TOOLS FOR PREDICTING MICROBIAL WATER QUALITY IN ESTUARIES USED FOR RECREATION AND SHELLFISH HARVESTING

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Abstract

RAUL ALEXANDER GONZALEZ: Tools for predicting microbial water quality in estuaries used for recreation and shellfish
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To reduce public health risks and associated economic costs, legislation has been passed to ensure that surface waters meet standards necessary for human contact. These guidelines recommend that states routinely monitor water quality and notify the public when waters are unsafe for recreational contact or shellfish harvesting. Traditional, culture-based methods require 18-24 hours for incubation. This long processing time causes delays in the time between sample collection and public notification, which is typically on a time scale longer than that of fecal contamination events themselves. To reduce this time lag, recent national and international recommendations have placed an emphasis on the use of rapid molecular and predictive methods as tools to improve public protection. In this dissertation, I developed and applied newly approved rapid methods to predict fecal indicator bacteria (FIB) in an eastern North Carolina (NC) estuary. *E. coli* and enterococci concentrations can be predicted using multiple linear regression (MLR) models and a combination of antecedent rainfall, climate, and environmental variables. *E. coli* and enterococci models accurately predicted a high percentage (>87%) of management decisions based on regulatory thresholds. The combined assessment of quantitative PCR (qPCR) and MLR models showed both methods can be used in tandem to provide rapid estimates of water quality in estuaries. Model equivalency was established for enterococci and *E. coli* MLR models using culture-

and qPCR-based data. Using time-frequency analysis, I determined that there is currently no optimal length of data needed for MLR model creation in eastern NC. Rather, managers can initiate their models with several weeks of data and then continually update models as new data become available. Lastly, I sought to understand the microbial dynamics of water quality across a range of hydrodynamic and meteorological conditions. Work here detailed a descriptive characterization of creeks to aid in variable selection during MLR development. Throughout the work, qPCR inhibition was the major complication. Therefore, I developed an approach to predict inhibition prior to sample processing. By using the tools outlined in this dissertation, managers in the region should be able to efficiently apply rapid methods and prediction tools in mid-Atlantic estuaries.

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List of Abbreviations

ΔC_T	Comparative C_T Method
ACF	Autocorrelation Functions
ANNs	Artificial Neural Networks
ATCC	American Type Culture Collection
BacHum	Human-associated *Bacteroides* spp.
BEACH	Beaches Environmental Assessment and Coastal Health
BMP	Best Management Practice
CCE	Calibrator Cell Equivalent
CE	Cell Equivalent
CFU	Colony Forming Unit
C_T	Cycle Threshold
CWA	Clean Water Act
DEM	Digital Elevation Model
DO	Dissolved Oxygen
E. coli	*Escherichia coli*
EC	*E. coli*
ENT	enterococci
FC	Fecal coliforms
FIB	Fecal Indicator Bacteria
MLR	Multiple Linear Regression
MPN	Most Probable Number
MST	Microbial Source Tracking
NC	North Carolina
NCDENR	North Carolina Department of Environment and Natural Resources

NCDMF	North Carolina Department of Marine Fisheries
NLCD	National Land Cover Database
NOAA	National Oceanic and Atmospheric Administration
NPRE	Newport River Estuary
NRE	Neuse River Estuary
NSSP	National Shellfish Sanitation Program
OLS	Ordinary Least Squares
PBS	Phosphate Buffered Saline
qPCR	Quantitative Polymerase Chain Reaction
RMSE	Root Mean Squared Error
RWQC	Recreational Water Quality Criteria
SD	Standard Deviation
SPC	Specimen Processing Control
SS	Sum of Squares
SST	Sea Surface Temperature
STV	Statistical Threshold Value
TCBS	Thiosulfate-Citrate-Bile salts-Sucrose
TMDL	Total Maximum Daily Load
USEPA	United States Environmental Protection Agency
VIB	Total *Vibrio* spp.
VIF	Variance Inflation Factor

Chapter 1

INTRODUCTION

Background

Pollution of environmental waters that are used for recreation, food production, and drinking water is a major concern worldwide. Within the group of potential contaminants, fecal pollution can account for 175 million infections annually in people who drink, bathe, or consume shellfish in polluted waters (Shuval, 2003). In addition to causing public health burdens, these fecal related infections have an added economic price in the form of health care costs and lost wages for infected individuals. One study in two California (CA) counties found that swimming related illness at 28 beaches corresponded to an annual economic loss of $21 to $51 million in health costs alone (Given et al., 2006), while another CA study found that the health burden at only 2 beaches was $3.3 million per year (Dwight et al., 2005).

To reduce public health risks and economic costs, the Clean Water Act (CWA) and the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) were passed to ensure that surface waters would meet standards necessary for human contact. Similarly, the National Shellfish Sanitation Program (NSSP), which is recognized by the Food and Drug Administration (FDA) and the Interstate Shellfish Sanitation Conference (ISSP), released the “Guide for the Control of Molluscan Shellfish,” to regulate waters

used for growing shellfish for human consumption to prevent illness in those that consume the shellfish produced. These guidelines recommend that states routinely monitor water quality and notify the public when waters are unsafe for contact or harvesting of shellfish.

The direct approach to monitoring water quality is enumeration of all microbial pathogens. However, since waterborne microbial pathogens can be viruses, protozoa, or bacteria, complete enumeration would require a large number of assays, time, and technical ability. Additionally, although some pathogens can still be highly infectious at low concentrations, these small densities can be sporadically distributed both spatially and seasonally and are difficult to culture. Therefore, it is not feasible to enumerate all or even an important subset of pathogens in a watershed or receiving water body. The current approach used globally for monitoring surface water quality is accomplished using the fecal indicator bacteria (FIB) system. FIB have been used successfully to manage waters for over a century in the United States. In recent decades, this approach has been supported by the results stemming from epidemiological studies that have demonstrated significant linkages between densities of FIB in water to adverse human health outcomes, such as GI illness (Cabelli et al., 1982; Pruss, 1998; Wade et al., 2003; 2008; Wiedenmann et al., 2006). The FIB system assumes that FIB have similar fate, transport, and higher densities than the pathogens themselves. When high densities of FIB are found in a watershed or receiving water body, managers conclude that fecal contamination and therefore fecally associated pathogens are likely present. In the US there are two main groups of FIB that are currently used in monitoring programs. For recreational waters, *E.coli* and enterococci are used for freshwaters and enterococci for

marine waters. For shellfish harvesting waters, fecal coliforms, of which *E. coli* is the dominant subset, are used as proxies for pathogens. The use of these FIB has evolved from monitoring and successful scientific investigation linking the use of these FIB to human health outcomes. Current US standards for recreational waters and shellfish harvesting waters are in Table 1.

There are two types of FIB enumeration methods that are currently used and recommended in the new criteria document released by the EPA—traditional, culture-based methods and new, rapid molecular methods using qPCR. Traditional culture-based methods, like membrane filtration and defined substrate technology tests such as IDEXX kits, require 18-24 hours for incubation. This long processing time causes a delay in the time between sample collection and public notification, which is typically on a time scale longer than that of fecal contamination events themselves (Boehm et al., 2002). During this time lag between sample collection and public notification the public can be exposed to pathogens in the water, or conversely elevated concentrations from yesterday's sample may no longer be present in the water resulting in an unnecessary closure. Rapid methods such as qPCR can reduce processing times to 3 hours (Leecaster and Weisberg, 2001; Noble et al. 2010, Griffith and Weisberg, 2011). Strong relationships have been demonstrated between qPCR-based FIB concentrations and human health outcomes and some studies have shown that this linkage is stronger than the relationship between culture-based FIB and health (Wade et al., 2008; Colford et al., 2012). So these molecular rapid methods are not only faster, but also more effective than traditional methods.

The new Recreational Water Quality Criteria (RWQC) document places emphasis on the increased use of rapid molecular methods and implementation of predictive

models as tools to improve public health protection. Both of these recommendations were aimed at reducing processing times and/or public notification of water closures. Well-developed predictive models can eliminate the delay between sample collection and results by providing real-time estimates of FIB concentrations at beaches. Frequently, multiple linear regression (MLR) models are used to predict recreational water quality (e.g. Olyphant et al., 2003; Olyphant and Whitman, 2004; Eleria and Vogel, 2005; Nevers and Whitman, 2005, 2011; Francy and Darner, 2007). MLR is an empirical statistical modeling approach that predicts FIB concentrations by relating water quality to antecedent rainfall, climate, and environmental parameters. When routine monitoring of beaches is not possible, MLR modeling is a useful tool for managers.

Water bodies that frequently do not meet water quality FIB standards are classified as impaired and must have a total maximum daily load (TMDL), which stipulates the maximum concentration of pathogens that the water body can naturally eliminate. The TMDL process (1) determines the existing and potential pollutant loads in the watershed (2) links the loads to contaminations sources and (3) outlines the best methods for ameliorating any problems. In order to determine contamination sources for TMDL, microbial source tracking (MST) can be used to determine if FIB are primarily of fecal origin and if the fecal pollution is human, which is more dangerous, or non-human, which is associated with less public disease risk. To do this, MST studies often document the presence of obligate anaerobes in the genus *Bacteroides* using qPCR, as these bacteria indicate a recent fecal contamination event in aerobic waters were they cannot survive long.

Although research on water quality monitoring tools is extensive for marine beaches and freshwater lakes, applications of these methods in estuarine waters, specifically in the southeast US are limited. In estuaries, fresh water from the land mixes with saline water from the ocean, creating a dynamic environment that can provide resources for commercially important fin and shellfish (Wetz and Yoskowitz, 2013). Because of the many recreational, commercial, and ecological services provided by estuaries, it is important that managers and researchers understand the application of rapid qPCR techniques and MLR modeling in these areas.

North Carolina (NC) is one of the top beach visitation destinations in the United States, ranking 6th in beach tourism (NC Dept. of Commerce, 2013). In eastern NC there are 240 recreational monitoring sites and over 1025 shellfish harvesting water sites monitored on a regular basis. In addition, some of the locations are known as "dual beneficial use" serving both designated uses concomitantly (NCDMF, 2013). The NC Department of Environment and Natural Resources (NCDENR) conducts the monitoring programs for both recreational and shellfish harvesting waters in the state and has expressed interest in the use of both rapid methods and predictive models to issue public health advisories in near real-time.

The overall goal of my dissertation was to develop and apply newly approved rapid methods in an estuarine environment that is used for both recreation and shellfish harvesting. My study sites in the Newport River Estuary (NPRE) and Neuse River Estuary (NRE) have been the sites of several previous studies on fecal contamination, which have demonstrated the importance of stormwater in contamination of the systems (Figure 1). My objectives were to generate data from a well-designed sampling approach

incorporating analyses of water samples for FIB using both culture-and qPCR-based methodologies applied across a range of meteorological conditions. This extensive data set then was utilized in chapter 2 to develop and validate traditional and alternative FIB MLR models using a combination of current and antecedent rainfall, climate, and environmental variables. This work has been published in *Water Research* (Gonzalez et al. 2012). The chapter 3 objectives were to optimize the use of rapid methods in estuarine samples and then subsequently use a similar MLR model development approach for qPCR-based data over a range of hydrological and meteorological conditions. This then permitted an ultimate comparison of MLR models developed using traditional culture-based and rapid methods, culminating in a submission to *Water Research* highlighting their similarities and differences. The objective of chapter 4 was to understand the microbial dynamics of water quality in the NPRE across a range of hydrodynamic and meteorological conditions. A detailed descriptive characterization of the creeks aided in variable selection during MLR development. The central objective of chapter 5 was to examine multiple time scales of data from the NRE using time-frequency analyses in order to determine the optimal length of data needed for robust MLR model creation. This time-frequency exercise was undertaken to further improve application of MLR models in this region.

Figures

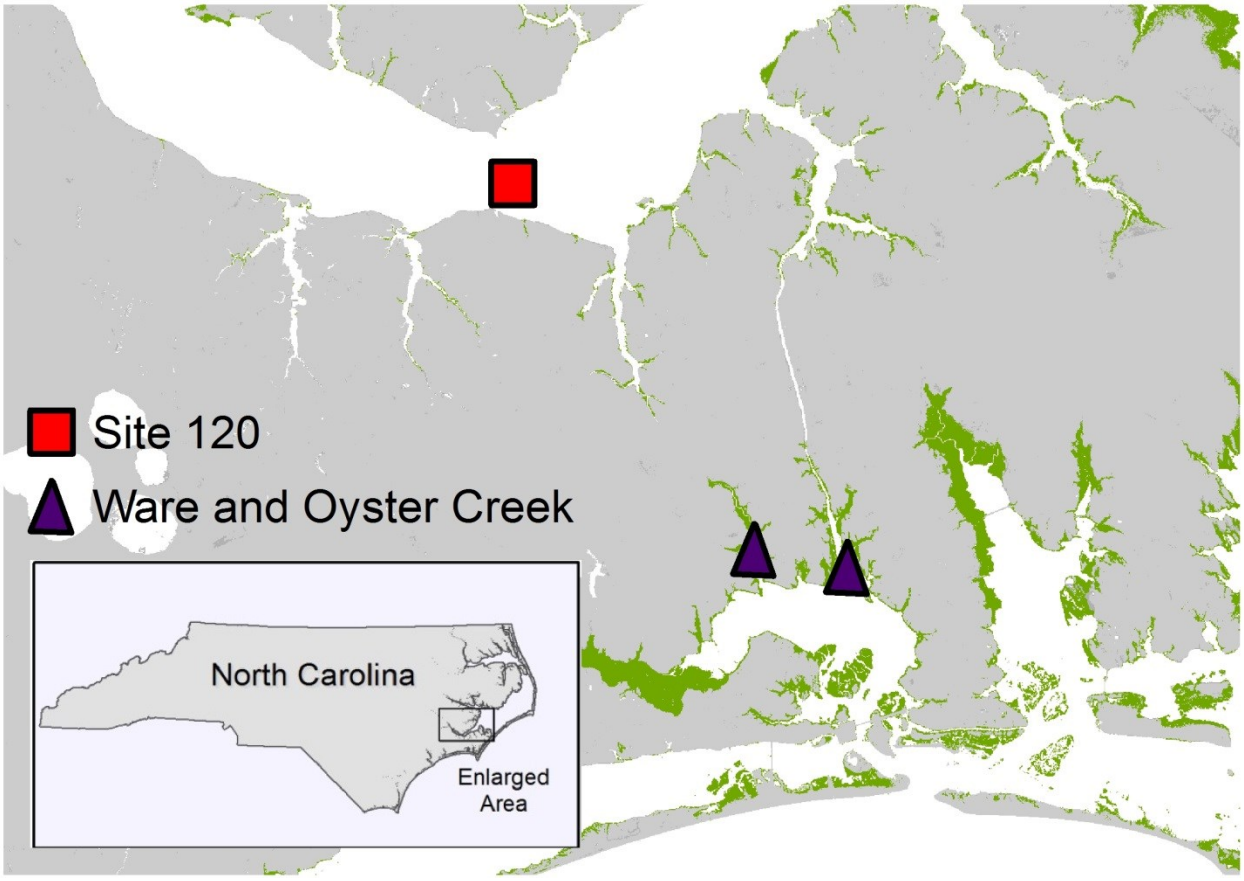

Figure 1.1. The Neuse River Estuary and Newport River Estuary sampling sites. Only data from site 120 of the Neuse River Estuary was used in this dissertation (chapter 5), while transects of Ware and Oyster Creeks were used for chapters 2, 3, and 4.

Tables

Indicator	GM CFU/100 ml	STV CFU/100 ml	GM CCE/100 ml	STV CCE/100 ml
RWQC				
enterococci (marine or freshwater)	30	110	-	-
E. coli (freshwater)	100	320	-	-
qPCR (enterococci)	-	-	300	1280
NSSP				
Fecal Coliform (harvesting waters)	14	31	-	-

Table 1.1. Current US standards for recreational waters and shellfish harvesting waters. The 2012 recreational water quality criteria (RWQC) and 2011 National Shellfish Sanitation Program (NSSP) documents recommend both a geometric mean (GM) and STV (statistical threshold value) for monitoring water quality. The waterbody GM should not be greater than the GM shown here in any 30-day period. The NSSP does not use the term STV but in both documents the STV presented here acts as a single sample threshold in the case of one sample, and 10% of samples should not exceed this value. Values are either in colony forming units (CFU) or calibrator cell equivalents (CCE).

References

Boehm, A., Grant, S., Kim, J., Mowbray, S., McGee, C., Clark, C., Foley, D., Wellman, D. 2002. Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environmental Science & Technology 36 (18), 3885-3892.

Cabelli, V. J., Dufour, A. P., McCabe, L., Levin, M. 1982. Swimming-associated gastroenteritis and water quality. American Journal of Epidemiology 115 (4), 606-616.

Colford Jr, J. M., Schiff, K. C., Griffith, J. F., Yau, V., Arnold, B. F., Wright, C. C., Gruber, J. S., Wade, T. J., Burns, S., Hayes, J. 2012. Using rapid indicators for *Enterococcus* to assess the risk of illness after exposure to urban runoff contaminated marine water. Water Research 46 (7), 2176-2186.

Dwight, R. H., Fernandez, L. M., Baker, D. B., Semenza, J. C., Olson, B. H. 2005. Estimating the economic burden from illnesses associated with recreational coastal water pollution—a case study in Orange County, California. Journal of Environmental Management 76 (2), 95-103.

Eleria, A. and Vogel, R. M. 2005. Predicting fecal coliform bacteria levels in the Charles River, Massachusetts, USA. Journal of the American Water Resources Association 41 (5), 1195-1209.

Francy, D. S. and Darner, R. A., 2007. Nowcasting beach advisories at Ohio Lake Erie beaches. Environmental Science & Technology 42 (13), 4818-4824.

Given, S., Pendleton, L. H., Boehm, A. B. 2006. Regional public health cost estimates of contaminated coastal waters: a case study of gastroenteritis at Southern California beaches. Environmental Science & Technology 40 (16), 4851-4858.

Gonzalez, R. A., Conn, K. E., Crosswell, J. R., Noble, R. T. 2012. Application of empirical predictive modeling using conventional and alternative fecal indicator bacteria in eastern North Carolina waters. Water Research 46 (18), 5871-5882.

Griffith, J. F. and Weisberg, S. B. 2011. Challenges in implementing new technology for beach water quality monitoring: Lessons from a California demonstration project. Marine Technology Society Journal 45 (2), 65-73.

Leecaster, M. K. and Weisberg, S. B. 2001. Effect of Sampling Frequency on Shoreline Microbiology Assessments. Marine Pollution Bulletin 42 (11), 1150-1154.

NC Department of Commerce, 2013. Fast facts, 2012 impact of visitor spending. http://www.nccommerce.com/LinkClick.aspx?fileticket=qwtGIIJxT28%3d&tabid=636&mid=4669.

NCDMF. 2013. North Carolina shellfish sanitation and recreational water quality section of the division of marine fisheries. http://portal.ncdenr.org/web/mf/shellfish-sanitation-and-recreational-water-quality.

Nevers, M. B. and Whitman, R. L. 2011. Efficacy of monitoring and empirical predictive modeling at improving public health protection at Chicago beaches. Water research 45 (4), 1659-1668.

Nevers, M. B. and Whitman, R. L. 2005. Nowcast modeling of *Escherichia coli* concentrations at multiple urban beaches of southern Lake Michigan. Water Research 39 (20), 5250-5260.

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., Weisberg, S. B. 2010. Comparison of rapid quantitative PCR-based and conventional culture-based methods for enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters. Applied and Environmental Microbiology 76 (22), 7437-7443.

Olyphant, G. A. and Whitman, R. L. 2004. Elements of a predictive model for determining beach closures on a real time basis: The case of 63rd Street Beach Chicago. Environmental Monitoring and Assessment 98 (1), 175-190.

Olyphant, G. A., Thomas, J., Whitman, R. L., Harper, D. 2003. Characterization and statistical modeling of bacterial (*Escherichia coli*) outflows from watersheds that discharge into southern Lake Michigan. Environmental Monitoring and Assessment 81 (1), 289-300.

Prüss, A. 1998. Review of epidemiological studies on health effects from exposure to recreational water. International Journal of Epidemiology 27 (1), 1-9.

Shuval, H. 2003. Estimating the global burden of thalassogenic diseases: human infectious diseases caused by wastewater pollution of the marine environment. Journal of Water and Health 1(2), 53-64.

Wade, T. J., Calderon, R. L., Brenner, K. P., Sams, E., Beach, M., Haugland, R., Wymer, L., Dufour, A. P. 2008. High sensitivity of children to swimming-associated gastrointestinal illness: results using a rapid assay of recreational water quality. Epidemiology 19 (3), 375-383.

Wade, T. J., Pai, N., Eisenberg, J. N., Colford Jr, J. M. 2003. Do US Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives 111 (8), 1102-1109.

Wetz, M. S. and Yoskowitz, D. W. 2013. An 'extreme'future for estuaries? Effects of extreme climatic events on estuarine water quality and ecology. Marine pollution bulletin 69 (1-2), 7-18.

Wiedenmann, A., Krüger, P., Dietz, K., López-Pila, J. M., Szewzyk, R., Botzenhart, K. 2006. A randomized controlled trial assessing infectious disease risks from bathing in fresh recreational waters in relation to the concentration of Escherichia coli, intestinal enterococci, *Clostridium perfringens*, and somatic coliphages. Environmental Health Perspectives 114 (2), 228-336.

Chapter 2

APPLICATION OF EMPIRICAL PREDICTIVE MODELING USING CONVENTIONAL AND ALTERNATIVE FECAL INDICATOR BACTERIA IN EASTERN NORTH CAROLINA WATERS[1]

Overview

Coastal and estuarine waters are the site of intense anthropogenic influence with concomitant use for recreation and seafood harvesting. Therefore, coastal and estuarine water quality has a direct impact on human health. In eastern North Carolina (NC) there are over 240 recreational and 1025 shellfish harvesting water quality monitoring sites that are regularly assessed. Because of the large number of sites, sampling frequency often is only on a weekly basis. This frequency, along with an 18-24 hour incubation time for fecal indicator bacteria (FIB) enumeration via culture-based methods, reduces the efficiency of the public notification process. In states like NC where beach monitoring resources are limited, but historical data are plentiful, predictive models may offer an improvement for monitoring and notification by providing real-time FIB estimates. In this study, water samples were collected during 12 dry (n = 88) and 13 wet (n = 66) weather events at up to 10 sites. Predictive models for *E. coli*, enterococci, and members

[1] This work has been published in:

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of the *Bacteroidales* group were created and subsequently validated. Our results showed that models for *E. coli* and enterococci (adjusted R^2 were 0.61 and 0.64, respectively) incorporated a range of antecedent rainfall, climate, and environmental variables. The most important variables for EC and ENT models were 5-day antecedent rainfall, dissolved oxygen, and salinity. These models successfully predicted FIB levels over a wide range of conditions with a 3% (EC model) and 9% (ENT model) overall error rate for recreational threshold values and a 0% (EC model) overall error rate for shellfish threshold values. Though modeling of members of the *Bacteroidales* group had less predictive ability (adjusted R^2 were 0.56 and 0.53 for fecal *Bacteroides* spp. and human *Bacteroides* spp., respectively), the modeling approach and testing provided information on *Bacteroidales* ecology. This is the first example of a set of successful predictive models appropriate for assessment of both recreational and shellfish harvesting water quality in estuarine waters.

2.1. Introduction

Fecal indicator bacteria (FIB) levels are monitored in recreational and shellfish harvesting waters as proxies for pathogens that can adversely impact human health upon ingestion of, or consumption of food from, contaminated waters. Because of the large number of sites that often are monitored with limited resources, sampling frequency typically is only on a weekly basis or less. This frequency, along with an 18-24 hour incubation time for FIB enumeration via culture-based methods, currently limits the ability of water quality managers to assess recreational water quality in a timely manner (Leecaster and Weisberg, 2003; Griffith and Weisberg, 2011). There is a need for

additional water quality management tools that do not require extensive expenditures in the form of personnel time for sample collection, processing, data analysis, and reporting.

Simple empirical predictor models can be used to maximize monitoring efficiency by providing real-time estimates of FIB concentrations. Predictor models can be used to supplement regular monitoring by identifying areas that need health warnings or more frequent monitoring, and are useful between sampling periods (USEPA, 2010). Furthermore, in the new draft ambient water quality criteria (USEPA, 2012), USEPA strongly recommends the use of predictive models as appropriate tools to boost the effectiveness of water quality monitoring programs. In states where beach monitoring resources are limited, but historical data are plentiful, these predictive models may offer a vital improvement for monitoring and notification.

Simple empirical predictor models have been used successfully to monitor recreational beaches in urban, populated areas (Olyphant and Whitman, 2004; Eleria and Vogel, 2005; Nevers and Whitman, 2005; Nevers and Whitman, 2010). Using different combinations of predictor variables, these models have been shown successful as a supplementary tool, but are not in widespread use. Published statistical models have typically focused on freshwater systems with large population centers focusing on areas that are subject to large scale point (e.g. Olyphant et al., 2003; Nevers and Whitman, 2005; Nevers and Whitman, 2008) and non-point (Francy et al., 2006; Francy and Darner, 2007; Francy et al., 2009) sources of contamination. Little work previously has been conducted in coastal and estuarine systems.

In eastern NC, coastal and estuarine waters are intensively used for both recreation and shellfish harvesting, with many locations simultaneously listed for both regulated uses. The NC Shellfish Sanitation and Recreational Water Quality Sections collectively monitor over 240 recreational and 1025 shellfish harvesting water locations (NCDMF, 2012). Previous work by Coulliette and Noble (2008) in the Newport River Estuary (NPRE) resulted in a presumptive rainfall closure model that recommended closing shellfish harvesting waters in the estuary after 2.54 cm of rain as opposed to the more liberal, currently used management action threshold of 3.81 cm. Additionally, Coulliette et al. (2009) created spatial/temporal maps using space-time modeling approaches that predicted elevated levels of *E. coli* (EC) based on rainfall and distance from land. While these models have provided useful guidance in specific conditions and in specific areas to water quality managers, they are not applicable to a wide range of estuarine and coastal locations, nor were they adequately developed over a wide range of season and conditions. The models created here are intended to provide managers with real-time estimates of FIB concentrations for dual beneficial use locations.

Due to the lack of host specificity of conventional indicators, measuring and predicting conventional FIB (EC, fecal coliforms (FC), and enterococci (ENT)) alone do not provide information regarding the source of microbial contamination. In the past decade, members of the bacterial group, *Bacteroidales*, have emerged as alternative fecal indicators that are both important for prediction of human health outcomes (Wade et al., 2010) and useful for tracking specific types of fecal contamination to receiving waters (e.g. Bernhard and Field, 2000; Layton et al., 2006; Kildare et al., 2007; Converse et al., 2009). Estimates of levels of fecal *Bacteroides* spp. (a subset of six species of

Bacteroidales, including *Bacteroides thetaiotaomicron*) can be indicative of a more recent fecal contamination event given that members of this family are obligate anaerobes (Kreader, 1995; Converse et al., 2009). Because they are obligate anaerobes, routine, culture-based quantification of members of the *Bacteroidales* group can be time consuming. However, advances in rapid molecular methods such as quantitative polymerase chain reaction (qPCR) have resulted in the development of many assays that estimate levels of total *Bacteroidales* (e.g. Total *Bacteroidales*: Dick et al., 2004; "Allbac": Layton et al., 2006; "BacUni": Kildare et al., 2007; "GenBac": Shanks et al., 2010) and human-associated *Bacteroidales* (Seurinck et al., 2005; Kildare et al., 2007; Converse et al., 2009). These qPCR assays for alternative FIB can be used in a tiered approach along with culture-based conventional FIB such as EC and ENT to provide further information on the sources of fecal contamination to receiving waters (Noble et al., 2006).

