

Bridging the Gap between Molecular and Traditional Assessments of Recreational Water  
Quality

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## **Abstract**

Reagan R. Converse: Bridging the Gap between Molecular and Traditional Assessments of

Recreational Water Quality

(Under the direction of Rachel T. Noble)

Recreational water quality monitoring of fecal indicator bacteria (FIB), such as *E. coli* and *Enterococcus* sp., currently relies on culture methods that require at least 18 hours for results. Given the short duration of many contamination events, regulatory agencies have demanded rapid methods for more accurate protection of public health. Recently, much work has focused on developing QPCR assays for FIB. Work presented examines the relationships between QPCR- and culture-based assays for FIB in recreational water from several perspectives.

Equivalency was compared between culture- and QPCR-measurements of *Enterococcus* sp. concentrations in beach water samples collected over two summers at two popular southern California beaches. QPCR- and culture-based concentrations were significantly correlated, and management decisions based on the two agreed up to 75% of the time. Persistence of DNA, the QPCR-measured endpoint, versus metabolically active cells, the endpoint of culture-based methods, was tested in seawater mesocosms inoculated with fecal material. QPCR and culture-based measurements conducted in replicate over time revealed that the DNA and cell signals were found to decay at similar rates with few and

short-lived significant differences, helping to alleviate concerns that extended DNA persistence may lead to overestimations of FIB concentrations.

Additional work was conducted to assess the use of a new QPCR assay to quantify the difficult to culture alternate marker of fecal contamination, fecal *Bacteroides* spp. The efficacy of the fecal *Bacteroides* spp. QPCR assay was compared to cultured FIB in lab-created samples and ambient (stormwater) samples. QPCR-measured fecal *Bacteroides* spp. concentrations were found to be as strongly correlated with contamination by sewage influent as current FIB. Findings also suggest that fecal *Bacteroides* spp. concentrations may be useful in distinguishing human from animal fecal contamination.

Overall, results demonstrate the promise of QPCR-based methods as an improved tool for water quality monitoring. However, technical hurdles remain before QPCR-based monitoring can be implemented. The most important of these is sample processing to remove QPCR-inhibitory compounds. Across this set of studies, approximately 30% of samples experienced some level of inhibition. QPCR should prove a useful tool for water quality management with the advent of standardized approaches for addressing inhibition.

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## **List of Abbreviations**

ATCC: American Type Culture Collection

CFU: colony forming units

Ct: cycle threshold

DNA: deoxyribonucleic acid

EPA: Environmental Protection Agency

FIB: fecal indicator bacteria

MF: membrane filtration

MPN: most probable number

MTF: multiple tube fermentation

NCDENR: North Carolina Department of Environment and Natural Resources

NPS: non-point source

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PS: point source

QPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

SE: standard error

SPC: specimen processing control

VBNC: viable but non-culturable

## **Chapter 1:**

### Introduction and Background

An estimated 120 million gastrointestinal illnesses are experienced globally each year due to contact with polluted coastal waters (Shuval 2003). Annually, 689,000 to 4,003,000 swimming-associated illnesses are experienced in southern California alone (National Resources Defense Council 2009). This can be quite expensive in terms of public health costs. Lost days at work and medical visits have been calculated to cost \$1.3 million annually from just two southern California beaches (Dwight *et al.* 2005).

In the interest of reducing public health risk and costs, the passage of the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) in 2000 extends the Clean Water Act to require coastal states to routinely monitor water quality and notify the public when use of recreational waters is unsafe. Due to the cost and difficulty of measuring a diverse suite of pathogens, monitoring of water quality depends upon the detection of indicator organisms that are used as proxies for the presence of fecal contamination and, therefore, the likely presence of fecal contamination-associated pathogens. This type of indicator-based system is employed globally for recreational, drinking, and shellfish harvesting water quality management. The indicator-based system relies on the use of several groups of fecal indicator bacteria (FIB) that have been shown to have significant relationships to human health after water contact or consumption. Ideally, these indicator organisms have similar survival and transport to pathogens, are present in greater numbers

than pathogens, are specific to a fecal source, and are correlated to health risk of exposure to contaminated waters (Bonde 1966).

While states generally implement their own monitoring standards and approaches, the United States Environmental Protection Agency (U.S. EPA) provides recommendations for the use of specific types of FIB. The U.S. EPA currently recommends the use of fecal coliforms (or the specific subgroup *E. coli*) and/or *Enterococcus* sp. as the approved FIB for use in recreational waters, though fecal coliform standards are often limited to freshwater due to short persistence in a saline environment. These bacteria are highly concentrated in fecal material, are strongly associated with gastrointestinal illness in swimmers and are relatively easy to detect (Cabelli 1982).

Current enumeration methods rely on the ease of culturing these bacteria using membrane filtration (MF), multiple tube fermentation (MTF), or defined substrate technology® tests. However, these methods can be susceptible to high rates of false-positives (Davies *et al.* 1994, Pisciotta *et al.* 2002, Ferguson *et al.* 2005) and suffer from demonstrated high levels of variability and analyst error (Noble *et al.* 2003, Griffith *et al.* 2006). Furthermore, culture-based methods are often slow, requiring 18 to 96 hours from sample collection to results. This delay makes a warning system based on these analyses inappropriate given that most beach contamination is episodic and of short duration (Leecaster and Weisberg 2001, Boehm *et al.* 2002). When detection methods have a long turn-around time, contaminated waters may remain open to the public, only to be closed after the contamination disappears.

The development of rapid, quantitative methods to distinguish and measure not only *E. coli* and *Enterococcus* but also markers of specific types of fecal contamination has the

potential to revolutionize the field of water quality monitoring. Combining traditional polymerase chain reaction with the use of specific fluorescence-emitting probes, quantitative PCR (QPCR) is one such technique that can be used. During each cycle of heating and cooling, DNA is denatured, bound to target-specific fluorescence-emitting probes, and amplified in vitro. There is a quantitative relationship between the amount of target DNA present at the start of the assay and the amount of amplified product (as detected by fluorescence) at the end of the assay, and algorithms can be applied to determine initial DNA concentrations from the changes in fluorescence (Saiki 1989, Bustin 2004). QPCR has become a mainstream medical technology, frequently used in clinical microbiology, diagnostic testing, pathology, and genetics research. When applied to water quality monitoring, QPCR allows the quantification of genes within the FIB cells. QPCR is especially attractive for monitoring programs because it could tremendously reduce the time taken between sample collection and results, giving results in two to three hours. Particularly exciting, the development of QPCR has opened the door to quantification of a wide array of non-culturable or difficult to culture microbes that are importance components of feces, such as *Bacteroides* spp.

Epidemiological studies in the Great Lakes have demonstrated a correlation between QPCR-measured *Enterococcus* sp. and health outcomes (Wade *et al.* 2008). However, epidemiological studies are expensive, and the conducted studies have focused on areas directly impacted by wastewater treatment plants. At this time, no large-scale studies have been published establishing a relationship between QPCR-enumerated FIB and health outcomes in marine recreational water, although a few studies have been conducted, and data analysis is underway. Furthermore, epidemiological studies alone are not enough for

widespread acceptance of this technology by water quality monitoring agencies.

Understanding the persistence of DNA signals in recreational waters, assessment of applicability of QPCR to different environments with various sources of fecal contamination, and large-scale standardization of QPCR techniques and quantification methods must be undertaken before QPCR can be implemented successfully as a tool for monitoring. The overall goal of the research described in this dissertation is to advance the understanding of the relationship between QPCR-based assays with current water quality methods, providing an understanding of the aforementioned issues. Chapter 2 presents a direct comparison of QPCR-based and culture-based enumeration of *Enterococcus* sp. in marine recreational waters, demonstrating that while the method results are correlated, they are not equivalent. To determine whether this is due to differences in the persistence of each method's endpoint, Chapter 3 examines the relationship between persistence of FIB DNA and metabolically active cells from a range of sources of fecal contamination. Results demonstrate that FIB DNA marker persistence is comparable to that of metabolically active cells. This chapter also highlights the advantages of using alternative, anaerobic indicators over traditional FIB for distinguishing between recent and old contamination. In Chapter 4, QPCR assays targeting these alternative bacterial indicators are assessed for human specificity in sewage-spiked and environmental samples. In particular, the fecal *Bacteroides* spp. QPCR assay is found to be useful in distinguishing human from bird fecal contamination. QPCR-based enumeration of alternative FIB and culture-based assessment of currently approved FIB are both used in Chapter 5 in order to establish their applicability to stormwater monitoring, which is often more complicated than monitoring waters affected by point sources.

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## Chapter 2:

Equivalency of culture- and QPCR-measurements of *Enterococcus* sp. in marine recreational waters

### Introduction:

Current fecal indicator bacteria (FIB) enumeration methods rely on the ease of culturing these bacteria using membrane filtration (MF), multiple tube fermentation (MTF), or defined substrate technology® tests. While cheap and relatively easy to perform, these methods are imperfect. The recommended methods suffer from unacceptable levels of variability and high rates of false positives (Davies *et al.* 1994, Pisciotta *et al.* 2002, Noble *et al.* 2003, Ferguson *et al.* 2005, Griffith *et al.* 2006). Furthermore, culture-based methods are often slow, requiring 18-96 hours from sample collection to results. Despite these problems, there is a demonstrated human health risk associated with *Enterococcus* sp. and *E. coli* as enumerated by culture-based methods (Cabelli 1982), and as a result, the most promising new FIB enumeration assays can be expediently identified by a correlation with current public health standards and protocols (Field *et al.* 2003).

New quantitative polymerase chain reaction (QPCR) assays for FIB are especially attractive because they tremendously reduce the time taken between sample collection and results, giving results in two to three hours. Though QPCR assays for FIB have shown promise, the absence of direct correlations between results of these methods and EPA-approved culture-based methods has thus far limited the utility of QPCR in monitoring

programs (Field and Samadpour 2007). Work to date has found different levels of equivalency between QPCR-based and culture-based methods for *Enterococcus* sp. in freshwater. In the Great Lakes, QPCR and MF results for *Enterococcus* sp. have a correlation value of  $r=0.68$  (Haugland *et al.* 2005). The correlation was observed to be much stronger in St. Marys and Frederica Rivers in Georgia,  $r = 0.93$  (Morrison *et al.* 2008). In laboratory experiments spiking sewage into marine waters, a significant correlation of approximately  $r=0.80$  has been demonstrated between EPA-approved culture-based counts of *Enterococcus* sp. and corresponding QPCR data (Noble *et al.* submitted). This relationship has not yet been demonstrated in a large-scale study of marine environmental samples.

The objective of this study was to directly compare QPCR-based and culture-based quantification of *Enterococcus* sp. in marine recreational waters, using samples collected over two summers at two California beaches. This is a first step toward assessing the potential implementation of QPCR for water quality monitoring.

## **Methods:**

### *Study Sites:*

Water samples were collected from two Southern California beaches: Avalon, on Catalina Island; and Doheny Beach, in Orange County. Both beaches have been cited for poor water quality and are frequently posted as being unfit for swimming, with more than 25% of samples exceeding daily national standards in 2008 (Natural Resources Defense Council 2009).

### *Sample Collection:*

Over weekends and holidays between May 28, 2007 and September 9, 2007 and May 24, 2008 and September 14, 2008, water was collected from four beach locations in Avalon and five locations at Doheny Beach three times daily. Grab samples were collected at approximately 0.5m depth in 5-gallon buckets that had been sterilized in 10% bleach and washed in 1% sodium thiosulfate. Samples were immediately mixed in 150 gallon tanks using 2ft x 2ft stir plates and 8in stir bars. After 15 minutes of stirring, subsamples were decanted into sterile 4L Cubitainers and either filtered onto 47mm, 0.4µm pore size polycarbonate filters for future QPCR analysis or used for culture-based enumeration.

*Enumeration of Enterococcus sp. by Culture:*

*Enterococcus* sp. were enumerated using Enterolert™ (Idexx Laboratories) following manufacturer's instructions and using the manufacturer-provided Most Probable Number (MPN) table. *Enterococcus* sp. concentrations were also measured by MF following EPA method 1600 (APHA 2005).

*QPCR Calibration Standards:*

*Enterococcus faecalis* (ATCC 29212) was used as a QPCR calibration standard. Cells were obtained from the ATCC and cultured overnight at 37°C in brain heart infusion broth. Cells were counted using EPA Method 1600 and Enterolert. Cell suspensions were diluted with PBS and filtered onto 47mm, 0.4µm pore size polycarbonate filters such that each filter held 100,000 cells. Filters were stored at -80°C until processing.

*Specimen Processing Control:*

A specimen processing control (SPC) was added to each sample in order to estimate PCR inhibition. Salmon sperm testes DNA (Sigma) was added at the beginning of the extraction step at a concentration of 100ng per 500 $\mu$ L for each sample, calibrator, and blank.

A QPCR assay targeting the SPC was developed by Haugland *et al.* (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. This SPC QPCR assay has been demonstrated to be less efficient than many other QPCR assays and thus provides conservative identification of inhibited samples (Haugland *et al.* 2005). All SPC QPCR sample reactions with a cycle threshold (Ct) value 0.5 log units higher than that of the calibrators and blanks were considered inhibited. Inhibited samples were diluted 1:10 and 1:100 with sterile water and reanalyzed.

#### *DNA Extraction:*

DNA was released from the polycarbonate filters by bead beating. Filters were transferred into 2mL semiconical screw-cap microcentrifuge tubes pre-loaded with 0.3g of acid-washed glass beads. Four hundred and ninety microliters of AE Buffer (Qiagen) and 10 $\mu$ L of the specimen processing control (10 $\mu$ g/mL salmon sperm testes DNA) were dispensed into each tube. Each tube was bead milled in an eight-position mini bead beater for 2 minutes, followed by centrifugation in an Eppendorf Microfuge for 1 minute at 12,000 x g. Supernatants were transferred to sterile 1.7 mL microcentrifuge tubes and centrifuged at 12,000 x g for 5 minutes. Supernatant was transferred to a sterile 1.7mL microcentrifuge tube, stored at 4°C, and used in lieu of DNA extract in the QPCR reactions.

*QPCR Analyses and Quantification of Target DNA:*

The SPC QPCR assays were conducted following Haugland *et al.* (2005). Twenty five microliter reactions were prepared using OmniMix beads (a lyophilized premix with 1.5 units of TaKaRa hot start Taq polymerase, 200 $\mu$ M of dNTPs, 4mM of MgCl<sub>2</sub>, and 25mM HEPES with a pH of 8; Cepheid), 1 $\mu$ M each of forward and reverse primers, 0.1 $\mu$ M of the TaqMan probe, and 5 $\mu$ L of sample DNA extract. Reactions were thermal cycled and monitored in a Smart Cycler II® (Cepheid). Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 30 seconds at 60°C.

For *Enterococcus* sp. QPCR, twenty-five microliter reactions were prepared following Noble *et al.* (submitted). Reactions were prepared using 5 $\mu$ L of sample DNA, OmniMix beads, and Total *Enterococcus* Species beads (lyophilized Scorpion primer and probe sets with 0.25 $\mu$ M of each primer and probe complex; Cepheid). Reactions were thermal cycled and monitored in a Smart Cycler II. Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 5 seconds at 95°C and 43 seconds at 62°C.

All samples were run in duplicate. After manually adjusting the threshold on the SmartCycler II, cycle threshold (Ct) was determined automatically by the instrument. A duplicate standard curve was run during each reaction using the calibrator and three serial 10-fold dilutions, and target DNA in each sample was quantified using the  $\Delta$ Ct method outlined by Pfaffl (2001). Amplification efficiency (E) was calculated using the slope of the log standard curve given by the SmartCycler software:  $E=10^{(-\text{slope})}$ . The ratio of target DNA in the samples was multiplied by the amount of target DNA in the calibrator to get the sample quantities in number of cells following Pfaffl (2001). When DNA extracts had to be

diluted due to inhibition seen in the SPC, sample quantities were also multiplied by the dilution factor to get a corrected quantity.

#### *Data Analysis:*

All sample concentrations were normalized to 100mL. Samples yielding a QPCR non-detect or below the culture methods' detection limits were assigned a value of 5 cells or MPN per 100mL. The data were not normally distributed so were transformed logarithmically and correlated using Spearman Rank Analyses in SPSS.

### **Results and Discussion:**

#### *Correlations:*

In total, QPCR and MF were performed on 1045 samples from Doheny Beach and Avalon, CA; Enterolert was used on 871 of these samples. When data across years and sites were pooled, there was a significant correlation between *Enterococcus* sp. concentrations measured by MF and Enterolert: Spearman coefficient = 0.704,  $p < 0.001$ . Abbott *et al.* (1998) observed a much stronger correlation between Enterolert and MF in marine water samples off New Zealand ( $r = 0.927$ ), but the correlation we observed was similar to that found freshwater (Kinzelman *et al.* 2003).

Significant but weaker correlations were observed between *Enterococcus* sp. concentration as measured by QPCR and the two culture-based methods. The Spearman correlation between MF and QPCR-measured *Enterococcus* sp. concentrations was 0.438 ( $p < 0.001$ ). This correlation was not as strong as was found in freshwater (Haugland *et al.* 2005) or in laboratory created marine samples (Noble *et al.* submitted). An even weaker

correlation was observed between *Enterococcus* sp. concentrations measured by Enterolert and QPCR (Spearman coefficient = 0.360,  $p < 0.001$ ).

The significant but low correlations between QPCR culture-based concentrations are likely due to the non-overlapping method limitations. Specifically, culture methods may underestimate bacterial densities when bacteria have entered a viable but non-culturable (VBNC) state. When enteric bacteria are exposed to stressful environmental conditions such as high levels of solar radiation and elevated oxygen concentrations, they enter an inactive state, during which they are still alive and remain infective but are not actively reproducing or respiring (Naganuma 1996, Pommepuy *et al.* 1996, Decamp and Rajendran 1998, Troussellier *et al.* 1998, Falcioni *et al.* 2008). As a result, culture methods that rely on physiological status will not detect these cells. On the other hand, QPCR may be susceptible to overestimation of bacterial populations because it measures a DNA endpoint, which can be problematic given the possibly prolonged persistence of DNA after cell death. This may be of particular importance when beaches receive treated wastewater: Griffith *et al.* (2007) demonstrated that cell death (following chlorination) had no effect on QPCR measurements. Conversely, QPCR inhibition may cause underestimates of QPCR measurements of bacterial densities. However, many researchers are using inhibition controls, diluting samples until no more inhibition is seen in the controls, and then multiplying sample FIB values by a dilution factor. While this may not be a problem when samples are uninhibited or require a low dilution factor, multiplication by a factor of up to 100 can lead to enormous error.

All samples were diluted 10-fold for QPCR analyses, and for most samples, this dilution factor was sufficient to eliminate inhibition in the SPC QPCR. However, approximately 30% of the samples continued to show inhibition and had to be diluted up to

100-fold. When excluding samples requiring a 100-fold dilution from analyses, correlations between QPCR and the two culture-based methods were improved. The Spearman correlation coefficient for QPCR and MF was 0.567,  $p < 0.001$  ( $n = 729$ ), and the correlation coefficient for QPCR and Enterolert was 0.514,  $p < 0.001$  ( $n = 691$ ). These correlations are closer to those observed in Haugland *et al.* (2005) and Noble *et al.* (submitted) but still lower. This is likely because these QPCR concentrations were still multiplied by a dilution factor of 10, while samples in Haugland *et al.* (2005) and Noble *et al.* (submitted) experienced less inhibition and did not require dilution and multiplication by a correction factor. The difference may also be due to increased likelihood of cells entering a VBNC state in marine environmental samples as compared with freshwater samples or samples created in the lab shortly before analyses.

