UNDERSTANDING FECAL CONTAMINATION DYNAMICS THROUGH THE INTEGRATION OF MOLECULAR PATHOGEN QUANTIFICATION AND LAND-WATER INTERFACE CHARACTERISTICS

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Science and Engineering.

Chapel Hill
2020

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ABSTRACT

Matthew T. Price: Understanding fecal contamination dynamics through the integration of molecular pathogen quantification and land-water interface characteristics
(Under the direction of Rachel T. Noble)

To reduce public health risks and related economic losses, federal guidelines have been established to ensure surface waters meet water quality standards. For example, the United States Environmental Protection Agency released criteria in 1986 that recommended state and local governments establish and enforce regulations to protect ambient waters against naturally-occurring or anthropogenic contaminants. Most of the regulations that were enacted were designed to address recreational water quality because of the risk of illness associated with contact and ingestion of contaminated recreational waters. It wasn't until 26 years after US EPA's 1986 release of criteria that new guidance was issued regarding updated tools for managing recreational surface waters. In this report, US EPA included updated recommended criteria for acceptable levels of fecal indicator bacteria, *E. coli* and enterococci, within surface waters, while also introducing recommended molecular tools. In this dissertation, I applied these molecular methods with current regulatory tools, in an eastern North Carolina (NC) estuary heavily influenced by tidal inundation to better understand potential environmental drivers of surface water contaminant transport. Additionally, enterococci, which is the FIB used for NC's regulatory assessment of surface water quality, can also be forecast using predictive modeling tools such as multiple linear regression (MLR) models. Similar to what was recommended with regards to incorporating molecular approaches, predictive modeling tools were also a newly

suggested monitoring tool recommended by US EPA in the 2012 update. Using a combination of *E. coli* concentration, tidal phase, and antecedent rainfall, the first part of this dissertation focused on the combined assessment of quantitative-PCR (qPCR), FIB and environmental parameters to show the practicality of using MLR in a regulatory framework to provide estimates of water quality in estuaries, specifically impacted by tidal inundation. Additionally, recent advancements towards the implementation of a fecal indicator virus (FIV), coliphage, have also been proposed as a monitoring tool for use in fresh and marine surface waters. However, the utility of coliphage as an additional water quality management criterion has yet to be fully evaluated. Using US EPA developed protocols for quantification of somatic and male specific coliphage, the second focus of this work looked at the applicability of using such a fecal indicator virus into a monitoring framework by comparing relationships of coliphages with FIB and qMST approaches in surface waters with diffuse source pollution. It was determined that coliphage enumeration in this system proved to be cumbersome, and expensive and, as such, it is suggested that for surface water monitoring, it may be useful to focus on a combination of qPCR and FIB approaches to identify hot spots, and better quantify specific sources of human fecal contamination. Finally, watershed-scale drivers of fecal contamination were assessed in the context of qMST and FIB molecular markers with environmental parameters such as elevation, land use and land cover. Work here was conducted in an urban watershed within the Washington DC metropolitan area and detailed a prioritization of sites across the sampling landscape based on qMST and FIB marker concentrations most associated with risk. This study also incorporated the use of predictive modeling with the ultimate goal of the research being to provide coastal managers approaches that may be incorporated in future water quality monitoring program designs across vast geo-spatial scales.

ACKNOWLEDGMENTS

There are so many people who have helped me throughout this process that I am not sure one page will suffice, but first and foremost thank you to my advisor and mentor, Rachel Noble. She has been an integral part in my academic growth during this madness we call graduate school. Through natural disasters and pandemics, she has remained patient and kind and has served as the mentor I strive to be for future students one day. I also would not be where I am today without the help of the Noble lab member, past and present. Thank you to Denene Blackwood for being such an amazing lab manager and to the rest of the crew for being there at a moment's notice, even if that notice requires 3 am sample processing. I thank all of my committee members for the many hours spent advising me to evolve my research into the best possible work it can be.

I am incredibly appreciative to have learned from the very best and I look forward to continued collaborations in the future. I am also thankful to my family: specifically, to my husband and to my parents for always being my biggest champions and for making me feel like my work means so much more than just what is on paper. And, finally, thank you to all the amazing friends I've met over the last few years: for your support, shoulders to cry on, and the many experiences we've shared, have made it all worth it. Thank you again to you all.

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LIST OF ABBREVIATIONS

BEACH	Beaches Environmental Assessment and Coastal Health
BMP	Best Management Practice
CCE	Calibrator Cell Equivalent
CDC	Centers for Disease Control
CE	Cell Equivalent
CFU	Colony Forming Unit
CSO	Combined Sewer Overflow
Ct	Cycle Threshold
CWA	Clean Water Act
ddPCR	Droplet Digital PCR
DEM	Digital Elevation Model
DO	Dissolved Oxygen
E. coli	*Escherichia coli*
EC	*E. coli*
ENT	enterococci
FIB	Fecal Indicator Bacteria
GI	Gastrointestinal illness
HA	Mixed Cellulose Ester
HUC	Hydrologic Unit Code
MD	Maryland
MD DoP	State of Maryland Department of Planning
MLR	Multiple Linear Regression

MPN	Most Probable Number
MST	Microbial Source Tracking
NC	North Carolina
NC DENR	North Carolina Department of Environment and Natural Resources
NC DMF	North Carolina Department of Marine Fisheries
NERRS	National Estuarine Research Reserve System
NLCD	National Land Cover Database
NOAA	National Oceanic and Atmospheric Administration
PBS	Phosphate Buffered Saline
PC	Polycarbonate
qMST	Quantitative Microbial Source Tracking
qPCR	Quantitative Polymerase Chain Reaction
RCR	Rachel Carson Reserve
RMSE	Root Mean Squared Error
RWQC	Recreational Water Quality Criteria
SAL	Single Agar Layer
SCCWRP	Southern California Coastal Water Research Project
SCM	Stormwater Control Measures
SD	Standard Deviation
SLR	Sea-Level Rise
SPC	Specimen Processing Control
SS	Sum of Squares
STV	Statistical Threshold Value

TC	Tidal Cycle
TH	Tidal Height
TP	Tidal Phase
TMDL	Total Maximum Daily Load
ToB	Town of Beaufort
UF	Ultrafiltration
US EPA	United States Environmental Protection Agency
WSSC	Washington Sanitation Suburban Commission

CHAPTER 1: INTRODUCTION

Contamination of fresh and marine surface waters used for contact recreation is a significant concern worldwide. Serving as a major contributor, fecal waste is a major causative agent of water degradation resulting in depleted ecosystem health, economic loss and illness risks, such as gastrointestinal illness (GI), respiratory and skin infections (Arnold et al., 2016; Boehm et al., 2015; Napier et al., 2018). It is estimated globally, that exposure to fecally-contaminated coastal waters results in approximately 120 million GI and 50 million severe respiratory illnesses per year (Boehm & Soller, 2013; Shuval, 2003a). Additionally, fecal-related illnesses have an economic burden in the form of hospital costs and lost income for afflicted individuals. Annual economic burden related to recreating in contaminated surfaces is believed to relate to costs between $2.2–3.7 billion, while only a fraction of these costs is allocated ($10 million) towards beach water protection programs (DeFlorio-Barker et al., 2018; US EPA, 2020).

To reduce the aforementioned effects of contaminated surface waters, in 1972 the US Environmental Protection Agency (US EPA) implemented the Clean Water Act (CWA) which sets guidelines for the discharge of pollutants to surface waters, including recreational water beaches. In 1986, these regulations were amended to include recommended fecal indicator bacteria (FIB) criteria for marine and fresh surface waters to protect beachgoers from diarrheal illness (Centers for Disease Control and Prevention, 2019). FIB, which serve as a cost-effective alternative to direct assay of microbial pathogens, have been used effectively to manage waters for decades throughout the US. FIB are widely available in the intestinal flora of warm-blooded vertebrates and while they may not directly cause human gastrointestinal illness, their occurrence

often correlates with adverse human health outcomes (Arnold et al., 2017; Cabelli, 1989; Lamparelli et al., 2015; Wade et al., 2010).

Enterococcus sp. (ENT) and *Escherichia coli* (EC) are used for fresh surface waters and *Enterococcus* sp. for marine surface waters. To enumerate FIB, there currently two methods employed, traditional, culture-based approaches and modern molecular methods. Traditional culture-based methods, like membrane filtration and defined-substrate technology tests such as IDEXX kits, while inexpensive and user-friendly, lack specificity with regards to fecal contaminant source (Wade et al., 2008). Alternative approaches, such as quantitative PCR (qPCR) and droplet digital PCR (ddPCR) can specifically determine whether the source of fecal waste are human or non-human derived (Noble et al. 2010, Griffith and Weisberg, 2011). As such, strong relationships have been demonstrated between qPCR-based concentrations and human health outcomes suggesting a strong link between the presence of certain molecular marker and illness (Warish Ahmed et al., 2018a; Colford et al., 2012; Napier et al., 2017).

In 2012, the US EPA revised the Recreational Water Quality Criteria (RWQC) to include additional tools for water quality management such as predictive modeling, quantitative microbial source tracking, and quantitative microbial risk assessment to name a few. Through the inclusion of predictive modeling tools, they opened the door for the incorporation of environmental parameter data from the land-water interface into modeling to understand drivers of fecal contamination at the local and watershed scale. It has been recognized that these types of efforts can save valuable money for routine monitoring, and focus resources on problem areas, but few have incorporated these approaches into assessments of complex estuarine/coastal systems. Frequently, multiple linear regression (MLR) models have been used to predict recreational water quality (Francy & Darner, 2007; Gonzalez & Noble, 2014; Molina et al.,

2014; Nevers & Whitman, 2011). MLR is an empirical statistical modeling approach that predicts FIB and MST concentrations by relating water quality to certain environmental factors such as antecedent rainfall, salinity or tidal height. When frequent monitoring of coastal waters is not possible, MLR modeling is a valuable tool for managers.

Stormwater runoff and sewage discharge remain the two largest contributors to surface water quality impairment nationwide. Flowing directly over pervious and impervious surfaces, stormwater picks up pollutants including potentially pathogenic bacteria and viruses from animal and human waste (Galfi et al., 2016; Griffin et al., 2003; Hathaway & Hunt, 2011; Mallin et al., 2009). Often times, this runoff enters stormwater distribution systems that then convey the untreated runoff into downstream receiving waterbodies, adversely impacting water quality. In this dissertation, a framework of traditional fecal indicator bacteria (FIB) quantification approaches and advanced molecular quantification tools was constructed to understand fecal contamination delivery in the context of different land-water interfaces.

The mid-Atlantic region of the US is the most densely inhabited in the country with approximately 40 million residents in the metropolitan areas between New York City and Washington, DC (US Census, 2013). The region, which is defined by its low elevation and gently sloping topography, can be impacted by episodic flooding due to intense storm events and tidal inundation. Storm events and tidal inundation will be compounded by sea-level rise (SLR), which is the global increase in the recorded level of the world's oceans due to the effects of global climate change (NOAA, 2020). Tide-gauge records throughout the region already indicate an enhanced increase in the rate of sea level rise with an average increase of approximately 3.8 mm per year (Ezer, 2019; Ezer et al., 2013; Kopp, 2013; Miller et al., 2013).

Chapter 2 of this dissertation focused on the microbial contaminants in stormwater and utilized an integrated FIB/qMST monitoring framework to understand tidal influence on stormwater delivery. The study focused on quantification of FIB using both culture and molecular approaches, as well as characterization of fecal contamination sources through the use of qMST approaches over a range of wet (storm), dry and tidal conditions. To account for tidal impact, samples were divided into three categories (inundated, transition and receding) based time as it related to the nearest recorded high tide. Additionally, a multi-sample, time-paced storm sampling strategy was employed during storm events to ensure samples were collected at various times along the hydrograph. Predictive models were generated using observed relationships across FIB and tidal phase to predict concentrations of *Enterococcus* sp. With this work, we hope to begin to place tidal characteristics into the context of stormwater of delivery.

Following the development of the Chapter 2 framework, fecal indicator virus (FIV), somatic and male-specific (F+) coliphages, were included in the study and evaluated for their utility as additional water quality criteria in the same complex, coastal, stormwater-driven system. Following US EPA Method 1642 protocol, whose method required dead-end hollow fiber ultrafiltration (UF) combined with single agar layer (SAL), the overall objectives of this study were to determine the prevalence of somatic and F+ coliphages in an estuarine tidal creek while also identifying key water quality and surface water parameters related with subsequent concentrations. By doing so, we were able to assess the applicability of US EPA 1642 within the context of a regulatory framework to efficiently measure FIV in systems less influenced by anthropogenic input.

Finally, Chapter 3 focused on the integration of FIB and qMST approaches at the watershed-scale across the Washington DC metropolitan area in order to better assess drivers of fecal

contamination trends in the context of parameters such as elevation, land use and land cover. While FIB and qMST dynamics have greatly advanced our ability to identify wastewater-impacted waters, diffuse sources of fecal pollution remain difficult to mitigate, especially across large spatial areas. As such, a comprehensive watershed approach may sometimes be necessary to manage water quality (Badgley et al., 2019; Bradshaw et al., 2016; Stewart et al., 2013). This approach requires identifying sources of fecal contamination that often occur simultaneously throughout the landscape and to consider environmental drivers influencing water quality. With this in mind, the primary objective for Chapter 3 was to address the applicability of a watershed-scale analysis in an urban landscape by examining various quantitative microbial source tracking marker concentrations in surface waters at varying watershed scales, under moderate elevation ranges and exhibiting different land use and land cover influences. By doing so, we may begin to develop prioritization efforts needed by water quality managers to better assist future mitigation strategies.

Taken collectively, the research outlined in this dissertation will provide useful tools for water quality researchers and managers to improve capabilities to understand drivers of water quality impairment. The advancements come at a time when little guidance is provided on the integration of existing FIB quantification approaches with newer, recommended tools for water quality management. As water quality managers improve their understanding of the implementation of the new tools, it is hoped that improvements will take place in the ability to mitigate stormwater, minimize the adverse impacts of tidal inundation on contaminant delivery in low-lying areas, and conduct municipal or regional prioritization of infrastructure repairs.

Figure 1.1. Sampling region used in this dissertation. Blue star indicates sampling area for Chapters 2 & 3 while red star indicates the sampling region for Chapter 4.

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CHAPTER 2: INTEGRATING CULTURE AND MOLECULAR QUANTIFICATION OF MICROBIAL CONTAMINANTS INTO A PREDICTIVE MODELING FRAMEWORK IN A LOW-LYING, TIDALLY-INFLUENCED COASTAL WATERSHED

2.1 SUMMARY

Contaminated stormwater runoff is a major causative agent of impairment in coastal receiving waters. There are significant public health risks associated with contaminated stormwater runoff impacting estuarine and coastal systems, however very few studies exist that have used a comprehensive monitoring framework as the foundation for an examination of the impacts of tidal inundation on stormwater conveyance and delivery. In the Town of Beaufort, North Carolina (NC), stormwater inputs adversely impact a prominently used estuarine reserve, the Rachel Carson Reserve (RCR) that lies proximal to the town and supports a diverse range of coastal habitats including tidal flats, salt marshes and maritime forests. We conducted field sampling multi-sample, time-paced storm event characterization paired with dry weather baseline monitoring program. All samples were analyzed using both conventional fecal indicator bacteria (FIB) enumeration approaches, combined with vetted quantitative microbial source tracking (qMST) assessments. Samples were collected over the course of one year from July 2017 to June 2018 and classified using tidal stage (Ex. inundated, receding and transition). Once fully analyzed, we used the generated data to develop a multiple linear regression model to predict concentrations of *Enterococcus* sp. related to tidal cycle, salinity and antecedent rainfall. Using this approach, we demonstrated that the concentration of *Enterococcus* sp. could be predicted by *E. coli* and tidal

phase ($Y_{ENT} = \beta_{EC} + \beta_{Rain} + (\beta_{Tidal\ Height} \times \beta_{Tidal\ Phase}) + (\beta_{Tidal\ Height} \times \beta_{Tidal\ Cycle})$). We also observed that FIB concentrations were significantly (<0.05) influenced by tide with higher concentrations observed in samples collected during receding (low) tides (EC: log 3.12 MPN/100 mL; ENT: 2.67 MPN/100 mL) compared to those collected during inundated (high) (EC: log 2.62 MPN/100 mL; ENT: 2.11 MPN/100 mL) or transition (EC: log 2.74 MPN/100 mL; ENT: 2.53 MPN/100 mL) tidal periods. Environmental parameters, such as salinity, were also found to significantly ($p<0.05$) correlate with *Enterococcus* sp. concentrations during periods of tidal inundation. Tide was shown to be a significant driver in explaining the variability in observed *Enterococcus* sp. concentrations, unlike precipitation, which was not determined to be a major driver of *Enterococcus* sp. concentration. This project demonstrated that water quality monitoring programs in low-lying coastal communities affected by tidal inundation should incorporate tidal parameters. It has also demonstrated that typical hydrograph-based evaluations conducted absent of knowledge of tidal inundation is likely an over-simplification of stormwater delivery to receiving waters.

2.2 INTRODUCTION

Stormwater runoff is one of the most important hydrological factors affecting surface water quality (Ahn et al., 2005; Mallin et al., 2009). Flowing directly overland, stormwater picks up pollutants including potentially pathogenic bacteria and viruses from animal and human waste (Griffin et al., 2003; Haile et al., 1999; Mallin et al., 2000; Prüss, 1998). Often times, this runoff enters stormwater conveyance systems that then carry the untreated runoff into downstream waterbodies, adversely impacting water quality and health for primary contact recreators.

Protection of public health is a key outcome of stormwater mitigation practices. Elevated levels of pathogenic bacteria and viruses represent the most common hazard to human health and have been significantly linked to disease outcome (Bichai & Ashbolt, 2017; Sinclair et al., 2009;

Soller et al., 2014). These are commonly found in stormwater runoff and carried to downstream surface waters via stormwater conveyance systems, combined sewer overflows, agricultural runoff and defecation of wild animals (Ahmed et al., 2019; Al Aukidy & Verlicchi, 2017; Noble et al., 2006). Additionally, pathogen loading to surface waters is often event-driven with increases of sewage contamination during rain events (Soller et al., 2015; Tolouei et al., 2019). Acute respiratory and gastrointestinal illnesses (GI) can result from ingestion or contact with contaminated water with these risks being highest when the fecal source is human-derived (Ex. sewage) (Arnold et al., 2016; Boehm et al., 2015; Cabelli et al., 1982). However, diverse sources of fecal contaminants (human and animal feces) are often discovered in stormwater, posing unique challenges in terms of identifying sources in addition to attributing human health risks.

The United States (US) Environmental Protection Agency (US EPA) has recommended the use of enterococci (ENT) and *Escherichia* coli (EC) as fecal indicator bacteria (FIB) to monitor both marine and fresh surface waters (US EPA, 2012). FIB serve as a proxy for the presence of microbial pathogens associated with feces. Ingesting water with high concentrations of FIB through recreation can lead to gastrointestinal and other illnesses (Colford et al., 2007; Haile et al., 1999; Soller et al., 2017). Additionally, FIB have been selected due to their low pathogenic potential and high concentrations in sewage and feces (Ahmed et al., 2008; Ahmed et al., 2019; Harwood et al., 2014; Sidhu et al., 2012). One major drawback towards the use of FIB, however, is their lack of source-specificity (Ex. human vs. non-human) regarding fecal contamination. As such, quantitative microbial source tracking tools (qMST) have been proposed.

Quantitative microbial source tracking methods aim to discriminate between human and non-human fecal sources in contaminated waterbodies (Lee et al., 2020; Nguyen et al., 2018; Shanks et al., 2015). The performance of human-specific (Ex. HF183) markers are of particular

interest to mitigate public health risks, given their utility and strong relationships to observed risk in sewage-impacted waters (Badgley et al., 2019; Haugland et al., 2010; Jothikumar et al., 2005). Additionally, US EPA has published recommendations for concentrations for *Enterococcus* sp. quantified via a qPCR-based approach in fresh and marine surface waters (Haugland et al., 2005; US EPA Method 1609 &1611, 2012). Previous epidemiological studies have indicated a stronger link between swimming-associated gastrointestinal illnesses and molecular approaches for *Enterococcus* sp. via qPCR compared to traditional culture-based methods (Arnold et al., 2016; Colford et al., 2012; Wade et al., 2008). Greater understanding of the concentrations of specific fecal qMST source markers relative to culture-based FIB enumeration used in routine water quality monitoring is necessary, especially within the context of coastal systems.

Significant research has been conducted relating EC and ENT concentrations to antecedent rainfall patterns finding greater FIB concentrations during peak hydrologic flows (Ahn et al., 2005; Lipp et al., 2001; Shehane et al., 2005; Stumpf et al., 2010). Additionally, the link between FIB prevalence and environmental parameters, such as salinity and water temperature, has also been established (Converse et al., 2011; Eregno et al., 2018; Gonzalez & Noble, 2014; Paule-Mercado et al., 2016). What has not been extensively studied, however, is the relationship between stormwater delivery and tide. A number of studies have reported on a dilution effect affecting stormwater during high tides, resulting in lower concentrations of fecal indicator bacteria (Coelho et al., 1999; Mallin et al., 1999; Mill et al., 2006; Wilhelm et al., 2002), but none have related this to stormwater delivery mechanisms across the tidal cycle.

Coastal North Carolina (NC) has over 5900 km^2 of land below 1-m elevation (Figure 1), making it the third largest low-lying region in the US (Poulter et al., 2009; Titus & Richman, 2001). Additionally, much of the coastal zone in NC has a low topographic slope increasing at

less than 0.09 m elevation for every horizontal mile (Corbett et al., 2008). As such, coastal NC remains susceptible to the effects of global climate change, including sea level rise, intensifying extreme storm events and increasing tidal ranges and sunny-day flooding (Hino et al., 2019). Sea level off the NC coast has increased 0.28 m as compared to 1950. The rate of rise accelerating over the last decade to now increasing by over 0.03 m every 2 years (NOAA, 2020; NC Coastal Resources Commission, 2015). This coupled with increased nuisance flooding frequency events suggest coastal surface waters along the coast of NC are at risk for continual impairment (King Tides Project, 2020; Sweet et al., 2014).