Most recreational water quality studies using statistical predictor models have been conducted in large urban marine beaches, or freshwater environments (Francy, 2009; Telech et al., 2009). This study evaluated the success of multiple linear regression (MLR) models in estuarine waters that have dual beneficial uses as both high-priority shellfish harvesting and recreational waters, primarily affected by non-point source stormwater runoff (Coulliette and Noble, 2008; Coulliette et al., 2009). To the best of our knowledge, this is the first presentation of strongly validated predictive statistical models to an estuarine system for the purpose of monitoring water quality. Furthermore, we present successful predictive models for both EC and ENT, which are the primary FIB types used nationally for shellfish harvesting and recreational water quality management,

respectively (NSSP, 2009; USEPA, 2012). Successful model development was accomplished through three phases. First, an intensive monitoring program was implemented at two estuarine locations, including collection of data on FIB and fecal *Bacteroides* spp. and human *Bacteroides* spp. (BacHum) concentrations, flow, loading, and a wide range of antecedent rainfall, climate, and environmental parameters during both wet and dry conditions. Second, a training data set based on the collected monitoring data was used to develop statistical predictor models. Third, using a second, independent data set the models for EC, ENT, fecal *Bacteroides* spp., and BacHum were subsequently assessed.

2.2. Materials and Methods

2.2.1. Site description

The data monitoring program was conducted at two shallow tributaries in the NPRE in eastern NC, Ware Creek and Oyster Creek (Figure 1). We selected these study sites because they are actively used for both recreation and shellfish harvesting. The area has been previously studied and described in Coulliette and Noble (2008) and Coulliette et al. (2009). Even though some monitoring has been conducted at these locations in the past, our aim was to capture a very wide range of conditions, both meteorologically and seasonally. Our sampling effort also included measurements of more water quality variables (including *Bacteroidales*-based molecular markers) at a higher number of study sites than these historical datasets. Because of the unbalanced and non-continuous nature of the previously conducted monitoring, we have included only recently collected data in our model development. Percent impervious cover and land-use data (Table 1) were estimated for the watershed of each tributary network using methods compiled from

Homer et al. (2004) and DiDonato et al. (2009). Sub-watersheds were defined in ArcGIS 9.3 based on a 1/9-arc second digital elevation model (DEM) obtained from the U.S. Geological Survey (USGS) National Map Seamless Server (http://seamless.usgs.gov). Impervious cover within each sub-watershed was calculated using the 2001 USGS National Land Cover Database (NLCD) Zone 58 Imperviousness Layer and land use data were calculated using NLCD surface imagery classified by the U.S. Department of Agriculture National Cartography & Geospatial Center (http://www.ncgc.nrcs.usda.gov/). Impervious cover was less than 2% in both watersheds (Table 1). The dominant land uses were emergent herbaceous wetlands and evergreen forest. In addition, row crop agriculture and developed open space (i.e. residential development) also were important land uses in the Ware Creek watershed.

2.2.2. Monitoring methods

Water samples were collected from Ware and Oyster tributaries in the NPRE (Figure 1) in the last three hours of ebb tide during 12 dry weather and 13 wet weather events between July 2009 and August 2010. Wet sampling periods were defined as events with 24 hour antecedent rain totals above 1.27 cm. This rainfall total typically results in overland flow contributions of stormwater runoff to the estuary and therefore concomitant increases in watershed flow above dry weather levels. At each of these two sites, sampling was conducted along an upstream-to-downstream transect of 4 to 6 sampling locations spanning roughly 1400 and 1800 m (Figure 1) for a total of 151 samples. Grab samples of water were collected in sterile 1L Nalgene bottles and a multi-parameter sonde (6920 V2, YSI International, Yellow Springs, OH) was used to measure *in-situ* environmental parameters including turbidity, dissolved oxygen, conductivity, and

water temperature. Headwater discharge was calculated from water velocity measurements (Flow Tracker Handheld ADV®, SonTek, San Diego, CA) and tributary cross-sectional area at sites Ware 1, Oyster 1a and Oyster1b. Samples were kept on ice until processing, which occurred no more than 6 hours after sample collection. Salinity (by refractometer) and pH (by ion-selective probe) were measured in the laboratory prior to processing.

Precipitation data were collected from a rain gauge (HOBO® Data Logging Rain Gauge-RG3, Onset, Cape Cod, MA) installed approximately 0.77 km from Ware Creek and 2.89 km from Oyster Creek (Fig. 1). The gauge reported precipitation accumulation in 0.025 cm increments. From this, a number of variables were calculated for MLR analysis. These included antecedent rain total (calculated for the 1, 2, 3, 5, 7, 14, and 28 days prior to sampling events), antecedent dry period (the amount of time prior to the sampling event that there was negligible rain), current rain total (which was determined by the addition of consecutive 0.025 cm increments of rainfall until no rainfall was recorded for at least 6 hours, thus dry weather totals were 0), and rain intensity (storm rainfall total / storm duration). Additional climate data was collected from a nearby personal weather station (Fig. 1, weather station ID: KNCBEAUF4) for MLR analysis. These variables included current and antecedent humidity, dew point, barometric pressure, and air temperature. Current values were taken at the time of sampling. Antecedent values were averaged for the 1, 2, 7, 14, and 28 days prior to sampling events, similar to Hathaway et al. (2010). In total, more than 50 rainfall and climate variables were defined for MLR analysis.

2.2.3. Enumeration of conventional and alternative fecal indicator bacteria

Defined Substrate Technology® kits were used to measure EC and ENT in all water samples using Colilert-18® and Enterolert™, respectively, per manufacturer guidelines (IDEXX Laboratories, Inc., Westbrook, ME). Samples were diluted at least 1:10 in deionized water. Quantification was conducted using a 97-well most probable number (MPN) Quanti-tray®/2000 along with algorithms as previously published by Hurley and Roscoe (1983).

The alternative indicators, fecal *Bacteroides* spp. and BacHum, were quantified using primers, probes, and qPCR assay according to the protocols in Converse et al. (2009) and Kildare et al. (2007), respectively. A specimen processing control (SPC) was used to measure the amount of inhibition and estimate DNA extraction efficiency in each sample. Ten ng of salmon testes DNA and 500 µl of AE buffer were added to each sample, calibrator, and negative control prior to DNA extraction. DNA was recovered from polycarbonate filters (previously stored at -80˚C after filtering 50-100 ml of sample) by bead-beating following the method of Converse et al. (2009). Assays were performed in a SmartCycler® II (Cepheid Inc., Sunnyvale, CA) with the following cycling conditions: 2 minutes at 95˚C, followed by 45 cycles of 15 seconds at 94˚C and 30 (60 for BacHum) seconds at 60˚C. Quantitative assessments of qPCR inhibition were conducted through the use of salmon testes DNA following the assay protocol of Haugland et al. (2005). Level of inhibition was assessed by calculating the difference between the cycle threshold (C_T) of the SPC in the unknown sample reaction and a reaction tube containing only the SPC (no target DNA). A sample with a difference of greater than 0.5 log units (2.32 C_T) was considered to be inhibited. All 154 samples

required dilution to resolve inhibition in undiluted samples. Therefore, we tested a range of dilution levels and determined that inhibition was adequately resolved at a 1:40 dilution for 149 samples and either a 1:60 or 1:100 dilution in the remaining 5 samples. Average amplification efficiencies of the standard curves for the inhibition (salmon testes DNA), fecal *Bacteroides* spp., and BacHum qPCR assays were 99.7% (n=7), 99.8% (n=5), and 99.8% (n=4), respectively.

2.2.4. Data and statistical analysis

All statistics were performed in SAS 9.2 (Raleigh, NC). Instantaneous loadings (calculated based on a grab sample) of all FIB were calculated by multiplying concentrations of EC, ENT, fecal *Bacteroides* spp., and BacHum (in units of either MPN/ 100 ml or cell equivalents (CE)/ 100 ml) and measured discharge (m^3/ s), and reported in units of MPN (or CE)/ hr. Yields were calculated by dividing instantaneous loadings by the area of the sub-watershed. Normality was checked using histograms. Concentrations and yields were $\log_{10}$-transformed to reduce skewness prior to any analysis. All data were pooled (n=151) to first examine between-creek differences and then the differences between dry and wet weather, as well as their interaction, using Scheirer-Ray-Hare tests.

Stepwise MLR that maximized adjusted R^2 was performed using the PROC GLMSELECT function in SAS. Variance inflation factors (VIF) were generated by the PROC REG function to measure collinearity in the MLR analysis. VIF values greater than 10 indicated collinearities among predictor variables that are strong enough to warrant corrective action. No more than five explanatory parameters were used to prevent over-parameterizing the models. EC, ENT, fecal *Bacteroides* spp., and BacHum models were created using a random subset of data for a training set (n=113) and validated using

a second randomly-selected independent set (n=41). Model performance was gauged using root mean squared error (RMSE), adjusted R^2, percent type I errors, percent type II errors, and percent correct (100 – percent errors). The RMSE measured the model's prediction capability using the independent variables, with a smaller RMSE value indicating a greater predictive capability. Adjusted R^2 described the proportion of the variation that the model's independent variables described. Type I and II errors are false positives and false negatives, respectively. An example of a type I error (false positive) is when model results recommend posting or closing a recreational or shellfish harvesting area when observed measurements indicate it should remain open. The single standard sample thresholds of 235 MPN/100 ml EC and 104 MPN/100 ml ENT for recreational water quality were used to judge error types. A threshold of 14 MPN/100 mL of EC was used to judge error types for shellfish water quality standards as a proxy for FC, a procedure which has been applied in prior work (e.g. Coulliette et al. 2009). Because there are no established recommended regulatory thresholds for fecal *Bacteroides* spp. and BacHum, the validation set was only judged based on RMSE and adjusted R^2.

To further understand relationships between the EC and ENT and the *Bacteroidales* group, we conducted an analysis of the Pearson's correlation coefficient on the pooled data set (n=151). Significance required a p-value ≤ 0.05 (alpha = 0.05). In addition, to try to understand if there were any linkages across specific concentration bins, the indicators were stratified based on concentration in a manner similar to Sauer et al. (2011). EC and ENT were stratified as follows: high (>10,000 MPN/100 mL), moderate (1000-10,000 MPN/100 mL), and low (<1000 MPN/100 mL). Fecal

Bacteroides spp. and BacHum were stratified as follows: high (>5000 CE/100 mL), moderate (1000-5000 CE/100 mL), and low (<1000 CE/100 mL).

2.3. Results

2.3.1. Summary statistics and loading for model development

The foundation for this modeling effort was an intensive sampling program over a wide range of environmental conditions, with culture-based quantification of EC and ENT and qPCR-based quantification of two important *Bacteroidales*-based markers (fecal *Bacteroides* spp. and BacHum). The total rainfall amounts during the wet weather events ranged from 0 to 20.3 cm and covered all seasons, making the data set broadly representative of this location. Environmental parameters and meteorological data summary are presented in Supplementary Table 1.

Means, medians, and ranges for concentrations of EC, ENT, fecal *Bacteroides* spp., and the BacHum marker are shown in Figure 2. Concentrations of EC and ENT often exceeded regulatory threshold limits at both tributaries even during dry weather conditions. During dry weather 55% (48/88) of samples exceeded the recreational water quality EC threshold and 52% (46/88) exceeded the ENT threshold. During wet weather 91% (60/66) of samples exceeded the recreational water quality EC threshold and 88% (58/66) exceeded the ENT threshold. The geometric mean threshold of 14 MPN/100 mL EC for shellfish water quality was exceeded 95% (84/88) of the time in dry weather and 100% (66/66) of the time in wet weather.

Analysis of the pooled dataset (n=151) using the Scheirer-Ray-Hare test indicated that EC concentrations were significantly different between the two sites (p=0.003)

irrespective of weather. *E. coli* concentrations were also significantly different between wet and dry weather conditions ($p<0.001$) irrespective of site. No interaction between site and weather was found ($p=0.308$). Similar to EC, ENT concentrations were significantly different between sites and weather conditions ($p<0.001$ for both), but there was also a significant interaction between site and weather for ENT concentration ($p=0.031$). Because there is an effect of one factor on the other, each factor was analyzed separately using a Kruskal-Wallis ANOVA. Results confirmed an ENT site ($p<0.001$) and weather ($p<0.001$) difference. For fecal *Bacteroides* spp. concentrations there were no significant differences between site, weather, or the interaction of site and weather ($p=0.155$, $p=0.240$, and 0.687 respectively). BacHum concentrations were significantly different between sites and weather ($p=0.005$ and $p<0.001$, respectively), and no significant interaction between site and weather were found ($p=0.304$). For those with observed significant differences (EC, ENT, and BacHum), concentrations were always greater at Ware Creek than Oyster Creek and during wet weather as compared to dry weather.

Yields (Figure 2) were calculated at the headwaters (i.e. Ware 1, Oyster 1a, Oyster 1b) during each sampling event. Yields at Oyster 1a and 1b were averaged. For EC, ENT, fecal *Bacteroides* spp. and the BacHum marker there was no difference in yields between the two creeks, but there was a significant difference in yields between wet and dry weather conditions (Figure 2). Yields of all indicators were three to four log higher during wet weather than dry weather (Figure 2). For example, mean EC yield in the Oyster Creek tributary increased from log 5.98 MPN/hr/km^2 during dry weather to log 9.18 MPN/hr/km^2 during wet weather. Mean fecal *Bacteroides* spp. and BacHum yields during dry weather were typically 1 log lower than that of EC and ENT, but also

increased significantly during wet weather. For example, mean BacHum yield to the Oyster Creek tributary increased from log 4.66 MPN/hr/km^2 to log 7.88 MPN/hr/km^2. No interaction effect between site and weather conditions was found for the four indicators. These summary statistics show the wide range of FIB concentrations, yields, flows, rainfall, and environmental parameters that was captured by the sampling effort, indicating the dataset is representative of this location.

2.3.2. Multiple linear regression models

Four models in total were created to predict concentrations of bacteria in a high priority estuarine system ─ one each for EC, ENT, fecal *Bacteroides* spp., and the BacHum marker. The models were created using data from all transect locations; therefore, the models are appropriate for any location spatially along each of the two tributaries of the NPRE. All models and their respective individual variables had significant p-values. All variables had VIFs much less than 10─ all were less than 4─ indicating no severe collinearity issues. Construction of the four concentration models, shown in Table 2, was done using a data training set (n=113). For all four concentration models, a combination of antecedent rainfall, climate (barometric pressure, air temperature), and environmental variables (DO, salinity, pH, turbidity, conductivity) were found to maximize the FIB variation explained by the model.

Models were evaluated by comparing the RMSE, adjusted R^2, and the error rates of a separate, randomly-selected validation set (n=41) to the training set models (n=113). Table 3 summarizes the model performances for the pooled data from both tributaries. The EC model created from the training set had 62% of its variation explained by five variables (in order of importance): 5-day antecedent storm total, dissolved oxygen,

salinity, water temperature, and barometric pressure. The RMSE was 0.4933. There was an error rate of 2% type I errors and 11% type II errors, resulting in 87% accuracy for the predictions made for recreational water quality management (Table 3). For shellfish water quality, there was an error rate of 1% type I errors and 2% type II errors resulting in 97% accuracy for model predictions (Table 3). Using this EC model on the validation set with the recreational water quality threshold, EC concentrations were predicted with 53% of the variation explained and a RMSE of 0.4954. The accuracy increased to 97%, with a 0% rate of type I errors and 3% rate of type II errors. Validating the EC model using the shellfish water quality threshold resulted in 100% accuracy in management decisions. Predictability using the EC model was considered successful based on a lower RMSE and a decrease in the error rate, even though the variance of the data set explained by the model reduced.

Similar success was found using the ENT model. The ENT model created using the training set had 64% of the data variation explained by five variables (in order of importance): 5-day antecedent rain total, salinity, dissolved oxygen, air temperature, and pH. The RMSE was 0.7466. There was an error rate of 3% type I errors and 4% type II errors, resulting in 93% accuracy in management decisions (Table 3). Using this model on the separate validation set resulted in insignificant reductions in the R^2 from 0.64 to 0.63 and a change and in the management decision prediction error rate from 93% to 91%. The RMSE decreased from 0.7466 to 0.6524.

The alternative indicators, fecal *Bacteroides* spp. and BacHum, were modeled successfully using the training set. The fecal *Bacteroides* spp. model had 56% of the data variation explained by five variables (in order of importance): antecedent dry period,

dissolved oxygen, salinity, water temperature, and turbidity. The BacHum model had 53% of the data variation explained by five variables (in order of importance): conductivity, 14-day barometric pressure, 1-day barometric pressure, pH, and antecedent dry period. However, the models did not perform as well using the validation data set. The RMSE of the fecal *Bacteroides* spp. model increased from 1.257 to 1.438 and the adjusted R^2 decreased 13%. Similarly the RMSE of the BacHum model increased from 0.8150 to 1.092 and the adjusted R^2 decreased 29%.

2.3.3. Relationships between the bacterial indicator groups

To more fully understand the relationships observed between the conventional (EC and ENT) and alternative (fecal *Bacteroides* spp. and BacHum) indicators, we used a combination of correlation analysis and FIB stratification. All FIB concentration comparisons had significant Pearson correlations (Table 4). The two conventional FIB were strongly correlated with each other ($r=0.79$, $p<0.01$). Fecal *Bacteroides* spp. had a weaker correlation to both EC and ENT ($r=0.45$, $p<0.01$ and $r=0.44$, $p<0.01$ respectively), while BacHum had a weak, but significant, correlation to both EC ($r=0.20$, $p=0.013$) and ENT ($r=0.36$, $p<0.01$). The correlation between the two *Bacteroides* spp. assays was weak ($r=0.18$, $p=0.027$). Table 5 shows results stratified by levels of conventional FIB and the *Bacteroides* spp. assays in an attempt to estimate their concordance according to concentration. Eighteen samples were stratified as high FIB concentrations (>10,000 MPN of both EC and ENT/100 ml), and are considered high priority samples (Sauer et al., 2011). Of these 18 high priority samples, 10 of the18 had moderate or high fecal *Bacteroides* spp. concentrations and 2 of the 18 samples had moderate or high concentrations of BacHum.

2.4. Discussion

While most water quality studies using statistical predictor models have been conducted in large urban beaches designated as recreational waters, this study evaluated the success of these models in estuarine waters with dual uses as both recreational and high priority shellfish waters. Programs that include monitoring for both uses using EC or FC (shellfish harvesting waters) and ENT (recreational waters) can put further resource strain on the monitoring agency. In estuarine systems, predictor models can conserve limited monitoring funds by identifying hot spots of chronic contamination that require more frequent monitoring while at the same time assessing contamination relationships to rainfall and identifying areas in need of presumptive rainfall closures.

2.4.1. Summary statistics and loading for model development

Samples were temporally and spatially collected over a broad range of weather conditions. Concentrations increased significantly for EC, ENT, and BacHum during wet weather conditions. The elevated concentrations of EC and ENT during wet weather as compared to dry weather agree with results from previous studies describing this difference throughout NC and specifically in this region (Characklis et al., 2005; Krometis et al., 2007; Hathaway et al., 2010; Krometis et al., 2010; Parker et al., 2010; Stumpf et al., 2010; Converse et al., 2011). The increase in wet weather BacHum concentrations also agrees with a previous study in a similar estuarine system in another region in eastern NC examining *Bacteroides* spp. concentrations (Converse et al. 2011). There was also a difference in concentrations between the two tributaries of the NPRE. Ware Creek had higher FIB concentrations than Oyster Creek, possibly due to differences in land use, including the presence of row crop agriculture and residential development

near Ware Creek and the larger percent of emergent herbaceous wetlands at Oyster Creek (67.4% as compared to 22.9 % at Ware Creek). Studies have shown that bacterial concentrations were greatly reduced in herbaceous wetlands (Karim et al., 2004; Sleytr et al., 2007). Moreover Hemond and Benoit (1988) argue that mechanistic detention of bacteria alone by wetland vegetation can remove bacteria by allowing natural die off. A reduction in water velocity will allow sediment to drop out of the water column. Bacteria capable of particle attachment, like ENT, might then persist and grow in sediment reservoirs. This persistent reservoir population of bacteria may contribute to the elevated FIB concentrations during dry weather.

The increase in storm fecal indicator yield, even with small amounts of precipitation, over dry weather yield also agrees well with studies conducted in other watersheds in the region (Krometis et al., 2007; Stumpf et al., 2010; Converse et al., 2011). While there was a difference in indicator concentrations between the two tributaries, there was no difference in yield between the two tributaries. The Ware Creek watershed had more impervious cover than the Oyster Creek watershed (1.48% vs. 0.15%), but both watersheds were well below the typical percent of impervious cover at which stream quality declines (10-15%, Schueler et al., 2009).

2.4.2. Multiple Linear Regressions

The five most influential independent variables differed for each of the four dependent variables (i.e. EC, ENT, fecal *Bacteroides* spp., and BacHum), but similarities between models were apparent. The importance of 5-day antecedent rainfall in both the EC and ENT models makes sense because prolonged rainfall can cause land-based runoff into receiving waters. This runoff may contain bacteria originating from a variety of

sources such as wildlife, pets, and improperly functioning septic systems (Conn et al., 2012). Interestingly, 5-day antecedent rainfall was more important in predicting EC and ENT concentrations than the storm total or 24-hour antecedent rainfall variables. This may be because 5-day antecedent rainfall incorporates the entire hydrograph accounting for the total wet weather bacterial load. A shorter time period may only account for portions of the hydrograph and may not account for hydrographic conditions of a recent antecedent storm (e.g. storms occurring in short succession). Moreover, high antecedent rainfall can increase soil saturation levels, thereby increasing overland transport (USEPA, 2010).

The importance of salinity or conductivity in all four models also supported the important role of overland stormwater runoff in tributary bacteria concentrations. This negative relationship between salinity/conductivity and indicator concentrations suggests that as the estuarine waters are impacted by freshwater inputs (stormwater and runoff), indicator bacteria concentrations increase.

Dissolved oxygen was the second-most important variable for the EC and fecal *Bacteroides* spp. models, and the third-most important variable for the ENT model. In all three models, the variable had a negative regression coefficient. Low dissolved oxygen might be indicative of a recent contamination event such as untreated wastewater that may contain elevated bacterial levels. Low dissolved oxygen levels in surface water may also be caused by oxygen consumption as bacteria decompose organic material in runoff.

Interestingly, the antecedent dry period was an important predictor variable in both the fecal *Bacteroides* spp. and the BacHum models, but with opposite associations.

The fecal *Bacteroides* spp. model had a positive relationship while the BacHum model had a negative relationship. This may be because the target host of the two assays differs. While it was designed to preferentially quantify members of the *Bacteroidales* group that are relevant to the human gut, the fecal *Bacteroides* spp. assay also detects fecal contamination from warm blooded animals, including wildlife. A larger number of antecedent dry days may allow a greater accumulation of fecal deposition by wildlife onto the watershed before being washed away during a storm event. On the other hand, BacHum more specifically targets human contamination. The most probable pathway of human waste into these tributaries is likely failing septic systems and therefore a signal may be more likely to occur when the antecedent dry period is short, the water table is higher, and the ground is saturated.

The prediction capabilities differed between conventional (EC and ENT) and alternative (fecal *Bacteroides* spp. and BacHum) indicators. The EC and ENT prediction models for recreational water quality behaved similarly to the training data set when they were applied to the validation data set. For EC, there was a slight decrease in error rate and little change in goodness-of-fit metrics for recreational water quality. For ENT, there was a decrease in RMSE and a decrease in overall error rate. By creating one model (per fecal indicator bacteria) across a diverse set of environmental conditions and spatially diverse locations, conventional FIB can now accurately be predicted in real-time at our study sites for recreational water quality standards. Future work will validate these models in the whole estuary and other similar estuaries nearby.

Bacteroidales model prediction within the same estuary allowed insight into what variables are important to their sources and fate in the environment. Through the larger

training data set, members of the *Bacteroidales* group were found to have strong linear relationships to many of the parameters tested (e.g. antecedent dry period, dissolved oxygen, conductivity, barometric pressure, water temperature, turbidity). Additionally the low prediction power of the qPCR-based models may be due to the elevated lower limit of method detection caused by sample inhibition. Because the estuarine samples were highly inhibited, samples for qPCR processing had to be diluted. This dilution weakened the performance of the model at lower concentrations and may be the reason for the lower amount of variation explained by the model. Future work including the use of commercial DNA extraction kits to reduce the presence of inhibitory compounds and the use of a larger validation dataset should be done to enhance *Bacteroidales* prediction.

2.4.3. Relationship between the bacterial groups

Results from the correlation analysis indicate a strong relationship between EC and ENT concentrations in these two tributaries of the NPRE. This suggests that EC and ENT likely originate from similar sources and have similar physical process affecting their ecology. However, there was a weak relationship between the conventional FIB (EC and ENT) and the *Bacteroides* spp. indicators, as indicated by low, but significant, Pearson correlation coefficients. The low concordance of high ranking EC and ENT to high ranking *Bacteroidales* members (Table 5) support the idea that other contamination sources exist besides warm-blooded mammals. Specifically, only 10 out of 18 high priority samples (EC and ENT > 10,000 MPN/ 100 ml) had moderate or high fecal *Bacteroides* spp. concentrations and merely 2 out of 18 high EC and ENT samples had moderate or high BacHum concentrations (Table 5). Members of the *Bacteroidales* group are obligate anaerobes with short survival times in aerobic environments, while EC and

ENT are facultative anaerobes that can survive, and even grow, in aerobic environments. EC and ENT likely originate from a variety of land-based sources that are delivered to the estuary by overland runoff, especially during storm events. Both EC and ENT may also persist in the environment (i.e. bed sediment) and contribute to elevated concentrations during dry weather, perhaps due to resuspension. Additionally, the weak relationship between *Bacteroides* spp. and conventional FIB may also be a byproduct of different enumeration methods. The conventional FIB were enumerated using culture based methods (measures metabolically active cells), while the *Bacteroidales* groups were enumerated through the amplification of target DNA. In order to rectify this problem, future work will compare EC, ENT, fecal *Bacteroides* spp., and the BacHum marker using only qPCR-based methods.

2.4.4. Application

EPA encourages the use of predictive models in situations where a water quality monitoring group does not have the capacity to otherwise provide timely information for making same day beach notification decisions (USEPA, 2012). In eastern NC, there are hundreds of recreational and shellfish harvesting water quality monitoring sites spread over wide geographic distances that require regular monitoring. Application of rapid methods for monitoring and same day notification of the public is simply not a viable option in the majority of these areas. Therefore, the predictor models offer a cost effective option to water quality management, and they have the potential to be applied elsewhere. The predictor models created in this study were developed in a region that has not been subject to extensive predictive modeling activities, but environmental parameter data are widely available and this approach could be adopted by a range of users locally

and regionally. Using parameters measured *in-situ* or retrieved online such as antecedent rainfall, dissolved oxygen, and salinity, real-time EC and ENT predictive models presented in this study can supplement monitoring and improve notifications of closures during both wet and dry weather conditions. Because of the robust sampling approach and data collection program, we anticipate that models like these could be relevant to both shellfish harvesting and recreational waters in similar regions in NC, Virginia, and other shallow, lagoon estuarine locations that are the prominent sites of much shellfish harvesting and recreation. Additionally, managers using these models can have the economic advantage of using a single model across locations while predicting a high percentage of correct management decisions.

2.5. Conclusions

- Water samples in the recreational and high priority shellfish harvesting waters of the NPRE often exceeded NC water quality thresholds, even during dry weather.

- Concentrations of the fecal indicator bacteria, EC and ENT, can be predicted using empirical statistical models and a combination of antecedent rainfall, climate, and environmental variables including 5-day antecedent rainfall, dissolved oxygen, and salinity.

- EC and ENT models accurately predicted a high percentage (>87%) of management decisions based on current regulatory thresholds.

- Models were not as successful in prediction of *Bacteroides* spp. concentrations using a validation set and the RMSE and adjusted R^2 performance metrics. However,

conducting the model development allowed insight into potentially important variables affecting their fate in the environment.

Figures

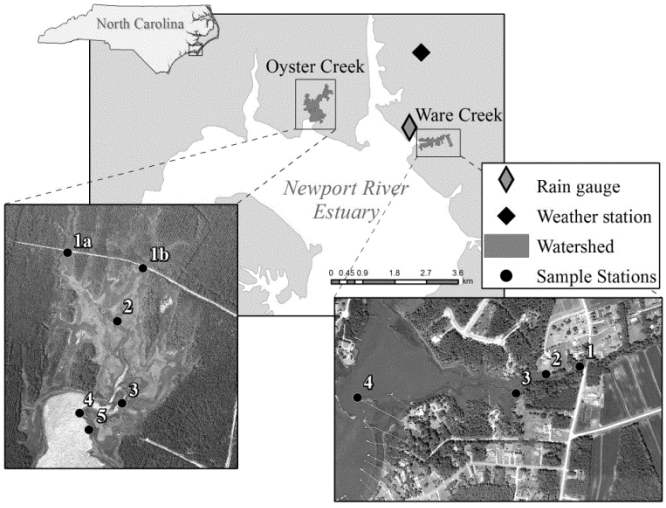

Figure 2.1. Ware Creek and Oyster Creek tributaries of the Newport River Estuary in eastern North Carolina, USA. Sampling sites are denoted by black circles, weather station denoted by a black diamond, and rain gauge by a grey diamond.