*Single Sample Recreational Water Quality Standard Agreement:*

When applying the current *Enterococcus* sp. single sample marine recreational water quality standard of 104 MPN or CFU/100mL and assigning the sample standard to *Enterococcus* sp. QPCR 104 cell equivalents/100mL, the method agreement for beach closure was 72% for MF and QPCR, 65.6% for Enterolert and QPCR, and 81.7% for Enterolert and MF when data from all sites and years was pooled. When excluding QPCR inhibited samples that required a 100-fold dilution, the methods agreement for beach closure improved: 75.8% for MF and QPCR ( $n = 641$ ) and 69% for Enterolert and QPCR ( $n = 603$ ).

QPCR-measured concentrations in this study were higher than culture-based concentrations, consistent with observations by He and Jiang (2005). In this study, most of the single sample standard disagreements were due to QPCR measurements above the single

sample standard while culture-methods were below the standard. A separate standard will likely be required for QPCR-based monitoring, which is expected given the different measured endpoints in QPCR and culture methods. Often the gene detected by the *Enterococcus* sp. QPCR assay has multiple copies per cell.

*Variability between Sites:*

When data were separated by site and year, there was substantial variability in the correlation coefficients (Tables 2.1 and 2.2). Method agreement was variable across years and sites. Single sample standard disagreement at Doheny Beach was very low in 2008: 7.5% disagreement between MF and QPCR (n=281); 6.76% disagreement between Enterolert and QPCR (n=281); 3.85% disagreement between MF and Enterolert (n=286). Similar percentages were observed in 2007 at Doheny Beach. In 2007 at Avalon, disagreement was greater: 22.95% between MF and QPCR (n=122); 26.2% between Enterolert and QPCR (n=84); and 26.2% between MF and Enterolert (n=84). In 2008 at Avalon, method disagreement was much higher: 44.5% for MF and QPCR, 61% for QPCR and Enterolert, and 21% for MF and Enterolert (n=238).

These differences between sites and years may be due to environmental impacts on culture methods. For instance, in water with high levels of total suspended solids and overall bacterial concentrations, overgrowth by background bacteria or the presence of filter-clogging substances have led to MF underestimations of FIB (Kinzelman *et al.* 2003). Changes in native bacterial communities may also lead to variation in incidence of false positives or negatives (Budnick *et al.* 1996, Kinzelman *et al.* 2003).

Site variability may also be explained by differences in DNA marker copy numbers across *Enterococcus* species (Blackwood, unpublished data) and variation in *Enterococcus* community composition between hosts (Ferguson *et al.* 2005). In waters affected by multiple sources of fecal contamination, some spatial and temporal variation in average copy numbers is expected, and it may be difficult to multiply the current single sample standard by a simple gene copy correction factor.

Incidence of inhibition was also extremely variable between sites and years. All samples collected at Doheny Beach in 2007 required only a 10-fold dilution, while 62.7% of samples collected in Avalon the same year required a 100-fold dilution. In 2008, the incidence of inhibition requiring a 100-fold dilution was similar in each site: 19.4% in Avalon and 15.7% in Doheny Beach. This is not surprising: there are several known PCR inhibitors whose concentrations have been shown to fluctuate widely (Brown 1987, Wilson 1997). Differences in levels of inhibition portend further problems in QPCR water quality standardization. Various modifications to DNA extraction and purification protocols are required for different types of PCR inhibition, and studies have shown that total yield and diversity of DNA in an extract depends heavily on the extraction techniques (Martin-Laurent *et al.* 2001, Luna *et al.* 2006, Kallmeyer and Smith 2009). Standardization of extraction, purification, and inhibition corrections will be vital to establishing QPCR-based water quality standards.

### **Conclusions:**

The correlations between QPCR and culture-based methods demonstrate the promise of QPCR-based monitoring for *Enterococcus* sp., corroborating epidemiological relationships

between QPCR-measured *Enterococcus* sp. and health effects in the Great Lakes (Wade *et al.* 2006 and 2008). Further epidemiological studies will be necessary to correlate *Enterococcus* sp. QPCR results with health outcomes in marine recreational water.

Additionally, PCR inhibition, corrections for this inhibition, and differences in *Enterococcus* community composition complicate the relationships between QPCR and culture based enumeration methods. Much work must be accomplished to improve extraction and purification methods and to correct inhibited QPCR results before QPCR can replace culture-based methods for water quality monitoring.

Table 2.1: Method correlations by site and year.

<b>Site</b>	<b>Year</b>	<b>Correlation between MF and QPCR (n and p)</b>	<b>Correlation between Enterolert and QPCR (n and p)</b>	<b>Correlation between MF and Enterolert (n and p)</b>
Doheny	2007	0.615 (n=103; p<0.001)	0.540 (n=103; p<0.001)	0.741 (n=103; p<0.001)
	2008	0.742 (n=337; p<0.001)	0.576 (n=331; p<0.001)	0.743 (n=332; p<0.001)
Avalon	2007	0.447 (n=324; p<0.001)	0.328 (n=237; p=0.004)	0.614 (n=237; p<0.001)
	2008	0.616 (n=294; p<0.001)	0.412 (n=294; p<0.001)	0.671 (n=294; p<0.001)

Table 2.2: Method correlations by site and year, excluding QPCR-inhibited samples.

<b>Site</b>	<b>Year</b>	<b>Correlation between MF and QPCR (n and p)</b>	<b>Correlation between Enterolert and QPCR (n and p)</b>	<b>Correlation between MF and Enterolert (n and p)</b>
Doheny	2007	0.615 (n=103; p<0.001)	0.540 (n=103; p<0.001)	0.741 (n=103; p<0.001)
	2008	0.526 (n=279; p<0.001)	0.542 (n=279; p<0.001)	0.699 (n=279; p<0.001)
Avalon	2007	0.528 (n=82; p<0.001)	0.312 (n=82; p=0.004)	0.403 (n=120; p<0.001)
	2008	0.690 (n=236; p<0.001)	0.536 (n=236; p<0.001)	0.684 (n=236; p<0.001)

Figure 2.1: Correlation and single sample marine recreational water quality standard agreement for all sites and years.

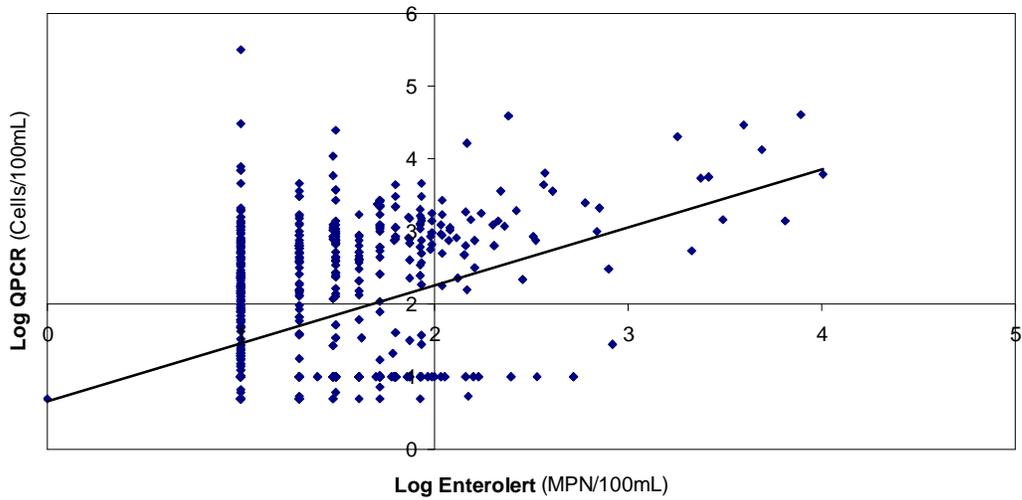
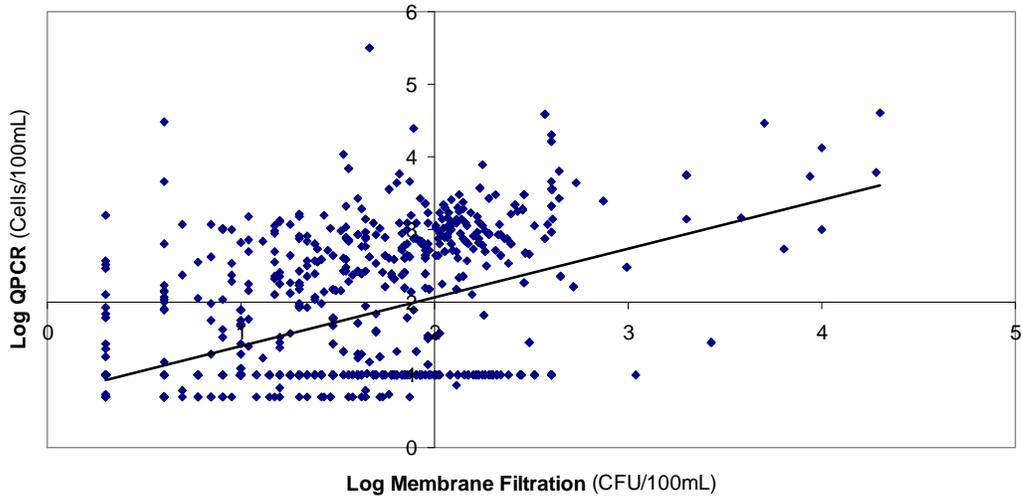
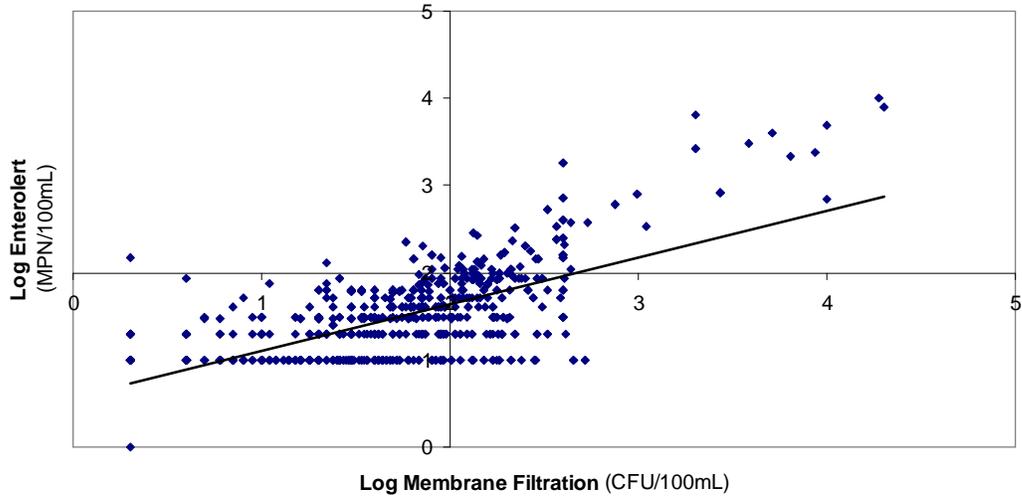
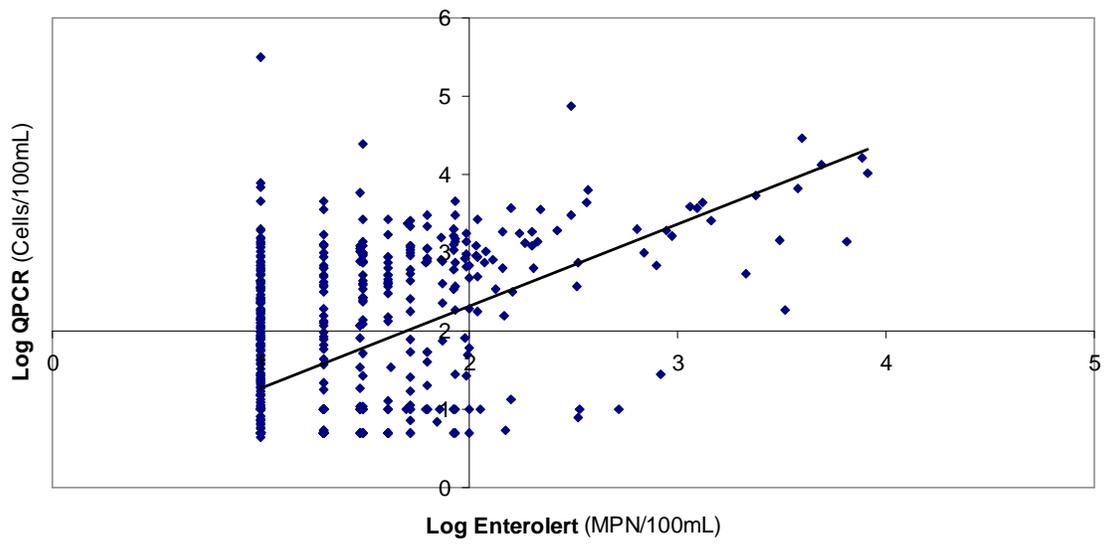
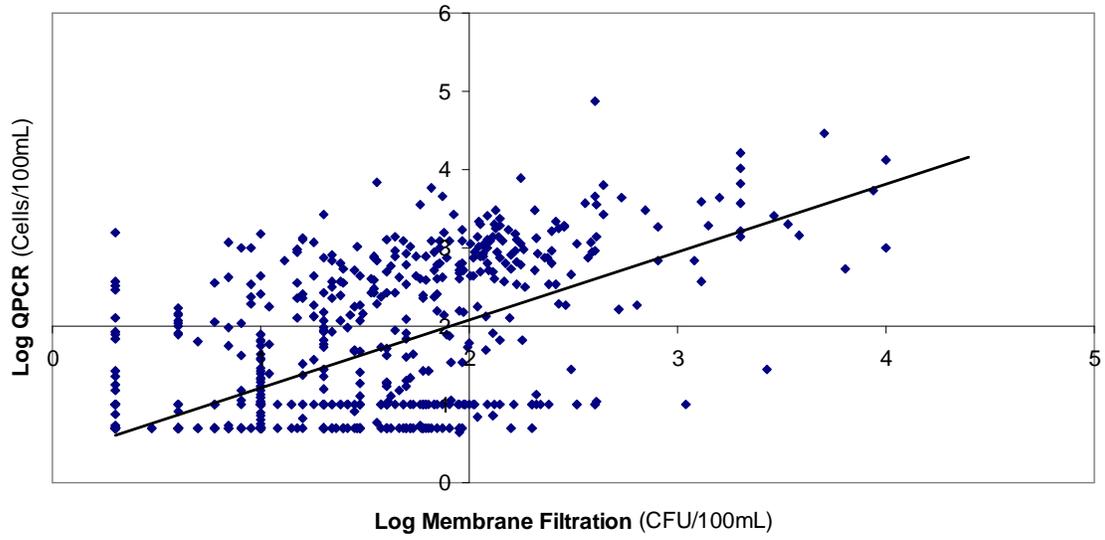


Figure 2.2: Correlation and single sample marine recreational water quality standard agreement for all sites and years, excluding QPCR inhibited samples.



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### **Chapter 3:**

Similar persistence of *Enterococcus* sp. and *E. coli* DNA markers and metabolically active cells in seawater

#### **Introduction:**

QPCR and culture-based measurements of a contamination event over time are expected to differ because the endpoints measured are different: DNA versus metabolically active cells. The potential for DNA to persist long after cell death is a primary concern for use of QPCR (Gedalanga and Olson 2009); DNA is quite stable. However, dead cells and free DNA have been shown to degrade on the scale of hours due to the activities of grazers and extracellular nucleases (Paul *et al.* 1987, Iriberry *et al.* 1994).

Also, rates of DNA marker degradation may not be constant across indicators: cells that survive longer in the marine environment are not expected to experience DNA degradation as quickly as cells that die quickly. As a result, anaerobic fecal indicator bacteria (FIB) may prove to be better indicators of recent contamination than *Enterococcus* sp. and *E. coli*. Until recently, anaerobic bacteria were impractical FIB because they were difficult to culture. With QPCR, monitoring of these indicators is practical, giving potentially improved water quality information.

In this study, we used a seeding experiment to examine the loss of FIB over time, as measured using both culture-based methods (with a metabolic endpoint) and QPCR (with a DNA-based endpoint). For the experiment, we used fecal material from humans, birds, and

dogs because we expected the loss of FIB to vary with source. We also examined differences in persistence of *Enterococcus* sp., *E. coli*, and fecal *Bacteroides* spp., which has recently been suggested as a good alternative indicator of fecal contamination (e.g. Kreader 1998, Dick and Field 2004, Layton *et al.* 2006). Because *Bacteroides* spp. are obligate anaerobes, their persistence was expected to be lower than *Enterococcus* sp. and *E. coli*.

## **Methods:**

### *Collection of Water and Fecal Samples:*

Samples of sewage influent were collected at the Morehead City, NC wastewater treatment plant (population 7,900, with secondary treatment). Dog feces were donated by a local dog owner. Guano from approximately 20 gulls was collected from a public boat ramp in Morehead City, NC. All fecal samples were collected in sterile 50mL tubes and kept on ice until seeded into seawater (within 3 hours). Seawater was collected from Atlantic Beach, NC wavewash at 0.5m depth.

### *Mesocosms:*

Eight 4L Cubitainers were filled with 2.2L of seawater. Fecal material was added such that each cube reached an approximate *E. coli* concentration of 1,000 to 5,000 cells/100mL. Preliminary Colilert-18™ (Idexx Laboratories) results on various masses of dog scat, gull guano, and sewage influent were used to make these calculations. To add fecal samples to seawater, 1g of dog scat, 0.12g of gull guano, and 1mL of sewage influent were added to 10mL, 10mL, and 9mL of phosphate buffered saline (PBS), respectively. Samples were vortexed until no visible aggregates remained intact. The gull guano slurry was diluted

1:10 again in PBS and vortexed. Ten milliliters of fecal slurry was added to the seawater Cubitainers (two Cubitainers with dog scat, two with gull guano, two with influent, and two with 10mL of PBS). Seeded water samples were shaken vigorously for 30 seconds twice daily and stored in the dark at room temperature (21°C).

At 0, 12, 24, 48, 72, 96, 120, and 144h, 220mL samples were taken from each Cubitainer. One hundred mL of water was filtered in replicate onto 47mm 0.45µm polycarbonate filters at each time point. Filters were stored in microcentrifuge tubes at -20°C until processed.

*Enumeration of Enterococcus sp. and Fecal Coliforms using Defined Substrate Testing:*

Samples at each time point were tested for fecal coliforms and total *Enterococcus* sp. using the defined substrate technology kits, Colilert-18 and Enterolert™ (Idexx Laboratories) following manufacturers instructions. Conversion of positive wells to a most probable number (MPN) was done using manufacturer-supplied MPN tables.

*QPCR Calibration Standards:*

*Enterococcus faecalis* (ATCC 29212), *E. coli* (ATCC 25922), and *Bacteroides thetaiotaomicron* (ATCC 29148) were used as a QPCR calibration standard. *E. faecium* and *E. coli* cell lines were obtained from the ATCC and cultured overnight at 37°C in brain heart infusion broth. Cells were counted using EPA Method 1600 and defined substrate testing. Cell suspensions were diluted with PBS and filtered onto 47mm, 0.4µm pore size polycarbonate filters such that each filter held 100,000 cells. Filters were stored at -80°C until extraction.

*B. thetaiotaomicron* was grown anaerobically in an overnight culture at 37°C in cooked meat medium. A portion of the cell suspension was removed and centrifuged for 5 minutes at 6,000 x g. The supernatant fluid was removed and aliquoted for use as a cell standard. Aliquots were frozen at -20° C. Cell counts were obtained by removing a portion of the cell suspension, serially diluting, fixing in formalin (1% v/v final) and counting cells using SYBR Green following the protocol of Noble and Fuhrman (1998). A known amount of cells (100,000) was filtered onto 47mm, 0.4µm pore size polycarbonate filters, and filters were stored at -80°C until extraction.

