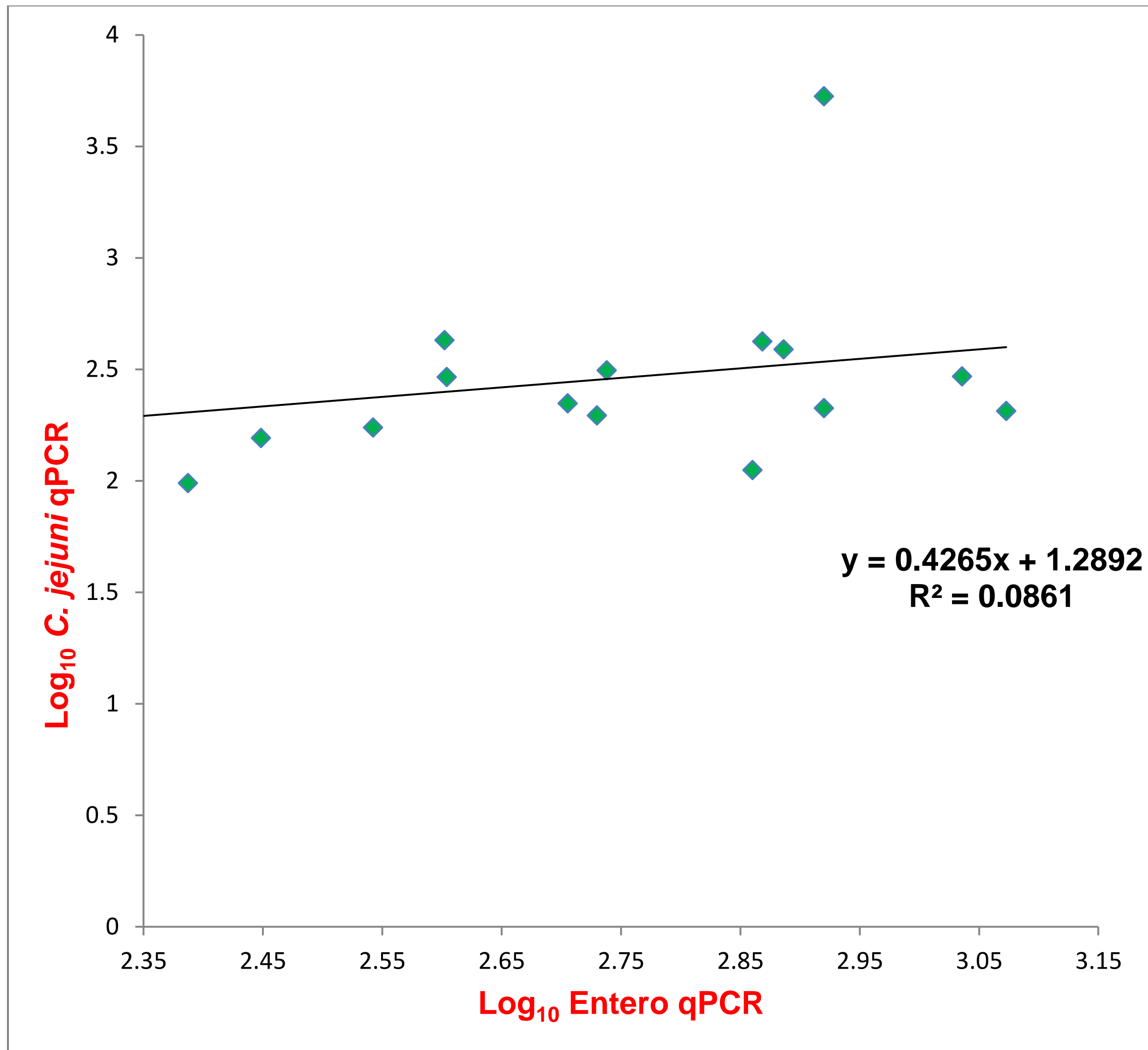

Figure 20: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Whitefish Dunes Park beach.