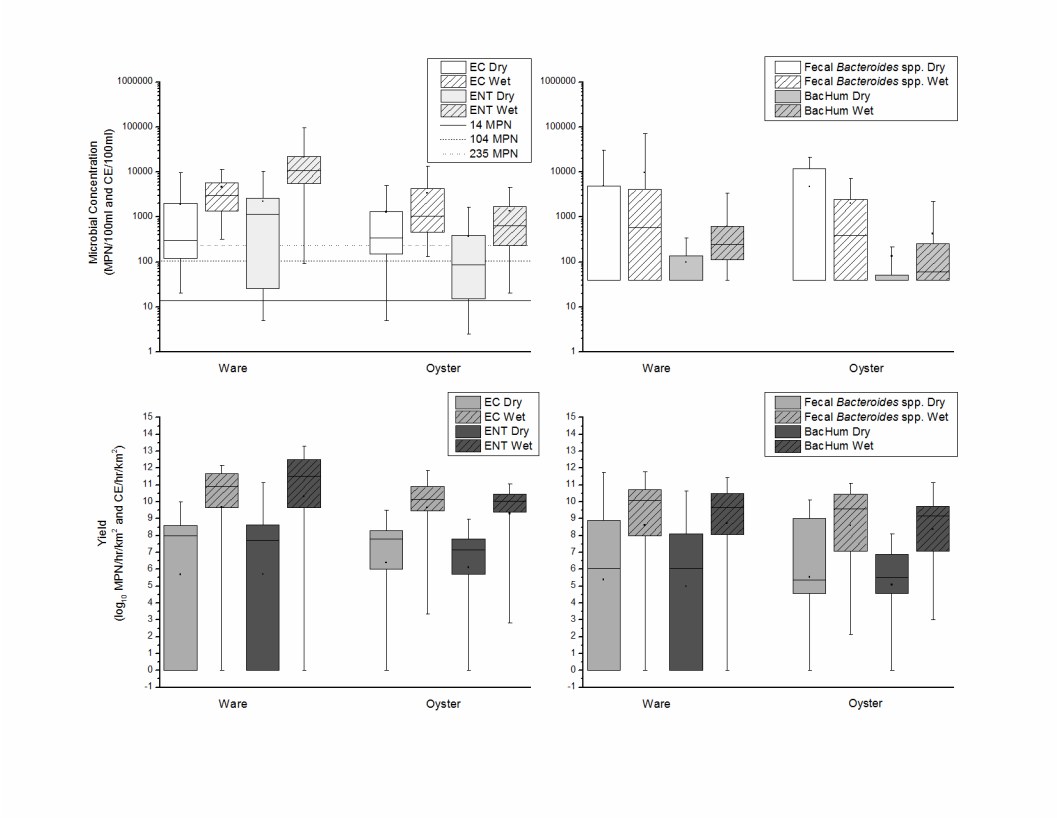

Figure 2.2. Box and whisker plots of *E. coli* (EC), enterococci (ENT), fecal *Bacteroides* spp., and human *Bacteroides* spp. (BacHum) concentrations (most probable number (MPN)/100 ml or cell equivalents (CE)/100 ml) and yields (MPN/hr/km^2 or CE/hr/km^2) as presented by site and weather. Box range is the 25th - 75th percentile. Whisker range is 5th-95th percentile. Means are depicted with a black square. Labeled lines indicate recreational and shellfish harvesting water quality thresholds.

Tables

	Ware Creek	Oyster Creek
Perimeter:	5,712 m	7,487 m
Area:	0.14 km2	0.31km2
Approx. creek length (inc. tributaries	1,778 m	1,407 m
Land Cover	%	%
Developed, Open Space	12.1	0
Evergreen Forest	19.3	21.3
Mixed Forest	0	0.58
Shrub/Scrub	0.6	0.58
Grassland/Herbaceous	11.5	6.71
Pasture/Hay	5.42	0
Cultivated Crops	21.7	0.58
Woody Wetlands	6.63	2.29
Emergent Herbaceous Wetlands	22.9	67.4
% Impervious Cover	1.48	0.15

Table 2.1. Percent impervious cover and land use data for Ware and Oyster Creek tributaries.

	Concentration Models (n= 113)			
	EC RMSE= 0.4933 R^2= 0.62	**ENT** RMSE= 0.7466 R^2= 0.64	**Fecal *Bacteroides* spp.** RMSE= 1.257 R^2= 0.56	**BacHum** RMSE= 0.8150 R^2= 0.53
Intercept	41.106	1.806	2.496	-34.987
Var. 1	**5-day Antecedent Storm Total**	**5-day Antecedent Storm Total**	**Antecedent Dry Period**	**Conductivity**
β1	0.098	0.129	0.002	-0.037
VIF	1.83	1.71	1.14	2.20
Var. 2	**Dissolved Oxygen**	**Salinity**	**Dissolved Oxygen**	**14-day [a]BP**
β2	-0.131	-0.087	-0.405	3.310
VIF	1.60	3.17	1.54	1.40
Var. 3	**Salinity**	**Dissolved Oxygen**	**Salinity**	**1-day BP**
β3	-0.045	-0.245	-0.149	-2.148
VIF	3.21	1.63	3.35	1.21
Var. 4	**Water Temp.**	**Air Temperature**	**Water Temperature**	**pH**
β4	0.088	-0.030	0.169	0.336
VIF	3.63	1.56	3.53	2.11
Var. 5	**Barometric Pressure**	**pH**	**Turbidity**	**Antecedent Dry Period**
β5	-1.306	0.611	-0.006	-0.001
VIF	2.13	2.29	1.12	1.16

[a]BP =Barometric Pressure

Table 2.2. Multiple linear regression models of *E. coli* (EC), enterococci (ENT), fecal *Bacteroides* spp., and human *Bacteroides* spp. (BacHum) concentrations using the training data set (n=113). Samples were collected during 12 dry and 13 wet weather events (0 – 20.3 cm of rain) from July 2009 to August 2010.

Model	Training/ Validation	RMSE	Adjusted R^2	Type I Error Rate (%)	Type II Error Rate (%)	Percent Correct
[a]EC	Training	0.4933	0.62	2	11	87
	Validation	0.4954	0.53	0	3	97
[b]EC	Training	0.4933	0.62	1	2	97
	Validation	0.4954	0.53	0	0	100
ENT	Training	0.7466	0.64	3	4	93
	Validation	0.6524	0.63	0	9	91
Fecal *Bacteroides* spp.	Training	1.2573	0.56	-	-	-
	Validation	1.4376	0.43	-	-	-
BacHum	Training	0.815	0.53	-	-	-
	Validation	1.0919	0.24	-	-	-

[a]EC model validated with recreational water quality threshold

[b]EC model validated with shellfish harvesting water quality threshold

Table 2.3. Summary of *E. coli* (EC), enterococci (ENT), fecal *Bacteroides* spp., and human *Bacteroides* spp. (BacHum) model performance using the independent validation set (n=41) as compared to the training set (n=113). Error rates and percentages correct are based on predictions of meeting or exceeding the standards for EC and ENT.

	EC	ENT	Fecal *Bacteroides* spp.	BacHum
EC	1			
ENT	0.79	1		
Fecal *Bacteroides* spp.	0.45	0.44	1	
BacHum	0.20	0.36	0.18	1

Table 2.4. Pearson correlation coefficients between *E. coli* (EC), enterococci (ENT), fecal *Bacteroides* spp., and human *Bacteroides* spp. (BacHum). All corrections have a p-value <0.05.

	No. samples with high FIB (>10,000 MPN/100 ml)	No. samples with moderate FIB (1000-10,000 MPN/100 ml)	No. samples with low FIB (<1000 MPN/100 ml)
No. samples with high *Bacteroides* spp. (>5000 CE/100 ml)	6(0)	16(1)	10(0)
No. samples with moderate *Bacteroides* spp. (1000-5000 CE/100 ml)	4(2)	12(6)	6(1)
No. samples with low *Bacteroides* spp. (<1000 CE/100 ml)	8(16)	26(47)	66(81)

Table 2.5. Number of samples distributed among different strata: *E. coli* and enterococci (FIB), and the *Bacteroides* spp. genetic markers. Number of fecal *Bacteroides* spp. samples shown first and number of human *Bacteroides* spp. (BacHum) samples shown in parenthesis.

	Salinity (ppt)	pH	Water Temp. (˚C)	Turbidity (NTU)	Dissolved Oxygen (mg/L)	Water Velocity (m/s)	24-hr Rain Total (cm)	Air Temp. (˚C)	Barometric Pressure (in Hg)
N total	149	149	129	137	134	120	151	151	151
Mean	15.9	7.31	23.6	80.5	6.29	0.17	3.38	23.4	29.9
Standard Deviation	12.6	0.65	6.52	98.1	2.54	0.27	5.16	5.09	0.19
Minimum	0	3.85	10.4	0.70	0.81	-0.11	0.00	7.22	29.4
Maximum	35.0	8.42	36.7	628	11.4	1.31	20.3	30.6	30.2
Interquartile Range	25.0	0.79	10.9	77.3	3.18	0.25	4.29	8.00	0.22
90th Percentile	32.0	8.07	31.6	205	9.88	0.59	9.25	29.7	30.12
95th Percentile	35.0	8.14	33.1	277	10.6	0.73	14.6	30.6	30.18
99th Percentile	35.0	8.39	35.6	470	11.3	1.27	20.3	30.6	30.18

Supplementary Table 2.1. Summary of environmental parameters collected alongside the 151 water samples.

References

Bernhard, A. E. and Field, K. G., 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Applied and Environmental Microbiology 66(4), 1587-1594.

Characklis, G. W., Dilts, M. J., Simmons III, O. D., Likirdopulos, C. A., Krometis, L. H., Sobsey, M. D., 2005. Microbial partitioning to settleable particles in stormwater. Water Research 39(9), 1773-1782.

Conn, K. E., Habteselassie, M. Y., Blackwood, A.D., Noble, R. T., 2012. Microbial water quality before and after the repair of a failing onsite wastewater treatment system adjacent to coastal waters. Journal of Applied Microbiology 112(1), 214-224.

Converse, R. R., Piehler, M. F., Noble, R. T., 2011. Contrasts in concentrations and loads of conventional and alternative indicators of fecal contamination in coastal stormwater. Water Research 45(16), 5229-5240.

Converse, R. R., Blackwood, A. D., Kirs, M., Griffith, J. F., Noble, R. T., 2009. Rapid QPCR-based assay for fecal *Bacteroides* spp. as a tool for assessing fecal contamination in recreational waters. Water Research 43(19), 4828-4837.

Coulliette, A. D. and Noble, R. T., 2008. Impacts of rainfall on the water quality of the Newport River Estuary (eastern North Carolina, USA). Journal of Water and Health 06(4), 473-482.

Coulliette, A. D., Money, E. S., Serre, M. L., Noble, R. T., 2009. Space/time analysis of fecal pollution and rainfall in an eastern North Carolina estuary. Environmental Science & Technology 43(10), 3728-3735.

Dick, L. K. and Field, K. G., 2004. Rapid estimation of numbers of fecal *Bacteroidetes* by use of a quantitative PCR assay for 16S rRNA genes. Applied and Environmental Microbiology 70(9), 5695-5697.

DiDonato, G. T., Stewart, J. R., Sanger, D. M., Robinson, B. J., Thompson, B. C., Holland, A. F., Van Dolah, R. F., 2009. Effects of changing land use on the microbial water quality of tidal creeks. Marine Pollution Bulletin 58(1), 97-106.

Eleria, A. and Vogel, R. M., 2005. Predicting fecal coliform bacteria levels in the Charles River, Massachusetts, USA. Journal of the American Water Resources Association 41(5), 1195-1209. Francy, D.S., Bertke, E.E., Finnegan, D.P., Kephart, C.M., Sheets, R.A., Rhoades, John, and Stumpe, Lester, 2006. Use of Spatial Sampling and Microbial Source-Tracking Tools for Understanding Fecal Contamination at Two Lake Erie Beaches: U.S. Geological Survey Scientific Investigations Report 2006-5298, 29 p.

Francy, D.S., and Darner, R.A., 2007. Nowcasting beach advisories at Ohio Lake Erie beaches: U.S. Geological Survey Open-File Report 2007–1427, 13 p.

Francy, D. S., 2009. Use of predictive models and rapid methods to nowcast bacteria levels at coastal beaches. Aquatic Ecosystem Health and Management Society 12(2), 177-182.

Francy, D.S., Bertke, E.E., and Darner, R.A., 2009. Testing and refining the Ohio Nowcast at two Lake Erie beaches—2008: U.S. Geological Survey Open-File Report 2009–1066, 19 p.

Griffith, J. F., and Weisberg, S. B., 2011. Challenges in implementing new technology for beach water quality monitoring: Lessons from a California demonstration project. Marine Technology Society Journal 45(2), 67-73.

Hathaway, J. M., Hunt, W. F., Simmons III, O. D., 2010. Statistical evaluation of factors affecting indicator bacteria in urban storm-water runoff. Journal of Environmental Engineering 136(12), 1360-1368.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., Dufour, A. P., 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research 39(4), 559-568.

Hemond, H. F. and Benoit, J., 1988. Cumulative impacts on water quality functions of wetlands. Environmental Management 12(5), 639-653.

Homer, C., Huang, C., Yang, L., Wylie, B., Coan, M., 2004. Development of a 2001 national landcover database for the United States. Photogrammetric Engineering & Remote Sensing 70(7), 829-840.

Hurley, M. A. and Roscoe, M. E. (1983) Automated statistical analysis of microbial enumeration by dilution series. Journal of Applied Microbiology 55(1), 159-164.

Karim, M. R., Manshadi, F. D., Karpiscak, M. M., Gerba, C. P., 2004. The persistence and removal of enteric pathogens in constructed wetlands. Water Research. 38(7), 1831-1837.

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B., Wuertz, S., 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research 41(16), 3701-3715.

Kreader, C. A., 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Applied and Environmental Microbiology 61(4), 1171-1179.

Krometis, L. H., Characklis, G. W., Drummey, P. N., Sobsey, M. D., 2010. Comparison of the presence and partitioning behavior of indicator organisms and *Salmonella* spp. in an urban watershed. Journal of Water and Health 8(1), 44-59.

Krometis, L. H., Characklis, G. W., Simmons III, O. D., Dilts, M. J., Likirdopulos, C. A., Sobsey, M. D., 2007. Intra-storm variability in microbial partitioning and microbial loading rates. Water Research 41(2), 506-516.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., Sayler, G., 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology 72(6), 4214-4224.

Leecaster, M. K. and Weisberg, S. B., 2001. Effect of sampling frequency on shoreline microbiology assessments. Marine Pollution Bulletin 42(11), 1150-1154.

Nevers, M. B. and Whitman, R. L., 2008. Coastal strategies to predict *Escherichia coli* concentrations for beaches along a 35 km stretch of southern Lake Michigan. Environmental Science & Technology 42(12), 4454-4460.

Nevers, M. B. and Whitman, R. L., 2010. Efficacy of monitoring and empirical predictive modeling at improving public health protection at Chicago beaches. Water Research 45(4), 1659-1668.

Nevers, M. B. and Whitman, R. L., 2005. Nowcast modeling of *Escherichia coli* concentrations at multiple urban beaches of southern Lake Michigan. Water Research 39(20), 5250-5260.

NCDMF, 2012. North Carolina shellfish sanitation and recreational water quality section of the division of marine fisheries. http://portal.ncdenr.org/web/mf/shellfish-sanitation-and-recreational-water-quality.

Noble, R. T., Griffith, J. F., Blackwood, A. D., Fuhrman, J. A., Gregory, J. B., Hernandez, X., Liang, X., Bera, A. A., Schiff, K., 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Applied and Environmental Microbiology 72(2), 1604-1612.

NSSP, 2009. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009 Revision. http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/ucm046353.htm

Olyphant, G. A. and Whitman, R. L., 2004. Elements of a predictive model for determining beach closures on a real time basis: the case of 63rd Street Beach Chicago. Environmental Monitoring and Assessment 98(1), 175-190.

Olyphant, G. A., Thomas, J., Whitman, R. L., Harper, D., 2003. Characterization and statistical modeling of bacterial (*Escherichia coli*) outflows from watersheds that discharge into southern Lake Michigan. Environmental Monitoring and Assessment 81(1), 289-300.

Parker, J. K., McIntyre, D., Noble, R. T., 2010. Characterizing fecal contamination in stormwater runoff in coastal North Carolina, USA. Water Research 44(14), 4186-4194.

Sauer, E. P., VandeWalle, J. L., Bootsma, M. J., McLellan, S. L., 2011. Detection of the human specific *Bacteroides* genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment. Water Research 45(14), 4081-4091.

Schueler, T. R., Fraley-McNeal, L., Cappiella, K., 2009. Is Impervious Cover Still Important? Review of Recent Research. Journal of Hydrologic Engineering 14(4), 309-315.

Seurinck, S., Defoirdt, T., Verstraete, W., Siciliano, S. D., 2005. Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology 7(2), 249-259.

Shanks, O. C., White, K., Kelty, C. A., Hayes, S., Sivaganesan, M., Jenkins, M., Varma, M., Haugland, R. A., 2010. Performance assessment PCR-based assays targeting *Bacteroidales* genetic markers of bovine fecal pollution. Applied and Environmental Microbiology 76(5), 1359-1366.

Sleytr, K., Tietz, A., Langergraber, G., Haberl, R., 2007. Investigation of bacterial removal during the filtration process in constructed wetlands. Science of The Total Environment 380(1–3), 173-180.

Stumpf, C. H., Piehler, M. F., Thompson, S., Noble, R. T., 2010. Loading of fecal indicator bacteria in North Carolina tidal creek headwaters: hydrographic patterns and terrestrial runoff relationships. Water Research 44(16), 4704-4715.

Telech, J. W., Brenner, K. P., Haugland, R., Sams, E., Dufour, A. P., Wymer, L., Wade, T. J., 2009. Modeling *Enterococcus* densities measured by quantitative polymerase chain reaction and membrane filtration using environmental conditions at four Great Lakes beaches. Water Research 43(19), 4947-4955.

USEPA, 2012. Draft recreational water quality criteria. http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/upload/recreation_document_draft.pdf

USEPA, 2010. Predictive tools for beach notification volume I: review and technical protocol. http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/upload/P26-Report-Volume-I-Final_508.pdf

Wade, T. J., Sams, E., Brenner, K. P., Haugland, R., Chern, E., Beach, M., Wymer, L., Rankin, C. C., Love, D., Li, Q., Noble, R., Dufour, A. P., 2010. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. Environmental Health 9(1), 66.

Chapter 3

COMPARISONS OF STATISTICAL MODELS TO PREDICT FECAL INDICATOR BACTERIA CONCENTRATIONS ENUMERATED BY QPCR- AND CULTURE-BASED METHODS IN EASTERN NORTH CAROLINA ESTUARIES

Overview

Recently, the EPA revised their recreational water quality criteria, in which improvements were made by approving enterococci (ENT) quantitative PCR (qPCR) as an alternative, rapid method and advocating the use of predictive models for water quality management. The implementation of qPCR–based methods and prediction models are meant to decrease the time between sample collection and public advisories and notifications. To date, few studies have compared qPCR-based models to culture-based prediction models and none of these studies have been conducted in coastal estuarine systems. In this study, we created prediction models using qPCR-based fecal indicator bacteria (FIB) data in dual-use recreational and shellfish harvesting waters and compared them to published ENT and *E. coli* (EC) culture-based prediction models in eastern North Carolina estuaries. Furthermore, an empirical statistical model was created to predict qPCR inhibition levels so that proper remediation techniques can be applied when it is a problem. Predictor variable selection in both qPCR- and culture-based ENT models was very similar; both models included 14-day rain total, dissolved oxygen, and salinity/conductivity, with 89 and 90% of qPCR and culture data described, respectively. Using ENT management action thresholds, qPCR and culture methods showed high

accuracy in management decisions. The qPCR model had 92 and 96% accuracy using the 110 and 1000 cell equivalents (CE)/100 ml thresholds, respectively, and the culture model had 90% accuracy in management decisions with the 110 MPN/100 ml threshold. EC models for qPCR- and culture-based concentrations used similar independent variables (14-day humidity, salinity/conductivity, a rain/storm variable, and a measure of air temperature), with each model explaining 26 and 55% of the data variation, respectively. When using different thresholds that were logs apart for management decisions, the two EC models accurately predicted management decisions; qPCR models correctly predicted management decisions 89 and 77% of the time (using 14 and 320 CE/100 ml, respectively) while culture models correctly predicted management decisions 99 and 88% percent of the time (with 14 and 320 MPN/100 ml, respectively). Equivalency between models was shown in our non-point source impacted estuaries, with ENT models performing slightly better than EC models. In addition, inhibition of the qPCR was a major issue that had to be addressed. An inhibition model was created with easily obtained meteorological data and accounted for a high level of data variability (adjusted R^2= 0.82).

Key words: multiple linear regression, quantitative PCR, *E. coli*, enterococci, monitoring, estuary

3.1. Introduction

As a result of the Beaches Environmental Assessment and Coastal Health (BEACH) Act in 2000, the US Environmental Protection Agency (USEPA) is required to endorse new or revised criteria recommendations for pathogens and fecal indicator bacteria (FIB) based on recent studies. The BEACH Act was an amendment to the Clean

Water Act (CWA), and provisions within the act led to the newly released 2012 recreational water quality criteria (RWQC) recommendations for protecting public health in marine and fresh waters designated for recreation. Prior to this latest amendment, the USEPA last issued recommended water quality criteria for recreation in 1986. The new criteria are based on the latest research and focus on two FIB—enterococci (ENT) for marine waters and *E. coli* (EC) for freshwater.

The new RWQC document places increased emphasis on the use of sound science for improving recreational water quality, including implementation of predictive models, rapid molecular methods, increased use of sanitary surveys, and other tools to improve protection of public health. The recommendation for use of rapid quantitative PCR (qPCR) based methods at appropriate beaches is focused on decreasing the time between sample to result, and therefore reducing the time to accurate public notification. To this end, the new RWQC includes specific guidance for the use of rapid ENT qPCR as an alternative to the more time-consuming conventional culture-based methods. Using qPCR for recreational water monitoring minimizes processing time, with results typically available in less than 3 hours (Leecaster and Weisberg, 2001; Noble et al. 2010, Griffith and Weisberg, 2011). The new RWQC also includes recommendations for the increased use of scientifically-based predictive modeling efforts. Well-developed predictive models can eliminate the delay between sample collection and results by providing real-time estimates of FIB concentrations at beaches. Multiple linear regression (MLR) models are frequently used to predict recreational water quality (e.g. Olyphant et al., 2003; Olyphant and Whitman, 2004; Eleria and Vogel, 2005; Nevers and Whitman, 2005, 2011; Francy and Darner, 2007; Gonzalez et al., 2012). MLR is an empirical statistical modeling

approach that predicts FIB concentrations by relating water quality to antecedent rainfall, climate, and environmental parameters.

The RWQC document currently recommends the use of qPCR for ENT monitoring only, and the USEPA recently assigned a threshold limit for this method (USEPA, 2012). However, the USEPA has given approval for the use of EC qPCR at two beaches in Racine, WI (Kinzelman, 2012). Therefore it may be likely that EC qPCR will be approved by the USEPA during future RWQC revisions, perhaps on a site-to-site basis. Fecal coliforms (FC), of which the EC are a significant subset, are also currently used to monitor shellfish harvesting waters. Also, since shellfish harvesting water guidelines frequently parallel recreational water quality criteria (Rees et al., 2010), new shellfish harvesting water criteria allowing alternative rapid methods may develop in the near future.

North Carolina (NC) is one of the top beach visitation destinations in the United States, ranking 6th in beach tourism (NC Dept. of Commerce, 2013). In eastern NC there are 240 recreational monitoring sites and over 1025 shellfish harvesting water sites monitored on a regular basis. In addition, some of the locations are known as "dual beneficial use" serving both designated uses concomitantly (NCDMF, 2013). The NC Department of Environment and Natural Resources (NCDENR) conducts the monitoring programs for both recreational and shellfish harvesting waters in the state and has expressed interest in the use of both rapid methods and predictive models to issue public health advisories in near real-time. During the summer of 2012 NCDENR initiated a demonstration project to decide whether qPCR could be implemented in NC. As part of this test, ENT qPCR and ENT culture methods were used side-by-side for the same

recreational water quality samples. This preliminary comparison study yielded highly comparable results between the conventional culture-based methods and qPCR. However, results from this study indicated that during periods of rainfall and high winds there was often PCR inhibition (Haines, unpublished data). PCR inhibition is a problem that is frequently encountered when analyzing water samples, not only in mid-Atlantic estuaries, but also in other regions (Dorevitch et al. 2010; Griffith and Weisberg, 2011; Gonzalez et al., 2012). Inhibition of the PCR can result in false negative results, including non-detect outcomes during periods of beach contamination, and delayed reporting time to the public due to the need for sample reanalysis. Therefore, strategies to accurately quantify and minimize inhibition are needed before widespread use of rapid qPCR-based methods can be initiated (Cao et al., 2012; Haugland et al., 2012; Cao et al., 2013).

Several studies have compared FIB concentrations as determined by rapid qPCR and culture-based methods (e.g. Morrison et al., 2008; Lavender and Kinzelman, 2009; Noble et al., 2010; Whitman et al., 2010; Converse et al., 2012). However, few studies have compared statistical model performance using qPCR and culture data and this comparison has only been conducted in freshwater systems (Telech et al., 2009; Byappanahalli et al., 2010). While public health and water quality monitoring agencies are interested in both rapid qPCR methods and predictive models, to our knowledge there has been no examination of predictive models generated using both qPCR and culture-based data for estuarine or marine coastal waters.

The objective of this work was to generate predictive models using qPCR-based FIB data in waters that are listed as “dual beneficial use”, i.e. they are actively used for both recreational and shellfish harvesting purposes. Secondarily, these models were

compared to published culture-based real-time prediction models of ENT and EC in NC estuaries (Gonzalez et al. 2012). Specifically, ENT and EC qPCR and culture models were compared with respect to their correlative relationships to environmental variables, model variable selection, and model performance in the context of management decisions. Furthermore, a highly predictive empirical statistical model was created to predict qPCR inhibition level so that proper remediation techniques can be applied when it is identified as a problem. Importantly, with this approach, the remediation techniques for inhibition can take place prior to initiation of sample analysis, saving valuable time and resources.

3.2. Materials and Methods

3.2.1 Study site description

Water samples were collected along two tributaries of the Newport River Estuary in eastern NC, Ware and Oyster Creeks. These tributaries are used for both recreation and shellfish harvesting. Site descriptions and monitoring methods have been described in detail in Gonzalez et al., (2012) and Coulliette and Noble (2008). Briefly, the watersheds of both tributaries have less than 2 percent impervious cover and are dominated by herbaceous wetlands and evergreen forest.

3.2.2 Sample collection and monitoring approaches

One liter samples were collected in sterilized containers along the tributaries during 17 dry and 10 wet weather events from 7/9/2009 to 9/1/2011. The sampling scheme captured a wide range of meteorological and seasonal conditions. In total, 171 grab samples were collected and processed, as described by Gonzalez et al. (2012), no more than 4 hours after sample collection.

Rain, climate, and environmental parameters were collected with water samples. A multi-parameter sonde (6920 V2, YSI International, Yellow Springs, OH) was used to measure the following *in-situ* environmental parameters—water temperature, dissolved oxygen, turbidity, and conductivity. Salinity and pH were measured in the laboratory prior to sample processing using a refractometer and ion-selective probe, respectively. Precipitation data were collected from a rain gauge (HOBO Data Logging Rain Guage-RG3, Onset, Cape Cod, MA) installed in the subwatershed of Ware Creek. From the rain gauge we calculated current and antecedent rain total (1, 2, 3, 5, 7, 14, and 28 days prior to sample collection), antecedent dry period, and rain intensity. Additionally, concurrent and antecedent humidity, dew point, barometric pressure, and air temperature data were collected from a weather station (weather station ID: KNCBEAUF4) approximately 2.5 km away. Antecedent data were averaged for 1, 2, 7, 14, and 28 days prior to sample collection. These rain, climate, and environmental parameters were used for correlation and MLR analysis.