*Specimen Processing Control:*

A specimen processing control (SPC) was added to each sample in order to estimate PCR inhibition. Salmon sperm testes DNA (Sigma) was added at the beginning of the extraction step at a concentration of 100ng per 500µL for each sample, calibrator, and blank.

A QPCR assay targeting the SPC was developed by Haugland *et al.* (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. All SPC QPCR sample reactions with a cycle threshold (Ct) value 0.5 log units higher than that of the calibrators and blanks were considered inhibited. Inhibited samples were diluted 1:10 and 1:100 with sterile water and reanalyzed.

*DNA Extraction:*

DNA was initially extracted from the polycarbonate filters using the DNA-EZ RW04 Extraction Kit (GeneRite). Filters were transferred into 2mL semiconical screw-cap

microcentrifuge tubes pre-loaded with 0.3g of acid-washed glass beads. Four hundred and ninety microliters of AE Buffer (Qiagen) and 10 $\mu$ L of the specimen processing control (10 $\mu$ g/mL salmon sperm testes DNA) were dispensed into each tube. Each tube was bead milled in an eight-position mini bead beater for 2 minutes, followed by centrifugation in a Eppendorf Microfuge for 1 minute at 12,000 x g. Supernatants were transferred to sterile 1.7 mL microcentrifuge tubes and centrifuged at 12,000 x g for 5 minutes. Supernatant was transferred to a sterile 1.7mL microcentrifuge tube, mixed by vortexing with 500 $\mu$ L of Binding Buffer, and applied to a DNAsure column. Columns were inserted into collection tubes and centrifuged at 12,000 x g for 1 minute. Columns were transferred to new collection tubes, and 500 $\mu$ L of EZ-Wash Buffer was applied to the columns. Columns were again centrifuged at 12,000 x g for 1 minute. After transferring to new collection tubes, columns were washed again with 500 $\mu$ L of wash buffer and 1 minute of centrifugation. Columns were transferred to sterile, low retention 1.7mL microcentrifuge tubes, and 50 $\mu$ L of DNA Elution Buffer was applied to the center of the columns and allowed to incubate at room temperature for 1 minute. Columns were again centrifuged for 1 minute at 12,000 x g. Extracted DNA in the microcentrifuge tube was stored at -20°C.

Replicate filters of the inhibited samples were extracted using the QIAamp DNA Stool Kit, an extraction kit specifically designed to remove inhibitors from stool samples. Only samples from dog-spiked mesocosms were sufficiently inhibited to require extraction with this kit. Manufacturer's instructions for isolation of DNA from stool for pathogen detection were followed using the filters rather than stool samples, using 1.39mL Buffer ASL mixed with 10 $\mu$ L of 10 $\mu$ g/mL salmon sperm testes DNA rather than 1.4mL Buffer ASL, and adding a 2 minute bead beating step after the first incubation at 70°C. DNA was finally

eluted to 50 rather than 200 $\mu$ L. Negative controls and calibrations standards were extracted simultaneously with the sample filters. Samples were stored at -20°C until processed. These samples were tested for the presence of inhibitors using the SPC QPCR reaction. No samples experienced inhibition of the SPC QPCR reaction after being subjected to the Stool Kit extraction.

*QPCR and Quantification of Target DNA:*

TaqMan chemistry was used for the SPC and fecal *Bacteroides* spp. primers and probes (Haugland *et al.* 2005, Converse *et al.* 2009). Twenty five microliter reactions were prepared using OmniMix beads (a lyophilized premix with 1.5 units of TaKaRa hot start Taq polymerase, 200 $\mu$ M of dNTPs, 4mM of MgCl<sub>2</sub>, and 25mM HEPES with a pH of 8), 1 $\mu$ M each of forward and reverse primers, 0.1 $\mu$ M of the TaqMan probe, and 5 $\mu$ L of sample DNA extract. Reactions were thermal cycled and monitored in a Smart Cycler II® (Cepheid). Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 30 seconds at 60°C.

Scorpion chemistry was used for the Total *Enterococcus* Species and *E. coli* QPCR reactions (Noble *et al.* submitted). Twenty-five microliter reactions were prepared using 5 $\mu$ l of sample DNA, OmniMix beads, and Total *Enterococcus* Species or *E. coli* beads (lyophilized Scorpion primer and probe sets with 0.25 $\mu$ M of each primer and probe complex; Cepheid). Reactions were thermal cycled and monitored in a Smart Cycler II. Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 5 seconds at 95°C and 43 seconds at 62°C.

All analyses of unknowns were run in duplicate. After manually adjusting the threshold on the Smart Cycler II, cycle threshold (Ct) was determined automatically by the instrument. Samples that yielded a “non-detect” QPCR result were assigned a concentration of 5 cells per 100mL. A duplicate standard curve was run concurrently with samples using the calibrator and three serial 10-fold dilutions. Amplification efficiency (E) was calculated using the slope of the log standard curve given by the SmartCycler software:  $E=10^{(-\text{slope})}$ . The ratio of target DNA in the samples was multiplied by the amount of target DNA in the calibrator to get the sample quantities in number of cells, following Pfaffl (2001). When DNA extracts had to be diluted due to inhibition seen in the SPC, sample quantities were also multiplied by the dilution factor to get a corrected quantity.

*Data Analysis:*

Data were scaled by dividing each sample concentration by the initial sample concentration (the sample concentration at time 0). Samples yielding a QPCR non-detect or below the defined substrate detection limit were assigned a value of 5 cells or MPN per 100mL and divided by the initial sample concentration. Scaled data were transformed using the natural logarithm. Overall decay rates (k) were estimated as the slope of the regression line. Only time points for which concentration was within the method detection limits were used in this regression analysis. Because indicators did not follow perfect exponential decay, decay rates were also calculated between each set of consecutive time points. Overall decay rates were compared using the Mann-Whitney U Test in SPSS. Decay rates between each set of consecutive time points were compared with a general linear mixed effects model using the GLIMMIX procedure in SAS 9.1.

Defined substrate and QPCR results were log normalized and correlated using Spearman rank correlation in SPSS.

## **Results:**

### *Rates of Decay by Method:*

In general, total *Enterococcus* sp. and *E. coli* concentrations from gull guano and sewage influent demonstrated exponential decay in a seawater matrix over the 7d period (Figures 1 and 2). In gull guano- and sewage-inoculated mesocosms, overall decay rates (k) for total *Enterococcus* sp. ranged between 0.0330 and 0.0797 for both Enterolert and QPCR. Overall *E. coli* decay rates for both sources and methods ranged between 0.0639 and 0.0783 (Table 3.1). Mann-Whitney U tests found no significant differences between QPCR-based and defined substrate-based decay rates for either total *Enterococcus* sp. or *E. coli* from gull-guano or sewage influent over time.

Because data did not exhibit perfect exponential decay, as shown by the  $R^2$  values in Table 3.1, simple linear regression analysis was not appropriate to compare decay rates over time between the methods. Instead, a general linear mixed effects model was used. There were no significant differences between defined substrate-based and QPCR-based decay rates for *E. coli* and total *Enterococcus* sp. in gull guano and sewage influent. There was a difference between QPCR-based and Colilert-18-based *E. coli* decay rates in influent-spiked mesocosm between 72-96 hours after contamination, with QPCR-measured concentrations showing a higher rate of loss than Colilert-18 measured concentrations, but this difference was not significant ( $p=0.0587$  as compared to Bonferroni-corrected significance level of 0.0125). No differences in *E. coli* QPCR or Colilert-18 decay rates were observed for gull

guano-spiked samples. Also in gull guano-spiked samples, the total *Enterococcus* sp. decay rate as measured by QPCR was marginally higher significance level than the Enterolert-based decay rate between 48 and 72 hours ( $p=0.0321$  as compared to Bonferroni-corrected significance level of 0.0167).

In water samples inoculated with dog feces, the general linear mixed effects model did not show any differences between decay rates for QPCR and Enterolert for total *Enterococcus* sp., excluding the first time points when results were above the Enterolert detection limits. However, *E. coli* concentration changes in mesocosms inoculated with dog feces were not well-described by exponential growth or decay over time: the QPCR assays were likely severely inhibited and unable to detect any *E. coli* much of the time. Colilert-18 did not detect any *E. coli* initially, but the concentration increased until 24 hours (Figure 3.2). As such, there was a difference in rates of *E. coli* concentration change between methods from 0 to 12 hours and from 12 to 24 hours, though significance was marginal ( $p = 0.0166$  and  $p=0.0148$ , respectively, as compared to Bonferroni-corrected significance level of 0.007). Because concentrations remained approximately constant after 24 hours, there was no significant difference in decay rates from 24-144 hours.

#### *Comparisons between Indicators:*

The general linear mixed effects model detected no differences between the overall rates of degradation of total *Enterococcus* sp. and *E. coli*, regardless of enumeration method or source of fecal material. The only significant difference during shorter periods of time was in influent-spiked QPCR samples between 72 and 96 hours ( $p=0.0049$ ), during which time *E. coli* degraded faster.

Fecal *Bacteroides* spp. were not detected in samples from the gull-inoculated mesocosms. In both sewage influent- and dog feces-spiked samples, fecal *Bacteroides* spp. signal decayed quickly (Figure 3.3). Overall, fecal *Bacteroides* spp. decayed significantly faster than total *Enterococcus* sp. and *E. coli* in both dog feces-seeded ( $p=0.0123$  and  $0.0159$ , respectively) and influent-seeded mesocosms ( $p < 0.0001$  and  $p = 0.0007$ , respectively).

#### *Comparison between Fecal Sources:*

There were no significant differences in overall rates of decay between gull guano-, dog feces- and influent-spiked samples for total *Enterococcus* sp. or *E. coli*. Fecal *Bacteroides* spp. decayed slightly faster in influent-spiked samples than in mesocosms spiked with dog feces with marginal significance ( $p=0.0835$ ).

#### *Method Correlations:*

In gull guano- and sewage influent-spiked mesocosms, there were significant correlations between log-normalized QPCR and Colilert-18 measurements of *E. coli* concentrations when data from all 7d was pooled (Spearman coefficient was  $0.979$ ,  $p < 0.001$ ). Strong correlation values were maintained when data were partitioned according to fecal source (Figure 3.5): Spearman coefficient in gull-spiked samples was  $0.980$ ,  $p < 0.001$ ; and Spearman coefficient in influent-spiked samples was  $0.929$ ,  $p < 0.001$ .

There was a significant but weaker correlation between Enterolert- and QPCR-based total *Enterococcus* sp. measurements in gull guano- and influent-inoculated water (Spearman correlation coefficient was  $0.426$ ,  $p = 0.048$ ; Figure 3.4). However, the relationship was much stronger when data were separated by source. Enterolert- and QPCR-concentrations

had a correlation coefficient of 0.952 ( $p < 0.001$ ) in samples with gull guano and 0.916 ( $p < 0.001$ ) in samples with influent.

There was substantial disagreement between defined substrate- and QPCR-based concentrations of *E. coli* in the dog-seeded mesocosms (Figures 3.4 and 3.5). QPCR detected little to no *E. coli* when water was spiked with dog feces, suggesting severe inhibition and resulting in no significant correlation between Colilert-18 and QPCR. Because total *Enterococcus* sp. concentrations were above the Enterolert detection limit for 48 hours in dog-seeded mesocosms and assigned a value of  $>24196$  MPN/100mL, data were scaled to the 48-hour concentration for which we have a quantitative measurement. There was not a significant correlation between Enterolert-based or QPCR-based concentrations of total *Enterococcus* sp. during the remaining time points.

## **Discussion:**

### *Decay Rates by Method:*

In general, QPCR-detectable DNA markers of FIB and metabolically active cells as measured by defined-substrate tests experienced exponential decay over time. The observed overall rates of decay were consistent with other studies using defined substrate analyses (Anderson *et al.* 2005) and QPCR (Walters *et al.* 2009) to examine the persistence of FIB in recreational waters. However, the decay rates were not necessarily expected to be similar between the methods. Differences were expected between QPCR and culture-based methods as they tracked a contamination event over time due to non-overlapping method limitations. Because QPCR can detect DNA from both viable and dead cells, QPCR measurements of indicators have sometimes been higher than results using culture methods (Josephson *et al.*

1993, Haugland *et al.* 2005, Griffith *et al.* 2007). While some studies have shown that DNA markers of FIB have outlasted culturable cells in seawater, it has also been demonstrated that DNA is quickly degraded in seawater (Josephson *et al.* 1993). Predation, primarily by protists, can eliminate up to 86% of enteric bacteria released into seawater (Iriberry *et al.* 1994). DNA released from cells by grazing, cell lysis, or DNA excretion has been shown to turn over in less than 7 hours due to the ubiquitous presence of DNase activity in seawater (Paul *et al.* 1987).

Results from this study demonstrated that, with few and minor exceptions, decay of QPCR-detectable DNA markers of FIB was comparable to the decay of metabolically active cells as measured by defined-substrate testing. With the exception of *E. coli* enumeration in dog scat, for which the difference was only marginally significant, the overall decay rates as measured by defined substrate testing and QPCR were not significantly different for either *E. coli* or total *Enterococcus* sp. Even when looking at changes in decay rates over the course of 7d, periods of difference were only marginally significant and lasted 24 hours or less. During most of these periods of difference, QPCR was experiencing a higher decay rate than defined substrate testing.

This experiment was conducted in the dark and at a constant temperature in order to elucidate the basic relationship between culture- and QPCR-based decay rates. Realistically, the relationship is not so simple: comparisons between methods have been complicated by unknowns about viable but non-culturable (VBNC) cells. The marine environment features a multitude of abiotic stressors to enteric bacteria including low temperatures, high salinity, solar radiation, elevated oxygen concentrations, and low concentrations of organic matter (Troussellier *et al.* 1998). Numerous studies have demonstrated that enteric bacteria may

quickly enter an inactive state after introduction to the stressful marine environment (for example, Naganuma 1996, Decamp and Rajendran 1998). Physiological status, as measured by reproduction and respiration, distinguishes active from inactive cells (Falcioni *et al.* 2008). These inactive VBNC cells are not dead, often remain infective, and may recover culturability under improved environmental conditions (Pommepuy *et al.* 1996). As a result, FIB monitoring using culture-based methods that rely on detection of reproduction or respiration underestimates the presence and magnitude of fecal contamination. QPCR, on the other hand, detects these VBNC cells, which have intact DNA, and likely provide improved measurements of fecal contamination. Walters *et al.* (2009) showed that though *Enterococcus* sp. lost culturability early, FIB QPCR signal persistence was similar to infectious enteroviruses when exposed to sunlight. Our experimental design eliminated the sunlight-induced VBNC state, simulating a best-case scenario for culture-based enumeration. Even in this case, degradation of DNA-markers was not significantly different from decay of metabolically active cells, suggesting that when culture-based decay is complicated by greater entry into the VBNC state, DNA-based decay can be taken to be more reliable indicators of presence of infective fecal pathogens.

#### *Persistence of Various Indicators:*

Results from our study showed no significant difference in overall rates of degradation of total *Enterococcus* sp. and *E. coli*. The short period of time in which there was a difference was not unexpected: *E. coli* have been found to be inactivated faster than *Enterococcus* spp. in saline water (Davies *et al.* 1995), and Gram-negative bacteria have been demonstrated to be eaten faster than Gram-positive bacteria due to differential digestibility of

the cell wall (Iriberry *et al.* 1994). In actuality, the *E. coli* decay rates by Colilert-18 may be underestimated in this experiment: fecal coliforms suffer greater inactivation from exposure to radiation than *Enterococcus* sp. (Davies-Colley *et al.* 1998). To determine whether or not the *Enterococcus* sp. decay rates vary with community composition, we also compared decay of *Enterococcus faecalis* and *Enterococcus casseliflavus*. *E. faecalis* is prevalent in human feces and much less so in animal waste (Leclerc *et al.* 1996). *E. casseliflavus* is assumed to be an “environmental” species, found in plants and soil (Leclerc *et al.* 1996, Pinto *et al.* 1999). We found no differences between the decay rates of either species and total *Enterococcus* sp. (data not shown), suggesting that total *Enterococcus* sp. decay is unaffected by species composition or source of *Enterococcus*.

Unfortunately, total *Enterococcus* sp. and *E. coli* are of limited value as FIB. Various limitations have been discussed at length in the literature, two of these weaknesses being that these FIB are shed by all warm-blooded animals and have been shown to grow and persist in beach sediments (e.g. Solo-Gabriele *et al.* 2000, Anderson *et al.* 2005). Much work has been undertaken to find improved, alternative indicators of fecal contamination, for example *Bacteroides* spp. These indicators have been shown to be more concentrated in human fecal contamination than animal and/or to have limited persistence in seawater (e.g., Dick and Field 2004, Okabe and Shimazu 2007, Bell *et al.* 2009).

Fecal *Bacteroides* spp. QPCR signal was found to degrade significantly faster than either total *Enterococcus* sp. or *E. coli*, suggesting that fecal *Bacteroides* spp. will be a useful tool for identifying recent fecal contamination. This is consistent with the findings of Fiksdal *et al.* (1985) and Walters *et al.* (2009), though persistence for each indicator was found to be shorter in this study. This may be because the mesocosms in this study used marine water,

while those in the other studies used freshwater at lower temperatures. The difference between fecal *Bacteroides* spp. and *Enterococcus* sp. and *E. coli* delay is likely to be even greater in recreational waters, where *Enterococcus* sp. and *E. coli* have been demonstrated to persist and reproduce (Anderson *et al.* 2005, Solo-Gabriele *et al.* 2000). Anaerobic fecal *Bacteroides* spp. are unlikely to reproduce in oxygenated waters. Still, it is important to remember that *Bacteroides* spp. cell loss is largely dependent upon predation: at colder temperatures when predator activity decreases, *Bacteroides* spp. persistence has been shown to increase (Kreader 1998, Okabe and Shimazu 2007). While total *Enterococcus* sp. and *E. coli* are also expected to experience decreased predation in colder temperatures, a thorough comparison of rates of predation on these indicators with fecal *Bacteroides* spp. will be necessary to extend the findings of this experiment to waters at colder temperatures.

#### *Method Correlations:*

New QPCR assays will need to be correlated with measures of human health, but because management decisions are currently based upon culture-based measures of FIB, correlation with current public health standards can be used to identify the most promising QPCR assays (Field *et al.* 2003). In general, defined substrate-based and QPCR-based measurements of total *Enterococcus* sp. and *E. coli* were highly and significantly correlated over the course of this experiment with Spearman coefficient estimates of 0.91 and above within sources. These findings corroborate the findings presented in Chapter 2 and from several other groups comparing currently-approved culture-based methods of FIB enumeration with QPCR (Haugland *et al.* 2005, Griffith *et al.* 2007, Noble *et al.* submitted).

However, there appear to be fecal-source specific differences in the correlations between methods. Though the correlation coefficient between Enterolert-based and QPCR-based total *Enterococcus* sp. concentrations was 0.952 ( $p < 0.001$ ) in gull-inoculated samples and 0.916 ( $p < 0.001$ ) in influent-inoculated samples, when these data are pooled, the correlation coefficient falls to 0.426 ( $p = 0.048$ ). This suggests that the relationships between Enterolert and total *Enterococcus* sp. QPCR differ among sources. This finding is not surprising: gene copy numbers are not constant among *Enterococcus* species; and *Enterococcus* community composition is known to differ between human and gull fecal material (Leclerc *et al.* 1996, Pinto *et al.* 1999, Wheeler *et al.* 2002, Ferguson *et al.* 2005). This may have management implications where gulls are a significant source of fecal contamination.