The study site for this research is located in Beaufort, NC, a coastal community situated in the coastal plain region of southeastern NC with a relatively small permanent population (4,391) that experiences seasonal growth given its proximity to coastal waters and productive tourism industry (US Census Bureau, 2020). The town sits proximal to the Rachel Carson Reserve (RCR), a series of islands and estuarine waters comprising approximately 2,000 acres within the NC National Estuarine Research Reserve System (NERRS). The RCR is strongly influenced by river and tidal dynamics and, as such, supports a diverse array of wildlife and coastal habitats, including tidal flats, salt marshes and maritime forests (NC DEQ, 2020). Therefore, methodologies incorporated within the framework of this research aimed to assess environmental surface water samples proximal to the RCR.

To our knowledge, this is the first study that has utilized a comprehensive microbial contaminant monitoring framework conducted over a wide range of climatic conditions to examine the importance of tidal phase on stormwater contaminant delivery. The primary objectives of this research were to 1) determine the concentrations and sources of fecal contaminants in discharge conveyed to receiving waters using a multi-sample, time-paced storm

sampling strategy employed during both storm events and ambient conditions at various times throughout the tidal cycle, 2) relate FIB and qMST marker concentrations to parameters such as tidal height, 24-h rainfall, salinity and total suspended solids (TSS) in an effort to understand potential environmental drivers of fecal contamination, and 3) use a predictive modeling tool to predict concentrations of *Enterococcus* sp. in the context of tidal height, cycle ad phase. The overall advancement associated with this work is to begin to understand patterns of delivery of microbial contaminants during storms to improve capabilities related to routine water quality monitoring.

2.3 MATERIALS AND METHODS

2.3.1 Study Sites and Sample Collection

Water samples were collected at three sampling locations throughout Beaufort (Figure 2): two at stormwater outfall locations (Orange St. and Marsh/Pollock) proximal to downstream receiving waters (Taylor's Creek) and a third site (Ann St.) one block inland that was selected to characterize watershed conditions. Nineteen sampling events were conducted seasonally over the course of 11 months from July 2017 – June 2018, with samples collected during both storm and ambient conditions. Storm sampling was initiated after a sustained period of moderate to heavy rainfall which produced accumulation of at least ~0.25 in until ~1 h after the storm ended. Dry weather samples were collected following three days without rainfall accumulation.

Samples were collected using both an automatic and grab sampling approach. Automatic grab sampling was conducted using an ISCO 6712 Portable Sampler where composite samples were collected every 3 hours and stored for up to 6 hours before processing. Following collection, samples were stored on ice and transported to the laboratory where they were analyzed within 2 hours of collection.

2.3.2 Environmental Parameters

Water temperature, dissolved oxygen (DO) and salinity were measured in situ using a YSI probe (YSI 6600 multiparameter probe, USA). Grab samples were filtered through Whatman GF/F filters (25 mm diameter, 0.7 µm nominal pore size), and analyzed for nitrate N (NO_x^-), ammonium (NH_4^+), phosphorus (PO_x^-) and total nitrogen (TN). Additionally, meteorological observations (Ex. 24-h antecedent rainfall, tidal height and air temperature) were collected from publicly available data provided by NOAA: Station (ID: 8656483). We were able to determine the relative meteorological conditions by rounding sample collection time to the nearest NOAA sampling point (6-minute increments).

2.3.3 Tidal Characterization

Similar to methods conducted in Boehm & Weisberg (2005), samples were classified into three tidal categories (Ex. receding, inundated and transition) classified by collection time as it related to the nearest recorded high tide. Given the semi-diurnal nature of tides within our system, samples were separated into three tidal categories: inundated (high tide), receding (low tide) or transition. Inundated samples were classified so if they had been collected within 2 hours of the previous high tide, while receding samples were collected >4 hours from the previous high tide. Transition samples were those collected in between the two groups (2-4 hours from nearest high tide). In addition, GPS locations and elevations were collected (Table 1) using a Trimble R8 RTK GPS relative to NAVD88 where average vertical error was ± 1.2 in. Outfall elevations were then used to verify coverage given NOAA verified tidal recordings.

2.3.4 Sample Preparation

FIB *E. coli* and enterococci were enumerated using Colilert-18® and Enterolert™ per manufacturer instructions (IDEXX Laboratories, Westbrook, ME). For downstream molecular

analysis, triplicate 100-150 ml samples were vacuum filtered through 0.45 μm pore size, 47 mm polycarbonate (PC) filters (HTTP, Millipore, Bedford, MA) using a six-place filtration manifold and vacuum pump assembly. The filters were placed into sterile, DNase/RNase-free microcentrifuge tubes and stored at -80 °C. DNA extractions were performed using the NUCLISENS® MINIMAG® extraction kit per manufacturer instructions, with extracts then stored at -20 °C. Consequent qPCR quantification of a total *Enterococcus* sp. FIB marker and human-specific fecal marker (HF183) was done using the primers, probes, and assays described in Table 2 below. Assays were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Extracted samples were processed using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts). Primers (100 μM) and probes (10 μM) were synthesized by LGC Biosearch Technologies (Petaluma, CA). Each reaction had a total volume of 25 μL, 20 μL including nuclease-free water, TaqMan® Environmental Master Mix 2.0, as well as appropriate primers and probes, and 5 μL of unknown sample, standard, or control. No template controls (NTCs) were processed with every plate.

2.3.5 Assessment of qPCR Specimen Processing Control and Inhibition Control

Performance of the qPCR assays through evaluation of recovery efficiency and qPCR inhibition was measured using β actin (*ACTB*) cDNA as a specimen processing control (SPC) as previously conducted by Conn et al. (2012). 5 μL of *ACTB* solution (4000 copies/μL) was pipetted into each of the samples, calibrators, and negative controls prior to processing. Following this, samples were extracted. Inhibition was determined by calculating the difference between the cycle threshold (Ct) of the SPC in samples with (experimental) and without (control,

only SPC) target DNA. Extracts were analyzed without dilution with samples having more than 0.5 log units (2.32 Ct) difference from control samples deemed inhibited (Lambertini et al., 2008). Since the total number of inhibited samples (11 out of 167 samples) constituted only 6.6% of total samples inhibited, no adjustment for inhibition was made. For all qPCR runs, appropriate controls were employed and showed no contamination: no template control (omission of DNA template from the qPCR reaction), and negative extractions control (inclusion of filter blank during DNA extraction). Plasmid standards were used for HF183 and *Enterococcus* sp. via qPCR assays. Standards were synthesized by GenScript (Piscataway, NJ). Gene sequences were synthesized and inserted into a linearized pUC57 vector which was cloned into DH5α competent cells. Plasmids were extracted using Wizard® Plus SV 10 Minipreps DNA Purification System (Promega Corp., Madison, WI) and linearized using Eco R1 digestion. They were then confirmed via a 1% agarose gel in Tris-Acetate-EDTA buffer. The weight of purified plasmids was then calculated spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Waltham, MA). Nanograms of plasmids were transformed to copy number by using a copy number calculator (SciencePrimer.com). Linearized plasmids were diluted and stored at a concentration of 1×10^8 copies per μL at -20°C.

2.3.6 Standard Curves

Standard curves for HF183 and *Enterococcus* sp. via qPCR consisted of the calibration standard and five 10-fold serial dilutions that were run in triplicate. For each of the molecular markers, standard dilution curves were aggregated to form a singular curve. The theoretical limit of detection (LOD) was the lowest concentration where the standard could be detected reliably in at least 50% of qPCR replicates. The limit of quantification (LOQ) for qPCR assays was defined as the lowest concentration above the lowest point on the standard curve where amplification

was observed in at least 50% of qPCR replicates. Curves, along with their respective total number of points, average amplification efficiencies, R^2 values, LOD and LOQ for the HF183 and *Enterococcus* sp. via qPCR assays are presented in Table 3.

2.3.7 Multiple linear regression models

Predictive modeling was also incorporated in the form of multiple linear regression (MLR) models. MLR is a statistical technique that uses several explanatory variables to predict the outcome of a response variable. For the purposes of our study, enterococci consistently served as our response variable, given its regulatory importance in surface water quality monitoring in NC. Additionally, FIB *E. coli* and 24-h antecedent rainfall were incorporated with three tidal variables: tidal height (TH), tidal phase (TP) and tidal cycle (TC). Tidal height was incorporated using verified tidal height data recorded by NOAA, while the tidal phase variable incorporated distance the sample was taken from the nearest high tide. An additional variable accounting for tidal cycle was also included in regression analysis. This was done using the sine and cosine functions to characterize the cyclical nature of tides:

$$\text{Sin}\left(2 \times \pi \times \left(\frac{Minutes\ from\ high\ tide}{Total\ minutes\ between\ high\ tides}\right)\right)$$

$$\text{Cos}\left(2 \times \pi \times \left(\frac{Minutes\ from\ high\ tide}{Total\ minutes\ between\ high\ tides}\right)\right)$$

$$\text{Tidal Cycle} = \text{Sin}\left(2 \times \pi \times \left(\frac{Minutes\ from\ high\ tide}{Total\ minutes\ between\ high\ tides}\right)\right) + \text{Cos}\left(2 \times \pi \times \left(\frac{Minutes\ from\ high\ tide}{Total\ minutes\ between\ high\ tides}\right)\right)$$

Using the regression model formula:

$$Y_i = \beta_0 + \beta_1 + \beta_2 x_1 + \beta_2 x_2$$

where Y_i is the log-transformed outcome ENT concentrations, β_k is the estimated coefficient (EC concentration, 24-h antecedent rainfall and tidal height) for variables X_1 (tidal phase) and X_2 (tidal cycle). Including the aforementioned terms, the final regression model was as follows:

$$Y_{ENT} = \beta_{EC} + \beta_{Rain} + (\beta_{Tidal\ Height} \times \beta_{Tidal\ Phase}) + (\beta_{Tidal\ Height} \times \beta_{Tidal\ Cycle})$$

2.3.8 Statistical Analysis

Log_{10} concentrations between FIB and qMST markers and environmental parameters were compared using matched paired t-tests for lognormally distributed samples or the nonparametric Wilcoxon Ranks-Sum Test for samples that did not fit a lognormal distribution. Non-detect samples were assigned a value of 5 copies/100 mL (log 0.7) with significance level set at 0.05 for all analyses. Analyses were conducted in OriginPro 8.5 (OriginLab, Northampton, MA).

2.4 RESULTS

2.4.1 Summary Statistics

In total, 137 samples were collected and analyzed using culture-based FIB enumeration, qPCR-based *Enterococcus* sp. enumeration and qMST marker enumeration using vetted, published qPCR-based approaches. Concentrations of EC (log 0.7 – 4.94 MPN/100 mL) and ENT (log 0.7 – 4.78 MPN/100 mL) were comparable to those of the molecular markers, HF183 (log 0.7 – 4.07 copies/100 mL) and *Enterococcus* sp. quantification via qPCR (log 0.7 – 5.03 copies/100 mL). Significant correlations were observed across combinations of FIB and qMST markers with significant positive correlations found between ENT and EC (r: 0.65; $p < 0.01$), *Enterococcus* sp. via qPCR (r: 0.71; $p < 0.01$) and HF183 (r: 0.45; $p < 0.01$).

In an attempt to understand stormwater conveyance as it relates to tidal cycle, samples were collected over a wide range of precipitation and tidal conditions (Figure 3). On average, log EC

and ENT concentrations in samples collected during storm events were 2.90 and 2.39 MPN/100 mL respectively, compared to average concentrations of 2.41 and 2.14 MPN/100 mL respectively during dry conditions. This was also true for qMST markers as HF183 and *Enterococcus* sp. quantified via qPCR were also found at mean higher concentrations in samples collected during storm conditions (HF183: log 2.08 copies/100 mL; *Enterococcus* sp. via qPCR: log 3.36 copies/100 mL) compared to those collected under ambient conditions (HF183: log 2.03 copies/100 mL; *Enterococcus* sp. via qPCR: log 2.70 copies/100 mL). When tested for significance, none of the differences in concentration between wet vs. dry conditions were found to be significantly different ($p<0.05$).

Salinity measurements also indicate a diverse array of samples were collected during storm and tidal conditions, as these values ranged from 0-35 parts per thousand (ppt) suggesting both fresh, stormwater samples along with marine, creek water samples were included in overall analysis. Additionally, a wide range of water temperatures that ranged from 9.0^{o}C during the winter months, to 28.2^{o}C during the summer months, indicate seasonality was also considered in sample collection.

2.4.2 Inter-Site Variability

On average, mean FIB and qMST marker concentrations where consistently higher at AS compared to those at the OS and M/P locations (Figure 4). Concentrations of EC, ENT and *Enterococcus* sp. via qPCR concentrations at the upstream, inland AS location averaged 3.62 MPN/100 mL, 3.10 MPN/100 mL and 3.96 copies/100 mL respectively, compared to average values of 2.15 MPN/100 mL, 1.76 MPN/100 mL and 2.19 copies/100 mL at OS and 2.69 MPN/100 mL, 2.39 MPN/100 mL and 3.08 copies/100 mL at M/P. The distributions of qMST marker and FIB marker concentrations measured across the sample sites were skewed, with

relatively low average EC and ENT concentrations observed for the two downstream locations (OS and M/P), and high concentrations at the inland location. As such, we wanted to assess FIB and qMST marker concentrations in samples that would exceed US EPA recommended criteria based on either molecular (*Enterococcus* sp. via qPCR: 1280 copies/100 mL (log 3.11)) or culture (EC: 320 MPN/100 mL (log 2.51); ENT: 104 MPN/100 mL) (log 2.04)) criteria defined in 2012 by US EPA and the NC Department of Environmental Quality (NC DEQ, 2020; US EPA, 2012). Previous reports in the literature have cross-linked the risk associated with *Enterococcus* sp. in sewage to measured concentrations of the qMST marker-HF183 (equivalent to 4200 copies/100 mL (log 3.62) (Boehm et al., 2015). Table 5 below summarizes the samples as they relate to recommended exceedance thresholds for each individual group of FIB and qMST markers.

Samples collected at the AS location consistently exceeded recommended concentrations for both culture- and qPCR-based quantification of FIB concentration. For ENT, 79% of samples collected during all environmental conditions exceeded the NC Department of Environmental Quality (DEQ) state threshold of 104 MPN/100 mL. This was also true when samples were analyzed for concentration of *Enterococcus* sp. via qPCR, which exceeded US EPA recommended criteria in approximately 83% of samples. When we compare these exceedances to the two downstream locations, which are influenced more greatly by tidal inundation, exceedance of FIB concentrations decreases. FIB exceedances were lowest at the OS outfall with approximately 32% and 27% of samples exceeding recommended EC and ENT concentrations respectively. This compares to an exceedance rate of 14% for samples analyzed for ENT concentrations via qPCR. HF183 concentrations, which are specifically associated with human fecal sources, only exceeded suggested thresholds (4200 copies per 100 mL, (Boehm et al.,

2015)) in approximately one-third of samples at AS and M/P with fewer samples (15%) exceeding suggested thresholds at OS.

2.4.3 Tidal Characterization

Descriptive statistics were calculated across sample sites as characterized by collection time within the tidal cycle (Table 6). Across the three tidal categories (inundated, transition and receding), FIB and qMST marker concentrations were consistently higher at the AS location when compared to the two downstream sites: OS and M/P. FIB and qMST marker concentrations were compared across tidal classifications using one-way ANOVA calculations with only EC concentrations significantly ($p<0.05$) differing between inundation and receding tidal periods. The same analyses were performed between FIB characterized by sites across the different tidal phases. At OS, significant ($p<0.05$) differences were found between ENT and HF183 concentrations between inundated (high) and receding (low) tides, while EC and *Enterococcus* sp. determined via qPCR concentrations were found to be significantly different at M/P. No significant differences in FIB concentrations were found at the AS location across the tidal classifications, which corroborates the inland location of this site.

A representative number of samples were collected across the tidal cycle in order to better represent FIB and qMST marker concentrations in the context of storm events and ambient (dry) conditions. Across the three tidal classifications, correlation coefficients were determined between ENT concentrations and EC, *Enterococcus* sp. concentrations determined via qPCR and HF183. A similar analysis was conducted with environmental parameters such as water temperature, salinity and TSS. Regardless of tidal cycle, ENT concentrations were found to significantly ($p<0.05$) correlate with other FIB concentration and qMST marker concentration, regardless of enumeration approach (culture vs. molecular). Only salinity measurements ($r = -$

0.448, p-value = 0.042) revealed a significant relationship, with regards to the environmental parameters measured, indicating negative correlation with ENT concentrations only during periods of tidal inundation.

2.4.4 Multiple linear regression models

Three models in total were created to predict concentrations of ENT in a tidally-influenced estuarine system. The models were created using data from all sampling locations, however only the two downstream location (OS and M/P) were significant ($p<0.05$) in their prediction of variation in ENT concentrations; therefore, the models are appropriate for locations regularly influenced by tidal inundation. For all three models, a combination of biological (EC concentrations) and environmental parameters (24-h antecedent rainfall, tidal height, tidal cycle and tidal phase) were found to maximize the ability to predict the observed variation in ENT concentrations explained. FIB and qMST markers, such as HF183 and *Enterococcus* sp. determined via qPCR, as well as environmental parameters, such as water temperature, salinity, TSS, NO_x^-, NH_4^+, PO_x^- and TN, were considered when making a data training set. However, the five variables used in our models that consistently performed the best across the three sites, when compared to other data training sets. Models were evaluated by comparing the p-value and adjusted R^2 values. Table 8 summarizes the model performances for the pooled data from the three sites. The OS model demonstrated that 55% of its variation could be explained by five variables, with EC concentration and tidal phase and cycle exhibiting significant influences on ENT concentrations. Similar results were observed for the M/P model with 63% of the variation in *Enterococcus* sp. concentration explained by the same variables. In this model, however, only EC concentration and tidal cycle were found to significantly contribute to ENT concentrations.

Interestingly enough, 24-h antecedent rainfall was not a significant contributor to the variation observed in *Enterococcus* sp. concentrations for any of the models.

2.5 DISCUSSION

Historically, rainfall has long been associated with elevated FIB concentrations in receiving waters (Coulliette & Noble, 2008; Hart et al., 2020; Silva et al., 2014). However, the influence of tide on contaminant delivery during storms is poorly understood, particularly in low-lying coastal plain systems. This study evaluated the relationships of both culture- and qPCR-based FIB and qMST markers in the context of tidal cycle in an estuarine system exposed to stormwater delivery across a wide range of storm and ambient weather conditions. To further evaluate relationships observed for ENT, EC and qMST marker concentrations according to tide, we developed a predictive modeling tool to better understand stormwater contamination dynamics in a complex, tidally-influenced estuarine system. These types of tools were recommended as part of the US EPA 2012 Update to the Recreational Water Quality Criteria, but few, if any, models have been developed in this area of research. Predictive modeling tools have previously shown their utility in estuaries such as the one in which we operated (Gonzalez et al., 2012; Gonzalez & Noble, 2014) and therefore may be used to better serve coastal water quality managers by better explaining microbial dynamics regarding the effect of tidal influence on contaminant transport and, when necessary, identifying areas of contamination that require further attention regarding stormwater engineering and retrofits. Through the work conducted in this research, we hope to provide a framework for stormwater researchers needing to incorporate a tidal parameter in their monitoring regimes, while also highlighting some of the major limitations associated with using such an approach.

2.5.1 Summary statistics

Samples were collected over a broad range of rainfall conditions and across the tidal cycle. While concentrations for FIB and qMST markers increased slightly during wet weather conditions, these values were not found to be significantly greater as compared to concentrations from samples collected during dry weather. Unlike previous studies that did find significant increases in FIB concentrations following rain events (Converse et al., 2011; Gonzalez et al., 2012; Parker et al., 2010; Stumpf et al., 2010), there appears to be a different driver of both FIB and qMST marker concentrations. To analyze this further, inter-site variability was studied with regards to FIB and qMST marker concentrations. On average, the upstream sampling location (AS) consistently had higher FIB and qMST marker concentrations compared to the downstream locations. We speculated that tidal inundation was occurring in the system and was the factor dictating the observed differences in concentrations. Lewis et al., (2013) observed a decrease in FIB concentrations with increases in tide stage dependent on the extent of the tidal height. They concluded that tidal shifts exceeding 1.5 m within the tidal range resulted in decreased FIB concentrations as the system is inundated and therefore diluted with seawater. Conversely, decreased tidal inundation was characterized by maximum inflows of freshwater which promote bacterial replication in systems with high concentrations of fecal contamination. This could explain why higher concentrations of FIB were observed at the AS location as compared to OS and M/P. Findings from this study support the idea of a dilution effect on FIB and qMST marker concentrations related to tidal mixing causing both dilution and bacterial cell rupture during high tide events that ultimately reduces measured FIB concentrations (Chen et al., 2019; De Brauwere et al., 2011; Kirchman et al., 1984; Pednekar et al., 2005).

Environmental parameters validated the observed, shifting dynamics across the various tidal classifications. Salinity measurements were found to be the highest during periods of tidal inundation (17 ppt) compared to transition (10 ppt) and receding (16 ppt) tidal periods. While not significantly different than average values during low tide events, significant correlations to ENT concentrations during high tide suggest the potential utility of such a parameter as has been reported in previous research (Byappanahalli et al., 2012; Dorsey et al., 2010; Sinton et al., 2002). Neither TSS nor water temperature exhibited strong relationships with either FIB or qMST indicators. This could be attributed to fewer measurements collected over the course of the study, which was the result of evolving research goals that emerged as the complexity of the system became apparent.