3.2.3 Fecal indicator bacteria enumeration

For this study, since the waters being assessed are used both for recreation and shellfish harvesting purposes, both ENT and EC measurements were conducted using both culture based and qPCR-based methods. Conventional, culture-based ENT and EC enumeration was conducted using the IDEXX defined substrate technology kits, Enterolert® and Colilert®-18, respectively (IDEXX Laboratories, Inc., Westbrook, ME). All samples were diluted 1:10 in deionized water as according to manufacturer instructions for ambient waters, and to generate quantitative information. Quantification

was conducted in 97-well most probable number (MPN) Quanti-tray®/2000 in conjunction with algorithms previously published by Hurley and Roscoe (1983).

Water samples of 50-100 ml volumes were filtered in duplicate using a six-place filtration manifold and vacuum pump assembly. The samples were filtered onto 0.45 µm pore size, 47 mm polycarbonate filters (HTTP, Millipore, Bedford, MA). Filtration was conducted to dryness and the filter funnel subsequently rinsed with 20 ml phosphate buffered saline (PBS) and then filtered to dryness. The polycarbonate filters were subsequently folded in half and placed into pre-labeled, sterile 2.0 ml screw-cap tubes containing 0.3 g of 1 mm zirconium silica beads (Biospec Corp., Bartlesville, OK) and were stored at -80 °C until batch analysis. Subsequent qPCR quantification of ENT and EC concentrations used primers, probes, and assays previously described by Cao et al., (2013) and Noble et al., (2010), respectively. Assays were performed in a SmartCycler II (Cepheid Inc., Sunnyvale, CA) with the following cycling conditions: 2 min at 95°C, followed by 45 cycles of 5 s at 94 °C and 43 s at 62 °C.

3.2.4 Assessment of qPCR inhibition

Performance of the qPCR assays through assessment of recovery efficiency and qPCR inhibition was measured using salmon testes DNA as a specimen processing control (SPC) as previously conducted by Haugland et al. (2005) and Cao et al. (2013). Stock solutions of salmon testes DNA were created to the final concentration of 10 ng/µl using AE buffer. The stock salmon testes DNA solution (100 ng or 0.2 ng/µl total) was pipetted into a 2.0 ml screw-cap tube containing 500 µl of AE buffer, to which filters were added from the samples, calibrators, and negative controls. Following this, mechanical lysis of the polycarbonate filters was conducted by bead-beating (Haugland et

al., 2005; Converse et al., 2009; Noble et al., 2010). Inhibition was assessed by determining the difference between the cycle threshold (C_T) of the SPC in samples with (experimental) and without (control, only SPC) target DNA. Samples with more than 0.5 log units (2.32 C_T) difference from control samples were deemed inhibited. All inhibited samples were purified with GeneRite DNA EZ-RW04 kit (GeneRite, New Brunswick, NJ) followed by a 1:5 dilution to resolve inhibition, which was re-tested.

For all qPCR runs, the following controls were implemented and showed no contamination: negative control, no template control, and negative extractions control. Calibration standards were created by growing *E. faecalis* (ATCC 29212) in brain heart infusion broth and *E. coli* (ATCC 25922) in tryptic soy broth overnight at 37˚C as described by Noble et al. (2010). Cell densities were enumerated by Enterolert® for ENT and Colilert®-18 for EC. Following quantification, the appropriate volume of stock target bacteria solution equating to 1 $x10^5$ cells per qPCR reaction was filtered onto polycarbonate filters as above, and cells were lysed using the same methodology as above. Standard curves for ENT and EC consisted of the calibration standard (at a concentration of 10^5 cells/5 µl) and four 10-fold serial dilutions that were run in duplicate. Average amplification efficiencies and standard curve R^2 values for the inhibition (salmon testes DNA), ENT, and EC qPCR assays are presented in Table 1.

The quantification model used to estimate qPCR concentrations was the comparative C_T method (ΔC_T) which is used to calculate results reported in units of cell equivalents (CE)/100 ml (Pfaffl, 2001). Multiple studies in California have used the ΔC_T quantification method to successfully implement rapid methods and improved rapid water quality notification at beaches based upon the existing water quality standards published

in California Assembly Bill AB411(Griffith and Weisberg, 2011; Cao et al., 2013). For both molecular assays, samples yielding C_T values below range of quantification on the respective calibration curve were designated as non-detects (detected but below the detection limit). For statistical analysis of qPCR-enumerated FIB results, non-detects were set to 5 CE/100 ml.

3.2.5 Data and statistical analysis

Statistics were performed in SAS 9.2 (Cary, NC) and OriginPro 8.5 (OriginLab, Northampton, MA). Normality was examined using histograms; it was deemed necessary to $\log_{10}$-transform MPN and CE based concentrations to reduce skewness prior to analysis. Data were pooled by analytical methods before checking for differences in means with student t-tests (culture methods n=173; ENT qPCR n=124; EC qPCR n=109).

Pearson's correlation coefficients (r) were calculated using OriginPro statistical software. To further understand the relationships between FIB groups and variables as well as the relationships among the FIB groups themselves, we pooled concentrations across analytical methods. Significance was set at alpha = 0.05.

Stepwise MLR models maximized adjusted R^2 and were calculated in SAS using the PROC GLMSELECT function. Each model was limited to five explanatory variables in order to prevent overparamatization. The PROC REG function was used to calculate variance inflation factors (VIF) in order to determine if collinearity issues were likely in the MLR analysis. When VIF values were greater than 10, we concluded that collinearity among predictor variables in the MLR model were strong enough to warrant corrective action.

Model performance was based on accuracy of predicted management decisions—did regression models accurately predict FIB concentrations above or below numeric concentration thresholds? Specifically, percent type I errors, percent type II errors, and percent correct (100 – percent errors) were examined. Type I and II errors are false positives and false negatives, respectively. A false positive (type I error) occurs if the MLR model predicts that bacterial levels are high enough to warrant posting or closing a recreational or shellfish harvesting area (exceeding a water quality threshold), but the actual bacterial levels do not justify such a closing. A false negative (type II error) occurs if the model recommends that an area should remain open (predictions fall below a water quality threshold), but actual contamination levels warrant closure.

The thresholds selected for our analysis are those new criteria values that closely approximate the old values being used, as these are likely to be selected for implementation based upon our interaction with water quality managers (e.g. J. D. Potts, personal communication; Dr. J. Griffith, personal communication). We did this to provide useful information to users during this period of transition. We judged culture-based ENT error types based on a 110 MPN or CFU/100 ml statistical threshold value (STV) (USEPA, 2012). The 2012 RWQC uses STV as a value where no more 10% of samples taken should exceed this threshold. In the case of only one sample being collected, however, it acts as a single sample threshold, which was used in the 1986 criteria. We used the more conservative of USEPA's culture-based ENT threshold (110 MPN/100 ml) that relates to an illness rate of 32 per 1000 primary contact recreators as opposed to 36 per 1000 primary contact recreators. We judged error types for ENT qPCR-generated data using two thresholds: 110 and 1000 CE/100 ml. The use of the 110

MPN or CFU/100 ml culture threshold on qPCR data is more conservative than USEPA's qPCR RWQC and has been previously used in CA monitoring programs (Griffith and Weisberg, 2011; Cao et al., 2013). The 1000 CE/100 ml threshold is also more conservative than the 2012 RWQC criteria 1280 calibrator cell equivalents (CCE)/100 ml STV that relates to an illness rate of 32 per 1000 primary contact recreators and utilizes the ΔC_T quantification method that the more conservative CA studies use.

Similarly, we judged culture-based EC based on a 320 MPN or CFU/100 ml STV that also relates to an illness rate in 32 per 1000 primary contact recreators (USEPA, 2012). To judge errors based on shellfish harvesting water standards currently used in the U.S., we used the median standard of 14 MPN or CFU/100 ml of EC as a proxy for FC (NSSP, 2011; Coulliette et al., 2009; Gonzalez et al., 2012). EC qPCR-generated data were analyzed using two thresholds (14 and 320 CE/100 ml). The 14 CE/100 ml is based on the 14 MPN or CFU/100 ml median National Shellfish Sanitation Program Guide standards. The 320 CE/100 ml is a conservative threshold based on USEPA's 320 MPN or CFU/100ml STV for fresh waters.

Inhibition is an important factor in the usefulness of qPCR in water quality monitoring (Cao et al., 2012; Haugland et al., 2012). It can cause loss of resources through the loss of usable qPCR data. Inhibition also can cause false negative results, causing a circumstance where the public is not adequately protected during periods of high inhibition and high contamination, which can occur often after storm events (Noble et al., in preparation). An inhibition MLR model was created, using the same procedure as above, to predict the level of an inhibition metric (C_T delay between sample and control using SPC assay) when analyzing a water sample. The larger the inhibition

metric, the more inhibited the sample. The inhibition metric was used as the independent variable and rain, climate, and environmental parameters were tested as predictor (dependent) variables.

3.3. Results

3.3.1 Summary statistics

Mean and ranges of qPCR and culture data are presented in Figure 1. Our data was collected over a wide range of environmental conditions, and spanned a wide range of concentrations for both ENT and EC. Mean log ENT concentrations as determined via qPCR analyses were 4.38 (SD= 4.95) with corresponding mean log ENT concentrations via culture-based analyses of 3.77 (SD=4.25). Mean log EC concentrations as determined by qPCR were 3.62 (SD =3.97) with corresponding culture-based concentrations of 3.45 (SD=3.69). There was a significant difference (p= 0.010) between the concentrations of ENT across method class (qPCR versus culture), but not between the EC analytical methods (p=0.101). There was a pattern of qPCR data having higher means and variances than culture-based data. The ENT qPCR concentrations exceeded the 110 and 1000 CE/100 ml threshold 78% (96/123) and 46.3 % (57/123) of the time, respectively, with the ENT culture concentrations exceeding the 110 MPN or CFU/100ml STV 64.9% (113/173) of the time. EC culture data exceeded the 320 MPN or CFU/100 ml recreational STV 66.5% (115/173) of the time. Using the EC culture data with the 14 MPN or CFU/100ml shellfish harvesting threshold, 97.7% (169/173) of samples exceeded. Using the EC qPCR generated data and two potential thresholds of 14 and 320 CE/100 ml produced 90.7% (98/108) and 62.0% (67/108) exceedances, respectively.

3.3.2 Correlations

All FIB concentration comparisons across method class were significantly correlated. The two ENT analytical methods had a strong, significant relationship (r=0.60, $p<0.0001$, n=123), while the two EC analytical methods has a slightly weaker but still significant correlation (r=0.54, $p<0.0001$, n=108). When comparing EC and ENT concentrations as determined by qPCR the correlation was strong (r=0.56, $p<0.0001$, n=108), while the two culture-based methods also showed a strong relationship (r=0.73, $p<0.0001$, n=173).

Correlations between FIB enumerated by the different analytical methods and select variables can be seen in Table 2. ENT qPCR concentrations were significantly correlated with 7 of the 12 variables tested. The strongest correlations occurred with rain intensity (r=0.56), barometric pressure (r=-0.51), and salinity (r=-0.47). ENT culture concentrations were significantly correlated with 6 of the 12 variables tested. The highest correlations were storm total (r=0.50), rain intensity (r=0.49), and salinity (r=-0.49). While most of the variables correlated with qPCR- and culture-based ENT were the same, there were some slight differences. The qPCR enumerated ENT concentrations were correlated with barometric pressure and water temperature while culture-based ENT was not. Culture-based ENT was correlated with dissolved oxygen, while it was not correlated with qPCR ENT concentrations.

EC qPCR concentrations were significantly correlated with 6 of the 12 variables tested. The strongest correlations were with rain intensity (r=0.39), barometric pressure (r=0.38), and storm total (r=0.36). EC culture concentrations were significantly correlated with 11 of the 12 variables tested. The strongest correlations were storm total (r=0.43),

dissolved oxygen (r=-0.43), and rain intensity (r=0.34). There were many cases where culture-based EC concentrations were correlated with variables that were not correlated with qPCR-based variables—water temperature, turbidity, dissolved oxygen, and dew point.

3.3.3 Model variable selection

All models and their predictor variables had significant *p*-values and all VIF were low, indicating no severe collinearity. In addition, each model used a salinity/conductivity variable and all but the EC qPCR model used dissolved oxygen. Also, some metric of current or antecedent rainfall was incorporated into all models (e.g. 14-day rain total, 28-day rain total, 5-day rain total, storm total). There were no changes in coefficient sign across analytical types but the amount of variability explained by the EC qPCR model was less than half that of the EC culture model.

Variable selection and parameter estimates for both the models generated using each ENT analytical method type are in Table 3. The ENT qPCR model showed 89% of the variability in ENT concentration explained by just five parameters; 14-day rain total, barometric pressure, 7-day air temperature, dissolved oxygen, and conductivity. Similarly, five variables explained 90% of the variation in the culture-based ENT concentrations: 28-day rain total, 14-day rain total, 28-day humidity, dissolved oxygen, and salinity. Fourteen day rain total, dissolved oxygen and salinity/conductivity were found to be important predictors for both analytical methods. Interestingly rain intensity was among the variables that had the strongest correlations with both analytical methods, but was not selected in either model.

Variable selection and parameter estimates for both EC analytical methods are in Table 3. The variables that explained 26% of the variation for EC qPCR endpoints were barometric pressure, 5-day rain total, 14-day humidity, 28-day air temperature, and salinity. The variables that explained 55% of the variability in EC culture-based model were storm total, dissolved oxygen, 14-day humidity, conductivity, and air temperature. Both models included 14-day humidity and salinity/conductivity in addition to rain/storm metrics (5-day rain total and storm total) and a measure of air temperature (28-day air temperature and air temperature). Similar to the ENT models, rain intensity was one of the variables most strongly correlated to EC concentrations, but was not selected in either EC model.

3.3.4 Model performance

The threshold analysis for all ENT and EC models across both analytical methods can be seen in Table 4. For each model, the percent of correct management decisions were all above 77%. The ENT predictive models had percent correct management decisions above 90%, with 92, 96 and 90%, based upon the ENT qPCR (110 and 1000 CE/100 ml) and culture (110 MPN or CFU/ 100 ml) thresholds respectively. The EC culture model, using the 14 MPN or CFU/100 ml shellfish harvesting threshold, had a 99% correct rate in management decision. The EC culture model, using the USEPA recommended STV of 320 MPN or CFU/100 ml, had an 88 % correct rate for management decisions. Using the same 2 thresholds as the EC culture model, the EC qPCR had 89 and 77% correct decisions, respectively. Type I error rates were higher than the type II error rates for all models except for the ENT qPCR model tested at the 110 CE/100 ml thresholds, and the EC culture model tested at the 14 MPN or CFU/100 ml

threshold. These cases had the same Type I and II error rates which were both low—all below 4%.

3.3.5 Inhibition model

Table 5 shows an empirical statistical model created to predict levels of inhibition. The inhibition model created explained 82% of the qPCR generated inhibition metric data variation by three variables (in order of importance): Rain intensity, turbidity, and 7-day air temperature.

3.4. Discussion

In this study we quantified EC and ENT levels in recreational waters using qPCR- and culture- based methods and then compared these levels to environmental variables. Statistical model performances created by qPCR- and culture-based concentrations were then compared to determine model equivalency. We found significant strong relationships between qPCR and culture calculated concentrations of both ENT and EC. For ENT measurements, both qPCR and culture methods had very similar correlations to the same environmental variables. In contrast, EC culture density measurements had more correlations with environmental variables, but only two-thirds of the strongest correlations were shared with qPCR measurements (i.e. storm total and rain intensity). Within ENT and EC MLR models, qPCR- and culture-based models selected similar explanatory variables.

ENT measurements and models across qPCR and culture methods showed high equivalency. Our finding that both qPCR and culture calculated ENT concentrations had similar relationships to environmental parameters was unlike that of Byappanahalli et al.

(2010) where the differences between the environmental responses of the two analytical method endpoints is emphasized by selected predictors and model results. The similarities between qPCR and culture method results for ENT measurements were also seen in model development and performance. The models for both methods had very similar variable selection; specifically they both included 14-day rain total, dissolved oxygen, and salinity/conductivity and did not include rain intensity even though this variable was strongly correlated to each method. The adjusted R^2 values for ENT models, 0.89 for qPCR and 0.90 for culture, explain almost the same amount of data variation. This trend was not seen by Telech et al. (2009) where they found that models created (using similar environmental variables) accounted for different amounts of variation across laboratory methods. Using the ENT qPCR thresholds and the standard culture threshold, the accuracy in management decisions was high. Both models performed well—the qPCR model had 92 and 96% accuracy with the 110 and 1000 CE/100 ml thresholds, respectively, and the culture model had 90% accuracy in management decisions.

For EC quantification methods, equivalency was not as well defined across all areas examined as ENT models were. Unlike ENT measurements, EC culture-based quantification had stronger and more numerous correlations to environmental variables than those for qPCR; however, the majority of the strongest correlated environmental variables for both methods were the same. With respect to EC model creation, the predictor variables were fairly similar (14-day humidity, salinity/conductivity, a rain/storm variable, and a measure of air temperature) but accounted for a major difference in the amount of variation explained by the model—qPCR model $R^2$was 0.26

whereas culture model R^2 was 0.55. Unlike our findings with ENT models comparison, this difference in the explanatory power of EC models between laboratory methods is similar to findings in other studies (Telech et al., 2009). The differences in correlations to select environmental variables and differences in model R^2 are probably due to the inhibition seen in the samples. At low concentrations, even small amounts of inhibition can cause high measurement variability due to the exponential nature of the delay in amplification of the DNA. In addition, low concentrations of target are associated with high levels of variability in qPCR-based analyses for a range of other reasons (e.g. Converse et al. 2012).

There is no currently recommended, USEPA-approved threshold for EC qPCR, but others are utilizing EC qPCR to manage specific beaches, so we adopted the threshold that is currently being used in those scenarios (Lavender and Kinzelman, 2009).When using thresholds that were orders of magnitude different, EC models accurately predicted management decisions a high percentage of the time. qPCR models predicted 89 and 77% correct decisions (using 14 and 320 CE/100 ml thresholds, respectively) while culture models predicted 99 and 88 % correct decisions (with 14 and 320 MPN or CFU/100 ml threshold, respectively). Additionally, a major finding is that when the two thresholds (each logs apart) were tested using the qPCR models, more exceedances occurred when moving from a higher threshold to a lower one, as expected. However, predictability did not decrease. This predictability could be due to the non-point sources of contamination and high background contamination at our study site throughout the year.

Like Telech et al. (2009), R^2 was not related to the percentage of correct management decisions. However, unlike Telech et al. (2009), we did not see a decrease in the percentage of correct management decisions as exceedances above the threshold increased. These results show that when judging models, researchers need to look at a combination of factors (adjusted R^2, errors, fit, accuracy) in order to effectively assess model performance.

Overall, equivalency between models was shown in our non-point source impacted estuaries. We did not see large differences between environmental responses across the two analytical methods like Telech et al. (2009) and Byappanahalli et al. (2010) but did see higher concentration and variance in qPCR results like He and Jiang (2005), Morrison et al. (2008), and Byappanahali et al. (2010). In addition, ENT correlations and models seemed to perform slightly better than EC models. When assigning EC qPCR threshold levels, the USEPA should take note of these differences. In fact, model development may help the local water quality manager interpret differences observed between advisories and closures in dual beneficial use waters, assisting the manager with appropriate protection of the public. Overall we saw high accuracy in management decisions for all models. The model equivalency between qPCR and culture data as well as the accurate management predictions based on these models could be due to the wide range of sampling conditions (0 to 20.3 cm of rain) covered during this study. It will be vital for others conducting model development to sample during a wide range of conditions and seasons to generate information representative of their estuaries and beaches. In addition, some of the model equivalency could be attributed to the fact that we did not include environmental factors like solar irradiance and wind that have been

shown to affect qPCR and culture based measurements differently (Noble et al., 2006; Telech et al, 2009; Shibata et al., 2010; Converse et al. 2012).

Although model equivalency was established in this study across analytical methods, inhibition was a major issue that had to be addressed. Inhibition is a problem in many environmental samples, but due to visibly high concentrations of dissolved and particulate organic matter and high residence time of mid-Atlantic estuaries, the problem can be compounded (Tsai and Olson, 1992; Paerl et al., 1998; Leuttich et al., 2000). NC waters are high in humic and tannic acid complexes, which are visibly apparent in the sample bottle when sampling in estuarine systems, especially during spring, summer and fall (Thurman and Malcolm, 1981). In qPCR samples, inhibition extends sample processing times due to the need for additional sample processing and reanalysis, accounts for high measurement variation at low concentrations, and causes excessive resource expenditures. Empirical models that predict inhibition magnitude prior to the use of the rapid qPCR-based methods and based on easy to access climate variables would therefore improve the rate of successful application of qPCR testing in estuarine and coastal waters. By using an SPC to calculate an inhibition metric in advance of the implementation of rapid qPCR-based methods, predicting inhibition will expedite the decision process on which inhibition remediation method would be most efficient for timely qPCR results and public notifications (Haugland et al., 2012). The inhibition model presented here accounted for a high level of data variability (adjusted R^2) and was created with easily obtained meteorological data. Its predictive power indicates that similar models may be widely applicable. Future work should more vigorously test this

model under a wide range of environmental and meteorological conditions, as well as different recreational site types (open beaches, enclosed beaches, estuaries).

The primary disadvantage of culture based methods is the long incubation time required, which delays the period of time to public notification and causes beaches to be closed after the contamination has already passed. More rapid monitoring methods such as qPCR and predictive modeling have been suggested for improved beach management in the newly released RWQC (USEPA, 2012). This work demonstrates a combined assessment of both, and shows that both approaches can be used in tandem to provide frequent estimates of water quality under changing conditions in estuaries and on beaches. It is therefore advisable that mangers in both NC (like NCDENR) and other states use a combination of rapid methods, like those presented in this study, to provide rapid water quality assessment for the communities they serve. Furthermore, this work demonstrates that it could be fruitful to mine historical environmental and meteorological data for previously conducted large scale water quality studies that have included measurements of both culture-based and qPCR-based FIB, for the generation of similar predictive models in other regions. This type of retrospective model development may assist the manager in the future implementation of the most appropriate tools during this important period of transition in water quality management.

3.5. Conclusions

- This combined assessment of qPCR and MLR shows that both approaches can be used in tandem to provide rapid estimates of water quality in non-point source impacted estuaries.

- Overall, equivalency between ENT and EC MLR models across analytical methods was shown.

- Although model equivalency was established, inhibition was a major issue that had to be addressed. The inhibition model presented here accounted for a high level of data variability and was created with easily obtained meteorological data. Its predictive power indicates that similar models may be widely applicable.

- Furthermore, this work demonstrates that it could be productive to mine historical environmental and meteorological data for previously conducted large scale water quality studies that have included measurements of both culture- and qPCR-based FIB, for the generation of similar predictive models in other regions.

Figures

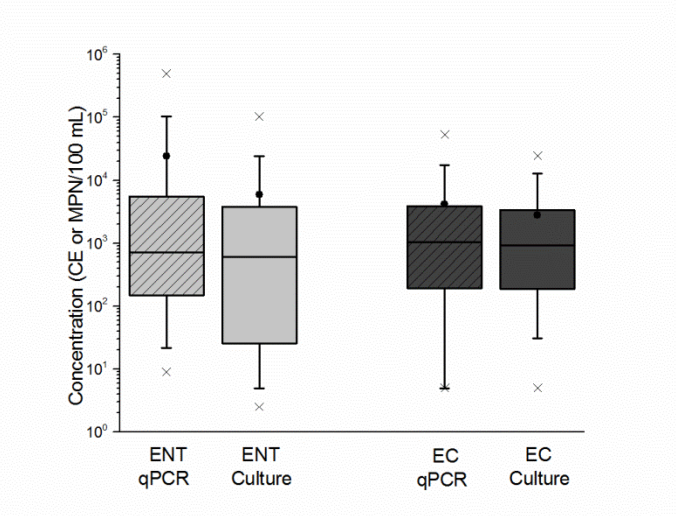

Figure 3.1. Box and whisker plots of enterococci (ENT) and *E.coli* (EC) (most probable number (MPN)/100 ml or cell equivalents (CE)/100 ml) as determined by quantitative PCR and culture analytical methods. Box range is the 25^{th} – 75^{th} percentile. Whisker range is 5^{th} – 95^{th} percentile. Means represented by solid black circles and medians are horizontal solid lines within each box.

Tables

Assay	Target	N	Amplification Efficiency (%)	R^2	Quantification Range
sketascorp	salmon testes DNA	1	0.95	0.98	NA
Total *Enterococcus*	enterococci	2	0.89	1.00	10 to 10^5
E. coli species	*E. coli*	2	0.97	0.99	10 to 10^5

Table 3.1. qPCR amplification efficiencies, standard curve R^2 values, and quantification range.

	log ENT		log EC	
Variables	**qPCR**	**Culture**	**qPCR**	**Culture**
Salinity	-0.47 (<0.0001)	-0.49 (<0.0001)	-0.28 (0.0036)	-0.17 (0.0254)
pH	-0.34 (<0.0001)	-0.34 (<0.0001)	-	-0.18 (0.0186)
Water Temperature	-0.38 (<0.0001)	-	-	0.20 (0.0167)
Turbidity	-	-	-	0.26 (<0.0001)
Dissolved Oxygen	-	-0.22 (0.0074)	-	-0.43 (<0.0001)
Storm total	0.46 (<0.0001)	0.50 (<0.0001)	0.36 (<0.0001)	0.43 (<0.0001)
Rain Intensity	0.56 (<0.0001)	0.49 (<0.0001)	0.39 (<0.0001)	0.34 (<0.0001)
Antecedent Dry Period	-	-	-	-
Humidity	0.22 (0.0138)	0.28 (<0.0001)	0.22 (0.0205)	0.23 (0.0022)
Dew Point	-	-	-	0.22 (0.0036)
Air Temperature	-	-	0.20 (0.0424)	0.31(<0.0001)
Barometric Pressure	-0.51(<0.0001)	-	0.38 (<0.0001)	-0.31(<0.0001)

Table 3.2. Pearson correlation coefficients between enterococci (ENT) and *E.coli* (EC) concentrations by different analytical methods (quantitative PCR [CE/100 ml] and culture [MPN/100 ml]) and select climate and environmental variables. All correlations shown have *p*-values < 0.05 (shown in parentheses).

	log ENT		log EC	
	qPCR	Culture	qPCR	Culture
Adj. R^2	0.89	0.90	0.26	0.55
RMSE	0.8927	0.8247	0.8836	0.5526
Intercept	98	-1.1	66	0.77
Var. 1	14-day Rain (0.20)	28-day Rain (0.10)	Barometric Press. (-2.2)	Storm Total (0.13)
Var. 2	Barometric Press. (-3.1)	14-day Rain (0.06)	5-day Rain (0.08)	Dissolved Oxy. (-0.16)
Var. 3	7-day Air Temp. (-0.50)	28-day Humidity (0.07)	14-day Humidity (0.03)	14-day Humidity (0.03)
Var. 4	Dissolved Oxy. (-0.14)	Dissolved Oxy. (-0.28)	28-day Air Temp. (0.05)	Conductivity (-0.41)
Var. 5	Conductivity (-0.01)	Salinity (-0.22)	Salinity (-0.02)	Air Temp. (0.05)

Table 3.3. Multiple linear regression models of quantitative PCR-based enterococci (ENT), culture-based ENT, quantitative PCR-based E. coli (EC), and culture-based EC. qPCR- and culture-based concentrations reported in CE or MPN/100 ml. Samples were collected during a wide range of meteorological and seasonal conditions from July 2009 to September 2011. Predictor variables remained untransformed during analysis and variable regression coefficients are in parentheses.