#### *Methodological Shortcomings:*

Findings from the dog-seeded microcosm highlight several shortcomings of both defined substrate testing and QPCR. First, the different QPCR assays were not affected equally by inhibition. Results from the SPC QPCR assay were not suggestive of inhibition, and indeed results from the total *Enterococcus* sp. QPCR reaction were in agreement with Enterolert results. However, the *E. coli* QPCR reaction appears to have been nearly completely inhibited. Previous work enumerating *E. coli* in dog feces samples using the same QPCR assays has found *E. coli* concentrations to be several orders of magnitude greater than fecal *Bacteroides* spp. or *Enterococcus* sp. per gram of feces (Converse *et al.* 2009), demonstrating that the nearly total absence of *E. coli* QPCR signal in this experiment was due to inhibition. Given their omnivorous diet, there are several likely QPCR inhibitors to be

found in dog feces: humic and fulvic acid, phenolic compounds, urea, and bile salts (Wilson 1997, Watson and Blackwell 2000). That these inhibitors could be adequately diluted out of samples for some QPCR reactions to work while others failed demonstrates that some QPCR assays are more susceptible to inhibition than others. As such, in situations where dogs contribute to fecal contamination, the currently used SPC QPCR assay is not a good measure of inhibition. Without a good measure of inhibition, QPCR could underestimate *E. coli* concentrations and be less protective of public health.

Colilert-18 results from dog feces-spiked water samples were also unusual with low initial concentrations growing several orders of magnitude in the first 24 hours before leveling. False positives could account for some of this concentration increase. However because the change was over orders of magnitude, it is highly unlikely that most of the change was due to false positives. Most likely, the growth was a result of the clumpy character of the dog feces. Several minutes of vortexing and manual mixing did not eliminate all visible aggregates of feces. Samples collected during the first time point probably contained fewer aggregates and therefore less *E. coli*. These aggregates may have also resulted in the relative stability of *E. coli* levels after 24 hours. Work with cowpats has demonstrated that manure-attached *E. coli* experience increased survival due to enhanced microsite habitat and the addition of nitrogen (Gagliardi and Karns 2000). Because dog scat could not be dissolved into seawater as well gull guano and sewage influent, it is likely that these fecal aggregates augmented *E. coli* survival in the dog-spiked mesocosms as compared with the other mesocosms. Fortunately, this is unlikely to be a concern when testing environmental water samples. Though inactivation kinetics of sorbed bacteria may differ from free, *E. coli* has been found to be transported from fecal material primarily as individual

cells. Only 8% of *E. coli* cells in runoff from cowpats, for instance, were attached to particles (Muirhead *et al.* 2005).

### **Conclusions:**

Microcosm results demonstrated that the decay rate of QPCR-based and culture-based measurements of *Enterococcus* sp. and *E. coli* were similar. This finding lends support to the potential for replacement of older, slower culture-based methods of water quality monitoring with rapid QPCR-based methods.

Besides rapidity, QPCR also allows detection of alternative, potentially superior indicators of fecal contamination. For instance, fecal *Bacteroides* spp. QPCR signal was found to degrade faster than either total *Enterococcus* sp. or *E. coli*, meaning that it may be a better indicator of recent fecal contamination than the currently recommended *E. coli* and *Enterococcus* sp.

However, results demonstrate that different QPCR assays are not affected equally by inhibition. Before QPCR can entirely replace culture methods, further work must be undertaken to find approaches to reduce inhibition or to ensure that quantitative information can be generated using QPCR-based methods on even the most complex of sample matrices.

Table 3.1: Overall decay rates (k). “All” refers to decay rate when data from all sources is pooled.

<b>Indicator</b>	<b>Source</b>	<b>Method</b>	<b>Overall Decay Rate</b>	<b>R<sup>2</sup></b>
<i>Enterococcus</i>	All	Idexx	-0.0198	0.3116
		QPCR	-0.0178	0.2374
	Gull	Idexx	-0.0421	0.9571
		QPCR	-0.0797	0.9104
	Influent	Idexx	-0.0393	0.9625
		QPCR	-0.033	0.9773
	Dog	Idexx	-0.0038	0.115
		QPCR	-0.0028	0.2728
<i>E. coli</i>	All	Idexx	0.0121	0.0293
		QPCR	-0.0199	0.143
	Gull	Idexx	-0.0731	0.9208
		QPCR	-0.0714	0.8815
	Influent	Idexx	-0.0639	0.9819
		QPCR	-0.0783	0.9395
	Dog	Idexx	0.0126	0.1179
		QPCR	N/A	
<i>Bacteroides</i>	Gull	QPCR	N/A	
	Influent	QPCR	-1.1372	0.8276
	Dog	QPCR	-0.0689	0.9415

Figure 3.1: Log average concentrations of *Enterococcus* spp. over time.

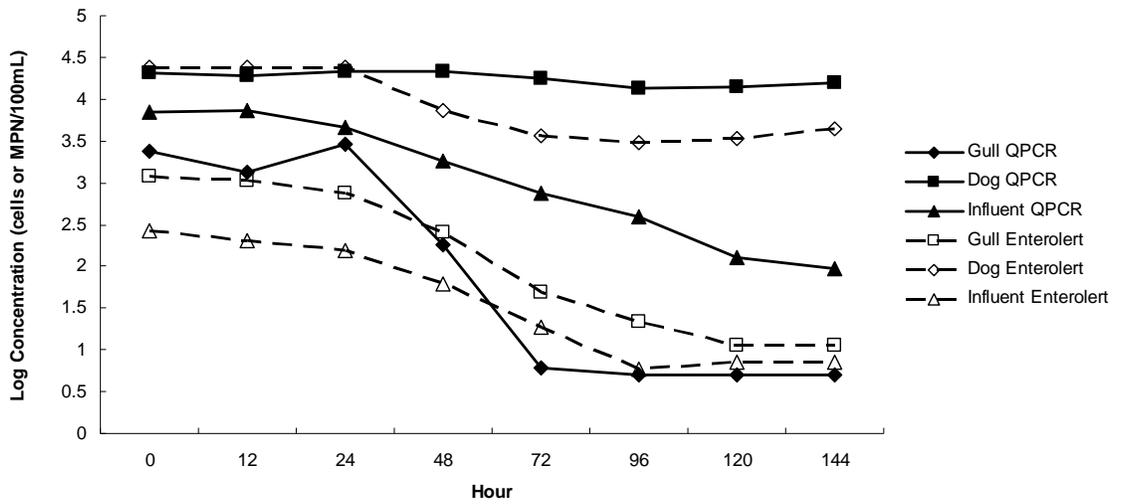


Figure 3.2: Log average concentrations of *E. coli* over time.

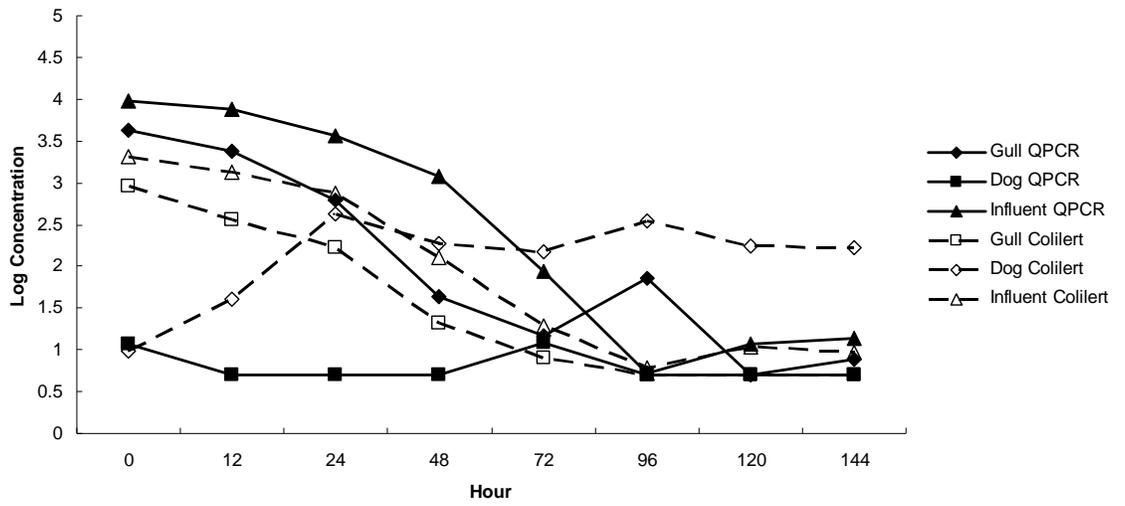


Figure 3.3: Log average concentration of fecal *Bacteroides* spp. over time.

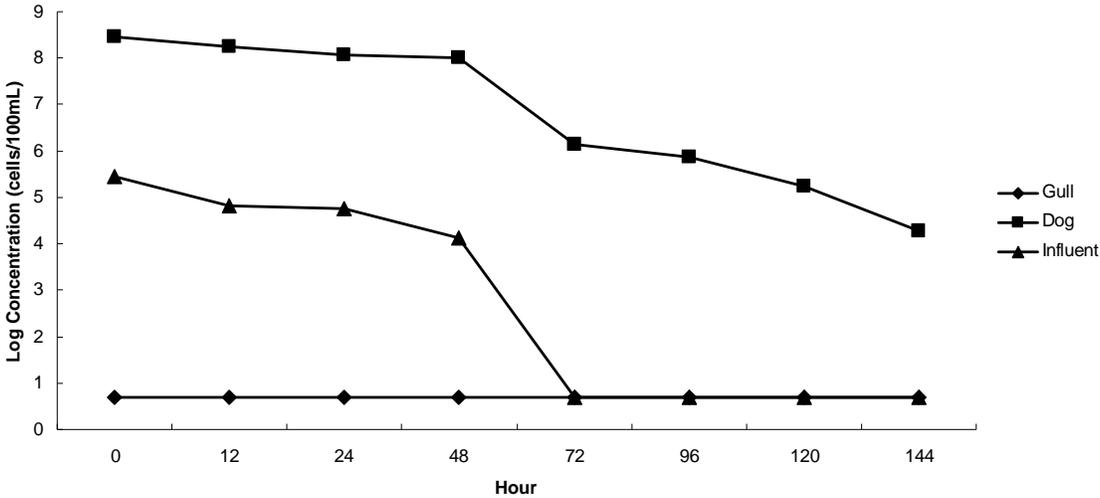


Figure 3.4: Log defined-substrate *Enterococcus* sp. results versus log QPCR results.

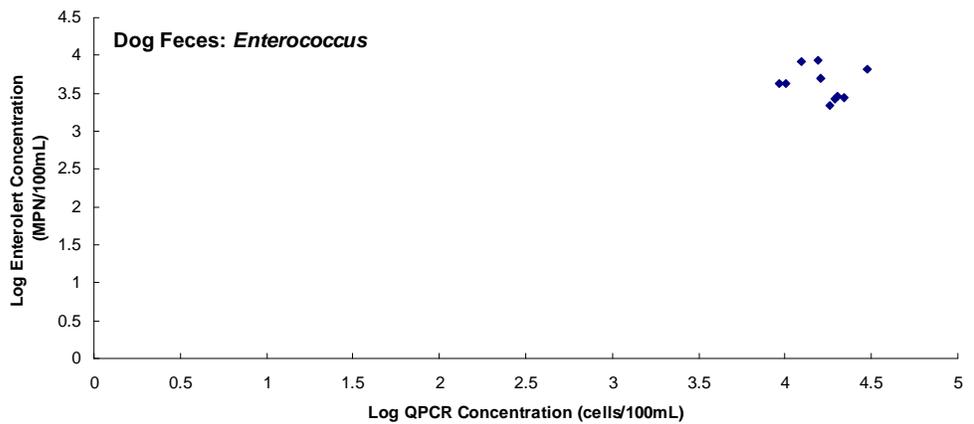
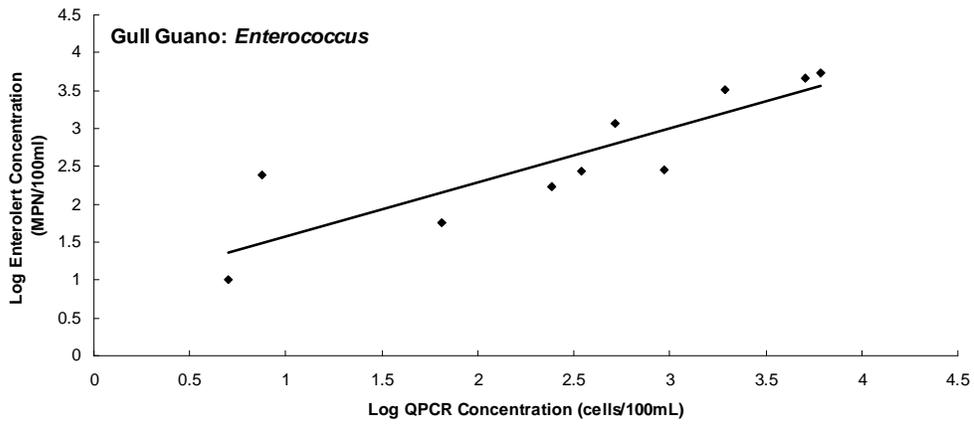
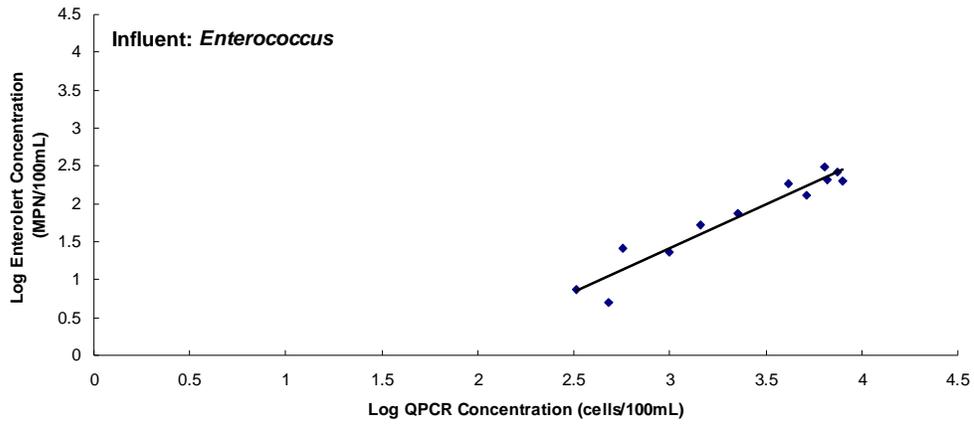
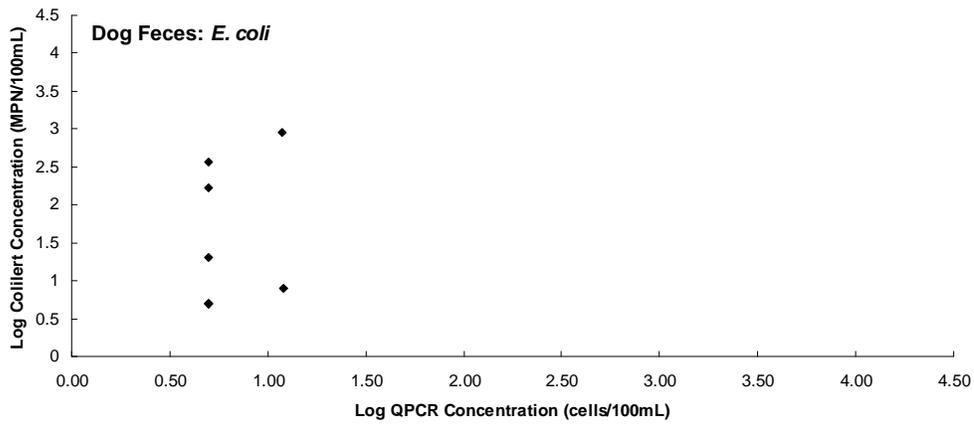
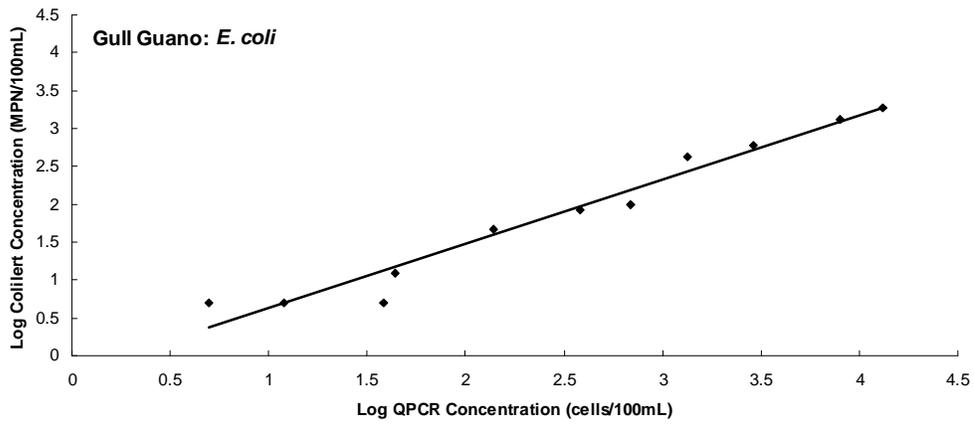
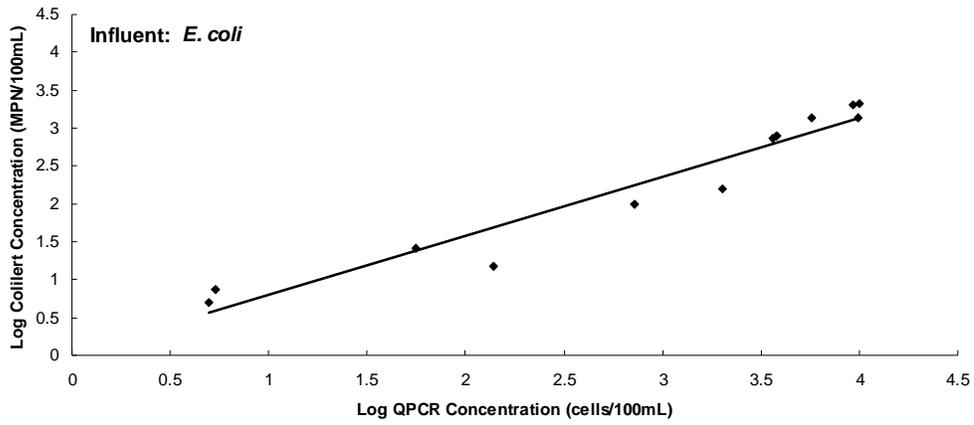


Figure 3.5: Log defined-substrate *E. coli*. results versus log QPCR results.



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## Chapter 4:

Rapid QPCR-based assays for fecal *Bacteroides* spp. and *Enterococcus* speciation as tools for assessing fecal contamination in recreational waters

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### Introduction:

While several studies have demonstrated a strong correlation between EPA-approved methods for fecal indicator bacteria (FIB) enumeration and rates of human illness after exposure to recreational water, these studies are not applicable to all waters because they have all been conducted only near sewage outfalls containing human fecal material (e.g. Cabelli *et al.* 1979, Dufour 1984, Wade *et al.* 2003). There is a paucity of data relating human health to contact with water contaminated by non-point source (NPS) runoff. This is particularly important because NPS runoff, specifically stormwater runoff, affects a majority of recreational beaches. NPS pollution differs fundamentally from point-source (PS) pollution in both composition and nature. Unlike PS pollution, for which rates of input and composition are typically known, NPS pollution is often diffuse, intermittent, and heterogeneous with agricultural, commercial, residential, and wildlife sources as potential

contributors of FIB and pathogens (Schwab 2007). As a result, stormwater runoff frequently carries with it a complex mixture of animal fecal material and/or human contamination. This mixture of contamination sources can be highly problematic because *Enterococcus* sp. and *E. coli* are often found in high concentrations in animal feces, and our understanding of the associated public health risk with contact or ingestion of animal feces is poor. Additionally, FIB have been documented to survive and grow in sand and other beach sediment, potentially persisting in the environment for longer periods and at greater concentrations than human and animal pathogens of concern (Solo-Gabriele *et al.* 2000, Anderson *et al.* 2005). Levels of *E. coli* and *Enterococcus* sp. have also been shown to have little or no relationship with the presence of human viral pathogens in NPS contaminated waters (Noble and Fuhrman 2001, Jiang *et al.* 2004). However, it has been demonstrated that NPS runoff can pose a serious threat to human health (Haile *et al.* 1999), but the human-health risk associated with exposure to water tainted by NPS pollution is not well understood.