2.5.2 Multiple linear regression models

To our knowledge, this was the first application of a predictive tool, such as MLR, that incorporated both qualitative and quantitative tidal variables. Previous modeling done by Gonzalez et al., (2014) was conducted in a neighboring system and demonstrated successful application of MLR. In this study, however, no tidal variable was incorporated to explain variation in either EC or ENT concentrations. Furthermore, rainfall was found to be a significant driver of FIB concentrations. The utility of our study is the incorporation of both well-established biological parameters (Hamilton et al., 2017; Jin et al., 2004; Parker et al., 2010) with less-understood environmental influences, such as tidal condition.

ENT and EC have long shown co-occurrence within fecal waste natural environment (Cabelli et al., 1982; Soller et al., 2010). Therefore, the relevance of EC concentration within the model makes sense due to its known positive correlation with ENT (Boehm & Sassoubre, 2014; Steele et al., 2018; Stumpf et al., 2010). We expect this to implicate shifting contaminant signals when

both FIB concentrations are found within samples. Tidal cycle, however, which has been studied much less frequently, also appeared to exhibit great influence on ENT concentration variation. We believe this implies that contaminant transport is more dependent on the timing of storm events as they relate to the state of the tide, compared to simply the extent, intensity of the storm event itself. If this is true, downstream waters could be susceptible to impairment long after a storm event ceases and related to the release of the system as the tide retreats. Thus, contaminated waterways remain open during contamination events increasing the likelihood of deleterious public health effects (Leecaster & Weisberg, 2001; Noble, Blackwood, Griffith, McGee, & Weisberg, 2010). Furthermore, in this framework, antecedent rainfall patterns would carry increased weight and value to future predictive model development. This is because long periods of increased rainfall will begin to favor higher surficial groundwater levels, as well as decreased infiltration capacity, potentially driving a compounded issue of stormwater delivery hampered by localized increased tidal elevation due to increased localized runoff (Yau et al., 2014).

2.5.3 Application

In low-lying, rural systems, such as Beaufort, NC, it is not uncommon to find some degree of spatial autocorrelation in water quality studies (Partyka et al., 2017; Tu & Xia, 2008) suggesting that the qualities under investigation are determined somewhat by unmeasured, and possibly external factors. If these influences are not taken into consideration, bias can be introduced into microbial water quality monitoring programs and the subsequent management decisions. In this particular study, we considered tidal variation, which is surprisingly understudied. Coastal communities across the entire NC coast sit at elevations around or below those found in Beaufort (E.g. Currituck (7 ft), Hatteras (3 ft), and Ocracoke (3 ft)) and, as such, experience similar

degrees of tidal inundation. By addressing this issue in more depth, stormwater researchers may have greater success in developing a more-inclusive framework for stormwater management that may be applied in susceptible coastal communities (Poulter et al., 2009; Pricope, Halls, & Rosul, 2019). We recognize the limitations of this study and the possible influence this may have on the reliability of model predictions. For instance, laboratory-based measures (e.g. salinity and TSS) not comprehensively conducted across all sample types throughout the study. Furthermore, it would have been of great interest to understand the elevation and pipe dimension and flow and discharge across the entire system, but these parameters were difficult to measure in practice and resulted in intermittent data collection. Additionally, sampling regimes varied between automatic and grab sampling, introducing bias related to sample collection frequency and type. Previous studies applying a tidal description in their sampling methods have primarily occurred during one tidal phase (Ex. low or high) which limits one's understanding of shifting FIB and qMST concentrations that change with the tide. Much of the previous literature shows geographic or socio-economic biases as many were conducted in the western US or in highly developed watersheds with lower tidal intrusion and greater financial resources to combat coastal flooding. With the greatest risks falling on low-lying, rural populations, accurate classifications of tidal inundation and its impact on microbial contaminant delivery in stormwater is necessary for future consideration.

We understand there is no "one-size-fits-all" model for the prediction of *Enterococcus* sp. concentration in discharge to coastal, surface waters. However, once baseline interactions between environmental parameters and microbial dynamics have been established through routine monitoring, data can then be interpreted in the context of tide. Without reliable spatial

and temporal knowledge of tidal cycle, we cannot fully rely on the results of published models to answer today's questions of acceptable water quality.

2.6 CONCLUSIONS

- Concentrations of culture FIB (*E. coli* and enterococci), *Enterococcus* sp. via qPCR and qMST (HF183) markers were significantly influenced by tide with higher concentrations found during receding (low) tides compared to those from inundated (high) or transition tidal periods.
- Environmental parameters, such as salinity, were found to significantly ($p<0.05$) correlate with ENT concentrations during periods of tidal inundation. Salinity is likely a valuable conservative marker for future dispersion studies.
- Study successfully showed the application of a predictive modeling tool by incorporating both qualitative and quantitative tidal variables in the context of observed variation in ENT concentrations. Tide was shown to be a significant driver in explaining variation in ENT concentrations, in addition to EC. However, 24-h antecedent rainfall was not determined to have major influence on contaminant concentration.
- Monitoring programs in low-lying coastal communities with tidal inundation issues must incorporate a tidal parameter in order to evaluate the impact of tidal inundation on stormwater conveyance.

2.7 ACKNOWLEDGMENTS

This work was funded by the National Estuarine Research Reserve System (NERRS) Science Collaborative, which is sponsored by the National Oceanic and Atmospheric Administration (NOAA). Many thanks go to the Town of Beaufort, NC for access and insight to the sampling

locations and for better understanding of the stormwater conveyance system. Thank you also to IDEXX Laboratories (Gil Dichter) for supplying Colilert-18® and Enterolert™ used for fecal indicator bacteria quantification. This research would not have been possible without the guidance and insight of Mike Piehler and his lab members, Suzanne Thompson and Adam Gold, who provided invaluable knowledge related to flow monitoring, field-based sampling knowledge and invaluable assistance in sample collection. Finally, thank you to fellow Noble lab members, Denene Blackwood and Thomas Clerkin, for countless hours spent helping collect and process samples.

2.8 FIGURES AND TABLES

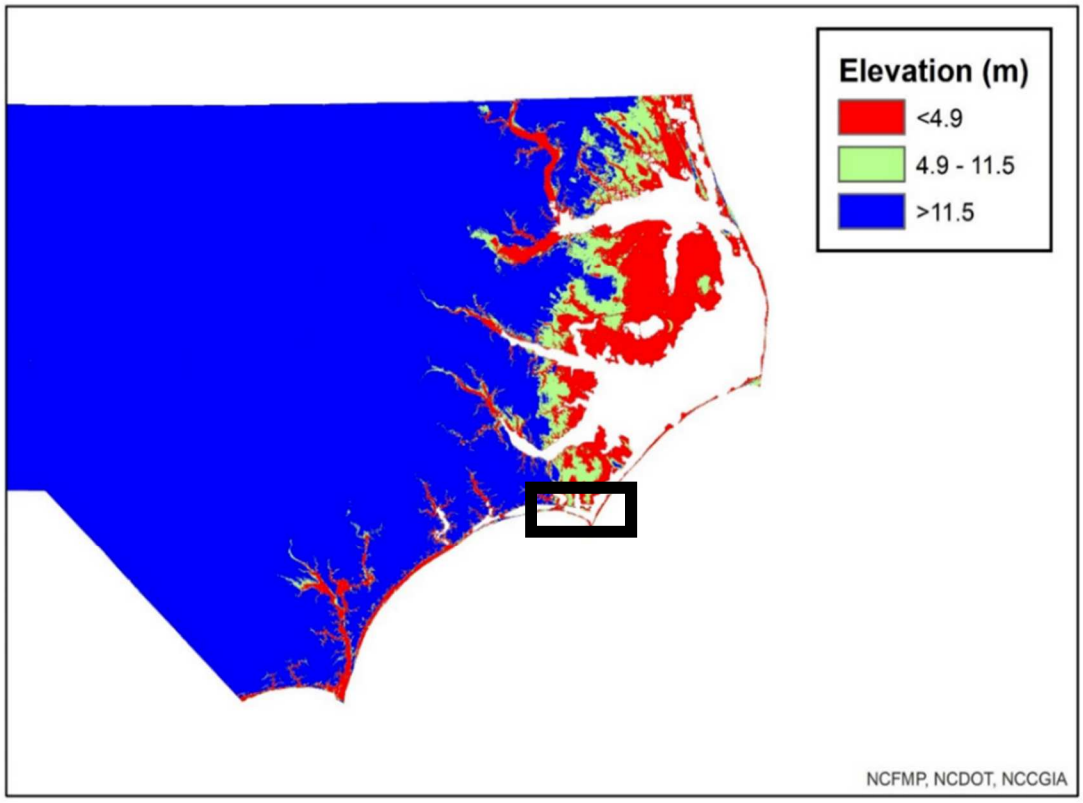

Figure 1: Digital Elevation Model (DEM) depicting elevation in coastal, eastern NC and sampling area.

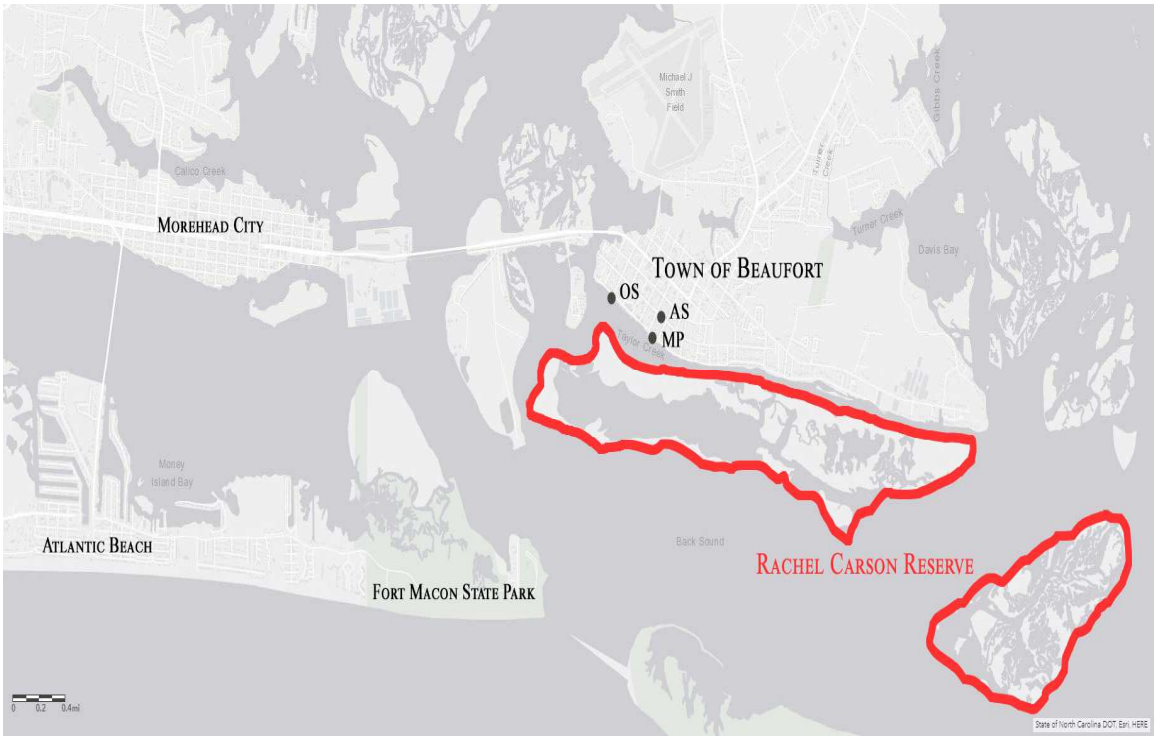

Figure 2: Three sampling locations: Orange St. (OS) and Marsh/Pollock (M/P) are located proximal to Taylor's Creek while Ann St. (AS) is one block inland.

Site	Latitude	Longitude	Elevation (m, NAVD88)	Pipe Radius (m)
Orange Street	34.71751	-76.66740	0.105	0.3
Marsh/Pollock	34.71454	-76.66190	-0.515	0.43
Ann Street	34.71613	-76.66070	0.446	0.46

Table 1: Latitude, longitude, elevation and pipe size for Orange Street (OS), Marsh/Pollock (MP) and Ann Street (AS) sampling locations.

Assay	Oligo ID	Sequence	Concentration	Reference
HF183	HF183	ATCATGAGTTCACATGTCCG	100 µM	Haugland et al. (2010)
	BFDRev	CGTAGGAGTTTGGACCGTGT	100 µM	
	BFDFAM	CTGAGAGGAAGGTCCCCCACATTGGA	10 µM	
Enterococcus sp. via qPCR	ECST748For	GAGAAATTCCAAACGAACTTG	100 µM	US EPA (2012)
	ENC854Rev	CAGTGCTCTACCTCCATCATT	100 µM	
	GPL813	TGGTTCTCTCCGAAATAGCTTTAGGG CTA	10 µM	

Table 2: Primer and probe sets for human-specific HF183 TaqMan assay and primer and probe sets for *Enterococcus* TaqMan 23S rRNA target gene sequence.

Target	# of Individual Standard Curves (Total # of Data Points)	Master Curve Formula	Amplification Efficiency (%)	R^2	Limit of Detection (copies/rxn)	Limit of Quantification (copies/rxn)
HF183	3 (55)	-3.11x + 45.01	1.10	0.99	4	43
ENT-qPCR	4 (69)	-3.58x + 46.89	0.90	0.98	88	588

Table 3: qPCR master curves, total number of points, amplification efficiencies, standard curve R^2 values, limit of detections (LOD) and limit of quantifications (LOQ).

	EC	ENT	HF183	*Enterococcus* sp. via qPCR	Tidal Height	Salinity	24-h Antecedent Rainfall
	Log MPN/100 mL	Log MPN/100 mL	Log copies/100 mL	Log copies/100 mL	Meters (m)	Parts per thousand (ppt)	Inches (in)
N Total	131	131	63	44	137	58	137
Mean	2.64	2.26	2.05	3.04	0.062	14.9	0.50
Standard Deviation	1.08	1.21	1.07	1.27	0.358	14.1	0.84
Range (Min-Max)	(0.7-4.94)	(0.7-4.78)	(0.7-4.07)	(0.7-5.03)	(-0.634-0.692)	(0-35)	(0-3.06)

Table 4: Indicator metrics for FIB (EC, ENT, and *Enterococcus* sp. via qPCR), qMST (HF183) and environmental (tidal height, salinity, 24-h rainfall) for all samples.

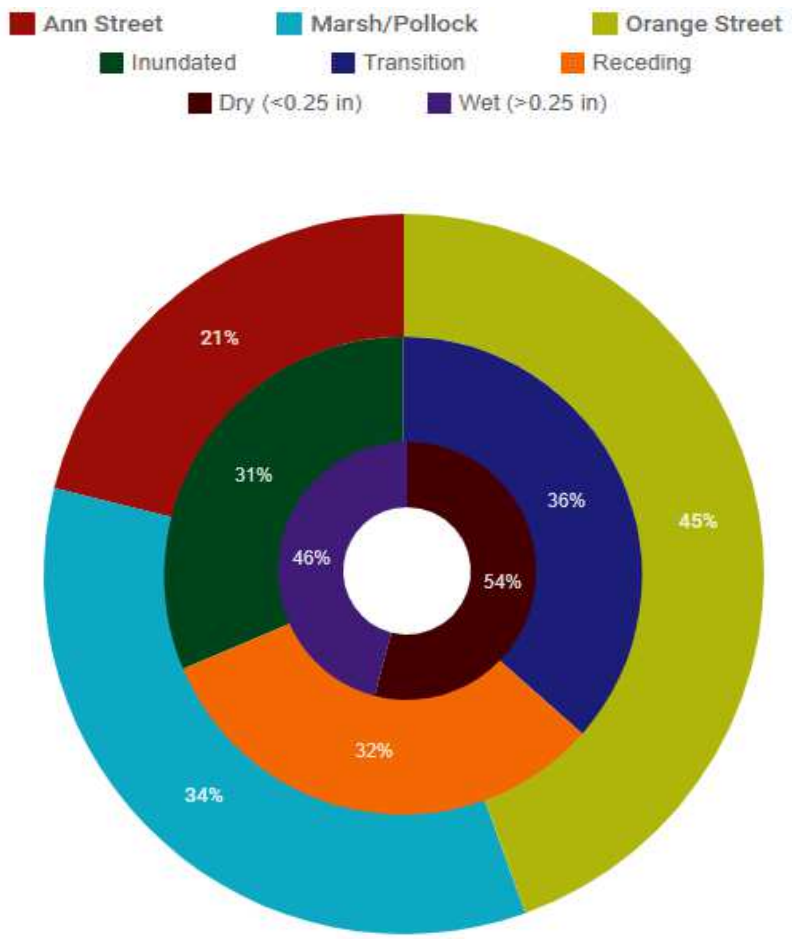

Figure 3: Number of samples collected at sampling sites: AS (n = 29), M/P (n = 47) and OS (n = 61), during tidal phases: inundated (n = 43), transition (n = 50) and receding (n = 44) and wet (n = 11) vs. dry (n = 15) conditions.

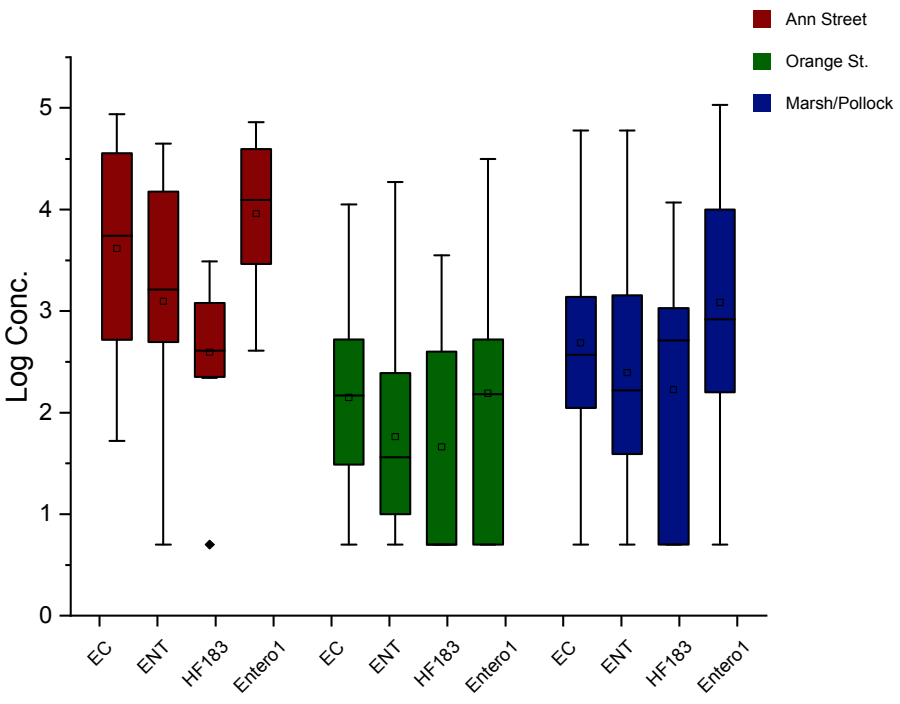

Figure 4: Boxplots of measured EC, ENT, HF183 and *Enterococcus* sp. via qPCR concentration distribution across the three sample sites: Orange Street (OS), Pollock/Marsh (M/P) and Ann Street (AS) across all samples collected. EC and ENT are in Most Probable Number (MPN) per 100 mL and HF183 and *Enterococcus* sp. via qPCR are displayed as copies per 100 mL.

	EC		ENT		HF183		Entero1	
	Mean (min-max) N	Above standard	Mean (min-max) N	Above standard	Mean (min-max)	Above standard N	Mean (min-max)	Above standard N
Site	Log MPN/100 mL	EC %[a]	Log MPN/100 mL	ENT %[b]	Log CCE/100 mL	HF183 %[c]	Log CCE/100 mL	Entero1%[d]
OS	2.15 (0.7 – 4.05) 59	32.2	1.76 (0.7 – 4.27) 59	27.1	1.66 (0.7 – 3.55) 27	14.8	2.19 (0.7 – 4.5) 14	14.3
M/P	2.69 (0.7 – 4.78) 44	54.5	2.39 (0.7 – 4.78) 44	61.4	2.22 (0.7 – 4.07) 25	32.0	3.08 (0.7 – 5.03) 18	38.9
AS	3.62 (1.72 – 5.64) 29	75.9	3.10 (0.7 – 4.65) 29	79.3	2.59 (0.7 – 3.49) 11	27.3	3.96 (2.61 – 4.86) 12	83.3

[a] US EPA 2012 FIB recommended threshold; [b] NC DEQ ENT threshold; [c] Haugland et al., 2010; [d] US EPA 2012 molecular marker recommended threshold

Table 5: Summarized data for EC, ENT, HF183 and ENT-qPCR concentrations at sampling sites (Orange St., Marsh/Pollock and Ann St.) including the distribution and prevalence of samples that exceeded recreational contact standards.

	Inundated (N = 43)			Receding (N = 44)			Transition (N = 50)		
Mean Value	**OS**	**M/P**	**AS**	**OS**	**M/P**	**AS**	**OS**	**M/P**	**AS**
Tidal Height (m)	0.42	0.40	0.46	-0.33	-0.32	-0.33	0.06	0.12	0.14
EC (MPN/100 mL)	1.98	2.31	3.58	2.50	3.19	3.67	1.99	2.68	3.56
ENT (MPN/100 mL)	1.37	1.93	3.04	2.06	2.85	3.09	1.77	2.52	3.30
***Enterococcus* sp. via qPCR (copies/100 mL)**	2.05	2.48	3.48	2.59	3.94	4.34	1.84	2.93	4.26
HF183 (copies/100 mL)	0.7	2.24	1.96	1.72	2.55	2.94	1.99	2.03	2.99

Table 6: Descriptive statistics of FIB characterized by tidal cycle (inundated, receding or transition) sampling location.