The equation of the line is $y = 0.4265x + 1.2892$ and $R^2 = 0.0861$. No correlation between x and y axes was observed.

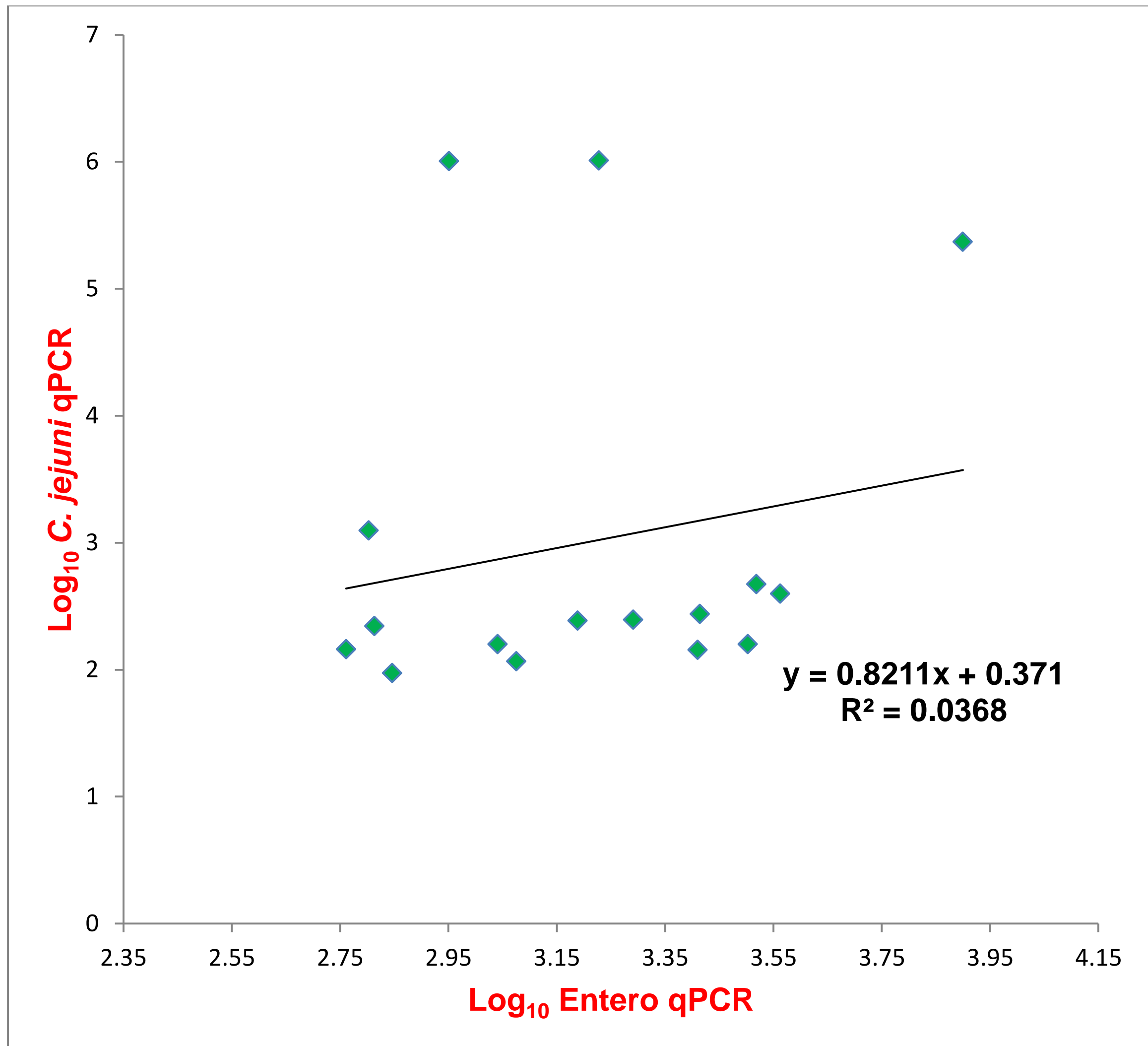

Figure 21: Plot shows the correlation between the qPCR enumeration of *Enterococcus* and *C. jejuni* at Menominee Park beach.