In order to better approximate human health risk and design appropriate best management strategies, the sources of fecal contamination in NPS runoff and recreational waters must be partitioned. The combined use of conventional and alternative indicators of fecal contamination, possibly in combination with a tiered approach, could enhance detection sensitivity and specificity (e.g. Savichtcheva and Okabe 2006). Identification of these new, alternative indicators for source identification is imperative and has received increasing attention in the research community. While there are many potential alternative indicators currently discussed in the literature, this study focused on two quantitative QPCR tools for assessing human-specific fecal contamination: measurement of *Bacteroides* concentrations

and the determination of the relative ratios of concentrations of three relevant *Enterococcus* species.

Differential partitioning of *Enterococcus* species among hosts has piqued interest in *Enterococcus* speciation as a potential method of bacterial source tracking (Ferguson *et al.* 2005, Wheeler *et al.* 2002, Pinto *et al.* 1999, Leclerc *et al.* 1996). *E. faecalis* and *E. faecium* are the most populous *Enterococcus* species in human feces, and they are much less prevalent in livestock (Leclerc *et al.* 1996). *E. casseliflavus*, on the other hand, is generally assumed to be an environmental species, inhabiting plants and soil (Pinto *et al.* 1999, Leclerc *et al.* 1996). The ratio of *E. faecalis* and *E. faecium* to *E. casseliflavus* should be useful in determining how much of the total *Enterococcus* is from human waste and how much comes from other sources that are less of a concern for human health. Until recently, speciation was impractical. For example, *E. casseliflavus* is difficult to differentiate from *E. faecium* using conventional biochemical tests (Devriese *et al.* 1996), but the development of species-specific QPCR assays makes *Enterococcus* speciation a practical possibility.

*Bacteroides* spp. have also received a great deal of attention as an alternative indicator of fecal pollution due to their favorable characteristics over currently used FIB, i.e. high concentrations of the marker in human feces and unlikely persistence or reproduction in aquatic environments. In the past, the utility of *Bacteroides* spp. as an alternative indicator was questioned due to difficulties involved in culturing anaerobic bacteria. At present, there exist several molecular assays that have been developed for the detection and/or quantification of *Bacteroides* spp. associated marker or genes (Kreader 1995, Reischer *et al.* 2007, Dick and Field 2004, Bernhard and Dick 2000, Layton *et al.* 2006, Okabe *et al.* 2007, Seurinck *et al.* 2005, Blackwood and Noble 2004, Carson *et al.* 2005, Kildare *et al.* 2007,

Chern *et al.* 2008, Shanks *et al.* 2008, Converse *et al.* 2009), and results from Chapter 3 of this dissertation demonstrated that *Bacteroides* spp. DNA signal decays faster than *Enterococcus* sp. and *E. coli* in marine water. Also, Savichtcheva *et al.* (2007) found positive correlations among total coliforms, fecal coliforms, and total and human-specific *Bacteroides* spp. genetic markers. Moreover, they also found that the genetic markers for *Bacteroides* spp. were predictive for the occurrence of enteric pathogens such as *E. coli* O-157 and *Salmonella* spp.

The objective of this study was to assess the relationships of several alternative FIB-targeting QPCR assays and currently used EPA-approved FIB measurements as tools to distinguish fecal contamination from humans and animals. We present the results from a laboratory-based blind experiment that was conducted by spiking sewage influent and gull guano into sterile and environmental samples in various proportions. EPA-approved FIB methods and rapid QPCR assays designed to target fecal *Bacteroides* spp., total *Enterococcus* sp., *E. faecalis*, *E. faecium*, and *E. casseliflavus* were used to analyze the samples, and the data was analyzed to assess the relationships among measured concentrations of the targets and the specificity of the different assays.

## **Methods:**

### *Sample Preparation:*

Briefly, fourteen samples were created in the laboratory by inoculating various levels of sewage influent (Orange County Sanitation District's Plant #1, Fountain Valley, CA) into seawater collected 11km offshore, from wavewash at Doheny Beach State Park (Dana Point, CA), and from a pond frequented by large populations of gulls also at Doheny Beach State

Park. Sewage was added in various dilutions to achieve final concentrations of approximately 50, 150, 500, 1,000, and 10,000 *Enterococcus* sp. cells per 100mL.

Four samples were created by inoculating gull guano (Wetland and Wildlife Care Center of Orange County, Huntington Beach, CA) into offshore seawater and Doheny Beach wavewash. For gull samples, approximately 1 gram of gull guano was added to 10L of seawater. Previous research conducted on similar fecal samples had shown that this inoculation should achieve a total *Enterococcus* sp. concentration of approximately 1,000 cells per 100mL (Griffith unpublished data).

Environmental water samples were collected from beaches with historically high concentrations of FIB: Imperial Beach in San Diego, CA; Doheny Beach State Park; Surfrider State Beach in Malibu, CA; Malibu Creek (Calabasas, CA); Ballona Creek (Culver City, CA); the Tijuana River in San Diego, CA. Sterile phosphate-buffered saline (PBS) and offshore seawater were run as negative controls.

#### *Sample Processing:*

One-hundred mL of samples were filtered onto 47mm diameter, 0.45µm pore-size polycarbonate filters using a six-place filtration manifold and vacuum pump assembly with disposable filter funnels. Filters were transferred into a sterile 2mL screw-cap tubes and stored at -80°C until extraction of DNA.

#### *Enumeration of Enterococcus sp. and E. coli by Membrane Filtration (MF):*

Total *Enterococcus* sp. and *E. coli* were enumerated by MF, following EPA Methods 1600 for *Enterococcus* sp. and 1103.1 for *E. coli*.

### *Preparation of Calibration Standard*

*Bacteroides thetaiotamicron* (ATCC 29148) was used as QPCR calibration standard for the fecal *Bacteroides* spp. QPCR. Cells were grown anaerobically in an overnight culture at 37°C in cooked meat medium. A portion of the cell suspension was removed and centrifuged for 5 minutes at 6,000 x g. The supernatant fluid was removed and aliquoted for use as a cell standard. Aliquots were frozen at -20°C. Cell counts were obtained by removing a portion of the cell suspension, serially diluting, fixing in formalin (1% v/v final) and counting cells using SYBR Green following the protocol of Noble and Fuhrman (1998). A known amount of cells (100,000) was filtered onto 47mm, 0.4µm pore size polycarbonate filters, and filters were stored at -80°C until extraction.

*Enterococcus faecalis* (ATCC 29212), *Enterococcus faecium* (ATCC 12952), and *Enterococcus casseliflavus* (ATCC 25758) were used as QPCR calibration standards for the *Enterococcus* speciation. Cell lines were obtained from the ATCC and cultured overnight at 37°C in brain heart infusion broth. Cells were counted using EPA Method 1600. Cell suspensions were diluted with PBS and filtered onto 47mm, 0.4µm pore size polycarbonate filters such that each filter held 100,000 cells. Filters were stored at -80°C until extraction.

### *Specimen Processing Control:*

A specimen processing control (SPC) was added to each sample in order to estimate PCR inhibition. Salmon sperm testes DNA (Sigma) was added at the beginning of the extraction step at a concentration of 100ng per 500µL for each sample, calibrator, and blank.

A QPCR assay targeting the SPC was developed by Haugland *et al.* (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. All SPC QPCR sample reactions with a cycle threshold (Ct) value 0.5 log units higher than that of the calibrators and blanks were considered inhibited. Inhibited samples were diluted 1:10 and 1:100 with sterile water and reanalyzed.

*DNA Extraction:*

DNA was extracted from the polycarbonate filters using the DNA-EZ RW04 Extraction Kit (GeneRite). Filters were transferred into 2mL semiconical screw-cap microcentrifuge tubes pre-loaded with 0.3g of acid-washed glass beads. Four hundred and ninety microliters of AE Buffer (Qiagen) and 10 $\mu$ L of the specimen processing control (10 $\mu$ g/mL salmon sperm testes DNA) were dispensed into each tube. Each tube was bead milled in an eight-position mini bead beater for 2 minutes, followed by centrifugation in a Eppendorf Microfuge for 1 minute at 12,000 x g. Supernatants were transferred to sterile 1.7 mL microcentrifuge tubes and centrifuged at 12,000 x g for 5 minutes. Supernatant was transferred to a sterile 1.7mL microcentrifuge tube, mixed by vortexing with 500 $\mu$ L of Binding Buffer, and applied to a DNAsure column. Columns were inserted into collection tubes and centrifuged at 12,000 x g for 1 minute. Columns were transferred to new collection tubes, and 500 $\mu$ L of EZ-Wash Buffer was applied to the columns. Columns were again centrifuged at 12,000 x g for 1 minute. After transferring to new collection tubes, columns were washed again with 500 $\mu$ L of wash buffer and 1 minute of centrifugation. Columns were transferred to sterile, low retention 1.7mL microcentrifuge tubes, and 50 $\mu$ L of

DNA Elution Buffer was applied to the center of the columns and allowed to incubate at room temperature for 1 minute. Columns were again centrifuged for 1 minute at 12,000 x g. Extracted DNA in the microcentrifuge tube was stored at -20°C.

*QPCR Analyses and Quantification of Target DNA:*

TaqMan® primers and probes were used to assay for SPC (Haugland *et al.* 2005), fecal *Bacteroides* spp. (Converse *et al.* 2009), *E. faecalis* (Santo-Domingo *et al.* 2003), *E. faecium* (Santo-Domingo, unpublished), and *E. casseliflavus* (Santo-Domingo, unpublished). Twenty five microliter reactions were prepared using OmniMix beads (a lyophilized premix with 1.5 units of TaKaRa hot start Taq polymerase, 200µM of dNTPs, 4mM of MgCl<sub>2</sub>, and 25mM HEPES with a pH of 8; Cepheid), 1µM each of forward and reverse primers, 0.1µM of the TaqMan probe, and 5µL of sample DNA extract. Reactions were thermal cycled and monitored in a Smart Cycler II® (Cepheid). Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 30 seconds at 60°C.

Scorpion chemistry was used for Total *Enterococcus* sp. QPCR assays. Twenty-five microliter reactions were prepared using 5µl of sample DNA, OmniMix beads, and Total *Enterococcus* Species beads (lyophilized Scorpion primer and probe sets with 0.25µM of each primer and probe complex; Cepheid). Reactions were thermal cycled and monitored in a Smart Cycler II. Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 5 seconds at 95°C and 43 seconds at 62°C.

All analyses of unknowns were run in duplicate. After manually adjusting the threshold on the SmartCycler II, cycle threshold (Ct) was determined automatically by the instrument. Samples that yielded a “non-detect” QPCR result were assigned a concentration

of 5 cells per 100mL. A duplicate standard curve was run concurrently with samples using the calibrator and three serial 10-fold dilutions. Amplification efficiency (E) was calculated using the slope of the log standard curve given by the SmartCycler software:  $E=10^{(-\text{slope})}$ . The ratio of target DNA in the samples was multiplied by the amount of target DNA in the calibrator to get the sample quantities in number of cells, following Pfaffl (2001). When DNA extracts had to be diluted due to inhibition seen in the SPC, sample quantities were also multiplied by the dilution factor to get a corrected quantity.

#### *Data Analysis:*

The mean concentrations of each target for all samples were log-transformed. All statistical correlations and differences were tested in SPSS statistical analysis software using Spearman-Rank analysis and the Mann-Whitney U test.

#### **Results:**

##### *Fecal Bacteroides spp. QPCR:*

Fecal *Bacteroides* spp. concentrations in the sewage-spiked samples were two orders of magnitude greater than those in the guano-spiked samples. The average fecal *Bacteroides* spp. concentration in gull-spiked samples was 25 cells per 100mL. The samples that contained the lowest amount of influent-spike had an average fecal *Bacteroides* spp. concentration of  $1.24 \times 10^3$  cells per 100mL. The fecal *Bacteroides* spp.: *E. coli* and fecal *Bacteroides* spp.: total *Enterococcus* sp. (as determined by MF) ratios ranged from  $2.5 \times 10^{-5}$  to  $3.5 \times 10^{-2}$  in samples with gull guano. In influent-inoculated samples, the fecal

*Bacteroides* spp.: *E. coli* (as determined by MF) ranged from 2 to 259, and the *Bacteroides*: total *Enterococcus* sp. (as determined by MF) ranged from 2 to 450.

The correlation of fecal *Bacteroides* spp. QPCR to *Enterococcus* sp. QPCR was 0.417 ( $p = 0.013$ ) for all samples. In sewage-spiked and environmental samples, this correlation was stronger ( $r = 0.851$ ,  $p < 0.001$ ). The fecal *Bacteroides* spp. concentrations retained their correlation with *Enterococcus* sp. and *E. coli* as measured by MF in environmental samples ( $r = 0.803$ ,  $p < 0.001$ ;  $r = 0.720$ ,  $p = 0.002$ ; respectively). In most offshore seawater and wavewash samples, *E. coli*, *Enterococcus* sp., and fecal *Bacteroides* spp. concentrations were low, most likely due to low inputs of fecal contamination. In Ballona Creek and Malibu Creek, fecal *Bacteroides* spp. concentrations were high while *Enterococcus* sp. and *E. coli* concentrations were low. In Tijuana River samples, fecal *Bacteroides* spp. levels were high ( $> 100,000$  cells per 100mL), but *Enterococcus* sp. and *E. coli* levels were orders of magnitude higher.

#### *Enterococcus* Speciation QPCR:

*Enterococcus faecalis* and *Enterococcus casseliflavus* concentrations averaged  $1.32 \times 10^4$  and 881 cells per 100 mL, respectively. The concentrations of both, normalized by the total amount of *Enterococcus* sp. in each sample, were significantly higher in gull-spiked samples than in sewage spiked.

*E. faecalis* concentrations were significantly correlated with *Enterococcus* sp. and *E. coli* concentrations ( $r = 0.615$ ,  $p < 0.001$ ;  $r = 0.669$ ,  $p < 0.001$ ; respectively). Weaker but still significant correlations were found between *E. casseliflavus* concentrations and *Enterococcus* sp. and *E. coli* concentrations ( $r = 0.459$ ,  $p = 0.006$ ;  $r = 0.467$ ,  $p = 0.005$ ; respectively).

*E. faecium* was only detected in one sample, offshore seawater spiked with approximately 1,000 total *Enterococcus* sp. cells.

*Sewage Spiked Samples:*

QPCR-based measurements of total *Enterococcus* sp., fecal *Bacteroides* spp., *E. faecalis*, and *E. casseliflavus* concentrations were similarly correlated to the relative amount of spiked influent ( $r = 0.891$ ,  $p < 0.001$ ;  $r = 0.848$ ,  $p=0.004$ ;  $r = 0.867$ ,  $p<0.001$ ; and  $r = 0.868$ ,  $p<0.001$ ; respectively). Total *Enterococcus* sp. and *E. coli* concentrations as measured by MF were also highly correlated with the amount of sewage influent spiked into seawater samples.

QPCR and MF measurements of total *Enterococcus* sp. were significantly correlated with  $r = 0.888$ ,  $p < 0.001$ . Total *Enterococcus* sp. as measured by QPCR was also significantly correlated with *E.coli* as measured by MF, and fecal *Bacteroides* spp., *E. faecalis*, and *E. casseliflavus* as measured by QPCR.

*Environmental Samples:*

Results from the total *Enterococcus* sp. QPCR and MF were significantly correlated in environmental samples ( $r = 0.561$ ,  $p = 0.024$ ). It is interesting to note that the correlation was much stronger when the two samples from the Doheny Pond were excluded from the comparison. These samples were so inhibited that even with 1:100 dilutions, none of the inoculated salmon sperm testes DNA was amplified. The magnitude of inhibition seen in the Doheny Pond samples was not observed in any of the other environmental samples analyzed and is unlikely to be observed in many beach recreational waters. Doheny Pond is a small,

shallow body of water that is visibly contaminated, is usually completely disconnected from the ocean, and supports a vast population of gulls. As an essentially closed-system, gull waste and sediment are highly concentrated in the pond. Both gulls and sediment bring with them a host of known inhibitors of PCR such as humic and fulvic acid, phenolic compounds, complex polysaccharides from algae, urea, and bile salts (Wilson 1997, Watson and Blackwell 2000). Most recreational water bodies experience greater flushing and support much lower densities of gulls, so PCR inhibitors would be unlikely to be found at the concentrations observed in Doheny Pond.

## **Discussion:**

### *Fecal Bacteroides spp. as an Indicator of Human-Specific Fecal Contamination:*

We present results for quantification of *Bacteroides thetaiotamicron* and related *Bacteroides* spp. using a rapid fecal *Bacteroides* spp. QPCR assay that shows specificity for human fecal contamination from a range of environmental and laboratory-created samples. The fecal *Bacteroides* spp. QPCR assay has a strong relationship to *Enterococcus* sp. and *E. coli* in sewage-spiked samples, even at relatively low copy numbers. Furthermore, the ratios of fecal *Bacteroides* spp. QPCR results to *Enterococcus* sp. measurements show a higher trend in samples impacted by human feces than in animal scat samples and samples contaminated with gull guano. These results, along with other concurrent assessments, indicate that this assay could be useful, especially if used in a tiered approach, to identify the relative presence of human fecal contamination.

Over the course of this study we have attempted to derive a set of ratios between fecal *Bacteroides* spp. and either *E. coli* or *Enterococcus* sp. as a preliminary step toward creating

a management tool using fecal *Bacteroides* spp. QPCR assay. We consistently observed ratios of greater than 2 from human sewage spike samples and less than 1 from samples inoculated with gull guano. This study involved analysis of a low number of samples, so further work to develop useful fecal typing ratios for animal fecal contamination is necessary. However, our preliminary results suggest that the fecal *Bacteroides* spp. assay could be useful in distinguishing runoff containing predominantly human contamination from that with predominantly animal, especially when the animal contamination is predominantly bird feces. That fecal *Bacteroides* spp. concentrations were low in samples spiked with gull guano is particularly advantageous: gulls contribute enormously to fecal contamination at recreational beaches with loadings of  $1.77 \times 10^8$  fecal coliforms per fecal deposit (Alderisio and DeLuca 1999). In the Great Lakes area, sequencing demonstrates that gulls are sometimes responsible for greater than 50% of *E. coli* in lakes (Ram *et al.* 2007). Additionally, *E. coli* and *Enterococcus* sp. deposited in gull guano are prime candidates for persistence and growth in beach sediments, inoculating beach waters when disturbed by waves or swimmers (Kleinheinz *et al.* 2006).