	Correlation coefficient	p-value
Inundated		
Water Temperature (ºC)	0.267	0.126
Salinity (ppt)	-0.448	**0.042**
TSS (mg/L)	-0.032	0.878
EC (MPN/100 mL)	0.635	**4.73E -6**
HF183 (CCE/100 mL)	0.538	**0.039**
ENT-qPCR (CCE/100 mL)	0.844	**5.62E -4**
Transition		
Water Temperature (ºC)	0.227	0.130
Salinity (ppt)	-0.259	0.372
TSS (mg/L)	-0.094	0.662
EC (MPN/100 mL)	0.825	**5.68E -13**
HF183 (CCE/100 mL)	0.426	**0.021**
ENT-qPCR (CCE/100 mL)	0.673	**0.002**
Receding		
Water Temperature (ºC)	0.319	0.070
Salinity (ppt)	-0.252	0.246
TSS (mg/L)	0.054	0.831
EC (MPN/100 mL)	0.492	**0.001**
HF183 (CCE/100 mL)	0.406	0.169
ENT-qPCR (CCE/100 mL)	0.742	**0.035**

Values in bold indicate significant relationship (p-value = $p<0.05$).

Table 7: Pairwise correlation analysis and tests of significance conducted between environmental parameters (water temp., salinity and total suspended solids), FIB (EC) and molecular markers (HF183 and *Enterococcus* sp. via qPCR) with ENT concentrations dependent on tidal cycle.

Factor	Coefficient	Std. Error	t-value	Prob>|t|
Orange St.				
R^2 = 0.55, p = 3.12 e-05				
Intercept	0.743	0.920	0.807	0.424
EC	0.680	0.123	5.513	1.46e-06***
24h Rainfall	0.102	0.108	0.946	0.349
Tidal Height	2.900	2.432	1.192	0.239
[a]Inundated	0.000	0.000	0.000	0.000
Receding	1.227	1.212	1.012	0.317
Transition	2.199	0.758	2.900	0.006**
Sin(TidalCycle)	-2.478	0.931	-2.662	0.011*
Cos(TidalCycle)	1.083	0.534	2.029	0.048*
Marsh/Pollock				
R^2 = 0.63, p = 2.01 e-04				
Intercept	1.408	1.072	1.314	0.198
EC	0.772	0.181	4.273	1.61e-04***
24h Rainfall	-0.035	0.168	-0.211	0.835
Tidal Height	5.167	2.590	1.995	0.055
[b]Inundated	0.000	0.000	0.000	0.000
Receding	0.675	1.333	0.506	0.616
Transition	1.731	0.962	1.800	0.081
Sin(TidalCycle)	-2.426	0.995	-2.439	0.020*
Cos(TidalCycle)	0.178	0.799	0.222	0.826
Ann St.				
R^2 = 0.62, p = 0.058				
Intercept	2.843	2.127	1.337	0.200
EC	0.325	0.235	1.385	0.185

24h Rainfall	-0.872	0.562	-1.553	0.140
Tidal Height	9.816	7.466	1.315	0.207
[c]Inundated	0.000	0.000	0.000	0.000
Receding	1.582	3.687	0.429	0.674
Transition	1.484	3.080	0.482	0.636
Sin(TidalCycle)	-2.702	2.702	-1.000	0.332
Cos(TidalCycle)	-1.121	2.393	-0.469	0.646

[a] Referent condition for the categorical variable, Orange St. model, effect is null; [b] Referent condition for the categorical variable, Marsh/Pollock model, effect is null; [c] Referent condition for the categorical variable, Ann St. model, effect is null.
* 0.05 significance level; ** 0.01 significance level; *** 0.001 significance level.

Table 8: Multiple regression model for the association of $\log_{10}$ Enterococci with biological and environmental characteristics by sampling location (Orange St., Marsh/Pollock and Ann St.). The regression model looks to better characterize the effect of tidal cycle on bacterial concentrations delivered with the system.

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CHAPTER 3: INTEGRATING NOVEL COLIPHAGE DETECTION METHODS IN THE FRAMEWORK OF EXISTING WATER QUALITY STANDARDS

3.1 SUMMARY

The use of somatic and male-specific (F+) coliphages as fecal indicator viruses (FIV) of contamination in groundwater has previously been established by the United States Environmental Protection Agency (US EPA). Recently, there has been increased interest in the development of a coliphage method to detect fecal contamination in fresh and marine surface waters and wastewater effluent. This study aimed to assess the applicability of US EPA Method 1642, which incorporates the use of a dead-end hollow fiber ultrafiltration (UF) step combined with single agar layer (SAL), in a regulatory framework to measure FIV. Ten sampling events were conducted seasonally over the course of a year (July 2019 – July 2020) from three sampling locations in Beaufort, NC. On average, F+ coliphage concentrations were significantly lower ($p < 0.01$) than those of somatic coliphages. Concentrations for FIV were low across all locations with average concentrations for somatic and F+ coliphage of log 1.48 PFU/100 mL and log 1.00 PFU/100 mL respectively. Somatic coliphage concentrations showed a wider range (0.3-3.1 log10 PFU/100 mL) across all samples when compared to F+ coliphages (non-detect-1.7 PFU/100 mL) but correlated poorly with culture-based and molecular measurements of *E. coli* and enterococci and HF183 concentrations. FIV, FIB and qMST concentrations were also assessed to evaluate the percent exceedance rate as defined by recommended water quality monitoring criteria and illness risk. On average, FIB (EC, ENT and *Enterococcus* sp. determined

via qPCR) exceeded US EPA recommended criteria in 36%, 56% and 68% of samples respectively. In these same samples, log concentrations for F+ and somatic coliphages were relatively low, however, with averages of 1.04 and 1.83 PFU/100 mL respectively. Finally, a cost assessment was performed between FIB (IDEXX Quanti-Tray®), qMST (qPCR) and FIV (UF-SAL) enumeration methods and it was concluded there to be more cost- and time-effective alternatives with regards to implementation within a routine water monitoring framework.

3.2 INTRODUCTION

Fecal contamination reduces surface water quality leading to potential public health risks attributed to the presence of enteric pathogens. Viral pathogens have been acknowledged as major causative agents of waterborne disease outbreaks in surface waters (Begier et al., 2008, Eftim et al., 2017, Sinclair et al., 2009, Yoder et al., 2008). However, direct enumeration of viral pathogens is problematic due to the expensive and time-consuming nature of testing procedures needed for routine testing. Instead, existing surface water quality guidelines employ the use of fecal indicator bacteria (FIB), such as *E. coli* (EC) and enterococci (ENT), to routinely assess recreational water quality and indicate the potential presence of pathogens. Nevertheless, research has demonstrated that FIB do not always directly indicate human health risks, especially when diffuse source pollution is the major driver of contamination (Colford et al., 2007; Gitter et al., 2020; McQuaig et al., 2012; Shrestha et al., 2020; Sinigalliano et al., 2010; Soller et al., 2010). As such, recent requests have called for the development of a fecal indicator virus (FIV) marker to be used in fresh and marine surface waters which may serve as a better indicator of human health risk.

Coliphages, which are a subset of bacteriophages that infect *E. coli*, are promising FIV for water quality monitoring. In particular, male-specific (F+) and somatic coliphage have been

proposed as an attractive alternative to testing for viral pathogens as FIV are nonpathogenic to humans, and share similar morphologies to enteric viruses (Jofre et al., 2016; Sobsey et al., 2004; Stetler, 1984). Additionally, coliphages are abundant in human fecal waste (Gantzer et al., 1998; Lucena et al., 2004; Zhang and Farahbakhsh, 2007), and are rarely found to replicate under ambient conditions (Muniesa and Jofre, 2004). Coliphages have recently been considered for use in regulatory applications, such as those required for routine monitoring of surface waters (US EPA, 2015).

The use of coliphages in the framework of water quality monitoring is not without criticism. Previous epidemiology studies have often reported high numbers of non-detects in environmental samples, potentially limiting our understanding of coliphage prevalence and public health risk as associated with human viral pathogens (Abdelzaher et al., 2011; Boehm et al., 2009; Colford et al., 2007; Viau et al., 2011; Wade et al., 2010). FIV are often times found at low ambient concentrations (< 1 log10 plaque forming units (PFU) per mL for F+ coliphages and 1 log10 PFU per 100 mL for somatic coliphages), and, as such, require high volumes (>1 L) of sample for enumeration (Boehm et al., 2009; McMinn et al., 2017b; US EPA Method 1602, 2001; Viau et al., 2011). Coliphages are enumerated using the plaque assay (US EPA Method 1601, 2001; US EPA Method 1602, 2001) which involves phage induced lysis of bacterial host cells (E.g. EC), indicated by cleared zones (plaques) in the bacterial lawn. The primary version of this assay, the single agar layer plaque assay (SAL), is perceived by some as “cumbersome” (Jofre et al., 2016). The method requires preparation of log phase bacterial host cells, autoclaving and tempering of agar, inoculation of molten agar with phages/sample and bacteria, then pouring agar plates followed by incubation of inoculated agar plates for 6–24 h (US EPA Method 1602, 2001). The long processing and preparation time required for sampling assay suggest the

possibility for contaminated water to go undetected for hours after a contamination event, leading to heightened risk for recreators in affected waterways (Noble et al., 2003). Therefore, the efficacy of employing such a labor- and time-intensive method must be considered in systems with a low wastewater signal.

In this study, we report the use of a dead-end hollow fiber ultrafiltration (UF) combined with SAL method, as outlined in US EPA Method 1642, to enumerate F+ and somatic coliphage in a North Carolina (NC) estuary with diffuse sources of human fecal contamination. The study site for this research is located in Beaufort, NC, a coastal community situated in the coastal plain region of southeastern NC with a relatively small permanent population (4,391) that experiences seasonal growth given its proximity to coastal waters and productive tourism industry (US Census Bureau, 2020).

The goal of this study was to assess the applicability of using FIV to identify potential fecal contamination in ambient surface waters without direct wastewater input. We 1) determined the prevalence of somatic and F+ coliphages using US EPA Method 1642; and 2) compared these concentrations with FIB and qMST marker concentrations using both culture- and molecular-based enumeration methods. Additionally, 3) we developed a cost assessment associated with sample processing to compare with other methods commonly employed in routine water quality sample processing. The overall advancement associated with this work is to begin to understand the practicality of employing a FIV method as an additional monitoring component of recreational water quality management.

3.3 MATERIALS AND METHODS

3.3.1 Study Sites and Sample Collection

Water samples were collected at three sampling locations throughout Beaufort: two at stormwater outfall locations (OS and M/P) proximal to downstream receiving waters (Taylor's Creek) and a third site (AS) one block inland that was selected to characterize watershed conditions upstream (Figure 1). Ten sampling events were conducted seasonally over the course of 12 months from July 2019 – July 2020, with greater emphasis placed on sampling during dry conditions within the summer months, correlating with heightened usage of Taylor's Creek. All samples were collected via grab sampling and transported on ice to the laboratory in sterile, wide-mouth, high-density polyethylene bottles on ice and analyzed within 6 h. Water temperature, dissolved oxygen (DO) and salinity (PSU) were measured in situ using a YSI 6600 multiparameter probe, Yellow Springs, OH, USA). Additionally, wind speed and direction, air temperature, barometric pressure and tidal height were collected from NOAA's Beaufort, NC Station (ID: 8656483) dataset. Precipitation data was collected from Weather Underground's Dakota Station (Weather Station ID: KNCBEAUF23).

3.3.2 Coliphage Stocks and Host Bacteria

Host bacteria for coliphage detection were *E. coli* CN13 [ATCC® #700609™] for somatic coliphages and *E. coli* F_{amp} [ATCC® #700891™] for F+ coliphages. Bacteria were grown in tryptic soy broth containing antibiotics for strains that were resistant (F_{amp}: ampicillin 15 µg/mL and streptomycin 15 µg/mL; CN13: nalidixic acid 100 µg/mL). Stock coliphage cultures of MS2 (ATCC® 15597-B1™) and somatic coliphage (ATCC® 13706-B1™) were also used.

3.3.3 Detection Methods and Recovery

3.3.3.1 Ultrafiltration and Elution

Samples were prepared following US EPA Method 1642 protocol (US EPA, 2018). This method includes the addition of an ultrafiltration and elution step compared to the traditionally used SAL approach, US EPA Method 1602. Ultrafiltration used a hollow-fiber ultrafilter (Dial Medical Supply, Rexeed 15S) which was washed with approximately 2 L of sample using a peristaltic pump (Masterflex L/S) at a flow rate of approximately 0.5 – 0.8 L/minute. Following ultrafiltration, 200 mL of elution solution was used to wash the filter both clockwise and counter-clockwise through four, one-minute cycles. Elution solution was prepared prior to the experiment by adding 0.1 g sodium polyphosphate, 0.1 mL Tween® 80 solution, 0.01 mL Y-30 antifoam to 1 L reagent-grade water and filter sterilizing thorough a 0.22 µm filter. The eluate final volume was then recorded and equally dispensed into two, sterile flasks 250 mL in capacity.

3.3.3.2 Sample Processing and Matrix Spikes

Initial precision and recovery analyses were conducted by spiking 2 L of PBS with a known concentration of F+ and somatic coliphage suspension and processing using US EPA Method 1642. Given the complex nature of estuarine water on coliphage recovery, matrix spike analyses were also performed (Table 2).

3.3.4 Sample Processing and qPCR Assay

EC and ENT were enumerated using Colilert-18® and Enterolert™ per manufacturer instructions (IDEXX Laboratories, Westbrook, ME). For downstream molecular analysis, triplicate 100-150 ml samples were vacuum filtered through 0.45 µm pore size, 47 mm polycarbonate (PC) filters (HTTP, Millipore, Bedford, MA) using a six-place filtration manifold and vacuum pump assembly. The filters were placed into sterile, DNase/RNase-free

microcentrifuge tubes and stored at -80 °C. DNA extractions were performed using the NUCLISENS® MINIMAG® extraction kit per manufacturer instructions, with extracts then stored at -20 °C. Consequent qPCR quantification of a total *Enterococcus* sp. FIB marker and qMST marker (HF183) was done using the primers, probes, and assays described in Table 3. Assays were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Extracted samples were processed using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts). Primers (100 µM) and probes (10 µM) were synthesized by LGC Biosearch Technologies (Petaluma, CA). Each reaction had a total volume of 25 µL, 20 µL including nuclease-free water (6.75 µL), TaqMan® Environmental Master Mix 2.0 (12.5 µL), as well as appropriate primers (0.50 µL) and probe (0.25 µL), and 5 µL of unknown sample, standard, or control. NTCs were processed with each plate.

3.3.5 Determination of PCR Inhibition in Samples

DNA from a halophilic, alkaliphilic archaeon (*Natronomonas pharaonis*) which does not naturally occur in surface waters or sewage served as the experimental sample processing control (SPC) and was added prior to extraction following previously published methods using US EPA Method 1611 (Haugland et al., 2005; Steele et al., 2019). Negative Extraction Controls (NEC) containing only lysis buffer and halophile DNA were processed for every extraction in the same manner as the samples. Samples were determined to be inhibited if the difference between the cycle threshold (Ct) of the SPC in samples with (experimental) and without (control, only SPC) target DNA was greater than 0.5 log units (2.32 Ct) (Lambertini et al., 2008). Since the total

number of inhibited samples (2 out of 27 samples) comprised only 7.4% of total samples, no adjustment for inhibition was made and the two samples were excluded from statistical analyses.

3.3.6 Standard Curve Determination

Standard curves for HF183 and *Enterococcus* sp. via qPCR consisted of the calibration standard and five 10-fold serial dilutions that were run in duplicate for HF183 and five 10-fold serial dilutions that were run in duplicate for *Enterococcus* sp. via qPCR. For each of the molecular markers, standard dilution curves were aggregated to form one single master standard curve which was then used for data extrapolation. The theoretical limit of detection (LOD) was the lowest concentration where the standard could be detected in at least 50% of qPCR replicates. The limit of quantification (LOQ) for qPCR assays was defined as the lowest concentration above the lowest point on the standard curve where amplification was observed in at least 50% of qPCR replicates. Curves, along with their respective total number of points, average amplification efficiencies, R^2 values, LOD and LOQ for the HF183 and *Enterococcus* sp. via qPCR assays are presented in Table 4.

3.3.7 Statistical Analysis

All coliphage data were log10 transformed and expressed as PFU per 100 mL for positive samples only, as ND samples were excluded in analysis. Statistical analyses were conducted using OriginPro 8.5 (OriginLab, Northampton, MA). A Levene's test for homogeneity of variance was used to evaluate variability within each group of measurements to determine eligibility for analysis of variance (ANOVA) testing. One-way ANOVA test was applied to somatic and F+ coliphage datasets from all three sites and simple linear regression was used to calculate correlation coefficients between indicator paired measurements.

3.4 RESULTS

3.4.1 Summary Statistics

Thirty-three samples were processed for F+ coliphage with seventeen samples paired with somatic coliphages. Summarized indicator metrics for FIV, FIB and qMST markers show that, on average, F+ coliphage concentrations were significantly lower ($p < 0.01$) than those of somatic coliphages, however, overall concentrations for both FIV were low. Somatic coliphages were found across a wider range (0.3-3.1 log10 PFU/100 mL) throughout the samples compared to F+ coliphages (non-detect-1.7 PFU/100 mL) with EC (0.95-3.6 MPN/100 mL) and ENT (1.0-4.1 MPN/100 mL) found in similar ranges. Overall, marker concentrations were skewed, with relatively low average concentration data across sites but with occasional, high concentrations.

FIV concentrations were greater at the upstream AS site (Figure 2) with the difference in concentration being significant ($p<0.05$) for somatic coliphages between the upstream site and the two downstream locations. Both cultured and molecular marker concentrations were higher at the AS location with indicator concentrations averaging log 2.69 MPN/100 mL, log 2.68 MPN/100 mL and log 4.05 copies/100 mL for EC, ENT and *Enterococcus* sp. via qPCR respectively. This compared to an average HF183 concentration in AS samples of log 2.61 copies/100 mL. Somatic coliphages were present in all samples while F+ coliphages had a 6% non-detect frequency. This compared to a 52% and 4% non-detection frequency ($n = 23$) for HF183 and *Enterococcus* sp. via qPCR respectively.

On average, coliphage concentrations were relatively low across sampling events. As such, coliphage concentrations exceeding US EPA recommended criteria based on either molecular (*Enterococcus* sp. via qPCR: 1280 copies/100 mL (log 3.11)) or culture (EC: 320 MPN/100 mL (log 2.51); ENT: 110 MPN/100 mL) (log 2.04)) criteria defined by heightened associations with

illness was assessed. HF183 risk was set at 4200 copies/100 mL (log 3.62) as previous research has focused primarily on human source contamination, as this matter tends to be the greatest concern for managers and regulators (Boehm et al., 2015). Table 6 below summarizes the samples as they relate to recommended exceedance thresholds as seen in US EPA criteria documents or in the peer-reviewed literature.

ENT exceeded US EPA recommended criteria in 56% and 68% of samples respectively, which compared to 0% of samples exceeding HF183 concentrations associated with heightened illness risk (Boehm et al., 2015). Correlation coefficients (r) were calculated across all sites using linear regression (Figure 3). EC showed significant positive correlation with ENT ($r = 0.823$; $p = 1.381e\ -7$) and somatic coliphage ($r = 0.520$; $p = 0.039$), however no other significant relationships were observed between FIV and corresponding FIB or qMST markers.

3.4.2 Environmental Parameters

Samples were collected across a range of environmental conditions (E.g. water temperature, salinity and dissolved oxygen) (Table 7). Correlation coefficients were calculated between the indicators and environmental parameters to determine significant influences on FIV, FIB and qMST concentrations throughout the system. With regards to FIV, neither F+ nor somatic coliphages significantly ($p<0.05$) correlated with any of the environmental parameters. Both EC and ENT did, however, show significant negative correlations with both dissolved oxygen (%) and salinity measurements.

3.4.3 Time and Cost

A cost assessment was conducted between FIB, qMST and FIV enumeration methods. When considering time and cost (Table 8) with each method, the assessment accounts for disposable items such as Petri-dishes and filters, as well as required chemicals and reagents (E.g.

agar, tryptic soy broth, nalidixic acid, Tween-80, etc.) but has excluded certain disposables (E.g. serological pipettes, pipette tips, gloves) as these are expected to be used for all of the methods. For the purposes of this evaluation, we have included costs per 10 samples to account for method controls and assumed that sample processing time is based off a single analyst familiar with the routine water quality assessment and has a basic understanding of microbiological processing techniques.

The processing of samples for FIB enumerated using the defined-substrate technology provided by IDEXX Quanti-Tray® offers the most cost-effective option of the three, while molecular tools, such as qPCR, serve as the most rapid tool, with regards to obtaining results. Coliphage detection methods, on average, require 1-2 days of sample preparation and processing with 16-24 hrs needed for overnight incubation.

3.4.4 Inter-Plate Variability

In order to account for the stochastic inter-plate variability inherent within the coliphage plating process, sample plate variance was performed between the five-plate replicates for each of the coliphage groups (Table 9). Variance, which measures the spread of variability between plates as measured by a plate's squared difference from the overall sample mean, was calculated using the formula in Equation 1:

(Equation 1)
$$V = \frac{\sum(x_i - \overline{x})}{n-1}$$

x_i = value of one observation
$\bar{x}$ = mean of all observations
n = number of observations

Variance values were then normalized into z-scores (Equation 2) and plotted (Figure 4) between the two coliphage groups.

(Equation 2) $$Z = \frac{x - \mu}{\sigma}$$

x = value of one observation
μ = mean of sample observation
σ = standard deviation of sample observation

Variations between sampling events were skewed, with relatively low average variation overall, but high single event variations within samples analyzed for both a singular coliphage (F+) and those analyzed for F+ and somatic coliphages. On average, greater variance was found in samples analyzed for F+ coliphages, however no significant ($p < 0.05$) differences were found between hosts in terms of application.