The equation of the line is $y = 0.8211x + 0.371$ and $R^2 = 0.0368$. A positive correlation between x and y axes was observed.

DISCUSSION

Specific Detection and Quantification of *C. jejuni* from the Selected Beaches

C. jejuni is an important pathogen that has been determined to be the most common cause of gastroenteritis in the world (Friedman *et al.,* 2000, Savill *et al.,* 2001). Therefore developing rapid methods to detect these pathogens is necessary when considering the risk to humans, animals and environment. In this study, a real time PCR analysis was developed and applied to the detection of this pathogen. The 86 base pair target region was a gene coding for the subunit of the oxidoreductase enzyme which plays a critical role in cell respiration (NCBI). This gene is very specific for *C. jejuni* species and had no homology with any of the recently updated bacterial databases on NCBI except for *Bacillus subtilis* strain 168 where the homology was 57.7% (Nogva *et al.,* 2000). Initially, a TaqMan assay was investigated to detect and quantify these species from the water sample. After several experiments, qPCR assay using TaqMan chemistry was discontinued due to the interference problems with the probe designed for the reaction. This could be due to improper absorption of the wave length emitted from the reporter dye by the quencher. At this point, a new set of qPCR assays using SYBR green chemistry were started. An $MgCl_2$ gradient-matrix experiment was conducted to adjust the concentration of the $MgCl_2$ in the reaction. With appropriate settings, qPCR assays were carried out on the ABI StepOne Plus

instrument using SYBR Green chemistry. SYBR Green chemistry comes with certain limitations. Unlike, TaqMan probes it does not just specifically bind to the target region but also binds to any double stranded DNA fragment in the reaction well such as primer dimers. Primer dimers are the unwanted PCR products that could be formed when there is self-complementarity within the primer sequences chosen. This problem can be reduced by designing the primers that have no repeat sequences or by conducting a gradient experiment with different concentrations of primers and finding out the appropriate concentrations of primers that should be used. The later was conducted in this study which was helpful in minimizing the primer dimer problem but not in eliminating it. After optimization of the assay using pure cultures, the standard curve experiments were conducted to gain confidence in the working of the assay. The best confidence in the assay achieved was 95% (R^2= 0.945). After gaining confidence in the working of the assay, the tests were conducted on the water samples collected from Lake Michigan and Lake Winnebago beaches. Out of the four beaches tested, it was determined that Menominee had the highest number of *C. jejuni* target sequences (theoretical calculation) with a mean of 2,136.33 per 100mL of the filtered water showing the highest contamination among four beaches, followed by Otumba and Sunset while Whitefish Dunes being the least contaminated, with 328.55 target sequences per 100mL of water sample (Table 21). Culture technique did not detect any *C. jejuni* except for the first week (22nd June) of sampling at Otumba Park.

Table 21: Mean of number of target sequences of *C. jejuni* quantified from the water samples (qPCR).

Beaches	Otumba	Sunset	W Dunes	Menominee
Mean- Tar. Seq's/ 100 mL	1417.90	596.61	328.55	2,136.33

To confirm that the Ct values obtained are not just primer dimer values, a melt curve analysis was done each time samples were run and the qPCR product was run on a 2.5% Ethidium Bromide gel. The Ct values obtained by performing SYBR Green qPCR were assumed to be partially affected by primer dimer formation only in case of Menominee Park beach as a different peak (at 70°C) apart from the amplification peak was observed (75°C). When a gel was run, a wide bright band at 100 bp's region and lighter band at around the 86 base pair region were observed after running the gel. This bright band was likely the primer dimer, whereas the lighter band was amplification product. These bands were observed with the 'No Template Control' of the Menominee Park Beach assay, which could indicate possible cross contamination with DNA from other bacterial species. The possibility of the contamination was eliminated, however, by starting with fresh primer stocks and reagents. The reason why the 'No Template Control' showed bands remained inconclusive. Errors like this could be avoided if strict qPCR sterile techniques are practiced.