It has been suggested previously that fecal *Bacteroides* spp. and other anaerobes could be useful tools in water quality management because the anaerobic bacteria would not be expected to survive in recreational waters and sediments for long periods of time (for example, Wang *et al.* 1996, Carson *et al.* 2005). At warm temperatures ( $\geq 24^\circ\text{C}$ ), PCR-detectable *Bacteroides* persists for only 1 to 2 days (Kreader 1998), and this was corroborated by findings presented in Chapter 3, which showed that fecal *Bacteroides* spp. signal degraded significantly faster than *Enterococcus* sp. and *E. coli* signal. Pathogens with limited persistence in marine water may not be well represented by *Enterococcus* sp. and *E.*

*coli*, which have been documented to persist and grow in the beach sands and marine wrack (Solo-Gabriele *et al.* 2000, Anderson *et al.* 2005). Poliovirus, for instance, can be inactivated in as few as 1 to 3 days in warm temperatures (Wait and Sobsey 2001). The shorter survival of *Bacteroides* spp. in environmental waters may improve estimations of the presence of fresh fecal contamination, and may therefore more accurately predict the presence of viral pathogens that are not capable of persisting or replicating in the environment. This study did not focus on the persistence of fecal *Bacteroides* spp. in water samples, but this is a necessary area for further research.

Because no single indicator may be a good proxy for all pathogens, a tiered approach may be best for quantification and characterization of human health risk. *E. coli* and *Enterococcus* sp. serve as good indicators of overall fecal contamination over sources and time. When there are exceedances of current FIB standards, the fecal *Bacteroides* spp. assay used in this study may be useful in apportioning human from non-human sources. Also because the *Bacteroides* spp. members are found in higher concentrations than either *E. coli* or *Enterococcus* sp. in gut flora, it may be that the ratio of *Bacteroides* spp. to either *E. coli* or *Enterococcus* sp. could be a useful tool for water quality management. With the exception of Tijuana River samples, influent-spiked seawater and environmental samples in this study had ratios of fecal *Bacteroides* spp.: *Enterococcus* sp. (as measured by MF), ranging from 6.26 to 152 when *Enterococcus* sp. concentrations exceeded single sample standards. Samples from the Doheny Pond (known to be contaminated with gull guano) or with gull guano-spikes had much smaller ratios:  $2.5 \times 10^{-5}$  to 0.16. The Mann-Whitney U test shows that these two ranges of ratios are significantly different, suggesting that a ratio between fecal *Bacteroides* spp. and *Enterococcus* sp. could indicate the presence of human fecal material.

This ratio will likely be most useful only when *Enterococcus* sp. concentrations exceed the standards; i.e. in a tiered approach context. In cases where the *Enterococcus* sp. or *E. coli* concentrations are very low and when fecal *Bacteroides* spp. concentrations would also likely be low, it would be difficult to determine whether the low concentrations are due to lack of human input, methodological variability, or dilution of contamination.

*Enterococcus Speciation as an Indicator of Human-Specific Fecal Contamination:*

Until recently, speciation was impractical given that *E. casseliflavus* is difficult to differentiate from *E. faecium* using conventional biochemical tests (Devriese *et al.* 1996). The development of QPCR assays for each species allows *Enterococcus* speciation to become a practical possibility for bacterial source tracking. In fact, Scott *et al.* have effectively designed an *E. faecium*-based assay for the *esp* gene that is often specific to human fecal contamination (2005). Unfortunately, the assays tested in this study provide only inconclusive results. Concentrations of *E. faecalis* and *E. casseliflavus* were each correlated with amounts of sewage additions. Concentration of both species were nearly equal in most sewage samples suggesting that *E. casseliflavus* may be a more important component of human sewage than previously thought. The strong detection of *E. faecalis* in samples spiked with gull guano also indicates that it is no more strongly related to human fecal contamination than to bird contamination, so at least in this context detection of *E. faecalis* would have no source predictive value. The *Enterococcus* speciation QPCR may have promise in another arena. The *E. faecalis* and *E. casseliflavus* QPCR assays related strongly to total *Enterococcus* sp. QPCR and MF methods in samples containing fresh contamination (i.e. those samples inoculated with fresh sewage or guano). It may be that

specific species of *Enterococcus* could be used to assess the presence of fresh contamination, thereby permitting partitioning of *Enterococcus* sp. stemming from reservoir populations (in sand and sediment) from fresh fecal contamination sources (like fresh sewage). This concept will require further refinement and testing but could represent an advancement in water quality management.

#### *QPCR versus MF for Total Enterococcus sp.:*

The correlation between total *Enterococcus* sp. concentrations as measured by QPCR and MF show that the QPCR assay yielded similar results to existing methods, corroborating findings from a previous study conducted in southern California waters by Noble *et al.* (submitted).

Total *Enterococcus* sp. as measured by QPCR were often orders of magnitude higher than those for *E. faecalis* and *E. casseliflavus*. This is likely the result of a combination of several factors. First, *Enterococcus* sp. cells may carry multiple copies of the gene targeted by the *Enterococcus* primers and probe. Second, QPCR could be measuring DNA from dead cells. Third, cells may be in a viable but non-culturable state due to environmental stress, leading to underestimations by culture-based MF.

#### **Conclusions:**

The rapid fecal *Bacteroides* spp. QPCR assay shows much promise as a tool for distinguishing human from animal fecal contamination in seawater, especially when used in a tiered-approach with currently recommended FIB. The strong correlation of the assay with amount of sewage influent in seawater samples and differential concentrations in influent and

animal scat allows not only detection of the presence of human fecal contamination but also determination of the relative ratio of human-specific contamination in heterogeneous runoff.

*Enterococcus* speciation as performed in this study was not useful in distinguishing human, gull, and environmental sources of contamination. Results suggest that speciation could be useful in distinguishing old from new fecal contamination, but this hypothesis will need further testing.

Figure 4.1: Correlations of *Bacteroides*, *E. faecalis*, and *E. casseliflavus* to amount of influent spike. Spearman rank coefficients listed.

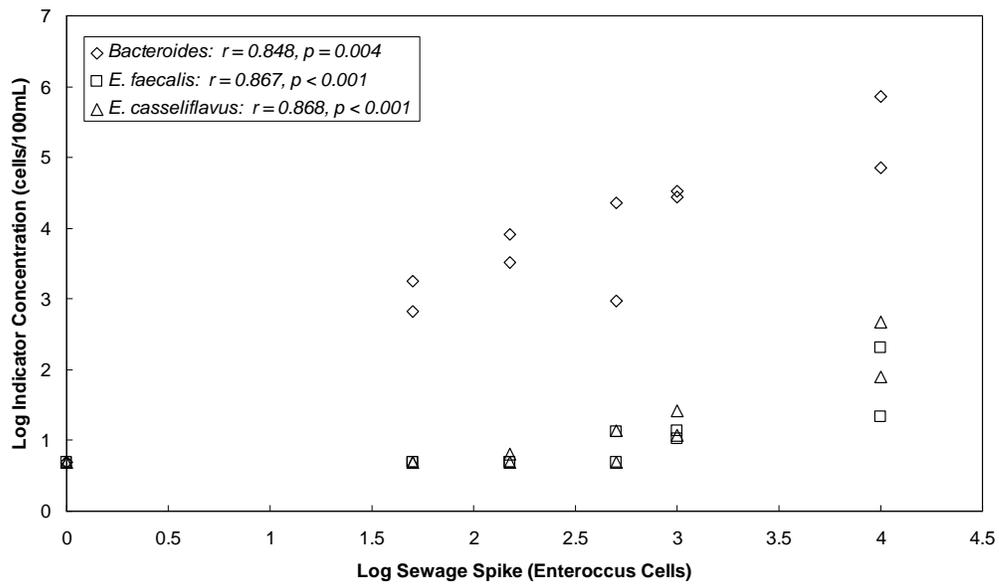


Figure 4.2: Correlation of *Enterococcus* concentrations as measured by QPCR and MF for all samples excluding those QPCR-inhibited samples from Doheny Pond ( $r = 0.921$ ,  $p < 0.001$ ).

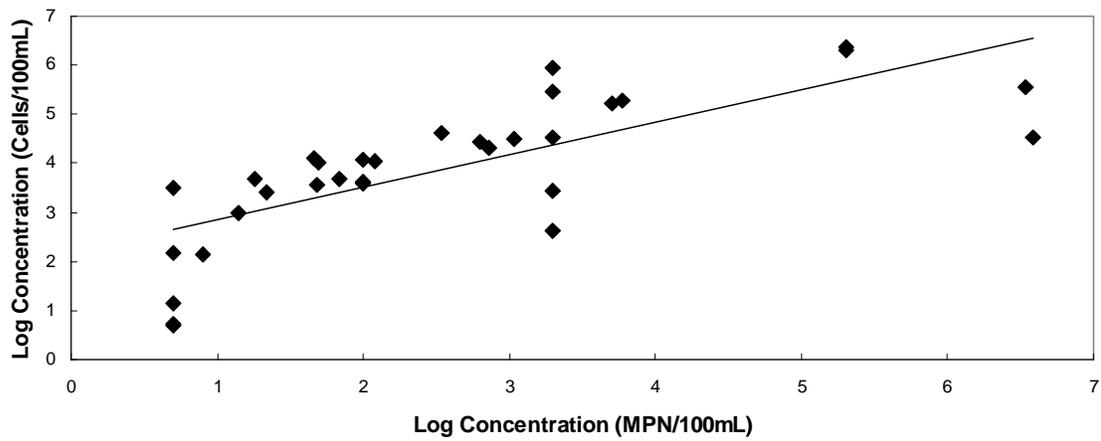
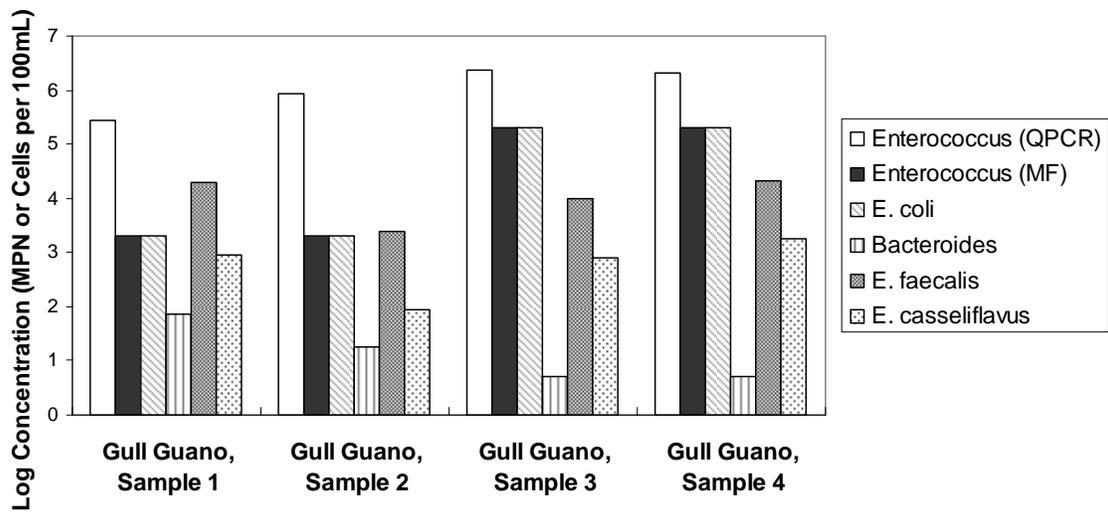


Figure 4.3: Concentrations of each indicator in samples spiked with full-strength gull-guano.



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## Chapter 5:

Application of traditional and alternative fecal indicator bacteria in stormwater under varying hydrologic conditions

### Introduction:

Fecal pollution in stormwater differs fundamentally from most point-source (PS) pollution delivered to recreational waters. In general, PS fecal pollution has been well-characterized and has known rates of input. Wastewater treatment plants are some of the most commonly known PS fecal pollution contributors to recreational fresh- and marine waters. Stormwater runoff is generally characterized as a non-point source (NPS) even if it is collected and distributed through a pipe or conduit to receiving water. Stormwater runoff can carry with it a range of fecal contamination: human, pet, livestock, wildlife, and waterfowl included. Land-use influences the type of contamination carried in stormwater runoff. Agricultural, commercial, and residential land may have different fecal contamination signatures. However, given the mixed land uses in most watersheds, this relationship can be quite complex (Schwab 2007). Because stormwater entrains fecal indicator bacteria (FIB) that have been accumulating on the land since the last rain event, the load of FIB from stormwater runoff to recreational waters during storms can be over 1000 times higher than loads during baseflow conditions (Krometis *et al.* 2007). However, the high magnitude of FIB contributed to receiving waters during a storm event is not necessarily indicative of human fecal contamination because animal feces can contain similarly high concentrations of EPA currently-approved FIB, fecal coliforms (or the dominant subset, *E. coli*) and

*Enterococcus* sp. These FIB have also been shown to grow in sediments (Anderson *et al.* 2005). While there is a demonstrated public health risk associated with recreating in water affected by NPS fecal contamination, most studies have been unable to demonstrate an association between FIB concentrations and human health outcomes (Noble and Fuhrman 2001, Jiang *et al.* 2004, Colford *et al.* 2007).

Sources of fecal contamination in stormwater must be identified and quantified in order to better approximate public health risk associated with recreation in receiving waters and to alleviate pollution problems. To that end, alternative indicators of fecal contamination with source tracking capabilities have been recommended (Savitcheva and Okabe 2006). Fecal *Bacteroides* spp. have been suggested as such an alternative indicator because molecular markers have been developed for the genus that are generally more concentrated in human fecal material than animal, they are obligate anaerobes, and they are expected to have limited persistence upon leaving the human gastrointestinal tract (Chapters 3 and 4, Fiksdal *et al.* 1985, Kreader 1998, Carson *et al.* 2005, Layton *et al.* 2006, Okabe and Shimazu 2007). Results from Chapter 4 demonstrate that fecal *Bacteroides* spp. can be used to distinguish human from avian fecal contamination in lab-created and environmental water samples. However, samples with heavy NPS fecal contamination, such as stormwater, may be more complicated. Differences are expected between fecal *Bacteroides* spp. and current FIB in terms of stormwater concentrations and persistence, and current research methodologies that attempt to characterize an entire storm based upon a single grab sample may be inappropriate when using fecal *Bacteroides* spp. as a source tracking indicator.

The objectives of this work were to: 1) examine patterns of currently used EPA-approved measures of FIB concentrations through the duration of storms, 2) examine basic hydrological characteristics of the storms in order to assess storm variability, 3) use QPCR-based methods to assess patterns of the alternative indicator fecal *Bacteroides* spp. in stormwater, and 4) pair the hydrologic conditions with numerical results for bacterial concentrations to assess loading and deduce information about the sources of contamination.

## **Methods:**

### *Sampling Location:*

Located on a barrier island in Dare County, North Carolina, Nags Head is a popular resort area with a peak visitor population of up to 50,000 (Esnard *et al.* 2001). Bordered on the east by the Atlantic Ocean and the west by Roanoke Sound, Nags Head spans 12 miles from north to south. The town has had poor water quality and swim advisories in the past (NCDENR 2003). Stormwater is a particular problem in Nags Head: forty hurricanes, tropical storms, tropical depressions, subtropical storms, and subtropical depressions came within 100km of the town between 1991 and 2006 (NOAA).

All 8 stormwater outfalls maintained by the North Carolina Department of Transportation (NCDOT) in Nags Head, locations shown in Figure 5.1, were chosen as study sites. These outfalls are named Sites 1 through 8. We also monitored a stormwater outfall draining into the Roanoke Sound at Whalebone Junction, labeled site 9. For site descriptions, see Table 5.1. At each site, the outfall catch basin was outfitted a Doppler flow meter (Teledyne ISCO 750 Area Velocity Module).

### *Field Sampling:*

Water samples were collected during five storms from June 2007 to November 2008. Sampling was initiated when storms seemed likely to produce at least 2.5cm (1 in) of rainfall, and 3 to 6 stormwater samples were collected over the course of each storm in sterile bottles following standard methods sampling techniques (APHA 2005). Samples were stored at 4°C until they were analyzed.

### *Enumeration of E. coli and Enterococcus sp. in Stormwater:*

*Enterococcus* sp. and *E. coli* concentrations were measured for each sample using defined substrate technology kits, Enterolert™ and Colilert-18™ (Idexx Laboratories) following manufacturer's instructions. Positive wells were converted to a most probable number (MPN) using manufacturer-supplied MPN tables.

### *Preparation of Stormwater Samples for QPCR:*

One-hundred mL water samples were filtered in triplicate onto 47mm 0.45µm polycarbonate filters and stored at -80°C until DNA extraction.

### *QPCR Calibration Standards:*

*Bacteroides thetaiotamicron* (ATCC 29148) was used as a QPCR calibration standard. Cells were grown anaerobically in an overnight culture at 37°C in cooked meat medium. A portion of the cell suspension was removed and centrifuged for 5 minutes at 6,000 x g. The supernatant fluid was removed and aliquoted for use as a cell standard. Aliquots were frozen at -20° C. Cell counts were obtained by removing a portion of the cell

suspension, serially diluting, fixing in formalin (1% v/v final) and counting cells using SYBR Green following the protocol of Noble and Fuhrman (1998). A known amount of cells (100,000) was filtered onto 47mm, 0.4µm pore size polycarbonate filters, and filters were stored at -80°C until extraction.

*Specimen Processing Control:*

A specimen processing control (SPC) was added to each sample in order to estimate PCR inhibition. Salmon sperm testes DNA (Sigma) was added at the beginning of the extraction step at a concentration of 100ng per 500µL for each sample, calibrator, and blank.

A QPCR assay targeting the SPC was developed by Haugland *et al.* (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. All SPC QPCR sample reactions with a cycle threshold (Ct) value 0.5 log units higher than that of the calibrators and blanks were considered inhibited. Inhibited samples were diluted 1:10 and 1:100 with sterile water and reanalyzed.

*DNA Extraction:*

DNA was extracted from the polycarbonate filters using the DNA-EZ RW04 Extraction Kit (GeneRite). Filters were transferred into 2mL semiconical screw-cap microcentrifuge tubes pre-loaded with 0.3g of acid-washed glass beads. Four hundred and ninety microliters of AE Buffer (Qiagen) and 10µL of the specimen processing control (10µg/mL salmon sperm testes DNA) were dispensed into each tube. Each tube was bead milled in an eight-position mini bead beater for 2 minutes, followed by centrifugation in an

Eppendorf Microfuge for 1 minute at 12,000 x g. Supernatants were transferred to sterile 1.7 mL microcentrifuge tubes and centrifuged at 12,000 x g for 5 minutes. Supernatant was transferred to a sterile 1.7mL microcentrifuge tube, mixed by vortexing with 500µL of Binding Buffer, and applied to a DNAsure column. Columns were inserted into collection tubes and centrifuged at 12,000 x g for 1 minute. Columns were transferred to new collection tubes, and 500µL of EZ-Wash Buffer was applied to the columns. Columns were again centrifuged at 12,000 x g for 1 minute. After transferring to new collection tubes, columns were washed again with 500µL of wash buffer and 1 minute of centrifugation. Columns were transferred to sterile, low retention 1.7mL microcentrifuge tubes, and 50µL of DNA Elution Buffer was applied to the center of the columns and allowed to incubate at room temperature for 1 minute. Columns were again centrifuged for 1 minute at 12,000 x g. Extracted DNA in the microcentrifuge tube was stored at -20°C.

*QPCR Analyses and Quantification of Target DNA:*

The SPC QPCR assays were conducted following Haugland *et al.* (2005). Fecal *Bacteroides* spp. primers and probes are described in Converse *et al.* (2009). Twenty five microliter reactions were prepared using OmniMix beads (a lyophilized premix with 1.5 units of TaKaRa hot start Taq polymerase, 200µM of dNTPs, 4mM of MgCl<sub>2</sub>, and 25mM HEPES with a pH of 8; Cepheid), 1µM each of forward and reverse primers, 0.1µM of the TaqMan probe, and 5µL of sample DNA extract. Reactions were thermal cycled and monitored in a Smart Cycler II® (Cepheid). Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 30 seconds at 60°C.