3.5 DISCUSSION

The occurrence of coliphages is generally associated with fecal contamination and the occurrence of enteric viruses. Therefore, coliphages have been suggested as a surrogate measure of enteric viruses in surface waters (Rezaeinejad et al., 2014; Savichtcheva & Okabe, 2006). This study evaluated the applicability of using F+ and somatic coliphages as a monitoring proxy in estuarine waters with diffuse source pollution. While previous studies have shown the applicability of using FIV in wastewater (Bailey et al., 2017; Hassaballah et al., 2020; Nappier et al., 2019; Sidhu et al., 2018) and urban coastal waters (Jiang et al., 2001; Rezaeinejad et al., 2014; Vergara et al., 2015), few have studied its pertinence in coastal surface waters.

3.5.1 Coliphage Occurrence and Site-Dependent Variability

In this study, the success of coliphages as FIV was assessed along with culture-based EC and ENT, molecular-based *Enterococcus* sp. and qMST marker HF183 in a southeastern, NC estuarine system to better gauge the implementation of such a monitoring tool as suggested by US EPA (US EPA, 2015). In this study, two stormwater outfall sites and one upstream pipe-access location were assessed over a 12-month period for fecal contamination. Somatic coliphages were found in all samples while F+ coliphages were found in 94% of samples. However, concentrations for both FIV were low with average concentrations of F+ coliphages at log 1.00 PFU/100 mL and log 1.48 PFU/100 mL for somatic coliphages. This matches earlier studies that have quantified ambient coliphage levels in environmental waters, as most often report a large percentage of samples with low coliphage concentrations (Abdelzaher et al., 2011, McMinn et al., 2017a, Medema et al., 1995, von Schirnding et al., 1992). With low coliphage concentrations, it difficult to truly understand the utility of their use as a water quality monitoring proxy.

EC, ENT and *Enterococcus* sp. via qPCR were detected during 97%, 79% and 65% of samples respectively and were present at consistently higher counts than coliphages. This is not surprising as FIB lack host specificity and have been shown to be persistent in both fresh- and marine water systems (Ferguson et al., 2005; Ferguson & Signoretto, 2011; Lin & Ganesh, 2013; Švec & Sedláček, 1999). Throughout our samples, the range and concentrations of both culture- and molecular-based indicator concentrations were consistently higher throughout the samples when compared to FIV concentrations. The qMST marker, HF183, which was quantified using qPCR, was only detected in 32% of samples. Specific to human-associated fecal contamination, the low prevalence of HF183 within our samples, taken during ambient conditions, makes the

inception of such a tool seem unnecessary unless known sewage-associated contamination is suspected.

Higher concentrations of somatic coliphages compared to F+ coliphages complement previous fresh- (Contreras-Coll et al., 2002, Jiang et al., 2001, Lucena et al., 2003, Rezaeinejad et al., 2014, Viau et al., 2011) and marine-water (Boehm et al., 2009, Contreras-Coll et al., 2002, Rodriguez et al., 2012) studies which have shown lower F+ coliphage abundance. This is further explained by the positive correlations between somatic coliphages and EC and ENT, with only the relationship between somatic coliphages and EC found to be significant ($p<0.05$). Several studies have also shown the positive relationship between coliphages and environmental conditions such as salinity (Boehm, Silverman, Schriewer, & Goodwin, 2019) and water temperature (Rezaeinejad et al., 2014; Liang et al., 2015), however, no significant correlations were found within our samples. This could be in attributed to the low sample size of the study with environmental parameters showing more significant relationships with FIV concentrations in larger data sets. Further studies are necessary to properly evaluate the abiotic and biotic factors affecting the replication and survivability of coliphages in estuarine water environments lacking direct wastewater input.

FIV, FIB and qMST concentrations were assessed to evaluate the percent exceedance rate as defined by recommended water quality monitoring criteria (EPA, 2012; Haugland et al., 2010). As samples were primarily taken during dry conditions, we wanted to assess the number of samples that would exceed recommended FIB and qMST thresholds and relate these values with coliphage concentrations. On average, EC, ENT and *Enterococcus* sp. via qPCR exceeded US EPA recommended criteria in 36%, 56% and 68% of samples respectively. Such high rates of exceedance indicate the presence of fecal contamination within the system, however, since

HF183 concentrations were relatively low, we cannot be certain the signal is specifically derived from human sources. Average somatic coliphage concentrations were greater in samples exceeding EC (log 1.96 PFU/100 mL), ENT (log 1.95 PFU/100 mL) and *Enterococcus* sp. via qPCR (log 1.58 PFU/100 mL) compared to F+ coliphage (EC: log 1.07 PFU/100 mL; ENT: log 1.09 PFU/100 mL; *Enterococcus* sp. via qPCR: log 0.95 PFU/100 mL), but values were consistently lower than those found in sewage (10^3 – 10^8 per 100 mL) (Harwood et al., 2005). A significant ($p < 0.05$) correlation existed between the two culture-based FIB, EC and ENT, however significant relationships were not found between the culture- and molecular-based *Enterococcus* sp. markers. Ervin et al., 2013 also found similar disparities between culture and method methods, raising questions related to the effect system dynamics and enumeration selection has on final concentration values.

3.5.2 Cost Assessment

To our knowledge, this is the first paper of its kind to assess method enumeration protocols commonly used in routine surface water quality monitoring. All estimates included costs for processing of 10 samples, preparation and processing times associated with both samples and relevant processing controls and standards, as well as wait time for results. We determined the defined-substrate technology method, provided by IDEXX Quanti-Tray®, to be the most cost-effective of the three methods analyzed. One limitation of this, however, is the 18-24 hour wait time needed before results can be obtained. QPCR by far is the best at providing results in the most-timely manner. This comes at a greater cost, however, as this study estimated total qPCR costs to average approximately $1300-$1500 for ten samples. Results using qPCR can be obtained within 4-8 hours depending on sample size and number of assays performed. Finally, the UF-SAL method recommended by US EPA (US EPA Method 1642) costs an average of

$800-$900 to process ten samples, however the biggest disadvantage to the method is its cumbersome nature with regards to sample processing. Method 1642 takes between 2-3 days to prepare and process samples with an additional 16-24 hours required for results. For weekly monitoring regimes, the time-consuming nature of the method may not be feasible with alternative methods likely to produce more time- and cost-effective outcomes.

3.5.3 Inter-Plate Variance

High degrees of variance were calculated in single-sample inter-plate differences both when using a single host (F_{amp}) and multiple hosts (F_{amp} and CN13), however these differences were not found to be significant ($p < 0.05$). Our study found great variability in coliphage concentrations (Brion, Meschke, & Sobsey, 2002), but low concentration totals throughout our system limited our understanding of this variability within individual samples. Further research must be conducted on surface waters with limited wastewater input during ambient conditions to determine method performance within estuarine samples. This must be done to ensure practical application of the method can be implemented on a regulatory scale in laboratories both familiar with microbiological sampling processes and those that may not be.

3.5.4 Limitations and Future Research

Because samples were taken during dry conditions, they may not necessarily capture the "worst case" scenario as expected following storm events. Additionally, we did not incorporate tide into our sampling regime and, as a result, the majority of samples were collected during low tide. This may have influences on FIV concentrations, even during dry conditions, as we know it has had previous influences on FIB concentrations within the same system (Hart et al., 2020). Lastly, optimized HF183 primers have been established that report high specificity and sensitivity to human sewage (Green et al., 2014). The implementation of more optimized primers

in this study may have improved HF183 performance and strengthened its correlation with other indicators.

The estimation of costs associated with enumeration methods that are relevant current choices for recreational water quality managers was a useful part of this study. We hope this will serve as a foundation for future laboratories to ascertain the practicality of implementing each of the methods given the resources available. While useful for studying human fecal contamination in waters directly impacted by sewage contamination, it is our recommendation that coliphages be excluded for analysis in systems with diffuse sources of fecal contamination due to their low prevalence and cumbersome enumeration method. We recommended instead, to incorporate a combination of culture-based FIB methods, which are less-costly, with molecular tools, such as qPCR, to utilize when source-specificity is needed. Greater understanding of ambient coliphage presence in fresh and marine surface waters is needed before large-scale implementation of such an FIV tool is incorporated in routine monitoring programs.

3.6 CONCLUSIONS

- Somatic and F+ coliphages were found in low concentrations across all sample sites with somatic coliphages exhibiting high inter-site variability between the upstream sampling location (AS) and the two downstream sites (OS and M/P).
- FIV correlated poorly with other FIB and qMST indicators. Other than a significant ($p<0.05$) positive correlation between somatic coliphages and *E. coli*, no other significant correlations were found between indicator bacteria/virus or between enumeration methods (culture vs. molecular).
- Coliphage preparation and processing of samples requires almost 3x as much time as those needed using other culture (IDEXX Quanti-Tray®) and molecular (qPCR) enumeration

methods. Alternative methods may provide more efficient results in the context of routine water quality monitoring.

- While F+ and somatic coliphage methods are well-validated in waste- and groundwater media, ambient concentrations in estuarine surface waters is poorly understood and must therefore be studied in greater depth before implementation of a FIV in surface water quality monitoring is done.

3.7 ACKNOWLEDGMENTS

This work was funded by the National Estuarine Research Reserve System (NERRS) Science Collaborative, which is sponsored by the National Oceanic and Atmospheric Administration (NOAA). Many thanks go to IDEXX Laboratories (Gil Dichter) for supplying Colilert-18® and Enterolert™ used for fecal indicator bacteria quantification. This research would not have been possible without the guidance and insight of my fellow lab mates Denene Blackwood and Tamara Bennett, as well as to Thomas Clerkin and David Zak, for time spent helping collect and process samples.

3.8 FIGURES AND TABLES

Figure 1: Map of sample sites: Orange St. (OS), Marsh/Pollock (MP) and Ann St. (AS) in Beaufort, NC.

Method	Phage	Initial Recovery (%)
Method 1642 (2 L)	Somatic (Phi-X174)	332%
	Male-specific (MS2)	5%

Table 1: Calculated initial recovery percentages for F+ and somatic coliphages.

Method	Matrix	Phage	MS Recovery (%)
Method 1642 (2 L)	Estuarine Water	Somatic (Phi-X174)	<1% - 17%
		Male-specific (MS2)	<1% - 57%

Table 2: Calculated matrix spike recovery percentages for F+ and somatic coliphages.

Assay	Oligo ID	Sequence	Concentration	Reference
HF183	HF183	ATCATGAGTTCACATGTCCG	100 µM	Haugland et al. (2010)
	BFDRev	CGTAGGAGTTTGGACCGTGT	100 µM	
	FAMDQ	CTGAGAGGAAGGTCCCCCACATTGGA	10 µM	
Enterococcus sp. via qPCR	ECST748For	GAGAAATTCCAAACGAACTTG	100 µM	US EPA (2012)
	ENC854Rev	CAGTGCTCTACCTCCATCATT	100 µM	
	GPL813	TGGTTCTCTCCGAAATAGCTTTAGGG CTA	10 µM	

Table 3: Primer and probe sets for human-specific HF183 TaqMan assay and primer and probe sets for *Enterococcus* sp. TaqMan 23S rRNA target gene sequence.

Target	# of Individual Standard Curves (Total # of Data Points)	Master Curve Formula	Amplification Efficiency (%)	R^2	Limit of Detection (copies/rxn)	Limit of Quantification (copies/rxn)
HF183	5 (50)	-3.37x + 42.20	0.98	0.99	147	819
ENT-qPCR	3 (28)	-3.41x + 45.02	0.96	0.99	325	659

Table 4: qPCR master curves, total number of points, amplification efficiencies, standard curve R^2 values, limit of detections (LOD) and limit of quantifications (LOQ).

	EC	ENT	HF183	ENT-qPCR	F+ Coliphages	Somatic Coliphages
	Log MPN/100 mL	Log MPN/100 mL	Log CCE/100 mL	Log CCE/100 mL	Log PFU/100 mL	Log PFU/100 mL
N Total	33	29	11	22	32	17
Mean	2.19	2.14	2.64	3.81	1.03	1.48
Standard Deviation	0.71	0.84	0.34	1.06	0.42	0.77
Range (Min-Max)	(1.0 – 3.6)	(0 – 4.1)	(2.2 – 3.1)	(2.5 – 6.2)	(0 – 1.7)	(0.3 – 3.1)

Table 5: Indicator metrics for EC, ENT, HF183, ENT-qPCR, F+ and somatic coliphage concentrations for all samples.

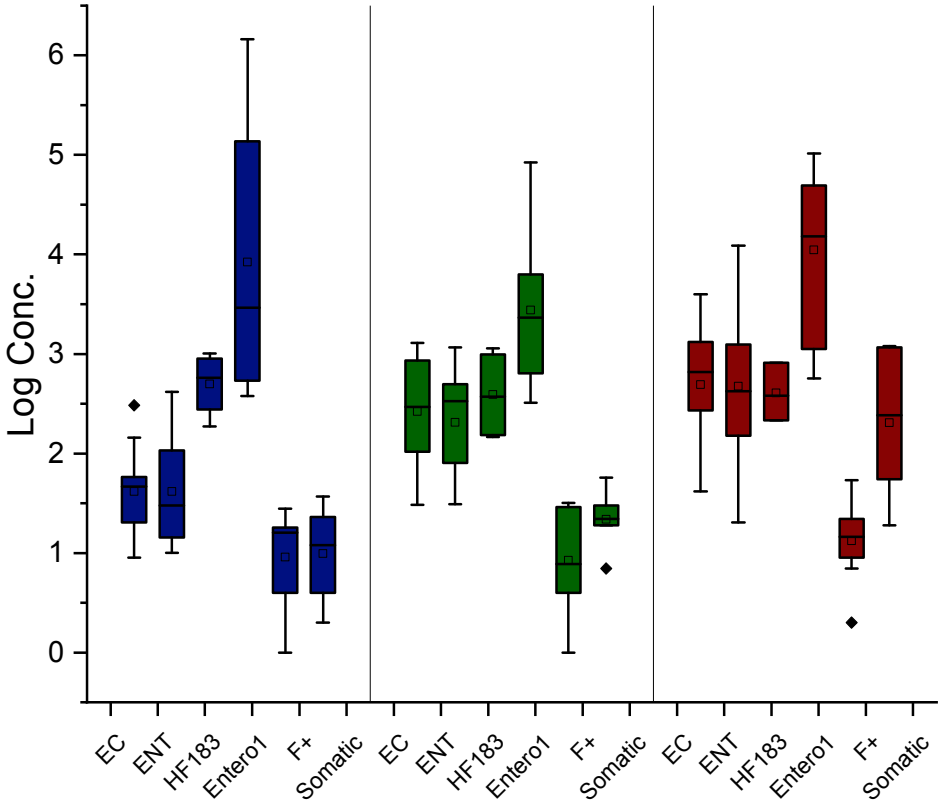
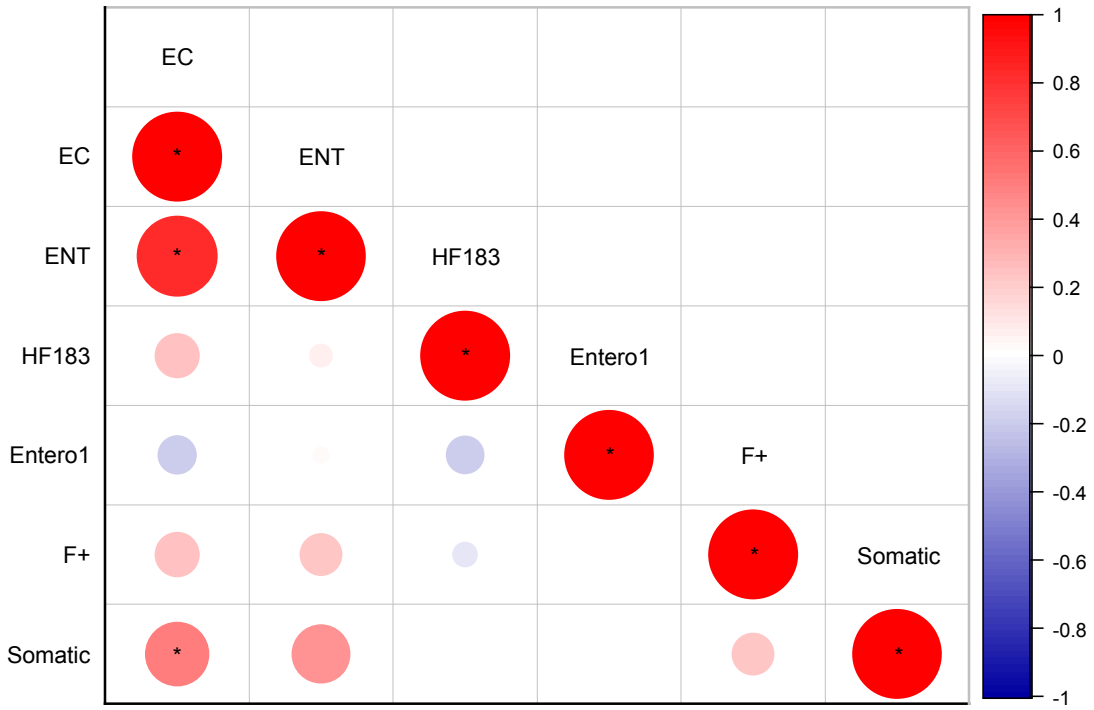


Figure 2: F+ and somatic coliphage concentrations across sampling sites: Orange St. (OS), Marsh/Pollock (M/P) and Ann St. (AS).

	EC[a] (n = 33)	ENT[a] (n = 27)	HF183[b] (n = 11)	*Enterococcus* sp. via qPCR[c] (n = 22)
% Exceedance	36%	56%	0%	68%

[a] US EPA 2012 FIB recommended threshold; [b] Haugland et al., 2010; [c] US EPA 2012 molecular marker recommended threshold

Table 6. Percent of samples that exceed recommended threshold concentrations associated with heightened illness risk.



* Correlation significant at the 0.05 level

Figure 3: Correlation coefficient values for coliphage and FIB/qMST marker pairings.

	Tidal Height (m)	Wind Direction (deg)	Water Temp. (°C)	Air Temp. (°C)	Dissolved Oxygen (%)	Salinity (ppt)	Ant. 24 h Rainfall (in)
N Total	34	23	20	34	20	20	34
Mean	-0.140	229	24	21	79	23	0.12
Standard Deviation	0.33	142	5.1	6.4	19	14	0.19
Range (Min-Max)	(-0.573-0.577)	(13-359)	(12-32)	(9-28)	(31-99)	(0-36)	(0-0.5)

Table 7: Descriptive statistics for environmental parameters measured with samples: tidal height, wind direction, water temp., air temp., dissolved oxygen, salinity and antecedent 24-hour rainfall.

Logistics	Method		
	IDEXX Quanti-Tray[b]	qPCR[c]	UF-SAL[d]
Cost per 10 samples[a]	$400 - $500	$1300 - $1500	$800 - $900
Prep. and Processing Time	20 – 30 min	2 – 4 hrs	16 – 24 hrs[e] 5 – 8 hrs
Time Until Results	18 – 24 hrs	2 – 4 hrs	16 – 24 hrs

[a] Based on current manufacturer pricing with prices for positive and negative controls as well as standard curve costs included for respective methods.
[b] Pricing includes costs for both Enterolert™ and Colilert-18® run in duplicate.
[c] Assay costs include the incorporation of both HF183 and ENT-qPCR markers run with four replicates.
[d] Costs include the assay of both F+ and somatic coliphages along with appropriate method controls.
[e] Time required for preparation of overnight host.

Table 8: Cost assessment for preparation and processing times for FIB, qMST and FIV enumeration methods.

Experiment Number	Date	Indicator	Mean (PFU/100 mL)	Variance	Std. Dev
1	07/03/19	F+ Coliphage	5.20	50.57	5.57
2	07/11/19	F+ Coliphage	5.20	18.90	3.10
3	09/19/19	F+ Coliphage	1.80	4.30	1.47
4	10/17/19	F+ Coliphage	1.87	3.63	1.66
5	10/23/19	F+ Coliphage	4.33	14.53	3.14
6	11/07/19	F+ Coliphage	4.55	16.03	3.20
		Somatic Coliphage	5.95	10.95	2.84
7	11/18/19	F+ Coliphage	2.45	1.75	1.05
		Somatic Coliphage	4.13	2.40	1.37
8	06/30/20	F+ Coliphage	1.07	1.93	1.21
		Somatic Coliphage	3.40	3.83	1.51
9	07/02/20	F+ Coliphage	1.53	2.60	1.31
		Somatic Coliphage	20.80	56.30	5.88
10	07/09/20	F+ Coliphage	0.67	1.40	1.04
		Somatic Coliphage	78.67	836.07	15.44

Table 9: Inter-plate variability between F+ and somatic hosts for the ten sampling events.

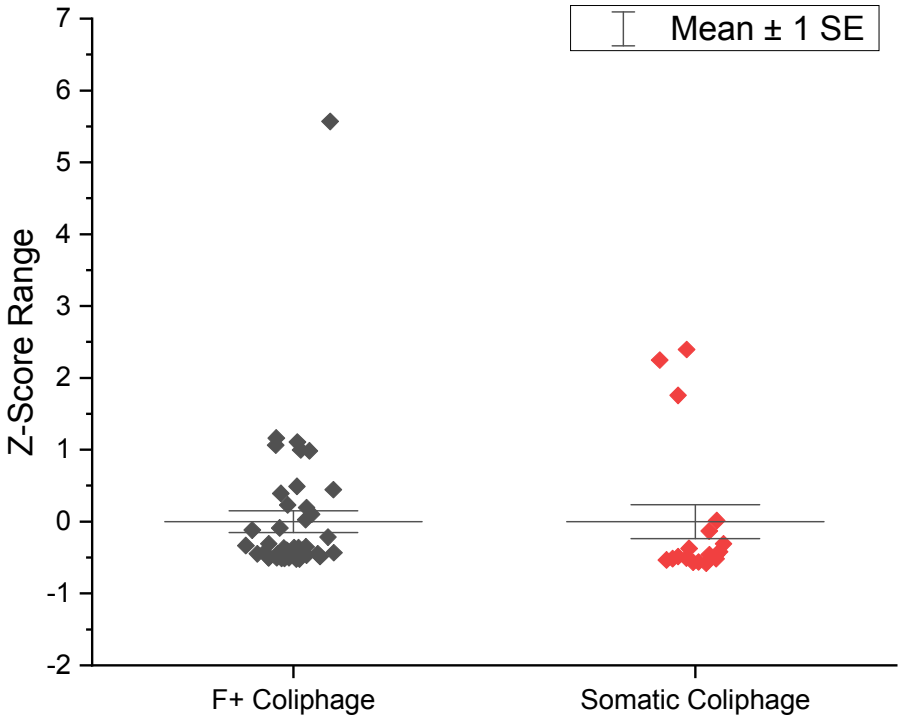


Figure 4: Z-scores showing inter-plate variability range for both F+ and somatic coliphage hosts.