Model	N	Threshold Tested	Type I Error Rate (%)	Type II Error Rate (%)	Percent Correct
ENT Culture	147	110	7	3	90
ENT qPCR	104	110	4	4	92
		1000	4	0	96
EC Culture	150	14	1	1	99
		320	10	2	88
EC qPCR	103	14	11	0	89
		320	15	9	77

Table 3.4. Summary of quantitative PCR-based enterococci (ENT), culture-based ENT, quantitative PCR-based *E. coli* (EC), and culture-based EC model performances using currently recommended, existing, or estimated FIB thresholds.

n= 107	RMSE= 0.6489	Adj. R^2= 0.82	
Parameter	Estimate ± s.e.	% Variation Described	*p* -value
Intercept	9.26 ± 0.842	-	< 0.0001
Rain Intensity	151 ± 75.2	57	0.0490
Turbidity	0.009 ± 0.001	35	< 0.0001
7-day Air Temp.	0.209 ± 0.090	7	< 0.0001

Table 3.5. Multiple linear regression model of ΔCT values to predict inhibition levels in water samples.

References

Byappanahalli, M. N., Whitman, R. L., Shively, D. A., Nevers, M. B., 2010. Linking non-culturable (qPCR) and culturable enterococci densities with hydrometeorological conditions. Science of the Total Environment 408 (16), 3096-3101.

Cao, Y., Raith, M. R., Griffith, J. F., 2013. Evaluation of alternative *Enterococcus* qPCR methods for recreational water monitoring. In: American Society for Microbiology, General meeting, Denver, CO, May 18-21, 2013.

Cao, Y., Griffith, J. F., Dorevitch, S., Weisberg, S. B., 2012. Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring *Enterococcus* in environmental waters. Journal of Applied Microbiology 113 (1), 66-75.

Converse, R. R., Griffith, J. F., Noble, R. T., Haugland, R. A., Schiff, K. C., Weisberg, S. B., 2012. Correlation between quantitative PCR and culture-based methods for measuring Enterococcus spp. over various temporal scales at three California marine beaches. Applied and Environmental Microbiology 78 (4), 1237-1242.

Converse, R. R., Blackwood, A. D., Kirs, M., Griffith, J. F., Noble, R. T., 2009. Rapid QPCR-based assay for fecal *Bacteroides* spp. as a tool for assessing fecal contamination in recreational waters. Water Research 43 (19), 4828-4837.

Coulliette, A. A. D. and Noble, R. T., 2008. Impacts of rainfall on the water quality of the Newport River Estuary (eastern North Carolina, USA). Journal of Water and Health 06 (4), 473.

Coulliette, A. D., Money, E. S., Serre, M. L., Noble, R. T., 2009. Space/time analysis of fecal pollution and rainfall in an eastern North Carolina estuary. Environmental Science & Technology 43 (10), 3728-3735.

NC Department of Commerce, 2013. Fast facts, 2012 impact of visitor spending. http://www.nccommerce.com/LinkClick.aspx?fileticket=qwtGIIJxT28%3d&tabid=636&mid=4669.

Dorevitch, S., Ashbolt, N. J., Ferguson, C. M., Fujioka, R., McGee, C. D., Soller, J. A., Whitman, R. L., 2010. Meeting report: knowledge and gaps in developing microbial criteria for inland recreational waters. Environmental Health Perspectives 118 (6), 871.

Eleria, A. and Vogel, R. M., 2005. Predicting fecal coliform bacteria levels in the Charles River, Massachusetts, USA. Journal of the American Water Resources Association 41 (5), 1195-1209.

Francy, D. S. and Darner, R. A., 2007. Nowcasting beach advisories at Ohio Lake Erie beaches. Environmental Science & Technology 42 (13), 4818-4824.

Gonzalez, R. A., Conn, K. E., Crosswell, J. R., Noble, R. T., 2012. Application of empirical predictive modeling using conventional and alternative fecal indicator bacteria in eastern North Carolina waters. Water Research 46 (18), 5871-5882.

Griffith, J. F. and Weisberg, S. B., 2011. Challenges in implementing new technology for beach water quality monitoring: Lessons from a California demonstration project. Marine Technology Society Journal 45 (2), 65-73.

Griffith, J. F., 2013. Personal communication. Southern California Coastal Water Research Program.

Haugland, R. A., Siefring, S., Lavender, J., Varma, M., 2012. Influences of sample interference and interference controls on quantification of enterococci fecal indicator bacteria in surface water samples by the qPCR method. Water Research 46 (18), 5989-6001.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., Dufour, A. P., 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research 39 (4), 559-568.

He, J. and Jiang, S., 2005. Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Applied and Environmental Microbiology 71 (5), 2250-2255.

Hurley, M. A. and Roscoe, M. E., 1983. Automated statistical analysis of microbial enumeration by dilution series. Journal of Applied Microbiology 55 (1), 159-164.

Kinzelman, J. L., 2012. qPCR monitoring data and the proposed criteria revisions - One local lab's journey towards implementation. In: Great Lakes Beach Association Annual Conference, Mackinac Island, MI, October 16, 2012 .

Lavender, J. S. and Kinzelman, J. L., 2009. A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of *Escherichia coli* and enterococci in municipal water quality monitoring programs. Water Research 43 (19), 4967-4979.

Leecaster, M. K. and Weisberg, S. B., 2001. Effect of sampling frequency on shoreline microbiology assessments. Marine Pollution Bulletin 42 (11), 1150-1154.

Luettich, R., ReynoldsFleming, J. V., McNinch, J. E., Buzzelli, C. P., 2000. Circulation characteristics of the Neuse River Estuary, North Carolina. Estuaries 20.

Morrison, C. R., Bachoon, D. S., Gates, K. W., 2008. Quantification of enterococci and bifidobacteria in Georgia estuaries using conventional and molecular methods. Water Research 42 (14), 4001-4009.

NSSP, 2011. National shellfish sanitation program guide for the control of molluscan shellfish.

http://www.fda.gov/downloads/Food/GuidanceRegulation/FederalStateFoodPrograms/UCM350344.pdf.

NCDMF, 2013. North Carolina shellfish sanitation and recreational water quality section of the division of marine fisheries. http://portal.ncdenr.org/web/mf/shellfish-sanitation-and-recreational-water-quality.

Nevers, M. B. and Whitman, R. L., 2011. Efficacy of monitoring and empirical predictive modeling at improving public health protection at Chicago beaches. Water Research 45 (4), 1659-1668.

Nevers, M. B. and Whitman, R. L., 2005. Nowcast modeling of *Escherichia coli* concentrations at multiple urban beaches of southern Lake Michigan. Water Research 39 (20), 5250-5260.

Noble, R. and Weisberg, R., 2005. A review of technologies for rapid detection of bacteria in recreational waters. Journal of Water and Health 3(4), 381-392.

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., Weisberg, S. B., 2010. Comparison of rapid quantitative PCR-based and conventional culture-based methods for enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters. Applied and Environmental Microbiology 76 (22), 7437-7443.

Noble, R.T., Gonzalez, R.A., and Blackwood, A.D., in preparation. Impact of stormwater discharge and intensity on qPCR inhibition.

Noble, R. T., Griffith, J. F., Blackwood, A. D., Fuhrman, J. A., Gregory, J. B., Hernandez, X., Liang, X., Bera, A. A., Schiff, K., 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Applied and Environmental Microbiology 72 (2), 1604-1612.

Olyphant, G. A. and Whitman, R. L., 2004. Elements of a predictive model for determining beach closures on a real time basis: the case of 63rd street Beach Chicago. Environmental Monitoring and Assessment 98 (1), 175-190.

Olyphant, G. A., Thomas, J., Whitman, R. L., Harper, D., 2003. Characterization and statistical modeling of bacterial (*Escherichia coli*) outflows from watersheds that discharge into southern Lake Michigan. Environmental Monitoring and Assessment 81 (1), 289-300.

Paerl, H. W., Pinckney, J. L., Fear, J. M., Peierls, B. L., 1998. Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA. Marine Ecology Progress Series 166, 17-25.

Pfaffl, M. W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 29 (9), e45-e45.

Potts, J. D., 2013. Personal communication. NC Department of Environment and Natural Resources.

Rees, G., Pond, K., Bartram, J., Santo Domingo, J. (eds.), 2010. *Safe management of shellfish and harvest waters*. IWA Publishing (International Water Association).

Shibata, T., Solo-Gabriele, H. M., Sinigalliano, C. D., Gidley, M. L., Plano, L. R., Fleisher, J. M., Wang, J. D., Elmir, S. M., He, G., Wright, M. E., 2010. Evaluation of conventional and alternative monitoring methods for a recreational marine beach with non-point source of fecal contamination. Environmental Science & Technology 44 (21), 8175.

Telech, J. W., Brenner, K. P., Haugland, R., Sams, E., Dufour, A. P., Wymer, L., Wade, T. J., 2009. Modeling Enterococcus densities measured by quantitative polymerase chain reaction and membrane filtration using environmental conditions at four Great Lakes beaches. Water Research 43 (19), 4947-4955.

Thurman, E. M. and Malcolm, R. L., 1981. Preparative isolation of aquatic humic substances. Environmental Science & Technology 15 (4), 463-466.

Tsai, Y. L. and Olson, B. H., 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Applied and Environmental Microbiology 58 (7), 2292-2295.

USEPA, 2012. Recreational Water Quality Criteria. http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/upload/RWQC2012.pdf.

Whitman, R. L., Ge, Z., Nevers, M. B., Boehm, A. B., Chern, E. C., Haugland, R. A., Lukasik, A. M., Molina, M., Przybyla-Kelly, K., Shively, D. A., White, E. M., Zepp, R. G., Byappanahalli, M. N., 2010. Relationship and variation of qPCR and culturable enterococci estimates in ambient surface waters are predictable. Environmental Science & Technology 44 (13), 5049-5054.

Chapter 4

FECAL BACTERIA FLUX INTO THE NEWPORT RIVER ESTUARY, NORTH CAROLINA: RELATIONSHIPS TO HYDRODYNAMICS AND MICROBIAL SOURCE TRACKING MARKERS

Overview

In order to limit exposure to disease causing pathogens, managers monitor fecal indicator bacteria (FIB) as proxies for watershed contamination. Water bodies that do not meet water quality standards are classified as impaired and must have a total maximum daily load (TMDL). The TMDL process (1) determines the existing and potential pollutant loads in the watershed (2) links the loads to contaminations sources and (3) outlines the best methods for ameliorating any problems. In order to determine contamination sources for TDML, microbial source tracking (MST) can be used to determine if FIB are primarily of fecal origin and if the fecal pollution is human, which is more dangerous, or non-human, which is associated with less public disease risk. To do this MST studies often document the presence of obligate anaerobes in the genus *Bacteroides* using quantitative PCR (qPCR), as these bacteria indicate a recent fecal contamination event in aerobic waters were they cannot survive long. In this study, we quantified traditional FIB (fecal coliforms (FC), *E. coli* (EC), and enterococci (ENT)) and three MST markers (fecal *Bacteroides* spp., human *Bacteroides* spp. (BacHum), and gull2) under a range of precipitation levels in a NC estuary. Using these measurements, we determined that during wet weather, all FIB markers were three to four log

concentrations higher than measured under ambient, dry conditions. In addition, FIB loading was significantly higher when precipitation during a wet weather event rose above 2.54 cm, a level that is lower than the current management action threshold (3.81 cm). Fecal *Bacteroides* spp. MST marker recovery was low, which may indicate environmental sources of traditional FIB within the estuary. In addition, BacHum MST markers, which indicated human contamination, were only detected during four storm events, where rainfall exceeded the 3.81 cm management action threshold. The gull2 MST marker was only detected in one sample, indicating that shorebirds are likely minimal contributors to estuarine fecal contamination. Our results highlight the need to identify and quantify potential *in situ* sources of FIB contamination in estuarine waters before TDML and BMP can be determined.

4.1. Introduction

Estuaries have a multitude of uses including shellfish harvesting and recreation. It is predicted that by 2020 coastal populations in the United States will grow by 9%, an increase equivalent to approximately 14.9 million people (NOAA, 2013). These coastal populations will be more subject to risks associated with poor microbial water quality, including contaminated shellfish beds and recreation areas. Accordingly, it is of the utmost importance to monitor the microbial water quality of estuarine waters to appropriately gauge human health risk to people coming into contact with coastal and estuarine waters.

Typically, microbial water quality is assessed by enumerating fecal indicator bacteria (FIB) as proxies for human pathogens that can be present in fecal contamination. Historically, the FIB used in monitoring regimes have been fecal coliforms (FC), *E. coli*

(EC), and/or enterococci (ENT), used either alone or in combination depending on the region, province, or state; with the water body location and intended use dictating the FIB that is most appropriate. Recreational freshwater and shellfish harvesting waters are generally regulated through the use of FC or EC standards. Coastal and estuarine swimming waters are typically monitored for and managed using ENT standards. Current FIB standards can be seen in Table 1 which has been modified from Myers et al. (2007).

While monitoring FIB in coastal waters provides information that can be used to protect the public from exposure to harmful bacteria and viruses, this monitoring alone, does not provide information on sources of contamination (Kinzelman et al., 2011). Human health risks associated with human fecal waste are considered to be higher when compared to non-human (i.e. wildlife or domesticated animals) fecal waste (Soller et al., 2010; Roslev and Bukh, 2011). Only with microbial source tracking (MST) techniques can the sources of fecal contamination be identified, and sometimes quantified. MST methods can generally be divided into library dependent and library independent methods (Field and Samadpour, 2007; Santo Domingo et al., 2007; Stoeckel and Harwood, 2007; Mott and Smith, 2011; Wuertz et al., 2011). Library independent methods include both chemical and molecular markers. These methods may be very different with regard to detection technique and their application, but their popularity has dominated over the library dependent methods (Mott and Smith, 2011). Library dependent methods require the creation of a library of fecal isolates from different known animal sources that are used for comparison with environmental isolates to determine sources of contamination (Wuertz et al., 2011). Although there has been an exponential increase in the number of MST related citations in the past 5 years, there is a clear recent trend toward the use of

qPCR techniques to quantify markers of fecal contamination from the *Bacteroidales* family.

Because they are obligate anaerobes, the presence of fecal *Bacteroides* spp., in oxygenated estuarine waters, is often indicative of recent fecal contamination (Kreader, 1995; Converse et al., 2009; Ahmed et al., 2009). Because culture-based quantification of members of the *Bacteroidales* group can be difficult and time consuming, rapid molecular methods such as quantitative PCR (qPCR) have been used consistently across the past decade as a useful approach for detecting members of the *Bacteroidales* group (e.g. Prslev and Bukh, 2011; Wuertz et al., 2011; Bernhard and Field, 2000; Layton et al., 2006; Kildare et al., 2007). When used in concert with culture-based conventional FIB, these qPCR assays can provide useful information about sources of fecal contamination to receiving waters and provide the best chance to minimize human health risks (Noble et al., 2006).

Under the Clean Water Act, a water body is designated as impaired when it exceeds the acceptable threshold for a given FIB. Besides public health impacts such as increased health care costs, other economic costs such as decreased tourism can occur as a result of either shellfish bed and/or recreational water closure (Given et al., 2006; Rabinovici et al., 2004; Landrum and Ache, 2000). In order to determine best management practices (BMP) to limit water body impairment, managers need to have up to date information on both conventional and alternative FIB concentrations and fluxes. As part of this, the total maximum daily load (TMDL) process defines contamination problems, sources, and loads from different areas and determines the best methods for ameliorating any problems (Kinzelman et al., 2011).

Our study site in eastern North Carolina, the Newport River Estuary (NPRE), is listed on the USEPA 303(d) list as an impaired water body due to exceedances of FC for shellfish harvesting waters. As part of a TMDL study of the NPRE, Coulliette and Noble (2008) found a clear relationship between stormwater runoff and degrading microbial water quality in the estuary. Specifically, they determined that based on FC concentrations, shellfish beds should be closed after 2.54 cm of rainfall, rather than the commonly accepted management action threshold of 3.81 cm (Coulliette and Noble, 2008). While this study provided a good initial metric for TMDL decisions, it lacked quantification of FIB fluxes into the system during wet and dry weather and no FIB source loadings were allocated using MST methods. Before BMP can be decided upon for this region, quantification and characterization of FIB fluxes will be necessary.

The goal of this study was to understand the microbial dynamics of water quality in the NPRE across a range of hydrodynamic and meteorological conditions. The major sources of fecal pollution to the NPRE were determined through the quantification of a combination of molecular markers specifically selected for their use in the estuarine system studied. Specifically FC/EC and ENT fluxes were examined in relation to rainfall. In addition, fecal *Bacteroides* spp. and human *Bacteroides* spp. (BacHum) concentrations were assessed using qPCR as a metric for total fecal and human fecal contamination, respectively. The relationships among measured FIB fluxes and microbial source tracking markers were also examined.

4.2. Methods

4.2.1. Site description

Two shallow tributaries of the NPRE that are often used for both recreation and shellfish harvesting, Ware Creek and Oyster Creek, were examined for 14 months as part of a routine monitoring program (Figure 1). Previous work done in these creeks has described general bacterial concentrations (Coulliette and Noble 2008, Coulliette et al. 2009), but information on bacterial concentrations under a wider range of meteorological and seasonal conditions are still needed. Percent impervious cover and land-use data were estimated for each watershed by Gonzalez et al. (2012). Briefly impervious cover was less than 2% in both watersheds. The dominant land uses were emergent herbaceous wetlands and evergreen forest. In addition, row crop agriculture and developed open space (i.e. residential development) also were important land uses in the Ware Creek watershed.

4.2.2. Hydrodynamics and sample collection

Precipitation measurements were collected in 0.025 cm increments using a HOBO ® Data Logging Rain Gauge-RG3 (Onset, Cape Cod, MA) that was 0.77 km from Ware Creek and 2.89 km from Oyster Creek (Figure 1). Rain total was determined by the adding consecutive 0.025 cm increments of rainfall until no rainfall was recorded for at least 6 hours, thus dry weather totals were 0.

Sampling was conducted in both tributaries (Ware and Oyster Creeks, Figure 1) between July 2009 and August 2010. Grab surface water samples were collected during 12 dry weather and 13 wet weather periods during the last three hours of ebb tide. We

classified a wet weather period as a sampling event that occurred after 24 hours of continuous rain that totaled at least 1.27 cm because this amount of rainfall would trigger overland flow of runoff and higher water flows in the tributaries (data not shown). Sampling dates and descriptions are given in Supplementary Table 1. Upstream to downstream sampling transects that were approximately 1400 m (Oyster Creek) and 1800 m (Ware Creek) were followed, with four to six sampling locations visited at each, for a total of 154 samples over the course of 14 months (Figure 1). Each water sample was collected in a sterile 1L Nalgene bottle and kept on ice until processing, no more than 6 hours after collection. Water velocity measurements were measured using a Flow Tracker Handheald ADV® (SonTek, San Diego, CA) and were used in concert with creek cross sectional area at sites Ware 1, Oyster 1a, and Oyster 1b to calculate headwater discharge.

4.2.3. FIB Enumeration

Traditional indicators, EC and ENT, were measured in all water samples (in duplicate and diluted 1:10 or 1:100 in deionized water) using the Colilert-18® and Enterolert™ Defined Substrate Technology® kits (IDEXX Laboratories, Inc., Westbrook, ME) according to manufacturer's guidelines . Quanti-tray®/2000 (97-well) trays were used to quantify bacterial concentrations using most probable number (MPN) algorithms from Hurley and Roscoe (1983).

4.2.4 Molecular sample preparation

Fifty to 100 ml of each water sample were filtered in duplicate onto 47 mm diameter polycarbonate filters (type HTTP, Millipore, Bedford, MA) with a 0.45 um pore-size. Upon filtration, they were immediately stored at -80˚C until DNA extraction and qPCR analysis.

4.2.5 Enumeration of molecular markers

The primer sequences for corresponding targets are shown in Table 2. Alternative indicator (fecal *Bacteroides* spp. and BacHum) concentrations were determined using a qPCR assay, with primers and probes outlined in Converse et al. (2009) and Kildare et al. (2007), respectively. Frozen polycarbonate filters were bead-beaten to release DNA into buffer solution (Converse et al. 2009). qPCR assays were completed on a SmartCycler® II (Cepheid Inc., Sunnyvale, CA) using the following conditions: 2 minutes at 95˚C, followed by 45 cycles of 15 seconds at 94˚C and 30 (60 for BacHum) seconds at 60˚C. An additional MST marker, gull2 assay, for the detection of gull fecal pollution was used to eliminate potential sources of contamination. The gull2 assay was run using the following conditions: 2 minutes at 95˚C, followed by 45 cycles of 15 seconds at 95˚C and 60 seconds at 62˚C.

4.2.6 Measurement of inhibition

Inhibition levels and DNA extraction efficiency were measured using ten ng of salmon testes DNA and 500 µl of AE buffer (2.5 ng/reaction) added to each sample, calibrator, and negative control prior to DNA extraction as a specimen processing control (SPC). Inhibition was calculated by subtracting the cycle threshold (C_T) of the qPCR reaction with the SPC only (no target DNA) from the C_T of the reaction with the SPC and unknown sample DNA (Haugland et al. 2005). A sample with a difference of greater than 0.5 log units (2.32 C_T) was considered to be inhibited. Based on these calculations, all 154 samples in this study required dilution to resolve inhibition problems; 149 samples were diluted 1:40 and the remaining 5 samples were diluted either 1:60 or 1:100.

4.2.7 qPCR calibration standards, assay detection limits, and amplification efficiencies.

Bacteroidales thetaiotamicron (ATCC 29148) was used as a calibration standard for the fecal *Bacteroidales* spp. qPCR assay similar to Converse et al. (2009). Briefly, cells were grown anaerobically overnight at 37° C in cooked meat medium (Difco). After centrifugation for 5 min at 6000 x *g*, the supernatant was removed, the cell pellet resuspended in water and aliquoted for used as a cell standard. Cell counts were determined following the protocol of Noble and Fuhrman (1998). Standard curves for BacHum and gull2 assays were created with plasmids containing their respective marker sequences as previously described by Kildare et al. (2009) and Lu et al. (2008). Standard curves for each qPCR assay consisted of the cell or plasmid standard and four 10-fold serial dilutions that were run in duplicate. All calibrator cell equivalents (CE) were calculated using the Pfaffl (ΔC_T) quantification method as described in Haugland et al. (2005).

Assay detection limits were calculated from the standard curves for each assay. The lowest concentration detected within the linear range of quantification during the TaqMan amplification was set as the assay detection limits. The detection limits for the fecal *Bacteroides* spp., BacHum, and gull2 assays were 41, 38, and 37 CE, respectively. After the appropriate dilution resolved inhibition, the limits of quantification for the fecal *Bacteroides* spp., BacHum, and gull2 assays were 1640, 1520, and 1480 CE, respectively.

Average amplification efficiencies and R^2 for the standard curves and quantification ranges of all molecular assays are in Table 3.

4.2.8 Data and statistical analysis

All statistics were performed in SAS 9.2 (Cary, NC) and OriginPro 9.0 (OriginLab, Northampton, MA). Normality was examined using histograms. Concentrations and loadings were $\log_{10}$-transformed to reduce skewness prior to any analysis. Homogeneity of variances was checked using Brown-Forsythe *F* tests.

Relationships between rainfall, discharge, and FIB were analyzed with linear regressions. Low and high discharge refer to levels below or above $0.15 m^3/s$. This baseline/threshold discharge was generated by averaging the discharge on days where there was no antecedent 5-day rain total. Instantaneous loadings (calculated based on a grab sample) of FIB were calculated by multiplying concentrations of FC and ENT (MPN/100 ml) and measured discharge (m^3/ s), and reported in units of MPN/ hr. Comparisons of instantaneous loading and FIB concentrations were completed using one-way ANOVAs with Scheffe post-hoc comparisons or Student's *t*-tests.

A "toolbox" approach was implemented for the determination of potential presence of human fecal contamination (Noble et al., 2006; Parker et al., 2010). The potential presence of human contamination was assumed when fecal *Bacteroides* spp. concentrations surpassed an "action threshold" of 5000 CE/100ml (Coulliette and Noble, 2008) coupled with a moderate to strong BacHum signal. The strength of the BacHum signal was gauged using a tiered system from Sauer et al. (2011) where they stratified the *Bacteroides* genetic marker as follows: High *Bacteroides* spp. (> 5000 CE/100 ml), Moderate *Bacteroides* spp.(1000-5000 CE/100 ml), and Low *Bacteroides* spp. (<1000 CFU/100ml).

Multiple linear regressions were used to determine the proportion of the FIB variation that the *Baceroidales* markers described. MLR models were created in the following form:

$$Y=\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k + e$$

Where Y is the dependent variable (e.g. ENT or EC), β_0 is a constant, β_k are regression coefficients for the predictors, X_k are the values of the predictors, and e is the residual error of the model. After checking for all MLR model assumptions, alternative FIB were used as independent variables. MLR that maximized adjusted R^2 were performed using the PROC REG function in SAS.

4.3. Results

4.3.1 Hydrodynamics and flux quantification

The linear regression of instantaneous discharge to rain showed a moderate, significant relationship (R^2= 0.44, r=0.66). In order to capture the entire rain hydrograph and incorporate a measure of soil saturation, we also did a regression of discharge to 5-day rain total. This linear regression improved slightly (R^2= 0.54, r=0.74).

FIB concentrations were compared between low (< 0.15 m^3/s) and high (> 0.15 m^3/s) discharge levels (Figure 2). FC were significantly greater at higher discharge (p=0.004). Similarly ENT showed a significant increase in concentration across higher discharges (p=0.045).

When comparing FC flux using the Coulliette and Noble (2008) 2.54 cm (1 in) rainfall threshold, there was no significant increase in FC flux when comparing negligible

precipitation to rain levels from 0.25 to 2.54 cm (p=1), However, there was a significant increase in flux when rainfall levels below 2.54 cm were compared to those above 2.54 cm (p=0.004; Figure 3). When using the traditional management threshold, there was also a significant increase in FC flux when the average below 3.81 cm (1.5 in) of rainfall was compared to that above the 3.81 cm threshold (p<0.001).

Similar results were seen with the relationship between ENT flux and rainfall levels (Figure 4). Rainfall caused a significant increase in ENT flux at the 2.54 cm threshold (p=0.002), yet no difference was seen when going from negligible rain amounts to 0.25 cm to 2.54 cm of rain (p=1). When using the management action threshold of 3.81 cm, rain caused a significant increase in ENT flux (p<0.001).

4.3.2 Microbial source tracking (MST)

The overall frequency of *Bacteroides* spp. marker detection was low. Overall samples with a fecal *Bacteroidales* spp. concentration > 5000 CE/100 ml occurred 21% (33/154) of the time, while samples with moderate to high BacHum concentrations (>1000 CE/100ml) occurred 6% (10/154) of the time. During dry weather the fecal *Bacteroides* spp. marker exceeded the "action threshold" of 5000 CE/100 ml 23% (20/88) of the time and the BacHum marker was moderate or strong 1% (1/88) of the time. During wet weather the fecal *Bacteroides* spp. marker exceeded the "action threshold" of 5000 CE/100 ml 18% (12/66) of the time and the BacHum marker was moderate or strong 14% (9/66) of the time. Figure 5, shows the frequency of the *Bacteroides* spp. markers across discharge—low versus high. For both discharge levels, *Bacteroides* spp. makers were low, with the frequency of the human-associated marker increasing 4 times when discharge was high. In addition, the total fecal *Bacteroides* spp. frequency

decreased when discharge was high. Of the 154 samples collected, only four have strong evidence (fecal *Bacteroides* spp. > 5000 CE/100 ml and BacHum > 1000 CE/100 ml) of human fecal contamination; all four of these samples were collected after 3.86 or 9.25 cm of rain.

Only one sample from Ware Creek had concentrations of the gull2 marker above the detection limit of 1480 CE/100 ml. This sample had a concentration of 32035 CE/100 ml, which was an order of magnitude higher than the corrected detection limit of the assay. A large number of sea birds were noted on the dock during this sample collection.

4.3.3 MST comparison to FIB

To understand the relationships observed between the conventional (EC and ENT) and alternative (fecal *Bacteroides* spp. and BacHum) indicators, we used a combination of correlation analysis and multiple linear regression. Fecal *Bacteroides* spp. had a significant correlation to both EC and ENT ($r=0.45$, $p<0.01$ and $r=0.44$, $p<0.01$ respectively), while BacHum had a weak, but significant, correlation to both EC ($r=0.20$, $p=0.013$) and ENT ($r=0.36$, $p<0.01$). The correlation between the two *Bacteroides* spp. assays was weak ($r=0.18$, $p=0.027$).