Wilcoxon t-test, a non-parametric test was conducted to find out the relation between culture and qPCR enumeration. No correlation was obtained as

expected, as the culture enumeration set did not have any comparable observations (Tables 6-9).

ANOVA was conducted on the qPCR enumeration of the four beaches to see the variance among the beaches. This analysis produced an F-value of 17.589 which determines that the variances differed among the four beaches which is true (Table 10).

Note: Conclusions drawn on the enumeration of *C. jejuni* from 'Menominee Park Beach' may not be reliable due to partial primer dimer effect on its quantification but statistical analysis was done in order to study the relative quantification of the pathogen when compared to the FIB for all the four beaches.

Detection and Quantification of Enterococci from the Selected Beaches

Enterococci are being used as indicators to estimate the fecal contamination of drinking and recreational water. The idea of enumerating these organisms along with the pathogens, such as *C. jejuni*, in Great Lakes beaches is a new approach. The other reason for using an indicator organism, along with a pathogen, is that the indicator does not always indicate risk, such as gastrointestinal illness in swimmers and may not mimic pathogen survival. The risk of acquiring a gastrointestinal illness upon exposure to the contaminated water can be better related to pathogen concentration in the water. USEPA

suggests the use of enterococci as an indicator of the fecal contamination in recreational water (Kinzelman *et al.,* 2003).

Both culture and qPCR techniques estimated the amount of the enterococci present in the water samples. The primers and probe of the enterococci qPCR assay targeted the lsrRNA gene of this bacterium. A standard curve experiment was conducted during the week of test sample analysis which derived a confidence of 98% or R^2 value of 0.9841 (Figure 15). This shows that the variation in the Ct values obtained was well explained by the variation in concentrations of target DNA used in the experiment. With the culture methods, Menominee showed highest indicator bacteria concentration with a mean of 116.59 target sequences per 100mL of the sample, followed by Otumba and Whitefish dunes while Sunset showing only 16.17 MPN per 100mL of water. With qPCR, Menominee had the highest indicator concentration with 141,941.4 target sequences per 100mL of the water sample followed by Sunset and Otumba while Whitefish Dunes showing least number of target sequences with a mean of 146.66 sequences per 100mL (Table 22). This was further confirmed by conducting a paired t-test which showed that the means of the four beaches are not equal (Table 18).

Table 22: Mean concentrations of enterococci quantified from water samples using culture and qPCR techniques.

Beaches	Otumba	Sunset	W Dunes	Menominee
Mean- MPN/ 100mL	98.02	16.17	40.2	116.59
Mean- target sequences/ 100 mL	9203.48	26171.56	146.66	1,41,941

ANOVA analysis was conducted to determine if the enterococci target sequence number varied among the four beaches which showed that the variances of the four beaches were similar. More complicated statistical analyses could be conducted in order to obtain stronger conclusions about this hypothesis (Table 20).

A linear regression analysis conducted with log transformed culture and qPCR enumeration for each beach. Figures 11, 15 and 17 show a positive correlation in case of Otumba, Whitefish Dunes and Menominee Park Beaches. In this case, as the MPN of enterococci increased the target sequences detected by qPCR increased. Whereas, Figure 13 for Sunset Park Beach shows a negative correlation between X and Y axis's which means that as the MPN of enterococci increased the target sequences detected using qPCR decreased ($p < 0.05$). This type of relationship can be observed in environmental samples due to possibility of the presence of varied inhibitory products (Opel *et al.,* 2010).