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CHAPTER 4: WATERSHED-SCALE DRIVERS OF FECAL CONTAMINATION RELATED TO LAND-USE, ELEVATION AND LAND COVER

4.1 SUMMARY

Fecal pollution of environmental waters is a leading contributor to water impairment throughout the United States. Human-associated contamination, which represents the greatest risk to human health, can be introduced into surface waters from a number of sources that include damaged sewer lines, faulty septic systems, and combined sewer overflows (CSOs). As a result, identifying sources of fecal contamination that are most likely to contribute pathogens is important to prioritize management strategies for mitigating fecal pollution. Work here was conducted in the Washington DC metropolitan area and detailed a descriptive characterization of waterbodies influenced by rapid human development in order to better understand drivers of fecal contamination as told by molecular qPCR markers across environmental parameters such as elevation, land use characteristics and watershed scale. Sampling for this study was conducted within thirteen watersheds in Prince George and Montgomery Counties, MD, USA over the course of 3 years from June 2017 – May 2020 with samples analyzed for both qMST (HF183, Gull2 and HAdV) and FIB (*Enterococcus* sp. via qPCR) molecular markers. Results of the study showed the distributions of qMST (HF183, Gull2 and HAdV) and FIB (*Enterococcus* sp. via qPCR) marker concentrations measured across the watersheds were skewed, with relatively low average concentrations at the watershed scale but with occasional, high concentrations individually. Molecular markers *Enterococcus* sp. via qPCR, HF183 and HAdV, which are most

associated with human-health risks, were found to exceed recommended thresholds 37%, 7% and 3% of the time respectively. Additionally, significant ($p < 0.05$) differences were found between HF183 concentrations between both elevation (Montgomery vs Prince's George counties) and land-use classifications (resource vs. developed) indicating the influence of environmental conditions on the delivery and persistence of human-associated fecal contaminants within the system. Lastly, predictive modeling indicated both HAdV and land-use to be significant contributors in explaining HF183 concentrations. This suggests the utility of using a qMST approach at a watershed-scale level. By utilizing a combination of qMST and FIB molecular markers, along with predictive modeling tools, as outlined in this dissertation, coastal managers may be better equipped to deal with fecally-polluted surface waters in the context of urban watersheds.

4.2 INTRODUCTION

Fecal pollution of environmental waters is a leading contributor to water impairment throughout the United States (US). It is widely understood that exposure to human feces represents a greater risk to human health due to the species-specificity of most pathogenic viruses and due to the "species barrier" attributed to infection (Dufour, 2013). Human excrement can carry millions of excreted pathogenic microorganisms, which are the causative agents of bacterial, viral and protozoan diseases (Shuval, 2003). Additionally, differentiations between species (human vs. non-human) result in the density of human-associated pathogens to be less collectively aggregated in animal feces, potentially signifying lower risk to human health (J. A. Soller et al., 2010; WHO, 1999, 2003).

Human fecal pollution can be introduced into water resources from a number of sources that include damaged sewer lines, faulty septic systems, combined sewer overflows (CSOs), illicit

dumping and recreational bathers (Shanks et al., 2015). As a result, identifying sources of fecal contamination that are most likely to contribute pathogens is important to prioritize management strategies for mitigating fecal pollution (Peed et al., 2011; Templar et al., 2016). Especially within urban watersheds, the ability to accurately classify the origin of fecal pollution is critical for the timely and cost-effective management of remediation efforts (Hajj-Mohamad et al., 2019; Seurinck et al., 2005).

Measuring enteric pathogens directly within the environment can help identify sources and potential exposure pathways, which can then be used to inform approaches towards reducing human exposure. However, there are many different fecal pathogens capable of causing disease, making it impracticable to measure them all (Fuhrmeister et al., 2019). Most pathogens are difficult to quantify due to their low concentrations in environmental matrices and costly and complex methods of detection (Leclercet al., 2001). Therefore, the solution to this problem has been to monitor for fecal indicator bacteria (FIB), which have been selected due to low pathogenic potential, high levels in sewage and feces, and relationships to pathogen presence (Harwood et al., 2014). The major FIB used throughout the US include *E. coli* (EC), and enterococci (ENT) (Sales-Ortells et al., 2015; Tallon et al., 2005; US EPA, 2012; WHO, 2003). Fecal indicator bacteria concentrations, however, have not been directly correlated with pathogen concentrations and, moreover, do not get at the origin of the pollutant sources (Aw & Rose, 2012; Girones et al., 2010; Korajkic et al., 2018; Purnell et al., 2020). Given this limitation of FIB, incorporation of qMST markers into a routine monitoring framework has become common practice (US EPA, 2019).

qMST approaches aim to discriminate between human and non-human fecal sources in fecally-contaminated waterbodies (Lee et al., 2020; Nguyen et al., 2018; Shanks et al., 2015).

The performance of human-specific (E.g. HF183) and viral markers (E.g. adenovirus) are of particular interest to mitigate public health risks associated with surface water quality, given the high risk from contamination associated with their occurrence within fecal waste matrices (Badgley et al., 2019; Haugland et al., 2010; Jothikumar et al., 2005). US EPA has published recommendations for thresholds for *Enterococcus* sp. via qPCR in fresh and marine surface waters (US EPA, 2012). Previous epidemiological studies have indicated a stronger link between swimming-associated gastrointestinal illnesses and molecular approaches for *Enterococcus* sp. via qPCR when compared to traditional culture-based methods (Wade et al., 2006, 2008). US EPA recommended implementation of rapid qPCR-based methods for improved water quality management in the 2012 Criteria, but little information was given on implementation of the method into an existing routine monitoring program.

While qMST has greatly advanced our understanding of fecal contamination in a site-specific framework, there is a need for more comprehensive watershed-based approaches. A comprehensive watershed approach is therefore necessary to manage water quality over large geo-spatial scales (Badgley et al., 2019; Bradshaw et al., 2016; Stewart et al., 2013). A watershed approach requires the identification of sources of pollution that often happen concurrently throughout the landscape and incorporates environmental parameters to assess potential influences on contaminant prevalence. To properly allocate resources necessary for infrastructure repairs and to prioritize mitigation strategies and stormwater control measures (SCMs), one must be able to identify areas that suffer from chronic human fecal contamination in order to ameliorate problems within the system.

Maryland (MD) is the nation's fifth-most densely populated state with an estimated population density of approximated 595 people per square mile (US Census, 2010). Two of the

counties most impacted by population concentration, Montgomery and Prince George's, lie within the greater Washington DC metropolitan area in southern MD and house close to 2 million residents (MD State Archives, 2019). With the rate of development continuing to outpace population growth, surfaces waters throughout the state have been affected. As of April 2019, approximately 10,000 miles of surface waters were classified as impaired (Class 4a or 5), with approximately 4,000 miles of these impaired waters specifically related to bacterial causative agents (MD Department of the Environment, 2019). These were classified as such because they did not meet the state's bacteria concentration criteria (geometric mean of 126 MPN per 100 mL and a single maximum of 410 MPN per 100 mL for EC and geometric mean of 35 MPN per 100 mL and a single maximum of 130 MPN per 100 mL for ENT) (MD Department of the Environment, 2019). Pathogens were the second leading causative agent for impairment in MD 303(d) listed waters accounting for approximately 20% (90 out 458 causes of impairment) reported impairments (US EPA, 2020).

Watersheds were defined by the US Geological Survey (USGS) using a national standard-ordered system based on surface hydrologic features. Known as a hydrologic unit code (HUC), these units are commonly incorporated in watershed-scale approaches to provide more site-specific interpretations or relationships between watershed characteristics and natural resources (USGS, 1999). The watershed approach has typically been focused on an 8-digit hydrologic unit basis which was also incorporated in this study. MD Department of the Environment (MDE) classifies 138 8-digit HUCs within the state (Figure 1) which we then used to identify individual watersheds.

This current study aims to address the applicability of a watershed-scale analysis in an urban landscape by examining the prevalence of qMST markers, both human and non-human, in

surface waters at two distinctly classified watershed scales, under moderate elevation ranges and exhibiting different land use and land cover influences. The effects of these classification metrics on qMST concentrations were analyzed and documented for use in watershed model validation and prioritization efforts for future mitigation strategies.

4.3 MATERIALS AND METHODS

4.3.1 Study Sites and Sample Processing

Samples were collected from 46 sites across thirteen watersheds in Prince George's and Montgomery Counties, MD, USA (Figure 1). Sampling locations exhibited an array of elevation (0 – 360 ft) and land use characteristics and were found in watersheds that ranged in size from 67 to 790 km^2 (Table 1). Seven sampling events were conducted bi-annually over the course of 3 years from June 2017 – May 2020 with a total of 304 samples collected per sampling event (1,216 total assays analyzed). Surface water samples were collected during ambient conditions with a minimum of three days separating precipitation events and collection date. Samples were collected in 1 L volumes using acid-washed, autoclaved polypropylene bottles and stored on ice until returned to the lab and processed within 6 h. Triplicate 100-150 ml samples were vacuum filtered through either a 0.45 μm pore size, 47 mm polycarbonate (PC) filter (HTTP04700, Millipore, Bedford, MA) or a 0.45 μm pore size, 47 mm polycarbonate Mixed Cellulose Ester (HA), filter (HAWP04700, Millipore, Bedford, MA) using a six-place filtration manifold and vacuum pump assembly. For the HA filtration, samples were adjusted to final concentration 25mM $MgCl_2$*6 H_2O pH 3 prior to filtration. The filters were placed into DNase/RNase-free microcentrifuge tubes and stored at -80 °C until downstream analysis.

4.3.2 qPCR Analysis

DNA extractions were performed using the NUCLISENS® MINIMAG® extraction kit per manufacturer instructions, with extracts then stored at -20 °C. Consequent qPCR quantification used a range of both human- and non-human associated qMST markers with primers, probes, and assays described in Table 2. Assays were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Extracted samples were processed using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts). Primers (100 µM) and probes (10 µM) were synthesized by LGC Biosearch Technologies (Petaluma, CA). Each reaction had a total volume of 25 µL, 20 µL including nuclease-free water (6.75 µL), TaqMan® Environmental Master Mix 2.0 (12.5 µL), as well as appropriate primers (0.50 µL) and probe (0.25 µL), and 5 µL of unknown sample, standard, or control. NTCs were processed with each plate.

4.3.3 ddPCR Analysis

A master mix was created by the addition of qMST marker primers and probes (Table 3), 12.5µL of 2X Supermix for Probes (no UTP, Bio-Rad Laboratories, Hercules, CA), 5µL template, and nuclease free water to a final volume of 25µL. Twenty microliters of the PCR master mix were pipetted into the samples wells of the DG8™ Cartridge (Bio-Rad,) using a Pipet-lite ™XLS+ manual 8-channel pipette with the range 5–50 µL (Rainin, Oakland, CA) and 70 µL of the Droplet Generation Oil for Probes (Bio-Rad, cat no. 186-3005) was added to the appropriate wells. The cartridges were covered with DG8™ Gaskets (Bio-Rad) and placed in a Droplet Generator (Bio-Rad) to generate the droplets. Afterwards, the droplets were gently transferred to a semi-skirted 96-well PCR plate (mTEC, Eppendorf,) using manual 8-channel

pipette with the range 5–50 µL (Rainin, L8-50XLS+). The PCR plate was sealed with pierceable foil (Bio-Rad) using a PX1™ PCR Plate Sealer (Bio-Rad). After sealing, the PCR plate was placed in a C1000 Touch™ Thermal Cycler (Bio-Rad). PCR amplification was performed with a C1000 PCR Thermal Cycler (Bio-Rad Laboratories), with the following temperature profile: 10 min at 95°C for initial denaturation, 40 cycles of 95°C for 30 s, and 58°C for 60 s, followed by 98°C for 10 min, then an indefinite hold at 12˚C. After PCR cycling was complete, the plate was placed in a QX200 instrument (Bio-Rad Laboratories) and droplets were analyzed according to manufacturer's instructions. Data acquisition and analysis were performed with QuantaSoft (Bio-Rad). The fluorescence amplitude threshold, distinguishing positive from negative droplets was set manually by the analyst as the midpoint between the average baseline fluorescence amplitude of the positive and negative droplet cluster. The same threshold was applied to all the wells of one PCR plate. Measurement results of single PCR wells were excluded on the basis of technical reasons in case that (i) the total number of accepted droplets was <10,000, or (ii) the average fluorescence amplitudes of positive or negative droplets were clearly different from those of the other wells on the plate. The numbers of positive and accepted droplets were transferred to an in-house developed spread sheet to calculate the copy number. Replicate wells were merged and a sample was considered positive only if there were three positive droplets.

4.3.4 Inhibition Control

Performance of the qPCR and ddPCR assays through evaluation of recovery efficiency and inhibition was measured using a halophilic, alkaliphilic archaeon (*Natronomonas pharaonis*), from Dr. Josh Steele at the Southern California Coastal Water Research Project (SCCWRP, unpublished data). This served as a specimen processing control (SPC) as previously conducted by Steele et al. (2019). 5 µL of SPC was pipetted into each of the samples, calibrators, and

negative controls prior to processing. Following this, samples were extracted. Inhibition was determined by calculating the difference between the cycle threshold (Ct) of the SPC in samples with (experimental) and without (control, only SPC) target DNA. Extracts were analyzed without dilution with samples having more than 0.5 log units (2.32 Ct) difference from control samples deemed inhibited (Lambertini et al., 2008). For all qPCR and ddPCR runs, appropriate controls were used and exhibited no contamination: no template control (omission of DNA template from the reaction), and negative extractions control (inclusion of filter blank during DNA extraction). Standards were synthesized by GenScript (Piscataway, NJ). Gene sequences were synthesized and introduced into a linearized pUC57 vector which was cloned into DH5α competent cells. Plasmids were extracted using Wizard® Plus SV 10 Minipreps DNA Purification System (Promega Corp., Madison, WI) and linearized using Eco R1 digestion. They were then confirmed via a 1% agarose gel in Tris-Acetate-EDTA buffer. The weight of purified plasmids was then calculated spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Waltham, MA). Nanograms of plasmids were transformed to copy number by using a copy number calculator (SciencePrimer.com). Linearized plasmids were diluted and stored at a concentration of 1×10^8 copies per µL at -20°C.

4.3.5 Environmental and SSO Event Data

All environmental data was collected from publicly available online sources. Elevation data was acquired from the USGS 3D Elevation Program National Map (Source: https:// apps.nationalmap.gov/3depdem). Land-use data was acquired from the State of MD Department of Planning website (Source: http://mdpgis.mdp.state.md.us/landuse/) while land cover was obtained from Chesapeake Conservancy (Source: https://www.chesapeakeconservancy.org/). Samples were classified binomially for each classification (Table 4). Watershed characteristics

were derived from the State of MD metadata 8-Digit watershed layer available through ArcGIS (Source: https://unc.maps.arcgis.com/home/webmap/).

4.3.6 Statistical Analysis

Correlation analyses were performed using Pearson product−moment correlation for dichotomous (developed/non-developed) data. The Pearson product−moment correlation measures the linear dependence of the variables. For each comparison, the experimental unit was the watershed represented by the water quality sampling site and for each comparison, graphical techniques were first used to compare the distribution of marker concentrations. Samples were log-transformed with non-detect samples calculated as 5 copies/100 mL (log 0.7). Higher percentages of Gull2 (90%) and HAdV (98%) were classified as non-detects compared to *Enterococcus* sp. via qPCR (17%) and HF183 (49%).

4.4 RESULTS

4.4.1 Descriptive Statistics

The distributions of qMST and FIB marker concentrations measured across the watersheds were skewed, with relatively low average concentration data at the watershed scale but with occasional, high concentrations. Samples were grouped by watershed and descriptive statistics (Table 5). On average, Oxon Creek watershed (n = 1) had the greatest watershed-scale averages for both HF183 (log 3.41 copies/100 mL) and HAdV (log 0.86 copies/100 mL), while the Anacostia River watershed had the highest watershed-scale averages (log 2.75 copies/100 mL) and single sample (log 5.27 copies/100 mL) totals for *Enterococcus* sp. via qPCR. Potomac River MO County had the highest single sample concentrations for both HF183 (log 8.40 copies/100 mL) and HAdV (log 2.73/100 mL).

Samples were plotted (Figure 3) to observe geo-spatial patterns across the sampling locations. Higher concentrations of *Enterococcus* sp. determined via qPCR and HF183 appear in locations closer to DC, with lower concentrations observed as one moves farther away from the city. As these two markers are commonly found in fecally-contaminated samples, higher concentrations of these markers could be attributed to an increasingly dense population surrounding the city with high human-associated inputs of runoff in water systems that becomes less pronounced with lowering population density. Locations with higher Gull2 and HAdV marker concentrations do not appear to exhibit a spatial pattern based on geo-spatial observations.

4.4.2 Site Prioritization

Average HF183, *Enterococcus* sp. via qPCR, Gull2 and HAdV concentrations were calculated for each site across the seven sampling events. Sites were then ranked from most contaminated to least contaminated for each marker and plotted against one another. In total, nine sites were consistently found to be on at least two of the four marker lists related to human-associated illness risk (Ex. *Enterococcus* sp. via qPCR, HF183 and HAdV). These sites, along with their respective watersheds, are listed in Table 6.

Five of nine sites with concurrently high HF183, *Enterococcus* sp. via qPCR, or HAdV concentrations were found within the Anacostia River watershed. Additionally, eight of the nine sites appeared simultaneously in both HF183 and HAdV lists. Correlation analysis was conducted for these nine sites with HF183 and HAdV showing significant ($p < 0.05$) positive correlations (r: 0.792). Additionally, analysis was performed between a combination of the remaining markers with no other significant relationships found.

4.4.3 Environmental Parameters

Elevation, land-use and land cover characteristics were mapped within the watersheds. On average, higher elevations (Figure 4) were observed in Montgomery County sites (221 ft), which are found upstream within the drainage area compared to sites found in Prince's George County (62 ft). Marker concentrations were averaged (Table 7) and statistical tests performed to determine significant differences between marker concentrations across the environmental classifications. Significant differences ($p < 0.05$) were found between HF183 concentrations between both elevation (Montgomery vs Prince's George counties) and land-use classifications (resource vs. developed). No significant differences, however, were found between marker concentrations when distinguished by land-cover (non-developed vs. developed).

4.4.4 Marker Concentration Exceedance

On average, marker concentrations were relatively low across the sampling events. To assess these values with US EPA recommended criteria (*Enterococcus* sp. via qPCR: 1280 copies/100 mL) or standards defined by previous research to indicate concentrations associated with heightened risk for human illness, this study assessed the percentage of samples exceeding commonly accepted threshold criteria for the qMST and FIB markers (Table 8). Due to the high infection potential of HAdV (Crabtree, Gerba, Rose, & Haas, 1997), exceedance in this case included simply the presence of the marker (1 copy/100 mL) within the sample. HF183 risk was set at 4200 copies/100 mL given previous research suggesting heightened illness risk when samples are in exceedance (Boehm et al., 2015). Gull2 marker concentrations were low across the sampling sites, indicating low gull-associated contamination. As such, Gull2 was excluded in this analysis. Concentration exceedances for HF183 (21 out of 304) and HAdV (7 out of 258)

were relatively low at 7% and 3% respectively, however, approximately 37% (113 out of 304) of samples exceeded US EPA recommended threshold values for *Enterococcus* sp. via qPCR.

4.4.5 Influence of Scale

It seemed necessary to better understand how watershed size related with measured concentrations. Similar to work conducted in Harmel et al. (2010), the watersheds were divided into two broad categories based on scale: river basin (<350 km^2) and small watershed (> km^2). Statistical analyses indicate significant differences ($p < 0.05$) between *Enterococcus* sp. via qPCR concentrations related to scale, with higher marker concentrations reported in larger scale (E.g. small watershed) distinctions compared to smaller scale (E.g. river basin) (Figure 5). While it is not appropriate to assume that increasing scale is the sole or major cause of higher *Enterococcus* sp. via qPCR marker concentrations, this correlative relationship is certainly present within our samples.

4.4.6 Multiple Linear Regression (MLR) Analysis

Predictive modeling was performed to forecast variation of HF183 concentration given the inclusion of our qMST and FIB markers (*Enterococcus* sp. via qPCR, Gull2 and HAdV) along with environmental parameters for elevation, land use and land cover. The model (Table 9) was created using averaged data for individual sites to create a singular entry. At the 0.05 significance level, the model was determined to be significant in predicting HF183 concentration given our explanatory variables. Within the model, both HAdV and land-use were found to be significant ($p < 0.05$) contributors in explaining HF183 marker concentration variation. This complements previously established findings showing significantly positive correlations between HF183 and HAdV marker concentrations as well as significant differences in marker concentrations when characterized across land use classifications.