Multiple linear regressions quantified the variance explained in the FIB parameters using the MST results (Table 4). For the whole dataset, 22% of FC and 28% of ENT data variation were explained by the two MST variables (fecal *Bacteroides* spp. and BacHum). A slight increases in R^2 was seen when partitioning the dataset according to discharge. At low discharges the MST markers combined to explain 36% of the FC data variation, while at high discharges 30% of variation was explained. Thirty-seven

percent of the ENT data variation was explained at low discharges by MST markers. At high discharges, 36% of the ENT data were described by the alternative indicators.

4.4. Discussion

4.4.1 Hydrodynamics and flux quantification

Gonzalez et al. (2012) showed significant increases in FIB fluxes occurred during wet weather in tributaries of the NPRE; fluxes of all indicators were three to four logs higher during wet weather versus dry weather. The motivation for our study was to relate microbial dynamics, discharge, and microbial flux to wet weather.

Based on our comparison of rainfall and discharge, we conclude that the hydrodynamics of Ware and Oyster Creeks were primarily modulated by rainfall activity in the region. Thus, increases in rainfall not only increased FC and ENT concentrations in the watershed, but also overall FIB loading to the system. In addition, our finding that FIB loading was significantly higher at rainfall levels over 2.54 cm, supports prior assertions by Coulliette and Noble (2008) that the currently used management action threshold of 3.81 cm is too liberal, if it is to be based on traditional indicators alone.

4.4.2 Microbial source tracking (MST) and comparisons to FIB

Low recovery of BacHum DNA from our qPCR analysis indicated that most of the contamination in these watersheds was not of human origin. These results should be taken into consideration when assessing the many studies that have shown bacterial levels in exceedance of management thresholds, even under dry conditions (Coulliette and Noble 2008, Gonzalez et al. 2012). It is possible that a non-human component of contamination alone is sufficient to cause noncompliance of recreation and shellfish

harvesting waters. In addition, the four likely human contamination events occurred after high discharges that were the result of rain above the currently used management action threshold of 3.81 cm. This is not surprising since research in the area has demonstrated that during periods of wet conditions wastewater effluent from on-site wastewater systems receive little additional treatment in the soil before reaching surface waters (Habteselassie et al., 2011; Conn et al., 2012). Thus, during storm events, septic systems can temporary fail and affect nearby waters. However the authors reported that surface water contamination measured from nearby failing septic systems suggested other sources of fecal material were also contributing to the elevated bacterial counts (e.g domestic pets, wildlife, and birds; Habteselassie et al., 2011; Conn et al., 2012). Due to the lack of definite source apportionment, the best use of the data is to rule out potential sources of contamination. With the low occurrence of potential human contamination and low occurrence of the gull2 assay, humans and shorebirds likely contribute minimally to contamination in the NPRE.

For many years, researchers have believed that fecal contamination of the NPRE was of wildlife origin (Coulliette and Noble, 2008; Habteselassie et al., 2011; Conn et al., 2012). However, the fecal *Bacteroides* concentrations were low in this study, correlations between FIB and alternative indicators (*Bacteroides* spp. markers) were low, and multiple linear regression models show that the two MST markers employed in this study did not sufficiently explain the variation encountered in the FIB. These results could be interpreted two ways: (1) other environmental sources may be dominant contributors of FIB or (2) FIB are persisting in the environment much longer than the obligate anaerobic fecal *Bacteroidales* spp. The idea of *in situ* environmental sources of FIB contamination

has been well documented in other systems; macroalgae in the Great Lakes (Whitman et al., 2003; Byappanahalli et al., 2003), beach sand (Yamahara et al., 2007; Whitman et al., 2009; Heaney et al., 2009), creek sediment (An et al., 2002; Obiri-Danso and Jones, 2000) and soil (Hardina and Fujioka, 1991; Byappanahalli and Fujioka, 2004) can all contribute significant amounts of FIB to the water column. The growth and survival of FIB in environmental sources can alter surface water quality through mechanical disturbance, water flow, and resuspension of FIB from sources within the tributaries and have the potential to contribute enough FC and ENT to the watershed to trigger recreational and shellfish bed closures.

4.4.3 MST in estuaries and study limitations

MST in estuaries can be difficult because estuaries, marshes, and wetlands can be affected by non-point sources of pollution and are often receptacles of multiple pollution sources. The multiple FIB sources in the NPRE can include livestock, pet waste, wildlife, birds, malfunctioning on-site wastewater systems, and various environmental sources (soils, sediment, sand, plant wrack, etc.). To distinguish naturally derived sources from human and animal sources, multiple MST techniques and/or markers would have to be used.

The greatest limitation to this study was the impact of PCR inhibition. Inhibitory compounds like humic and fulvic acids and complex polysaccharides can disrupt the amplification of the target nucleic acids through several mechanisms (Radstrom et al., 2008). There are multiple methods to control PCR inhibition, of which dilution is economical, easy, and was used in this study. Unfortunately the level of dilution required to diminish the inhibition effect caused a dilution of the bacterial concentrations,

subsequently causing the detection limit to increase by 2 orders of magnitude. The high detection limit of the *Bacteroides* spp. assays may have artificially lowered the R^2 of the multiple linear regressions by not accurately accounting for the bacterial variation at low concentrations. Therefore, in estuaries that have high magnitudes of inhibition, dilution may not be the most effective answer. It might be better to pursue a true competitive internal positive control like Gregory et al. (2006), or better yet, process the samples using additional nucleic acid extraction and techniques (like purification kits, gel filtration resin, or PCR facilitators) to avoid co-concentrating inhibitors (Gibson et al., 2012).

4.4.4 Management applications and research needs

Here we provided evidence to support that, based on FIB concentrations and loading levels alone, adoption of a management action threshold level of rainfall at 2.54 cm may be warranted. However, it is also important to note that before determining TMDL and the appropriate BMP for a watershed, managers must understand not only the amount of contamination in the water, but also the potential sources of this contamination. Using both traditional and alternative indicators during routine monitoring of the NPRE, we have shown that basing recreational and shellfish bed closures on only FIB, without knowledge of contamination sources may be problematic. It is evident that more work to identify and quantify potential *in situ* sources of FIB contamination to this region are still needed before BMP can be determined.

Figures

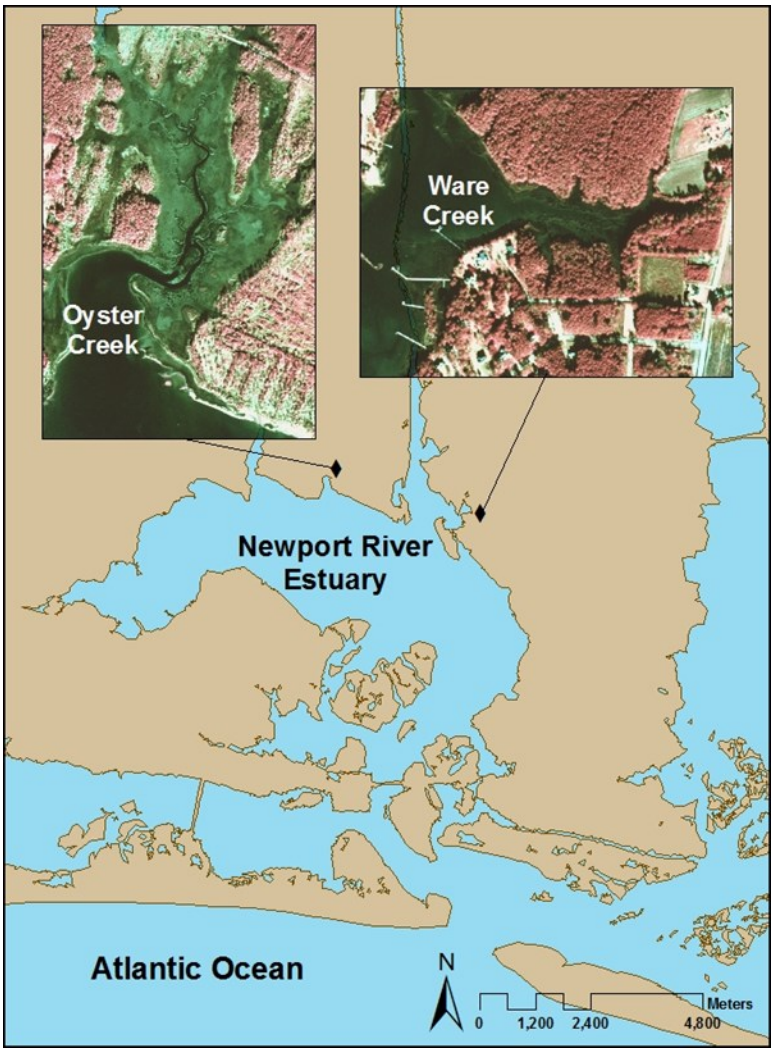

Figure 4.1. Ware and Oyster creek tributaries of the Newport River Estuary in eastern North Carolina.

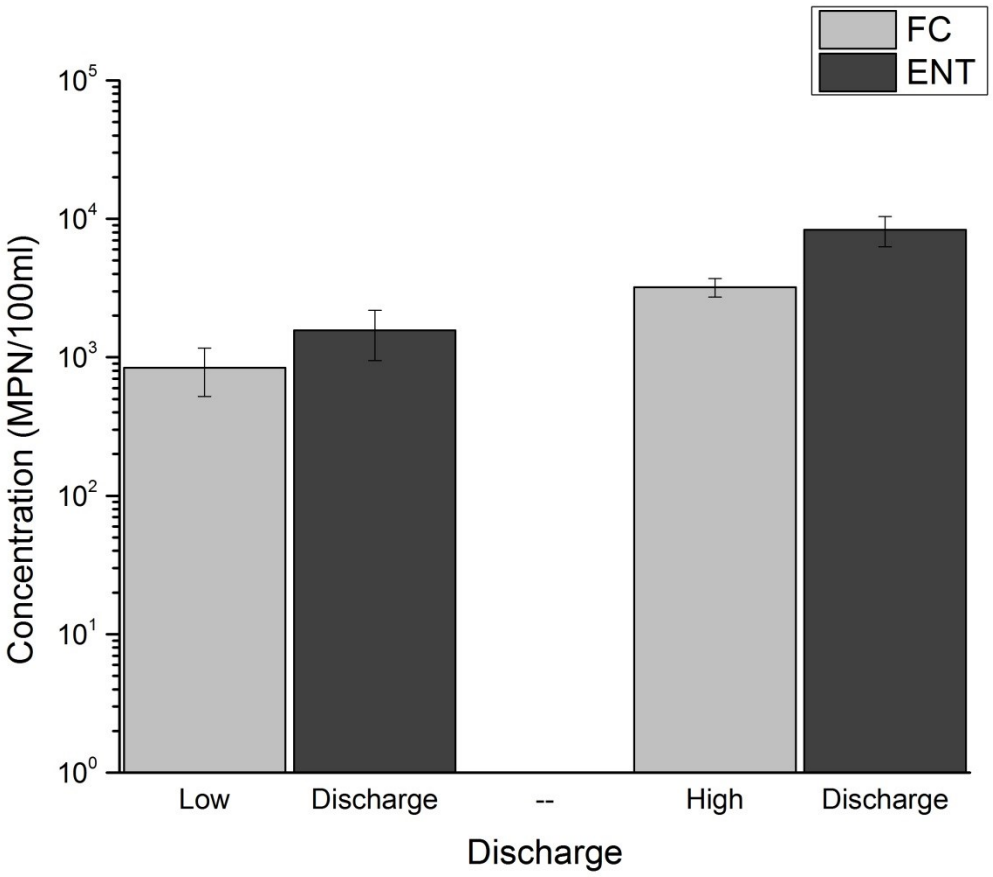

Figure 4.2. Mean fecal coliform (FC) and enterococci (ENT) concentrations according to low and high discharge. Column error bars are $\pm$ 1 standard deviation.

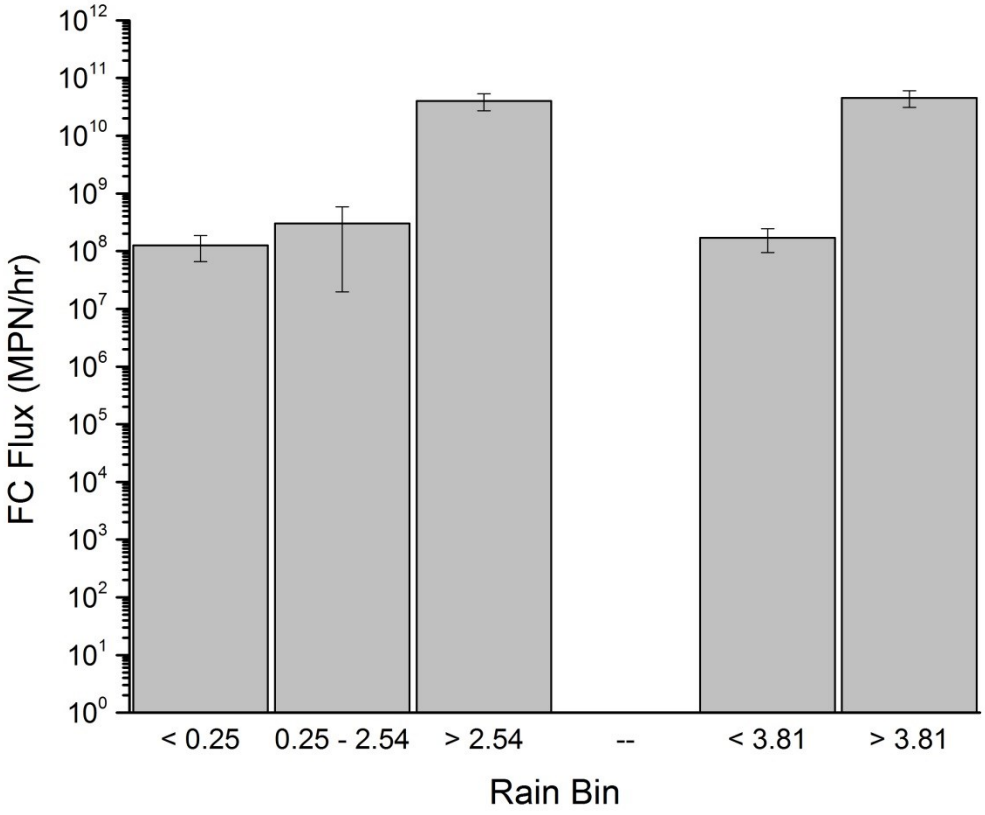

Figure 4.3. Fecal coliform (FC) flux at Newport River Estuary headwaters by the general rainfall categories of < 0.25 cm, > 0.25 to < 2.54 cm, and > 2.54 cm and then by the management action plan of < 3.81 cm and > 3.81 cm. Column error bars are $\pm$ 1 standard deviation.

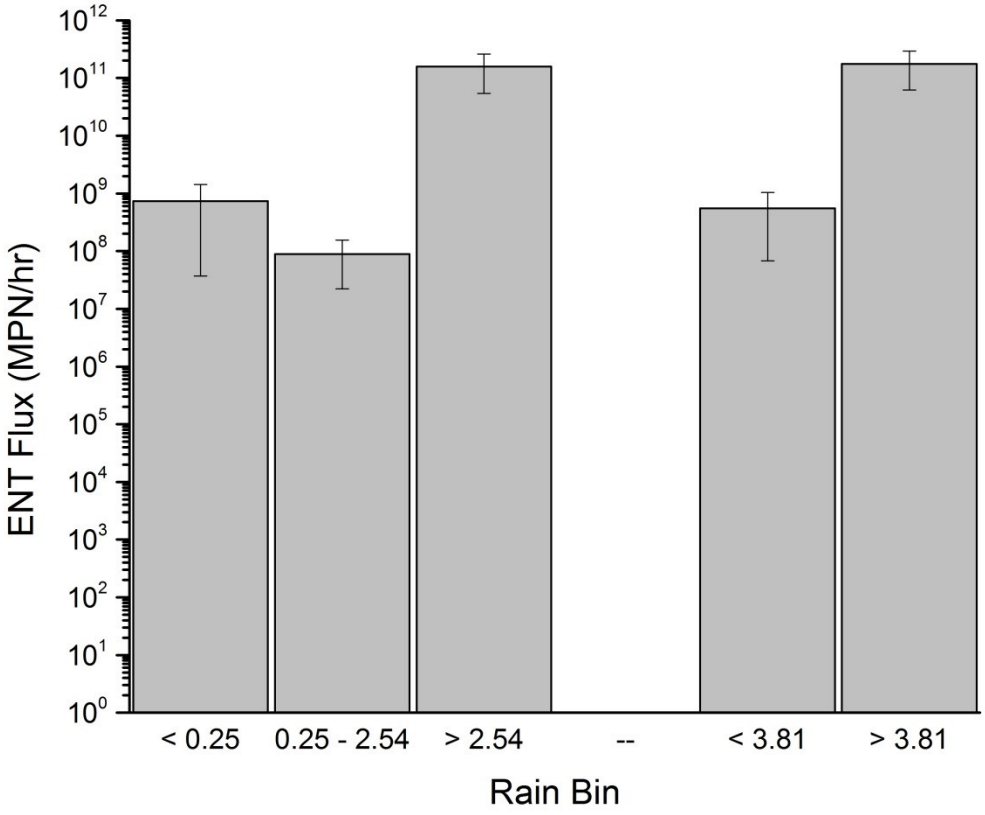

Figure 4.4. Enterococci (ENT) flux at Newport River Estuary headwaters by the general rainfall categories of < 0.25 cm, > 0.25 to < 2.54 cm, and > 2.54 cm and then by the management action plan of < 3.81 cm and > 3.81 cm. Column error bars are $\pm$ 1 standard deviation.

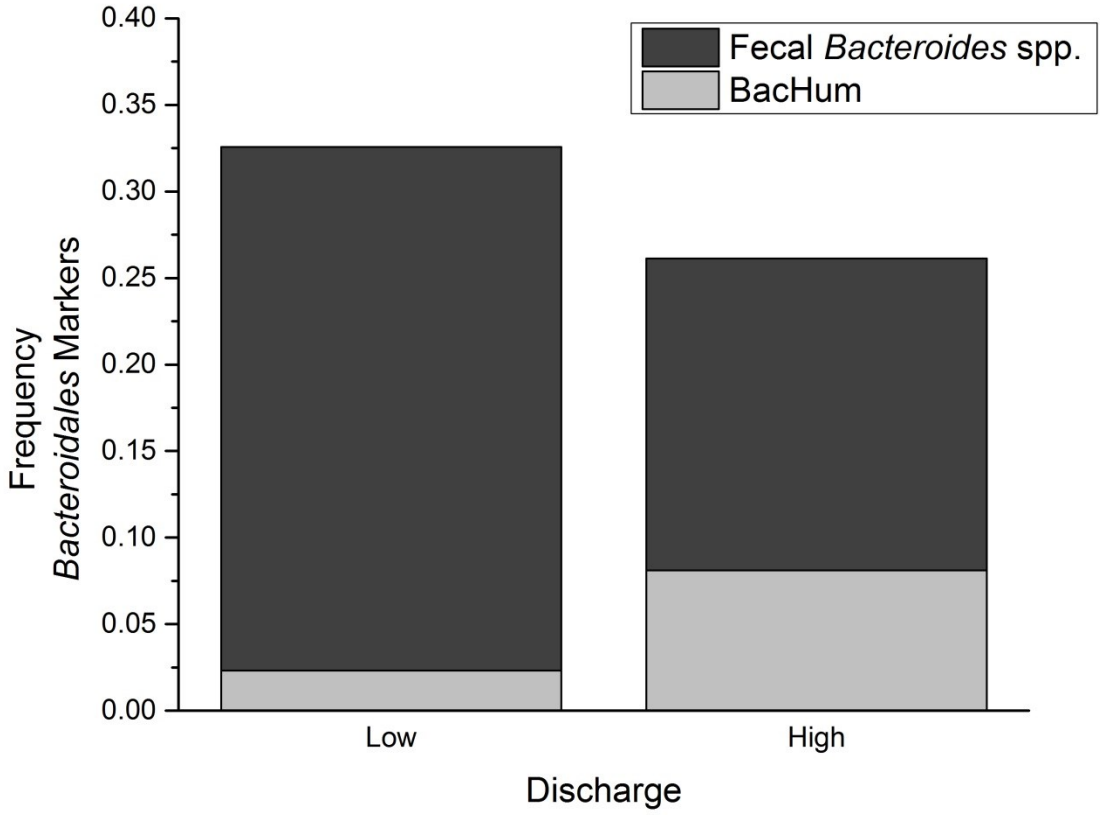

Figure 4.5. Frequency of the fecal *Bacteroides* spp. and human-associated *Bacteroides* spp. (BacHum) microbial source tracking markers across low and high discharge.

Tables

Indicator	Water Type	Geometric mean (density/100 ml)	Single-sample maximum (density/100 ml)			
			Designated Beach Area	Moderate Use	Light Use	Infrequent Use
	Freshwater					
EC		126	235	298	410	576
ENT		33	62	78	107	151
	Marine					
ENT		35	104	158	276	501

Table 4.1. Recreational water criteria under the BEACH Act of 2000. Recommended indicators are *E. coli* (EC) and enterococci (ENT).

Assay	Primer Sequence (5' - 3')	Reference
sketa22	F - GGTTTCCGCAGCTGGG R - CCGAGCCGTCCTGGTCTA	Haugland et al., 2005
fecal *Bacteroides* spp.	F - CGTTCCATTAGGCAGTTGGT R - CGTAGGAGTTTGGACCGTGT	Converse et al., 2009
BacHum	F - TGAGTTCACATGTCCGCATGA R - CGTTACCCCGCCTACTATCTAATG	Kildare et al., 2007
gull2	F - TGCATCGACCTAAAGTTTTGAG R - GTCAAAGAGCGAGCAGTTACTA	Lu et al., 2008

Table 4.2. Forward and reverse primer sequences of the sketa22, fecal *Bacteroides* spp, human-associated *Bacteroides* spp. (BacHum), and gull2 microbial source tracking assays.

Assay	Target	N	Amplification Efficiency (%)	Average R^2	Quantification Range
sketa22	salmon testes DNA	7	0.99	99.7	N/A
fecal *Bacteroides* spp.	total fecal *Bacteroidales*	5	0.91	99.8	10^1 - 10^5
BacHum	human-associated *Bacteroidales*	4	0.96	99.8	10^1 - 10^3
gull2	*Catellicoccus marimammalium*	3	1.05	99.5	10^1 - 10^4

Table 4.3. qPCR amplification efficiencies, R^2 values, and quantification ranges of the sketa22, fecal *Bacteroides* spp, human-associated *Bacteroides* spp. (BacHum), and gull2 microbial source tracking assay standard curves.

		Discharge	
	Total (n= 154)	Low (n= 43)	High (n= 111)
FC	0.22	0.36	0.30
ENT	0.28	0.37	0.36

Table 4.4. Fecal indicator bacteria (fecal coliforms (FC) and enterococci (ENT)) percent variation explained by the microbial source tracking markers (fecal *Bacteroides* sp. and human-associated *Bacteroides* spp.)

References

Ahmed, W., Goonetilleke, A., Powell, D., Gardner, T. 2009. Evaluation of multiple sewage-associated *Bacteroides* PCR markers for sewage pollution tracking. Water Research 43 (19), 4872-4877.

An, Y., Kampbell, D., Breidenbach, P. 2002. *Escherichia coli* and total coliforms in water and sediments at lake marinas. Environmental Pollution 120 (3), 771-778.

Bernhard, A. E. and Field, K. G. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Applied and Environmental Microbiology 66 (10), 4571-4574.

Byappanahalli, M. and Fujioka, R. 2004. Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. Water Science and Technology 50 (1), 27.

Byappanahalli, M. N., Shively, D. A., Nevers, M. B., Sadowsky, M. J., Whitman, R. L. 2003. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga Cladophora (Chlorophyta). FEMS Microbiology Ecology 46 (2), 203-211.

Conn, K. E., Habteselassie, M. Y., Denene Blackwood, A., Noble, R. T. 2012. Microbial water quality before and after the repair of a failing onsite wastewater treatment system adjacent to coastal waters. Journal of Applied Microbiology 112 (1), 214-224.

Converse, R. R., Blackwood, A. D., Kirs, M., Griffith, J. F., Noble, R. T. 2009. Rapid QPCR-based assay for fecal *Bacteroides* spp. as a tool for assessing fecal contamination in recreational waters. Water Research 43 (19), 4828-4837.

Coulliette, A. D. and Noble, R. T. 2008. Impacts of rainfall on the water quality of the Newport River Estuary (eastern North Carolina, USA). Journal of Water and Health 6 (4), 473.

Coulliette, A. D., Money, E. S., Serre, M. L., Noble, R. T. 2009. Space/Time Analysis of Fecal Pollution and Rainfall in an eastern North Carolina Estuary. Environmental Science & Technology 43 (10), 3728-3735.

Field, K. G. and Samadpour, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research 41 (16), 3517-3538.

Gibson, K., Schwab, K., Spencer, S., Borchardt, M. 2012. Measuring and mitigating inhibition during quantitative real time PCR analysis of viral nucleic acid extracts from large-volume environmental water samples. Water Research 46 (13), 4281-4291.

Given, S., Pendleton, L. H., Boehm, A. B. 2006. Regional public health cost estimates of contaminated coastal waters: a case study of gastroenteritis at southern California beaches. Environmental Science & Technology 40 (16), 4851-4858.

Gonzalez, R. A., Conn, K. E., Crosswell, J. R., Noble, R. T. 2012. Application of empirical predictive modeling using conventional and alternative fecal indicator bacteria in eastern North Carolina waters. Water Research 46 (18), 5871-5882.

Gregory, J. B., Litaker, R. W., Noble, R. T. 2006. Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. Applied and Environmental Microbiology 72 (6), 3960-3967.

Habteselassie, M., Kirs, M., Conn, K., Blackwood, A., Kelly, G., Noble, R. 2011. Tracking microbial transport through four onsite wastewater treatment systems to receiving waters in eastern North Carolina. Journal of Applied Microbiology 111 (4), 835-847.

Hardina, C. and Fujioka, R. 1991. Soil: The environmental source of *Escherichia coli* and enterococci in Hawaii's streams. Environmental Toxicology and Water Quality 6 (2), 185-195.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., Dufour, A. P. 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research 39 (4), 559-568.

Heaney, C. D., Sams, E., Wing, S., Marshall, S., Brenner, K., Dufour, A. P., Wade, T. J. 2009. Contact with beach sand among beachgoers and risk of illness. American Journal of Epidemiology 170 (2), 164-172.

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B., Wuertz, S. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research 41 (16), 3701-3715.

Kinzelman, J., Kay, D., Pond, K. 2011. Relating MST Results to Fecal Indicator Bacteria, Pathogens, and Standards. In: Anonymous Microbial Source Tracking: Methods, Applications, and Case Studies, Springer, 337-359.

Kreader, C. A. 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Applied and Environmental Microbiology 61 (4), 1171-1179.

Landrum, K. E. and Ache, B. 2000. The economic impact of oyster closures and their implications for the shellfish challenge in the Baratariaterrebonne national estuary, Louisiana. Proceedings of the Water Environment Federation 2000 (2), 796-807.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., Sayler, G. 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology 72 (6), 4214-4224.

Lu, J., Santo Domingo, J. W., Lamendella, R., Edge, T., Hill, S. 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Applied and Environmental Microbiology 74 (13), 3969-3976.

Mott, J. and Smith, A. 2011. Library-dependent source tracking methods. In: Microbial source tracking: methods, applications, and case studies, Springer, 31-59.

Myers, D., Stoeckel, D., Bushon, R., Francy, D., Brady, A. 2007. Fecal indicator bacteria (ver. 2.0): US Geological Survey Techniques of Water-Resources Investigations, book 9, chapter A7, section 7.1, Accessed May 2013 from http://water.usgs.gov/owq/FieldManual/Chapter7/7.1.html.

National Ocean Service, NOAA. 2013. The National Coastal Population Report: Populations Trends from 1970 to 2020. Available from: http://stateofthecoast.noaa.gov/features/coastal-population-report.pdf.

Noble, R. T. and Fuhrman, J. A. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquatic Microbial Ecology 14 (2), 113-118.