All analyses of unknowns were run in duplicate. After manually adjusting the threshold on the SmartCycler II, cycle threshold (Ct) was determined automatically by the instrument. Samples that yielded a “non-detect” QPCR result were assigned a concentration of 5 cells per 100mL. A duplicate standard curve was run concurrently with samples using the calibrator and three serial 10-fold dilutions. Amplification efficiency (E) was calculated using the slope of the log standard curve given by the SmartCycler software:  $E=10^{(-\text{slope})}$ . The ratio of target DNA in the samples was multiplied by the amount of target DNA in the calibrator to get the sample quantities in number of cells following Pfaffl (2001). When DNA extracts had to be diluted due to inhibition seen in the SPC, sample quantities were also multiplied by the dilution factor to get a corrected quantity.

#### *Data Analysis:*

All indicator concentrations were log-transformed. All statistical correlations and differences were tested in SPSS statistical analysis software using non-parametric statistical tests: Spearman-Rank analyses, the Friedman Test, and the Wilcoxon Signed Rank Test.

#### **Results:**

*Enterococcus* sp. and fecal coliform concentrations in Nags Head’s stormwater outfalls were consistently high. Average log *E. coli* concentrations in stormwater ranged from 2.5543 MPN/100mL (S.E. 0.1588) to 3.3156 MPN/100mL (S.E. 0.1509) during the five study storms. Average log *Enterococcus* sp. concentrations were similarly high, ranging from 2.5643 MPN/100mL (S.E. 0.1594) to 3.7197 MPN/100mL (S.E. 0.1032). Average

indicator concentrations and flow rates across all storms are summarized in Table 5.2.

Averages across sites are presented in Table 5.3.

#### *Flow and FIB Loads:*

Changes in indicator concentrations and flow revealed no predictable patterns in indicator loading over the course of a storm event. For instance, Figure 5.2 presents these changes through a representative storm (Sept 2008). In general, indicator concentrations remained high throughout the entire storm, regardless of changes in flow, as shown in Figure 5.3, which shows data pooled from all storms and sites. When data are pooled, only *E. coli* concentrations were significantly correlated with flow (Spearman correlation coefficient = 0.223,  $p=0.009$ ). Even when data are separated by site and/or storm, there was often no correlation between flow and indicator concentrations. Fecal *Bacteroides* spp. concentration was not significantly correlated with flow during any storm, and *E. coli* and *Enterococcus* sp. concentrations were only significantly correlated with flow during 2 of the 5 storms. Significant correlations when data are separated by site are summarized in Table 5.4.

#### *Differences between Storm Events:*

Results from Friedman tests show that there were significant differences in fecal *Bacteroides* spp. concentrations, *Enterococcus* sp. concentrations, and flow between storm events ( $p=0.001$ ,  $<0.001$ , and  $<0.001$ , respectively). There were no significant differences in *E. coli* concentrations between storms. Wilcoxon Signed Rank tests were performed to determine which storms were significantly different pairwise for fecal *Bacteroides* spp. and *Enterococcus* sp. concentrations, using a Bonferroni corrected significance level of 0.005.

Results are presented in Figure 5.4. Friedman tests did not detect a significant effect of storm event on the ratio of each indicator to flow. However, storm events did have an effect on the ratios between indicators. Significant differences between storms are shown in Figure 5.

#### *Differences between Sites:*

Friedman tests were also used to look at the effect of site on several measured factors. Because we did not have information about drainage area and water table height for site 9 at Whalebone Junction, this site was excluded from the analyses. Overall, site had a significant effect on *E. coli* and *Enterococcus* sp. concentrations and flow:  $p = 0.018$  for *E. coli*;  $p = 0.033$  for *Enterococcus* sp. ; and  $p < 0.001$  for flow. Site had a marginally significant effect on fecal *Bacteroides* spp. concentration ( $p = 0.057$ ). Friedman test results also show that site had a significant effect on the ratio of *E. coli* to *Enterococcus* sp. ( $p = 0.001$ ). Site had no significant effects on the ratio of fecal *Bacteroides* spp. to either *E. coli* or *Enterococcus* sp. When using the Wilcoxon Signed Rank test to compare sites pairwise, no significant differences were found at the Bonferroni corrected significance level of 0.0033.

#### *Correlations:*

When data were pooled, there was not a significant correlation between concentrations of fecal *Bacteroides* spp. and concentrations of *E. coli* or *Enterococcus* sp. ( $n = 118$ ). *E. coli* and *Enterococcus* sp. concentrations were correlated with a Spearman coefficient of 0.473 ( $p < 0.001$ ,  $n = 145$ ).

*E. coli* concentrations were found to be significantly correlated with flow (Spearman coefficient=0.223,  $p=0.009$ ,  $n=137$ ), but neither fecal *Bacteroides* spp. nor *Enterococcus* sp. concentrations significantly correlated with flow.

*Enterococcus* sp. and fecal coliform concentrations correlated with the number of days since the last storm event: for *Enterococcus* sp., the Spearman coefficient was 0.454 ( $p<0.001$ ,  $n=126$ ); and for *E. coli*, the Spearman coefficient was 0.316 ( $p<0.001$ ,  $n=126$ ). Fecal *Bacteroides* spp. concentration was not significantly correlated with the number of days since the last storm event.

Concentrations of all three indicators and flow were significantly correlated with the size of the outfall drainage areas. Fecal *Bacteroides* spp. and fecal coliform concentrations were similarly correlated with drainage area, with Spearman coefficients of 0.307 ( $p=0.001$ ,  $n=117$ ) and 0.289 ( $p=0.001$ ,  $n=125$ ), respectively. *Enterococcus* sp. concentration had a slightly weaker but still significant correlation with drainage area (Spearman coefficient=0.176,  $p=0.049$ ,  $n=125$ ).

Finally, fecal *Bacteroides* spp., *E. coli*, and flow were correlated with relative water table height, which was measured on three occasions during dry weather in the summer of 2005. Fecal *Bacteroides* spp. concentrations were negatively correlated with water table depth (Spearman correlation coefficient of -0.212,  $p=0.021$ ), while fecal coliform concentrations and flow were both positively correlated with water table depth (Spearman correlation coefficients of 0.379,  $p<0.001$  and 0.321,  $p<0.001$ , respectively)

These correlations are not always equal or significant when the data are separated by storm event. During the June 2007 storm, fecal *Bacteroides* spp. concentration was significantly correlated with both *E. coli* and *Enterococcus* sp. concentrations (Spearman

coefficient = 0.679,  $p=0.022$ ,  $n=11$ ; and Spearman coefficient = 0.771,  $p=0.006$ , and  $n=11$ ; respectively). However, fecal *Bacteroides* spp. concentration was not significantly correlated with either *E. coli* or *Enterococcus* sp. during any other storm event. *E. coli* and flow were only significantly correlated during the June and December 2007 storms (Spearman coefficient = 0.81 with  $p=0.001$  and Spearman coefficient = 0.33 with  $p=0.022$ , respectively). *Enterococcus* sp. concentrations were also significantly correlated with flow only during the 2007 storms, but the correlation coefficient differs strongly between storm events: 0.662,  $p=0.019$ ; and -0.439,  $p=0.022$ .

Correlations are also not always consistent or significant when data are separated by site. Significant correlations by site are summarized in Table 5.4.

### **Discussion:**

Stormwater runoff in Nags Head consistently carries high concentrations of FIB, indicating the presence of fecal contamination. *Enterococcus* sp. concentrations exceeded the single sample standard of 104 MPN or CFU per 100mL during each storm and at all sites. These findings were consistent with findings of the NCDENR Recreational Water Quality Program, which measured *Enterococcus* sp. levels in the same outfall catch basins beginning in the fall of 2003 through the spring of 2004. Of 14 sampling occasions, each site exceeded the single sample standard 3 to 11 times. Also, even though the State of North Carolina does not manage recreational water quality using fecal coliforms, the average *E. coli* concentrations exceeded the single sample fecal coliform marine recreational water quality standard of 400 MPN per 100mL during 4 of the 5 storms and at 6 of the 8 sites.

### *FIB Loading Patterns:*

In general, *Enterococcus* sp. and *E. coli* concentrations in stormwater started high and remained so throughout the course of a storm. This is consistent with the findings of Surbeck *et al.* (2006), who hypothesized that in urban environments FIB are ubiquitous and FIB loads will remain constant. Basically, this hypothesis assumes that there is uniformity and high concentrations of FIB in the environment so we cannot see a wash-off effect as indicators are entrained by stormwater.

Because EPA-approved FIB are found at consistently high concentrations, alternate indicators with greater host-specificity and shorter persistence were expected to give improved loading and source information. Due to its limited persistence in aerobic environments and relatively lower concentrations in animal scat than human waste (Converse *et al.* 2009), we did not expect fecal *Bacteroides* spp. concentrations to behave like *E. coli* and *Enterococcus* sp. over the course of a storm event. We expected that concentrations of fecal *Bacteroides* spp. from surface deposits of fecal material would be low and would likely experience a first flush effect. Our findings were not consistent with this hypothesis: fecal *Bacteroides* spp. concentrations were consistently high throughout storm events. Because surface depositions come almost exclusively from animals with lower fecal *Bacteroides* spp. concentrations than humans, this suggests that septage is contributing significantly to the fecal *Bacteroides* spp. load in stormwater. Indeed, septic systems are the primary form of wastewater treatment in Nags Head, despite consistently high water tables that are often less than 1m from the surface even in dry conditions.

### *Storm Effects on FIB Variability:*

Magnitude of contamination was shown to be different across storm events, in particular in fecal *Bacteroides* spp. and *Enterococcus* sp. concentrations. These differences are to be expected, accompanying the various physical characteristics of storms. Rainfall intensity and duration affect turbidity, dissolved oxygen levels, and flow of water in outfalls, all of which have been shown to be significantly correlated with FIB concentrations (Mallin *et al.* 2001, Coulliette *et al.* 2008, Stumpf *et al.* 2009). In North Carolina tidal creeks, FIB concentrations have been shown to be significantly correlated with streamflow (Stumpf *et al.* 2009). Though magnitude of flow was found to vary significantly across storm events in this study, flow significantly correlated with neither fecal *Bacteroides* spp. nor *Enterococcus* sp. concentrations when data were pooled. Only *E. coli* concentrations in outfalls were found to be significantly correlated with flow, though the strength of the correlation varied from storm to storm. When data were separated by storms, we also observed a correlation between flow and *Enterococcus* sp. concentrations during 2 of the 5 storms. These differing correlations between indicator concentrations and flow demonstrate that the relationship between flow and FIB is not constant and that FIB concentrations are likely influenced by other factors.

Antecedent rainfall, for instance, was found to be significantly correlated with both *E. coli* and *Enterococcus* sp. concentrations in the stormwater outfalls. Though this was not consistently the case in rural North Carolina tidal creeks (Stumpf *et al.* 2009), this study shows that FIB become increasingly concentrated on land in developed areas when there has been no rain to wash the indicators downstream. The correlation between *Enterococcus* sp. concentrations and days since the last rain event was found to be stronger than the correlation between *E. coli* and antecedent rainfall. This relationship has not been found in similar studies (e.g., Stumpf *et al.* 2009). The correlation is likely due to the character of the

landscape: Nags Head is flat but for deep stormwater ditches. Indicators deposited on the flat land will not easily be transferred to the ditches without a rain event. The difference between the indicators is likely due to differential die-off of the bacteria; specifically, *Enterococcus* sp., which has been shown to survive longer than *E. coli* in most sediments (Howell *et al.* 1996). Still, the concentration of neither is likely to decline quickly: work with dog feces has shown that fecal coliform density does not decline up to 30 days after deposition (Weiskel *et al.* 1996). Fecal *Bacteroides* spp. concentrations were not significantly correlated with antecedent rainfall, and this is likely due to the limited persistence of the anaerobic bacteria after exposure to oxygen. This also supports the hypothesis that fecal *Bacteroides* spp. in stormwater comes from septage or septage-contaminated groundwater, not surface depositions. Otherwise, we would expect to see some relationship, though not as strong as those with *Enterococcus* sp. and *E. coli*, between fecal *Bacteroides* spp. concentrations in stormwater from surface depositions and length of time since the last rain event. Septic systems and groundwater have less oxygen exposure than land, allowing greater fecal *Bacteroides* spp. persistence than at the oxygenated surface.

*Site Effects on FIB Variability:*

Magnitude of contamination was also significantly different among stormwater outfalls even though the sites were geographically close, all sites within 10km of one another except site 8, which was approximately 13km south of the other sites. In accounting for these differences, drainage area and water table height were significantly correlated with indicator concentrations. The correlation between all three indicator concentrations and

drainage area was expected: with greater area, there is greater opportunity for fecal contamination, and hence concentration of FIB in outfalls.

Fecal *Bacteroides* spp. concentrations were negatively correlated with water table depth: areas with deeper water tables had smaller fecal *Bacteroides* spp. concentrations. This again suggests that much of the fecal *Bacteroides* spp. signal comes from septage or groundwater contaminated with septage that has surfaced after ground saturation. This hypothesis will require further testing, as we did not monitor water table height through each storm event and are rather using it as a relative term and assuming that the differences in depth between sites is constant.

Of course there are other site specific factors that may influence FIB concentration in stormwater. Land use can be particularly important: development, population, and impervious surface coverage are all strongly correlated with high FIB concentrations (Mallin *et al.* 2001). All sites in this study drained predominantly developed residential areas, and accordingly had the high FIB concentrations. Soil type can also be an important factor affecting stormwater FIB, with lower mortality rates observed in finer and clay-based sediments (Howell *et al.* 1996). Given the proximity of the study sites and their location on a barrier island, difference in soil type was likely not an important factor in this study.

### **Conclusions:**

Though fecal *Bacteroides* spp. loading patterns were found to be similar to those of *Enterococcus* sp. and *E. coli*, indicator loads were affected differently by site- and storm-specific factors, such as flow and water-table height. Many of these differences were likely due to fecal source heterogeneity. Though work from Chapter 4 demonstrated that fecal

*Bacteroides* spp. can be used to distinguish human from animal contamination when water is affected by few sources of contamination, stormwater represents a special and difficult case for distinguishing human from non-human fecal contamination, and highlights the importance of considering many site-specific factors in microbial source tracking.

Table 5.1: Site Descriptions.

<b>Site # (Street Name)</b>	<b>Drainage Area (acres)</b>	<b>Land Use</b>	<b>Water Table Height in meters</b>
1 (Baum)	192	Mostly residential, some commercial and wooded areas, 3 schools.	1.36779
2 (Martin)	366	Mostly residential, some commercial and wooded areas.	1.538478
4 (Gallery)	214	Mostly residential, some commercial and undeveloped areas.	.88392
5 (Curlew)	164	Mostly residential, some commercial.	1.431798
6 (Conch)	53	Mostly residential, some commercial and undeveloped areas.	1.207008
7 (Soundside)	31	Residential and commercial.	1.316736
8 (South Nags Head)	99	Residential.	0.577596

Table 5.2: Average log indicator concentrations (cells/100mL or MPN/100mL) and flow rate (m<sup>3</sup>/s) by storm.

<b>Storm</b>	<b><i>Bacteroides</i> (SE)</b>	<b><i>E. coli</i> (SE)</b>	<b><i>Enterococcus</i> (SE)</b>	<b>Flow (SE)</b>
June 2007	2.5099 (0.3766)	2.9319 (0.1507)	3.3951 (0.1464)	117.7074 (57.3358)
Dec 2007	4.7217 (0.0976)	2.5543 (0.1588)	2.5643 (0.1594)	103.1052 (24.54)
April 2008	3.1378 (0.463)	2.6343 (0.1225)	2.62 (0.0804)	149.1675 (26.176)
Sept 2008	3.9129 (0.4359)	3.3156 (0.1509)	3.7197 (0.1032)	143.4293 (39.6842)
Nov 2008	4.6642 (0.0783)	3.1266 (0.1012)	3.2085 (0.1134)	354.0306 (66.393)

Table 5.3: Average log indicator concentrations (cells/100mL or MPN/100mL) and flow rate (m<sup>3</sup>/s) by site.

<b>Site</b>	<b><i>Bacteroides</i> (SE)</b>	<b><i>E. coli</i> (SE)</b>	<b><i>Enterococcus</i> (SE)</b>	<b>Flow (SE)</b>
1	4.9148 (0.0638)	3.0615 (0.1480)	2.9558 (0.1422)	85.231 (17.8899)
2	4.1579 (0.2678)	3.3233 (0.0812)	3.0908 (0.1811)	366.0988 (43.6452)
4	4.9892 (0.2346)	2.6741 (0.1924)	3.0977 (0.1629)	225.579 (99.3309)
5	3.2939 (0.6536)	3.2940 (0.1284)	2.9881 (0.1665)	135.4231 (68.7076)
6	3.9235 (0.3555)	2.8553 (0.1453)	2.9959 (0.1494)	207.8789 (45.7562)
7	2.9296 (0.4795)	2.3080 (0.2737)	2.4780 (0.2225)	54.3964 (25.6614)
8	4.1903 (0.5434)	1.7646 (0.4537)	3.6010 (0.2851)	54.8885 (33.5387)
9	3.4585 (0.5735)	2.6333 (0.1682)	2.5699 (0.2579)	85.7582 (25.2198)

Table 5.4: Significant correlations when data are separated by site.

<b>Site</b>	<b>Correlating Variables</b>	<b>Spearman Coefficient</b>	<b>p</b>	<b>n</b>
2	<i>E. coli</i> and <i>Enterococcus</i> sp.	0.429	0.029	26
	<i>E. coli</i> and flow	0.462	0.018	26
4	Fecal <i>Bacteroides</i> spp. and <i>Enterococcus</i> sp.	0.632	0.021	13
	Fecal <i>Bacteroides</i> spp. and flow	-0.627	0.022	13
5	Fecal <i>Bacteroides</i> spp. and flow	-0.786	0.036	7
6	<i>E. coli</i> and <i>Enterococcus</i> sp.	0.814	<0.001	19
	<i>Enterococcus</i> sp. and flow	-0.542	0.017	19
7	<i>E. coli</i> and <i>Enterococcus</i> sp.	0.832	<0.001	20
9	Fecal <i>Bacteroides</i> spp. and <i>E. coli</i>	-0.790	0.007	10
	<i>E. coli</i> and <i>Enterococcus</i> sp.	0.651	0.012	14

Figure 5.1: Map of sampling locations.