4.5 DISCUSSION

Fecal sources can be clearly identified using innovative molecular methods and predictive modeling. Detection of these contaminants, however, depends on the resolution of analysis as broad applications developed based on at sewage-impacted coastal sites may not be universally appropriate (Dorevitch et al., 2010; Liao, Krometis, & Kline, 2016; Wade, Pai, Eisenberg, & Colford, 2003). In expansive urban systems, predictor models can help to better explain microbial dynamics where direct testing would require costly and time-consuming procedures. This study aimed to better understand the dynamics between fecally-associated quantitative molecular markers and certain environmental parameters that may be relevant to specific marker prevalence in surface water samples. Additionally, this study sought to develop a framework with which coastal resource managers may be better equipped to prioritize problem sites across a large geo-spatial scale and create a predictive model that may be useful in aiding resource management.

4.5.1 Sample Patterns

Samples were collected over a vast geo-spatial area spanning two counties in southern MD, Montgomery and Prince's George. Average marker concentrations were low when analyzed collectively, however, marker concentrations linked with heightened illness risk (E.g. HF183 and HAdV) were found in high concentrations throughout individual samples. This may suggest levels of diffuse pollution are event-driven within the system as *Enterococcus* sp. via qPCR and HF183 markers were detected in approximately 82% and 51% of samples respectively. When translated to increased risk, only approximately 37% and 7% of *Enterococcus* sp. via qPCR and HF183 exceeded recommended thresholds. Detection frequencies are even lower for the viral (HAdV) and non-human (Gull2) qMST marker, with 10% and 3% of samples found to contain

these markers respectively. Correlations between HF183 and HAdV marker concentrations show a direct positive relationship and suggest strong associations that may better indicate heightened risk when concurrently found in water samples. This compares to poor correlations with FIB *Enterococcus* sp. via qPCR marker which has previously been found to have poor correlations with human-associated molecular markers (Ahmed et al., 2016; Hughes et al., 2017; McQuaig et al., 2012). This could indicate *Enterococcus* sp. via qPCR marker prevalence in water samples may not always indicate human-specific contamination and, as such, may not be associated with heightened illness risk.

Additionally, environmental parameters such as elevation, land-use and land-cover were also evaluated within the context of qMST and FIB marker concentrations. Both elevation and land-use appear to have influences on HF183 marker concentrations with non-developed sites at lower elevations, such as those found in Montgomery County sites, found to have significantly ($p < 0.05$) higher concentrations. Jent et al., (2013) found significant associations between land use and HF183 marker concentrations during wet weather, however these same correlations were not found during dry sampling events. As all of the samples collected throughout this study were collected during dry conditions, this could suggest land-use associations with HF183 marker concentrations could be strengthened with the addition of a rainfall parameter within the sampling framework. Additionally, Jent et al., (2013) found neither *Enterococcus* sp. via qPCR or HF183 markers to be significantly correlated with elevation during either dry or wet weather. However, a ruminant-specific marker, CF128, was found to be significantly correlated with elevation during both dry and wet weather. As this was a more agriculturally-dominated landscape compared to the anthropogenically-influenced nature of the system in this study, this

could explain the significant relationship between the HF183 marker and elevation parameter found throughout the samples.

This study also aimed to assess the effect influence of scale had on patterns of qMST and FIB marker concentrations. *Enterococcus* sp. via qPCR marker concentrations significantly ($p < 0.05$) differed between the two scale classifications (E.g. river basin vs. small watershed). Potential reasons may include environmental persistence, sporadic-growth in nutrient rich waters, and differences in the relative amounts of baseflow and surface runoff (Byappanahalli et al., 2012.; Dickenson & Sansalone, 2012; Sinton et al., 2007). It therefore may be necessary to operate at smaller watershed scales to ensure site specificity is accounted for, specifically as it relates to differing qMST and FIB molecular marker concentrations. In any case, this spatial trend should be incorporated into watershed□scale predictive modeling as down-scaling could improve the accuracy of downstream qMST and FIB predictions.

4.5.2 Multiple linear regression models

The application of a predictive modeling approach was incorporated into our study using a combination of qMST and FIB molecular markers as well as environmental variables to predict variation in HF183 concentrations. HF183 has been shown to be a sensitive and accurate analytical marker of human fecal contamination due to its high host-specificity and abundance in human waste (Ahmed et al., 2019; Boehm et al., 2013; Sidhu et al., 2012; Staley et al., 2012). In the model generated in this study, it was discovered that both the HAdV marker and land-use characterization components were significant explanatory variables of HF183 marker concentration variation within the system. HAdV has been shown to be an acceptable indicator of human-health risk (Ahmed et al., 2018; Girardi et al., 2019) and, as such, the correlation found in the study between HF183 and HAdV marker concentrations could indicate the utility of using

HF183 within the framework of a watershed-scale approach towards monitoring elevated risk potential in surface water samples. Because qMST studies can be expensive and resource-intensive, it is also important to understand environmental influences on contaminant prevalence. Evaluating human-associated qMST concentrations along with watershed characteristics might aid in identifying likely sources or at least "hot spots" of elevated contamination to focus management or further investigation (US EPA, 2011). Additionally, significant effort must be placed on the proper characterization and update of land-use types within urban areas in order to properly understand corresponding MST and FIB concentrations.

4.5.3 Implications for Managing and Regulating Contamination

Many of the qMST and FIB marker analyses in this study illustrate the need for improved understanding of contaminant transport in the environment and highlight the difficulties in managing and regulating contamination of surface waters within a vast catchment area. First, the influence of watershed-scale was important regardless of land use or background contaminant sources within the watersheds, as indicated by qMST and FIB marker concentrations. This supports the requirement for a targeted approach to establish clear linkages between bacterial sources and receiving waters and the need to integrate a watershed-scale parameter into predictive modeling applications. Secondly, episodic events, as measured using qMST and FIB markers, indicate periods of water impairment across the study area. While not in the majority of samples, the fact qMST and FIB marker concentrations were found across sites during ambient environmental conditions, indicate the possibility of continued contamination outside the scope of the study's sampling regime. Lastly, in spite of the variability and uncertainty in measured qMST and FIB marker concentrations, the influence of elevation and land-use were observed at all sites, with or without distinguishable contamination sources. This indicates the need for

enhanced understanding of anthropogenic and background contamination sources as they relate to the fate, transport, and survival of qMST and FIB molecular markers in upland environments with a scope land use characteristic.

4.6 CONCLUSIONS

• The distributions of qMST (HF183, Gull2 and HAdV) and FIB (*Enterococcus* sp. via qPCR) marker concentrations measured across the watersheds were skewed, with relatively low average concentration data at the watershed scale but with occasional, high concentrations. Molecular markers *Enterococcus* sp. via qPCR, HF183 and HAdV, which are most associated with human-health risks, were found to exceed recommended thresholds 37%, 7% and 3% of the time respectively.

• A ranking of sites based on qMST and FIB concentrations found that at the "worst" sites HF183 and HAdV showing significant ($p < 0.05$) positive correlations with one another with five of the nine sites showing increased concentrations of both.

• Significant differences ($p < 0.05$) were found between HF183 concentrations between both elevation (Montgomery vs Prince's George counties) and land-use classifications (resource vs. developed) indicating the influence of environmental parameters on the delivery and persistence of human-associated fecal contaminants within the system.

• Predictive modeling indicated both HAdV and land-use to be significant contributors in driving HF183 human-specific marker concentrations. This suggests the utility of using such an approach at a watershed-scale to better allocate resource delivery and mitigation strategies for water quality managers and researchers.

4.7 ACKNOWLEDGMENTS

This work was funded by the Washington Suburban Sanitary Commission (WSSC Water). Many thanks go to Michael Durbano and Michael Powell at EA Consulting for their hard work in collecting and distributing the samples. Also, thank you to Martin Chandler at WSSC who provided invaluable knowledge and data that made this research possible. Finally, thank you to my fellow Noble lab members, Thomas Kiffney, Kelsey Jesser, Rachel Canty and Thomas Clerkin, for countless hours spent helping collect and process the samples.

4.8 FIGURES AND TABLES

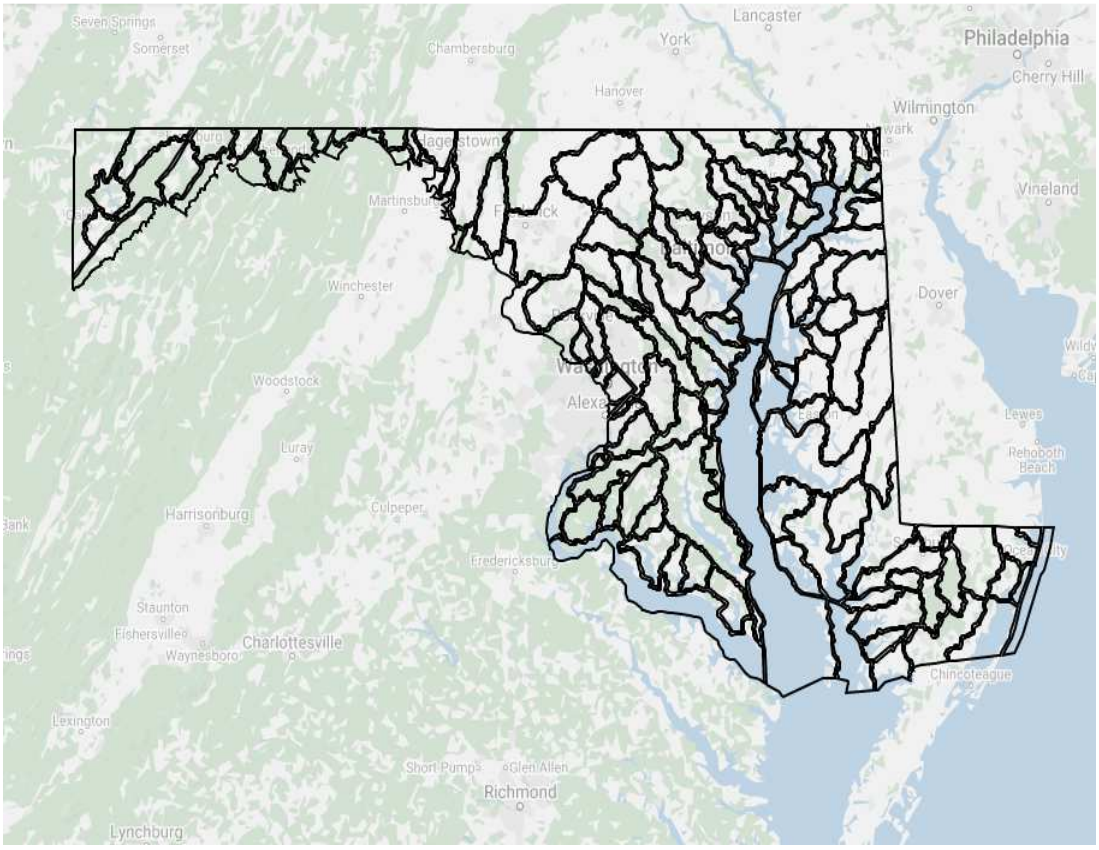

Figure 1: Map of sub-watersheds in MD delineated by MDE 8-digit HUC value.

Site	Watershed	Scale	Land Use	Land Cover	Area (km^2)	No. of samples
ANA001	Anacostia River	River Basin	Industrial	Impervious Surface	376	27
ANA002	Anacostia River	River Basin	Other Developed Lands	Herbaceous Vegetation	376	27
BRC001	Potomac River U Tidal	Small Watershed	Commercial	Tree Canopy	147	27
BRC002	Potomac River U Tidal	Small Watershed	Forest	Herbaceous Vegetation	147	27
CBJ001	Cabin John Creek	Small Watershed	Institutional	Tree Canopy	67	27
CBJ002	Cabin John Creek	Small Watershed	Forest	Tree Canopy	67	27
DSI001	Potomac River MO County	River Basin	Low Density Residential	Tree Canopy	357	15
HSP001	Patuxent River upper	Small Watershed	Very Low Density Residential	Tree Canopy	229	27
HSP002	Patuxent River upper	Small Watershed	Forest	Tree Canopy	229	27
INC001	Anacostia River	River Basin	Commercial	Impervious Surface	376	27
INC002	Anacostia River	River Basin	Forest	Tree Canopy over Impervious Roads	376	27
LBD001	Anacostia River	River Basin	Forest	Tree Canopy	376	27
LBD002	Anacostia River	River Basin	Industrial	Tree Canopy	376	27
LFS001	Potomac River MO County	River Basin	Forest	Herbaceous Vegetation	357	27
LFS002	Potomac River MO County	River Basin	Forest	Tree Canopy	357	27
MCY001	Lower Monocacy River	River Basin	Institutional	Herbaceous Vegetation	790	15
MDB001	Potomac River MO County	River Basin	Low Density Residential	Tree Canopy	357	27
MDB002	Potomac River MO County	River Basin	Medium Density Residential	Herbaceous Vegetation	357	27
MTW001	Mattawoman Creek	Small Watershed	Forest	Herbaceous Vegetation	252	15

NEB001	Anacostia River	River Basin	Forest	Tree Canopy	376	27
NEB002	Anacostia River	River Basin	Water	Water	376	27
NWA001	Anacostia River	River Basin	Other Developed Lands	Herbaceous Vegetation	376	27
NWA002	Anacostia River	River Basin	Forest	Tree Canopy	376	27
OXN001	Oxon Creek	Small Watershed	Forest	Herbaceous Vegetation	28	27
OXN002	Anacostia River	River Basin	High Density Residential	Tree Canopy	376	27
PKY001	Patuxent River upper	Small Watershed	High Density Residential	Tree Canopy	229	27
PKY002	Patuxent River upper	Small Watershed	Low Density Residential	Herbaceous Vegetation	229	27
PNT001	Anacostia River	River Basin	Commercial	Impervious Surface	376	27
PNT002	Anacostia River	River Basin	Transportation	Impervious Roads	376	27
PSW001	Piscataway Creek	Small Watershed	Forest	Tree Canopy	180	27
PSW002	Piscataway Creek	Small Watershed	Forest	Tree Canopy	180	27
PTC001	Patuxent River upper	Small Watershed	Forest	Tree Canopy	229	15
PTN001	Rocky Gorge Dam	Small Watershed	Very Low Density Residential	Tree Canopy	139	15
RCM001	Potomac River MO County	River Basin	Low Density Residential	Tree Canopy	357	15
RKC001	Rock Creek	Small Watershed	Forest	Tree Canopy	159	27
RKC002	Rock Creek	Small Watershed	Forest	Impervious Roads	159	27
SLC001	Anacostia River	River Basin	Forest	Water	376	27
SLC002	Anacostia River	River Basin	Forest	Tree Canopy	376	27
SNC001	Seneca Creek	Small Watershed	Forest	Water	334	27
SNC002	Seneca Creek	Small Watershed	Agriculture	Tree Canopy	334	27
UBD001	Anacostia River	River Basin	Forest	Herbaceous Vegetation	376	27
UBD002	Anacostia River	River Basin	Forest	Herbaceous Vegetation	376	27
WNB001	Western Branch	Small Watershed	Agriculture	Herbaceous Vegetation	290	27
WNB002	Western Branch	Small Watershed	Forest	Tree Canopy	290	27

WTB001	Potomac River MO County	River Basin	Other Developed Lands	Herbaceous Vegetation	357	27
WTB002	Potomac River MO County	River Basin	Very Low Density Residential	Tree Canopy	357	27

Table 1: Descriptive statistics for the watersheds defined by scale, land-use, land-cover and sampling totals.

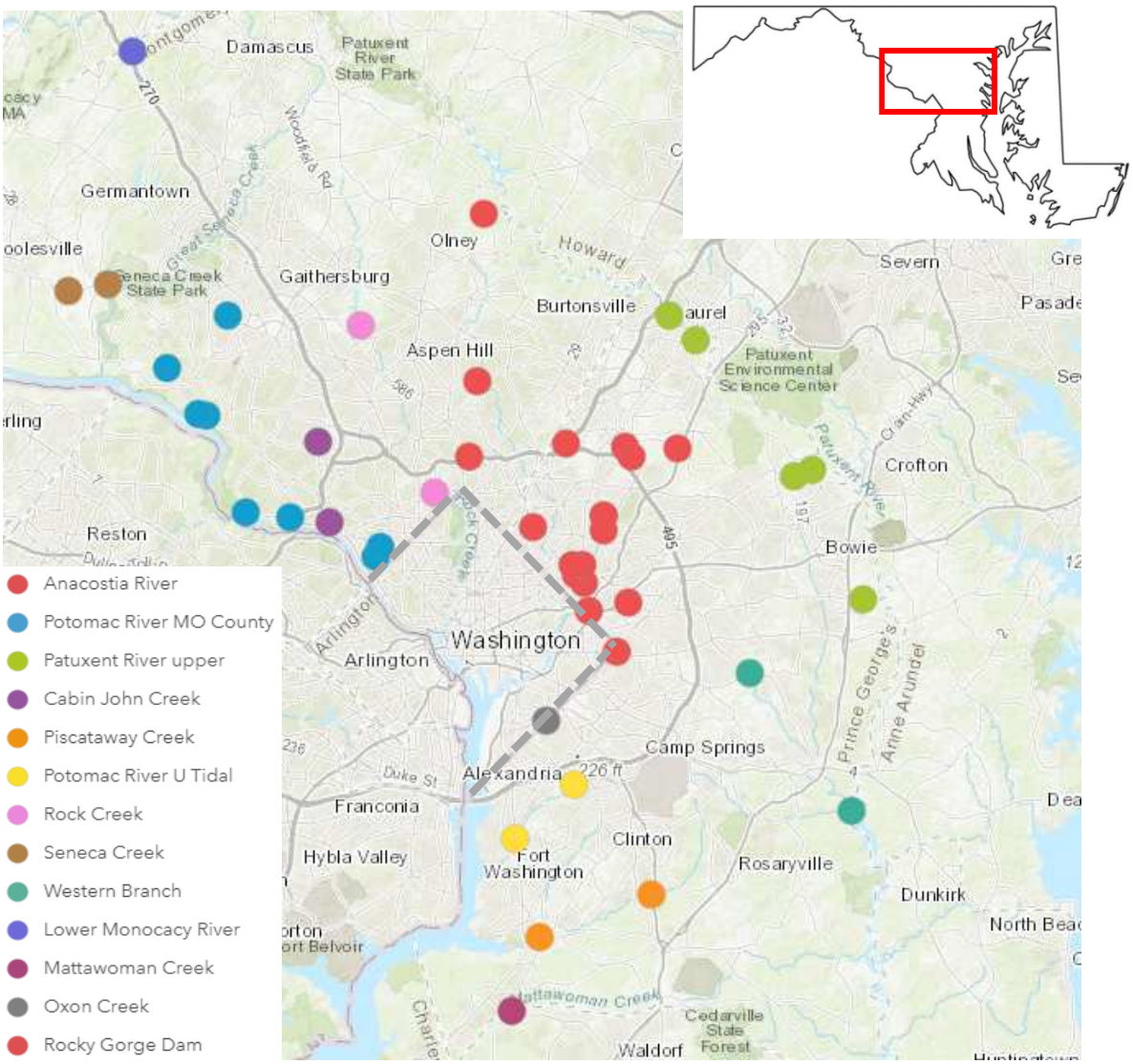

Figure 2: Sampling locations in Prince's George and Montgomery Counties (MD, USA). Their respective watersheds are color coded (see inset).

Assay	Oligo ID	Sequence	Concentration	Reference
Adenovirus	JTVXF	GGACGCCTCGGAGTACCTGAG	1 µM	Jothikumar et al. (2005)
	JTVXR	ACIGTGGGGTTTCTGAACTTGTT	1 µM	
	JTVXP	CTGGTGCAGTTCGCCCGTGCCA	0.1 µM	
HF183 TaqMan	HF183	ATCATGAGTTCACATGTCCG	1 µM	Haugland et al. (2010)
	BFDRev	CGTAGGAGTTTGGACCGTGT	1 µM	
	FAMDQ	CTGAGAGGAAGGTCCCCCACATTGGA	0.1 µM	
Gull2 TaqMan	Gull For	TGCATCGACCTAAAGTTTTGAG	1 µM	Shibata et al. (2010) Sinigalliano et al. (2010)
	Gull Rev	GTCAAAGAGCGAGCAGTTACTA	1 µM	
	Gull TM FAM BHQ	6-FAMCTGAGAGGGTGATCGGCCACATTG GGACT-BHQ-1	0.1 µM	
***Enterococcus* sp. via qPCR**	ECST748For	AGAAATTCCAAACGAACTTG	1 µM	US EPA (2012)
	ENC854Rev	CAGTGCTCTACCTCCATCATT	1 µM	
	GPL813	TGGTTCTCTCCGAAATAGCTTTAGGGCTA	0.1 µM	

Table 2: Primer and probe sets for human-specific HF183 TaqMan, *Enterococcus* sp. via qPCR, Gull2 TaqMan and HAdV gene sequence.

Assay	Oligo ID	Sequence	Concentration	Reference
HF183 TaqMan	HF183	ATCATGAGTTCACATGTCCG	0.9 μM	Haugland et al. (2010)
	BFDRev	CGTAGGAGTTTGGACCGTGT	0.9 μM	
	FAM	CTGAGAGGAAGGTCCCCCACATTGGA	0.25 μM	

Table 3. Name and sequences of primers and probes used for HF183 TaqMan assay: ddPCR sample analysis.

Classification	MD Department of Planning Classification	Classification Value
Land Use		
Resource Lands	Agriculture Forest Water	1
Developed Lands	Very Low Density Residential Low Density Residential Medium Density Residential High Density Residential Commercial Industrial Institutional Other Developed Lands	2
Land Cover		
Non-developed	Herbaceous Vegetation Tree Canopy Water	1
Developed	Impervious Roads Impervious Surface Tree Canopy over Impervious Roads	2

Table 4. Binomial classification of land cover and land use data.