Noble, R. T., Griffith, J. F., Blackwood, A. D., Fuhrman, J. A., Gregory, J. B., Hernandez, X., Liang, X., Bera, A. A., Schiff, K. 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Applied and Environmental Microbiology 72 (2), 1604-1612.

Obiri-Danso, K. and Jones, K. 2000. Intertidal sediments as reservoirs for hippurate negative campylobacters, salmonellae and faecal indicators in three EU recognised bathing waters in north west England. Water Research 34 (2), 519-527.

Parker, J. K., McIntyre, D., Noble, R. T. 2010. Characterizing fecal contamination in stormwater runoff in coastal North Carolina, USA. Water Research 44 (14), 4186-4194.

Rabinovici, S. J., Bernknopf, R. L., Wein, A. M., Coursey, D. L., Whitman, R. L. 2004. Economic and health risk trade-offs of swim closures at a Lake Michigan beach. Environmental Science & Technology 38 (10), 2737-2745.

Rådström, P., Löfström, C., Lövenklev, M., Knutsson, R., Wolffs, P. 2008. Strategies for overcoming PCR inhibition. Cold Spring Harbor Protocols 3 (3), 1-10.

Roslev, P. and Bukh, A. S. 2011. State of the art molecular markers for fecal pollution source tracking in water. Applied Microbiology and Biotechnology 89 (5), 1341-1355.

Santo Domingo, J. W., Bambic, D. G., Edge, T. A., Wuertz, S. 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Research 41 (16), 3539-3552.

Soller, J. A., Schoen, M. E., Bartrand, T., Ravenscroft, J. E., Ashbolt, N. J. 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Research 44 (16), 4674-4691.

Stoeckel, D. M. and Harwood, V. J. 2007. Performance, design, and analysis in microbial source tracking studies. Applied and Environmental Microbiology 73 (8), 2405-2415.

Whitman, R. L., Shively, D. A., Pawlik, H., Nevers, M. B., Byappanahalli, M. N. 2003. Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Applied and Environmental Microbiology 69 (8), 4714-4719.

Whitman, R., Przybyla-Kelly, K., Shively, D., Nevers, M., Byappanahalli, M. 2009. Hand-mouth transfer and potential for exposure to E. coli and F coliphage in beach sand, Chicago, Illinois. Journal of Water and Health 7 (4), 623-629.

Wuertz, S., Wang, D., Reischer, G. H., Farnleitner, A. H. 2011. Library-independent bacterial source tracking methods. In: Microbial source tracking: methods, applications, and case studies, Springer, 61-112.

Yamahara, K. M., Layton, B. A., Santoro, A. E., Boehm, A. B. 2007. Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. Environmental Science & Technology 41 (13), 4515-4521.

Chapter 5

USING TIME-FREQUENCY ANALYSIS TO DETERMINE MINIMUM TIME LENGTH FOR BACTERIAL STATISTICAL PREDICTION MODELS

Overview

Monitoring of fecal indicator bacteria (FIB) in coastal waters as a proxy for pathogen presence, is limited by sample processing times, which can be as long as 18 to 24 hours for culture based techniques. This lag time inhibits rapid water closures and forces managers to make decisions based on samples collected the previous day. In response, multiple linear regression (MLR) prediction models have been commonly employed in different types of watersheds (freshwater, coastal beaches, estuaries) and areas with different uses (shellfish harvesting and recreation) to predict real-time FIB concentrations. Researchers often use time series datasets that are at least 2 years long for MLR model creation, with an additional year for model validation. However, recent studies have shown that it is possible to use shorter length time series for MLR modeling. Therefore, the central objective of this study was to examine multiple time scales of data from a North Carolina (NC) estuary using time-frequency analyses in order to determine the optimal length of data needed for robust MLR model creation. To do this, we first employed simple ordinary least squares (OLS) regression analysis to look for trends in the FIB time series data and then used periodogram analysis to examine which time periods comprised significantly large proportions of the time series variation. As a

contrast to the FIB data, we monitored and modeled levels of native *Vibrio* spp., a bacterial genus that contains potential human pathogens, but is not related to fecal contamination. We found that the fecal coliform time series had no patterns, trends, or periods. However, there was a significant, but weak linear trend in the total *Vibrio* spp. dataset, which was detected using OLS regression. Using periodogram analysis, we found no important periods in the FIB dataset, but we were able to detected regular periods in the total *Vibrio* spp. dataset that accounted for large amounts of the time series variance. These periods were found at 11.6 and 104 months and accounted for 28.5 and 16.8% of the data variance, respectively. Using the periodogram results and its relationship to mean stabilization of the data set, we determined that the optimal dataset length for a robust total *Vibrio* spp. MLR model was 19.3 months. Because there were no significant trends in the FIB data, we concluded that, based on the current data available, there is no optimal minimum time frame for data collection for FIB MLR models. Managers who want to create MLR models for NC estuarine waters should collect some data, model, and then actively update the model as more data becomes available.

5.1. Introduction

Exposure to excessive human or animal pathogens from contact with coastal waters or contaminated seafood can result in human illness and is therefore an important public health concern. Microbial water quality monitoring of these waters, especially those areas where recreation and shellfish harvesting are common, is extremely important. Because it is not feasible to detect and quantify every waterborne pathogen, the USEPA has recommended that managers use fecal indicator bacteria (FIB) as proxies for relative contamination in recreational and shellfish harvesting waters. Typically,

Enterococci (ENT) are used for monitoring marine waters, while fecal coliforms and *E. coli* (EC) are used in freshwaters.

Currently, monitoring of FIB in coastal waters is limited by sample processing times. Current culture techniques for enumerating FIB can take as long as 18 to 24 hours for sample incubation. This delay inhibits rapid management decisions from being made and forces managers to assess water quality and area closures based on samples collected the previous day. In response to these processing time concerns, more rapid methods have emerged, like quantitative PCR, which can reduce the delay from sample collection to management decisions down to 3 hours (Leecaster and Weisberg, 2001; Noble et al. 2010, Griffith and Weisberg 2011). In addition, prediction models can be used to provide real-time estimates of FIB. Several statistical prediction methods like artificial neural networks (ANNs), regression, and rainfall-based models are currently being used, but multiple linear regression (MLR) models are the most commonly employed in different types of watersheds (freshwater, coastal beaches, estuaries) and areas with different uses (shellfish harvesting and recreation).

Conventionally, researchers use at least 2 years of data for MLR model creation and an additional year for model validation. However, recent studies have challenged this multi-year structure. Comparison of four modeling studies conducted at Huntington Beach, Ohio present some noteworthy results for MLR model creation, validation, and performance. Two of these studies used multiple years of USGS EC concentration data to model, monitor, and predict when advisories were necessary (Francy et al., 2003; Francy and Darner, 2006). The predictive capability of these models, as measured by adjusted R^2 values were variable, but all were under 0.38 (Francy et al., 2003; Francy and Darner,

2006). In a separate study, Frick et al. (2008) looked at MLR model performance using datasets with 21, 28, 35, 42, and 49 days of data for model creation. They found model performance varied with the lowest adjusted R^2 value at 28 days (adj. $R^2 = 0.46$) and the highest R^2 value measured at 35 days (adj. $R^2 = 0.61$). Based on these results, Frick et al. (2008) proposed that this type of 'nowcasting' using shorter time periods of data to create MLR models would be a feasible option for locations with limited datasets. Because it was evident that researchers at Huntington Beach could use a variety of data time scales to create relatively reliable MLR models, Ge and Frick (2009) used time-frequency analysis to determine the optimal time frame needed in order to make the most accurate management decisions in a watershed.

Currently, MLR models with different dataset lengths and time-frequency analyses for FIB monitoring have not been investigated in estuarine waters. Eastern North Carolina (NC) presents a novel estuarine study site to test the ideal dataset length for MLR model creation because multiple, long term datasets are readily available. The NC Department of Environment and Natural Resources (NCDENR) conducts the monitoring programs for both recreational and shellfish harvesting waters in the state and therefore has a large historical database of fecal coliforms concentrations from their shellfish harvesting monitoring sites as well as historical datasets of ENT concentrations from recreational beach monitoring. In addition, research laboratories on the North Carolina (NC) coast like the University of NC have monitored several bacteria groups for more than ten years. NCDENR has expressed interest in the use of predictive models to issue public health advisories in near real-time for the 240 recreational monitoring sites

and over 1025 shellfish harvesting water sites that they currently monitor on a regular basis.

Therefore, the central objective of this study was to examine multiple time scales of data from a NC estuary using time-frequency analyses in order to determine the optimal time frame of monitoring for MLR model creation. Specifically, we first employed simple ordinary least squares (OLS) regression analysis to look for trends in a fecal coliform time series data and then used periodogram analysis to examine which time periods comprised significantly large proportions of the time series variation. To contrast the periodicity in anthropogenic fecal coliforms at NC study sites, we also monitored and modeled levels of native *Vibrio* spp., a common and ubiquitous bacterial genus that also contain potential human pathogens, but are not related to fecal contamination. By looking for important time scale patterns in a fecal coliform time series, decisions about which time lengths of data were most appropriate for MLR creation in NC estuaries will be discussed.

5.2. Methods

5.2.1. Site description and water collection

The Neuse River Estuary (NRE), which extends from New Bern, NC to the Pamlico Sound, is part of the second largest estuarine complex in the United States (Luettich et al., 2002). This estuary is microtidal due to enclosure by barrier islands; thus, flow within the system is controlled by fresh water input from the Neuse River, wind magnitude, and wind direction (Leuttich et al., 2000; Peierls et al., 2012). The NRE has been extensively monitored for two bacterial groups—fecal bacteria and *Vibrio*—for

a decade as part of the ecology of infectious disease program, funded by NSF. Through this program, water samples were collected from the NRE at five stations covering a length of over 73 km from May 2004 through December 2011. The locations of the stations (Stations 0, 30, 70, 120, and 180) are shown in Figure 1, however only samples from station 120 were used in our study since station 120 is adjacent to NCDENR shellfish monitoring sites. Surface water samples were collected in 10 L acid rinsed containers at an approximate depth of 0.2 meters below the water surface. Measurements of salinity (ppt), water temperature (°C), dissolved oxygen (mg/l), and pH were measured *in situ* using a YSI 6000 multiprobe sonde (Yellow Springs Instruments, Yellow Spring, OH).

5.2.2. Water processing and the time series data

Bottles containing water from the NRE were kept in shaded storage and were processed for bacterial concentrations within six hours of collection. Enumeration of *E. coli,* which we used as a proxy for fecal coliforms, was determined using the IDEXX defined substrate technology kit, Colilert®-18 (IDEXX Laboratories, Inc., Westbrook, ME). All samples were diluted 1:10 in deionized water, according to manufacturer instructions for ambient waters, and to generate quantitative information. Quantification was conducted using 97-well most probable number (MPN) Quanti-tray®/2000 in conjunction with algorithms previously published by Hurley and Roscoe (1983). Total *Vibrio* spp. enumeration was determined by diluting water samples with phosphate buffered saline, and then filtering through a 0.45µm cellulose fiber filter (Pall, Port Washington, NY). Filters were placed onto Thiosulfate-Citrate-Bile salts-Sucrose (TCBS, BD, Franklin Lakes, NJ) agar and incubated for 24 h at 35°C. After incubation,

the total number of yellow and green colonies were tabulated and adjusted for dilution to be expressed as colony forming units (cfu)/100 ml.

We used a 104 month data set that spanned from May 2004 to December 2012 for our time-frequency analysis. Because the monitoring was conducted twice a month, every month except for the winter, monthly concentrations were averaged on months when sampling occurred twice. This ensured that the data was evenly spaced so that period analysis was done on periods that did not overlap. Prior to analysis, the data had to be transformed to achieve normality. The fecal coliform time series required reciprocal root transformation ($-1/X^{0.5}$, where X is each observation), while the total *Vibrio* spp. time series required $\log_{10}$- transformation.

5.2.3. Lagged autocorrelation functions

Before carrying out more complex pattern analyses, we used lagged autocorrelation function tests to examine significant trends/patterns in the datasets. 'Lag 1' autocorrelations were obtained by correlating an observed value (X_t) with the value observation one time point prior (X_{t-1}). SAS 9.2 (Cary, NC) statistical software provided the lagged autocorrelation functions using the IDENTIFY statement of the ARIMA procedure and a 95% confidence interval. Plots were created in SAS with asterisks denoting the level of lag autocorrelation and gray shading denoting 95% confidence intervals. If several lagged correlations fell outside of the confidence interval, we concluded that there was evidence of a pattern in the time series. If no autocorrelations fell outside of the confidence interval, we concluded that the time series consisted of white noise.

5.2.4. Linear trend assessment

Before looking for cycles in time series data, it was necessary to identify and remove any trends in the time series. We used ordinary least squares (OLS) regression trend analysis to fit a trend to the data. Using the REG procedure in SAS, we used observation number as the independent variable and the observed time series as the dependent variable. The R^2 determined the amount of data variation accounted for by the trend. When a significant trend was found, the residuals from this trend were saved and used in the periodogram analysis.

5.2.5. Periodogram analysis

Periodogram analysis was used to break up time series variance within each dataset by periods and identify which, if any, periodic components explained a large percentage of the data variance. The SPECTRA procedure in SAS generated periodograms for the time series data as well as peak intensities and their Sum of Squares. Periodogram analysis estimated the percentage of variance that was accounted for by a series of different sinusoids, starting with a period equal to the total sample size (N) and continuing with incrementally smaller periods until N/(N/2) was reached. Variance was analyzed by partitioning the Sum of Squares for the overall time series into Sum of Squares that were accounted for by the periods, with the percent variation of each peak described by its individual Sum of Squares divided by the overall time series Sum of Squares.

Equations for each sinusoid tested were computed in SAS used the Fourier transform to decompose the time series sets into a sum of sine and cosine waves of

different amplitudes and wavelengths. The Fourier transform decomposition of the series x_t is

$$x_t = \frac{a_0}{2} + \sum_{k=1}^{m} (a_k \cos(\omega_k t) + b_k \sin(\omega_k t))$$

where *t* is the time subscript, x_t are the equally spaced time series data, *m* is the number of frequencies in the Fourier decomposition (in this case *m*=N/2), a_0 is the mean term, a_k are the cosine coefficients, b_k are the sine coefficients, and ω_k are the Fourier frequencies (ω_k=2πk/N).

Periodogram plots were constructed with period length on the x-axis and intensity (J_k) on the y-axis, which were calculated as:

$$J_k = \frac{N}{2} (a_k^2 + b_k^2)$$

Periods of interest had significantly larger intensities (J_k) than all other periods, which were statistically confirmed using a Fisher test.

5.2.6. Multiple linear regression time length

Our study applied periodogram analysis to estimate the time length over which statistically robust regression models could be established. In order for MLR models to be robust, variance in a dataset should be minimal after independent variables are accounted for, and the time length of the dataset used should be long enough for the mean to stabilize. Mean stabilization of a dataset can be thought of as the point at which the mean of a dataset does not change considerably when more time points are added in. Here, we computed the optimal length of data needed for MLR modeling by determining

the period within datasets that accounted for the most variance, and then multiplied that by the proportion of time relative to that period that was needed for mean stabilization (Ge and Frick, 2009).

5.2.7. Multiple linear regressions

In order to verify the minimum time length results, MLR were created with the GLMSELECT procedure in SAS using data from all 5 NRE monitoring sites. This was done to create the largest sample size for the analysis. Model performance was gauged using root mean squared error (RMSE) and adjusted R^2. The RMSE metric measured the model's prediction capability using the independent variables, with a smaller RMSE value indicating a greater predictive capability. Adjusted R^2 described the proportion of the variation that the model's independent variables described. Salinity, water temperature, dissolved oxygen, and pH were used as independent variables during the fecal coliform MLR creation. Salinity and water temperature were the only variables examined during the total *Vibrio* spp. modeling exercise since those variables have been found to strongly predict *Vibrios*.

5.3. Results

5.3.1. Preliminary data screening and data description

Monthly fecal coliform and total *Vibrio* spp. data sets spanned from 5/2004 to 12/2012 with 104 time points in the series. The mean, median, variance, standard deviations, and ranges of the two time series can be seen in Table 1. Transformations reduced skewness, leaving normally distributed datasets that met time-frequency analysis statistical assumptions. Outliers were detected in both datasets, but the discrepancy in the

data could not be attributed to collection or sample processing error, so they were not removed. Visual examination of each data set versus time showed some preliminary trends in each data set (Figure 2). Neither regular repetitions, nor linear or non-linear trends in the fecal coliform data were visually detected. Preliminary examination of the total *Vibrio* spp. dataset revealed a potential cyclic trend of troughs occurring approximately every 12 months and a slight positive increase in total *Vibrio* spp. densities over time.

5.3.2. Lagged autocorrelation functions

Lagged autocorrelation functions can be used as a preliminary, simple test to determine if there are any trends in the data before conducting more complex tests for patterns. Figure 3a shows the lagged autocorrelation functions for the fecal coliform time series. Most of the autocorrelations fall within the 95% confidence interval, indicating that variation in the time series is likely white noise and the observations are uncorrelated with each other. Figure 3b shows that one or two of the lagged autocorrelation functions fall outside of the 95% CI for the total *Vibrio* spp. time series, which may indicate there is periodicity in the data. This periodicity, combined with the oscillation in the direction and magnitude of the autocorrelations, may indicate that a cycle is present in the total *Vibrio* spp. dataset.

5.3.3. Linear trend assessment

OLS analysis found no significant trends in the fecal coliform time series and visual inspection of the data did not show any evidence of curvilinear trends, so we did not attempt to fit any to the data. OLS regression found a weak, but significant positive linear trend in the total *Vibrio* spp. data ($p = 0.04$, $R^2 = 0.04$). The raw time series data

with the fitted linear trend superimposed on it can be seen in Figure 4a; the equation that describes this trend was $y = 2.5E^{-4}x - 618$. Regression results showed that the linear trend accounted for 4% of the variance in the time series. After the trend analysis and removal, the residuals from the OLS analysis were plotted and saved for periodogram analysis (Figure 4b).

5.3.4. Periodogram analysis

The periodogram for the fecal coliform time series can be seen in Figure 5. While there are several prominent peaks, none of these peaks accounted for a large proportion of the variance in the time series (Table 2). The largest periodogram intensity accounted for 9.5% of the data set variance but this was not significantly larger than the other peaks. The Fisher's Kappa metric (4.864) did not surpass the critical value (alpha=0.05) of 6.567, and thus was not significantly different from white noise.

The periodogram for the total *Vibrio* spp. time series is shown in Figure 6. Two large peaks are visible at a period of 11.6 months and 104 months (Table 2). The 11.6 month cycle accounted for 28.5% of the variation and the 104 month cycle accounted for 16.8% of the time series variation. In addition, the Fisher's Kappa metric (14.55423) surpassed the critical value (alpha=0.05) of 6.567, and thus the largest peak, at 11.6 months, was significantly different from white noise. Also the second peak, at 104 months, accounted for a significant proportion of the time series variance when evaluated using a Fisher's test.

5.3.5. Minimum length of time for maximized MLR robustness

There was no computed minimum time length for data collection for fecal coliforms because the fecal coliform data was no different than white noise. Because strong signals in the fecal coliform time series were sporadic, the mean of the time series did not appear to stabilize. For total *Vibrio* spp. we estimated that the mean stabilized at a time length greater than 5/3 times its period. Since the major pattern in this dataset had a period of 11.6 months, we estimated the minimum length of time for maximum MLR robustness to be 19.3 months.

5.3.6. Multiple linear regressions

Table 3 summaries the results of MLR models using four different time lengths for both datasets. MLR model performance on fecal coliform data, based on adjusted R^2 and RMSE, was similar for all time lengths. The adjusted R^2 ranged between 0.42 and 0.49 and the RMSE varied between 0.1705 and 0.2000. In contrast, the total *Vibrio* spp. MLR regression models showed more variation in their performance. The range of adjusted R^2 was from 0.37 to 0.54, while the RMSE ranged from 0.6020 to 0.9793.

5.4. Discussion

NC managers have expressed interest in predictive modeling for the NRE in order to provide real-time estimates of pathogen densities in the watershed. Regional supervisors like NCDNER have long term measurements of fecal coliforms in recreational and shellfish harvesting waters and long term total *Vibrio* spp. datasets are also available for the NRE, making it an ideal estuarine study site for time-frequency analysis and MLR optimization for both types of bacteria. Our analysis showed that fecal coliform and total *Vibrio* spp. datasets had different patterns over time.

Using multiple statistical tests including autocorrelation functions, OLS regression, and periodogram analysis, we showed that the fecal coliform time series had no patterns, trends, or periods. This was unlike other studies in freshwater and marine systems that looked at periods in FIB time series data. In the Great Lakes, wavelet analysis of time series data, showed two distinct, repeating periods that explained large amounts of data variance; one at 7-8 days and a second at 21 days (Ge and Frick, 2009). Unfortunately, the fecal coliform measurements from the NRE that we used for our analyses were not collected at such a high frequency, and thus our analysis could not examine trends at this resolution. A separate study in Orange County, CA found significant 6 month and 1 year periods in FIB time series data using periodograms (Lim and Jeong, 2012). In addition, they found a decreasing trend in FIB over the 10 year time series (Lim and Jeong, 2012). It is possible that Lim and Jeong (2012) found fecal bacteria patterns and our study did not because of differences in the surface cover and precipitation around the watersheds. Orange County beaches are surrounded by a completely urbanized watershed, while the NRE has a mixed watershed with more rural areas. The urbanized watersheds in Orange County have more impervious cover and therefore precipitation mediated runoff, which occurs during winter months, likely caused the seasonal trends in the data.

In contrast to the fecal coliform time series, there was a weak, but significant linear trend in the total *Vibrio* spp. dataset, which was detected using OLS regression. Once the trend was removed, periodogram analysis detected regular periods in the dataset that accounted for large amounts of variance in the time series. Specifically, significant periods were found at 11.6 and 104 months that accounted for 28.5 and 16.8% of the data

variance, respectively. Similar results have been found when looking at cholera incidence, of which the infectious agent is *Vibrio cholerae*. Ohtomo et al. (2010) saw multiple, significant periods in a cholera time series, two of which they took special note of at 1 year and 11 years. The authors hypothesized that, because the cholera dataset and sea surface temperature (SST) data both fluctuated on an annual cycle, there may have been a relationship between the two datasets (Ohtomo et al., 2010). Indeed, other researchers have found positive correlations between SST and *Vibrio* disease incidence (Baker-Austin et al., 2010, 2012). Ohtomo et al. (2010) also postulated that the 11 year period found in the cholera dataset had an inverse relationship to sunspot number, which can be thought of as a metric for UV radiation, and also fluctuated on an 11 year period. Thus, it is possible that as solar intensity decreased, there was a concomitant increase in cholera incidence (Ohotomo et al., 2010). This inverse relationship with UV radiation could be modulating the 104 month total *Vibrio* spp. period.

Using Ge and Frick's (2009) approach, we needed two variables to calculate the minimum dataset length for robust MLR modeling: the period of the major pattern in the dataset and the length of time that the mean stabilizes relative to that major period. Because the fecal coliform dataset had no distinguishable pattern, we could not calculate an optimal minimal time length of data collection. However, it is possible that if fecal coliform data were measured more frequently, as was done in Ge and Frick (2009), some distinguishable patterns and minimum time lengths could emerge. For the total *Vibrio* spp. dataset, the major pattern had a period of 11.6 months and the mean stabilized at a time length greater than 5/3 times this period. By multiplying these two numbers, we

calculated that at least 19.3 months of data would be needed to create the optimal MLR model.

The results found in this study can have important management implications. The NRE watershed encompasses one of the fastest growing population centers in the US, however, the time-frequency analysis in this study showed that fecal coliforms and the pathogens they represent, might not be the largest public health problem in NC coastal areas, as they are not increasing linearly over time. However, there does seem to be a linear trend in total *Vibrio* spp. over time, indicating that this genus might represent more of a public health concern in the coming years as climate change and other anthropogenic factors continue to change densities of native pathogens within the genus (Colwell, 1996; Patz et al., 2005; Lipp et al., 2002). Based on the monthly data collections, there does not appear to be a minimum time length of data needed to create robust MLR models based on fecal coliform data and only 19.3 months of total *Vibrio* spp. data are needed. Therefore, managers wanting to create MLR models for NC estuaries do not need large historic databases; they can collect some data and actively add more data as additional measurements are available. Until more frequent fecal coliform data measurements are available in the NRE, conservative managers may want to continue using at least 2 years of FIB data, as this is the unofficial convention.

More frequent monitoring of NRE fecal coliforms are needed to determine if there is periodicity in the data on a time scale that is shorter than the current monthly measurement increments. If shorter periods are present, this data can be used to calculate optimal dataset length needs for MLR creation. In addition, future work should analyze different environmental parameters and climate data that are associated with this dataset

using the time-frequency analysis to determine if there are any similarities in periodicity. Using this method, we may be able to determine which factors could be the most relevant variables to include in MLR models.

Figures

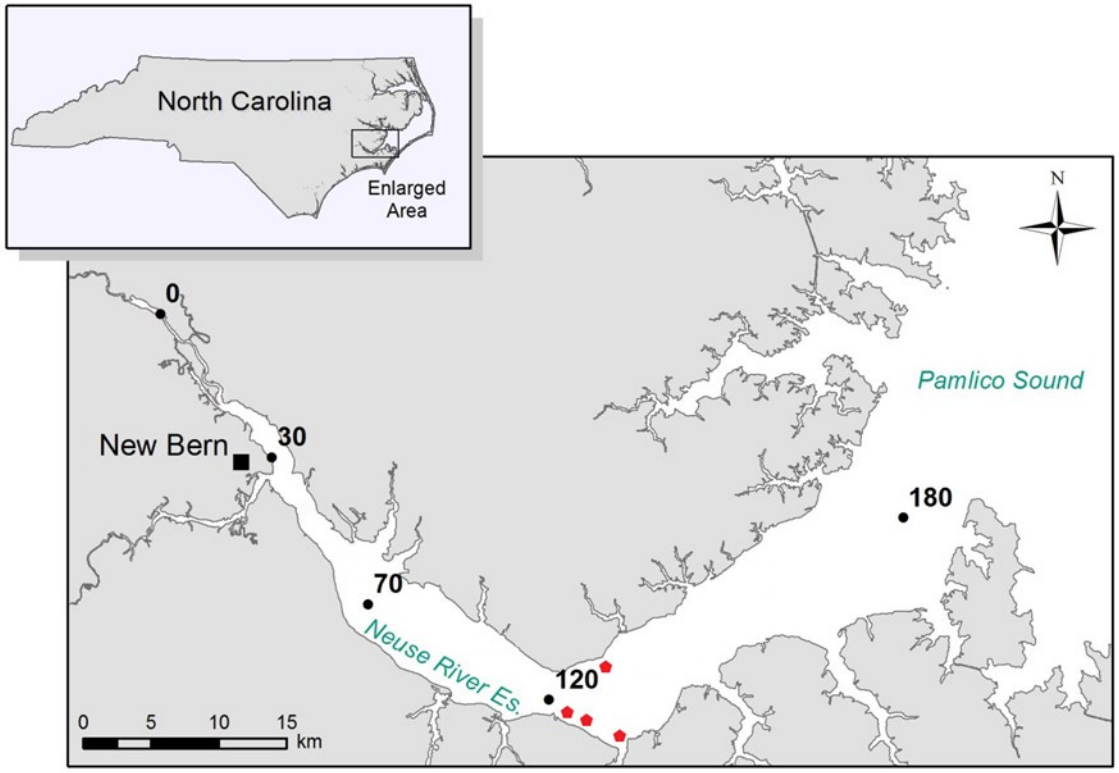

Figure 5.1. Ecology of infectious disease monitoring program sampling sites in the Neuse River Estuary. Only samples from station 120 were used in our study due to the proximity to NCDENR shellfish monitoring sites (red pentagons).