Comparison of *C. jejuni* and Enterococci Concentrations from Beach Waters

C. jejuni is sensitive to specific growth conditions and can enter into a "viable but non-culturable state" with environmental stress. This could explain our inability to culture *C. jejuni* by traditional microbiological techniques. qPCR being a sensitive technique was able to detect the *C. jejuni* target sequences even when culture technique was unable to detect the organism. Linear regression analysis of enterococci and *C. jejuni* concentrations obtained by qPCR showed interesting results. There was a negative correlation between FIB and pathogen concentration during same weeks of sampling at Otumba and Sunset beach (Figures 18 and 19). This means that as the FIB concentration increased, the pathogen concentration decreased. For Whitefish Dunes, no correlation between the pathogen and the indicator levels was observed which means that their concentrations were relatively equal (Figures 20). Whereas for Menominee, positive correlation was observed which means that as the FIB concentration increased, the pathogen concentration also increased (Figure 21). High concentrations of both the pathogen and indicator were observed at the Menominee Park. Sunset Park Beach had high number of enterococci target sequences when compared to the *C. jejuni*. The same is true for the Otumba and Whitefish Dunes Park beaches.

These findings indicate that relative quantities of the pathogens and indicators can be highly variable in number between different water bodies during the same season of sampling and potentially can be enough to cause gastroenteritis (considering mean of the pathogen concentrations being higher than 500 organisms in three of the tested beaches). This brings up the necessity to develop rapid multiplex methods that can better detect and analyze different pathogen concentrations (*Campylobacter, Salmonella*, *E. coli* O157:H7) in recreational water. These methods should include assays that can detect other pathogens besides bacterial pathogens, such as viruses (such as *Norovirus*) and protozoans (such as *Cryptosporidium*, *Giardia* etc.) which also cause GI sickness to keep the public safe.

CONCLUSIONS

Considering the potential health risks associated with exposure to recreational water-borne pathogens, such as *C. jejuni*, progress should be made in developing assays that can detect pathogens that can cause GI illness in swimmers. While there are different types of culture and molecular methods available, the qPCR methods can detect lower concentrations of water-borne organisms. The qPCR method in this study was able to detect bacterial concentrations that were ten-fold greater than detected by culture. Additionally, qPCR assays with TaqMan chemistry should be preferred over SYBR Green chemistry as TaqMan has high specificity of target DNA detection hence producing more reliable values (TaqMan method was discontinued in this study due to the interference issues). The limitation of the qPCR assay, to detect the DNA of the non-viable cells can be partially overcome by adding DNases step to the assay. In conclusion, well optimized qPCR assays will continue to be rapid and reliable in quantification of the *Campylobacter jejuni* (and perhaps other pathogens) and FIB from environmental samples.

FUTURE WORK

This study has shown that recreational water can be a potential reservoir for the *C. jejuni* and there is need to develop standard methods that can accurately measure the microbiological burden in water.

- Taking advantage of the rapid detection methods such as qPCR, assays can be developed to detect the concentration of others pathogens (bacteria, viruses, protozoans) in the environmental water samples.
- In situations where recreational water contamination with pathogens occurred, it would be important to identify the source of the contaminant organisms by determining the bacterial strain profiles in the water and comparing them to isolated strains from possible sources such as cattle feces, POWTS, avian feces etc.
- However, additional refinement of this assay is required to obtain more reliable results and to nullify the effect of qPCR inhibition (if any) on target sequence detection. The limitation of qPCR, to detect the extracellular DNA can be overcome by designing assays using DNases that can degrade the DNA of non-viable cells.
- Further longitudinal studies with higher sample size over longer period of time are necessary to establish a precise relationship between culture and molecular techniques, and also to estimate the magnitude of any

microbiological risk on public health, associated with the recreational water exposure.

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