Watershed	No. of Sites	Average *Enterococcus* sp. via qPCR (copies/100 mL)	Average HF183 (copies/100 mL)	Average Gull2 (copies/100 mL)	Average HAdV (copies/100 mL)
Anacostia River	17	2.75 (n = 119)	1.86 (n = 119)	0.78 (n = 119)	0.74 (n = 102)
Cabin John Creek	2	2.41 (n = 14)	1.01 (n = 14)	1.11 (n = 14)	0.70 (n = 12)
Lower Monocacy River	1	2.48 (n = 4)	1.33 (n = 4)	0.70 (n = 4)	0.70 (n = 3)
Mattawoman Creek	1	2.28 (n = 4)	1.09 (n = 4)	1.09 (n = 4)	0.70 (n = 3)
Oxon Creek	1	2.70 (n = 7)	3.41 (n = 7)	0.79 (n = 7)	0.86 (n = 6)
Patuxent River upper	5	2.65 (n = 32)	1.41 (n = 32)	0.94 (n = 32)	0.70 (n = 27)
Piscataway Creek	2	2.29 (n = 14)	1.75 (n = 14)	0.77 (n = 14)	0.70 (n = 12)
Potomac River MO County	8	2.70 (n = 50)	1.46 (n = 50)	0.88 (n = 50)	0.77 (n = 42)
Potomac River U Tidal	2	2.62 (n = 14)	2.20 (n = 14)	0.87 (n = 14)	0.70 (n = 12)
Rock Creek	2	2.67 (n = 14)	1.51 (n = 14)	0.70 (n = 14)	0.70 (n = 12)
Rocky Gorge Dam	1	2.32 (n = 4)	0.70 (n = 4)	0.70 (n = 4)	0.70 (n = 3)
Seneca Creek	2	2.46 (n = 14)	2.28 (n = 14)	0.89 (n = 14)	0.70 (n = 12)
Western Branch	2	2.46 (n = 14)	1.81 (n = 14)	0.85 (n = 14)	0.70 (n = 12)

Table 5. Descriptive statistics for average *Enterococcus* sp. via qPCR, HF183, Gull2 and HAdV concentrations defined by watershed.

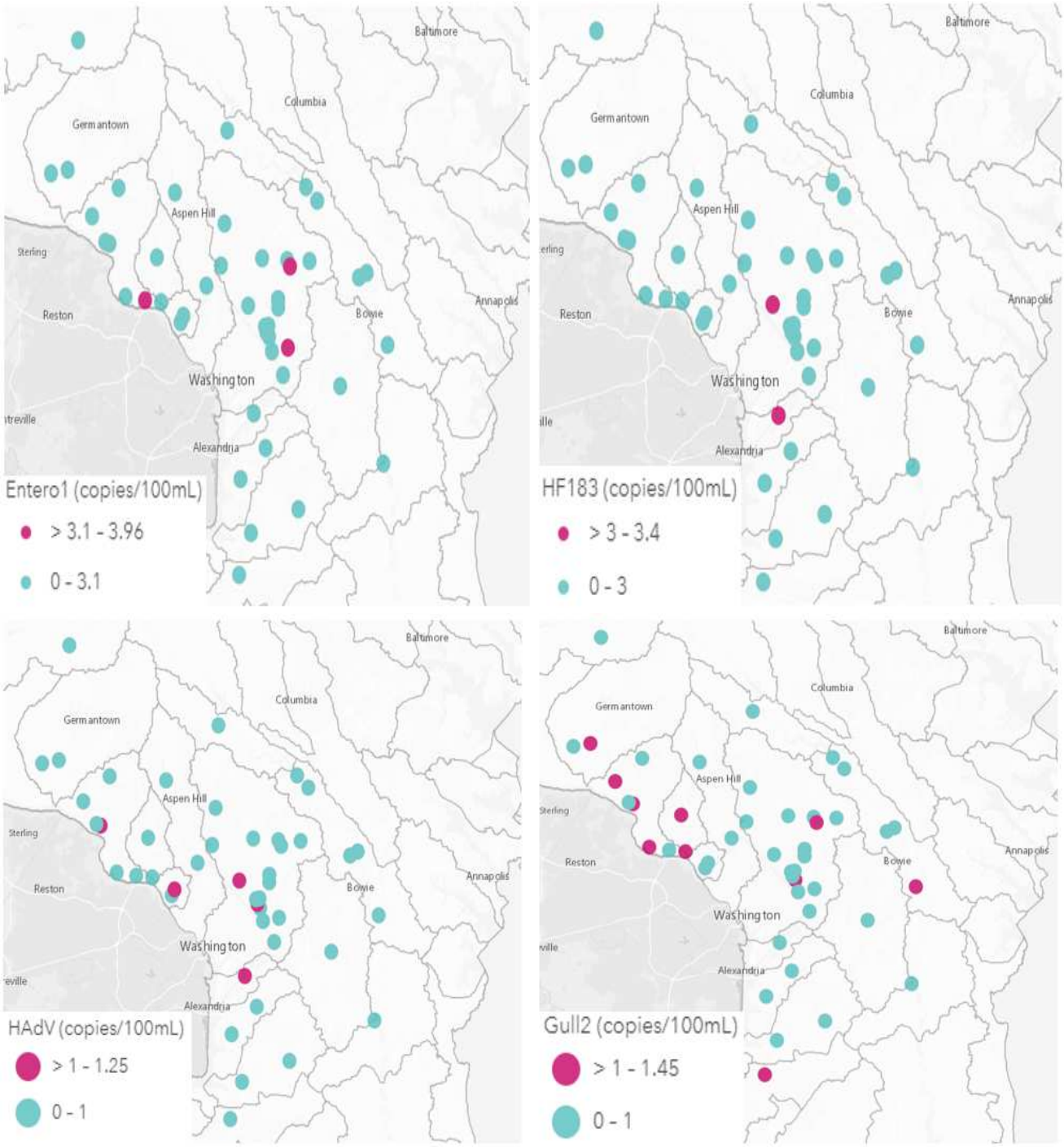

Figure 3: Geo-spatial distribution of A) *Enterococcus* sp. via qPCR, B) HF183, C) Gull2 and D) HAdV markers across watersheds.

Site	Watershed	Average *Enterococcus* sp. via qPCR (copies/100 mL)	Average HF183 (copies/100 mL)	Average Gull2 (copies/100 mL)	Average HAdV (copies/100 mL)
ANA002	Anacostia River	2.17	2.61	0.70	1.26
BRC002	Potomac River U Tidal	2.89	2.30	0.87	0.93
LFS002	Potomac River MO County	2.73	2.90	0.70	1.16
NEB001	Anacostia River	2.84	2.43	0.70	0.95
NWA001	Anacostia River	2.84	2.47	0.70	0.95
OXN001	Oxon Creek	2.70	3.41	0.79	1.23
SLC001	Anacostia River	2.70	3.11	0.70	1.18
SNC001	Seneca Creek	2.68	2.42	1.08	0.95
UBD001	Anacostia River	3.96	2.46	1.05	0.95

Table 6. Descriptive statistics for "worst" sites determined by averaged concentrations across the three qMST and one FIB markers and correlation between at least two of the four markers related to human-health risk (Ex. *Enterococcus* sp. via qPCR, HF183 and HAdV).

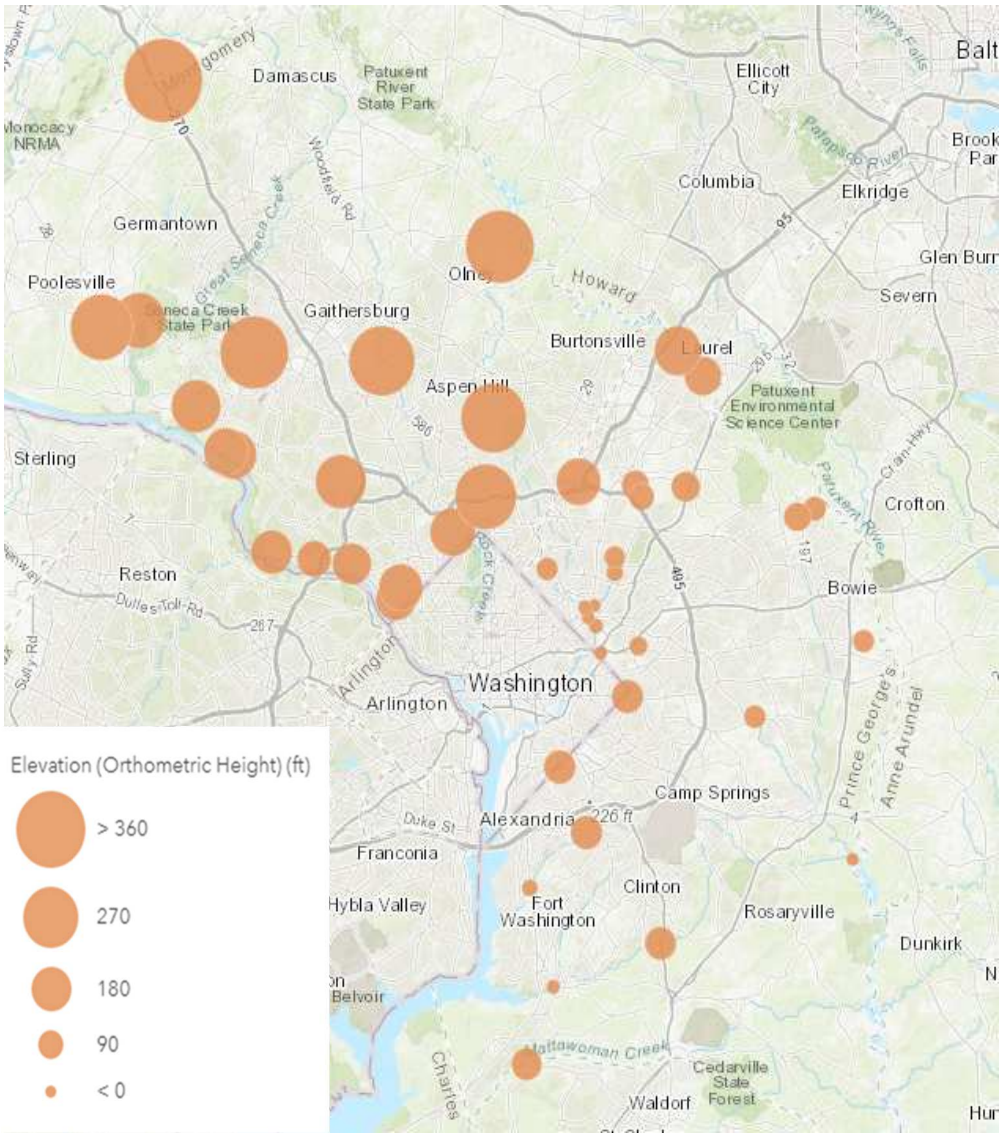

Figure 4: Geo-spatial patterns of elevation across sample sites.

	Average *Enterococcus* sp. via qPCR (copies/100 mL)	Average HF183 (copies/100 mL)	Average Gull2 (copies/100 mL)	Average HAdV (copies/100 mL)
Elevation				
Montgomery County (n = 18)	2.62	1.41	0.87	0.83
Prince's George County (n = 28)	2.65	1.87	0.83	0.90
Land-Use				
Resource (n = 25)	2.65	1.93	0.85	0.90
Developed (n = 21)	2.62	1.40	0.85	0.84
Land-Cover				
Non-developed (n = 40)	2.66	1.75	0.86	0.88
Developed (n = 6)	2.53	1.29	0.80	0.80

Table 7. Descriptive statistics for elevation, land-use and land cover data across averaged *Enterococcus* sp. via qPCR, HF183, Gull2 and HAdV concentration data for individual sites.

	Enterococcus sp. via qPCR	HF183	HAdV
% Exceedance	37%	7%	3%

Table 8. Percent of samples that exceed recommended threshold concentrations associated with heightened illness risk.

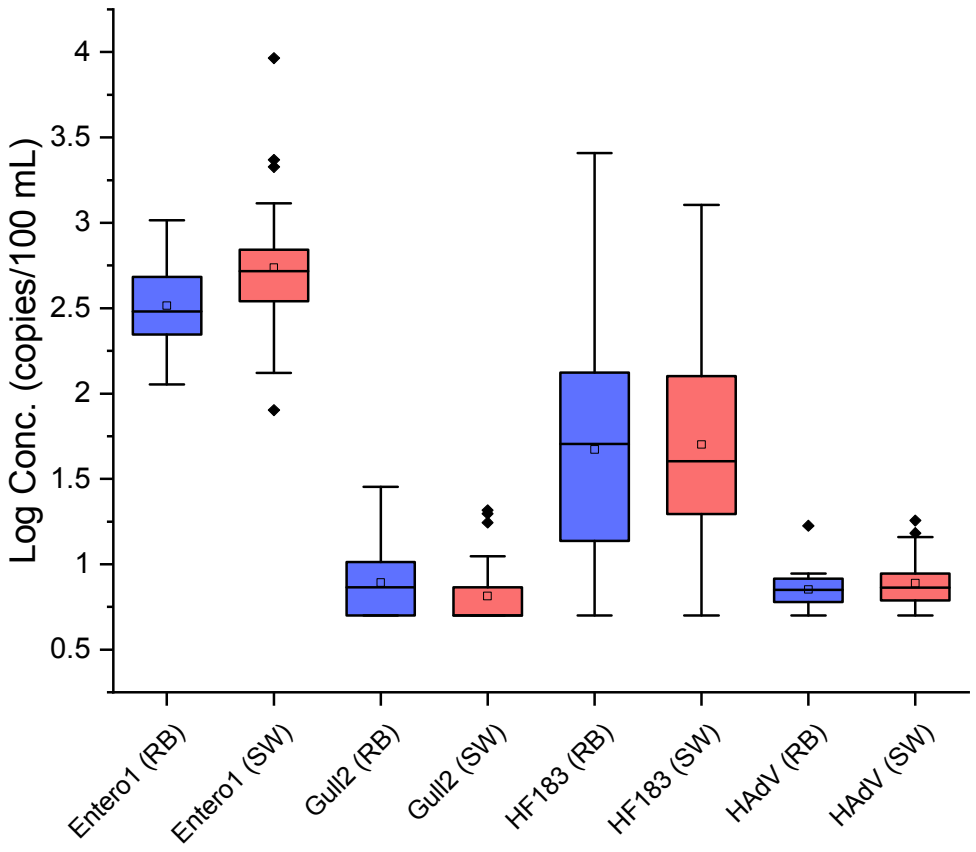

Figure 5: Distribution of qMST marker concentrations as they related to watershed size. Samples were distinguished between river basin (<350 km^2) and small watershed (> km^2) groups based on watershed sizes. Average concentrations of qMST markers are summarized with blue boxes indicating qMST concentrations in river basins and red boxes indicating concentrations in small watersheds.

Factor	Coefficient	Std. Error	t-value	Prob>|t|
R^2 = 0.81, p = 1.24e-12				
Intercept	-1.23	0.693	-1.78	0.083
***Enterococcus* sp. via qPCR**	0.175	0.130	1.34	0.186
Gull2	-0.300	0.234	-1.28	0.208
HAdV	3.79	0.401	9.43	**1.30e-11****
Elevation	-8.89e-4	5.26e-4	-1.69	0.099
Land Use	-0.254	0.096	-2.64	**0.012***
Land Cover	-0.098	0.146	-0.676	0.503

* 0.05 significance level
** 0.01 significance level

Table 9: Multiple regression model for the association of $\log_{10}$ HF183 concentrations with molecular markers *Enterococcus* sp. via qPCR, Gull2 and HAdV and environmental parameters: elevation, land-use and land-cover.

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CHAPTER 5: CONCLUSIONS

Surface waters across the US provide valuable natural resources with significant recreational and economic benefits (US EPA, 2010). Fecal waste contributions degrade these resources and, as such, create serious public health risks. To reduce the deleterious effects of contaminated surface waters throughout the country, US EPA established tools for states and localities to monitor and manage marine and fresh surface waters. The most recent update to these criteria came in 2012 with the 2012 Recreational Water Quality Criteria (RWQC). In addition to the incorporation of fecal indicator bacteria (FIB), *E. coli* and enterococci, emerging molecular tools were also recommended for use in monitoring surface waters (US EPA, 2012). While the link between molecular tools, such as qPCR, and human risk is expanding, there is room in the regulatory framework to incorporate molecular and predictive modeling tools to better understand drivers of fecal contamination across diverse coastal land/water interfaces. Predictive tools such as multiple linear regression (MLR) have shown useful in making real-time estimates of FIB concentrations within marine and fresh surface waters, by relating water quality to certain environmental factors (Ex. rainfall or tidal height) that may be exerting influence on the system (Francy & Darner, 2007; Gonzalez et al., 2012; Gonzalez & Noble, 2014b; J. Soller et al., 2015b).

Coastal communities along the mid-Atlantic, are especially susceptible to the impacts of global climate change, due to increasing urbanization and population densities up and down the coast (US Census, 2010). Routine flooding related to more episodic and intense storm events,

along with rising tidal ranges and sea level rise, pose serious concerns for managers in need of preserving water quality (Ezer, 2019; Ezer, Atkinson, Corlett, & Blanco, 2013; Kopp, 2013). Until now, little work has been conducted throughout the region that tie land use and environmental factors with levels of fecal contamination as understood through the integration of culture and molecular pathogen quantification. Impacting both urban and rural communities alike, research outlined in this dissertation will help improve our understanding of drivers of water quality impairment across a range of environmental conditions and comes at a time when little guidance is provided in the management of real-world problems such as stormwater mitigation, tidal inundation in low-lying areas, and municipal or regional prioritization of infrastructure repairs.

In Chapter 2, we provided an evaluation of stormwater dynamics in the context of a tidally-influenced coastal setting through the use of MLR analysis. This study was successful in its examination of the impact of sampling regime, environmental features and tidal characterization on our ability to predict the concentrations of the fecal indicator bacteria, enterococci. An important distinction of this study from other published coastal stormwater monitoring studies is the classification of tidal cycle (Ex. inundated, receding or transition) during periods of sample collection. Previous studies applying a tidal description in their sampling methods have primarily occurred during one tidal cycle with little emphasis on stormwater delivery (Martin et al., 2005; Sanders et al., 2005). Additionally, many of these studies were conducted in the western US or in highly developed watersheds, coastal areas with lower tidal intrusion. Across our sampling sites, we found that tidal cycling can be associated with increased concentrations of both enterococci and HF183. This is significant considering these findings were consistent between culture and molecular-based enumeration methods. Additionally, this study successfully applied a predictive

modeling tool, as suggested by US EPA, to better understand drivers of enterococci concentration. We discovered tide, not rainfall, to be one of the primary drivers of contaminant transport. As such, we would recommend, as others have detailed before (Boehm & Weisberg, 2005; Jovanovic et al., 2017), that within tidally-influenced communities, tide should be considered in coastal water quality monitoring designs and those simply monitoring water in the context of rainfall alone are problematic.

Following the development of the Chapter 2 framework, Chapter 3 evaluated the applicability of using fecal indicator viruses (FIV), F+ and somatic coliphages, as monitoring tools in the management of coastal surface waters with diffuse source pollution. Previous studies have shown the applicability of using FIV in wastewater (Bailey et al., 2017; Hassaballah et al., 2020; Nappier et al., 2019; Sidhu et al., 2018) and urban coastal waters (Jiang et al., 2001; Rezaeinejad et al., 2014; Vergara et al., 2015), however, few have actually studied their pertinence in surface waters lacking direct wastewater input. While both coliphage groups were found in a large percentage of samples, overall concentrations for both were low and showed significant variability in occurrence. Additionally, when compared to both culture- (*E. coli* and enterococci) and molecular-based (*Enterococcus* sp. via qPCR) FIB as well as qMST marker HF183, we found poor correlations across all sample sites. These findings are supported by previous studies that also found weak relationships between coliphage concentrations and FIB (Love et al., 2010; Wanjugi et al., 2018). Perhaps the greatest contribution of this study is the estimation of costs determined for commonly used enumeration methods in routine surface water monitoring. On average, we found the coliphage enumeration method (US EPA Method 1642) to require the greatest sample preparation and processing time, with other enumeration methods offering less-expensive and timely alternatives. Coliphages have been well-validated in

wastewater and groundwater, however additional research is needed to better understand their utility in routine water quality monitoring in systems with diffuse source pollution.

My final chapter (Chapter 4) aimed to address the applicability of a watershed-scale analysis in an urban landscape by examining various qMST marker concentrations in surface waters at varying watershed scales, under moderate elevation ranges and exhibiting different land use and land cover influences. An important distinction of this study from other published water quality monitoring studies is the use of smaller sub-watersheds, known as hydrologic unit codes (HUC). Given the vast spatio-temporal scale we were operating at, we believe that by operating at a smaller scale, this will provide more site-specific interpretations or relationships between watershed characteristics and contaminant prevalence. Over the course of the study, we concluded that the distributions of microbial-source tracking (HF183, Gull2 and HAdV) and fecal indicator (*Enterococcus* sp. via qPCR) marker concentrations were skewed, with relatively low average concentration data at the watershed scale but with occasional, high concentrations. Molecular markers *Enterococcus* sp. via qPCR, HF183 and HAdV, which are most associated with human-health risks, were found to exceed recommended thresholds 37%, 7% and 3% of the time respectively, however. Additionally, a ranking of sites based on qMST and FIB concentrations found that at the "worst" sites HF183 and HAdV showing significant ($p<0.05$) positive correlations with one another with five of the nine sites showing increased concentrations of both. This could suggest the utility of HF183 in identifying heightened human health risks in samples contaminated with fecal waste. Lastly, predictive modeling, using MLR, indicated both HAdV and land-use to be significant contributors in driving HF183 concentrations. This suggests the utility of using such an approach at a watershed-scale to better allocate resource delivery and mitigation strategies for water quality managers and researchers.

We believe that findings from this study may be useful for resource managers and local governments working to improve water quality as the methods provided in our study may better identify approaches towards identifying contaminated waters and aid in the prioritization of resources needed to improve these impacted systems.

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