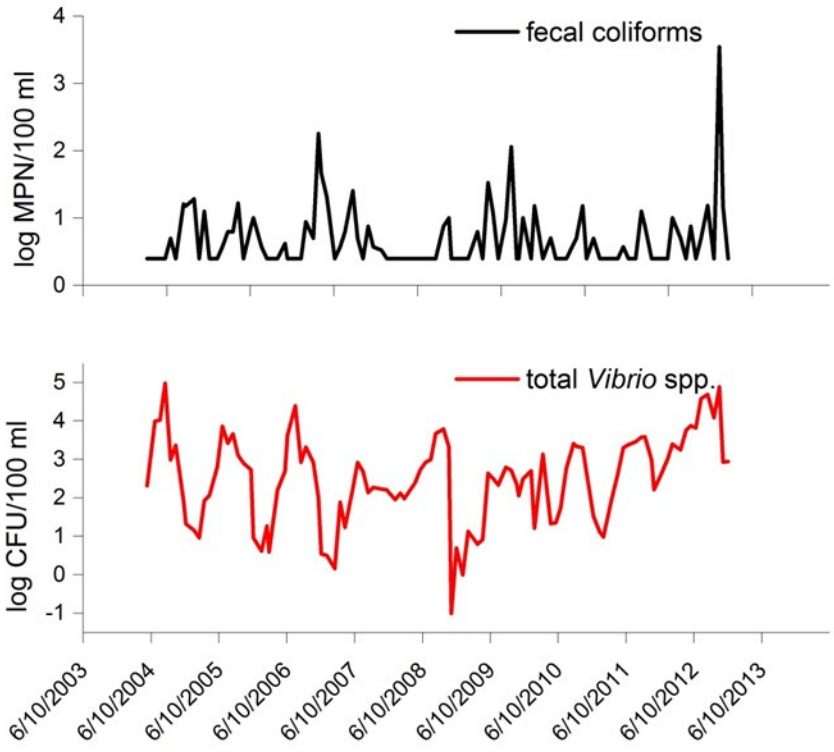

Figure 5.2. Graphs of fecal coliform and total *Vibrio* spp. time-series data. The 104 month data set spanned from May 2004 to December 2012.

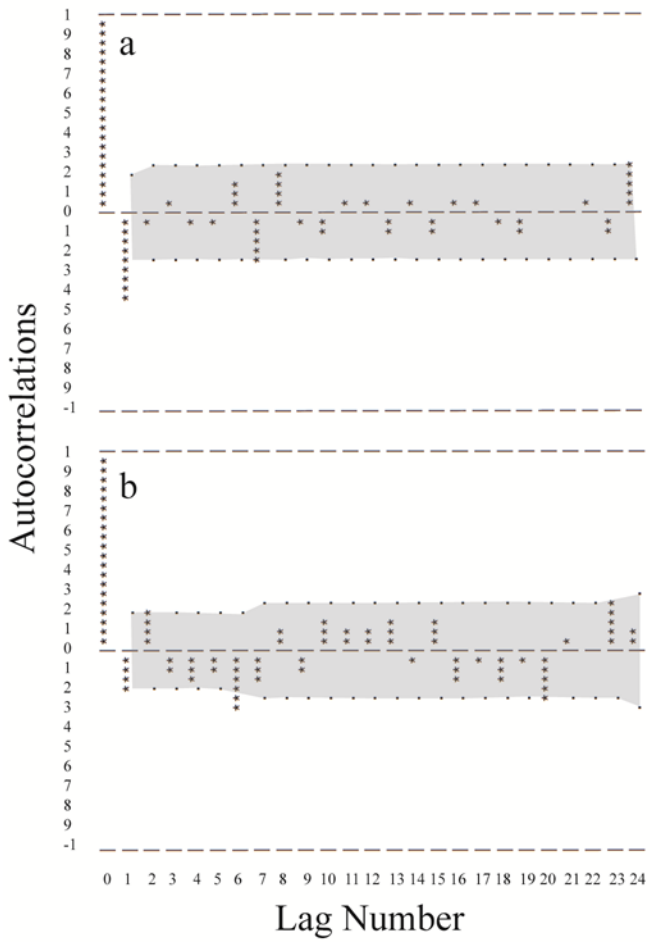

Figure 5.3. Lagged autocorrelation functions (ACF) for (a) fecal coliforms and (b) total *Vibiro* spp. The shaded grey area is the 95% confidence interval.

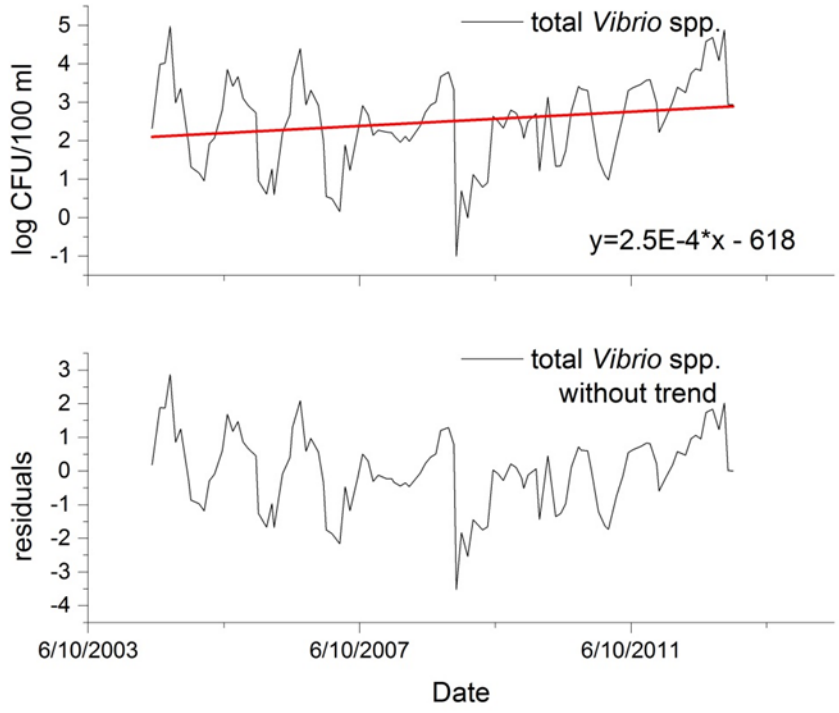

Figure 5.4a. The raw total *Vibrio* spp. time series data with the superimposed fitted linear trend. The equation describing this trend is $y = 2.5E^{-4}x - 618$. Figure 4b shows the residuals from the OLS analysis after trend removal.

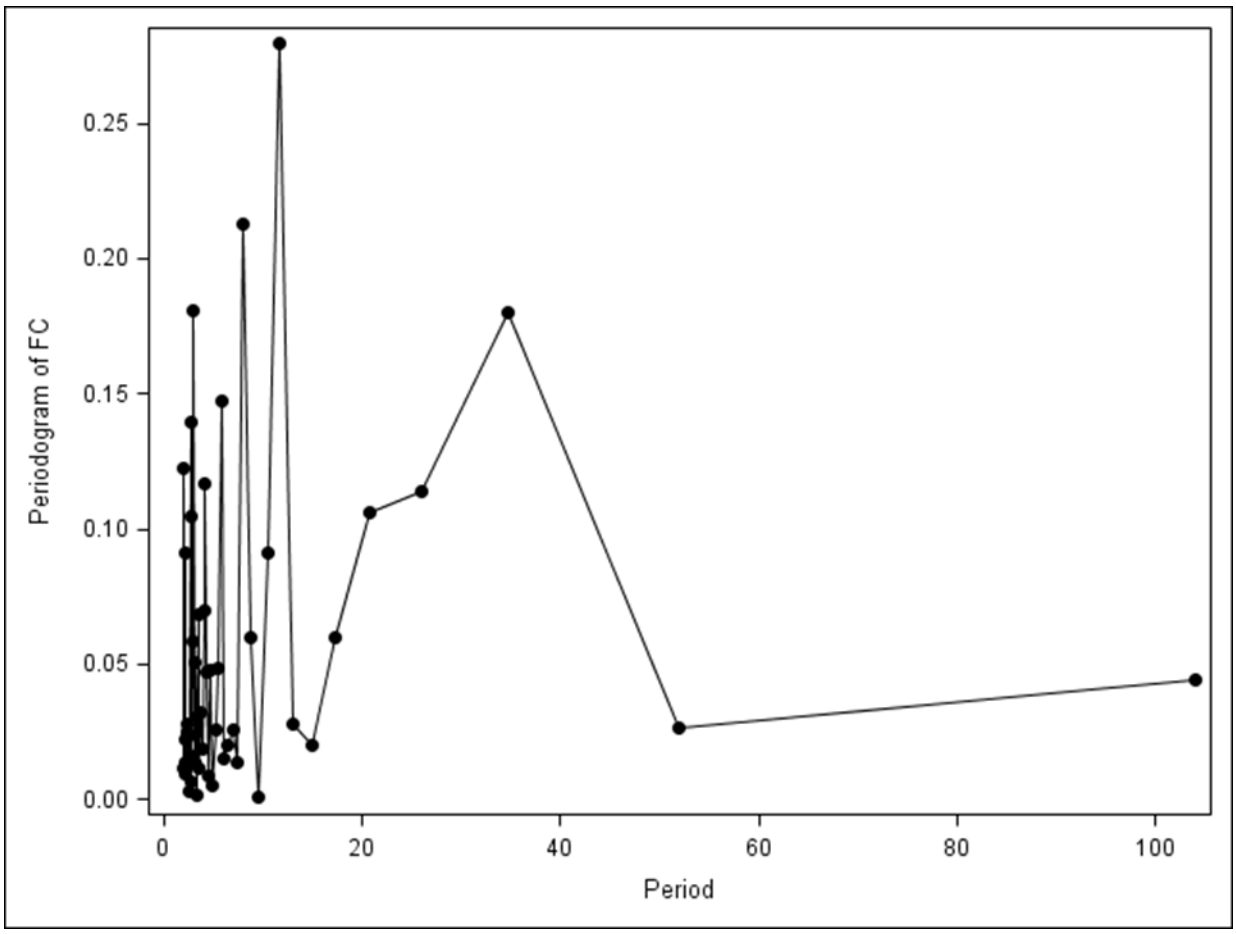

Figure 5.5. The fecal coliform (FC) time series periodogram. The y-axis is periodogram intensity (sum of squares). No significant large peaks are apparent.

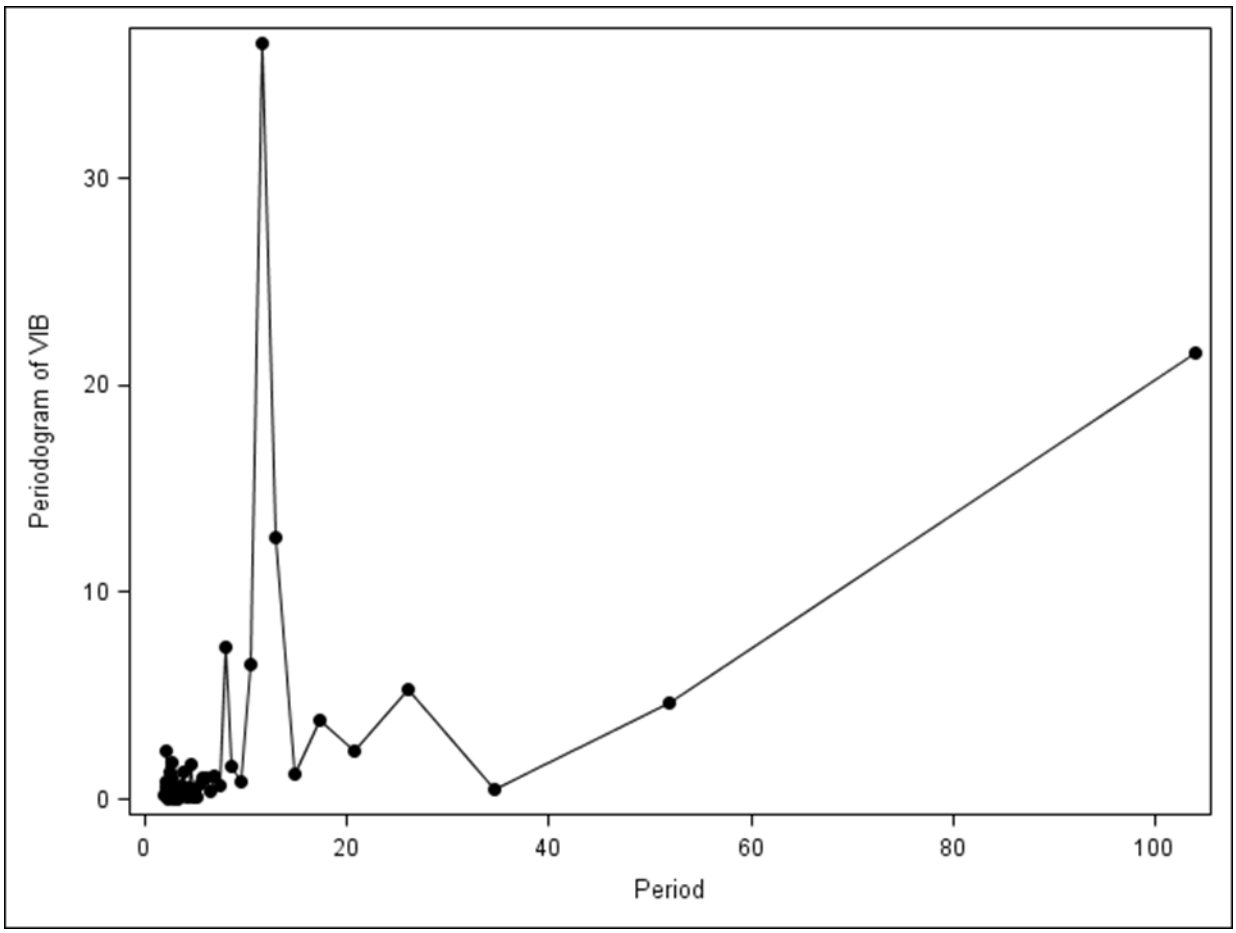

Figure 5.6. The total *Vibrio* spp. (VIB) time series periodogram. The y-axis is periodogram intensity (sum of squares). Two large peaks are visible at a period of 11.6 months and 104 months.

Tables

Bacterial Group	N	Mean	Median	SD	Variance	Minimum	Maximum
Fecal Coliforms	104	43	3	345	1.2E+05	3	3.5E+03
Total *Vibrio* spp.	104	4057	470	13388	1.8E+08	0	9.5E+04

Table 5.1. The mean, median, variance, standard deviations, and ranges of the fecal coliform and total *Vibrio* spp. time series. The monthy data sets spanned from 5/2004 to 12/2012.

	Fecal Coliforms			Total *Vibrio* spp.		
	Period	**Periodogram Intensities (SS)**	**% Variance**	**Period**	**Periodogram Intensities (SS)**	**% Variance**
1	.	0	0.0	.	0	0.0
2	104.0	0.04421	1.5	104.0	21.5711	16.8
3	52.0	0.02626	0.9	52.0	4.636	3.6
4	34.7	0.18007	6.1	34.7	0.4573	0.4
5	26.0	0.11399	3.9	26.0	5.3054	4.1
6	20.8	0.10601	3.6	20.8	2.3675	1.8
7	17.3	0.05987	2.0	17.3	3.8139	3.0
8	14.9	0.02001	0.7	14.9	1.1727	0.9
9	13.0	0.02768	0.9	13.0	12.6497	9.9
10	11.6	0.27968	9.5	11.6	36.5029	28.5
11	10.4	0.09147	3.1	10.4	6.5253	5.1
12	9.5	0.00069	0.0	9.5	0.8388	0.7
13	8.7	0.05985	2.0	8.7	1.5611	1.2
14	8.0	0.21291	7.2	8.0	7.3085	5.7
15	7.4	0.0137	0.5	7.4	0.6571	0.5
16	6.9	0.02552	0.9	6.9	1.1295	0.9
17	6.5	0.02013	0.7	6.5	0.4173	0.3
18	6.1	0.01511	0.5	6.1	1.0658	0.8
19	5.8	0.14752	5.0	5.8	0.9907	0.8
20	5.5	0.04826	1.6	5.5	0.7809	0.6
21	5.2	0.02588	0.9	5.2	0.12	0.1
22	5.0	0.00512	0.2	5.0	0.3583	0.3
23	4.7	0.04755	1.6	4.7	0.1086	0.1
24	4.5	0.00855	0.3	4.5	1.6526	1.3
25	4.3	0.04708	1.6	4.3	0.6129	0.5
26	4.2	0.11689	4.0	4.2	0.1186	0.1
27	4.0	0.06998	2.4	4.0	0.5061	0.4
28	3.9	0.0186	0.6	3.9	1.2863	1.0
29	3.7	0.03195	1.1	3.7	0.6822	0.5
30	3.6	0.01149	0.4	3.6	0.5795	0.5
31	3.5	0.06844	2.3	3.5	0.3622	0.3
32	3.4	0.0304	1.0	3.4	0.1499	0.1
33	3.3	0.00179	0.1	3.3	0.0202	0.0
34	3.2	0.05039	1.7	3.2	0.0345	0.0
35	3.1	0.01365	0.5	3.1	0.4914	0.4
36	3.0	0.18054	6.1	3.0	0.1519	0.1
37	2.9	0.05852	2.0	2.9	0.6085	0.5
38	2.8	0.00621	0.2	2.8	0.0503	0.0
39	2.7	0.10482	3.6	2.7	0.07	0.1
40	2.7	0.13937	4.7	2.7	1.0062	0.8
41	2.6	0.00281	0.1	2.6	1.7867	1.4
42	2.5	0.02369	0.8	2.5	0.2243	0.2
43	2.5	0.01629	0.6	2.5	1.3496	1.1
44	2.4	0.02792	0.9	2.4	0.3864	0.3
45	2.4	0.02506	0.9	2.4	0.7152	0.6
46	2.3	0.02364	0.8	2.3	0.0096	0.0
47	2.3	0.02377	0.8	2.3	0.5418	0.4
48	2.2	0.00939	0.3	2.2	0.2407	0.2
49	2.2	0.0911	3.1	2.2	0.8384	0.7
50	2.1	0.02189	0.7	2.1	0.5706	0.4
51	2.1	0.01386	0.5	2.1	2.3066	1.8
52	2.0	0.12261	4.2	2.0	0.2198	0.2
53	2.0	0.011785	0.4	2.0	0.21003	0.2

Table 5.2. Periodogram analysis for the fecal coliform and total *Vibrio* spp. time series using N=104 observations.

	Fecal Coliforms				Total *Vibrio* spp.		
Time Length	**N**	**RMSE**	**adj. R^2**	**Variables**	**RMSE**	**adj. R^2**	**Variables**
1 year	180	0.2000	0.49	Salinity, DO, pH	0.6020	0.38	Salinity, Temp.
2 year	239	0.1928	0.47	Salinity, DO, Temp.	0.7170	0.54	Temp., Salinity
4 year	429	0.1808	0.45	Salinity, pH, DO	0.9802	0.37	Salinity, Temp.
8 year	1531	0.1705	0.42	Salinity, DO, pH	0.9793	0.44	Salinity, Temp.

Table 5.3. Multiple linear regression models using four different time lengths for both fecal coliform and total *Vibrio* spp. datasets.

References

Baker-Austin, C., Trinanes, J. A., Taylor, N. G., Hartnell, R., Siitonen, A., Martinez-Urtaza, J. 2012. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nature Climate Change 3 (1), 73-77.

Baker-Austin, C., Stockley, L., Rangdale, R., Martinez-Urtaza, J. 2010. Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. Environmental Microbiology Reports 2 (1), 7-18.

Colwell, R. R. 1996. Global climate and infectious disease: the cholera paradigm. Science 274 (5295), 2025-2031.

Francy, D. S., Darner, R. A., County, C. 2006. Procedures for developing models to predict exceedances of recreational water-quality standards at coastal beaches: U.S. Geological Survey Techniques and Methods 6–B5, 1-34.

Francy, D., Gifford, A., Darner, R. 2003. *Escherichia coli* at Ohio bathing beaches—distribution, sources, wastewater indicators, and predictive modeling: US Geological Survey Water-Resources Investigations Report 02–4285, 1-117.

Frick, W. E., Ge, Z., Zepp, R. G. 2008. Nowcasting and forecasting concentrations of biological contaminants at beaches: A feasibility and case study. Environmental Science & Technology 42 (13), 4818-4824.

Ge, Z. and Frick, W. E. 2009. Time-frequency analysis of beach bacteria variations and its implication for recreational water quality modeling. Environmental Science & Technology 43 (4), 1128-1133.

Griffith, J. F. and Weisberg, S. B. 2011. Challenges in implementing new technology for beach water quality monitoring: Lessons from a California demonstration project. Marine Technology Society Journal 45 (2), 65-73.

Hurley, M. A. and Roscoe, M. E. 1983. Automated statistical analysis of microbial enumeration by dilution series. Journal of Applied Microbiology 55 (1), 159-164.

Leecaster, M. K. and Weisberg, S. B. 2001. Effect of Sampling Frequency on Shoreline Microbiology Assessments. Marine Pollution Bulletin 42 (11), 1150-1154.

Lim, S. and Jeong, Y. 2012. Decadal Trend of Coastal Water Quality in Orange County Beaches and Management Efficacy at Improving Public Health Protection. Journal of Environmental Science and Engineering A 1 (8), 967-979.

Lipp, E. K., Huq, A., Colwell, R. R. 2002. Effects of global climate on infectious disease: the cholera model. Clinical Microbiology Reviews 15 (4), 757-770.

Luettich Jr, R. A., McNinch, J. E., Paerl, H. W., Peterson, C. H., Wells, J. T., Alperin, M. J., Martens, C. S., Pinckney, J. L. 2000. Neuse River Estuary modeling and monitoring project stage 1: hydrography and circulation, water column nutrients and productivity, sedimentary processes and benthic-pelagic coupling, and benthic ecology.

Luettich, R. A., Carr, S. D., Reynolds-Fleming, J. V., Fulcher, C. W., McNinch, J. E. 2002. Semi-diurnal seiching in a shallow, micro-tidal lagoonal estuary. Continental Shelf Research 22 (11), 1669-1681.

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., Weisberg, S. B. 2010. Comparison of rapid quantitative PCR-based and conventional culture-based methods for enumeration of Enterococcus spp. and Escherichia coli in recreational waters. Applied and Environmental Microbiology 76 (22), 7437-7443.

Ohtomo, K., Kobayashi, N., Sumi, A., Ohtomo, N. 2010. Relationship of cholera incidence to El Nino and solar activity elucidated by time-series analysis. Epidemiology and infection 138 (1), 99.

Patz, J. A., Campbell-Lendrum, D., Holloway, T., Foley, J. A. 2005. Impact of regional climate change on human health. Nature 438 (7066), 310-317.

Peierls, B. L., Hall, N. S., Paerl, H. W. 2012. Non-monotonic Responses of Phytoplankton Biomass Accumulation to Hydrologic Variability: A Comparison of Two Coastal Plain North Carolina Estuaries. Estuaries and Coasts 35 (6), 1376-1392.

Chapter 6

CONCLUDING REMARKS

Predictive modeling

Surface waters of the Unites States provide valuable natural resources, including recreational and food benefits (EPA, 2010). Fecal pollution inputs contaminate these resources and pose health and economic risks. To reduce water contact risks, national legislation (i.e. the BEACH and Clean Water Acts) required federal assistance for water monitoring and notification programs. National recreational and shellfish harvesting water criteria have been updated based on new scientific studies, and international countries and organizations have been quick to follow suit (Gareth et al., 2010; NSSP, 2011; EPA, 2012). Long incubation times, man-power, and financing required by traditional enumeration techniques make predictive modeling a recommended alternative for monitoring agencies that need real-time estimates of water quality. Specifically, multiple linear regression (MLR) models have been used to make real-time estimates of fecal indicator bacteria (FIB) in marine and freshwater bodies, generating empirically-based beach notices that either provide advice about water usage or close the water location for use (USEPA, 2010). Estuaries, where marine and freshwaters meet, are often used for both recreation and shellfish harvesting. Until now, little work has been conducted in estuaries in the southeastern US, like eastern North Carolina (NC), to determine if MLR models can be effectively used. There are thousands of monitoring

sites and limited resources in this region, so it is an ideal location to implement MLR models. Successful predictive model development and validation can be highly relevant globally. While models are site specific, successful estuarine model development here can guide international organizations that have expressed interest in coastal predictive models by demonstrating which combination of easily obtained variables should be mined and tested.

Research findings

This is the first presentation of strongly validated predictive statistical models to an estuarine system for the purpose of monitoring real-time water quality.

In chapter 2, I showed concentrations of the FIB, *E. coli* and enterococci, can be predicted using empirical statistical models and a combination of antecedent rainfall, climate, and environmental variables including 5-day antecedent rainfall, dissolved oxygen, and salinity. *E. coli* and enterococci models accurately predicted a high percentage (>87%) of management decisions based on current regulatory thresholds. Models were not as successful in prediction of alternate indicators of the *Bacteroidales* group using a validation set and the RMSE and adjusted R^2 performance metrics. However, conducting the model development allowed insight into potentially important variables affecting their fate and transport in the environment.

In chapter 3, I showed that quantitative PCR (qPCR) and MLR models can be used in tandem to provide rapid estimates of water quality in non-point source impacted estuaries. Overall, model equivalency was established for enterococci and *E. coli* MLR models using culture- and qPCR-based data. Although model equivalency was

established, inhibition was a major issue that had to be addressed. The inhibition model presented in this chapter accounted for a high level of data variability and was created with easily obtained meteorological data.

In chapter 5, I examined multiple time scales of data from a NC estuary using time-frequency analysis in order to determine the optimal length of data needed for robust MLR model creation. Because there were no significant trends in the FIB data, we concluded that, based on the current data available, there were no optimal minimum time frames for FIB multiple linear regression model data collection. Managers who want to create prediction models for NC estuarine waters should collect some data, model, and then actively update the model as more data becomes available.

In developing and validating the prediction models in chapters 2, 3, and 5, water quality exceedances were frequent. Thus, in chapter 4, I sought to understand the microbial dynamics of water quality in the Newport River Estuary across a range of hydrodynamic and meteorological conditions. This chapter detailed a descriptive characterization of creeks to aid in variable selection during MLR development. We determined that FIB loading was significantly higher when precipitation during a wet weather event rose above 2.54 cm (1 in), a level that is lower than the current management action threshold of 3.81 cm (1.5 in). This finding corroborates other studies in the area that show that stormwater runoff begins to affect water bodies at 2.54 cm (1in) of rainfall. While storm water runoff is a dominant source of fecal pollution, I found that reservoir *in-situ* populations of FIB are important contributors of FIB to the water column. Fecal *Bacteroides* spp. source tracking marker recovery was low, which may indicate environmental persistence of FIB within the estuary. In addition, BacHum

microbial source tracking markers, which indicate human contamination, were only detected during four storm events, where rainfall exceeded the 3.81 cm management action threshold. The gull2 microbial source tracking marker was only detected in one sample, indicating that shorebirds were likely minimal contributors to estuarine fecal contamination.

The models created in this dissertation are globally relevant empirical predictive models for use in recreational and shellfish harvesting waters. My work will impact national and international agencies (e.g. WHO, European Union Water Initiative) by stressing the usefulness of a combination of current and antecedent rain, climate, and environmental parameters. Additionally, since I have found evidence that a major source of FIB may be environmental sources, agencies wanting to create estuarine predictive models should pursue variables that affect the survival and transport of not just runoff contamination but also *in-situ* sources as well.

Future work

When taken in combination, the chapters in this dissertation can be used by managers to supplement their 'toolbox' for water quality assessment. During my research, two major future research needs were discovered. Sample inhibition proved to be a major hindrance to qPCR, so I created a model to predict inhibition magnitude before sample processing, saving time and money. However, this model, which was based on meteorological variables, needs to be vigorously tested at different site types and under a wide range of conditions. Secondly, in chapter 4, I was able to eliminate many potential sources of human fecal contamination. Future work using microbial source tracking should focus on *in situ* environmental sources of fecal contamination.

References

National Shellfish Sanitation Program, NSSP. 2011. National shellfish sanitation program Guide for the control of molluscan shellfish. http://www.fda.gov/downloads/Food/GuidanceRegulation/FederalStateFoodPrograms/UCM350344.pdf.

Rees, G., Pond, K., Bartram, J., Santo Domingo, J. (eds.), 2010. *Safe management of shellfish and harvest waters*. IWA Publishing (International Water Association).

USEPA, 2012. Recreational Water Quality Criteria. http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/upload/RWQC2012.pdf.

USEPA. 2010. Predictive Tools for Beach Notification Volume I: Review and Technical Protocol. http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/upload/P26-Report-Volume-I-Final_508.pdf.