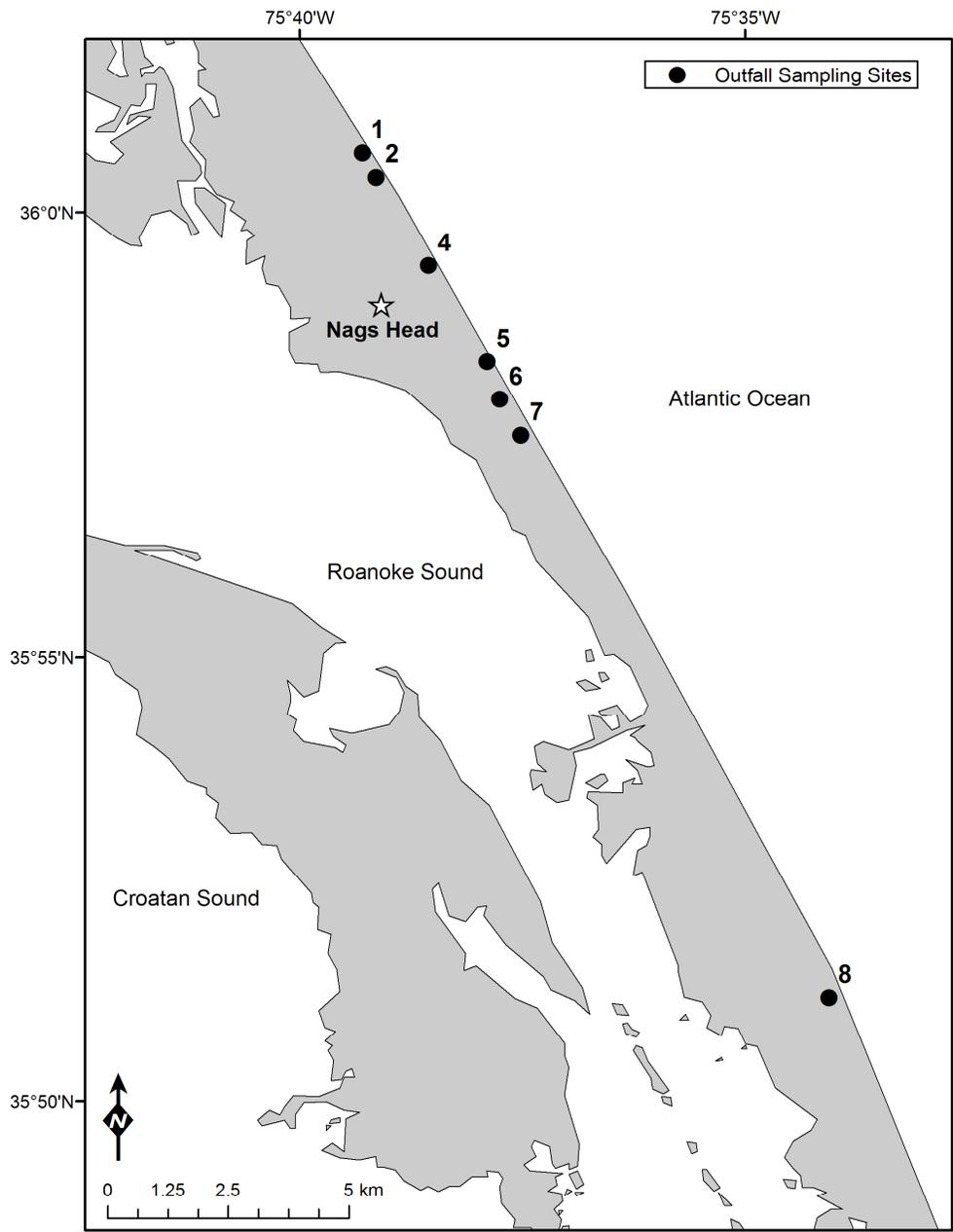
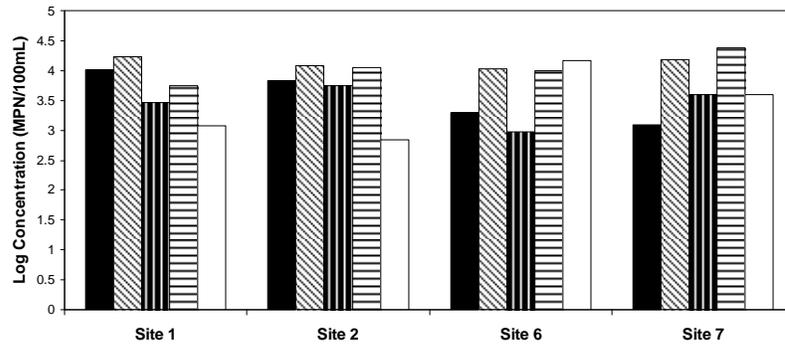
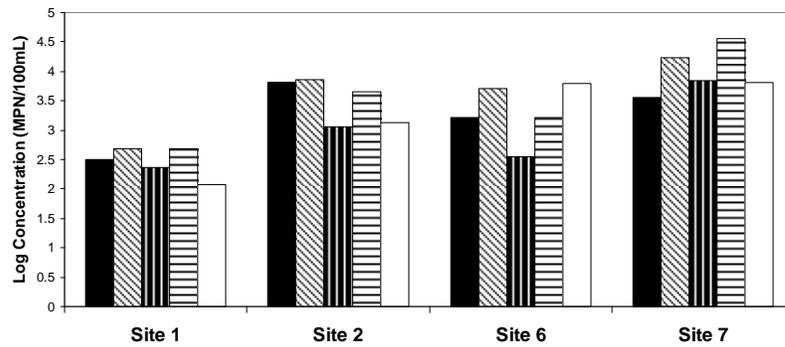


Figure 5.2: Log indicator concentrations and flow over the course of the September 2008 storm.

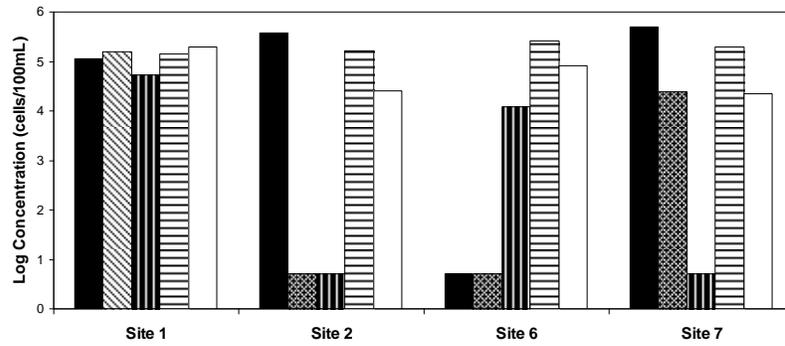
*Enterococcus* sp. Concentration



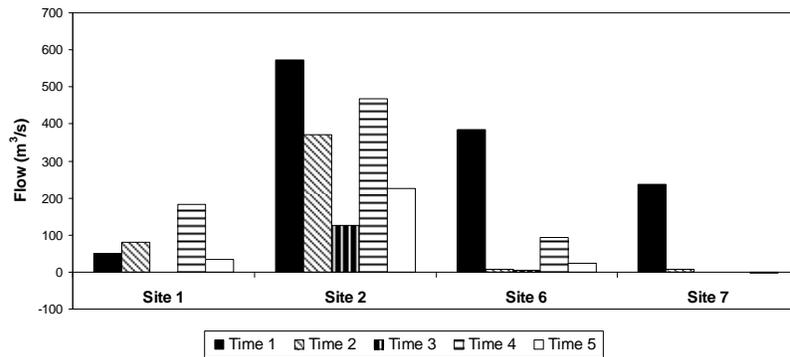
*E. coli* Concentration



Fecal *Bacteroides* spp. Concentration



Flow



■ Time 1   ▨ Time 2   ■ Time 3   ▤ Time 4   □ Time 5

Figure 5.3: Correlation between indicator concentrations and flow. Data pooled from all sites and years.

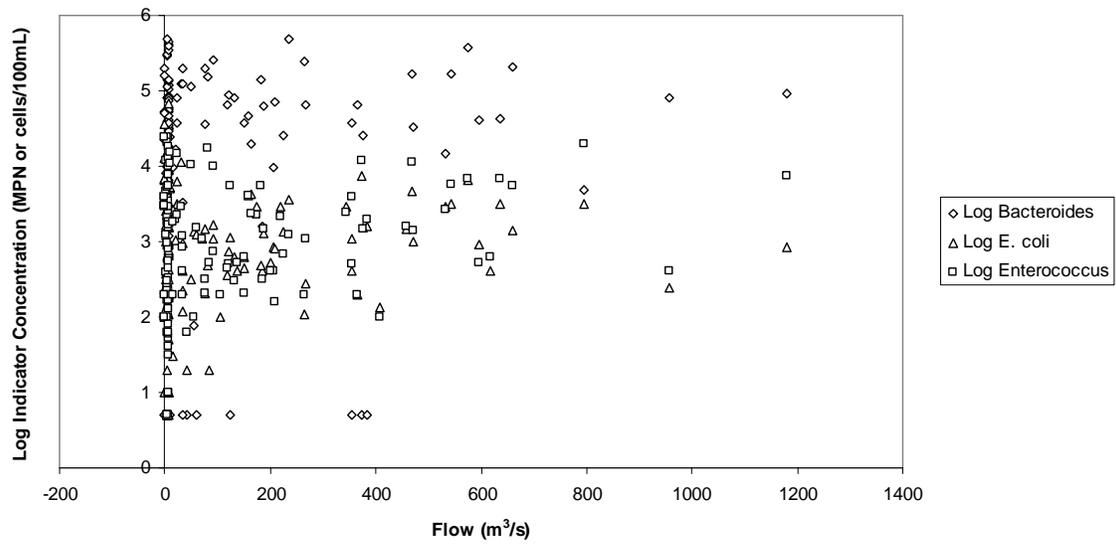


Figure 5.4: Log average *Enterococcus* sp. and fecal *Bacteroides* spp. during each storm.

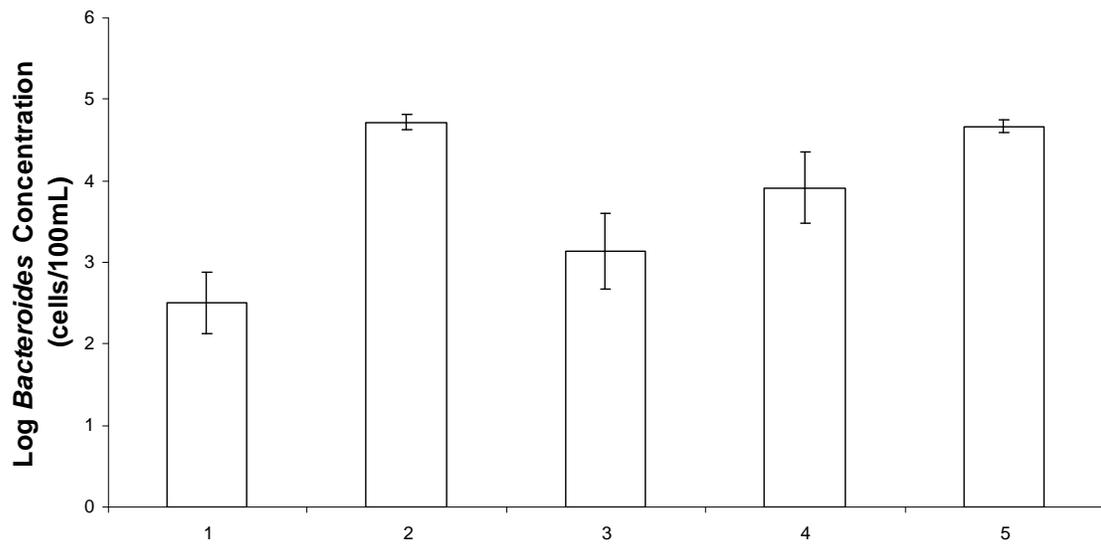
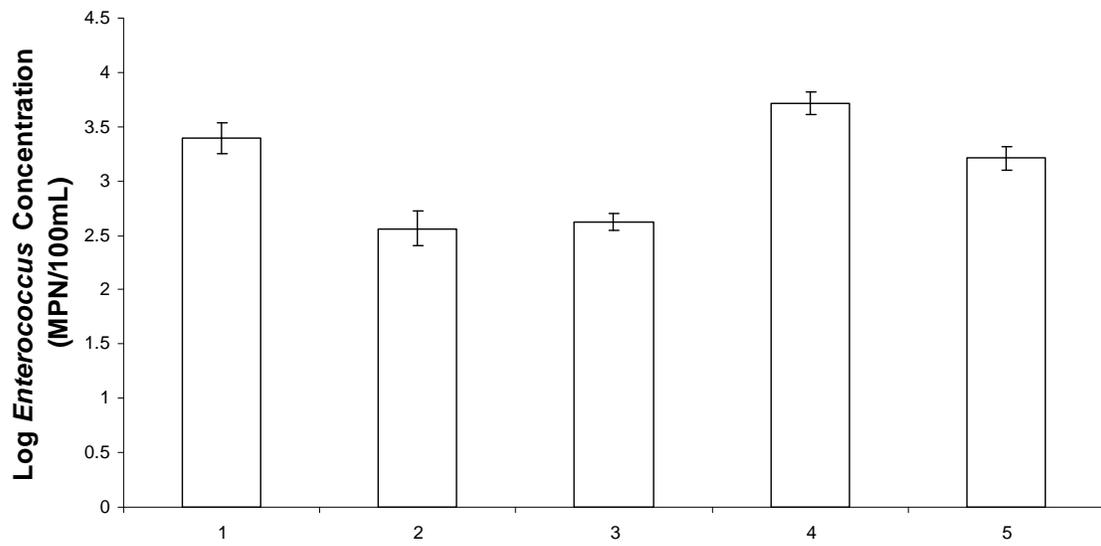
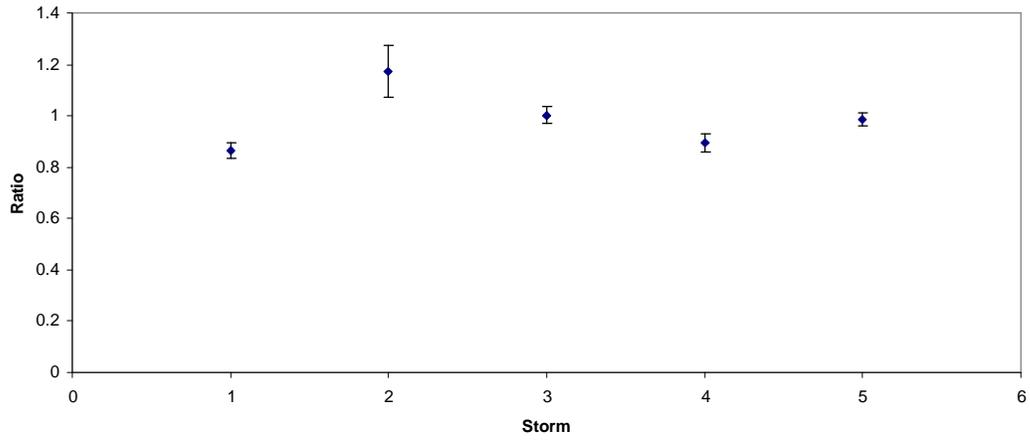
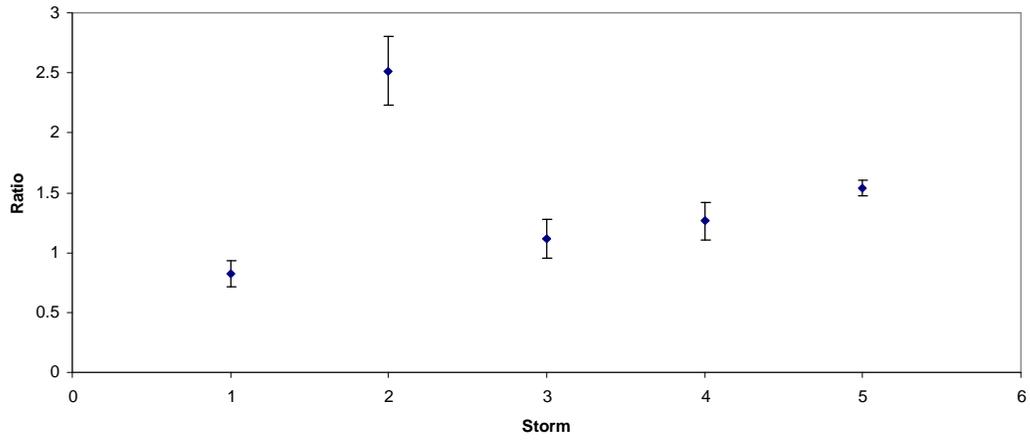


Figure 5.5: Ratios between log average indicators during each storm.

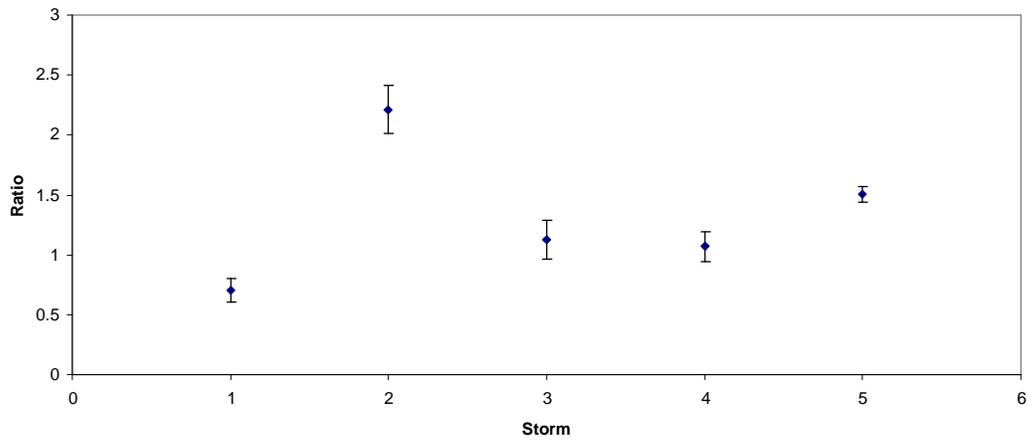
**Log *E. coli* : Log *Enterococcus* sp.**



**Log *Bacteroides* : Log *E. coli***



**Log *Bacteroides* : Log *Enterococcus* sp.**



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## Chapter 6:

### Concluding Remarks

The potential of QPCR to improve water quality monitoring cannot be overstated. With rapid results and a suite of new source-specific markers, QPCR allows real-time assessment of water quality and identification of the health threat posed by specific sources of contamination. The implementation of QPCR-based monitoring should not only significantly reduce the delay before beach closures, but managers should be able to focus on mitigating the most important contributors of fecal contamination to specific water bodies.

The work in this dissertation sought to advance the understanding of the relationship between QPCR-based assays with current culture-based water quality methods. In general, results demonstrated the promise of QPCR-based monitoring. QPCR results for currently approved FIB were significantly correlated with defined-substrate test and membrane filtration results. Results from the seeding experiment should allay some questions about the question of extended DNA persistence in recreational water, one of the primary concerns about adoption of QPCR. The study demonstrated that the persistence of DNA markers (the measured endpoint of QPCR) is similar to persistence of metabolically active cells (the measured endpoint of culture-based methods).

Results from this dissertation also demonstrated the efficacy of a new alternative indicator, easily detectable by QPCR but not culture methods. In cases of point source pollution, fecal *Bacteroides* spp. serves as a good indicator of human fecal contamination,

especially when used in conjunction with traditional FIB. Additionally, the QPCR marker for fecal *Bacteroides* spp. was found to degrade faster than that of *Enterococcus* sp. and *E. coli*, suggesting that it may help distinguish between new and old fecal contamination.

Nevertheless, there is a long way to go before widespread adoption of QPCR-based water quality monitoring. Work described in this dissertation highlighted two weaknesses of QPCR as a monitoring tool: inhibition and differences in DNA marker copy numbers across FIB species. QPCR inhibition is particularly problematic because it does not affect all assays equally. Improved extraction protocols or specimen processing control will need to be developed in order to standardize QPCR results nationwide. Also, differences in DNA marker copy numbers across species prevent an easy translation of current FIB water quality standards to new QPCR standards. Epidemiology studies will be required to address this challenge.

On an even more basic level, managers and technicians are simply not prepared to replace cheap and simple culture methods with QPCR. QPCR machines and supplies are expensive and beyond the reach of many monitoring agencies. Thankfully, reaction prices are falling, but government regulatory agencies will likely need to assist state and local managers with initial cost-sharing. Also, QPCR requires more technical expertise than currently-used culture methods. Managers and technicians will need to be trained to use molecular methods, which will not be possible until there is large-scale protocol standardization. Policy-makers, researchers, and monitoring agencies must work more collaboratively in order for QPCR to be implemented as a rapid method for recreational water quality testing.